Motivated behaviour – the role of GABA receptor subtypes in the nucleus accumbens

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For Danny

Declaration

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.

Signature:……

Abstracts and Publications arising from work reported in this thesis

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MOTIVATED BEHAVIOUR – THE ROLE OF GABA RECEPTOR SUBTYPES IN THE NUCLEUS ACCUMBENS

SUMMARY

The role of γ-aminobutyric acid (GABA) in modulating nucleus accumbens (Acb) function was investigated using feeding in rats as a model of motivation. Feeding encompasses processes associated with 'appetite', 'satiety' and 'reward'. Distinct neurotransmitter systems in the Acb have been demonstrated to control specific behavioural mechanisms subserving these motivational processes.

The Acb has been described as an interface between 'motivation' and 'action'. It is involved in the modulation of feeding, sexual behaviour, defensive behaviours and drug seeking. Over 97% of Acb neurons are GABAergic and GABA mimetics robustly increase food intake in satiated animals. One current hypothesis suggests that $GABA_A$ and $GABA_B$ receptors gate control of ingestive motor responses via the same circuit but this circuit cannot control more complex goal-directed behaviours (Kelley et al., 2005).

Temporal changes in the feeding related 'behavioural satiety sequence' (BSS) correlate with mechanisms that override satiety. The BSS is sensitive to effects on consummatory behaviour. Satiated rats given chow following intra-Acb infusions of a $GABA_B$ receptor agonist fed voraciously and the BSS was delayed but all other behaviours were still present. The pattern was similar with fasting and a systemically administered benzodiazepine but, with a μ-opioid agonist (postulated to increase the incentive value of food), the peak in feeding was delayed. A $GABA_A$ receptor agonist induced feeding but all other behaviours were significantly reduced at all effective doses.

In a second order operant schedule that independently measured appetitive and consummatory behaviour, the $GABA_A$ and opioid receptor agonists had no effect on responding but the $GABA_B$ receptor agonist increased reinforced responding in a dose dependent manner. Several other behavioural indices of motivation also increased, suggesting behaviourally selective effects. These results are inconsistent with the hypothesis described above.

Intra-Acb GABA receptor subtype stimulation led to significant differences in the location and magnitude of activity in motivation related brain structures, particularly the lateral hypothalamus and amygdala, which were revealed using Fos-like immunoreactivity as a marker. The differential modulation of feeding behaviour via GABA receptor subtypes in the Acb is used to construct a modified model to explain endogenous inhibitory motivational control.

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List of Abbreviations

2AG	2-arachidonoylglicerol
5-HT_{2a}	Serotonin 2a receptor
ABC	Avidin-Biotin Complex
Acb	Nucleus accumbens
AcbC	Nucleus accumbens core
AcbSh	Nucleus accumbens shell
Ach	Acetylcholine
ACHE	Acetylcholinesterase
AMPA	Alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid
ANOVA	Analysis of variance
A-O	Action outcome
AP	Anterior-Posterior
Arc	Arcuate nucleus
BCC	Behavioural control column
BLA	Basolateral nucleus of the amygdala
BNST	Bed nucleus of the stria terminalis
BSS	Behavioural Satiety Sequence
BZs	Benzodiazepines
c.m.s	Central motive state
CA1	Region of the hippocampus
Ca^{2+}	Calcium ion
CaBP	Calbindin D28K protein
CART	Cocaine- and amphetamine-regulated transcript
CB1	Cannabinoid receptor 1 (brain)
CCK	Cholecystokinin
CeA	Central nucleus of the amygdala
c-fos	Cellular fos
c-Fos	Fos protein
ChaT	Choline acetyltransferase
CNS	Central nervous system
CR	Conditioned reinforcement
CRf	Conditioned reinforcer
CS	Conditioned stimulus
CVLM	Caudal ventrolateral medulla
DA	Dopamine
DAB	Diaminobenzidine stain
DAMGO	[D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin
DMH	Dorsomedial hypothalamus
DV	Dorsoventral
EAAs	Excitatory amino acids
EPSP	Excitatory postsynaptic potential
ESLH	Electrical stimulation of the lateral hypothalamus
FI	Fixed interval
FLI	Fos-like immunoreactivity
Fos	Fos protein
FR	Fixed ratio
GABA	γ–aminobutyric acid
$GABA_A$	A type GABA receptor
$GABA_B$	B type GABA receptor
$GABA_BR1$	R1 subunit of B type GABA receptor

$GABA_BR2$	R2 subunit of B type GABA receptor
$GABA_C$	C type GABA receptor
GAD	Glutamate decarboxylase
GP	Globus pallidus
i.p.	Intra-peritoneal
IAAs	Inhibitory amino acids
IEG	Immediate early gene
ILI	Inter lever press interval
IPLI	Inter pellet / lever press interval
IPSP	Inhibitory postsynaptic potential
LC	Locus coeruleus
LH	Lateral hypothalamus
MCH	Melanin-concentrating hormone
MFB	Medial forebrain bundle
ML	Mediolateral
mRNA	Messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone
MSN	Medium spiny neuron
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-D-aspartate
NOS	Nitrous oxide synthase
NPY	Neuropeptide Y
NS	Non significant
NSAID	Non-steroidal anti-inflammatory drug
NTS	Nucleus of the solitary tract
PAG	Periaqueductal gray
PB	Parabrachial nucleus of the pons
PBN	Parabrachial nucleus
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PFC	Prefrontal Cortex
PIT	Pavlovian to instrumental transfer
PO	Per os (by mouth)
POMC	Pro-opiomelanocortin
PPE	Preproenkephalin
PR	Progressive ratio
PVN	Paraventricular hypothalamic nucleus
PVT	Paraventricular thalamic nucleus
RF	Reticular formation of the medulla
RRF	Retrorubral field
RT	Reaction time
s.c.	Subcutaneous
SEM	Standard error of the mean
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
S-R	Stimulus - response
TTX	Tetrodotoxin
US	Unconditioned stimulus
VMH	Ventromedial hypothalamic nucleus
VP	Ventral pallidum
VTA	Ventral tegmental area

Chapter 1

Introduction

Overview

The nucleus accumbens (Acb) is a critical locus for the control of motivated behaviours. It is anatomically well placed to serve as an interface between circuits subserving motivational processes and motor behaviours (Mogenson et al., 1980). The majority of neurons in the Acb are GABAergic (Meredith, 1999) and activation of GABA receptors in the shell (AcbSh) stimulates feeding in satiated rats (Stratford and Kelley, 1997b). However, activation of $GABA_A$ receptors does not stimulate instrumental responding for food (Zhang et al., 2003) or potentiate acquisition of Pavlovian associations (Hanlon et al., 2004). The underlying mechanisms for the contrasting effects of activating $GABA_A$ receptors on feeding and on food seeking have not been explored although there is evidence that the lateral hypothalamus is critically involved (Stratford and Kelley, 1999). Thus there are limitations in our understanding of the role of inhibitory neurotransmission in the Acb in the context of feeding, and by extension, in motivation. In particular, it is not known yet whether stimulation of AcbSh GABA receptors affects processes subserving appetite or satiety or indeed if the results of inhibitory neurotransmission could be $GABA_A$ or $GABA_B$ receptor specific.

In attempting to distinguish appetite and satiety, we need to bear in mind that appetitive motor behaviours precede the initiation of consummatory responses. It is possible that the behavioural transitions between appetitive to consummatory responses and from consummatory responses to non-motivated behaviours either depends on fluctuations in a single variable or on multiple processes at the level of the Acb. These questions will be addressed by investigating further the contribution of inhibitory neurotransmission in the Acb to the phasic expression of food motivated behaviours and the consequent activation of associated neural circuitry. Understanding the role of the Acb in motivated behavioural output is particularly important given evidence that obesity, drug addiction and affective disorders all involve pathological over-expression of goal orientated actions and consummatory behaviours.

Structure of this chapter

First of all, in this chapter, I will introduce a theoretical framework for investigating different phases of food motivated behaviour. Initially this will involve a discussion of the development of motivational theories to explain the contribution of internal and external factors and associative learning to the initiation and termination of feeding. I will then review evidence for the contribution of a variety of regions of the brain in food motivated behaviours including the Acb. To some extent an historical approach will be taken to illustrate how some of the ideas and arguments that will crop up throughout this thesis have developed and to provide context for those concepts that remain dominant in understanding food motivated behaviour. Next I will describe the internal organisation of the Acb and both the afferents to this region and the efferents that project from it. I will focus in particular on the evidence for subdividing the Acb into core (AcbC) and shell (AcbSh) regions.

The next part of the chapter will cover in more detail the functional and behavioural evidence for the contribution of the AcbSh to the control of feeding and food motivated behaviours. This will focus in particular on the role of GABA and hence I will consider in brief the different subtypes of receptors for this transmitter in the central nervous system. I will then review the behavioural evidence for a specific role for endogenous GABA in the AcbSh in controlling ingestive behavioural output in terms of food intake and appetitive responses. I will also consider briefly evidence for the contribution of other local neurotransmitters and neuromodulators in the AcbSh that may act synergistically or compete with GABAergic signalling to control ingestive behaviour. Next I will look at the evidence for additional behavioural effects of modulating AcbSh GABA neuronal activity.

Finally I will review the effects of modulating AcbSh GABA neuron activity on activity in other functionally linked brain regions. This chapter will conclude with a discussion of one current theory to explain the role of GABA sensitive neurons in the Acb in feeding and the relevance of this mechanism to understanding broader control over motivated behaviours. I will highlight questions that this theory raises, particularly with

reference to the role of GABA receptor subtypes in the AcbSh, and describe the aims of the studies undertaken in this thesis.

Motivation

The term motivation has been applied to behaviour elicited by a variety of natural goals that can initiate motor output in animals and humans including feeding, drinking, sexual behaviour, exploration, fear and aggression/defence, sodium appetite and temperature control. In addition it is possible to identify numerous categories of species-specific motivated behaviours such as territory acquisition and defence, nest building, parental behaviours towards offspring, defensive burying etc. Humans are also motivated to self-administer drugs and alcohol although animals do show a natural affinity for stimulants and opiates (Weeks, 1962, Thompson and Schuster, 1964, Headlee et al., 1955, Spragg, 1940, Deneau and Seevers, 1964, Pickens and Harris, 1968). Many difficulties have arisen in attempting to formulate a single motivational theory that adequately explains the complex behavioural output for such varied goals (Toates, 1986). In this thesis feeding will be used as a model system because it has been demonstrated that stimulation of GABA receptors in the Acb increases feeding but not drinking (e.g. Ward et al., 2000).

Motivated behaviour can be described as following three sequential phases 1) initiation, 2) procurement and 3) consummatory (Swanson and Mogenson, 1981). Furthermore animal and human behaviour has historically been discussed in terms of appetite, when a particular goal is actively sought, and satiety following consummation which is characterised by eventual termination of behaviour. To understand how motivated behaviour is controlled in the CNS we need to understand what processes contribute to each of these phases.

At the start of the 20th century it was believed that complex innate or voluntary behaviours consisted of a series of individual reflex components; the reflex chain hypothesis (for a review of the multiple origins of this hypothesis see (Clower, 1998). Of course this was in part due to the huge impact of the work of Pavlov on conditioned reflexes (e.g. see Pavlov and Anrep, 1927). However animals would need an extensive repertoire of reflexes controlled by a multitude of dedicated synaptic pathways for each stimulus type. These synaptic pathways would be in constant competition and would be

poorly suited to adapt to a changing environment or producing new behaviours on encountering novel stimuli.

A strict behaviourist view (Thorndike, 1933b, Thorndike, 1933a, Skinner, 1938) was that an animal's behaviour could be explained by the acquisition of fixed reflexive connections between specific external stimuli (S) and the response (R) without higher order cognitive processing (S-R association). The problem with this, pointed out as early as 1917 by Craig, (Craig, 1917, Craig, 1918, Craig, 1919, Craig, 1920) is that many active behaviours in motivated animals appear before there is any clear stimulus i.e. they are not stimulus bound reflexes and, apart from during the eventual congress with an incentive goal, do not take the form of stereotypical chained reflexes.

Homeostatic theories of motivation

For decades motivation has been considered almost exclusively in terms of drives and often in terms of homeostatic drives. However the conceptual framework that grew up out of a homeostatic approach is inadequate in explaining much of what can be observed and measured about behaviour in reality. Modern motivational theory also takes into account processes governing affective responses, associative learning and cognitive inputs to voluntary behaviour as well as innate goal directed responses. Some of the evidence that has accumulated for the multiple processes that subserve motivated behaviour will be (necessarily) briefly considered here.

Walter Cannon proposed an explanation for motivation whereby behavioural output was dependent on the influence of physical signals from the peripheral nervous system (e.g. gastric contractions) on the central modulation of autonomic and reflex responses (Cannon, 1932). These reflex responses were necessary to maintain a constant internal state that he termed 'homeostasis'(Cannon, 1926, Cannon, 1932). His student Curt Richter suggested that animals could express organised motor behaviours not just reflexes, to maintain a constant internal state (e.g. Richter, 1941).

Cannon and Richter did not consider learned behaviours to be conducive to homeostatic regulation and thus the integration of information about the consequences of expressing homeostaticaly driven behaviours was not considered important (Smith, 2007, Smith, 2008). In contrast, Hull (1943) believed that associative learning was intrinsically

involved in the expression of a specific hierarchy of different habits depending on stimulus strength. Homeostatic imbalance or deficit was a driving force for learning which habits to express. Drive reduction was believed to be rewarding and physical drive reducers such as food and water could initiate and reinforce the expression of motivated behaviours.

Central to homeostatic models of motivation and drive reduction theory is that deprivation is intrinsic to motivation and animals cease to be motivated once they are replete or in a state of 'satiety' (Moran, 1975). It was later hypothesised that such a model requires a 'set point' or optimum physiological parameter, detectors to compare deviations in current physiological state from the goal value and a mechanism such as motivational drive to trigger behavioural responses (Toates, 1986(Berridge, 2004). The model predicts that an animal will engage in an appropriate category of motivated behaviour until a set point has been reached. For example a rat injected with salt to induce dehydration will drink exactly the right amount of water to return body fluid concentration to normal (Fitzsimons, 1963).

The termination of homeostatic behaviour driven by internal deficits is an example of negative feedback whereby motivational drive is eliminated when consummatory behaviour returns the internal state to the set point. At a basic level ingestive behaviour is an example of unambiguously homeostatic mechanisms that maintains body fluid levels and energy balance and it is possible to experimentally induce behaviours that are terminated by negative feedback. There is a vast body of literature exploring the neurophysiological mechanisms that subserve this feedback. Beyond circulating levels of nutrients many candidate 'satiety' signals have been identified including cholecystokinin (CCK) (Harper and Raper, 1943), melanocyte-stimulating hormones (α-, β-, and γ-MSH) (Bittencourt et al., 1992), leptin (Rowland et al., 1996), cocaine and amphetamine regulated transcript (CART) (Thim et al., 1998, Kristensen et al., 1998). The search for satiety factors also resulted in the discovery of hormones and peptides that can apparently increase ingestive behaviours including neuropeptide Y (Rudski et al., 1996, Dhillo et al., 2002), agouti gene related transcript (AgRP) (Fan et al., 1997, Dhillo et al., 2002) and ghrelin (Kojima et al., 1999, Wren et al., 2000, Date et al., 2000). The homeostatic model of motivation has led to the identification of areas of the brain that process information about deviations from optimal conditions and signals

that can initiate and terminate autonomic responses particularly in relation to feeding and drinking (Berridge, 2004). These will be discussed in detail later in this chapter.
The problem with a homeostatic view of motivated behaviours is that, although it explains goal-direction it does not adequately explain all the conditions that precede the initiation of consummatory responses or shifts in the magnitude, speed and persistence of behavioural output in response to external and internal stimuli. Equally homeostatic explanations do not take into account the fact that non-human animals and humans will vigorously pursue a multitude of specific goals when there is no apparent internal imbalance or need for homeostaticaly driven behaviours. For example, animals will overfeed when the normal sensory experience associated with ingestion is bypassed (Rowland and Nicolaidis, 1976) or eat even when satiated if presented with conditioned cues that predict reward (Weingarten, 1983), highly palatable meals (Wirtshafter and Davis, 1977, Gilbert and Sherman, 1970, Carper and Polliard, 1953, Carper, 1953, Guttman, 1953) or novel / varied diets (Kushner and Mook, 1984, Clifton et al., 1987, Treit et al., 1983). Conversely they fail to consume enough to meet physiological needs if the flavour is aversive (Nicolaidis and Rowland, 1975).

Furthermore, outside of a laboratory setting feeding and drinking is often anticipatory, initiated prior to any homeostatic deficit (Bindra, 1968), but controlled via the same cues from detector systems that would trigger motivation if a deficit were present (Berridge, 2004). It has been suggested that rather than motivation being driven by a set point it is the result of an ever shifting balance between a multitude of opposing endocrine and neuronal processes and innate behaviours (Bolles, 1980, Wirtshafter and Davis, 1977, Pinel et al., 2000).

Drive reduction theory also suffered following the discovery that stimulation of neurons in the brain that could elicit feeding was rewarding in and of itself i.e. drive and reward appeared to be the same process subserved by the same local circuits in the CNS (e.g. see Valenstein et al., 1970, Valenstein, 1975). It was demonstrated that high levels of electrical stimulation in various regions of the brain could be rewarding, there was a clear anatomical dissociation between rewarding and punishing mechanisms and stimulation that increased consummatory responses to natural rewards was most effective in the same regions where self stimulation was rewarding (Olds, 1958b, Olds, 1958a, Olds et al., 1960, Margules and Olds, 1962).

Section summary

To summarise, regardless of whether behaviours are subserved by drive to reach a set point or fluctuations in drive engendered by competing systems, homeostatic models only really explain the expression of basic goal-specific behaviours like ingestion, and then only under specific sets of circumstances. These processes do not explain more complex strategies to procure access to a goal in anticipation of needs, the flexibility of behavioural selection in the face of changing or novel environments or the persistence of behaviours beyond satisfying physiological needs. A major advance in the approach to studying motivation was the assertion that motivation systems must encompass processes that allow the acquisition of new, flexible operant responses that can be changed appropriately in light of changing circumstances (Berridge, 2004). A conceptual framework that contributed to this more inclusive theory of motivation was based on the idea of appetitive and consummatory phases in behaviour (Craig, 1917).

Appetitive and consummatory behaviour

On the basis of extensive observation of natural behavioural patterns Craig (1918) described motivated behaviours as occurring in a series of phases that appeared in cycles. He described two key phases as consisting of "appetitive" and "consummatory" behaviours. An appetite described a state of agitation and restlessness in the absence of the "appeted" stimulus that was characterised by a "readiness to act" and included "incipient consummatory action" (Craig, 1918). The strategy that the animal would employ during the appetitive phase to gain access to the stimulus depended on its success in using the strategy previously to make consummation of the act possible.

Craig noted that appetitive behaviours became quicker and more efficient over time and thus must be learnt by trial and error. Importantly Craig (1918) suggested that "readiness" to perform some actions excluded the performance of others because 1) activity in some neurons would inhibit activity in others, 2) the "condition of the internal secretions" meant the animals was unready for alternatives and 3) some instincts were mutually exclusive due to "incompatibility of motor components" (Craig, 1918). In particular condition two suggests a similar level of insight into the underlying

neural mechanisms as Hebb more than 30 years later (Hebb, 1949). Craig (1918) also suggested that while he believed appetite was the outward expression of internal causes and physiological states a stimulus from the environment could be an "immediate excitant of an appetite".

The consummatory phase was initiated by congress with appeted stimuli and activity at this stage often took the form of chained reflexes to consummate the appetitive act. Consummation of the appetitive act would usually rapidly "discharge" the appetite. Consummation could lead to further appetite and these first two phases (appetitive and consummatory) could alternate for some time. Nevertheless consummatory behaviour would eventually terminate the appetitive phase. Craig also suggested that once the animal reached a state of "satisfaction" the appetitive stimuli might actually become aversive and actively avoided (phase 3). The final phase (4) was a state of rest in the absence of the activities expressed in the preceding phases.

Taking into account the importance of the appetitive phase in Craig's model it was not enough to explain motivation in terms of drives subserving consummatory behaviour via homeostatic mechanisms. However it was many years before theorists attempted to construct a parsimonious model that included factors that could subserve the appetitive phase. Epstein (1980) suggested that motivation included (1) flexible goal directedness or means – end readiness, (2) goal expectation, and (3) affect (Berridge, 2004). The important shift on emphasis was that motivated behaviour must be subserved by processes that that could initiate appetitive behaviours before the goal object initiated consummatory responses. In Epstein's criteria this would require more than simple S-R type learning to organise flexible procurement strategies to gain access to the goal under different circumstances. This learning component was further important to confer expectation whether this was in the form of simple conditioned anticipatory responses or a more cognitive declarative process. Finally it would also include an affective component expressed as a result of interactions with a hedonically potent goal.

The suggestion of such criteria to define a motivated behaviour lead to a new model – incentive motivation. The premise of this new conceptual framework was that reward was not due to drive reduction but related to the positive value of the goals and appetitive-consummatory behaviours would not always be satisfied and terminated

simply by physiological drive reduction. Models of motivation needed to take into account learning, expectation and affective (emotional) components.

Hedonic reward, affective processes and learning

One possible explanation for non-homeostatic motivation is that animal behaviour can be driven by the hedonic properties of rewards, cues predicting hedonically rewarding goal stimuli and learning about the value of external cues. The parallel development of motivational theory and learning theory has been inextricably linked for decades and it is beyond the scope of this introduction to discuss the vast literature. The early controversies surrounding the conflict between single process theories (Hull, 1943, Guthrie, 1935, Tolman, 1932, Pavlov and Anrep, 1927) and two process learning theory first suggested by Miller and Konorski (1928, in (Rescorla and Solomon, 1967) were discussed in detail by Rescorla and Solomon (1967) over 30 years ago. The legacy of these arguments however was evidence that reinforcement of behaviours was fundamental to learning and that affective components of the learning process contributed to an animal's experience and consequent behaviour.

Alcaro et el., (2007) suggest that a clear distinction between motivational systems that determine future behaviour and learning systems that retroactively strengthen associations between preceding events is often made. Thus approach to incentive stimuli is considered an expression of motivation whereas the attribution of value to previously neutral stimuli is considered an expression of learning. However, although this serves a useful purpose when attempting to measure the magnitude and persistence of innate behaviours it sidesteps the issue that animals are continually adapting their behavioural responses to their environment on the basis of experience. Little behaviour expressed even by non-human animals will be purely innate reflexes, instincts and unconditioned actions.

Tolman proposed an incentive learning theory whereby the motivational effects of physiological drive states were indirect due to the incentive value that an animal assigns to an instrumental reinforcer or outcome(Tolman and Gleitman, 1949a, Tolman and Gleitman, 1949b). The animals learnt through consummatory interactions with the goal to assign a higher value when in a state of homeostatic deficit and hence will work harder to attain that goal when in the same state. This incentive value constitutes a non-

homeostatic form of motivation. Many incentive motivation theories have built upon the concept of incentive value associated with reward but I will consider the contribution of some key authors who, in the 1970s and 1980s attempted to bring some of the old learning theory into perspective to form a more parsimonious motivation theory.

A shift away from drive reduction theories and the strict behaviourism of researchers such as Skinner began as alternative incentive concepts of motivation were proposed. In particular the concepts developed by Bolles, Bindra and Toates (e.g. Bolles, 1958, Bolles, 1972, Bolles, 1979, Bindra, 1969, Bindra, 1974a, Lajoie and Bindra, 1978, Toates, 1981, Toates, 1986) were critical to the development of incentive motivation theory.

Bolles (1972) suggested that behaviour was motivated by incentive expectancies rather than by homeostatic drives (or a need for drive reduction). He asserted that previously neutral cues that had been associated with hedonically potent goals could induce a state of expectation and would be perceived as predictors of future reward (Bolles, 1972). However Berridge (2004) pointed out that it is not clear why expectation would necessarily be motivating enough to induce active behaviour when an animal could passively wait for the reward the cue lead them to expect.

Bindra (1979) suggested that stimuli in the environment could be "affectively" (hedonically) potent or neutral and that those stimuli that were hedonic could be positive or negative. It is probably important to note that, at the time this theory was developed, it did not explicitly describe the neurobiological representations of internal 'motivation' but simply referred to internal 'homeostatic' status (Nader et al., 1997). A "central motive state" (c.m.s.) (Morgan, 1943) was produced by the interaction between the central representation of incentive stimuli (such as food) with internal, physiological states (such as hunger) (Bindra, 1974a, Bindra, 1978). This idea was not new (e.g. for a review of Kornorski's ideas that shaped such theories see Zielinski, 2006) but Bindra went further in suggesting that if an animal experienced a neutral stimulus followed by a rewarding incentive such as food the former could take on some of the incentive properties of the reward and motivate behaviour itself (Bindra, 1974b, Lajoie and Bindra, 1978).

Bindra (1979) talked of "organismic variables" determining *how* excitable motivation related brain circuits were and neural activity due to affective or hedonic stimuli determining *which* circuits would be excited. The problem with this explanation is that it might predict that once a neutral stimulus acquired incentive value it would always elicit the response whether the animal was internally motivated or not. Neither does it explain how incentive stimuli such as food encountered when an animal is not in an appropriate state of deprivation can have hedonic value, particularly if not consumed.

Toates (1981, 1986) added the caveat that the incentive value of a neutral stimulus, gained via association with a rewarding stimulus, was subject to modulation by the current internal state of the animal, thus hungry animals might lick a cue light but treat it as a neutral signal when satiated. Toates (1986) also suggested that the animal may not encounter the stimuli in a state that will confer positive hedonic value to it (e.g. water is not particularly arousing when an animal is not thirsty) but they assimilate information about the original incentive stimuli that can become hedonically arousing at a later date. Finally Toates (1986) suggested that, although novel objects may appear to have hedonic properties because they arouse exploration or avoidance, these responses are short lived (animals habituate to them) whereas animals do not become permanently habituated to food and transfer of incentive to neutral stimuli increases rather than decreases with experience (Toates, 1986). The c.m.s cannot be generated by internal variables alone but determines how effective an external incentive will be in arousing a c.m.s. which in turn will lead to behavioural output (Toates, 1986).

Incentive learning theory

Dickinson and Balleine (1994) condensed many of these concepts into an incentive learning theory to explain the motivational control of goal directed actions. This was based on concepts originally discussed by Tolman. The basic premise of the incentive learning theory is that the motivational effect of states of deprivation is indirect because state determines the incentive value of a reinforcer or action outcome. The assignment of such value occurs once an animal has learnt the value of a particular outcome in a given motivational state only by experiencing it in that state. This process of learning was referred to as incentive learning by Dickinson and Balleine (1994) who suggested that animals learnt both the value of reinforcers under states of deficit ("motivational

determination") and that the high value in this state was dependent on being in that state ("motivational control")(Dickinson and Balleine, 1994, Dickinson and Balleine, 1995, Dickinson et al., 1996). These two types of learning represented the basic incentive motivation associated with hedonic goals and the cognitive aspects of expectancy and prediction that were discussed by Toates (1986). Central to the understanding of incentive learning theory is the demonstration that different neuronal substrates subserve the assignment of incentive value and the cognitive processes of expectancy and prediction (Balleine and Dickinson, 1998, Belin et al., 2009).

Saliency attribution - Wanting versus liking

Researchers such as Young (Young, 1952, Young, 1966) and Sheffield (Sheffield et al., 1951, Sheffield et al., 1954) consistently demonstrated that pleasure and positive affective experience, hedonic processes, were important instigators and modulators of behaviour when other investigators were concentrating exclusively with concepts of drive reduction and associative learning (Berridge, 2007a). Berridge and colleagues suggested an important dichotomy between these important hedonic processes which they termed 'liking' and non-hedonic saliency attributed to reward which they termed 'wanting' (Berridge and Valenstein, 1991). This was termed the incentive salience model (Berridge, 1996). In this model liking and wanting, affective states that human subjects can articulate but that can only be measured indirectly in rats, are taken as two dissociable components of reward, mediated by separate brain systems (Berridge et al., 1989, Berridge, 1996).

Liking was defined as the immediate evaluation of how 'pleasurable' a stimulus is, whereas wanting was defined as a process that underlies our attraction toward stimuli in the environment (Berridge et al 1989, Berridge 1996). It is the approach to a stimulus (and instrumental responses to it) that constitutes a measure of 'wanting' (Berridge 1996). Berridge and Valenstein (1991) hypothesised, for example, that electrical stimulation of the lateral hypothalamus (ESLH) specifically increases incentive salience attribution (wanting) via associative learning but without directly activating circuits involved in affective responding. Liking and wanting often occur together but can be dissociated under certain circumstances and are subserved by different neuronal circuitry (Berridge, 1996, Berridge, 2001, Berridge, 2004, Berridge et al., 2009).

Section summary

While early homeostatic models of motivation have contributed to our understanding of the role of internal signals to both initiate and terminate some classes of behaviour the importance of external influences must be accounted for. Although a motivated animal does not always express obvious goal directed behaviours, given the appropriate external stimuli it will actively seek congress with or avoid an incentive object e.g. positive food reward or aversive prey animal. Increases in motivation support increases in the speed and strength of both the acquisition and expression of operant responses. Motivated behaviours are flexible and although they may become habitual, strategies can be consciously adapted. Motivation during learning can result in an increase in the ability of external factors to influence the active expression of motivated behaviours at lower thresholds of internal drive or even when internal drive is lacking. Any model of motivation must thus take into account complex interactions between the impact of signals associated with internal state and arousal levels, external stimuli and the saliency attributed to them through learning and affective, emotional and cognitive processing. A schematic summary of this integration is shown in Fig. 1.1.

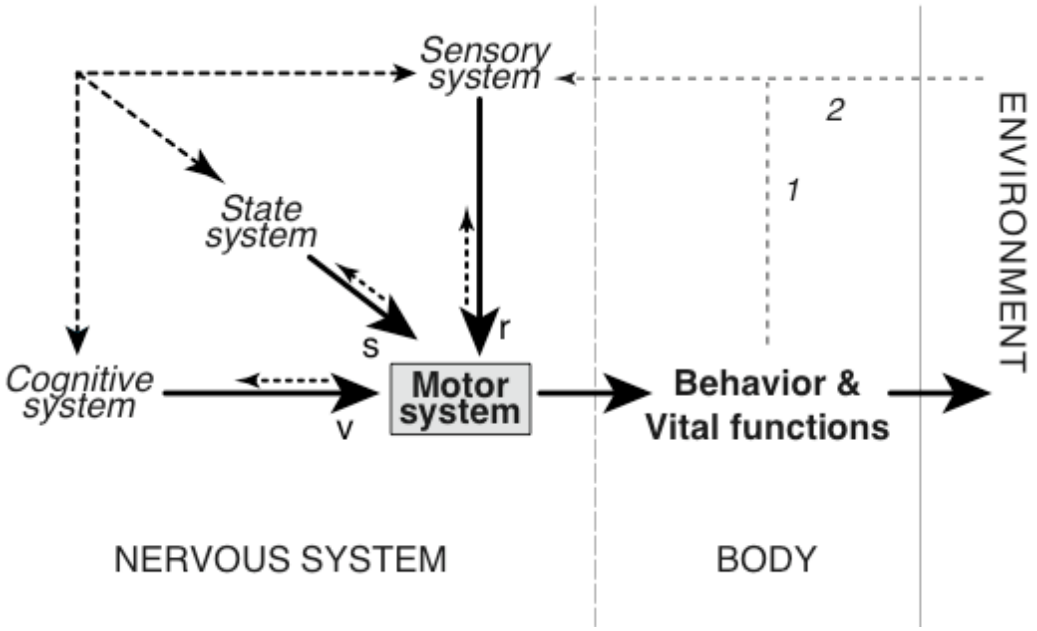


Figure 1.1. "A basic schematic for nervous system functional organization. This model assumes that the motor system controls behaviour and bodily vital functions, and that there are three classes of inputs to the motor system—cognitive, which is responsible for voluntary (v) control; sensory, which is responsible for reflex(r) control; and behavioural state, which is responsible for

state(s) control. Note that the sensory, state, and cognitive systems share bidirectional connections, and that the results of internal (1) and external (2) behaviours feed back through the sensory system to influence future behaviours". (Figure and legend copied from (Swanson, 2000)

Regions of the brain subserving motivated behaviour

While early research into the control of motivated behaviours focused on peripheral mechanisms controlling homeostatic drives, authors such as Lashley (1938) took a centralist view that an excitatory state had to be aroused in a hypothetical neural system in the brain to cause a switch from non-motivated to motivated behaviour. This switch must involve the integration of a variety of inputs including endocrine signals, physiological state, internal and external stimuli and that the central nervous system could organise specific goal directed outputs (Lashley, 1938). The challenge was to find where in the brain the appropriate information was integrated and such a switch occurred. I will focus here particularly on areas of the brain identified as being necessary for the expression of food ingestion and behavioural responses that food and food related stimuli engender.

Hypothalamus and food intake

Since the 19th century it has been recognised that damage (e.g. tumours) in the region of the hypothalamus-pituitary complex in humans can lead to overeating and obesity (Mogenson and Huang, 1973). However diseases such as Babinski-Frölich Syndrome encompass a variety of other symptoms because tumours are rarely restricted to a single structure. With the development of neurosurgical techniques to target lesions to discrete regions of the brain in non-human animals it was possible to explore this phenomenon further. Electrolytic lesions of the medial and particularly ventromedial hypothalamus (VMH) caused hyperphagia, increased fat deposition and weight gain in rats (Brobeck et al., 1943, Brobeck, 1946, Hetherington and Ranson, 1939, Hetherington and Ranson, 1940, Hetherington and Ranson, 1942b, Hetherington and Ranson, 1942a). VMH lesioned animals would not work to gain access to food (Miller et al., 1950).

In contrast electrolytic destruction of the extreme lateral portion of the lateral hypothalamus (LH) caused total abolition of spontaneous intake of food and water, the effect was so extreme that animals would starve to death and the lesions blocked the hyperphagia induced by VMH lesions (Anand and Brobeck, 1951a, Anand and

Brobeck, 1951b). These authors suggested that the LH constituted a "feeding centre" and the VMH a 'satiety centre' in the brain (Anand and Brobeck, 1951a, Anand and Brobeck, 1951b, Brobeck, 1955).
Stellar (1954) took the idea of feeding centres further suggesting that the hypothalamus could be the seat of the central motive state that had been described over a decade before (Morgan, 1943). He theorised that "the amount of motivated behaviour is a direct function of the amount of activity in certain excitatory centres of the hypothalamus" (Stellar, 1954). He postulated that excitatory centres (in the LH) were modulated by activity in inhibitory centres (in the VMH). The inhibitory centres could control neural mechanisms for the satiation of motivation. Furthermore different classes of behaviour could be subserved by anatomically discrete areas within these centres.

Around this time it was also reported that electrical stimulation in the LH directly evoked feeding responses and increased food intake (Hess, 1954 in (Miller, 1957, Anand and Dua, 1955, Smith, 1956). The use of electrode implantation also revealed that animals found stimulation of some regions of the hypothalamus intrinsically rewarding and would press a lever for this stimulation (Olds, 1956a). More precise topographical studies revealed that stimulation in the region of the LH elicited approach whereas in the VMH only escape could be produced (Travis and Olds, 1959, Olds et al., 1960, Olds, 1960).

Despite the compelling evidence from both lesion and electrical stimulation studies problems with the dual centres hypothesis of hypothalamic modulation of motivated behaviour soon became evident. It was clear from the start that lesions in the VMH caused a number of metabolic and behavioural effects collectively known as the Ventral Hypothalamic Syndrome. This included reduced drinking, increased blood insulin levels, decreased oxygen consumption, decreased utilisation of amino acids, heightened reactivity to stimuli, "finickiness" in diet selection, reluctance to work for food and inhibition of self stimulation in the lateral hypothalamus (Hoebel and Teitelbaum, 1962, Hoebel, 1965) (Miller et al., 1950).

Likewise, lesions of the LH did not cause permanent aphagia or adipsia but did cause profound reductions in general motor functions and arousal (Teitelbaum and Stellar, 1954). Rats did not respond appropriately to physiological challenges such as injection

of saline or to food or water deprivation and only consumed food if it was particularly palatable and presented with water (Teitelbaum and Epstein, 1962). Electrical stimulation of the lateral hypothalamus, as well as producing hyperphagia, causes licking, chewing, swallowing described as 'eating automisms', which suggests that the hypothalamus is linked to other brain regions such as the posterior brain stem (Bell, 1971). This combination of multiple responses following destruction or stimulation of the LH was referred to as the Lateral Hypothalamic Syndrome.

Some authors suggested that the profound effects on feeding and drinking that followed lesions or electrical stimulation of the hypothalamus could actually be due damage or stimulation of the medial forebrain bundle (MFB) that courses through this region of the brain (Morrison and Mayer, 1957, Morrison et al., 1958). Positive responses to electrical stimulation coincided with stimulation in the MFB rather than in discrete centres in the hypothalamus and Hess's "parasympathetic" regions (Hess, 1957 in (Olds et al., 1960) fell within an extended continuum from the telencephalon to tegmentum rather than specifically within the hypothalamus (Olds et al., 1960, Olds, 1960). Morgane (1961)(Morgane, 1961e) noted that the MFB connected the hypothalamus to other areas of the brain for which evidence was emerging for their role in the control of feeding including the amygdaloid complex and regions of the cortex.

Stimulation of the MFB in the region of the hypothalamus both elicited food seeking and consummatory responses and was intrinsically rewarding (Olds et al., 1960, Olds, 1960, Margules and Olds, 1962). Lesions, knife cuts or stimulation of the MFB anterior or posterior to the 'feeding' and 'satiety' centres did not alter basic consummatory ingestive responses or intake (Morgane, 1960, Morgane, 1961b, Paxinos and Bindra, 1972, Paxinos and Bindra, 1973, DiCara and Wolf, 1968). In addition, lesions of the MFB anterior to the hypothalamus did not reduce the rewarding effects of self stimulation in the hypothalamus (Olds and Hogberg, 1964).

Nevertheless the MFB did appear to be necessary for the expression of motivational aspects of ingestion (Morgane, 1961b, Morgane, 1961d). It was acknowledged that despite the lack of effects of MFB lesions immediately adjacent to the hypothalamus damage to other structures along the pallidofugal extent of the MFB e.g. in the globus pallidus (GP) did produce some aspects of hypothalamic syndromes (Morgane and

Kosman, 1960, Morgane, 1961c, Morgane, 1961a, Morgane, 1961d, Morgane, 1961e, Morgane, 1961b). Although Morgane (1961e) thought the MFB was not essential for the control of feeding he acknowledged that the lateral hypothalamic nucleus was "so firmly imbedded in this bundle at the level of the 'feeding center" that it cannot be separated from it by any method of destruction".

In the 1960s a method was developed to visualise catelcholamine expression in neurons (Carlsson et al., 1961, Carlsson et al., 1962, Bjorklund et al., 1968) and dopamine containing pathways through the MFB were identified and characterised (Dahlstroem and Fuxe, 1964, Anden et al., 1965, Fuxe et al., 1965, Fuxe, 1965a, Fuxe, 1965b, Anden et al., 1966c, Anden et al., 1966a, Hillarp et al., 1966, Anden et al., 1966b, Fuxe et al., 1966, Anden et al., 1967, Bjorklund and Nobin, 1973, Lindvall et al., 1974). With the development of techniques to selectively destroy these dopaminergic fibres of passage it became possible to study their involvement in feeding and food motivated behaviour.

In the 1970s it was suggested by many investigators that the importance of the LH in feeding was minimal because effects could be due instead to the incidental destruction of the dopaminergic fibres of the nigrostriatal or mesolimbic pathways since many of the characteristics of the LH syndrome could be mimicked by DA fibre destruction (Ungerstedt, 1970, Ungerstedt, 1971, Ungerstedt et al., 1974, Marshall and Teitelbaum, 1973, Marshall et al., 1974) (Fibiger et al., 1973a, Fibiger et al., 1973b, Oltmans and Harvey, 1972, Oltmans and Harvey, 1976). At the same time Grossman and colleagues showed that knife cuts to fibres entering or leaving the hypothalamus laterally (that connected the brainstem and basal ganglia) also mimicked the hypothalamic syndromes (Grossman and Grossman, 1971, Grossman, 1972, Grossman and Grossman, 1973).

The problem with the assertion that many of the effects of lesioning the hypothalamus could be reproduced by damaging fibres of passage was that while it was possible, for example, to destroy dopaminergic fibres selectively, it was not possible to destroy cell bodies and spare fibres in the hypothalamus and hence test the unique contribution of this region to any of the effects demonstrated by electrolytic lesions. With the discovery of excitotoxins such as ibotinic acid the importance of the LH in feeding was reconfirmed because excitotoxin induced fibre sparing lesions still resulted in hypophagia (Grossman et al., 1978, Stricker et al., 1978).

Animals with excitotoxic lesions of the LH showed less drastic deficits in feeding, drinking and in the maintenance of body weight but were unable to respond appropriately to experimentally induced physiological challenges (Winn, 1984, Clark, 1990, Winn, 1990). Nevertheless animals with excitotoxic lesions of the LH were capable of regulating ingestion when food or water deprived, responded appropriately to adulteration of their diet and showed no deficits in locomotion, sensorimotor responses, or in willingness to perform an operant response for food (Winn et al., 1984, Dunnett et al., 1985, Clark et al., 1990, Winn et al., 1990, Clark et al., 1991, Winn, 1995)

The re-assertion of the role for the lateral hypothalamus in feeding and drinking was supported by electrophysiological studies that showed activity in the hypothalamus in direct response to the sight and taste of food (Burton et al., 1976, Rolls et al., 1976, Rolls et al., 1977, Rolls et al., 1979). Furthermore on a purely anatomical basis the direct connections to the cortex and to the autonomic systems of brainstem of spinal chord suggested the hypothalamus as a point of integration (Saper et al., 1979, Tucker and Saper, 1985, Saper, 1985, Saper et al., 1986). Since the 1980s specific regions of the hypothalamus e.g. the paraventricular nucleus (PVN) have been shown to be the primary sites where metabolic hormones exert their effect (Gao and Horvath, 2007)

Section summary

The evidence discussed in this section demonstrates that multiple regions in the hypothalamus play a fundamental role in the control of food intake and that, contrary to beliefs of the first half of the 20th century, activity in feeding related brain regions is intrinsically rewarding. The recognition that damage or stimulation of the MFB might be responsible at least in part for some of the symptoms associated with hypothalamic syndromes suggests that other areas of the brain that are connected via these fibres of passage may be critical to the control of food motivated behaviour. In particular, in the 1970s it was demonstrated that the integrity of the dopaminergic fibres that pass through the LH is critical in the organisation of multiple aspects of behavioural control.

The role of other areas of the brain in feeding

Despite the predominance of a hypothalamocentric approach to understanding food motivated behaviours for over 40 years (from the mid 1940s to mid 1980s) there was ample evidence for the involvement of multiple brain regions in ingestion. The acknowledgment that the integrity of the MFB could be important in the regulation of motivation and, more specifically the nigrostriatal and mesolimbic dopamine pathways, also suggested that regions that were either the sources of ascending or descending fibres or the site of termination of these neurons might in turn be necessary and or adequate for the expression of motivated behaviours. These included the amygdala, accumbens (Acb), septum and prefrontal cortex (PFC) anterior to the hypothalamus and the ventral tegmental area (VTA), substantia nigra (SN), periaqueductal grey (PAG) and reticular formation (RF) posterior to the hypothalamus (Nauta, 1961, Morgane and Stern, 1972).

Before Olds and colleagues explored the effects of electrical stimulation in the LH they had shown that stimulation in the subthalamic nucleus (STh), cingulate gyrus (CG) in the cortex and (most robustly) in the septum was rewarding and facilitated acquisition of lever pressing as effectively as a primary reinforcer such as food (Olds and Milner, 1954, Olds, 1956b). Conversely in some locations the stimulation appeared to be aversive, was vigorously avoided and reinforced learning of avoidance responses (Delgado et al., 1954, Miller, 1957). Stimulation in the basomedial forebrain facilitated learning of a maze and runway behaviour more robustly than giving food reward (Olds, 1956b). Animals were more willing to overcome aversive events to self stimulate than to gain access to food but response rates varied with fluctuations in natural drive states e.g. hunger, suggesting that areas that were intrinsically involved in mediating the effects of primary rewards were being stimulated (Olds, 1958b, Olds, 1958a).

Robust decreases in ingestion but with varying degrees of persistence were also demonstrated following electrolytic lesions of the GP (Morgane, 1961a), midbrain tegmentum (Blatt and Lyon, 1968), SN (Ungerstedt, 1971), septum and amygdaloid complex (Pubols, 1966), the brainstem (Parker and Feldman, 1967, Zeigler and Karten, 1974) or thalamic regions (Zeigler and Karten, 1974). Increased ingestion was reported following lesions of, for example, the mamillary nuclei (Graff and Stellar, 1962), septum (Harvey and Hunt, 1965) and PAG in the brainstem (Skultety and Gary, 1962).

The fundamental role for the brainstem in many aspects of the basic control of feeding was demonstrated by the finding that decerebrate rats could ingest food and fluid placed in the mouth and stop ingestion if the taste was aversive, when gastrointestinal signals suggest satiety had been reached or if the food was noxious or toxic (Grill and Kaplan, 2002, Norgren and Leonard, 1971, Grill and Norgren, 1978a, Grill and Norgren, 1978b, Grill and Norgren, 1978d, Grill and Norgren, 1978c). Nuclei in the brainstem are connected to peripheral nerves involved in ingestion, digestion, nutrient absorption and parasympathetic and sympathetic responses but, while the brainstem can mediate acute food responses it cannot respond to long term homeostatic challenges (Berthoud et al., 2001, Berthoud, 2002, Berthoud, 2004). Electrical stimulation in 'higher' regions of the brain such as the VTA, MFB and areas of the cortex can initiate stereotypical, coordinated movements that are activated by direct stimulation of "pattern generators" in the spinal chord and brainstem (Stein, 1978, Dubner, 1970, Sessle et al., 1976)

One group of structures that was particularly well investigated by the 1970s was those constituting the limbic system and particularly the amygdaloid complex (Mogenson and Huang, 1973). Following lesioning of the amygdala, investigators reported profound induction of hyperphagia and obesity (Morgane and Kosman, 1959, Morgane and Kosman, 1957, Morgane and Kosman, 1960) or conversely aphagia and adipsia (Brutkowski et al., 1962, Fonberg, 1966, Collier and Gault, 1969) or transient effects (Crow and Whitaker, 1970). The amygdaloid complex consists of multiple distinct nuclei and lesions in dorsomedial sites appeared to be responsible for aphagia (Fonberg, 1966) whilst destruction of basolateral sites caused hyperphagia (Fonberg, 1968, Fonberg, 1971). Early investigations indicated that lesions might exert their effects due to decreased sensitivity to stimuli and to reinforcement contingencies (White, 1971, Schwartzbaum, 1960) and to increased sensitivity to the context (Sclafani et al., 1970) and deprivation state of the animal (Cole, 1974). Electrical stimulation in the amygdala, other forebrain limbic regions and the VTA were shown to elicit ingestive responses (Mogenson and Huang, 1973) and influenced the behaviour elicited by stimulating the hypothalamus (Sibole et al., 1971, Siegel and Flynn, 1968, Siegel and Skog, 1970).

By the 1970s it was acknowledged that feeding and drinking was probably subserved by complex circuits (e.g. Mogenson and Huang, 1973) but there was little direct evidence for how these circuits might be organised. There was much focus on the inter-

relationship between activity in limbic structures and the hypothalamus and between the hypothalamus and the midbrain (for a review see (Mogenson and Huang, 1973). There was a general consensus that hypothalamic nuclei were well placed to serve an integratory role, limbic structures modulated hypothalamic output and this output signalled to midbrain and brainstem regions that subserved the motor execution of behavioural responses (Mogenson and Huang, 1973).

However there was no satisfactory evidence to show how all these regions were connected or related "anatomically or physiologically to neural, humoral and other signals to which they apparently respond(ed), nor to the autonomic, endocrine and somatic efferent systems that they influenced" (Mogenson and Huang, 1973). While the hypothalamus was shown to influence downstream regions in the midbrain and brainstem it also caused changes in activity in rostral regions of the brain suggesting a more complex level of integration (Mogenson and Huang, 1973). Additionally while the hypothalamus clearly served as a point of integration within circuits that controlled biologically relevant behaviours this did not mean that it subserved all motivated behaviour. Mogenson et al (1980) pointed out that, while stimulation or lesioning of brain regions could suggest a role in the execution of specific classes of motor responses, these experiments did not elucidate how movements were planned and initiated or how the 'cognitive' or 'emotive' areas of the brain affect these processes.

An interface between limbic and motor regions of the brain

In 1980 Mogenson and colleagues published a seminal paper in which they proposed that the nucleus accumbens, part of the ventral striatum, could be the point of integration between neurons of the limbic and motor regions of the brain on the basis of newly identified anatomical connections reported by Graybiel at a meeting in 1976. In fact prior to this Ungerstedt had already suggested "the anatomical connections of the striatum makes it well suited to be concerned with the final sensory-motor integration, i.e. the efferent organization of behaviour" (Ungerstedt and Ljungberg, 1974).

At the time only a small proportion of the afferents and efferents of the Acb were known. The model of Mogenson and colleagues was based on the evidence that it received direct inputs from the amygdala, hippocampus and other limbic structures and indirect inputs from limbic regions via the VTA and that it projected directly and

indirectly (via the SN) to the GP (Mogenson et al., 1980). The advance in tracing techniques over the following decades has shown that the Acb is even better placed anatomically to act as an interface than these authors originally thought. The additional afferent and efferents since identified will be discussed later in this chapter.

Mogenson and colleagues (1980) provide anatomical, electrophysiological and behavioural evidence for the interconnectivity of key components of their model. They also pointed out that while the "emotive" brain i.e. regions of the limbic system innervated the Acb, the "cognitive" brain (regions of the frontal lobes) was connected to the dorsal striatum and the two regions could filter or gate converging signals to determine their influence over downstream regions such as the GP. While Mogenson and colleagues demonstrated that the Acb was involved in the control of locomotor responses via its connections to the GP, and pointed out that locomotion is a prerequisite to more complex behaviours, there was no functional evidence for the role of the Acb in motivated behaviours e.g. feeding, agonistic responses etc (Mogenson et al., 1980). These authors noted that Judson Herrick (1926) had suggested that the Acb could be involved in both locomotor and "facial reflexes involved in feeding" (Herrick, 1926 in Mogenson et al., 1980).

Section summary

A large number of regions in the brain from cortical structures to the brainstem have been implicated in the control of food motivated behaviour. Advances in techniques in the 1970s to identify anatomical connections between regions of the brain lead Mogenson and colleagues to propose a model that included the Acb as an interface between motivation and action because of its connectivity with limbic and motor regions. While evidence for the role of the Acb in mediating reward and reward related actions was emerging it was becoming increasingly obvious that it was not a homogenous structure (see below) and that its position within circuits that might modulate motivation was determined by regional variations in afferents and efferents. Before the role of the Acb in motivation is further discussed, using feeding as a model, the anatomy and position of the Acb in distributed macrocircuits will first be described.

Anatomy of the Acb

The striatum was first suggested to be a unified complex consisting of dorsal and ventral divisions by Heimer and Wilson (1975). The term "ventral striatum" was put

forward by these authors to denote the area encompassing the Acb and olfactory tubercles (Heimer and Wilson, 1975). Broadly speaking the Acb resembles the rest of the striatum in terms of cytoarchitecture, subcortical connections and local neurotransmitters (Meredith et al., 1992) but a unique combination of afferents and efferents of this ventral portion distinguish it from the rest of the striatum. Furthermore, although the Acb was originally viewed as a unitary structure both anatomically and functionally, it became evident that there were distinct regional differences in neuronal connections of the Acb, particularly in their relationship to the extended amygdala and LH (Groenewegen and Russchen, 1984).

Core and shell subdivisions

With advances in neuroanatomical methodology it was suggested that the Acb can be divided into at least two distinct subregions, the "core" (AcbC) and "shell" (AcbSh) (Zaborszky et al., 1985). The AcbC and AcbSh differ in terms of their histochemistry (Voorn et al., 1989, Jongen-Relo et al., 1993, Jongen-Relo et al., 1994), cytoarchitecture (Meredith et al., 1989, Meredith et al., 1992, Meredith et al., 1993, Wright et al., 1996) and differences in the hodology of projections to and from these regions (Zaborszky et al., 1985, Heimer et al., 1991b, Wright and Groenewegen, 1995, Wright and Groenewegen, 1996, Groenewegen et al., 1999b, Brog et al., 1993). The AcbC and AcbSh can also be dissociated on the basis of metabolic responses (Pontieri et al., 1994, Orzi et al., 1996, Pontieri et al., 1998), patterns of neurotransmitter release (Deutch and Cameron, 1992, Pontieri et al., 1995, Jones et al., 1996, Ladurelle et al., 1994, Sorg et al., 1995) and gene expression (Beck and Fibiger, 1995, Dilts et al., 1993) (Chergui et al., 1996) following a range of stimuli.

The AcbC consists of the tissue that surrounds the anterior commissure that is, in turn, enveloped in an L-shaped band of tissue, the AcbSh, along its ventral and medial aspects and laterally in caudal regions. There are transitional zones of organisation between the alleged subregions and no clearly defined boundaries exist (Zahm, 1999). A third region where AcbC and AcbSh cannot be clearly distinguished, termed the 'rostral pole', has also been proposed (Zahm and Brog, 1992, Zahm and Heimer, 1993) but has only been identified in rats (Meredith et al., 1996). In contrast homologous divisions between AcbC and AcbSh subregions have been clearly identified in non-

human primates and in humans (Meredith et al., 1996, Voorn et al., 1994, Voorn et al., 1996, Haber and McFarland, 1999, Prensa et al., 2003). In addition to the gross distinguishable subregions, complex patterns of compartmentalisation exist within each region (Meredith et al., 1989).

Structural characteristics of the AcbSh

Evidence has accumulated that the AcbSh is the most heterogeneous area of the striatum in terms of its neurochemistry (Voorn 2004), synaptic wiring and morphology of its neurons (Meredith et al., 1992) and complexity of compartmental organisation (Hiroi, 1995, Herkenham et al., 1984). Some authors suggest that this region can be further split into at least three subterritories or domains: the caudal dorsomedial shell (sometimes referred to as the "septal pole" or "cone" region), the rostral shell and the lateral shell (Zahm and Brog, 1992). It has also been suggested that the AcbSh is not a unitary region of the Acb but rather a collection of segregated neuronal "ensembles" (Pennartz et al., 1994, O'Donnell et al., 1999, O'Donnell, 2003). Some authors believe that the caudal medial portion of the shell is a transitional division of the extended amygdala while the core is an integral part of the basal ganglia (Meredith, 1999, Alheid and Heimer, 1988, Heimer et al., 1991a, Meredith and Totterdell, 1999).

Although the way in which the Acb is placed within the networks of neurons and circuits that can control behavioural output is critical to understanding its role it is also important to take into account the internal workings of this structure. It is has been recognised for over a decade that "segregated territories" within the Acb receive multiple converging inputs and could differentially activate output neurons depending on which subset of neuronal substrates and transmitters are in play (Meredith, 1999). This is particularly important given the focus on GABA receptors because their function in the Acb may depend on their local interaction with other neurotransmitter systems.

Medium spiny projection neurons

The large majority of Acb neurons, up to 98% in the rodent brain, (Rymar et al., 2004) are GABAergic and >90% of these are medium spiny neurons (MSNs) that constitute the projections to extrinsic targets (Meredith et al., 1993, Meredith, 1999). The MSNs in the AcbSh are morphologically distinct from those in the dorsal striatum being smaller and, in the medial portion of the AcbSh, significantly less spiny (Meredith et al., 1992).

These projection neurons (MSNs) are GABAergic and immunoreactive for the calcium binding protein calbindin D28K (CaBP) and predominantly fall into two classes (Meredith et al., 1993, Zahm and Heimer, 1993, Hussain and Totterdell, 1994, Zahm and Brog, 1992) (See Table 1.1).

The first group are immunoreactive for enkephalin (Groenewegen and Russchen, 1984, Graybiel, 1984, Graybiel and Chesselet, 1984) and the second a combination of dynorphin and substance P (Christensson-Nylander et al., 1986, Fallon and Leslie, 1986, Gerfen and Young, 1988, Van Bockstaele et al., 1994, Zahm and Heimer, 1988, Pickel et al., 1988). Cocaine- and amphetamine-regulated transcript (CART) peptide immunoreactivity is also localised to GABAergic, substance P/dynorphin containing MSNs and these subtypes are denser in the rostral shell than the caudal region (Smith et al., 1997, Smith et al., 1999, Dallvechia-Adams et al., 2002, Hubert and Kuhar, 2006).

Both types of projection neurons are immunoreactive for neurokinin and neurotensin (Zahm and Heimer, 1988, Martin et al., 1991, Ikemoto et al., 1995, Delle Donne et al., 1996). MSNs in the Acb are organized in clusters (Berendse et al., 1992b, Heimer et al., 1997). Axon collaterals from MSNs repeatedly synapse onto neighbouring MSNs within the AcbSh and these connections are mediated by $GABA_A$ receptors (Chang and Kitai, 1985, O'Donnell and Grace, 1993, Taverna et al., 2004). It has been hypothesised that “lateral inhibition” between MSNs may provide a fast mechanism for selecting neuronal ensembles competitively involved in processing the heterogeneous flows of information” from brain regions that converge in the Acb (Taverna et al., 2004).

GABAergic interneurons

2-3% of all GABAergic neurons are interneurons (Rymar et al., 2004). GABAergic interneurons can be further divided into at least 3 subtypes; 1) parvalbumin positive 2) calretinin positive and 3) somatostatin (SS) / neuropeptide Y (NPY) / nitrous oxide (NO) positive populations (Hussain et al., 1996, Meredith, 1999) (Hidaka and Totterdell, 2001, French et al., 2005). Calretinin interneurons are densest in the medial AcbSh (Hiroi, 1995, Sadikot et al., 1996). Regionally parvalbumin interneurons are less dense in the medial and particularly caudal medial shell (Sadikot et al., 1996, Bennett and Bolam, 1994). Some axonal processes from interneurons synapse onto other interneurons and MSNs but SS/NPY/NOS positive interneurons rarely synapse onto MSNs or other interneurons but they do innervate each other (Meredith, 1999).

Cholinergic interneurons

The cholinergic interneurons account for 1-2% of all neurons in the Acb (Phelps and Vaughn, 1986). Cholinergic interneurons are immunoreactive for a combination of choline acetyltransferase (ChaT), acetylcholine (Ach) and acetylcholinesterase (ACHE) (Graybiel and Ragsdale, 1978, Herkenham and Pert, 1981, Herkenham et al., 1984, Gerfen, 1988, Phelps and Vaughn, 1986). Cholinergic interneurons are densest in the caudal medial shell, three times less so in core and five times less in the rostral pole region (Meredith et al., 1989). Cholinergic interneurons synapse onto GABAergic interneurons and extend axon collaterals to innervate other cholinergic interneurons and they rarely receive inputs from MSN axon collaterals (Meredith and Chang, 1994).

Table 1.1. Principal neurochemical and receptor phenotypes of projection and local circuit neurons in the nucleus accumbens (after Meredith, 1999). Abbreviations: ChaT, choline acetyltransferase; $D_1/D_2/D_3$, dopamine receptor subtypes 1, 2 and 3; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; NADPH, nicotinamide adenine dinucleotide phosphate; NMDA, N-methyl-D-aspartate; NOS, nitrous oxide synthase, 5-HT_{2a}, serotonin 2a receptor.

Projection neurons		Local circuit neurons			
Medium spiny neurons		ChAT	Parvalbumin	Calretinin	NOS
GABA Enkephalin Preproenkephalin D_1 receptor D_2 receptor D_3 receptor 5-HT_{2a}	GABA Dynorphin Substance P Preprotachykinin D_1 receptor D_2 receptor D_3 receptor 5-HT_{2a}	Acteylcholine Acetylcholinesterase D_1 receptor D_2 receptor	GABA GAD	GABA GAD	NADPH diaphorase GABA GAD Somatostatin Neuropeptide Y Acetylcholinesterase NMDA subunit R1 Calbindin D28k
Not specific to MSN type					
Calbindin D28k NeurokininB Neurotensin Adenosine 2A receptor NMDA receptor subunit R1 μ-opioid receptor δ-opioid receptor					

GABA receptors in the AcbSh

$GABA_A$ receptors containing the subunits α2, α4, β3 and δ are located postsynaptically on the dendrites of the MSNs while receptors containing the α1, β2 and γ2 subunits are mostly restricted to the interneurons (Schwarzer et al., 2001). $GABA_B$ receptors are found presynaptically either as auto- or heterorecptors, they are sparse postsynaptically but most common extrasynaptically (Galvan et al., 2006, Bettler and Tiao, 2006).

Broadly speaking the postsynaptic location of $GABA_A$ receptors is consistent with the current view that they mediate fast phasic inhibition and because the $GABA_B$ receptors are mostly extrasynaptic they are thought to mediate slow long term inhibition, while presynaptic $GABA_B$ receptors inhibit neurotransmitter release (Otis and Mody, 1992, Bettler and Tiao, 2006, Bowery and Enna, 2000, Mody et al., 1994, Mody, 2001). It has been suggested that connections between MSN axon collaterals are mediated by $GABA_A$ receptors (Taverna et al., 2004). The location of $GABA_A$ and $GABA_B$ receptors is depicted in Fig. 1.2.

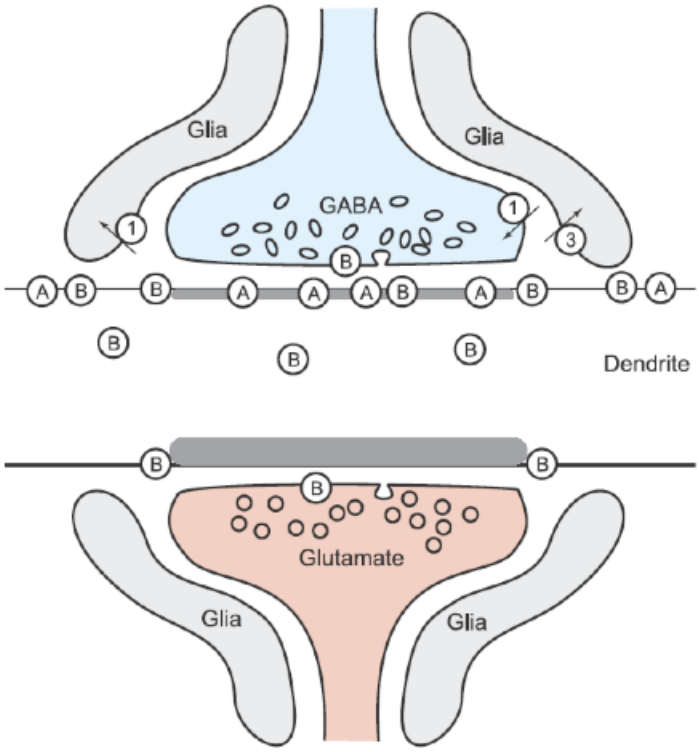


Figure 1.2. Summary figure of the localisation of GABA receptors (and transporters) in the striatum. The schematic shows exemplary glutamate and GABA terminals that form axo-dendritic synapses. Ⓐ$GABA_A$ receptors, Ⓑ$GABA_B$ receptors, ①GABA transporter 1, ③GABA transporter 3. (copied from (Galvan et al., 2006)

Characteristics of $GABA_A$ and $GABA_B$ receptors

Separate A and B GABA receptors were originally classified on the basis of sensitivity to bicuculline and baclofen (Hill and Bowery, 1981). The ionotropic $GABA_A$ receptors are ligand gated chloride channels formed by five subunits (Nayeem et al., 1994, Macdonald and Olsen, 1994). The subunits α1-6, β1-4, γ1-3, ρ1-3, δ, ε, θ, π have so far been identified (Galvan et al., 2006) and the most common stoichiometry is two α, two β and one γ subunit (Sieghart, 1995, Barnard et al., 1998, Sieghart and Sperk, 2002)

(See fig 1.3). The different combinations of subunits confer different pharmacology and physiology and there is regional heterogeneity in their distribution throughout the brain (Sieghart, 1992, Pirker et al., 2000, Martin and Olsen, 2000, Johnston, 1996). $GABA_A$ receptors mediate the fast hyperpolarising actions of GABA (Martin and Olsen, 2000). $GABA_A$ receptors in the Acb are found postsynaptically on MSNs and on GABAergic interneurons at both GABAergic and non-GABAergic synapses (Fujiyama et al., 2000, Galvan et al., 2006).

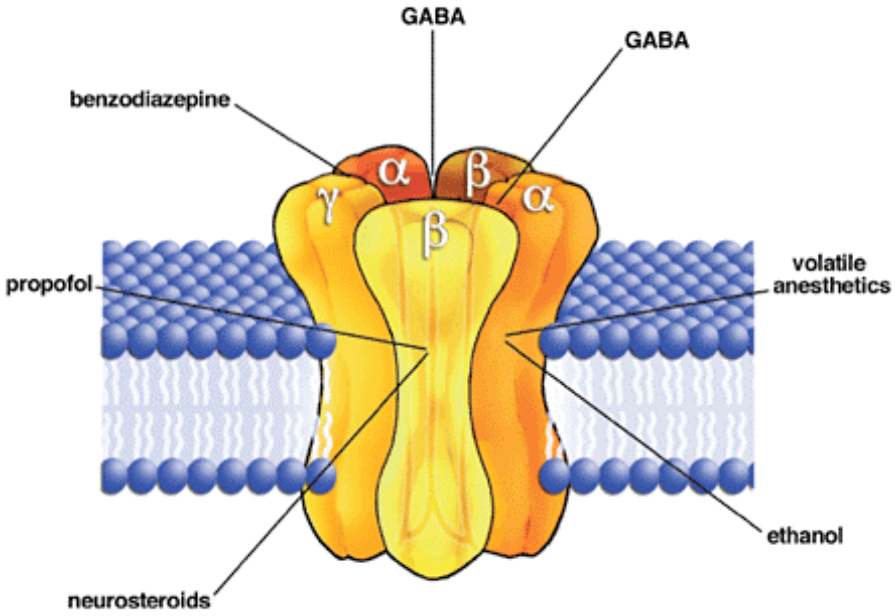


Figure 1.3. Schematic drawing of the $GABA_A$ ligand-gated ion channel complex. The receptor molecule is formed by five subunit proteins. In this case, two subunits are of the α type, two β, and one γ. Globular regions of the protein stick out from the membrane on the extracellular side, and the interfaces between these are targets for GABA, benzodiazepines and related drugs. Protein domains that span the cell membrane are depicted as cylinders. These regions are thought to be targets for anaesthetics, neurosteroids, and alcohol. In the middle of the subunits is the ion channel pore. (Figure and legend copied from (Lovinger, 2008).

$GABA_B$ receptors are G-protein linked metabatropic receptors consisting of two subunits, $GABA_BR1$ and $GABA_BR2$ (Bowery, 1993, Bowery and Enna, 2000). The subunits each have seven transmembrane domains and must be co-expressed as heteromeric complexes for the formation of a functional receptor (Kaupmann et al., 1998, Marshall et al., 1999). The heteromeric receptors are associated with both potassium and calcium channels (Jones et al., 1998). The $GABA_B$ receptor has a unique "Venus Fly Trap" mode of activation whereby activation requires conformational changes (Calver et al., 2002, Galvez et al., 1999) (See Fig. 1.4). These receptors are

found in abundance pre- and extra-synaptically but are less prevalent postsynaptically (Galvan et al., 2006). Presynaptically they function as autoreceptors on GABAergic neurons and as heteroreceptors on glutamatergic afferents (Galvan et al., 2006).

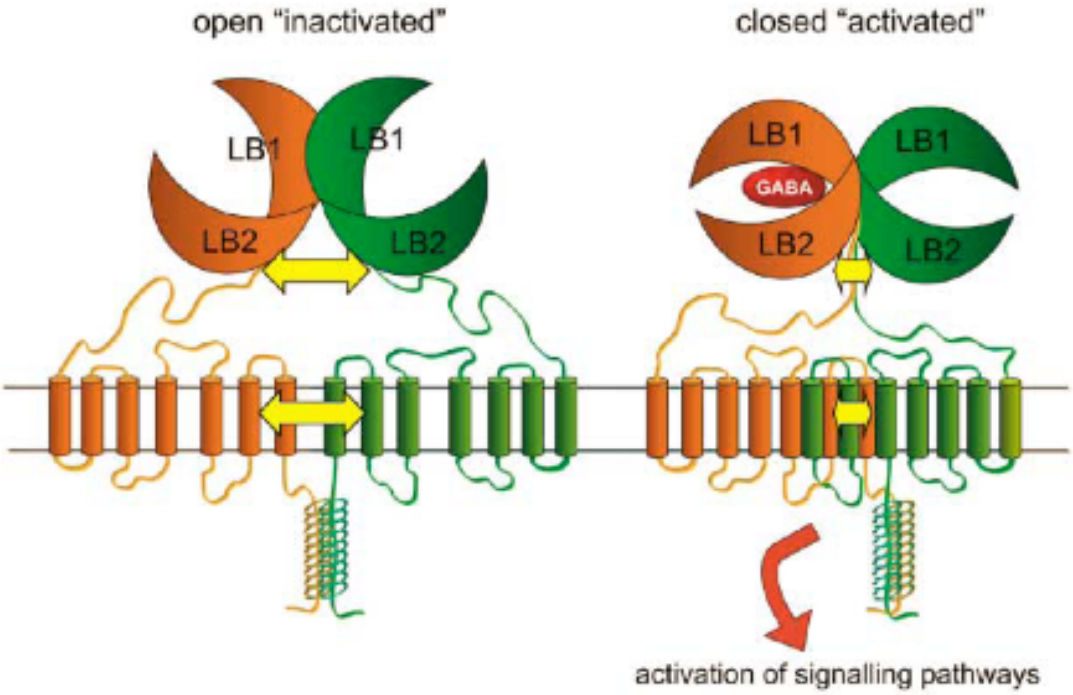


Figure 1.4. Schematic drawing of the heteromeric $GABA_B$ G-protein coupled receptor. Lobes 1 (LB1) and 2 (LB2) make up a single protomer in $GABA_B1$ (orange) and $GABA_B2$ subunits (green). In the inactivated 'open' state, the ligand-binding pocket situated in the $GABA_B1$ extracellular binding domain is open and the extracellular and transmembrane domains of subunits are apart. Agonist binding to $GABA_B1$ induces 'closing' of the ligand-binding pocket and an 'activated' receptor state. This conformational change results in the extracellular and transmembrane domains of the subunits coming closer together (yellow arrows) and is key in the inception of downstream signalling cascades. (Figure copied, and legend modified from (Calver et al., 2002).

Other classes of receptors in the AcbSh

Glutamate AMPA receptors are predominantly located postsynaptically on MSNs whereas a higher proportion of NMDA and kainate receptors are located on presynaptic axon terminals of hippocampal and cortical afferents as well as on postsynaptic sites (Tarazi, 1998). Some NMDA receptors are presynaptic on dopaminergic terminals which could provide a site for modulation of DA release by glutamatergic afferents (Gracy and Pickel, 1996). D2 receptors are present in enkephalinergic neurons (Le Moine and Bloch, 1995), half of the cholinergic interneurons (MacLennan et al., 1994) but not in the somatostatin interneurons (Jongen-Relo et al., 1995). D1 receptors are more abundant in dynorphinergic neurons and cholinergic interneurons (Jongen-Relo et

al., 1995, Le Moine et al., 1991). μ-opioid receptors are the most abundant opioid receptor subtype in the Acb and are found postsynaptically in the Acb (Churchill et al., 1991). A small number of μ-opioid receptors are co-localised with the NMDA receptor R1 subunit (Gracy et al., 1997). δ-opioid and κ-opioid receptors are predominantly found presynaptically (Svingos, 2001, Svingos et al., 1999b, Svingos et al., 1999a). A number of other receptor types in the AcbSh have been identified including cannabinoid receptors (Herkenham et al., 1991a, Herkenham et al., 1991b, Herkenham, 1991, Herkenham, 1992, Hohmann and Herkenham, 2000, Pickel et al., 2004, Pickel et al., 2006, Matyas et al., 2007), NPY receptors (Goldberg et al., 1975) (Meredith, 1999, Migita et al., 2001), adrenergic receptors (Rommelfanger et al., 2009) and CCK receptors (Kombian et al., 2004).

Section summary

The majority of neurons in the AcbSh are GABAergic and can be split into 2 classes of MSNs and three classes of interneurons. In addition there are a small number of cholinergic interneurons. Receptors for GABA, glutamate, dopamine, opioids and variety of other neurotransmitters are distributed both pre- and postsynaptically on MSNs and interneurons. The functional implications of the location and co-localisation of the various receptor subtypes both pre and postsynaptically in the AcbSh is discussed in detail in a number of reviews (e.g. Meredith, 1999, Meredith and Totterdell, 1999).

Inputs to and outputs from the AcbSh

As Stratford (2007) points out the AcbSh receives a particularly wide variety of inputs from afferent connections originating in cortical and subcortical areas of the brain but none of these afferents are exclusively related to the AcbSh (Groenewegen et al., 1999b). Nevertheless some regions that project to the AcbSh may in reality send only a very small number of afferents to the AcbC if any (Brog et al., 1993, Zahm, 1999). In some cases the information that reaches the Acb does so via indirect pathways including other brain regions that may or may not themselves project directly to this region. In the following section I will review what is known about both direct and indirect pathways by which the AcbSh is innervated. Characterising the indirect pathways is important because it shows the sort of information that reaches the Acb and the level of processing

that occurs before it gets there. I will also consider the degree to which afferents to the AcbSh overlap and converge, particularly where they do so on the same neurons.

Afferents to AcbSh

Excitatory amino acid inputs – direct pathways

The majority of inputs to the AcbSh are coded by excitatory amino acids (EAAs) (McGeer et al., 1977, Lopes da Silva et al., 1984, Fuller et al., 1987, Robinson and Beart, 1988, Walaas and Fonnum, 1979, Fonnum et al., 1981, Pennartz and Kitai, 1991, Albin et al., 1992, Finch et al., 1995). Glutamatergic inputs synapse primarily onto the spines of MSNs (Tallaksen-Greene et al., 1992, Sesack and Pickel, 1990, Meredith et al., 1990). In other words they bypass local circuit neurons (interneurons). However these interneurons are found postsynaptic to the terminal boutons of neurons that may also be extrinsic glutamatergic inputs (French and Totterdell, 2004). Although there is some evidence that glutamatergic afferents synapse onto interneurons in the dorsal striatum (Vuillet et al., 1989, Dimova et al., 1993, Lapper and Bolam, 1992, Lapper et al., 1992) very little is known about this relationship in the Acb. In the caudomedial shell afferent and intrinsic synapses are mixed from distal to proximal locations (Meredith and Totterdell, 1999) and proximal inputs can selectively gate distal signals (Mogenson et al., 1980, Pennartz et al., 1994).

The AcbSh receives glutamatergic afferents from the prefrontal cortex (PFC) (Berendse et al., 1992a, Sesack et al., 1989), entorhinal cortex (Finch et al., 1995, Krayniak et al., 1981), CA1 region and subiculum of the hippocampus (Groenewegen et al., 1987, Kelley and Domesick, 1982, Brog et al., 1993), basal amygdaloid complex (Wright et al., 1996), midline, intralaminar and paratenial nucleus of the thalamus (Brog et al., 1993, Berendse et al., 1988, Berendse and Groenewegen, 1990, Robinson and Beart, 1988, Vertes and Hoover, 2008).

Projections from regions within the PFC are topographically arranged with respect to specific compartments within the AcbSh and this arrangement appears to be determined by layer of the PFC from which the projections originate (Berendse et al., 1992a, Wright and Groenewegen, 1995). The medial entorhinal cortex projects to the AcbSh and the lateral region to the AcbC although there is some cross over (Totterdell and

Meredith, 1997). The projections of the hippocampus target predominantly the medial ventral and rostral regions of the AcbSh (Brog et al., 1993, Groenewegen et al., 1987). Different nuclei of the basal amygdaloid complex project to specific regions of the AcbSh (Wright et al., 1996). For example the caudal basolateral amygdala (BLA) projects to caudal regions of the AcbSh but there do not appear to be direct projections from the central nucleus of the amygdala (CeA) (Brog et al., 1993, Phillipson and Griffiths, 1985, Wright et al., 1996). Projections that arise from either midline or intralaminar thalamic nuclei terminate in distinct regions of the AcbSh (details in Groenewegen 1999)(Brog et al., 1993, Berendse and Groenewegen, 1990)

Excitatory amino acid inputs – indirect pathways

There are many interconnections between cortical, thalamic, and limbic inputs of the Acb which are often reciprocal (Groenewegen et al., 1999b). Taste and visceral information can reach the AcbSh via indirect pathways that terminate in glutamatergic inputs to this region (Kelley et al., 2005b). The agranular insular cortex, which receives gustatory and visceral sensory input from brainstem regions, has dense projections to the infralimbic and prelimbic portions of the PFC and hence influences the AcbSh (Berendse et al., 1992a, Hurley et al., 1991, Shi and Cassell, 1998, Vertes, 2004). The gustatory cortex also connects via the parabrachial nucleus (PB) of the pons to the ventroposterior thalamus which projects to the AcbSh (Ricardo and Koh, 1978, Saper, 1982) and via the BLA to the AcbSh (McDonald and Jackson, 1987). However the BLA also projects to the LH and hence influences LH connections back to the AcbSh (Petrovich et al., 2001). Homeostatic signalling between the LH and the arcuate nucleus (Arc) reaches the AcbSh via a link between the LH and midline thalamic nuclei (Wright and Groenewegen, 1996, Berendse et al., 1988, Berendse and Groenewegen, 1990).

The dorsal region of the paraventricular thalamic nucleus (PVT) from which the majority of projections to the AcbSh emanate is heavily innervated by neurons from the LH, dorsomedial hypothalamus (DMH), Arc and dorsal raphe nucleus (Guy et al., 1981, Parsons et al., 2006, Kirouac et al., 2005, Otake, 2005, Freedman and Cassell, 1994). This part of the PVT is also innervated by projections from infralimbic and prelimbic cortices, amygdala, suprachiasmatic nucleus, median raphe nucleus, PB, LC, PAG and nucleus of the solitary tract (NTS) (Krout and Loewy, 2000a, Krout and Loewy, 2000b, Krout et al., 2001, Krout et al., 2002, Risold et al., 1997, Chen and Su, 1990). Thus the

PVT acts as a relay to the AcbSh for unprocessed and highly processed information about energy balance, visceral signals, arousal and circadian status (Stratford, 2007).

Inhibitory amino acid inputs

While the majority of GABAergic inputs to Acb MSNs are from interneurons and local axon collaterals (Meredith, 1999, Taverna et al., 2004) the VP sends reciprocal GABAergic afferents to both the AcbC and AcbSh (Churchill and Kalivas, 1994). There are also GABAergic projections from the VTA to Acb (Van Bockstaele and Pickel, 1995) but these authors do not indicate where in the Acb these neurons terminate. The regions of the VP and VTA that innervate the Acb are themselves reciprocally connected (Haber et al., 1985). GABAergic inputs from axon collaterals of MSNs at proximal sites is responsible for lateral inhibition within the AcbSh (Meredith, 1999).

Dopaminergic and cholecystokinin containing inputs

VTA (A10) dopaminergic neurons project to the entire striatum but primarily to the medial and ventral AcbSh however cell clusters receive hardly any dopaminergic fibres whereas the 'cone-shaped' area is densely innervated (Voorn et al., 1986). Another cluster of A10 dopaminergic neurons from the raphe nucleus in the brainstem also reaches the AcbSh (Ikemoto, 2007) The A8 group of dopaminergic neurons from the retrorubral field (RRF) project to lateral regions of the AcbSh but, in contrast to the AcbC region, the AcbSh does not receive dopaminergic inputs from the SN (A9) (Brog et al., 1993, Beckstead et al., 1979, Jimenez-Castellanos and Graybiel, 1987, Gerfen et al., 1987, Zahm, 1992). CCK is co-expressed in some VTA dopaminergic neurons (Lanca et al., 1998) which terminate in the medial AcbSh (Zaborszky et al., 1983, Zaborszky et al., 1985) (Lanca et al., 1998). CCK immunoreactive neurons from the SNc may also terminate in the AcbSh (Zaborszky et al., 1983, Zaborszky et al., 1985) although these neurons primarily innervate the AcbC (Lanca et al., 1998). Indirect pathways that terminate in dopaminergic inputs to the AcbSh include a connection from the NTS via a pathway between the PB to the CeA then to the VTA which innervates the AcbSh (McDonald, 1991). The CeA sends GABAergic afferents to the VTA, SN, RRF and hence may influence dopamine efflux in the AcbSh via the projections from these regions (Wallace et al., 1992, Phillipson and Griffiths, 1985, Fudge and Haber, 2000, Kim et al., 2004, Ahn and Phillips, 2002, Will et al., 2004).

Peptidergic inputs

Direct projections from the LH to the AcbSh have been identified (Baldo et al., 2003, Peyron et al., 1998). More than 50% of CART immunoreactive neurons from the arcuate and perifornical lateral hypothalamus project directly to the AcbSh (Yang et al., 2005) as do melanin-concentrating hormone (MCH) containing fibres from the LH (Peyron et al., 1998). Orexin is exclusively synthesised by neurons in the LH and the AcbSh has orexin neurons suggesting that there are orexinergic projections from the LH to the Acb (Sakurai et al., 1998, Nambu et al., 1999, Trivedi et al., 1998, Marcus et al., 2001, Cluderay et al., 2002). There are a small number of projections from the VMH to the Acb (Canteras et al., 1994). The PAG sends enkephalin immunoreactive fibres to AcbSh (Li et al., 1990). The majority of opioid input to the AcbSh is from local axon collaterals of enkephalin and dynorphin immunoreactive MSNs (Herkenham et al., 1984).

Noradrenergic inputs

The AcbSh receives a very small number of noradrenergic inputs from the LC and the A1 region of the caudal ventrolateral medulla (CVLM) but robust innervation from the noradrenergic cell group (A2) in the NTS (Ricardo and Koh, 1978, Brog et al., 1993, Berridge et al., 1997, Delfs et al., 1998). There are however other projections from the LC that are not immunoreactive for noradrenaline that innervate the AcbSh (Brog et al., 1993, Delfs et al., 1998). The rostral NTS also influences the AcbSh via the PB nucleus (Brog et al., 1993). Cell groups in the NTS and CVLM process gustatory signals and input from chemo and baroreceptors in the nervous system (Delfs et al., 1998).

Serotonergic inputs

The AcbSh receives dense serotonergic inputs from the median raphe nucleus (Rodriguez et al., 1999, Van Bockstaele and Pickel, 1993, Moore et al., 1978, Azmitia and Segal, 1978, Hensler et al., 1994, Vertes, 1991, Brog et al., 1993, Stratford and Wirtshafter, 1990, Compan et al., 1996). The rostral raphe nucleus receives inputs from the LH (Date et al., 1999) and thus may represent an indirect pathway via which LH mediates arousal and behavioural state signals to the AcbSh (Stratford, 2007).

Convergence of inputs

Afferents from the ventromedial PFC and BLA overlap and often synapse onto the same neurons but those from the PVT terminate on a different group of cells (Johnson et al., 1994, Wright and Groenewegen, 1995). Afferents from the ventral subiculum of the hippocampus, PFC and BLA terminate on the same output neurons of the AcbSh (French and Totterdell, 2002, O'Donnell and Grace, 1995, Groenewegen et al., 1999a, Groenewegen et al., 1999b, French and Totterdell, 2003, Johnson et al., 1994) (Totterdell and Smith, 1989, Kelley et al., 1982, Wright and Groenewegen, 1995, Wright and Groenewegen, 1996, Fudge et al., 2002). Acb MSNs are usually physiologically "silent" in a "down state" characterised by a very hyperpolarised membrane potential and will only fire when in a relatively depolarised "up state" (Groenewegen et al., 1999b, O'Donnell and Grace, 1995). It has been suggested that excitatory inputs from the hippocampus gate the upstate (O'Donnell and Grace, 1995, Mulder et al., 1998, Groenewegen et al., 1999a) but activation of the PFC alone has been shown to sustain these up states also (Gruber and O'Donnell, 2009).

Simultaneous excitation from multiple sources may be necessary to depolarise projection neurons enough to influence AcbSh output (Pennartz et al., 1992) (O'Donnell and Grace, 1995, O'Donnell et al., 1999, Goto and Grace, 2008). Dopaminergic inputs also terminate in apposition to convergent glutamatergic inputs on MSNs (Meredith, 1999, Meredith and Totterdell, 1999). Dopaminergic inputs to AcbSh projection neurons have been shown to inhibit GABAergic neuronal activity at the D2 receptor and potentiate glutamatergic influence at the D1 receptor (West and Grace, 2002, West et al., 2003) thus modulating the influence and integration of afferent inputs to the region (Sesack and Grace, 2009).

Section summary and functional implications

Afferents from cortical and subcortical regions that have previously been implicated in food motivated behaviours terminate in the AcbSh. Information reaching the AcbSh via such connections from the neocortex, allocortex and thalamus will already have been highly processed (Stratford, 2007). There are also multiple inputs from areas of the brain that monitor homeostatic and energy balance, taste and visceral functions and levels of arousal (Stratford, 2007). It has recently been suggested that cortical inputs promote goal directed behaviours, the ventral subiculum adds spatial and contextual

information, the PFC contributes executive control signals with regards to task switching and response inhibition and the BLA provides information about conditioned associations and affective value (Sesack and Grace, 2009). Stratford (2007) postulates that the major innervation from the thalamus might be the most important excitatory input for the control of intake and consummatory responses (Stratford, 2007). The thalamostriatal afferents might also direct attention to "behaviourally significant events" (Smith et al., 2004).

Efferents from AcbSh to other regions of the brain

While the AcbC and AcbSh broadly receive the same afferents, albeit topographically arranged within the various compartments, there are striking differences in the efferent projections from AcbC and AcbSh subregions (Heimer et al., 1991b, Zahm and Heimer, 1990, Zahm and Brog, 1992, Zahm and Heimer, 1993). The Acb as a whole projects predominantly to midbrain mesotelencephalic dopamine neurons (Zahm and Brog, 1992). However the AcbC connects to classic basal ganglia output targets while the AcbSh projects predominantly to limbic structures and midbrain dopamine neurons (Maldonadoirizarry et al., 1995). The projection neurons of the Acb are GABAergic but can be separated into two major classes depending on co-expression of substance-P, enkephalin and D1 or D2 receptors (See Table 1.1). For the majority of locations that receive AcbSh efferents no data is available yet as to which subtype of GABAergic MSNs are involved.

Efferent projections from the AcbSh terminate primarily in the LH and preoptic area, the VTA, the VP and the extended amygdala with less dense projections to the bed nucleus of the stria terminalis (BNST), substantia innominata, substantia nigra pars compacta (SNc) and reticulata (SNr), RRF, the LC, the mesencephalic and mesopontine tegmentum, the raphe nucleus and the PAG (Nauta et al., 1978, Mogenson et al., 1983, Groenewegen and Russchen, 1984, Rye et al., 1987, Zahm et al., 1987, Zahm and Heimer, 1990, Haber et al., 1990, Heimer et al., 1991b, Berendse et al., 1992b, Usuda et al., 1998, Zahm, 2000). The density of projections appear to diminish with increasing distance from their origin to the most distal structures (Zahm, 2000).

There appears to be a large degree of mediolateral and rostrocaudal topographical organisation of AcbSh outputs (Ikemoto, 2007, Usuda et al., 1998, Mogenson et al., 1983). Projections from ventromedial portions of the AcbSh innervate the posteromedial VTA and lateral VTA, and from the ventrolateral AcbSh innervate the lateral VTA and SNc (Berendse et al., 1992b, Heimer et al., 1991b, Usuda et al., 1998, Zhou et al., 2003). The medial AcbSh projects to the ventromedial VP (Zahm and Heimer, 1990, Heimer et al., 1991b, Usuda et al., 1998) while the lateral AcbSh innervates the ventrolateral VP (Usuda et al., 1998). VP neurons that receive inputs from the AcbSh project to the LH suggesting an indirect pathway through which activity in the AcbSh can influence the LH (Groenewegen et al., 1993). The large majority of neurons that project to the VTA from the AcbSh are immunoreactive for D1 receptors and substance P whereas projections to the VP primarily express D2 receptors and enkephalin but also D1 and substance P (Lu et al., 1997, Lu et al., 1998, Robertson and Jian, 1995).

Some of the brain regions that contain terminal fields of AcbSh efferents only receive inputs from restricted origins in this structure. Only the rostral ventromedial AcbSh projects to the paramedian raphe nucleus, the caudal dorsomedial AcbSh uniquely projects to the BNST, the caudal ventromedial AcbSh uniquely projects to the LC and the caudal ventrolateral shell uniquely projects to the ventrolateral part of the VP (Usuda et al., 1998).

Section summary and functional implications

The AcbSh primarily innervates the LH and lateral preoptic area the VTA and the VP. It has access to thalamo-prefrontal and hypothalamic circuits via the VP. The AcbSh efferents do not reach brainstem or spinal chord motor pattern generators but do predominantly terminate in the basal forebrain, diencephalon and rostral brainstem in regions that can more directly contribute to the initiation and motor control of adaptive behaviours (Zahm, 2000). Although it is difficult to define clear direct and indirect pathways from the AcbSh that could be involved in behavioural activation and response inhibition because of the unusual variety of connections compared to classic basal ganglia regions (Sesack and Grace, 2009) some theories have been recently proposed.

First of all these pathways might be relayed through the VP with the direct pathway involving pallidothalamic connections and the indirect pathway reaching the subthalamic nucleus (Nicola et al., 2000, O'Donnell et al., 1997). Secondly the LH and regions in the basal forebrain may directly control some motor functions and direct innervation from the AcbSh would constitute the inhibitory pathway while indirect connections via the VP cause disinhibition (Nicola et al., 2000). Finally the VTA could be the relay with the direct pathway connecting via this region to the thalamus and the indirect pathway involving an extra set of synapses with the VP and then the VTA which would require GABAergic projections to the thalamus (Sesack and Grace, 2009). The pattern of involvement of D1 and D2 receptors in AcbSh efferent projections to the VP and VTA add support to such models (Sesack and Grace, 2009).

Connections between AcbSh and AcbC subregions

The AcbSh is robustly connected to the AcbC via a multisynaptic indirect feed-forward, striatopallido-thalamocortico-striatal pathway that terminates in the AcbC (Zahm, 1999, Zahm, 2000). While the AcbSh sends efferents to the VP this region in turn projects to the mediodorsal nucleus of the thalamus which innervates both the dorsal prelimbic and agranular insular cortex (Groenewegen, 1988, Zahm et al., 1996, O'Donnell et al., 1997, Groenewegen et al., 1993). These cortical regions send dense projections to the AcbC and the caudate putamen (Wright and Groenewegen, 1996, Sesack et al., 1989, Berendse et al., 1992a). The AcbSh is also strongly connected to the AcbC via its connection to the VTA (and to some extent the SNc), which in turn send massive projections to the AcbC (Haber et al., 2000, Otake and Nakamura, 2000). It has recently been suggested that the two subregions are reciprocally connected by a complex series of ascending spirals (Haber et al., 2000).

Heimer et al., (1991) suggested that fibres within the Acb cross AcbC/AcbSh boundaries. It has recently been demonstrated that the AcbC and AcbSh are directly and reciprocally connected by axons from both MSNs and interneurons with specific topographic distribution shown in Fig. 1.5 (van Dongen et al., 2005). These authors suggest that “GABA may be a major neurotransmitter in inter-Acb communication to control behavioural selection”.

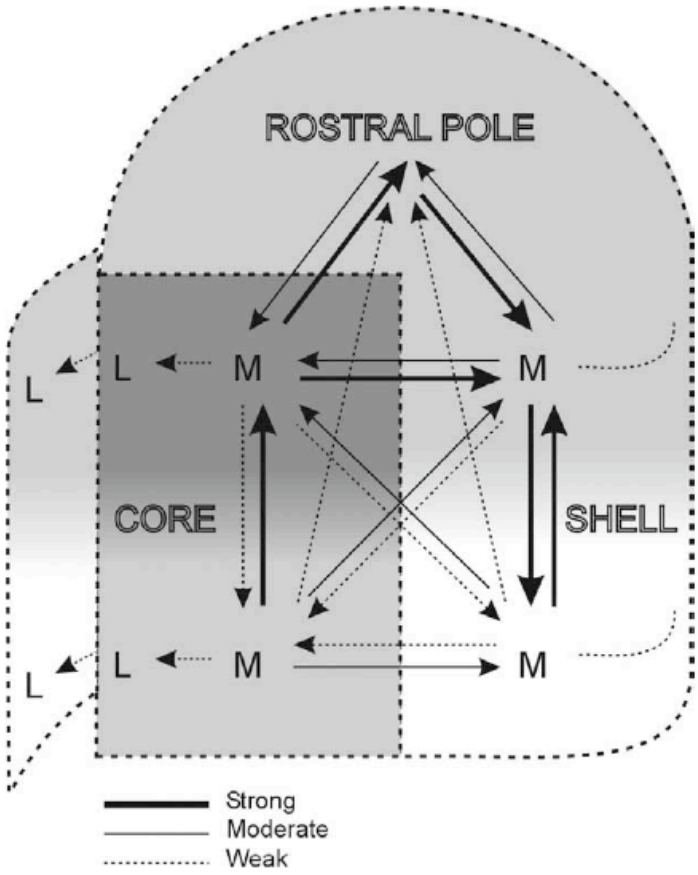


Figure 1.5. Schematic representation of the intra-accumbens projection patterns. Thick lines indicate strong projections and thinner lines moderate (straight) to weak (dashed) projections. There appears to exist a predominant caudal to rostral stream originating in the caudal core, reaching the rostral core and, subsequently, the rostral pole and rostral shell. Rostral and caudal shells are reciprocally connected. Several other projections are indicated between the rostral pole and different subareas in shell and core as well as between these subareas. These projections are in general weaker. Abbreviations: M, medial; L, lateral. (Copied from (van Dongen et al., 2005).

Anatomical subregions of the Acb are functionally dissociable

The evidence discussed so far points to specific functional specialisation of the AcbC and AcbSh and associated circuits. Since the AcbC and AcbSh subregions were identified the behavioural responses elicited by comparable manipulations have been extensively studied and, despite 1) the multiplicity of the levels of internal organisation, 2) the poorly delineated transitional areas between the two and 3) the presence of pathways by which they can directly influence each other there is ample evidence that these regions play distinct and totally dissociable roles in some aspects of the integrated output of the Acb.

It has been suggested that the Acb is essential for "adaptive" responding whereby an organism integrates external information with internal cues to produce behaviours consistent with survival in a changeable environment (Zahm, 2000). In general it is thought that the AcbC is predominantly involved in the learning and execution of voluntary motor functions whereas the AcbSh plays a role in affective and motivational aspects of the behavioural output (Kelley, 2004). It has been suggested that the AcbC is critical for mediating the impact of external cues that an animal has learnt are relevant to both stimulate and guide actions (Cardinal et al., 2002a). In contrast the AcbSh is critical to the modulation of the expression of unconditioned behaviours including feeding (Kelley, 1999a, Cardinal et al., 2002a).

The AcbSh, however, also appears to invigorate some types of learned behaviours that are coordinated through the AcbC (Voorn et al., 2004). The AcbC and AcbSh are part of interacting and intimately linked neuronal networks (Corbit et al., 2001) (Parkinson et al., 1999, van Dongen et al., 2005) and are directly linked to each other (van Dongen et al., 2005). It is possible that any true functional differentiation between the regions is only apparent in the context of their divergent connections to other structures with which they form anatomical macrocircuits that control specific processes. Ultimately Voorn et al. (2004) suggest that what really distinguishes the AcbSh from the AcbC is its unique role in the modulation of innate behaviours such as food intake.

A role has been demonstrated specifically for the AcbSh in a variety of "innate" unconditioned behaviours such as responses to availability of sexual reward (Moncho-Bogani et al., 2005, Jenkins and Becker, 2001), defensive responses (Reynolds and Berridge, 2001, Reynolds and Berridge, 2002, Reynolds and Berridge, 2003, Beck and Fibiger, 1995, Reynolds and Berridge, 2008, Saul'skaya and Marsden, 1998, Inoue et al., 1994), maternal responses to pups (Li and Fleming, 2003, Parada et al., 2008, Olazabal and Young, 2006) prey killing (Albert et al., 1982, Albert et al., 1984, Albert et al., 1985), pair bonding (Aragona et al., 2006, Aragona and Wang, 2007) and to drugs of abuse or alcohol (Pontieri et al., 1994, Pontieri et al., 1995, Sorg et al., 1995, Orzi et al., 1996, Pontieri et al., 1998, Di Chiara et al., 1999, Ito et al., 2000, Cadoni and Di Chiara, 2000, Filip and Siwanowicz, 2001, Di Ciano and Everitt, 2001, Bassareo et al., 2003, Ito et al., 2004, Cadoni et al., 2005, Lecca et al., 2006, Lecca et al., 2007) but far the most widely studied is its role in short-term intake of freely available food.

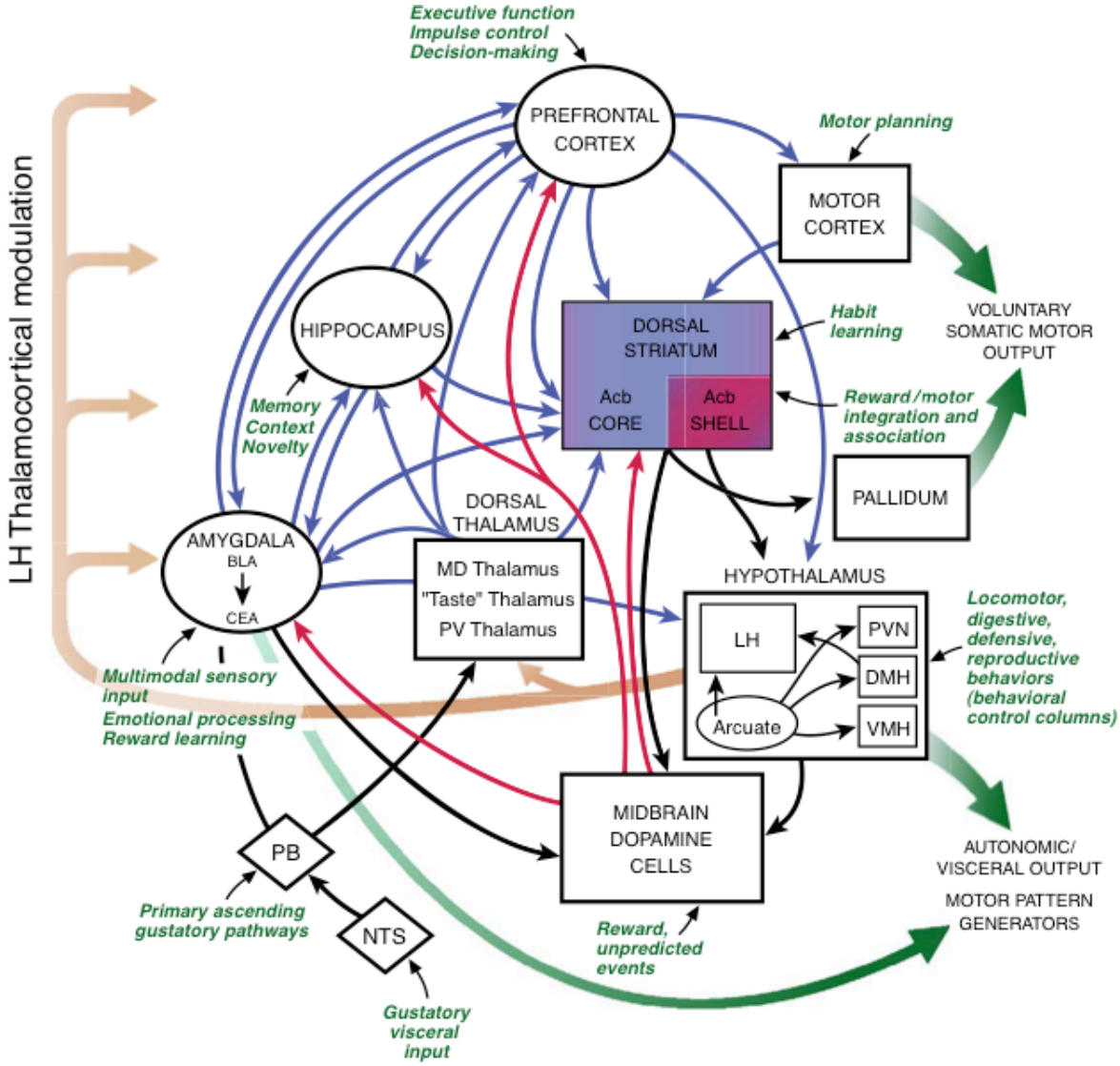


Figure 1.6. A schematic view of brain circuitry involved in food intake and motivation. Pathways coded by glutamate as the main neurotransmitter are shown in blue, while dopamine pathways are shown in red. Tan lines arising from the lateral hypothalamus (LH) indicate widespread direct and indirect projections from hypothalamus to neocortex and forebrain limbic structures. Arrows in black indicate projections that are coded by GABA (from striatal regions and the central nucleus of the amygdala), or that are unknown. Primary gustatory inputs arise from the nucleus of the solitary tract (NTS) and the parabrachial nucleus (PB), prior to converging on cortical and thalamic regions involved in affect, memory, and behavioural inhibition. Other abbreviations: Acb, nucleus accumbens; MD, mediodorsal; PV, paraventricular; BLA, basolateral amygdala; CEA, central nucleus of amygdala; PVN, paraventricular nucleus of hypothalamus; DMH, dorsomedial hypothalamus; VMH; ventromedial nucleus of hypothalamus. (Copied from Kelley et al., 2005b).

The AcbSh as part of a complex circuit subserving feeding

While the preceding sections have considered the individual afferents and efferents of the AcbSh and some indirect pathways between the various structures it still remains to consider how the Acb is placed within functional macrocircuits known to subserve motivated behaviour. While a vast number of schemes have been published I will concentrate here on a scheme produced by Kelley and colleagues on the basis of their extensive exploration of the functions of this structure. Fig. 1.6 depicts many of the direct and indirect connections to and from the AcbSh that have been discussed so far. This model will be used throughout the remainder of the thesis when exploring how the AcbSh might exert influence over other regions in the circuit that subserve motivation.

Evidence that the Acb as a whole is involved in feeding

Given the possibility that aspects of the LH syndrome could be partially reproduced by damage to the nigrostriatal bundle which depleted DA in the striatum it was suggested that the nigrostriatal bundle was also involved in feeding, (Marshall, 1974). Systemic administration of DA agonists reinstated feeding and drinking in animals with nigrostriatal DA bundle lesions that reduced DA in striatum (Ljungberg and Ungerstedt, 1976, Marshall, 1976). It was also recognised that, like the septum which was involved in feeding responses, the Acb contributed descending fibres to the MFB, received afferents from the VTA and hypothalamus through the MFB and was connected reciprocally to the septum (Lorens et al., 1970).

Lorens (1970) reported that electrolytic lesions of the whole Acb caused a transient increase in feeding, no effect on drinking, increased spontaneous activity but did not increase responding for food on a conditioned reinforcement or fixed ratio schedule (Lorens et al., 1970, Lorens, 1971). In another study Acb lesions caused hyperphagia, had no effect on drinking, but did not increase in body weight and the authors concluded that the increased intake was due to increased restlessness, agitation and hyperactivity (Smith and Holland, 1976).

Selective dopaminergic lesions caused mild hyperphagia for wet mash and dry food pellets in an experimental photocell cage but not in the home cage and these authors attributed increased intake to an inability to switch behaviours (Koob et al., 1978).

While it was consequently shown that feeding increased DA transmission in the Acb and hypothalamus (Heffner et al., 1980) dopaminergic lesions of the Acb did not appear to affect body weight (Winn and Robbins, 1985). Nevertheless other researchers showed that dopaminergic lesions of the Acb caused changes in feeding behaviour after locomotor effects had attenuated decreasing the latency to feed, increasing meal length, bout size and total intake (Evenden and Carli, 1985) The organisation of feeding behaviour was also disrupted by non selective electrolytic lesions of the whole Acb (Clifton and Somerville, 1994).

Alternative lines of evidence for the role of the Acb in motivated processes in general (and by extension its importance in food motivated behaviour) relate to its response to reward and reward related cues. For example animals will self stimulate in the Acb (Rolls et al., 1980), neurons in the Acb fire in response to reward and reward related cues and novel stimuli (Williams et al., 1993, Rolls, 1994). Animals will also self administer a variety of drugs into the Acb including stimulants (Hoebel et al., 1983) injections of which induce conditioned place preference (Carr and White, 1983), opiates and opioid receptor agonists (Olds, 1982, Goeders et al., 1984), NMDA antagonists (Carlezon and Wise, 1996) and cholinergic agonists (Ikemoto et al., 1998). Electrical stimulation of the mesolimbic DA pathway induced feeding which was blocked by application of a DA antagonist in the Acb (Mogenson and Wu, 1982, Apicella et al., 1991. Electrical stimulation in the MFB or VTA resulted in an increase in the release of DA in the Acb (Nakahara, 1989) and feeding also increased DA turnover in the Acb (Heffner et al., 1980). Instrumental responding for food reward and self stimulation in the LH also increase DA in the Acb (Hernandez and Hoebel, 1988).

Section summary

From the late 1970s through to the early 1990s evidence was emerging that the Acb as a whole structure is involved in reward related processes, motivation and specifically in feeding. These multiple lines of evidence included the behavioural effects of non-selective and selective lesions, the electrophysiological responses of the region, the release of neurotransmitters and the effect of pharmacological agents. However there was some conflicting evidence as to the degree to which the Acb was specifically involved in feeding.

Regulation of free feeding by amino acids in the AcbSh

Although a role for the Acb (as a unitary structure) in feeding was established by the 1980s it was not until the mid 1990s that the potential for functional specialisation of AcbC and AcbSh subregions in motivated behaviour was investigated. Because a large proportion of afferents to the Acb are glutamatergic the behavioural role for excitatory amino acid (EAA) receptors in both regions was the focus of these early experiments (Kelley and Maldonado-Irizarry, 1995).

Excitatory amino acids in AcbSh and feeding

Intra-AcbC but not AcbSh infusions of an NMDA antagonist have been shown to reduce open field activity, exploration of novel objects, psychostimulant induced locomotor activity, spatial learning and spatial behaviours directed at procuring food (Maldonado-Irizarry and Kelley, 1994, Kelley and Maldonado-Irizarry, 1995, Maldonado-Irizarry and Kelley, 1995). NMDA antagonists however have no apparent effects on *ad libitum* feeding in either region (Maldonado-Irizarry and Kelley, 1994, Kelley and Maldonado-Irizarry, 1995, Maldonado-Irizarry and Kelley, 1995).

The AMPA receptor was also shown to be involved in spatial learning and the expression of spatial behaviour but the pattern of the effects were distinguishable from those of the NMDA receptor (Maldonado-Irizarry and Kelley, 1995). Importantly the authors reported the incidental observation, made during these studies, that blockade of EAA input to AMPA/kainate receptors in the AcbSh but not the AcbC stimulated feeding when the animals were returned to the home cage (Maldonadoirizarry and Kelley, 1995). They also reported that NMDA lesions of the AcbC resulted in a decrease in body weight post operatively whereas lesions of the AcbSh caused an increase, suggesting a role for glutamatergic inputs to this region in ingestive behaviour (Maldonadoirizarry and Kelley, 1995).

The same group went on to confirm a specific role for AcbSh but not AcbC AMPA/kainate receptors in feeding; a variety of antagonists caused rapid onset, robust and dose dependent increases in intake of freely available food that was reduced by systemic administration of D1 and D2 antagonists but not naloxone suggesting a modulatory role for dopamine in the response (Maldonadoirizarry et al., 1995).

(Stratford and Kelley, 1997b, Kelley and Swanson, 1997, Stratford et al., 1998, Kelley, 1999a, Basso and Kelley, 1999). Conversely low doses of AMPA infused into the AcbSh reduced deprivation induced feeding and intake induced by a sucrose solution (Stratford et al., 1998). The effects of manipulation of AMPA/kainate receptor function were specific to food intake not water intake and receptor blockade did not induce non-specific consummatory responses such as gnawing of non-food objects (Stratford et al., 1998).

It has since been reported that stimulation of AMPA receptors by infusion of high doses of AMPA into the AcbSh can increase feeding in a dose dependent manner although the effect is much delayed compared to the rapid onset of feeding responses seen with AMPA/kainate antagonists (Echo et al., 2001). Infusion of high doses of NMDA into the AcbSh produced a delayed onset increase in feeding that was shorter in duration than the AMPA effect, less robust and with an inverted-U-shaped dose response function (Echo et al., 2001). These authors suggest that a specific pattern of AcbSh activity is required to maintain "normal feeding" and that perturbation in this pattern by both significant increases or decreases in glutamatergic transmission can elicit feeding (Echo et al., 2001). Stratford (2007) offers the alternative hypothesis that the latency of the effect suggests that a high dose causes transmitter depletion or "neuronal fatigue" that results in delayed onset but temporary suppression of activity in AcbSh output neurons.

Given the importance of the LH in feeding and the unique projections from the AcbSh to this region it was postulated that there could be a functional link between the two regions (Maldonadoirizarry et al., 1995). Concurrent blockade of activity in the LH inhibited the increase in intake induced by intra-AcbSh infusions of AMPA/kainate antagonists suggesting that the behavioural effects of blocking glutamate transmission in the AcbSh were dependent, at least in part, on this region (Maldonadoirizarry et al., 1995). It was hypothesised that this effect was due to the disruption of tonic excitatory inputs to the AcbSh resulting in a decrease in the firing rate of a local population of neurons that were postulated to exert an inhibitory effect over neurons in the LH when active although, at the time, the neurotransmitter content of this pathway had not been identified (Maldonadoirizarry et al., 1995).

Inhibitory amino acids in AcbSh and feeding

The establishment of a role for EAAs in feeding inevitably led to the hypothesis that stimulation of inhibitory amino acid (IAA) receptors would also have effects on feeding due to acute inhibition of neural activity similar to that caused by blocking glutamate transmission (Stratford and Kelley, 1997b). As a result a number of studies were published that used either the $GABA_A$ receptor agonist muscimol or the $GABA_B$ receptor agonist baclofen to inhibit activity of the AcbSh.

Muscimol ($GABA_A$ agonist)

The $GABA_A$ agonist muscimol has been extensively used to explore the role of accumbens GABA receptors in feeding (for review see Stratford 2007 or Kelley 2005a, 2005b) and is one of the most widely used exogenous agonists of this receptor (Johnston, 1996). Muscimol (MW 114.10g) is a conformationally restricted analogue of GABA (Johnston, 2000) originally extracted from the fungus Amanita muscaria and demonstrated to be pharmacodynamically active in the mid 1960s (Mueller and Eugster, 1965). It was confirmed to be a GABA analogue three years later (Johnston et al., 1968) and its actions were attributed to binding to synaptic GABA receptors (Beaumont et al., 1978).

In 1986 it was shown that it did not bind at the same site as the benzodiazepines (Deng et al., 1986). The GABA receptor was reported to include at least two subtypes designated A and B (Bowery et al., 1981) and it was soon established that muscimol was a potent agonist at the $GABA_A$ receptor subtype with a much higher binding affinity than GABA itself (Curtis et al., 1971, Krogsgaard-Larsen and Johnston, 1978). It also acts as a partial agonist at $GABA_C$ receptors but $GABA_C$ receptors are not present in the accumbens. Muscimol is thought to be inactive at $GABA_B$ receptors although there is some recent evidence that it may interact with these receptors – See Discussion, Chapter 5. It is a week inhibitor of GABA uptake and hence must be recognised by the endogenous GABA transport system but it is not recognised by GABA aminotransferase (Johnston, 1971, Krogsgaard-Larsen and Johnston, 1978, Johnston et al., 1978).

Baclofen ($GABA_B$ agonist)

Baclofen is a conformationally flexible analogue of GABA (Kerr and Ong, 1995). In 1979 it became evident that there were GABA receptors on presynaptic membranes that reduced the release of neurotransmitter but which were insensitive to a range of GABA receptor antagonists and agonists already in use (Bowery et al 1979). Baclofen (MW 213.66g) would selectively bind to these novel receptors and they were eventually defined as bicuculline-insensitive baclofen-sensitive $GABA_B$ receptors (Hill and Bowery, 1981, Bowery et al., 1981, Bowery et al., 1983). It was the use of this GABA analogue that led Hill and Bowery (1981) to classify GABA receptors into subtypes A and B. Baclofen is equipotent to GABA at the $GABA_B$ receptor (Bowery and Hudson, 1979). The limited alteration of the GABA conformation exhibited by baclofen results in its specificity as an agonist only at the $GABA_B$ receptor (Kerr and Ong, 1995). It reduces the amount of transmitter released at a variety of synapses (Bowery, 1993) and reduces the amplitude of EPSPs and IPSPs evoked by electrical stimulation via a pre-synaptic mechanism (Misgeld et al., 1995).

Effects of GABA receptor agonists in AcbSh on feeding

Intra-AcbSh but not AcbC infusions of either the $GABA_A$ agonist muscimol or the $GABA_B$ agonist baclofen significantly increases intake of laboratory chow in satiated animals (Stratford and Kelley, 1997b, Stratford and Kelley, 1997a, Basso and Kelley, 1999, Stratford and Kelley, 1999, Ward et al., 2000, Soderpalm and Berridge, 2000, Znamensky et al., 2001, Reynolds and Berridge, 2001, Reynolds and Berridge, 2002).

The effects of $GABA_A$ or $GABA_B$ receptor agonists on feeding could be selectively blocked by the respective $GABA_A$ (bicuculline) and $GABA_B$ (saclofen) receptor antagonists (Stratford and Kelley, 1997b, Znamensky et al., 2001). However infusion of GABA receptor subtype antagonists into the AcbSh does not alter food intake (Znamensky et al., 2001). Increasing local levels of endogenous GABA by infusing γ-vinyl-GABA, an inhibitor of the metabolic enzyme GABA transaminase, also increased feeding in a dose related manner (Stratford and Kelley, 1997b). Infusion of the benzodiazepine agents (BZs) diazepam and midazolam into the AcbSh did not increase intake, consistent with the assertion that the increase in feeding is due to an increase in

endogenous GABA because BZs require GABA activity to exert their effects (Soderpalm and Berridge, 2000).

The effects of $GABA_A$ and $GABA_B$ agonists were specific to nutrient consumption as there was no concomitant effect on water intake but infusions of muscimol did generalise to both solid dry food and a variety of macronutrient containing solutions, suggesting a very specific and fundamental role for a subset of AcbSh neurons in feeding rather than in general motivational processes (Stratford et al., 1998, Ward et al., 2000, Basso and Kelley, 1999). Muscimol was however shown to have no effect on macronutrient selection and did not increase the intake of saline or saccharin solutions, which was taken to indicate that it could not modulate the palatability and hence the rewarding properties of food (Basso and Kelley, 1999). Elsewhere it has even been reported that muscimol can actually reduce the intake of saccharin solutions of varying concentrations (Stratford and Wirtshafter, 2007).

Reynolds and Berridge (2002) demonstrated that, not only did intra-accumbens $GABA_A$ receptor stimulation increase intake of a sucrose solution, it also produced a positive correlation between intake and hedonic or 'liking' facial reactions at rostral locations in the shell. These authors argued however that, although GABA stimulation appeared to elicit orofacial responses normally associated with subjective experience of liking, a dissociation between negative affective taste reactions elicited in caudal regions of the shell co-expressed with increased intake indicated that GABAergic neurotransmission may be separately responsible for determining the valence of affective behaviours and the amount of food intake (Reynolds and Berridge, 2002).

The effects of GABA receptor stimulation in the AcbSh did not however cause any increases in oral behaviours directed at non-food objects such as gnawing of wooden blocks (Stratford et al., 1998, Ward et al., 2000, Stratford and Kelley, 1999). Animals also preferentially increased consumption of the most easily available food source but would feed from a less accessible source if no food was easily available or if food was replaced with wooden blocks (Ward et al., 2000). No oral stereotypies or vacuous oral movements have been reported for either GABA agonist or AMPA/kainate antagonist infusions into the AcbSh region.

GABAergic and glutamatergic effects on food intake were demonstrated to be regionally specific within the AcbSh, which consequently dictated the sites of infusion in further studies carried out by Kelley's laboratory (Basso and Kelley, 1999). Lopes et al., (2007) reported that infusions of muscimol or baclofen made in more rostral regions than those investigated by Kelley and colleagues were just as effective in increasing free food intake. However examination of their diagrams of infusions sites shows that they actually infused within the same region. The relevance of these coordinates is that other laboratories have reported a more complex profile of behaviours elicited by both GABA agonists and AMPA/kainate antagonists depending on where along an extended rostrocaudal gradient they are infused.

Reynolds and Berridge (2001, 2002) published evidence that GABA effects on intake and positive orofacial responses were restricted to rostral regions of the medial shell whilst, in caudal regions, defensive behaviours, negative affective responses and negligible effects on intake were produced. Between these rostral and caudal poles infusions into mid regions predominantly caused positive effects although a small proportion of infusions (<10%) caused mixed feeding and fear responses (Reynolds and Berridge, 2001, Reynolds and Berridge, 2002). The negative effects that Reynolds and Berridge (2001, 2002) reported included defensive treading and burying, fearful vocalisations and either escape behaviour from or direct aggression (biting) towards the handler. It does seem rather odd that none of the other authors mentioned this in any study using intra-AcbSh infusions of GABA agonists but it is possible that such responses were put down to the stressfulness of the infusion procedure.

Despite the fact that distinct locations have been suggested for the sensitivity to infusions of GABA agonists and AMPA/kainate antagonists, between the various laboratories that have reported increased feeding, infusions have been effective across an extended rostro-caudal gradient. Furthermore these laboratories used different strains and sexes including male or female Sprague Dawleys, female Wistars and male Hooded Listers. Thus it is quite possible that there could more variability in the rostrocaudal distribution of positive vs. negative valence, particularly between strains, than Reynolds and Berridge suggest.

Recent research indicates that there are also structural and functional gradients along a rostrocaudal axis in terms of positive or aversive taste responses to food (Reynolds and Berridge, 2002, Roitman et al., 2005, Taha and Fields, 2005b) place preference or avoidance (Reynolds and Berridge, 2002, Reynolds and Berridge, 2003) appetitive positive motivation and fear elicited negative motivation (Reynolds and Berridge, 2002, Reynolds and Berridge, 2003) and sexual experience (Bradley and Meisel, 2001). It has been suggested that previous emotional experience in an environment can reversibly increase the size of zones along this gradient in a context specific manner (Reynolds and Berridge, 2008) and that DA transmission is essential to the expression of both appetitive and aversive responses along this gradient (Faure et al., 2008)

Other neurotransmitters/peptides in the AcbSh and feeding

It was reported that DA but not selective DA receptor agonists could also increase feeding in AcbSh but no the AcbC, although the effect was modest compared to that of AMPA/kainate receptor blockade (Swanson et al., 1997). However DA antagonists did not reduce overall intake in hungry animals nor were the reported effects on the pattern of feeding specific to either subregion (Baldo et al., 2002). Endogenous DA transmission is increased significantly more in the AcbSh than in the AcbC during consummatory responses induced by un-cued presentation of food but pre-exposure to conditioned stimuli associated with food inhibits this response.

There is no clear differentiation between the AcbC and AcbSh in terms of the robust increase in ingestion induced by infusion of μ-opioid receptor agonists and the most sensitive regions were reported to fall within ventrolateral aspects of these subregions i.e. not the same area that was most sensitive to GABA agonists or AMPA/kainate receptor blockade (Zhang and Kelley, 2000). However, regional mapping of the effects of microinfusions of opioids into the Acb suggests that there is a hedonic “hot spot” in the rostromedial shell where liking is mediated despite the observation that the effects on intake are widely distributed (Pecina and Berridge, 2005, Pecina et al., 2006, Smith and Berridge, 2007, Pecina and Berridge, 2000).

The dissociable effects of intra-Acb opioids appears to be consistent with the contention that they also subserve feeding related functions other than palatability (Glass et al.,

1999). Stratford (2007) states that the evidence that areas of maximum efficacy of amino acid or opioid receptor manipulation are regionally distinct suggests that the two systems could also be functionally discrete. However, although areas that subserve maximum intake fall in medial (GABA/glutamate) vs. lateral (opioids) plains the opioid hot spot for liking occupies a $1mm^3$ area that overlaps with the region where positive orofacial responses are elicited by GABA/glutamate modulation (Pecina et al., 2006).

Opioid induced feeding is not initiated as rapidly as $GABA_A$ induced feeding, is less intense but lasts longer (Bakshi and Kelley, 1993b). Stratford (2007) suggests that opioids affect intra-meal processes that determine the persistence of already initiated feeding whereas GABA can directly initiate ingestive behaviour. However intra-Acb opioid receptor stimulation has net inhibitory effect on the excitability of local neurons (McCarthy et al., 1977, Hakan and Henriksen, 1989, Yuan et al., 1992, Yuan, 1996, Brundege and Williams, 2002) suggesting that opioid induced feeding is also due to disinhibition of neurons in terminal regions due to inhibition of GABAergic output (Zhang and Kelley, 2000).

The potential role for a variety of other endogenous neuroactive substances in AcbSh in the control of feeding responses has only begun to be investigated in the last five or so years. Melanin concentrating hormone (MCH) infused into the AcbSh increases intake of food and the effect is blocked by a specific MCH receptor antagonist (Georgescu et al., 2005, Guesdon et al., 2009). Ghrelin infused into AcbSh also increases feeding although no ghrelin receptors have been located in this region as yet (Naleid et al., 2005). Nevertheless there are neuronal processes in the AcbSh that express ghrelin (Cowley et al., 2003). It has been reported that hypocretin/orexin in the AcbSh has no effect on feeding (Baldo and Kelley, 2001) but in a more recent study it stimulated feeding which was blocked by a selective orexin receptor antagonist (Thorpe and Kotz, 2005). AcbSh infusions of the endocannabinoids anadamine and 2AG results in potent increases in food intake, a decrease in latency to begin feeding and the effect is blocked by pre-treatment with a specific CB1 selective antagonist (Mahler et al., 2007, Soria-Gomez et al., 2007, Williams and Kirkham, 2002, Kirkham et al., 2002).

CART infused into AcbSh inhibits spontaneous, deprivation induced and muscimol induced feeding and food deprivation decreases CART in the AcbSh (Yang et al., 2005,

Osei-Hyiaman et al., 2005). CCK can act directly to suppress inhibitory and excitatory postsynaptic potentials in the Acb but the total effect appears to be to be excitation of the MSNs (Kombian et al., 2004, Kombian et al., 2005). As indicated earlier the VTA sends dense CCK projections to the AcbSh co-expressed with DA (Lanca et al., 1998). However CCK infused into the Acb does not suppress mild deprivation induced feeding (Blevins et al., 2000) but it does decrease sucrose intake in animals that have a high baseline intake (Sills and Vaccarino, 1996). Antagonists of CCK receptors increases sucrose consumption in animals that previously had low baseline intake but paradoxically decreases intake of high baseline consumers (Sills and Vaccarino, 1996).

The role of the Acb subregions in food motivated learning

There is a vast literature on the effects of manipulating the function of the entire Acb using electrolytic lesions, excitotoxic lesions (e.g. ibotinic acid), cell type specific lesions (e.g. 6-OHDP), or infusing a variety of agents that act at specific receptors, on the acquisition and expression of conditioned responses. Nevertheless, with the realisation that this structure consisted of distinct AcbC and AcbSh regions, the potential for functional dissociations in their contribution to basic mechanisms subserving motivated learning and performance of Pavlovian associations or instrumental responses or the interaction between the two was explored.

It would appear that there is no direct role for the AcbSh in modulating Pavlovian approach behaviour but AcbC lesions disrupt acquisition, depress responding post acquisition and disrupt discrimination between conditioned and unconditioned cues (Parkinson et al., 1999, Hall et al., 2001, Parkinson et al., 2000, Corbit et al., 2001, Cardinal et al., 2002b). The CeA is also involved in the acquisition of approach and it is believed that this region may influence the role of the Acb by controlling DA in the VTA (Cardinal et al., 2002b).

Intra-Acb DA enhances conditioned reinforcement whilst dopaminergic lesions abolish it (Taylor and Robbins, 1984, Taylor and Robbins, 1986, Robbins et al., 1989). The AcbSh is necessary for the potentiating effects of amphetamine on conditioned reinforcement but the AcbC is necessary for response accuracy suggesting a role for the AcbSh in the ability of Pavlovian cue salience to potentiate instrumental responses and the AcbC to organise motor output (Parkinson et al., 1999).

Intra-AcbSh DA also enhances Pavlovian to Instrumental Transfer (PIT) (Wyvell and Berridge, 2000). Excitotoxic lesions of the AcbC but not the AcbSh have been shown to abolish PIT, as did lesions of the CeA but not the BLA suggesting that a dopaminergic circuit via the VTA between the CeA and AcbC was responsible for the transfer (Hall et al., 2001). In another study published in the same month however it was reported that lesions of the AcbSh but not the AcbC abolished PIT (Corbit et al., 2001). It has emerged since these studies that there are two forms of PIT; generalised and outcome-specific that might recruit different circuits within the brain (Corbit and Balleine, 2005). The conflicting results probably arose out of procedural differences with the former study using a generalised procedure and the latter an outcome-specific procedure (Belin et al., 2009). The role of the amygdala is also somewhat dependent on the type of PIT procedure in question and it is possible that a CeA/AcbC circuit is dominant in generalised PIT whereas a BLA/AcbSh circuit takes over in outcome-specific PIT (Corbit and Balleine, 2005, Holland and Gallagher, 2003).

Lesions of the Acb that abolish PIT do not, however, affect the ability of animals to evaluate instrumental outcomes suggesting that the Acb is a critical part of the circuit that subserves the attachment of incentive value to cues but not to the reward (de Borchgrave et al., 2002). Further work on the relationship between PIT and the value of the reinforcer suggested that this depends to some degree on the amount of training and on whether a general or outcome-specific form of PIT has been learnt which could explain why either the AcbC or the AcbSh can modulate PIT (Holland, 2004).

To summarise both DAergic and GABAergic mechanisms in the AcbC are involved in the acquisition and expression of Pavlovian approach but the AcbSh is not directly involved. In conditioned reinforcement of instrumental responses the AcbC organises motor output while the AcbSh is involved in the modulation of the salience of the Pavlovian cue. The AcbC is necessary for the expression of generalised PIT whilst the AcbSh is involved in outcome specific PIT. It has been suggested that the AcbSh can potentiate responding for reward related cues and is responsible for salience attribution whereas the AcbC confers directionality of these behaviours (Wickens et al., 2007).

Role of AcbSh in expression of conditioned behaviours

On the basis that modulation of neurotransmitter signals in AcbSh had been implicated in both appetitive responses to food and in defensive behaviours as well as being activated by feeding, noxious stimuli and stress (for a review of the evidence see Reynolds and Berridge, 2001) the effects of intra-AcbSh GABAergic and glutamatergic signalling on conditioned place preference/avoidance has been investigated. Reynolds and Berridge (2002) found that animals developed place preference for the chamber paired with rostral infusions of muscimol and place avoidance for the chamber paired with caudal infusions. In contrast AMPA/kainate receptor blockade induced a univalent gradient of place avoidance from mild but significant avoidance in rostral regions to more robust avoidance at caudal extremes (Reynolds and Berridge, 2003).

Following the serendipitous establishment of a functionally specific role for GABA in free feeding behaviour, Kelley and colleagues turned their attention to investigating both the putative extended circuitry underlying the effect and the specific behavioural mechanisms by which this circuitry regulated feeding. In seeking to determine the behavioural mechanisms, investigators in Kelley's laboratory began to characterise the effects of muscimol on what they termed the "feeding central motivational state" based on the theoretical construct described by Bindra (1974).

When considering the problem couched within these terms, it was reasonable for Kelley's group to hypothesise that if muscimol increased intake it might do so via direct effects on the incentive motivational properties of the reward or on external reward related stimuli. Despite the apparent lack of evidence of effects of GABA stimulation in the Acb on the perceived incentive value of freely available food reward (Basso and Kelley, 1999), increases in intake were so large and animals ate so voraciously that it was hypothesised that it might also affect food seeking behaviours, for example operant responding for food (Kelley et al., 2005b).

In 2003 Zhang et al. employed a Progressive Ratio (PR) schedule to investigate food-seeking behaviour following intra-Acb $GABA_A$ receptor stimulation. In a PR schedule an increase in willingness to work for a palatable food reward i.e. an increase in break point is taken to indicate increased motivation for the reward (Hodos, 1961, Hodos and

Kalman, 1962). In this case the $GABA_A$ agonist muscimol failed to increase the rate of lever pressing, accuracy of pressing on the reinforced lever or the break point (Zhang et al., 2003).

In a later study, Kelley's laboratory attempted to specifically address the problem of whether or not GABAergic stimulation of the Acb resembled a state of 'hunger'. They posed the question; does this manipulation mimic "a motivational state of food deprivation that commonly enables animals to learn a new operant response to obtain food?" (Hanlon et al., 2004). Their data indicated that stimulation at the $GABA_A$ receptor was insufficient alone to potentiate the acquisition of lever pressing for food in satiated animals whereas deprivation significantly increased the rate at which they learnt relative to non-deprived animals (Hanlon et al., 2004). In other words muscimol did not appear to be mimicking the natural internal motivational state associated with hunger and/ or the potential re-evaluation of the rewarding properties of food or food cues when hungry (Dickinson and Balleine, 1995).

Section summary

There is now a large body of evidence demonstrating that the AcbSh plays a critical role in regulating both consummatory and appetitive responses to food reward. It would appear that inhibition of activity in AcbSh projection neurons ether via blockade of glutamate receptors or stimulation of GABA receptors induces feeding in satiated animals. A variety of other neurotransmitter systems in the AcbSh have been implicated in incentive motivation processes that may subserve food seeking. In addition the role of the AcbSh in learning responses to food availability and food seeking strategies appears to depend on the form of learning (Pavlovian vs. instrumental) and the complexity of the task. However stimulating $GABA_A$ receptors neither potentiates Pavlovian conditioning, nor expression of an instrumental response to food.

Current hypotheses to explain the role of GABAergic neurons in the AcbSh in motivated behaviour

It has been variously argued that the Acb processes outcome predictions (e.g. see Wilson, 2005, Day et al., 2007), or that it processes signals regarding the saliency of stimuli that allow behavioural switching (Redgrave et al., 1999), that it gates convergent inputs (Goto and Grace, 2008, Taha and Fields, 2005a, Taha and Fields, 2006) that it is

part of an action selection circuit (Wickens et al., 2007, Nicola, 2007) or a feedback circuit that modulates behavioural initiation (Balleine and Ostlund, 2007). Much of the controversy surrounding the role for the Acb in motivation has arisen because of the prevalence of papers that continue to treat it as a unitary structure.

Kelley and colleagues hypothesised that the AcbSh, which has unique access to "feeding selective nodes" in a behavioural control column in the LH (Swanson, 2000), usually inhibits activity in these areas. However when GABA receptors in the AcbSh are stimulated the GABAergic MSNs are inactivated thus disinhibiting the LH and activating the nodes, which in turn recruit motor pattern controllers (Kelley et al., 2005b). They also propose that only a subset of downstream motor pattern generators are eventually recruited, thus fragments of feeding behaviour only would be expressed, biasing animals towards consummatory responses.

Kelley's theory precludes the possibility of effects on motivational components of food seeking or consummatory processes (Kelley et al., 2005b). Furthermore they state that this pharmacological manipulation actually bypasses "higher corticolimbic networks" that subserve the more complex responses and computations underlying operant responding (Kelley et al., 2005b). The hypothesis specifically excludes the possibility that pharmacological manipulation of GABA tone has effects on the hedonic response to food (Kelley et al., 2005b, Basso and Kelley, 1999) or on behavioural output when more complex food-seeking strategies e.g. lever pressing, are required (Kelley et al., 2005b). AcbSh neurons predominantly seem to be involved in release of predetermined behaviour patterns in relation to unconditioned stimuli (Meredith et al., 2008).

This hypothesis raises a number of questions. For example is the site of action of GABA (at A or B receptors) critical to the expression of food motivated behaviours and what other areas of the brain are activated as a consequence? Does the feeding behaviour elicited by stimulating GABA receptors resemble physiologically induced feeding? Does GABA in the Acb modulate intake via effects on the initiation of feeding, in the maintenance of the consummatory act or in satiety processes that terminate feeding? Alternatively will the evidence point to a single underlying process? These questions will be investigated using behavioural measures that can dissociate effects on appetite and satiety in free feeding animals or instrumental responding.

Aims and chapter overviews

This thesis will further explore the behavioural impact of activating subtypes of GABA receptors in the AcbSh on both food intake and food seeking behaviours. On the basis of the findings reported a model of the functional organising principles of neuronal networks that encompass the Acb will be discussed with respect to endogenous inhibitory motivational control in this region. This thesis investigates the role of GABA in the Acb in the organisation of innate consummatory responses and instrumental appetitive behaviour. Studies reported in data chapters 3-6 utilised methods that can separate out the stages of feeding behaviour i.e. initiation of appetitive food seeking behaviour, maintenance of consummatory behaviour and the termination of feeding as satiety is reached.

Throughout the thesis the effects of intra-Acb $GABA_A$ and $GABA_B$ receptor agonists were measured in animals that were pre-satiated and hence not driven to feed by physiological mechanisms. The behavioural paradigms chosen (see Chapter overviews below for details) provide measures that dissociate between innate consummatory responses to food and instrumental responding for food reinforcement. The methodological approach is described in Chapter 2, which also covers details of the animals used, welfare considerations, health and ethical issues associated with animal experiments involving central administration of drugs. The surgical procedure to implant bilateral cannulae for infusions into the Acb is described and I provide details of the drugs that will be administered centrally or systemically.

In Chapter 3 the results from experiments using the first paradigm, the behavioural satiety sequence (BSS) are reported. The hypothesis put forward by Kelley (2005a, 2005b) suggests that GABA in the Acb contributes to the expression of innate consummatory responses through control of motor behaviours. However the effects on feeding of the $GABA_A$ agonist muscimol and the $GABA_B$ agonist baclofen have only previously been measured in terms of total *ad libitum* intake. The BSS is sensitive to manipulation of the organisation of the consummatory response but also to potential disruption of the natural sequence of associated feeding related behaviours (Clifton and Cooper, 1996). Thus it is a useful measure of feeding specific versus non-specific effects of pharmacological treatments. These studies therefore further characterise the

effects of intra-Acb $GABA_A$ and $GABA_B$ receptor agonists on free feeding. Because Kelley suggests that GABA receptor activation does not cause changes in motivational processes these results were compared with the BSS expressed following physiological and pharmacological manipulations known to affect motivation. To this end the BSS was also recorded following fasting, intra-Acb administration of a μ-opioid agonist or systemic administration of a benzodiazepine.

In Chapter 4 I report the results of a set of studies that used a second order operant schedule designed to measure both appetitive and consummatory responding for food reinforcement (Thornton-Jones et al., 2005). The effects of intra-Acb administration of the $GABA_B$ receptor agonist baclofen on instrumental responding were recorded across a range of doses and, once again, compared with the effects of a μ-opioid agonist known to affect motivational processes. Free intake was recorded both prior to and following operant test sessions to confirm the behavioural efficacy of the doses tested and to confirm that the drug was targeting a region of the Acb that is involved in control of feeding responses. Complimenting these two approaches (operant responding vs. free intake) was an extensive analysis of videos recorded during operant testing to identify and quantify behaviours other than appetitive instrumental and consummatory responses that might be modified by manipulation of accumbens function.

Chapter 5 covers a second set of studies using the second order schedule and a range of doses of the $GABA_A$ receptor agonist muscimol. Once again free intake was recorded prior to and following the operant test sessions and videos were analysed for any effects on other behaviours.

The penultimate chapter, Chapter 6, gives details of a standard immunohistochemical approach for identifying the presence of the protein Fos in the nuclei of neurons, which can be used as a marker for the immediate early gene *c-fos* (Morgan and Curran, 1991). This is widely used as a tool to highlight areas of neuronal activation (Herrera and Robertson, 1996). In this case it was used to identify and compare the elements of the macrocircuit activated by accumbens GABA receptor subtype stimulation. The final set of studies reported in this Chapter used Fos-like immunoreactivity (FLI) as a marker of neuronal activation following infusion of either baclofen or muscimol in the same animals for which free intake and instrumental responding had been measured in

Chapters 4 and 5. This approach meant that it was not possible to directly compare the activity of brain regions following behaviour under the influence of drug. However it was important to establish if there was any difference in the profile of activation with baclofen or muscimol prior to initiation of behavioural responses because this had not been done before. The behavioural data confirmed that the doses of baclofen and muscimol use to induce *c-fos* expression were also functionally active.

Finally Chapter 7 is a general discussion. In this chapter I will consider potential explanations for the results reported in the four preceding data chapters when all of the data is considered together. I will set these findings within the context of what is already known about both the structure and neurochemistry of the AcbSh and its place within the wider circuitry that subserves motivated behaviour. I will then consider the implications of the results for understanding the role of Acb GABA in the integration of motivation and action with reference to current hypotheses. As far as is possible within the scope of this thesis I will construct a modified model to explain endogenous inhibitory transmission effects in the Acb on motivational control. I will suggest future directions for this research in order to further explore the conclusions I have come to and to test the validity of the modified model mentioned described.

In summary, the main aims of this thesis are:

1) To characterise and compare feeding and related behaviours or instrumental appetitive behaviours following GABA receptor subtype stimulation in the AcbSh.
2) To compare the effects of GABA receptor subtype stimulation in the AcbSh with other pharmacological manipulations that affect specific phases of ingestive behaviour.
3) To compare the effects of GABA receptor subtype stimulation in the AcbSh with physiological manipulations that increase feeding and food motivated behaviours.
4) To investigate the effects of GABA receptor subtype stimulation in the AcbSh on neuronal activity in other areas of macrocircuits subserving motivational processes.
5) To further investigate the hypothesis that GABA receptor stimulation in the AcbSh simply results in increased intake because of disinhibition of downstream motor pattern generators.
6) To set all of the above in the context of their relevance to the ‘natural’ effects of hunger and satiety on food motivated behaviours and hence motivation in general.

Chapter 2

Methods

Introduction

This chapter covers essential information with regards to the animals used for this thesis, their welfare, health and ethical considerations. Given that most of the studies reported involve central administration of drugs, there is then a description of the surgical procedure to implant the necessary guide cannulae and histological methods used for subsequent identification of infusion sites. The majority of the subsequent experiments relied on behavioural testing in two key paradigms; the recording of a behavioural satiety sequence (BSS) and a 2nd order operant schedule. The methods used for both are described in detail below. Where relevant, additional methodology or modifications to either the BSS study or testing on the 2nd order operant schedule will be described in Chapters 3, 4 and 5.

Also included is a description of the method used to measure intake in response to freely available food, which was tested in parallel with the operant work reported in both Chapters 4 (2nd order studies with baclofen) and 5 (2nd order studies with muscimol). Because video footage was recorded for the operant experiments in Chapters 4 and 5 it was subsequently possible to look at incidental behaviours not measured at the time of testing. The method and categories used to assess these behaviours are described here. Finally I provide details of the drugs used throughout the various studies presented. Chapter 6 reports studies using an immunocytochemical labelling method to visualise areas of the brain activated as a consequence of drug infusions. Given that this method was only used for those studies the detailed methodological description will be included within the chapter itself.

Animals and housing conditions

Outbred male Lister hooded rats (strain HsdOla:LH, Harlan UK) were used in all the studies reported in this thesis. Starting weights for each batch of animals were chosen on the basis of the procedure through which the animals were to go. Those destined for

a prolonged period of training were bought in relatively small so as to be an appropriate size for stereotaxic surgical techniques at the time of guide cannulae implantation (e.g. for the 2nd order schedule described below). Those that went through a short period of habituation prior to direct behavioural observations were bought in at a larger size so as to be an equivalent weight to the surgery groups at the time of testing. Specific animal weights for each experiment are indicated in the relevant chapters.

The animals were housed in a Home Office approved unit at the University of Sussex following the appropriate code of practice from the Home Office (ASPD, 2005). Holding and test rooms were adequately ventilated for the stocking density and maintained at controlled temperature (19-22°C) and humidity (45-55% saturation) under a 12hr light / dark cycle. Animals had *ad libitum* access to water throughout the study but periods of restricted access to food were imposed, as detailed in each chapter.

Acclimation to the new environment, handling procedures and to the presence of an observer took a minimum of one week. Animals were group housed but, when surgical procedures were involved, they were habituated to single housing in Perspex walled, solid bottom cages (See Fig. 2.1) for at least 7 days prior to any procedure. Apart from the fact that the Home Office states that rats should be group housed unless the experiment requires otherwise (ASPD, 2005) group housing also appears to speed up operant training (Greenhalgh, 2007).

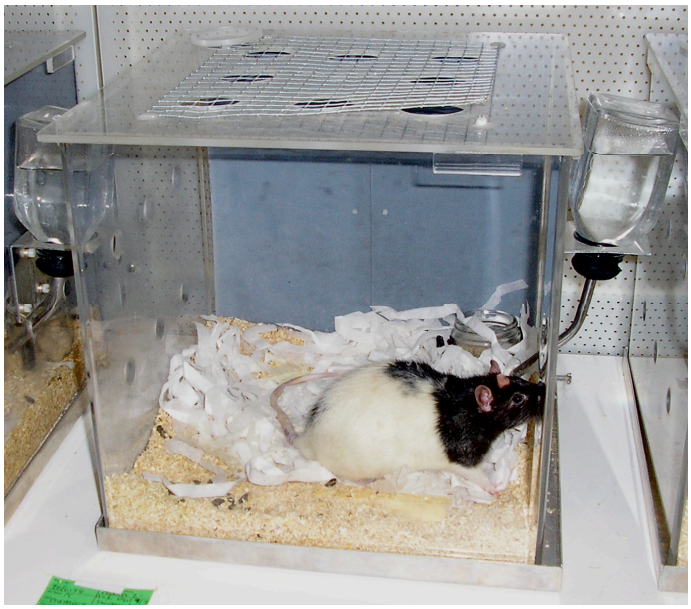


Figure 2.1. Perspex cages used to house single animals undergoing surgery.

Welfare and health

Housing

When animals were kept in groups of three or four (depending on their size) the largest standard rat cage available was used. This was a solid bottomed cage width 36cm, length 53cm and height 25cm (model RC2R, North Kent Plastic Cages LTD., UK). A cage of this size provided a floor area of 400cm^2, adequately meeting the required floor area for animals as large as 550g. The lid height also exceeds the recommended minimum of 20cm. When animals were housed singly the Perspex cages were not visually isolated and perforations in the walls (for ventilation) meant that they were also able to smell and hear the presence of other animals, which is believed to reduce the stress associated with isolation (Lawlor, 1984, Lawlor, 2002). When animals were taken out for training they were allowed brief periods of contact with other animals and as much time as possible playing with and being handled by myself.

Both group and singly housed animals were provided with enough sawdust bedding to allow natural hoarding and defensive behaviours, shredded dust free tissue nesting material and wooden blocks to chew on. In the group cages they also had a plastic tube big enough for all animals to fit in. In the single Perspex cages extra nesting material was provided to allow the animal to burrow into it and thus express natural behaviour.

Monitoring of health

Animals provided by Harlan had been screened for clinical or sub-clinical disease. Once at Sussex they were regularly handled and checked for signs of disease, stress or distress by both the unit staff and myself. Occasional signs of non-specific stress were observed with the occurrence of porphyrin around one or both eyes. This only occurred in animals post-operatively. In each case the eyes were bathed with sterile 0.9% saline to prevent blockage of the tear duct. This was often enough to prevent it happening again. No animal that developed persistent signs of disease, infection or non-surgical wounds was used for this thesis. At the time of testing only those animals that had apparently fully recovered from surgery were used and if animals showed signs of stress such as porphyrin they were tested when these were no longer apparent.

Topical medications

There was only one case of noticeable self-directed behaviour with one of the animals licking a forearm until raw. This was treated twice daily with a topical agent, Viatop (Boehringer Ingelheim, UK) purchased through the University of Sussex designated vet. The antiseptic gel (containing the natural ingredient raffinose, to relieve itching) rapidly stopped further licking of the affected area and allowed fur re-growth within a week.

Ethical considerations

All animal care and experimental work was carried out in accordance with the Animals (Scientific Procedures) Act 1986. Unit staff and the Project and Personal Licence holders also followed the guidelines set out in the Home Office codes of practice. The UK Government is particularly keen on promoting the application of 'the 3Rs' - the replacement of procedures with others which do not use animals, the reduction of the number of animals used and the refinement of procedures to minimise pain and suffering. The 3R's have been revisited throughout the course of this studentship and a variety of steps have been taken to meet these principles in practice.

No animal study was undertaken that could be replaced by non-animal alternatives such as tissue culture, computer modelling or molecular biological techniques. Clearly, given that this thesis addresses fundamental questions about behavioural neuroscience, this would not be possible at this time and the only way to advance our understanding of the link between brain function and behaviour is to study it directly *in vivo.* To meet the requirements for reduction all studies were carefully designed to gain the most power and validity using the smallest possible number of animals. To this end I followed the guidelines and advice found in Festing et al. (2002).

All experiments were also designed to control variability as much as possible. Although Festing et al., (2002) recommend that one of the best ways to achieve this is to use isogenic inbred rats outbred Lister Hooded rats are routinely used for behavioural studies throughout the world. The Lister Hooded strain used for these studies has been maintained as a closed colony since 1932 at Harlan UK and individuals tend to be similar but their genotype at a given locus is unknown.

Subjects were bought in at a uniform weight, housed under optimum conditions (see above), fed standardised diets and handled on specific days by the unit staff for cage cleaning and for the rest of the time only by myself. Experiments were planned and designed prior to any animals being bought in to minimise numbers of rats used.

The need for refinement under the Act was addressed by the use of appropriate medications to reduce pain and to avoid post-operative infection (see below) and by adhering to aseptic techniques for the preparation of all solutions to be used during surgery (i.e. saline for cleaning the wound) or to be administered centrally. The introduction of a rapid setting dental resin during the surgical procedure significantly reduced the amount of time each animal was anaesthetised. Care was taken to ensure animals were very well habituated to the operator prior to testing to reduce the stress associated with the infusion process.

Methods for central administration of drugs

Surgical procedure to implant guide cannulae

Rats were anaesthetised using 4% Isoflurane (Abbott Animal Health, UK), 0.4L/min oxygen and 0.4L/min nitrous oxide in an induction chamber. Gas flow was switched to a nose cone and subjects transferred to the stereotaxic apparatus (Stoelting Co., USA). Isoflurane concentrations were adjusted gradually from 4% down to 1% to maintain a constant rate of anaesthesia during the subsequent procedure.

The surgical technique was based on a standard flat skull technique employed by Ward et al. (2000) with the incisor bar set at IO – 5mm. In this case narrow thin wall, 26-guage, 16mm stainless steel cannulae (Coopers Needleworks, UK) were implanted bilaterally aimed 2.2mm above the AcbSh. Coordinates were initially based on those used previously in this laboratory for a University colony of Lister hooded rats bred from Charles River UK stock (Ward et al., 2000). These coordinates were aimed 2.2mm above the AcbSh at anteroposterior (AP), + 1.2mm, mediolateral (ML), ± 1.5mm relative to bregma and dorsoventral (DV), -5.8mm relative to skull surface. Histological examination revealed core involvement in infusion sites in some animals (excluded from the final analysis).

For subsequent experiments coordinates were adjusted on the basis of a review of the range of coordinates reported in the literature for various strains of rat (Stratford and Kelley, 1999, Stratford and Kelley, 1997b, Basso and Kelley, 1999, Zhang et al., 2003, Hanlon et al., 2004, Stratford, 2005, Reynolds and Berridge, 2002, Lopes et al., 2007). These were compared to the initial placements in these Lister Hoods from Harlan and, relating the brain anatomy to photomicrographs in an appropriate stereotaxic atlas (Paxinos and Watson, 1998), new coordinates were chosen that placed the infusion point in the medial caudal shell region only.

For cannula implantation a trepan, 3mm in diameter, was used to make two holes in the bone directly above the target area. The 26-guage cannulae were consequently aimed 2.2mm above the AcbSh at AP + 1.4mm, ML ± 0.9mm and DV-5.8mm. A burr drill bit was used to make shallow guide holes and three 3.2 mm stainless steel bone anchor screws (Stoelting Co., USA) and Geristore dental resin (Dkap International Ltd., UK) were used to anchor the cannulae to the skull. This resin anchor was reinforced by the addition of a plastic collar filled with Simplex self-cure acrylic dental resin (Associated Dental Products Ltd., UK). 33-gauge stainless steel obdurators (Coopers Needleworks, UK) were inserted to prevent occlusion. After closure of the initial incision around the collar it was lightly dusted with the topical antibiotic powder Cicatrin (GlaxoSmithKline, UK), which contains neomycin as the active ingredient. This was also employed post-operatively if the wound showed signs of infection.

Post-operatively, recovering animals were placed in absorbent paper lined cages on heat mats. Cages were covered to provide a dark, warm sheltered environment and animals were immediately provided with water and wet mash containing an analgesic and antibiotics (see below for details of products used). Post-op analgesia was provided for a minimum of 48 hours and animals were allowed to recover for at least 7 days. Weight was monitored and, when all subjects were gaining weight rapidly, they were put back on to restricted diet for a minimum of two days prior to re-habituation to the apparatus.

Use of analgesia post-operatively

Analgesia was employed immediately post operatively for all animals undergoing surgical procedures. I used Metacam Oral Suspension for Dogs (Boehringer Ingelheim, UK) containing the equivalent of 0.05 mg per drop when delivered from the integral

dropper. Meloxicam is a non-steroidal anti-inflammatory drug (NSAID) of the oxicam class which acts by inhibition of prostaglandin synthesis, thereby exerting anti-inflammatory, analgesic, anti-exudative and antipyretic (reduces body temperature in the case of fever) effects. It is recommended for rats *per os* (PO - by mouth) (Flecknell and Waterman-Pearson, 2000). In this case the analgesia was given immediately post operatively in wet mash. Flecknell and Waterman-Pearson (2000) state that, in rats, a dose of up to 1mg per kg is appropriate and the named vet suggested a dose rate of 0.1mg per kg. For the purposes of post-operative analgesia in this laboratory a dose rate of 0.2mg per kg was used. A second dose was administered at 24 hours post surgery and a third at 48 hours. Analgesia was discontinued after 3 days to avoid renal problems (Flecknell and Waterman-Pearson, 2000).

Use of antibiotics post-operatively

Rats were given a broad-spectrum antibiotic, Oxytetracycline (Sigma Aldrich, UK) also known as Terramycin, post-operatively. This was used to prevent postoperative infection and is indicated in particular for mild upper tract respiratory problems and soft tissue infections. The recommended dose range is 10-20mg /kg PO for rats (Poole et al., 1999). There was some evidence that the animals found the taste in their drinking water aversive so it was dissolved in the water with the analgesic, used to make up the wet mash and administered for three days. If animals showed signs of ill health (see below) after 3 days the antibiotic regime was continued until their condition improved

Dealing with weight loss and/or dehydration post operatively

In a small number of animals unusual weight loss was observed (more than ~ 2.5% body mass) within the first few days post-operatively. This was sometimes accompanied by listlessness and obvious signs of dehydration and/or a lack of appetite. These animals were treated with antibiotics as above and, where there was obvious discomfort, one or more additional doses of analgesia was administered. They were also placed on a heat-pad in the home cage and were hand fed a 0.9% / 5% sucrose solution using a syringe. Between feeding with the nutritive solution they were regularly offered water in the syringe and wet mash was left in the cage. Once the animals looked healthier and had gained weight they were given prolonged access to the wet mash to encourage quick recovery and weight gain. They were not considered for testing until they weighed more than they had done pre-operatively.

Infusions and maintenance of guide cannulae patency

The stainless steel obdurators used to occlude the cannulae between infusions were rotated and cleaned daily. If cannulae became sticky or blocked, animals were anaesthetised (as above) and the obdurators were carefully removed with the head held stable in the ear bars. Clean obdurators were inserted. If obdurators were lost in the cage they were replaced as soon as the loss was discovered. For the infusions 31g stainless steel injectors were made in house. These had a stainless steel collar fitted such that the remaining length of injector extended 2.2mm beyond the tip of the guide cannulae to reach the target structure. These injectors were connected via number 10 PPE tubing (Harvard Apparatus LTD., UK) to 10µl Hamilton microsyringes. A microinfusion syringe pump model 802 (Univentor, Malta) operated the injectors, which held two microsyringes facilitating simultaneous bilateral infusions. During post-op re-training, subjects underwent two habituations to the infusion procedure (which involved gentle restraint in the hands) using dummy injectors that did not extend beyond the tip of the guide cannulae. One sham infusion of sterile 0.9% saline was administered two days prior to testing.

On test days intra-AcbSh infusions of drug or vehicle were made bilaterally and simultaneously at a rate of 0.5µl per side over 30 seconds (1µl of drug solution infused in total). Injectors were left in for a further minute to allow diffusion of drug away from the tip. Any discharge from the cannulae post infusion was noted and blotted away with a medical wipe (Kimberly-Clark, UK). The obdurators were cleaned in ethanol, dried and replaced. Subjects were then placed in the test cage immediately post infusion. Between animals the infusers were dipped in ethanol to clean and sterilise them and then the drug solution was run through for a couple of seconds to get rid of any contamination, air bubbles or blockage. Infusion sites were later verified histologically.

Perfusion method

At the end of each experiment subjects were deeply and terminally anaesthetised using an overdose, via the i.p route, of 0.8ml of Euthatal (Merial Animal Health Ltd., UK), a sodium pentobarbital solution (200mg/ml) used exclusively for euthanasia in small animals. Rats were transcardially perfused using a peristaltic pump, model 205U (Watson and Marlow, UK). Approximately 150mls of 0.1M phosphate buffered saline (PBS) was pumped through over a period of five minutes followed by 200mls of 10%

formal saline. Alternatively, if the brains were to be run through an immunohistochemical procedure paraformaldehyde (PFA) was used as a fixative instead of formal saline. A guillotine was used to decapitate the animal post perfusion and the cerebellum was exposed using bone Rongeurs to peel away the skull. Heads were post-fixed in formal saline/PFA for at least 24 hours. Brains were then dissected out and returned to the fixative for a further 24 hours. Finally the brains were blocked and cryoprotected in phosphate buffered 30% sucrose solution by immersing them for a minimum of 48 hours prior to freezing at -80^{o}C.

Histology and placement verification

Brains were sectioned coronally at 60μm on a freezing microtome. Relevant sections were mounted on gelatinized slides and, following alcohol dehydration, run through a standard Nissl staining procedure using Thionin. By comparing key landmarks with the appropriate figures in Paxinos and Watson (1998), the location of infusion sites was determined. The length of the infuser extending beyond the cannula tip (2.2mm) was scaled down to the final section size and the infusion site taken as being the point at which this ended within the lowest area of gliosis.

Only animals with correctly placed cannulae were included in the final analysis i.e. those that had no core involvement and little or no involvement of more medial or ventral structures. A correct placement was defined as being within the shell region mediolaterally and between anterior-posterior co-ordinates of 1.4mm ± 0.5mm. Animals with gliosis that appeared to have spread too extensively dorsoventrally (into other brain structures) were excluded from the final group for analysis. Drawings of the infusion sites are shown for each experimental group in the relevant chapters.

Key methods and apparatus for behavioural testing

Simple measure of increases in intake:

For the BSS experiments (described below), intake was measured within the test apparatus used for the main study. In all other studies intake in response to freely available food within the same apparatus was recorded prior to testing the animals on the main schedule to verify behavioural activity of the infusion sites. Following testing on some of the operant schedules an equivalent dose response curve for intake in response to freely available food was established after the main study (see data chapters for details).

To this end animals were habituated to receiving normal laboratory chow pellets in an easily accessible glass dish in an environment that was distinguishable from the home cage. The 'test cage' was a standard solid bottom cage (RB3R, North Kent Plastic Cages Ltd., UK) width 28cm, length 45cm and height 20cm. A small amount of sawdust (~10mm depth) was present but not enough to allow hoarding of pellets. Food was removed from the home cage one hour before testing and replaced with 5 fresh pellets to which rats had access for 30 minutes. Animals were then transferred to the test cage for a further 30 minutes to compensate for initial arousal and finally given access to a pre-weighed pot of fresh laboratory chow for a set period in the presence of an observer. Habituation continued until intake was stable over a period of 4 days. On test days, following administration of drug, animals were either returned to the test cage in the absence of food for an appropriate amount of time to allow optimum drug effect (in the case of i.p. injections) or, in the case of infusions of drug, the test meal was immediately provided and the session began (see Fig. 2.2 for timeline).

Where practical the operator was present and recorded gross behaviour directly, otherwise the session was filmed for later behavioural analysis. Food pots were retrieved and the sawdust was fingertip searched for any spillage. Checks run with paper in the bottom of the cage instead of sawdust demonstrated that crumbs and dust too small to be collected with a fingertip search weighed < 0.2g and hence were not considered to be a significant loss in the sawdust filled cages. Where water was provided the bottles were weighed prior to and post test session and the total amount of water consumed was calculated.

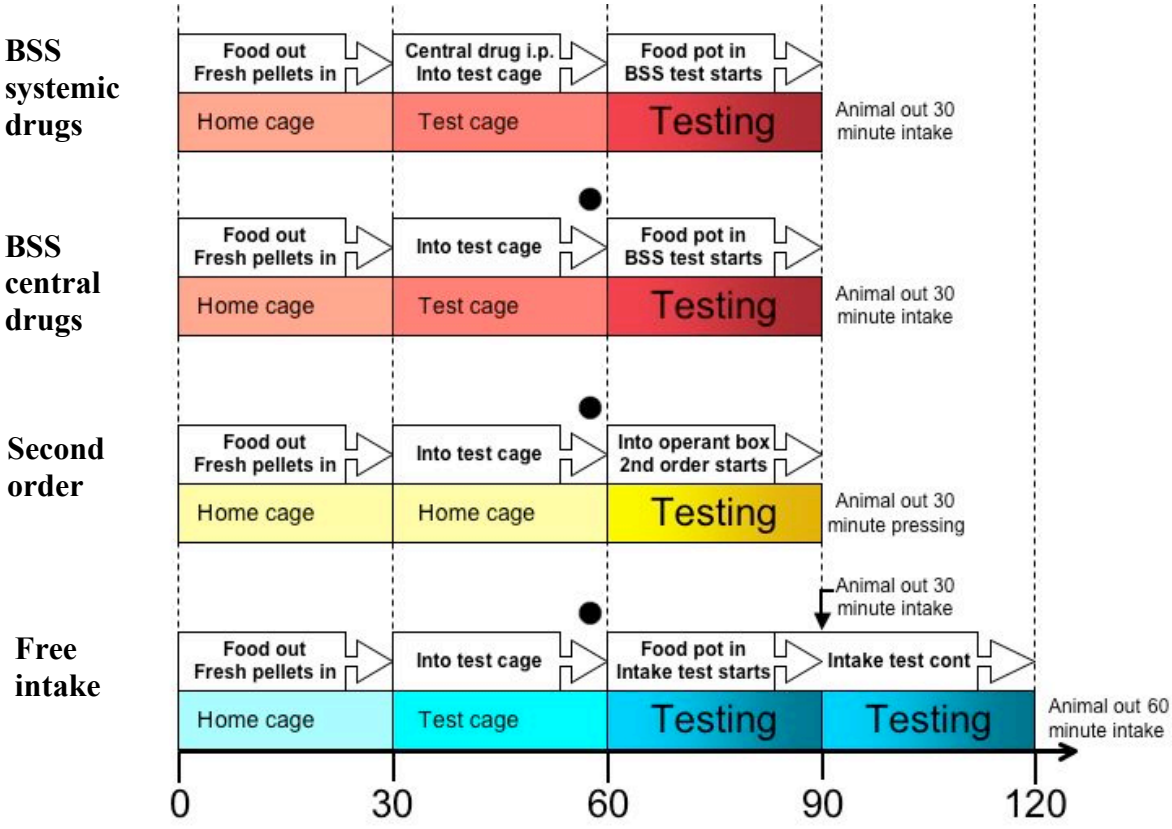


Figure 2.2. A comparison of the timelines for the behavioural satiety sequence, 2nd order and free intake test schedules. ● indicates the point at which infusion of drug into the AcbSh took place.

Behavioural Satiety Sequence (BSS):

Apparatus

All subjects were habituated to the test cages in a test room isolated from the holding room. The cage rack was designed to allow behavioural assessment in cohorts of 12. A microprocessor was programmed to illuminate LEDs, mounted adjacent to each test cage, at consecutive 2.5 s. intervals. These provided a signal for the observer to press the key on the keypad that corresponded with the behaviour the animal was engaged in at the time that the LED came on. The press turned the LED off but the next LED would illuminate 2.5s later irrespective of when the observer pressed the key.

Habituation and training

The method was based on procedures previously described (Clifton et al., 1989; Vickers et al., 1996). Habituation was identical to that described for simple measures of increases in intake (above). The length of access to food during habituation was determined by the final test period to be used (see data chapters for details). As expected, consumption was low but acclimation to the test environment continued until there was no significant difference in the mean meal size over four consecutive days.

Testing procedure

Following habituation some of the test groups underwent surgical implantation of guide cannulae, were allowed to recover for a minimum of seven days and then reintroduced to the test cages and given access to food on a daily basis until responding was stable. On test days, as with the habituation period, food was removed from the home cage one hour before testing and replaced with 5 fresh pellets (~7g) to which rats had access for 30 minutes. Animals receiving i.p injections were given drug at this stage and then transferred to the test cage for a further 30 minutes to compensate for initial arousal. For those animals receiving centrally administered drugs it was after this 30 minute period in the test cage that the infusions took place (see Fig. 2.2 for timeline).

Animals were administered vehicle or drugs at various doses according to a within subjects procedure using a Latin square design then returned to the test cage. They were then presented with a pre-weighed dish of test diet for the designated test session period. Their behaviour was either videoed and then analysed at a later date or, at the time, using the keypad. During feeding, individuals were observed at 30 second intervals (the time taken for the LEDs to illuminate for every member of the cohort) across the total test period. Behaviour was subdivided into four mutually exclusive categories (based on those employed by a variety of laboratories) for scoring: *1) Ingest, 2) Active, 3) Groom and 4) Inactive* (See Table 2.1 for detailed definitions).

Table 2.1. Categories employed to record the behavioural satiety sequence in rats. The first four columns show categories that have been used in the past and the final column lists the categories developed from previous studies for this thesis.

Bolles 1960	Antin et al. 1975	Clifton et al. 1989	Halford & Blundell 1996	Categories in this thesis
Grooming Licking the fur.	**Grooming** Biting or licking the coat, feet, genitals or tail; scratching head or body with hind leg; stroking whiskers or face with one or both front paws.	**Groom** Activities like grooming and licking that were directed at the body surface.	**Grooming** Scratching, licking, or biting of the coat, whiskers, feet, or genitals.	**Groom** Grooming, biting or licking of head, body or tail using mouth or limbs.
Grooming Scratching with hind limb.				
Grooming Washing the face with paws.				
Eating	**Feeding** Licking food tube.	**Ingest** Eat from food dish or, very occasionally with wet mash, drink from water bottle.	**Eating** Biting, gnawing, or swallowing food from dish or from front paws.	**Ingest** Retrieval of food with mouth or paws, holding, chewing and ingesting food.
Drinking	**Drinking** Licking water tube	NA	**Drinking** Licking bottle nozzle.	NA
Sleeping Animal motionless and had its eyes shut.	**Resting** A relaxed position with the head curled under the chest; curled on the side with the head resting on the bottom of the cage: stretched out on the bottom of the cage either on the side or upright. Resting could occur with eyes open or closed.	**Rest** Resting with head or body lowered, perhaps with the eyes closed.	**Resting** Sitting or lying in a relaxed position or resting.	**Inactive** Absence of movement in a resting posture (head and/or body lowered) with or without eye closure.
Miscellaneous Any and all behaviour not otherwise categorised.	**Locomotion** Walking to front or back of the cage or circling.	**Explore** Moving around the cage, rearing, sniffing or standing alert.	**Locomotion** Moving, rearing and other behaviour patterns not defined elsewhere.	**Active** Moving around cage, rearing, sniffing, standing alert and any other behaviour not already defined.
	Rearing Front paws raised off the bottom of the cage and either placed on the sides of the cage or held in front of the body.		**Rearing** Front paws rose from cage bottom. Can be supported by the tank side.	
	Sniffing Rapid wrinkling of the nose directed at environmental surface.		**Sniffing** Head movements with rear limbs immobile; twitching of vibrissae at an aspect of the environment.	

Second-order operant responding (2nd order)

Apparatus

Animals were trained and tested in standard operant chambers (width 25cm x length 25cm x height 21cm) controlled by the Paul Fray operating system (Paul Fray Ltd., UK) run on an Archimedes computer, using Arachnid. Each chamber was housed in a sound-attenuating box with a fan to maintain adequate ventilation and to reduce the impact of ambient noise. The boxes were housed separately from the holding room, eliminating general procedural noises and olfactory or auditory stimuli from rats not undergoing testing.

Chamber illumination came from ceiling mounted house lights and a separate cue light, 6cm above the food magazine, could be independently programmed. Presses on one of two, 4cm wide, levers inserted on either side of the food magazine could activate the cue light, deliver pellets or both. These pellets were delivered from an external pellet dispenser into a recessed well accessed by pushing a Perspex flap mounted at the front of the magazine. 10mm x 10mm galvanised wire mesh was used as flooring. Below this flooring was a waste pan containing sawdust.

Habituation and training

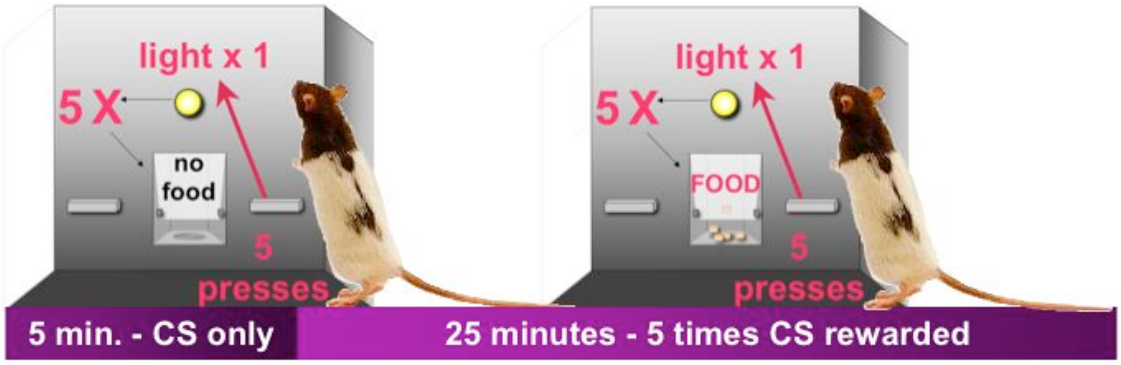
Initially rats were put onto a restricted diet of 14-16g of chow per animal (depending on average body weight calculated for each cage of 4 animals). After an initial weight loss down to 85-90% of free feeding weight this meal size was maintained as it provided adequate calories to support slow but steady weight gain. Once growth rate was stabilized on this restricted diet (<7 days), 45mg standard Noyes pellets (Formula A/I) were introduced with their meal for two days prior to the start of training.

Rats initially received 2 days of habituation during which pellets were automatically delivered on a 120 s random time (RT) schedule until a total of 15 pellets had been delivered over a 30 minute period. Levers were retracted during the habituation phase. Once subjects were removed, the magazine was checked to make sure pellets had been consumed. With all subjects taking all of the pellets by the second session, training commenced the following day. Training sessions were 30 minutes long. Animals were initially trained on an FR1 schedule; one press on the correct lever (reinforced) resulted

in the stimulus light coming on for 8s and, 2s into this period, one pellet was delivered. Pressing on the other lever (incorrect) had no programmed consequences.

The 8s centre light illumination was coincident with pellet delivery and hence the two became associated resulting in the light becoming a CS. The 8s period of illumination also indicated that the FR requirement had been fulfilled and any further lever presses on the correct lever after pellet delivery had no programmed consequences (referred to as non-reinforced presses). Levers were inserted for a total of 30 minutes. The position of the actively reinforced lever, left or right, was balanced for all experimental groups. When all animals were responding to the reinforced lever around 100 times (~ 3 days) the response requirement was increased to an FR5; five presses = CS and delivery of one pellet. They were trained on this contingency for 2 days. Next it was increased to an FR5 (2) where the animals had to complete a set of 5 presses for the CS twice for delivery of 2 pellets.

At this stage training continued until all animals made 90% of their responses on the reinforced lever. The response requirement was then increased to an FR5 (3) for two days before the introduction of a five minute period (Fixed interval: FI5) at the start of the session in which responding on the reinforced lever resulted in a CS but no pellet delivery. Again, 90% correct responding was required before moving on to the final schedule, an FR5 (5) such that 5 reinforced presses = CS and 5 CSs (total of 25 presses) were consequently rewarded with delivery of 5 pellets (see Fig. 2.3). During this period animals were split up and re-housed in Perspex cages (as described above) but surgery was only undertaken when responding on the FI5/FR5 (5) schedule was stable over four consecutive days following the split.



Appetitive phase **Consummatory phase**

Figure 2.3. Detailed timeline for the 2nd order operant schedule. In the first 5 minute period 5 presses on the reinforced lever = cue light for 8s but none of the presses are rewarded. In the following 25 minutes 5 presses = cue light and 5 cue light illuminations are rewarded with 5 pellets.

Once animals were fully trained they were returned to an *ad libitum* feeding regime. They were also habituated to palatable wet mash, which maintains hydration in post op animals and facilitates efficient oral administration of medication. Following recovery from surgery food was once again restricted to 14-16g / animal / day. After four days, food restricted subjects were re-trained on the FI5/FR5 (5) schedule until responding was not significantly different from pre-operative levels and remained stable for at least four consecutive days.

Testing procedure

On test days animals were pre-fed 1 hour prior to drug infusions to reduce baseline food reward responses to a low level. During pre-feeding, animals were allowed access to 5 pellets of standard laboratory chow (~7g) for 30 min as for the BSS experiments (see Fig. 2.2 for timeline). The rats were infused in the holding rooms to minimise arousal caused by moving them to the test rooms. Animals were well habituated to the procedure and did not make any noise or show signs of distress that might affect the other animals in the room. Infusions were made as described above following a counterbalanced Latin Square design such that each animal served as its own control and subjects were tested in pairs to counter the potential effects of the lag time associated with administration. The drugs and doses used are specified in the data chapters.

Once a pair of animals had been infused they were moved to the testing room and placed in the operant boxes. Testing started at approximately 11:00 am so that the session finished well before the dark cycle began and before the expected feeding time for restricted animals. At least 48 hours was left between infusions and animals were re-trained drug free on the days in between testing.

Gross behavioural analysis

Apparatus

Miniature webcams were inserted into the door of each operant chamber. These were linked into a circuit board that tessellated the 4 images before they were fed to a VCR machine to record all four camera outputs simultaneously.

Table 2.2. Behavioural categories used for analysis of videos recorded during second operant studies.

Behavioural category	Definition
Active	Moving around cage, rearing, sniffing, standing alert, circling, jumping and any other behaviour not defined elsewhere.
Lever	Pressing, touching or holding the lever.
Hopper	Poking of nose or paws into the pellet delivery hopper or touching of the Perspex flap covering the entrance to the hopper
Pellet	Handling or actively chewing pellets outside the hopper
Rearing	Front paws raised off the floor and nose directed at point above paw level.
Groom	Grooming, biting or licking of head, body or tail using mouth or limbs.
Oral	Oral stereotypy expressed as licking or chewing, predominantly of flooring but may be directed at walls of operant box.
Inactive	Absence of movement in a resting posture (head and/or body lowered) with or without eye closure.
Off screen	Completely off screen or obscured to the point that behaviour cannot be defined.

Testing procedure

Behaviour in the 2nd order operant schedule was videoed and later analysed (blind) with a keyboard that triggers a computer to interrogate the video in terms of time and number of frames for the category of key pressed. The categories are *1) Active, 2) Lever, 3) Hopper, 4) Pellet, 5) Rearing, 6) Groom, 7) Oral, 8) Inactive and 9) Off screen* (see Table 2.2). These are based on those used by Greenhalgh et al. 2007 and modified to best accommodate the behaviours exhibited by animals in these studies (see Chapter 4 for detailed explanation). Each half hour segment of footage for the 2nd order test period was analysed in real time. A keypad with 8 keys was linked, with the VCR machine to a computer. 'VideoTranscriber' software, written in house by Prof. Pete Clifton, was used to record key presses and produce an output file that contained data points relative to the time and duration of key presses. Unix shell scripts were used to extract data for individual behaviours, providing numbers for total duration and frequency of occurrences of each behaviour under various drug conditions.

Drugs, doses and chemical designations

Muscimol ($GABA_A$ agonist)

Drug preparation and storage

Muscimol (Sigma, UK) was dissolved in 0.9% sterile saline, which gave a solution with a pH of around 7.5 without any adjustment (Table 2.4). Initially a 10 times (10X) concentration stock solution of 1 mg/ml was made up. The stock was filtered using a Millex GV 0.22μm sterile syringe filter unit (Millipore, USA) in a laminar flow cabinet. Further dilution and aliquoting was carried out in this sterile environment. All glassware, tips etc had been previously autoclaved. Behavioural efficacy of this dose in terms of intake was initially tested in a pilot study (not reported here) at a dose of 880ρmols/μl^{-1}, made up by further dilution of the 10X stock. This dose was chosen because it has been shown to cause a highly significant increase in chow consumption in previous experiments, being the most effective dose used by Stratford and Kelley (1997).

Observations of behaviour indicated significant motor impairment in animals given 880ρmols/μl^{-1}, of muscimol. Although muscimol is used as a sedative when administered peripherally it does not follow that it would have sedative effects when

administered in the accumbens. Nevertheless it has been reported that infusions of the drug may induce catalepsy, muscular rigidity and postural asymmetry or a more general reduction in motor activity (Turski et al., 1984, Anden et al., 1979). A range of more appropriate doses for this strain was used for further tests by serially diluting the 10X stock to give solutions of 55, 110, 220, 440 and 660ρmols/μl^{-1} concentration. Table 2.3 indicates how this range of doses compares with dose ranges tested previously by other laboratories. Aliquots were stored at -20^{o}C. The doses used for each set of tests are indicated in the relevant chapters.

Baclofen ($GABA_B$ agonist)

Drug preparation and storage

Baclofen (Sigma, UK) was dissolved in 0.9% sterile saline and the pH of the solution adjusted to around 7.5 using 1M sodium hydroxide (Table 2.4). Initially a 10 times (10X) concentration stock solution of 1.88mg/ml was made up and pHing was carried out prior to any further dilution of this stock. Further dilution and aliquoting was carried out in this sterile environment as described above. Intake was tested in a pilot study (not reported in thesis) at a dose of 880ρmols, which was made up from the 10X stock. This dose was chosen because it has been shown to cause a highly significant increase in chow consumption in previous experiments (Stratford and Kelley, 1997; Ward et al, 2000) and is equimolar to Stratford and Kelley's (1997) most effective dose of muscimol.

Observations of behaviour indicated significant motor impairment in animals given 880ρmols of baclofen and it has been reported that the drug may affect motor activity or, more specifically, have a myorelaxant effect when administered into the accumbens (Lorenc-Koci et al., 1994, Jelen et al., 1994, Wachtel and Anden, 1978). The range of doses that were consequently used in the rest of the experiments was 110, 220, 440 and 660ρmols/μl^{-1}. Table 2.3 indicates how this range of doses compares with dose ranges tested previously by other laboratories. Aliquots were stored at -20^{o}C. The doses used for each set of tests are indicated in the relevant chapters.

Table 2.3. Table to show molar equivalents of baclofen and muscimol doses used by other laboratories compared to doses used for this thesis.

Kelley and Stratford 1997		Ward et al. 2000	Reynolds & Berridge 2002	Zhang et al. 2003	Hanlon et al. 2004	Dose range used in this thesis		Molar equivalent
Baclofen (ng)	Muscimol (ng)	Baclofen (ng)	Muscimol (ng)	Muscimol (ng)	Muscimol (ng)	Baclofen (ng)	Muscimol (ng)	pmols
							6.25	55
						23.5	12.5	110
38	20							175
						47	25	220
74	40			40				350
						94	50	440
						141	75	660
188	100	188		100		188	100	880
			150					1320
376	200			200	200			1750

Bretazenil (benzodiazepine)

Drug preparation and storage

Bretazenil (RO16-6028, Roche, UK) was made up at a concentration of 1mg/ml in 0.9% saline with 1 drop of Tween 20 added for every 5ml of saline (Table 2.4). The solution was sonicated for ~ 45minutes to fully dissolve the drug. Bretazenil was administered i.p. at a volume of 1ml/kg and hence a dose of 1mg/kg body weight. The bottle was shaken well between animals as there was a tendency for the bretazenil to come out of solution and for fine sediment to settle at the bottom. Testing did not begin until 30 minutes post injection and during this time animals were returned to the home cage without access to their regular chow pellets. An appropriate volume of fresh drug solution was made up on each test day.

DAMGO (μ-opioid agonist)

Drug preparation and storage

DAMGO (Sigma, UK) was made up in 0.9% sterile saline (Table 2.4) in sterile conditions and filtered as described previously. Initially a dose of 0.25ng/μl DAMGO was chosen having reviewed the range of doses utilised by a number of other laboratories. This dose was appropriate for the BSS study (Chapter 3) but Kelley's

laboratory suggests that some locomotor depression may occur which impairs lever pressing but wears off prior to the timing of maximum drug effect on feeding 30-60 min post-infusion (Zhang and Kelley, 1997; Zhang et al., 2003). It was decided, therefore, that their lower dose of 0.025ng/μl would be more appropriate to increase lever pressing in the consummatory phase of the 2nd order schedule (Chapter 4) with no confounding locomotor impairment and no need for a pre-treatment period prior to testing. Aliquots were stored at -20^{o}C. The doses used for each set of tests are indicated in the relevant chapters.

Table 2.4. Chemical designations and suppliers of compounds used in this thesis.

Drug name	Chemical designation	Carrier	pHing to ~ 7.4	Supplier
Baclofen	(±)-Baclofen (±)-β-(Aminomethyl)-4-chlorobenzenepropanoic acid Lioresal	0.9% saline	+ 1M NaOH	Sigma UK (Poole)
Muscimol	3-Hydroxy-5-aminomethyl-isoxazole 5-Aminomethyl-3-hydroxy-isoxazole 5-Aminomethyl-3-isoxazolol	0.9% saline	NA	Sigma UK (Poole)
DAMGO	[D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin acetate salt Tyr-D-Ala-Gly-N-methyl-Phe-Gly-ol	0.9% saline	NA	Sigma UK (Poole)
Bretazenil	Ro 16-6028 9H-Imidazo[1,5-a]pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylic acid	0.9% saline Tween 20	NA	Roche UK

Data analysis and statistics

Although group housing was based on the order in which animals came out of the transport boxes, the order in which they were consequently allocated to experimental conditions, operated on and given infusions or injections of drugs was randomised using numbers generated in Excel. Animals were numbered and caged consecutively as they came out of the transport box but which test cage location or operant box in which they were consequently trained was randomised. Using fully counterbalanced, within subject experiments where each animal acted as its own control the day on which they underwent surgery was also allocated randomly. Animals that underwent surgery on different days were then randomly allocated to each dose group for the first day of an experiment. Each study followed a formal experimental design, for example a Latin Square design, and statistical analysis was planned in advance using appropriate reference texts to supplement the basic approach recommended by Festing et al., (2002) (Howell, 2007, Howell, 2001, Field, 2000). Basic analysis procedures and statistics are described below and, in further detail, in each data chapter.

All data were analysed in Genstat (version 9.1, VSN International, UK) using ANOVA with a repeated measures within subject design where appropriate and an alpha level of 0.05. Data sets were checked for homogeneity of variance and a normal distribution of residuals using SPSS 11 for Mac OS X. Where results departed from normality or unequal variances were identified the analysis was adjusted to take this into account. Post hoc analyses were carried out using the Dunnett's t test to compare treatments with the control condition. Where there was a reason to compare between treatments the Bonferroni test was applied. Any other statistical procedures used will be indicated in the data chapters.

Chapter 3

GABA receptor subtype stimulation in the accumbens: effects on the behavioural satiety sequence (BSS).

Introduction

In 1997 Stratford and Kelley reported that GABA agonists infused directly into the Acb shell (AcbSh) robustly increased intake of solid food and had "specific effects on feeding behaviour". This led to their suggestion that GABA-sensitive cells in this brain region may constitute an "important component of the central mechanisms controlling food intake". Both $GABA_A$ and $GABA_B$ receptors present in the Acb have, over the following decade, been amply demonstrated to play a role in the control of feeding (see introduction) but the exact nature of that role has yet to be firmly established. GABA receptor manipulation in the shell has been described as producing "behaviourally selective" effects on feeding (Stratford, 2007) but, as Kelley's hypothesis suggests, this may only represent a release of basic motor response components of the ingestive process (Kelley et al., 2005b, , 2005a).

In the introduction to this thesis I presented evidence that, although the orexigenic effects of GABA receptor agonists in the Acb are undisputed, there is actually little additional data published on the complete feeding behavioural profile or functional specificity of this manipulation. There is no research available on the macrostructure or microstructure of the behaviourally selective effects associated specifically with eating solid food. In particular, there is only a minimal amount of incidental information pertaining to the effects of intra-Acb GABA agonists on specific phases of feeding i.e. appetitive, consummatory and satiety stages (see Introduction for details). There is also no report that demonstrates a simultaneous assessment of the effects of GABA receptor agonists on total intake when food is freely available, feeding behaviours and non-feeding behaviours.

To date researchers appear to have relied on the evidence that the manipulation of endogenous levels of GABA and the blockade of glutamate transmission in the Acb also induces hyperphagia to support the assertion that the effects of GABA agonists on food intake are functionally significant and physiologically relevant (for a review see

Stratford 2007). Another compelling factor appears to be that stimulation of GABA sensitive cells does not increase in the intake of water or non-caloric solutions (Stratford et al., 1998, Basso and Kelley, 1999, Ward et al., 2000). It has also been demonstrated that intra-AcbSh GABA receptor stimulation has no effects on locomotor activity or motor coordination whilst animals are feeding (Stratford and Kelley, 1997b, Lopes et al., 2007). This certainly does not exclude the possibility that intra-Acb GABA receptor stimulation simultaneously disrupts the expression of other species specific non-feeding behaviours (Tallett et al., 2008).

When animals stop feeding it might not be a specific sign of satiety (Antin et al., 1975) given that they may do so to engage in other behaviours such as drinking, grooming, exploring, resting, social interaction and defensive behaviours (Bolles, 1960, Richter, 1922, Barnett, 1956) or because of other factors such as nausea or malaise (Blundell et al., 1985). It has long been recognised that an undisrupted meal progresses in a predictable manner. A complete temporal profile that directly compares multiple behaviours associated with the progression of a meal under the influence of central GABA agonists or vehicle could allow distinctions to be made between functionally specific and non-specific drug effects. For this thesis a well established time sampling method will be used to record such a behavioural profile, which is described in more detail below.

The transition from feeding to post-prandial behaviours is termed the Behavioural Satiety Sequence (BSS). Richter first described the predictable pattern of activity that follows feeding (the BSS) in 1922, observing that there was a distinct profile of behaviours displayed by his rats when given access to food. This was characterized by an initial period of feeding followed by a considerable amount of exploratory and general activity once feeding had terminated. Animals would spend much time grooming themselves and eventually would either cease all other activity and ‘rest’ or fall asleep. Richter designed a two-chamber cage with very basic pressure sensors attached to a smoke drum to record a trace representing activity in each chamber. In the smallest chamber food was accessible but not removable so that only feeding activity was recorded in this area. Recordings of activity in the other chamber were supplemented by observations of what the animal was actually doing during each period. His work highlighted the value of observing an extensive component or

repertoire of species-specific behaviour as well as measuring discrete indices of a response to external manipulation.

Bolles (1960) expanded on Richter's method by using a modified time-sampling procedure based on that described by Bindra and Blond (1958) to visually scan the behaviour of rats feeding on pellets in their home cage. By allowing the animals to manipulate and carry the food where they wanted to a more natural BSS could be recorded. He separated out observations into three categories of 'grooming' behaviour and four other categories: eating, drinking, sleeping and a general 'miscellaneous' category encompassing exploring, sniffing and 'being active'; active behaviours which could not be reliably distinguished by different observers (Bolles, 1960). The results indicated that grooming usually occurred "after other kinds of directed behaviour", for example after eating, and that it was "predictable from its behavioural context" (Bolles, 1960). Thus the idea of a predictable sequence of events following feeding began to emerge. It was not until the mid 1960's however that careful behavioural observations based on Richter's work were regularly used in conjunction with manipulations of feeding behaviour (Smith, 1997).

By 1974 Smith et al. were following up the concept suggested by Bolles that it was specifically the onset of satiety that was characterised by this clear sequence of behaviours and, in 1975, this group confirmed the existence of a "specific behavioural sequence of satiety" that could be manipulated by exogenous cholecystokinin (CCK), a gut hormone thought to be a mediator of satiety (Antin et al., 1975). The BSS, as it became known, was shown to develop with the gradual cessation of normal feeding but did not progress if the food did not reach the stomach or if it was made unpalatable using quinine (Antin et al., 1975, Liebling et al., 1975, Young et al., 1974, Antin et al., 1977). Halford (1998) suggests that the credibility of the BSS was established in particular through G.P. Smith's work, a strong proponent of Richter's seminal research (Smith, 2007). Antin et al. (1975) also used a modified time sample method to measure behavioural components of feeding in response to presentation with a liquid diet. Their behavioural categories were much more specifically defined than those employed by Bolles (1960) and inter-rater reliability was high (Antin et al., 1975).

It is now widely accepted that the BSS can be observed with the natural operation of satiety (Bolles, 1960) and better reflects the process than cessation of feeding alone (Blundell et al., 1985). The BSS is particularly sensitive to the organization of consummatory components of feeding and related behaviour patterns (Clifton and Cooper, 1996). The sequence is preserved but temporally shifted after treatments that enhance or decrease satiety and those that specifically affect appetite, for example, via palatability estimation (Halford et al., 1998). Observation of this consistent sequence of events in response to a meal is now regularly used as a tool to study the physiological relevance of peripheral and central manipulations of feeding on the basis that this pattern can be modified or even totally disrupted by various orexigenic and anorexigenic compounds (Halford et al., 1998).

In the context of this thesis two potential gross effects on the BSS will be considered. First of all, if the treatment causes a behaviourally selective increase in feeding but does not directly affect other behaviours a temporal shift of the whole BSS to the right is expected. All four behaviours specified in the methods section i.e. ingestion, activity, grooming and inactivity will most likely still be present but, the proportion of the latter three behaviours may be relatively reduced if they are yet to be fully expressed due to the delay in the onset of satiety. One might predict that, for example, if the BSS was very delayed resting behaviour may not appear within the standard test period.

In contrast to this temporal shift it might also be possible to see a significant enhancement or reduction in non-feeding behaviours such that the order of progression of the BSS is actually disrupted. For example if one or more of the behavioural components was robustly reduced or even absent the BSS could be described as being disrupted. The same would hold true if one or more of the behavioural components was significantly enhanced at the expense of the appearance of another behaviour and again the BSS would be described as disrupted.

There are some more subtle shifts that will be considered on a case by case basis when interpreting the results reported here, for example if the BSS is preserved but the timing of its onset rather than the termination of the meal is shifted. Nevertheless, where the BSS is described as “temporally shifted” or “disrupted” the meaning of these terms should be taken as they are described here i.e.: Temporal shift = presence of all four

behaviours but shifted to the right (with the possible loss of the final stages of the BSS i.e. inactivity); Disruption = Significant reduction or absence of one or more behaviours without effects on other categories or significant reduction or absence of one or more behaviours because of an increase in other categories. Care must be taken with this latter interpretation of disruption as, without extending the test period, this could represent a major temporal shift and this will be taken into account when interpreting the findings.

To illustrate what we would expect to see in a control group of animals maintained on an *ad libitum* diet but habituated to gaining direct access to fresh chow for a limited period each day, I have shown a "typical" BSS (see Fig. 3.1). This is based on the average proportions of behaviour recorded from a group of animals given a wet mash diet (from Halford et al. 1998). Pilot studies carried out prior to the experiments reported in this chapter indicated that animals given access to chow follow a similar BSS despite the smaller volume of food consumed. Indeed Antin et al. (1975) reported a recognisable BSS with a liquid diet and the value of using the BSS would be questionable if the general pattern only held for one meal type. Note that in Fig. 3.1. some grooming behaviour is present throughout the session but there is a trend towards an increase as feeding trails off.

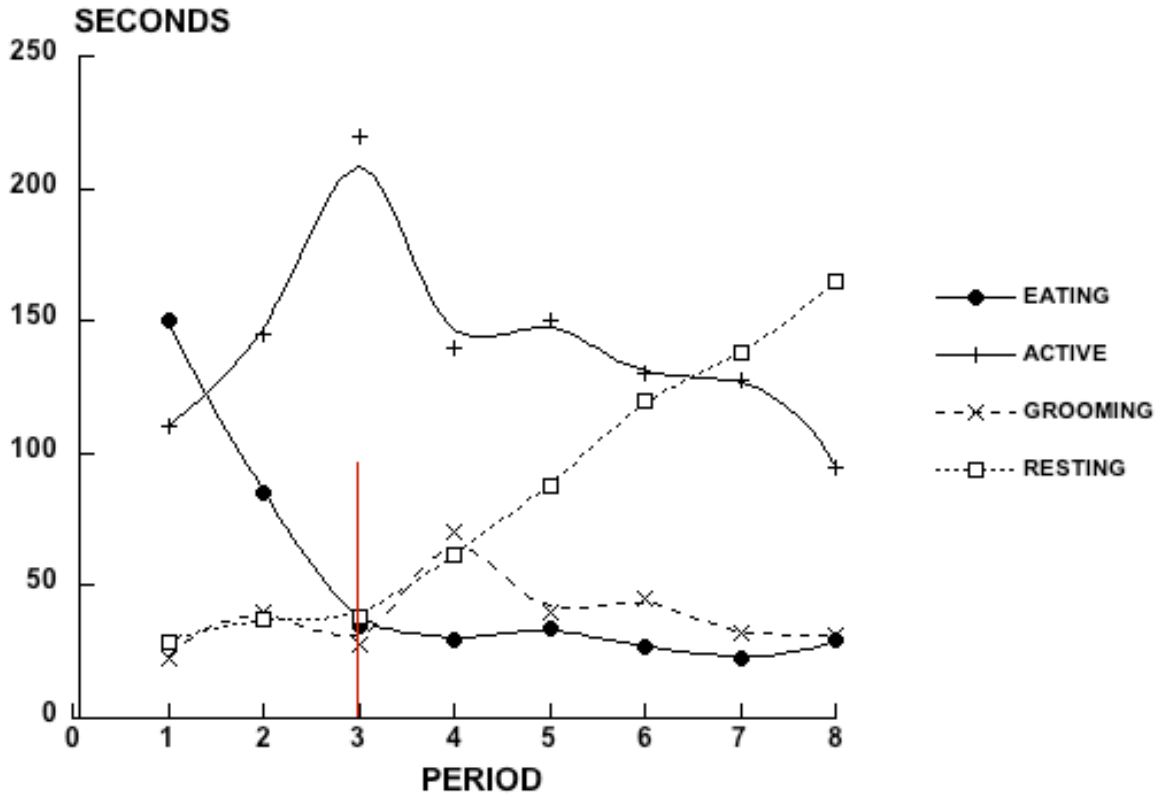


Figure 3.1. Example of a 'typical' behavioural satiety sequence (BSS) expressed by rats given access to a wet mash diet for 40 minutes (based on Figure 2, Halford et al. 1998). The vertical line indicates the point at which there is a transition from ingestion to inactive behaviour.

The methodological approach for recording the BSS has changed somewhat over the years. Although the behaviours recorded predominantly reflect the main categories described by Richter (1922) there appears to be no clear consensus on the precise definitions (refer to Table 2.1, Chapter 2, page 72). There also remain differences in opinion as to how the incidence of each behaviour should be recorded. In general, however, data is currently collected in one of two ways. First of all, total time spent engaging in each behaviour can be recorded after the event from videos/DVDs of test sessions using a continuous monitoring technique (e.g. Ishii et al., 2003a/b). The data is usually then parsed into time bins e.g. of 5 minutes and duration is plotted against period of test session.

The second method involves direct observation during the test session and recording of the current behaviour at discrete time points for a number of animals simultaneously (Clifton, 2005). Although, in essence, it is a form of one-zero sampling (such that a behaviour is absent or present at the sampling time) the instantaneous or scan sampling method (for groups) involves recording the current behavioural state of the animal assuming that the sample period is much shorter than the average duration of that behaviour (Altmann, 1974).

The method described by Clifton (2005) is also referred to as a Momentary Time Sampling (MTS) procedure (Powell et al., 1977). Altmann (1974) concludes that this method is particularly useful for estimating the proportion of time spent engaged in non-social behaviours using easily distinguishable categories, very short observation periods and evenly distributed sampling points. It has been suggested that the latter method permits an increase in statistical power because more subjects and treatment conditions can be handled in one study (Clifton, 2005) and this method is the one used regularly in this laboratory (See Chapter 2, page 70). This is the method described in detail in Chapter 2.

Halford et al. (1998) describe the BSS as a "powerful biobehavioural assay of drug action on appetite" and have employed this approach extensively within the laboratory at Leeds over the last two decades. Consequently the characterisation of the BSS elicited by the manipulation of $GABA_A$ or $GABA_B$ receptor function in the Acb constituted one of the primary methods used to answer the questions posed in this thesis. Building a record of the BSS is a useful approach to characterising the full profile of behaviours associated with ingestion induced by $GABA_A$ or $GABA_B$ agonists because the animals can express natural behaviour without restraint. The effects of both can be compared and it will also allow analysis of the microstructure of behaviours over time. The BSS should help to highlight any drug effects that are not specific to feeding.

One of the other key aims stated in the introduction, Chapter 1 (page 59), is to compare the effects of GABA receptor subtype stimulation in the Acb with other pharmacological manipulations believed to independently affect specific phases of ingestive behaviour. In this Chapter the actions of intra-Acb GABA agonists (which were introduced in detail in the introduction to this thesis); muscimol ($GABA_A$) and

baclofen ($GABA_B$) on the BSS are compared to those associated with intra-Acb injections of an opioid agonist, systemic administration of a benzodiazepine (both of which increase intake) and to natural effects of hunger induced by periods of fasting.

It was deemed prudent to include a drug comparison using a compound that also robustly increases intake (but via different mechanisms) when injected directly into the same region of the Acb as the GABA agonists. A large body of evidence would suggest that the release of the opioid peptide enkephalin and stimulation of opioid receptors within the striatum is associated with increased food intake (Kelley et al., 2002). In this case the obvious choice was a selective μ-opioid agonist. The effects of peripheral and central administration of these compounds has been widely studied (see Chapter 1, page 50) and, in particular, their effects in the AcbSh are well characterised. The μ-opioid receptor in the Acb has been implicated in increases in intake (Bakshi and Kelley, 1993b) particularly for highly palatable and preferred meals including sucrose, non-caloric saccharin, dilute saline solution and lipids (Mucha and Iversen, 1986, Kelley et al., 1996, Kelley et al., 2002, Zhang and Kelley, 2002, Zhang and Kelley, 2000, Zhang and Kelley, 1997).

DAMGO ([D-Ala2,N-Me-Phe4-Gly5-ol]-enkephalin) is a synthetic opioid peptide that has a high affinity for the μ-opioid receptor (Handa et al., 1981). It would appear that opioid signalling in the Acb promotes consummatory behaviours via the perceived palatability of the food and that these effects are amplified in previously more hedonically rewarding food types which subsequently reinforces responding in operant schedules and shapes food preferences (Gosnell and Patel, 1993, Hanlon et al., 2004, Bodnar, 2007, Bodnar, 2004). Nevertheless DAMGO also increases intake of chow (Bakshi and Kelley, 1993b) and would be expected to produce a BSS similar to that associated with enhancement of palatability e.g. by adding sucrose to the meal (Ishii et al., 2003b). This can then be directly compared to the BSS recorded with baclofen or muscimol to establish if there are any signs of appetitive processes being mediated by GABA in the Acb.

It has also been important to keep in mind that another aim was to elucidate how closely GABA receptor stimulation alone can been seen as necessary and adequate for the expression of naturalistic, physiologically driven feeding responses. Ishii et al. (2003)

point out that, to adequately interpret the effects of pharmacological manipulations on the BSS, it is important to use a reference profile of natural motivational manipulations of the behavioural repertoire. In this case an experiment will be included that will involve subjecting animals to systematically varied periods of fasting prior to a free-feeding test period with chow. The effects of hunger on the BSS have been demonstrated before using a mash meal (Ishii et al., 2003a) and, as with the control conditions, it is predicted that the BSS in response to chow following fasting will fit much the same general pattern.

Systemic BZs have also been shown to robustly and consistently increase intake via interactions with a variety of brain regions but probably not the Acb (Soderpalm and Berridge, 2000). The use of BZs could provide an interesting counterpoint to the data from the experiments using centrally administered drugs. The effects of BZs are also believed to be due to palatability enhancement (Cooper, 2005, O'Hare et al., 2006) as is the case with DAMGO but the structures involved are different to those that subserve the latter effect.

One of the difficulties with using BZs to study hyperphagia however is the prevalence of common and robust side effects such as sedation (Haefely et al., 1993) and the possibility that, at higher doses, full agonist effects increase intake due to anxiogenesis (and/or reduction in neophobia) rather than via effects on appetite (Shephard and Estall, 1984). Nevertheless it is now widely accepted that the hyperphagic effect is independent of other behavioural effects and is directly mediated by BZs (Cooper, 2005). The discovery of a range of compounds that act as partial BZ agonists led to the realisation that there is a sliding scale of efficacy for various behavioural measures with some partial agonists exhibiting a limited effect on motor responding but a robust effect on intake (Martin et al., 1993).

One of these partial agonists, bretazenil, is more behaviourally selective than the full agonists (Pieri et al., 1988, Martin et al., 1988, Haefely, 1984) and causes a robust increase in intake when administered peripherally (Yerbury and Cooper, 1987). Cooper (2005) goes as far as to quote Martin et al (1993) who say that it is “an example of an *appetite-selective* drug for which the hyperphagic activity can be completely dissociated from the major side-effects of full agonists”. The distinct profile of the BSS associated

with this increase in intake has been characterised, as have the longer term effects on meal patterning (Clifton and Cooper, 1996). Bretazenil also facilitates conditioned place preference (Di Scala et al., 1992) in a manner consistent with a number of other orexigenic compounds (Spiteri et al., 2000, Tzschentke, 1998, Schechter and Calcagnetti, 1998). As such it is an ideal candidate as a point of comparison between hyperphagic effects that are behaviourally specific and attributed to enhanced appetite mediated by structures other than the Acb and the effects of GABA agonists that may or may not encompass an appetite-enhancing component.

The results reported in this Chapter will begin, therefore, to address four of the key aims of this thesis. The first two experiments, 3.1 and 3.2, will provide a record of the BSS elicited by intra-Acb baclofen or muscimol which will contribute to the characterisation and comparison of the effects of GABA receptor subtype stimulation on feeding related behaviours. Both of these experiments will also test the hypothesis put forward by Kelley and colleagues that GABA receptor stimulation in the Acb simply results in increased intake because of the consequent release of downstream motor pattern generators. On the basis of their hypothesis it would be predicted that the increase in motor behaviours specifically directed at food consumption could limit the expression of the rest of BSS behavioural repertoire in both cases.

In experiments 3.3 and 3.5 the use of other pharmacological manipulations i.e. intra-Acb DAMGO and peripheral bretazenil will help to elucidate whether the effects of GABA receptor stimulation are specific to different phases of ingestion e.g. anticipatory responding, consummatory behaviour and satiety mediated by the Acb. By comparing hyperphagia induced by intra-Acb baclofen and muscimol with that induced by hunger in experiment 3.4 it will help to put the role of GABA in the context of naturally elicited feeding and motivational control in general.

The final experiment compares the effects of testing a peripherally administered drug with a chow meal (as used in the first five experiments) or a mash meal. This experiment was carried out because, as will be demonstrated in experiment 3.5 below, the BSS recorded with bretazenil and chow does not mimic that previously reported for bretazenil and a mash meal. This could raise questions about the use of chow as the test diet in the first five experiments, which will be discussed at the end of this chapter.

To summarise, previous research shows that rats presented with a familiar and freely accessible meal exhibit a distinct profile of behaviour as they eat. Initially their behaviour is dominated by ingestion, and then succeeded by periods of activity, grooming and finally rest. The pattern of this sequence is preserved but temporally shifted in specific ways by experimental manipulations of initial appetite, palatability or satiety (Ishii et al., 2003a, Halford et al., 1998, Ishii et al., 2003b). The sequence is disrupted by drugs that reduce intake through nausea, general malaise, sedation or hyperactivity (Halford et al., 1998). The six experiments reported in this chapter:

1) Characterise and compare the effects of $GABA_A$ and $GABA_B$ agonists on feeding related behaviours (Experiments 3.1 and 3.2). Do the GABA agonist subtypes produce similar BSS profiles with a chow meal?
2) Test the hypothesis that the effects are due to the release of downstream motor pattern generators (Experiments 3.1 and 3.2). Do the GABA agonist subtypes shift or disrupt the BSS?
3) Compare the effects of GABA agonists in the Acb to those of other drugs injected into the same region and known to affect specific phases of ingestive behaviour (Experiment 3.3). Do the GABA agonist subtypes enhance appetite, increase consummatory behaviour or delay satiety?
4) Put these results in the context of the known effects of hunger, and hence, the broader category of motivated behaviour (Experiment 3.4). Do the effects of the GABA agonist subtypes mimic the effects of hunger on the BSS?
5) Compare the effects of Acb receptor manipulation with peripheral drug effects also known to affect specific phases of ingestive behaviour but via different mechanisms (Experiment 3.5). Do the effects of GABA subtype agonists represent an Acb specific control of feeding or a more general effect mediated by multiple brain regions?

6) Begin to address potential issues raised by using different meal types to characterise a BSS (Experiment 3.6).

On top of these key questions the overarching question asked in this chapter is:

Does intra-Acb administration of the GABA receptor subtype agonists baclofen and muscimol modify responses to food in a manner consistent with behaviourally selective effects on appetite and/or satiety rather than simply via the release of downstream motor components that perpetuate the physical act of consumption?

Experiments presented in this chapter

Experiment 3.1
BSS with chow following bilateral intra-Acb infusions of the $GABA_B$ agonist baclofen.

Experiment 3.2
BSS with chow following bilateral intra-Acb infusions of the $GABA_A$ agonist muscimol.

Experiment 3.3
BSS with chow following bilateral intra-AcbSh infusions of the μ-opioid agonist DAMGO.

Experiment 3.4
BSS with chow following periods of food restriction (4 and 8 hours).

Experiment 3.5
BSS with chow following peripheral administration of the benzodiazepine bretazenil.

Experiment 3.6
BSS with mash following peripheral administration of the benzodiazepine bretazenil.

Materials and methods

Animals

A group of drug naïve animals (n=12) were used for Experiment 3.1 (BSS with baclofen). After a break the same group were then run through the BSS test again for experiment 3.3 (BSS with DAMGO). Experiment 3.2 (BSS with muscimol) was carried out whilst measuring dose effects on intake in a group of animals that had previously undergone testing in a 2nd order operant experiment (experiment 5.2, Chapter 5). A second group of drug naïve animals (n=12) was used for experiments 3.4 (BSS with fasting) and 3.5 (BSS with bretazenil). These two experiments were run together using a counterbalanced design (see procedures below). Finally, a third batch of drug naïve animals (n=12), were used for experiment 3.6 (BSS with bretazenil and mash meal). Figure 3.2. illustrates the order in which groups of animals were used. Subjects were bought in weighing between 250-275g.

Given the relatively short period of habituation required before surgery the first naïve group brought in for experiments 3.1 / 3.3 were housed singly in Perspex cages upon arrival. For experiment 3.2 animals that came from the 2nd order experiment (carried out in experiment 5.2, Chapter 5) were already housed in Perspex cages. The second naïve cohort used for both experiments 3.4 and 3.5 (that did not undergo surgery) were initially housed in groups of three in standard cages then transferred to standard single cages for 7 days prior to testing. The third and final naïve group used for the systemic drug administration in experiment 3.6 were housed singly in standard cages upon arrival.

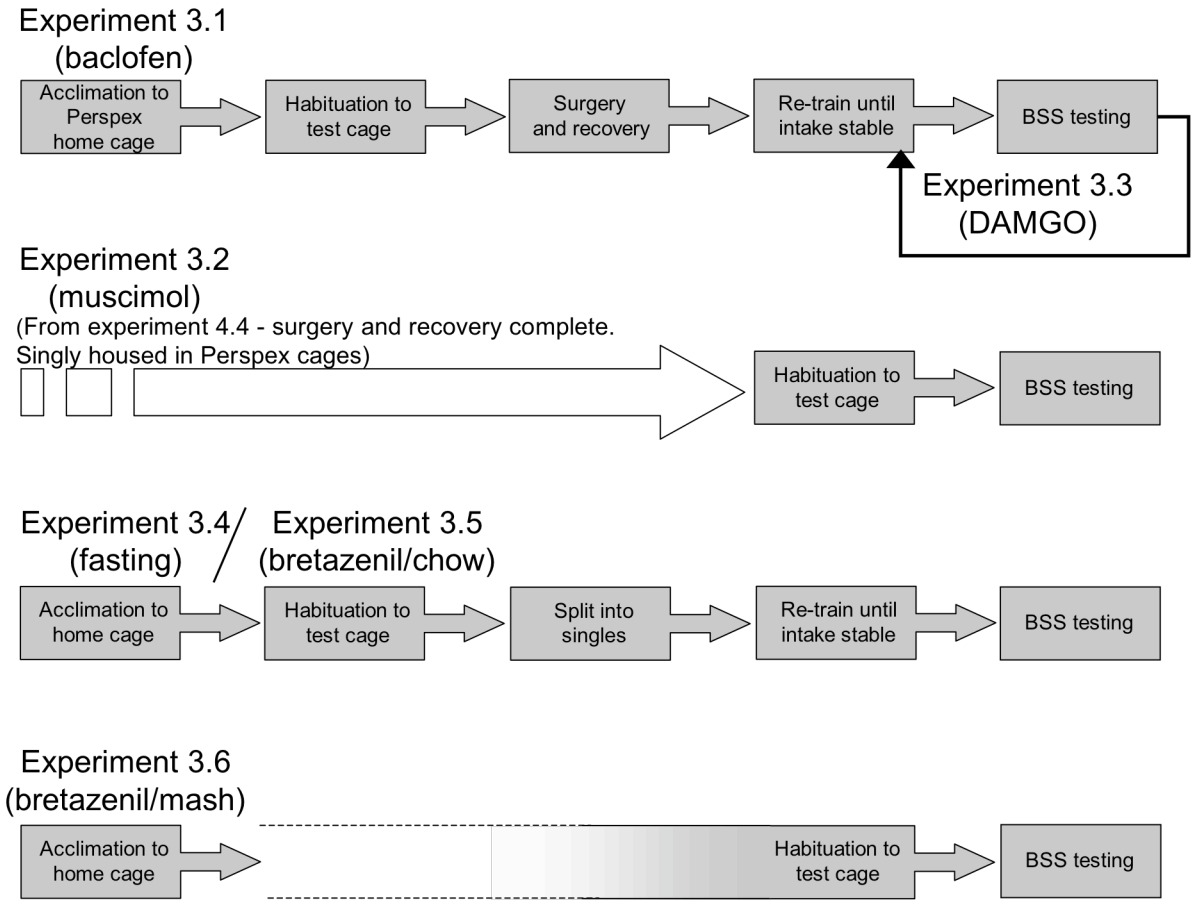


Figure 3.2. Diagram to show which groups of animals were used for more than one test procedure within experiments 3.1 - 3.6 and the timeline for each experiment.

Apparatus and habituation

The apparatus and habituation procedure for the BSS experiments are described in detail in Chapter 2 (page 70). Irrespective of their origin (naïve from Harlan or used following on from a previous experiment) animals were habituated to the test cages and testing procedure in the same way. The only caveat is that the animals in experiment 3.2 (BSS with muscimol) were habituated whilst they were still food restricted for the 2nd order experiment. They received 5 fresh pellets at the same stage in the habituation procedure as all other groups so as to reduce intake to a low baseline level (refer to training and testing timeline in Fig. 2.2, Chapter 2, page 70). Groups that did not undergo surgery were given an extra four days habituation to parallel the re-training period that postoperative animals received (indicated by the extended habituation phase depicted in Fig. 3.2). In experiment 3.6 (BSS with bretazenil and mash) animals were given access to a glass pot of mash in the home cage for 2 days prior to introduction of

this meal in the test cage during habituation. Animals undergoing infusions in experiments 3.1, 3.2, and 3.3 were habituated to the procedure as described in Chapter 2, (page 67). Animals in experiments 3.4/3.5 and 3.6 were habituated to receiving i.p. injections using a 0.9% saline sham on two occasions prior to testing.

Experiments 3.1 – 3.5 tested the BSS in response to standard laboratory chow pellets (10 fresh pellets in a glass pot) whilst in 3.6 the BSS with wet mash was tested. Testing procedures for the BSS experiments are described in Chapter 2, (page 71). In all cases a within subject, counterbalanced, Latin square design was used such that each subject acted as its own control. Animals were not put in test cages between test days.

Specific procedural details – central drug administration

Only two animals were tested at a time when drugs were centrally infused (experiments 3.1, 3.2 and 3.3) to compensate for the time taken for each infusion. The total test period for central experiments was 30 minutes so that all 6 pairs could be tested over as short a period as possible between 10:00 and 16:00. This meant that behaviour for each of the two animals was recorded every 2.5 seconds and therefore a total of 360 observations were recorded for each. There was a minimum of 48 hours between infusions.

Experiment 3.1

In experiment 3.1 animals were infused with a dose range of 110, 220 and 440 ρmols/μl^{-1} baclofen or vehicle (0.9% sterile saline – used throughout) immediately before being transferred to the test cages. After testing with baclofen these animals were given a few days drug free and then this experiment was followed by a BSS test using DAMGO (see details for Exp. 3.3 below).

Experiment 3.2

In experiment 3.2 a dose range of 220, 440, 660 ρmols/μl^{-1} muscimol plus vehicle was tested. This higher dose range was used because this was the range that had been previously tested in the 2nd order experiment. The BSS data were collected at the same time as measuring the effect of this dose range on total intake.

Experiment 3.3

In experiment 3.3 only one dose of 0.25ng/μl^{-1} of DAMGO or vehicle was tested. This dose has previously been shown to be behaviourally effective in terms of increased intake when infused into the Acb (Hanlon et al., 2004).

Specific procedural details – other BSS studies

The test period for systemic experiments or those using physiological manipulation was 40 minutes as used in this laboratory previously (Clifton et al., 1989). In this case, where animals were tested as a cohort of twelve, each animal's behaviour was observed every 30 seconds and 80 observations were recorded in total for each. For experiments 3.4 (BSS with fasting) and 3.5 (BSS with bretazenil) a counterbalanced design was used so that each animal experienced both fasting and bretazenil treatment categories as well as the control condition over the course of testing. There was a minimum of 72 hours between i.p. injections.

Experiment 3.4 and 3.5

In these experiments animals received five possible treatment options encompassing either bretazenil doses or periods of fasting. The treatment categories were:

1) vehicle + no fast = control condition
2) vehicle + 4hr fast = 1st condition Exp. 3.4
3) vehicle + 8hr fast, = 2nd condition 3.4
4) 0.3mg/kg^{-1} bretazenil + no fast = 1st condition Exp. 3.5
5) 1.0mg/kg^{-1} bretazenil + no fast. = 2nd condition Exp. 3.5

For the fasting treatment categories all food was removed at a) 7.30am and animals were tested at 15.30 (8 hour fast) or b) 11.30am and animals were tested at 15.30 (4 hour fast). Thirty minutes prior to recording the BSS fasted animals or un-fasted control animals were given a vehicle injection i.p.. Those animals receiving bretazenil were also injected at this time. Note that in every condition animals received an injection even if it was only vehicle to ensure that the level of stress experienced was equivalent.

Experiment 3.6

In experiment 3.6, the BSS in response to mash was tested with vehicle or 1.0mg/kg^{-1} bretazenil only.

Data analysis

The final analysis groups were decided on the basis of histological verification of infusion sites. The cumulative data for the proportion of time point samples represented by each behaviour were extracted for these animals from the raw output data then amalgamated into five minute time bins using Unix Shell scripts. The results parsed into time bins provided 60 recording of behaviour per animal per bin in experiments 3.1, 3.2 and 3.3. For experiments 3.4/3.5 and 3.6 there were 10 recordings of behaviour per animal per bin.

The mean proportion of each behaviour in each time bin and the relative changes in these proportions over time were compared between doses using a repeated measures, mixed design ANOVA with dose, behavioural category and time-bin as factors. The effects on total intake was analysed using a within subjects, repeated measures ANOVA with dose as factor. Data were plotted as stacked columns for each 5 minute bin to show the relative proportions of each behaviour and the same data were also represented in line plots to better illustrate the change in the proportion of individual behaviours across the test session.

Results

***Experiment 3.1*: BSS with chow following bilateral intra-AcbSh infusions of the $GABA_B$ agonist baclofen at 110, 220 & 440ρmols/μl $^{-1}$.**

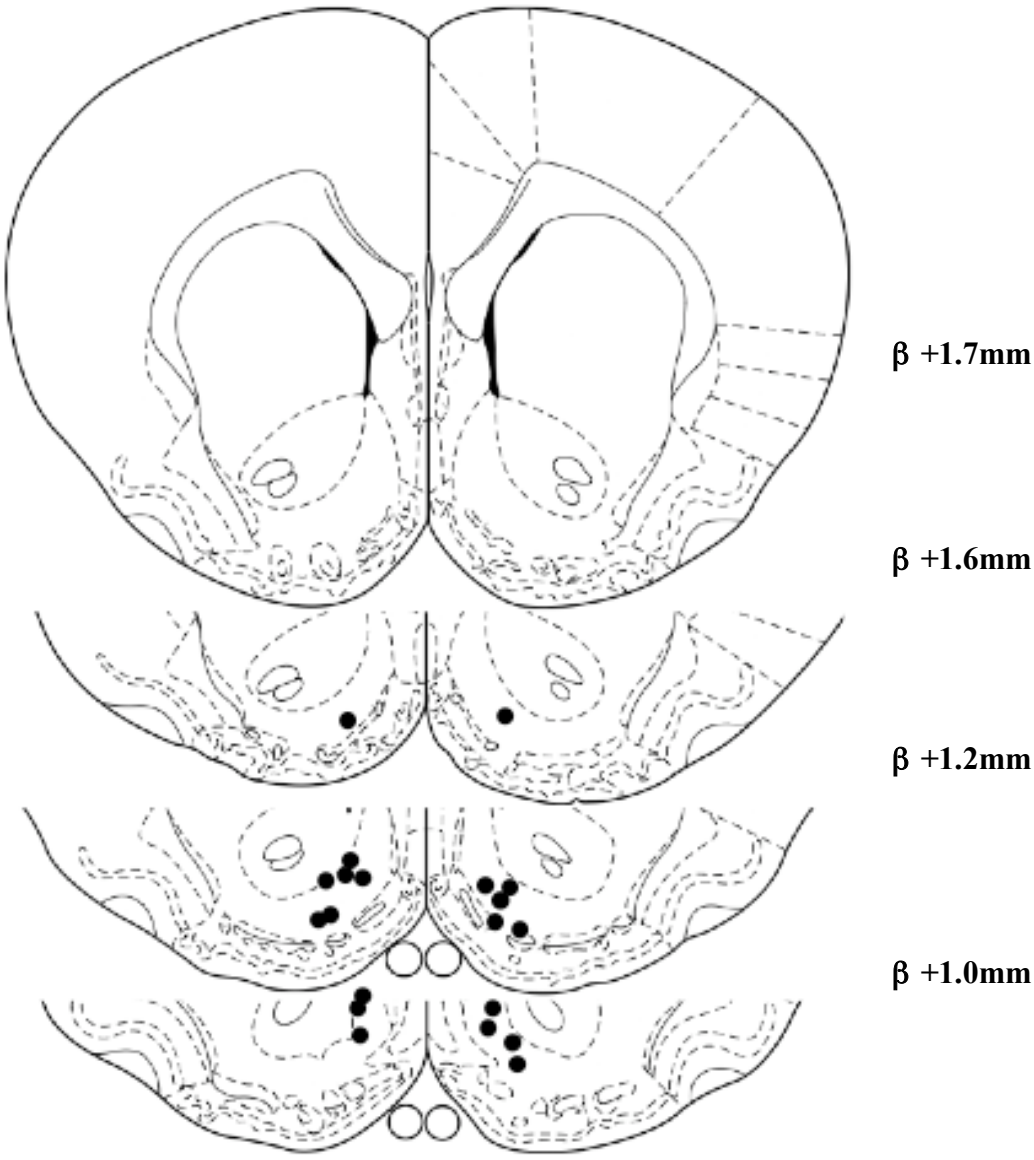


Figure 3.3. Injection sites plotted on drawings taken from Paxinos and Watson (1998); sections are anterior relative to bregma. Bilateral target coordinates (n=10) were (AP), + 1.2mm, mediolateral (ML), ± 1.5mm relative to bregma and dorsoventral (DV), -7.8mm relative to skull surface.

A schematic illustration of Acb infusion site placements is given in Fig. 3.3. A total of n=10 animals were found to have placements that fell within the acceptable target area defined in Chapter 2 (page 68) .

Baclofen infused into the Acb of pre-satiated animals dose dependently increased chow intake relative to intake with vehicle [Fig. 3.4; $F(3,27) = 15.48$, $p < 0.001$], significantly so at 110ρmols/μl^{-1} ($p < 0.05$), 220 and 440ρmols/μl^{-1} ($p < 0.001$).

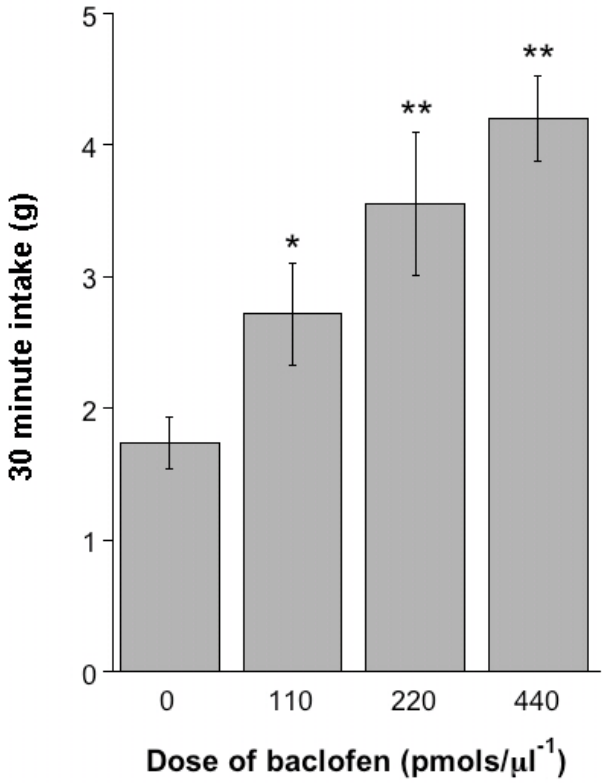


Figure 3.4. The effects of bilateral infusions of saline and a range of doses of baclofen into the Acb in pre-fed rats (n=10) given access to laboratory chow over a 30 minute test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by * p<0.05, ** p < 0.01.

The BSS was also affected in a dose dependant manner (Fig. 3.5). Overall there was a highly significant interaction between drug and time manifested as an increase across the early part of session for the category 'Ingest' [$F(15,135)=1.87$, $p=0.032$]. The significant interaction for the 'Active' category [$F(15,135)=3.78$, $p < 0.001$] reflected both a decrease and consequent increase, and the 'Inactive' category saw a reduction [$F(15,135)=8.64$, $p < 0.001$]. There was no significant effect on grooming.

Planned post-hoc analysis using Dunnett's test indicated that the increase in feeding behaviour was only significant 5-10 minutes into the session at the lowest dose of 110ρmols ($p < 0.05$), between 0-15 minutes at 220ρmols (0-5 minutes, $p < 0.05$; 5-15 minutes, $p < 0.01$) and throughout the last 25 minutes of the session at the highest dose of 440ρmols (5-15 minutes, $p < 0.01$; 15-20 minutes, $p < 0.05$; 20-25, $p < 0.01$; 25-30 minutes, $p < 0.05$).

Active behaviour was significantly lower during the first 0-15 minutes at doses of 220ρmols (0-10 minutes $p < 0.01$ and 10-15 minutes, $p < 0.05$) and 440ρmols (0-15 minutes, $p < 0.01$) of baclofen. It was also lower during this period at 110ρmols but only significantly so between 5-10 minutes ($p < 0.05$). There was no significant difference in activity at any dose between 15-25 minutes but the proportion was slightly but significantly higher in the last 5 minutes at 220ρmols ($p < 0.05$).

Inactive behaviour was almost completely absent over the entire 30 minute test period at all three doses. In the last 10 minutes inactive behaviour was significantly lower than with vehicle at all doses of baclofen (20-30 minutes, $p < 0.01$). Fig 3.6 shows that the transition from feeding to inactivity occurred between 15 and 20 minutes (but closer to 20 minutes) with vehicle. There was no transition within the 30 minutes with drug treatment.

Whilst recording the BSS it was noted that animals treated with baclofen increasingly engaged in some additional behaviour including consumption of sawdust or faeces and licking the floor for crumbs, particularly at the highest dose. This was recorded as part of the 'Ingest' category.

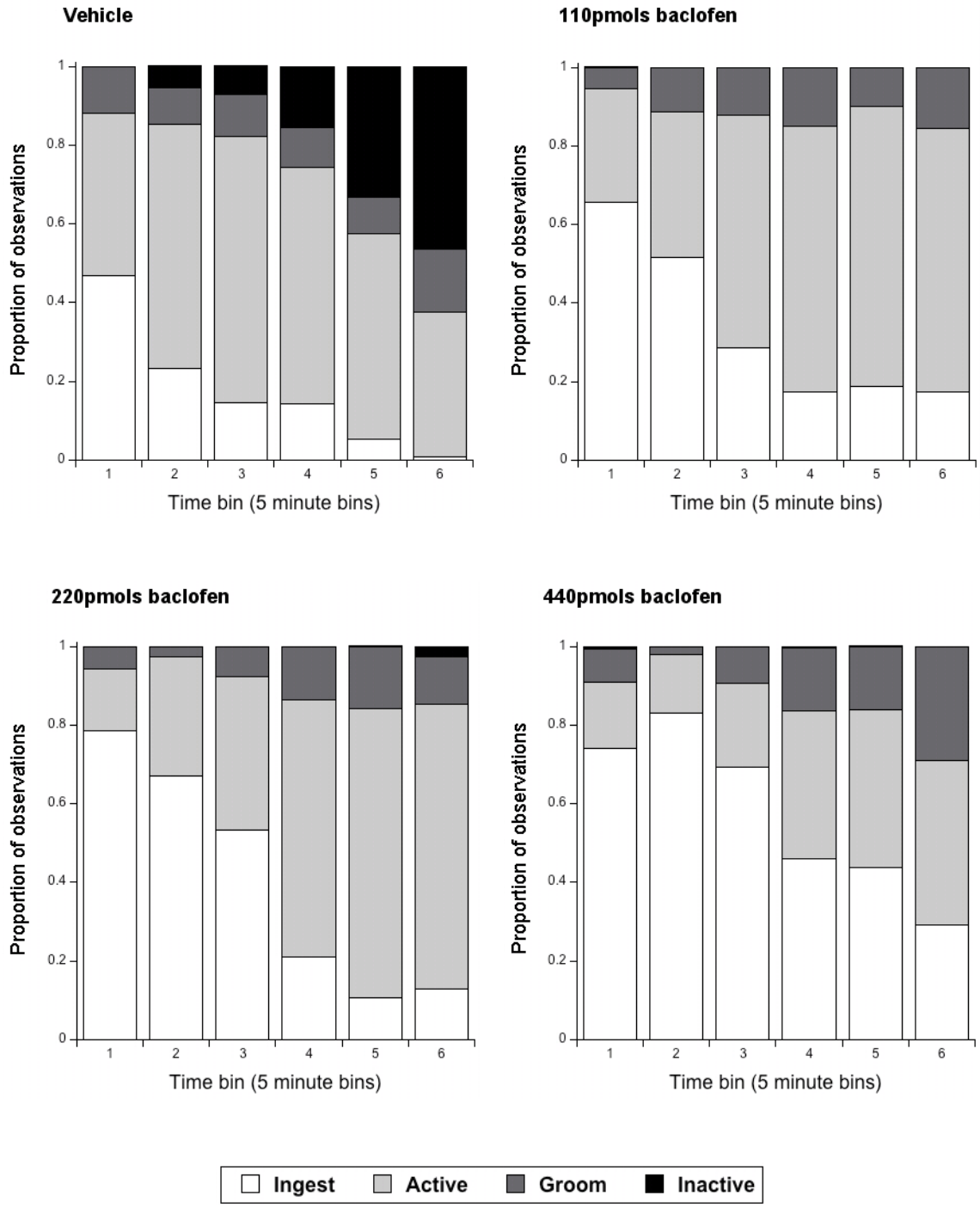


Figure 3.5. The behavioural satiety sequence (BSS) with a range of doses of baclofen compared to vehicle (n=10). The relative proportions of each behaviour are shown in 5 minute time bins over a 30 minute period of free access to chow.

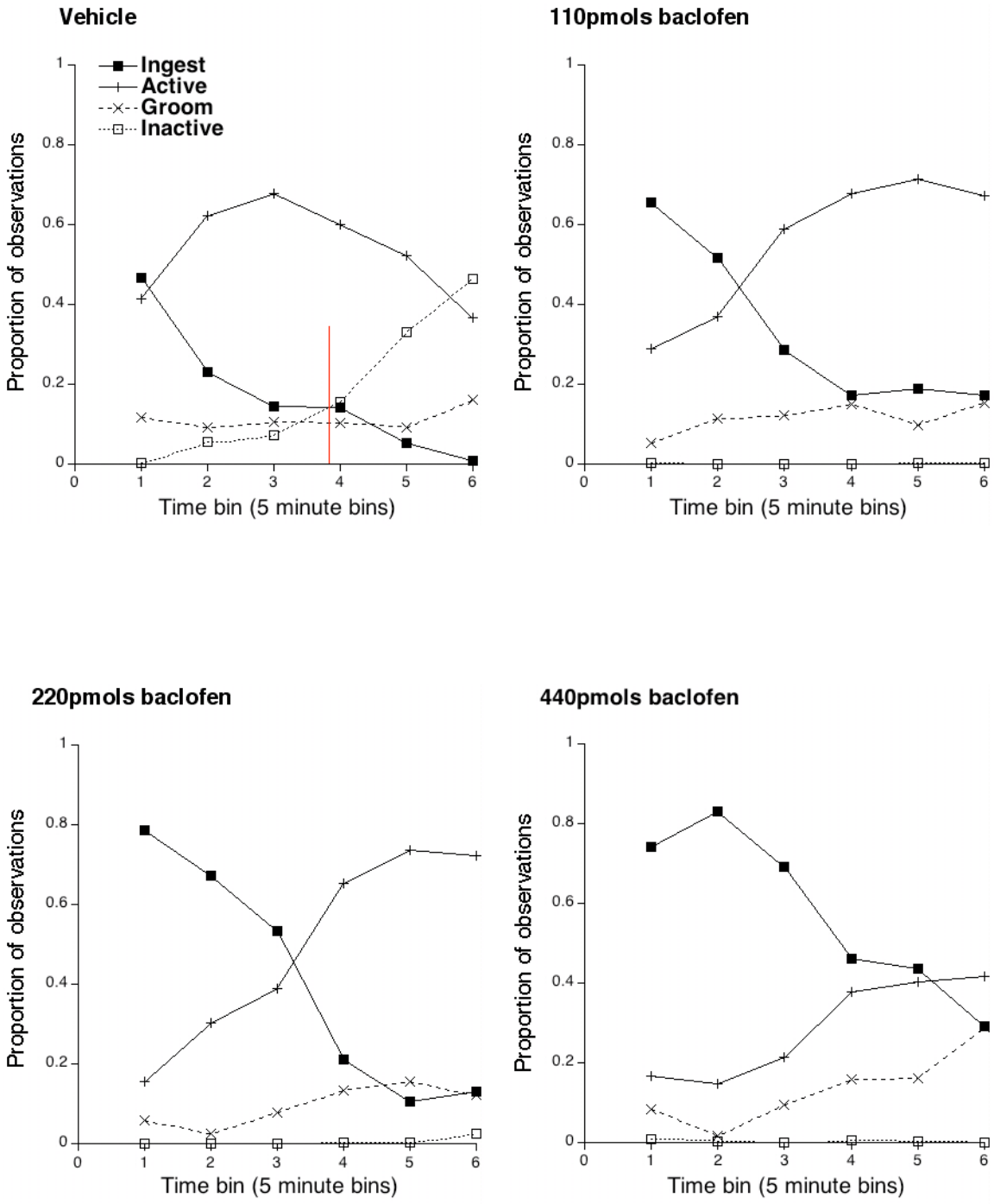


Figure 3.6. The effects of various doses of baclofen compared to vehicle on the time course of ingestive, active, grooming and inactive behaviours split into 5 minute bins (n=10). The vertical line on the 'vehicle' chart indicates the point at which there is a transition from ingestion to inactive behaviour. This does not occur within the 30 minute time frame at any of the doses of baclofen.

***Experiment 3.2*: BSS with chow following bilateral intra-AcbSh infusions of the $GABA_A$ agonist muscimol at 220, 440, 660ρmols/μl^{-1}.**

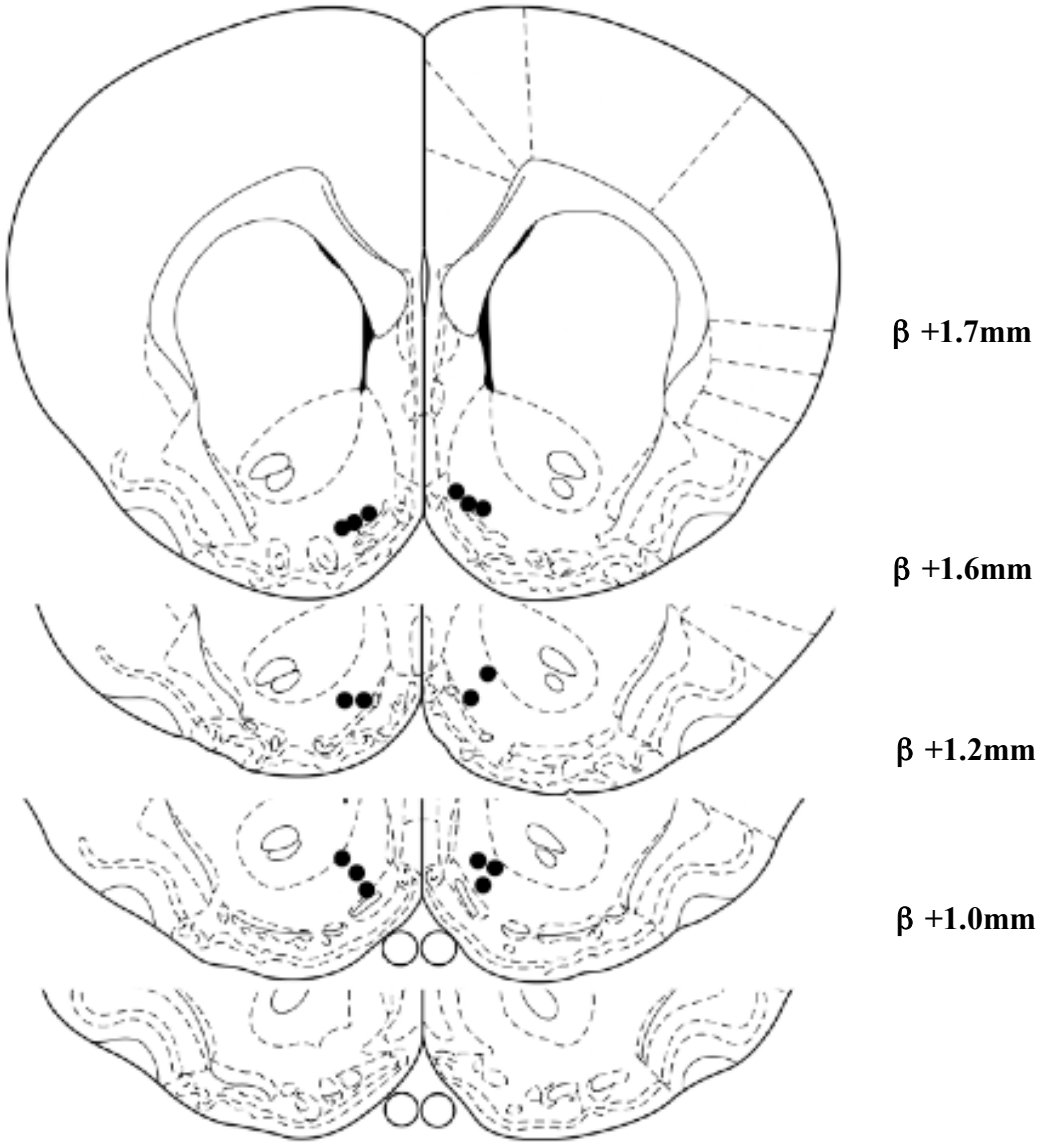


Figure 3.7. Injection sites plotted on drawings taken from Paxinos and Watson (1998); sections are anterior relative to bregma. Bilateral target coordinates (n=8) were (AP), + 1.4mm, mediolateral (ML), ± 0.9mm relative to bregma and dorsoventral (DV), -7.8mm relative to skull surface.

A schematic illustration of Acb infusion site placements is given in Fig. 3.7 A total of n=8 of the original n=12 animals were found to have placements that fell within the acceptable target area defined in Chapter 2 (page 68).

Muscimol infused into the Acb of satiated animals increased chow intake relative to intake with vehicle [Fig. 3.8; $F = 3,20(1)$, $p < 0.001$], significantly so at all doses ($p < 0.001$) but there was no difference in the total amount of chow consumed between doses.

The BSS was significantly different from vehicle with all drug treatments but, again, there was no apparent dose dependant effect (Fig. 3.9). Overall there was a highly significant interaction between drug and time manifested as an increase in the category 'Ingest' [$F (15,100(5))=2.01$, $p=0.021$], across the session. The interaction for the 'Active' category [$F (15,100(5))=3.09$, $p < 0.001$] reflected a decrease as it did for the category 'Inactive' [$F (15,100(5))=4.32$, $p < 0.001$]. There was an effect of drug on grooming [$F(3,20(1))=15.2$, $p < 0.001$] but there was no interaction between drug and time.

Planned post-hoc analysis using Dunnett's test indicated that the increase in feeding was significant at all doses across all time bins ($p < 0.01$). Active behaviour was significantly lower at all doses for the first 20 minutes (0-20 minutes, $p < 0.01$ at all doses). There was no significant difference in activity at any dose during the last 10 minutes. Inactive behaviour was significantly lower at all doses in the first 5 minutes ($p < 0.05$). There was no inactive behaviour at any dose between 5–15 minutes, no significant difference at 15-20 minutes and it was significantly lower than with vehicle at all doses in the final 10 minutes ($p < 0.05$). The lack of interaction between drug and time for grooming behaviour reflects a proportional decrease across every time bin with all three doses of muscimol.

Fig. 3.10 shows that the transition from feeding to inactivity occurred between at approximately 20 minutes with vehicle. There was no transition within the 30 minutes with drug treatment. Whilst recording the BSS it was noted that animals treated with muscimol were in contact with the food much of the time although not necessarily eating. Additionally animals forced so much food into their mouths that they would

often chew and gag for long periods before collecting the next pellet, animals appeared to be less aware of their surroundings or non responsive to cues that were not in their immediate vicinity. They were seemingly oblivious to the observer. This was not true for animals previously observed under the effects of baclofen.

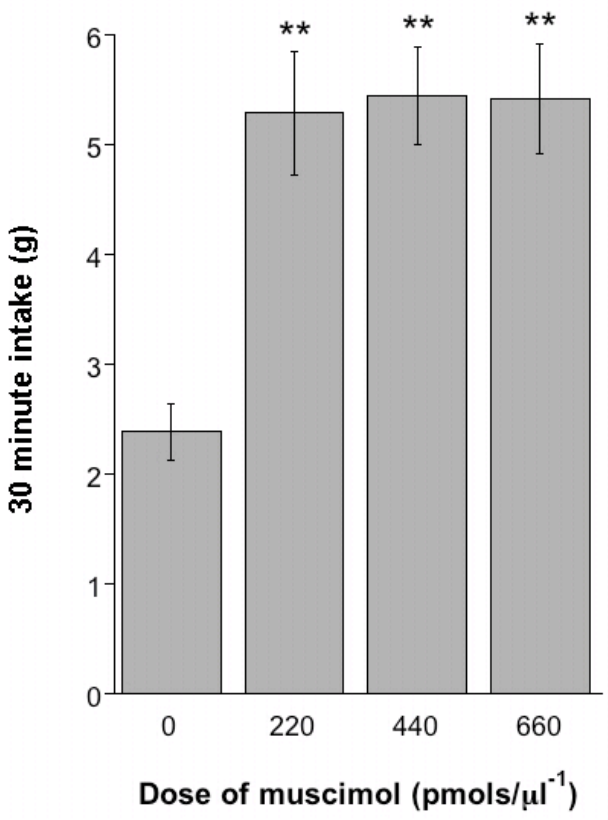


Figure 3.8. The effects of bilateral infusions of saline and a range of doses of muscimol into the Acb in pre-fed rats (n=8) given access to laboratory chow over a 30 minute test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ** $p < 0.01$.

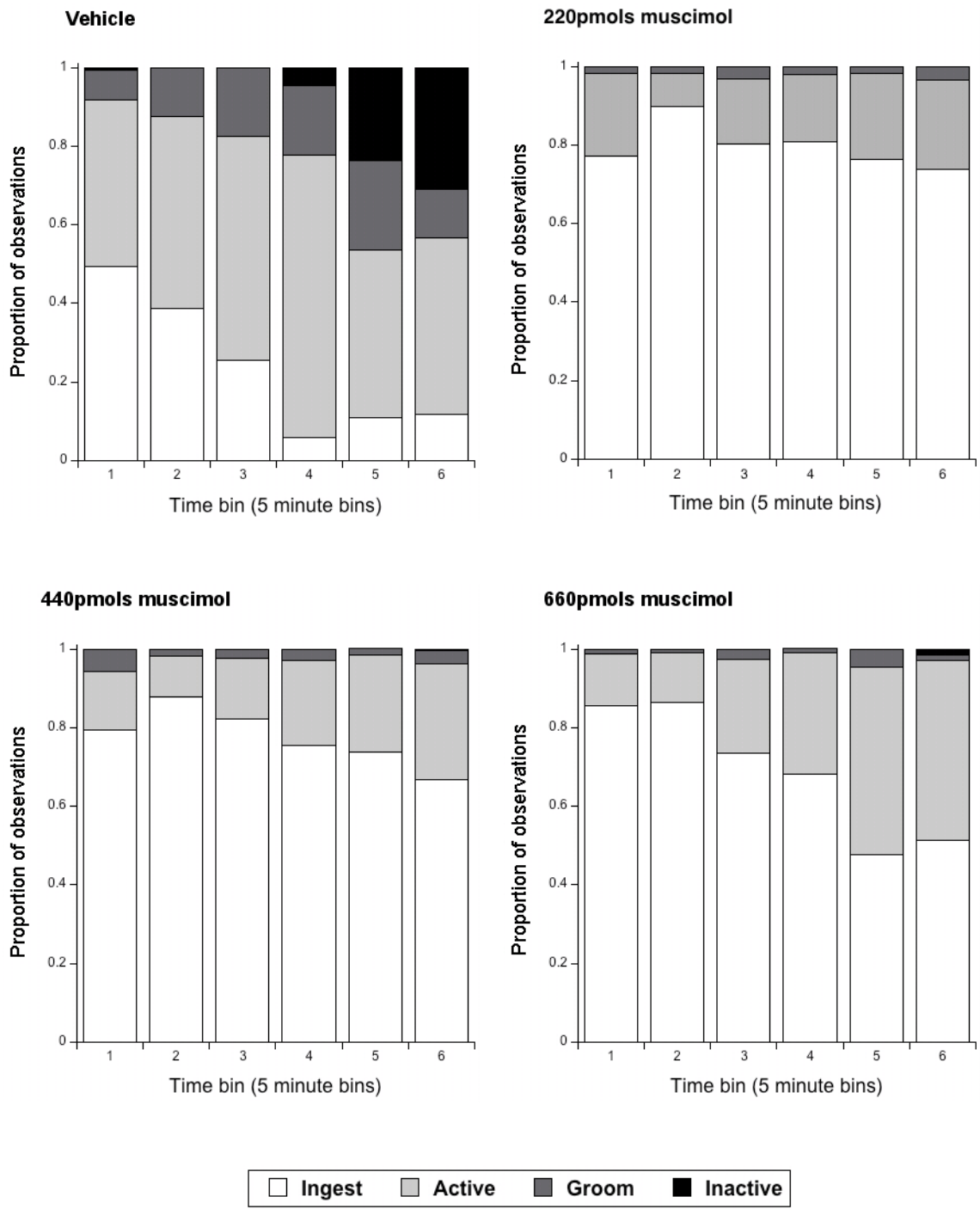


Figure 3.9. The behavioural satiety sequence (BSS) with a range of doses of muscimol compared to vehicle (n=8). The relative proportions of each behaviour are shown in 5 minute time bins over a 30 minute period of free access to chow.

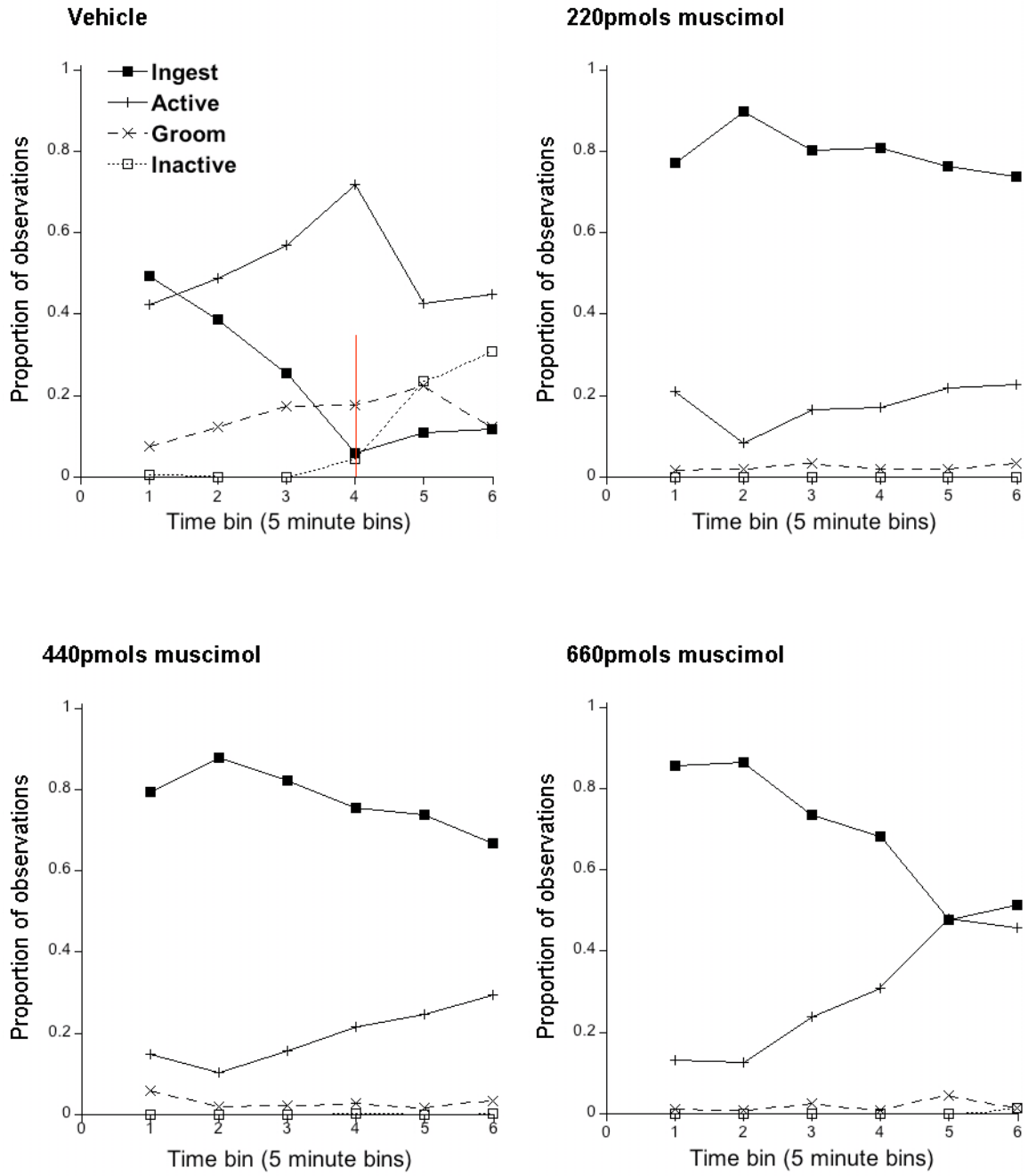


Figure 3.10. The effects of various doses of muscimol compared to vehicle on the time course of ingestive, active, grooming and inactive behaviours split into 5 minute bins (n=8). The vertical line on the 'vehicle' chart indicates the point at which there is a transition from ingestion to inactive behaviour. This does not occur within the 30 minute time frame at any of the doses of muscimol.

Experiment 3.3: BSS with chow following bilateral intra-AcbSh infusions of the μ-opioid receptor agonist DAMGO (0.25ng/μl^{-1})

Refer to Fig. 3.3 experiment 3.1 for a schematic illustration of Acb infusion site placements. A total of n=7 animals from the original 10 that were found to have placements that fell within the acceptable target area were used in this experiment. One animal from the group used for the final analysis in experiment 3.1 was eliminated from this experiment because one of the guide cannulae became blocked. Two others were eliminated because they were unable to feed or express any other behaviours due to an apparent sedative effect post infusion. In both cases the drug may have ended up more posterior unilaterally than the average placement seen in the other animals included in the analysis.

DAMGO infused into the Acb of satiated animals increased chow intake relative to intake with vehicle [Fig. 3.11; F (1,6) =12.84, p=0.012].

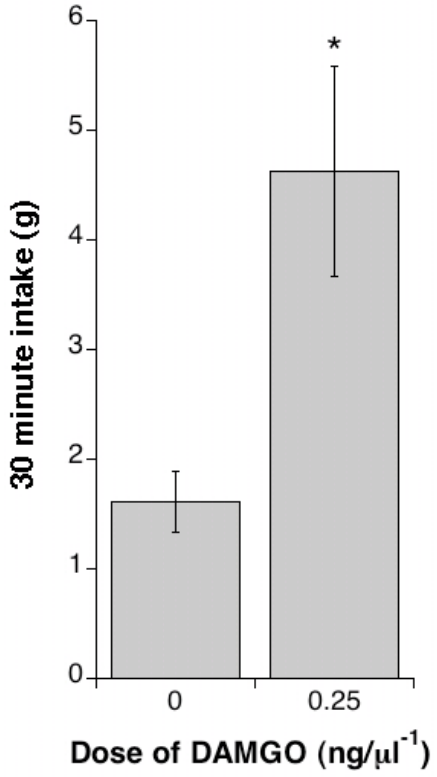


Figure 3.11. The effects of bilateral infusions of saline or DAMGO into the Acb in pre-fed rats (n=7) given access to laboratory chow over a 30 minute test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by * p<0.05.

The BSS was significantly modified by bilateral infusion of DAMGO (see Fig. 3.12). There was no significant effect of drug on 'Active', 'Groom' or 'Inactive' behavioural categories. However, there was a significant effect of drug [$F(1,6)=12.43$, $p=0.012$] and time [$F(5,30)=8.16$, $p < 0.001$] on the 'Ingest' category. This was reflected in a significantly higher proportion (relative to that elicited by vehicle) of this behaviour in the last 15 minutes of the session (15-20 minutes, $p < 0.01$; 20-30 minutes, $p < 0.05$). For the vehicle treatment the transition from feeding to inactive behaviour occurred between 20 and 25 minutes into the test session. There was no transition with drug within the 30 minute period (see Fig. 3.13).

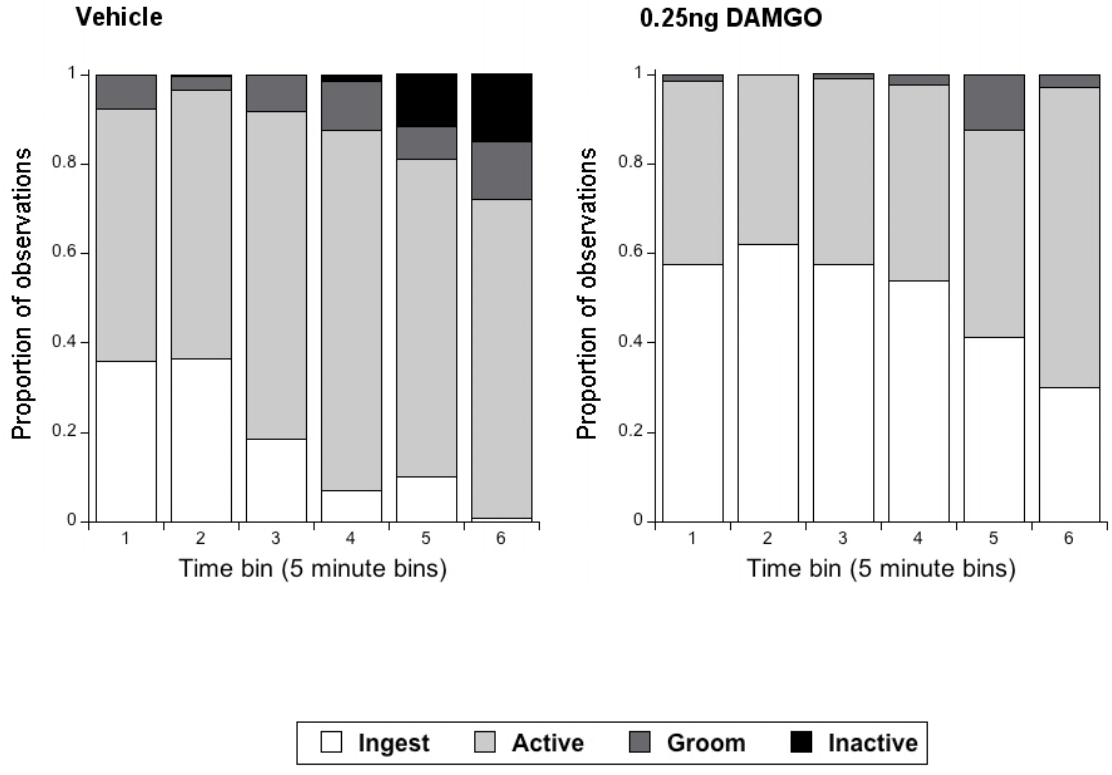


Figure 3.12. The behavioural satiety sequence (BSS) with vehicle and DAMGO (n=7). The relative proportions of each behaviour are shown in 5 minute time bins over a 30 minute period of free access to chow.

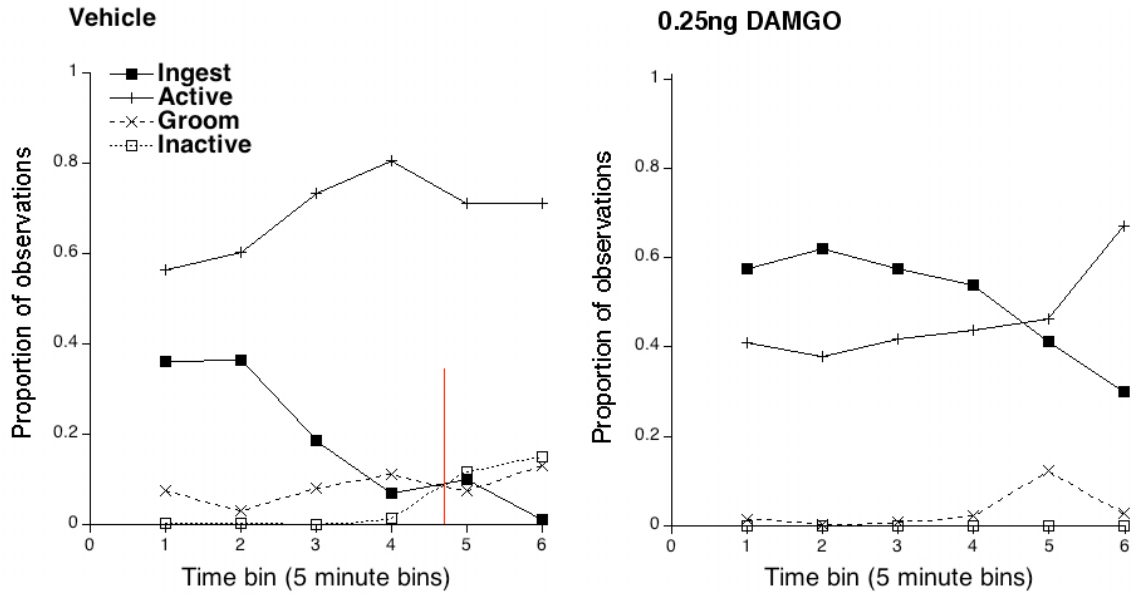


Figure 3.13. The effects of vehicle and DAMGO on the time course of ingestive, active, grooming and inactive behaviours split into 5 minute bins (n=7). The vertical line on the 'vehicle' chart indicates the point at which there is a transition from ingestion to inactive behaviour. This does not occur within the 30 minute time frame with drug.

Experiment 3.4: BSS following periods of food restriction of 4 or 8 hours.

The whole cohort n=12 were used in the analysis for this experiment. Fasting significantly increased intake [Fig. 3.14; $F(2,22)=57.93$, $p = < 0.001$) and this increase was significant at both 4 and 8 hours ($p < 0.01$). There was no significant difference between the amount consumed at 4 and 8 hours.

There was a significant effect of length of fasting on the BSS expressed (Fig. 3.15). Overall fasting significantly increased the proportion of behaviour in the category 'Ingest' [$F(2,22)=19.23$, $p < 0.001$] and the proportion changed over time [$F=7,77=39.25$, $p < 0.001$]. There was no interaction between fasting period and time. There was a significant interaction between fasting period and time for the category 'Active' [$F(14,154)=2.94$, $p < 0.001$] which both decreased and subsequently increased across the session. There was a significant increase in the 'Groom' category with time [$F(7,77)=4.74$, $p=<0.001$] but no significant effect of drug. The same was true for the 'Inactive' category, which showed a significant increase over time [$F(7,77)=27.38$ $p=<0.001$] but no significant effect of drug.

Planned post-hoc analysis using Dunnett's test indicated that both periods of fasting increased the time spent feeding in the first 20 minutes ($p < 0.001$). Significantly less time was spent in active behaviours in the first 15 minutes with a 4 or 8 hour fast (0-10 minutes, $p < 0.05$ for both; 10-15 minutes, $p < 0.01$ for both). Over the following 10 minutes there was no significant difference between the treatments but activity rose steadily with a 4 hour fast so that between 25-30 minutes it was significantly higher ($p < 0.05$). Activity then declined but there was no significant difference by the end of the session. Grooming was unaffected by fasting but inactive behaviour was decreased relative to vehicle (NS).

Fig. 3.16 demonstrates a transition from feeding to inactive behaviour in the *ad lib* fed condition between 20 and 25 minutes. The transition was delayed by approximately 5 minutes with fasting for 4 and 8 hours.

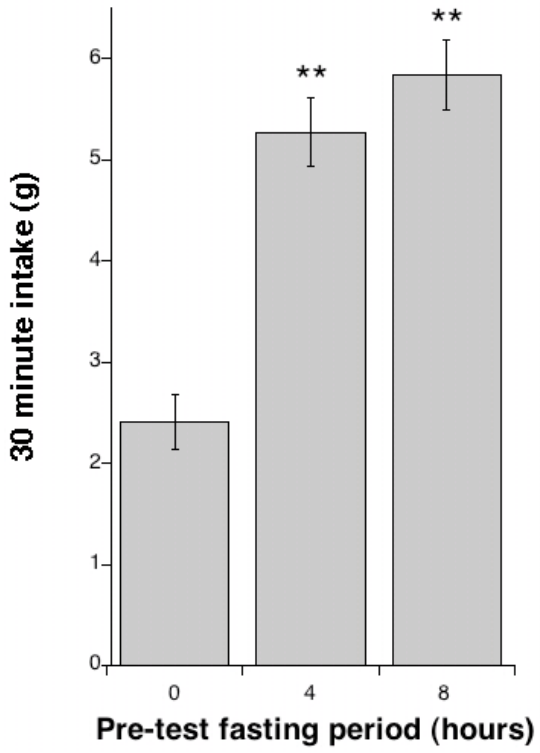


Figure 3.14. The effects of periods of fasting on intake in rats (n=12) given access to laboratory chow over a 40 minute test session. Error bars represent ±SEM. Significant differences from vehicle treated group are denoted by ** $p < 0.01$.

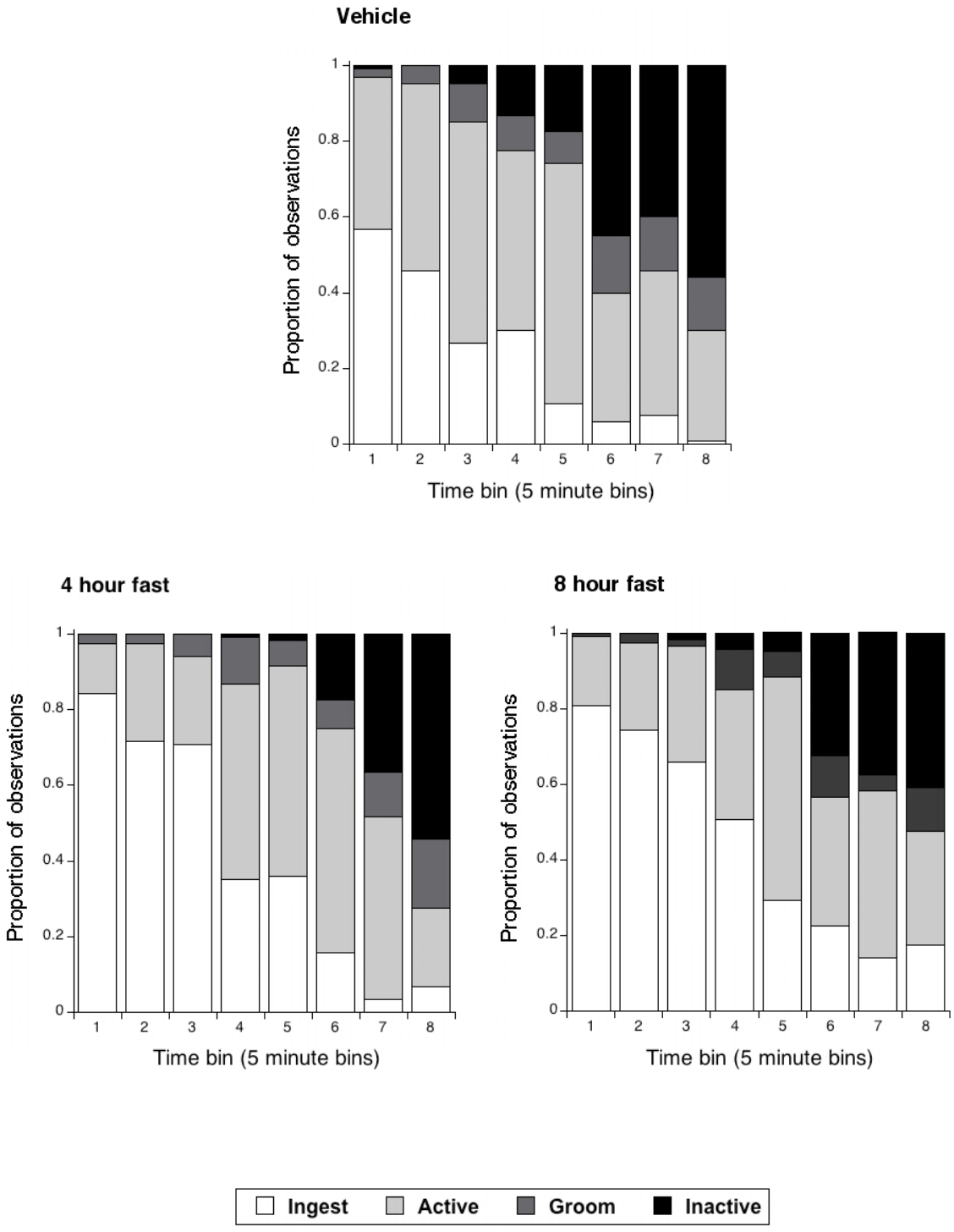


Figure 3.15. The behavioural satiety sequence (BSS) with no fast compared to 4 and 8 hours food restriction (n=12). The relative proportions of each behaviour are shown in 5 minute time bins over a 40 minute period of free access to chow.

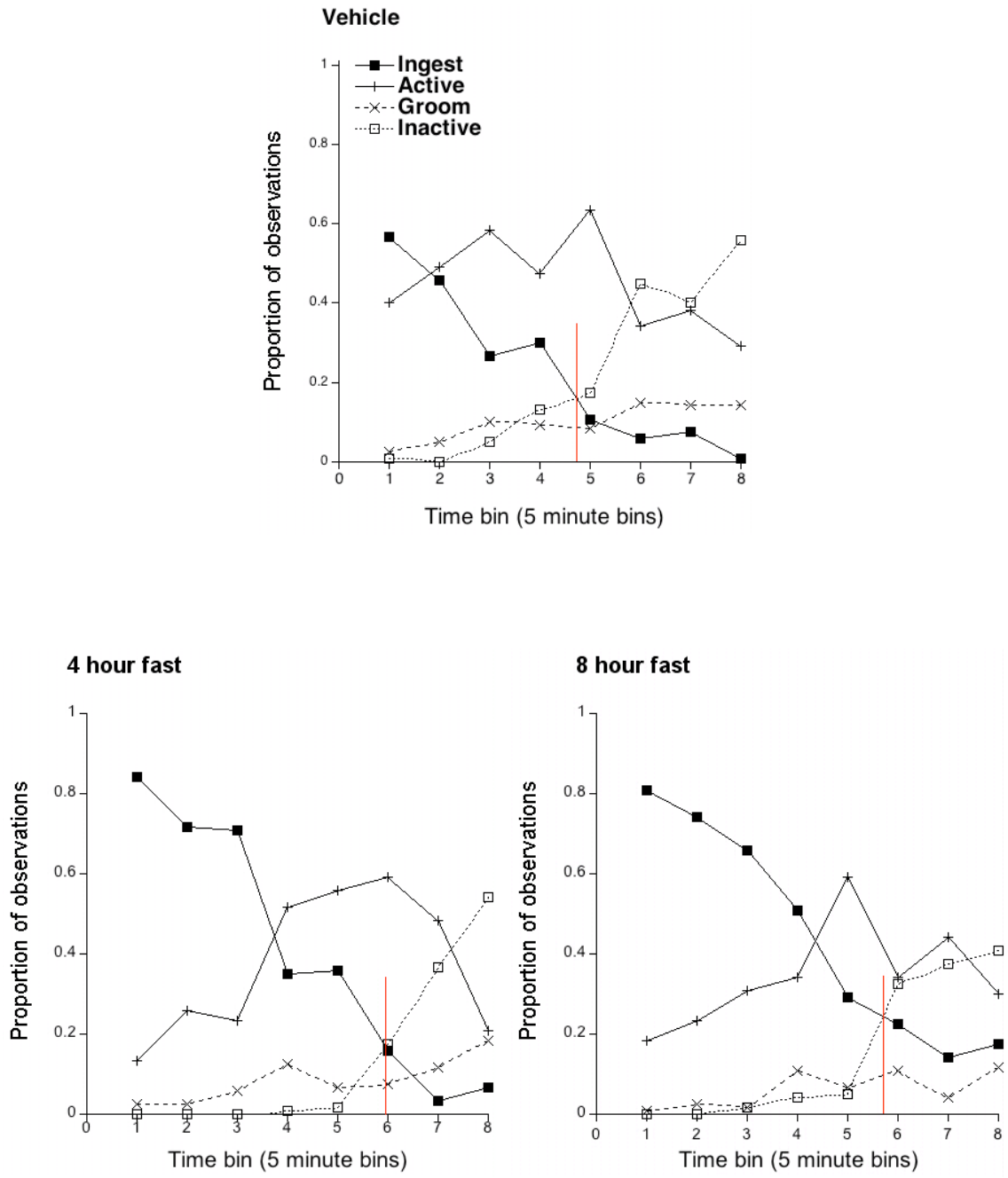


Figure 3.16. The effects of no food restriction compared to periods of 4 and 8 hours fasting on the time course of ingestive, active, grooming and inactive behaviours split into 5 minute bins (n=12). The vertical lines on the charts indicate the point at which there is a transition from ingestion to inactive behaviour. This is shifted to the right in fasted animals.

Experiment 3.5: BSS with chow following peripheral administration of the benzodiazepine bretazenil at 0.3 and 1.0mg/kg^{-1}.

The whole cohort n=12 were used in the analysis for this experiment. Bretazenil increased intake [Fig. 3.17; F=(2,22)=6.66, p=0.005] and this was dose dependent, significant at 0.3mg/kg ($p < 0,05$) and 1.0mg/kg ($p < 0.01$).

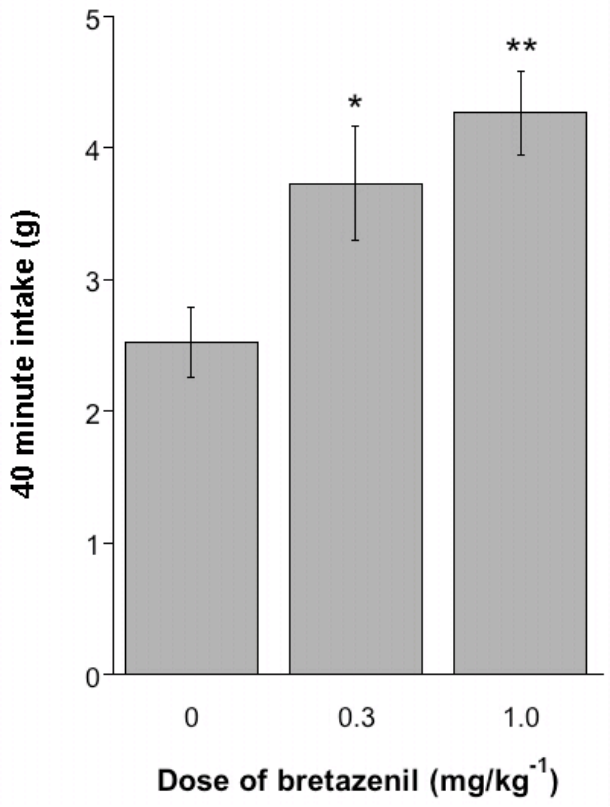


Figure 3.17. The effects of vehicle and two doses of bretazenil administered systemically on intake in rats (n=12) given access to laboratory chow over a 40 minute test session. Error bars represent ±SEM. Significant differences from vehicle treated group are denoted by * $p < 0.05$ and ** $p < 0.01$.

Bretazenil also dose dependently affected the BSS (see Fig. 3.18). Overall there was a significant effect of drug significantly increased the proportion of observations in the 'Ingest' category [$F(2,22)=12.69$, $p < 0.001$] and the proportion decreased over time [$F(7,77)=32.58$, $p < 0.001$]. There was no interaction between drug and time. 'Active' behaviour was significantly reduced as a consequence of drug [$F(2,22)=13.98$, $p < 0.001$] and time [$F(7,77)=3.23$, $p=0.005$] but again there was no interaction. There was a significant effect of drug decreasing observations in the 'Groom' category

[F(2,22)=4.08, p=0.031] but no significant effect of time and finally the 'Inactive' category was not significantly effected by drug but increased over time [F(2,22)=31.78, $p < 0.001$].

Planned post-hoc analysis using Dunnett's test indicated that the proportion of ingestive behaviour was significantly higher at both doses over the first 15 minutes of the test session (for 0.3mg and 1.0mg of bretazenil at 0-5 minutes, $p < 0.01$; 5-10 minutes, $p < 0.05$; 10-15 minutes, $p < 0.01$). For the remainder of the session the lower dose had no significant effect on feeding but 1.0mg of bretazenil resulted in a continued elevation in ingestive behaviour between 20 and 30 minutes ($p < 0.05$). There was no significant effect of either dose of drug on intake in the last 10 minutes.

Active behaviour was significantly reduced at both doses for the first 15 minutes (0-10 minutes, $p < 0.01$ for both doses; 10-15 minutes, $p < 0.05$ at 0.3mg and $p < 0.01$ at 1.0mg). This extended through the next 5 minutes for 1.0mg (15-20 minutes, $p < 0.05$) and then, as active behaviour increased in the vehicle condition activity again became significantly lower at both doses (20-25 minutes, $p < 0.01$ for both doses). Finally for the last 15 minutes of the session there was no significant difference in activity between the treatment groups. The effect of drug on grooming only became apparent at the end of the session with a significantly lower proportion at both doses from 30-35 minutes ($p > 0.05$).

In Fig. 3.19 the transition from feeding to inactive behaviour under vehicle treatment occurred between 15 and 20 minutes. The transition was delayed to between 25 and 30 minutes with both drug treatments.

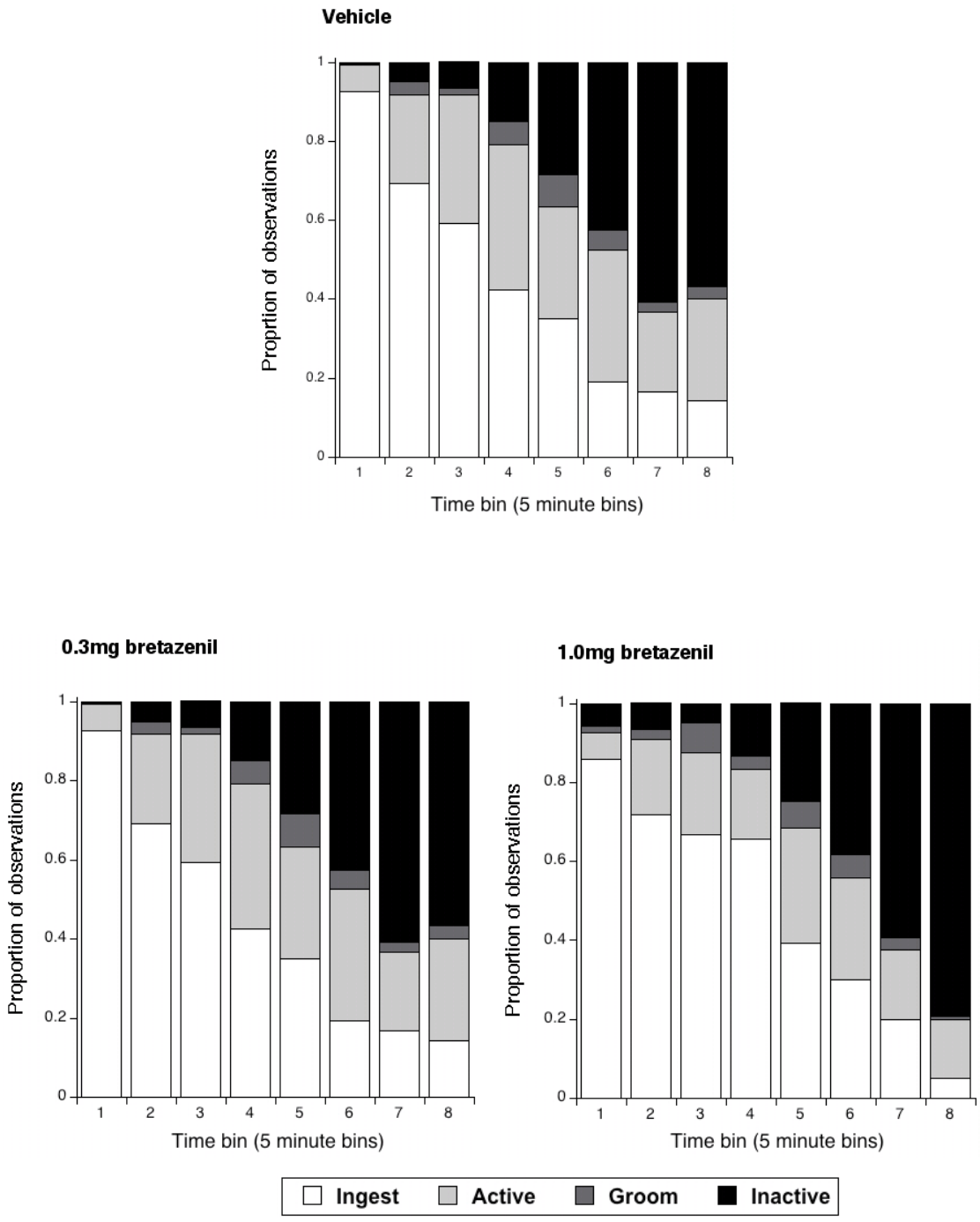


Figure 3.18. The behavioural satiety sequence (BSS) with vehicle and two doses of bretazenil administered systemically (n=12). The relative proportions of each behaviour are shown in 5 minute time bins over a 40 minute period of free access to chow.

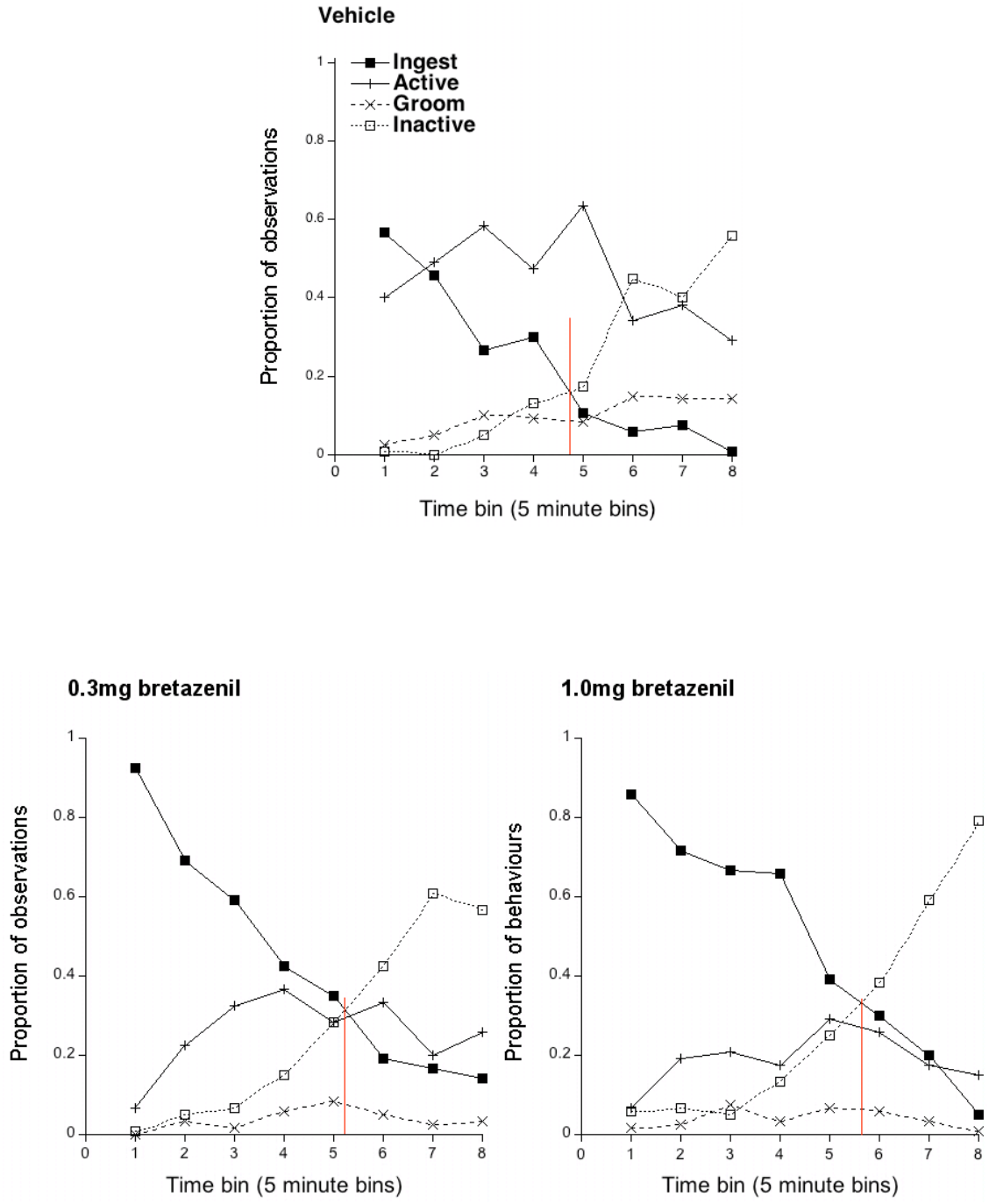


Figure 3.19. The effects of vehicle and two doses of bretazenil administered systemically on the time course of ingestive, active, grooming and inactive behaviours split into 5 minute bins (n=12). The vertical lines on the charts indicate the point at which there is a transition from ingestion to inactive behaviour. This is shifted to the right in drug treated animals.

Experiment 3.6: BSS with mash following peripheral administration of the benzodiazepine bretazenil at 1.0mg/kg^{-1}).

The whole cohort n=12 were used in the analysis for this experiment. A dose of 1.0mg of bretazenil significantly increased intake of wet mash relative to vehicle [Fig. 3.20; $F(1,11)=37.9$, $p < 0.001$]**.** Bretazenil also affected the BSS (see Fig. 3.21). Overall there was a significant increase in the proportion of observations and an interaction between drug and time in the category 'Ingest' [$F(7,77)=5.6$, $p < 0.001$]. There was a significant reduction in the proportion of observations in the 'Active' category [$F7,77)=9.8$, $p < 0.001$] and the 'Groom' category [$F(7,77)=2.41$, $p=0.028$]. There was a significant increase in the 'Inactive' category due to drug [$F(1,11)=5.77$, $p=0.035$] which changed over time [$F(7,77)=65.47$, $p < 0.001$] but there was no interaction.

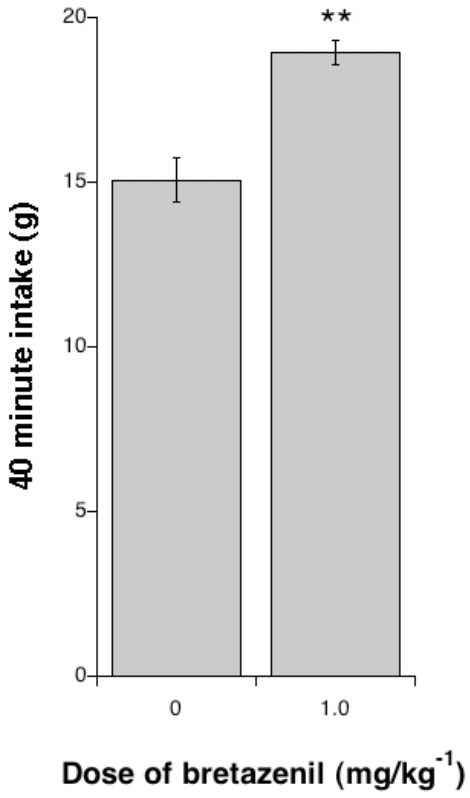


Figure 3.20. The effects of vehicle or bretazenil administered systemically on intake in rats (n=12) given access to mash over a 40 minute test session. Error bars represent ±SEM. Significant differences from vehicle treated group are denoted by ** p < 0.01.

Planned post-hoc analysis using Dunnett's test indicated that the proportion of ingestive behaviour was significantly higher between 5-20 minutes (5-10 minutes, p=0.002; 10-15 minutes $p < 0.001$ and 15 – 20 minutes, p=0.028). Active behaviour was significantly lower from 5 to 35 minutes (5-10 minutes, p= 0.032; 10-20 minutes, $p < 0.001$; 20-25 minutes, p=0.003, 25-30 minutes, p=0.017 and 30-25 minutes, p=0.043).

The effect of drug on grooming resulted in a significant reduction between 5 and 15 minutes (5-10 minutes, p=0.013; 10-15 minutes p=0.012). Grooming was not significantly lower between 15 and 20 minutes but by 25 minutes this difference was significant again ($p < 0.001$). Grooming remained lower for the rest of the session but this was not a significant difference. From 15 minutes onwards the proportion of inactive behaviour displayed with bretazenil was higher than with vehicle in every time bin but the difference was only significant between 30-35 minutes (p=0.029). Fig. 3.22 shows the transition from feeding to inactive behaviour occurred between 20-25 minutes with and without drug treatment.

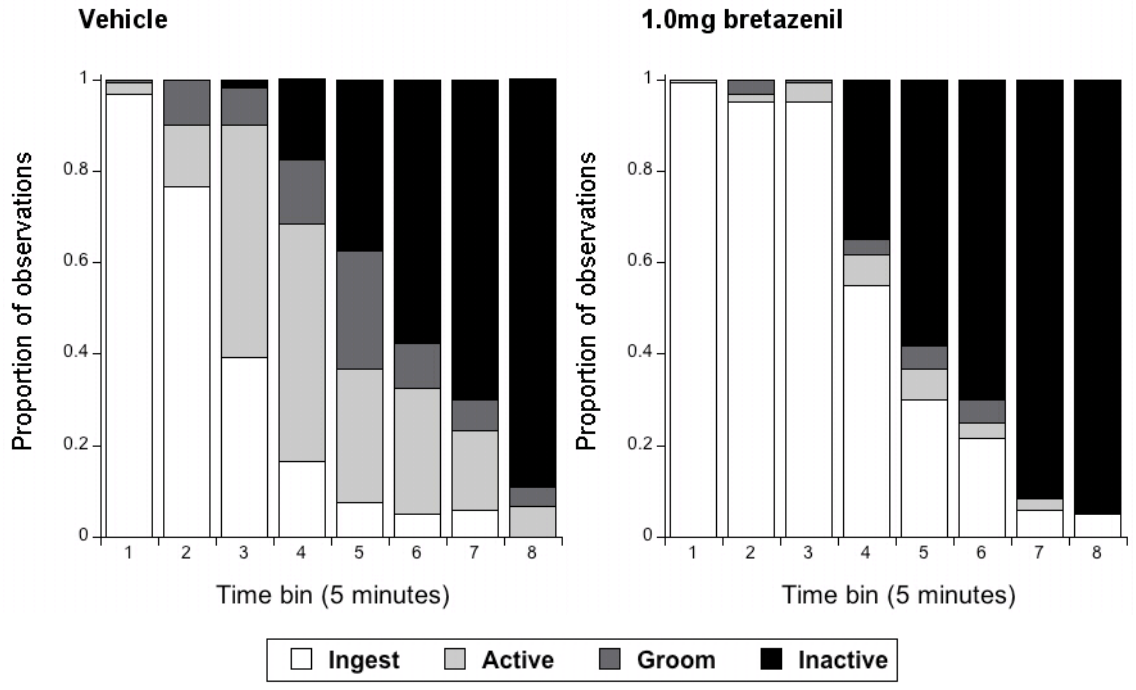


Figure 3.21. The behavioural satiety sequence (BSS) with vehicle or bretazenil administered systemically (n=12). The relative proportions of each behaviour are shown in 5 minute time bins over a 40 minute period of free access to mash.

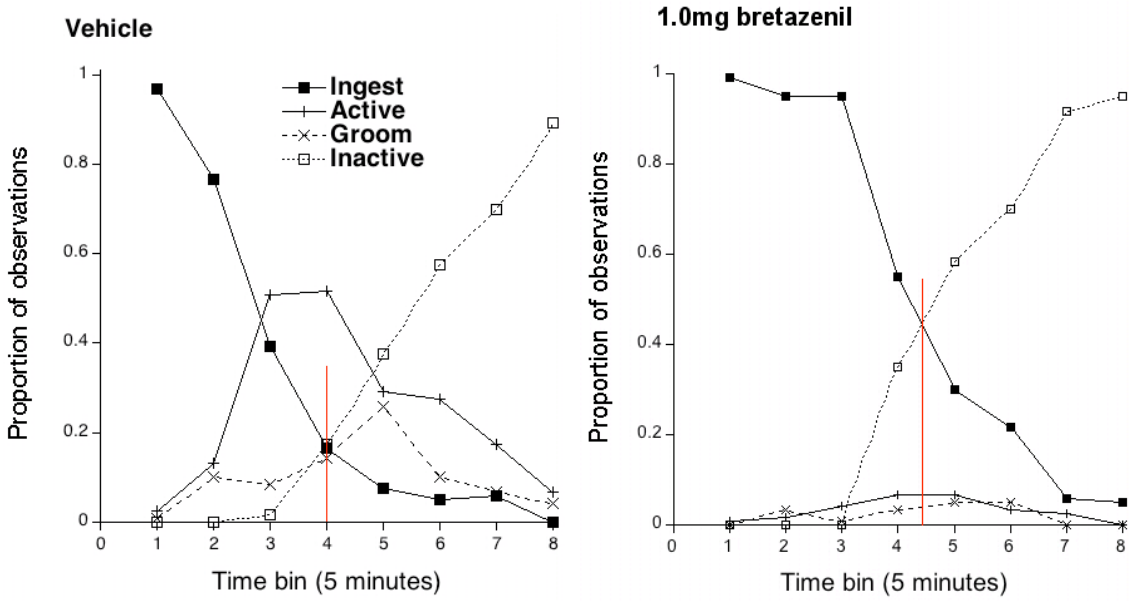


Figure 3.22. The effects of vehicle or bretazenil administered systemically on the time course of ingestive, active, grooming and inactive behaviours split into 5 minute bins (n=12). The vertical lines on the charts indicate the point at which there is a transition from ingestion to inactive behaviour. This is shifted very slightly to the right in drug treated animals.

Discussion

The experiments presented in this chapter addressed the lack of data with regards to the immediate and short term effects of intra-Acb GABA receptor subtype stimulation on the progression of feeding and associated non-feeding behaviours. Specific behaviours were recorded as part of the BSS in response to standard laboratory chow. In addition, BSS data were collected as a point of comparison for a number of other manipulations, both pharmacological and physiological, known to modify specific phases of ingestion.

A recognisable BSS was expressed in response to a chow meal eaten during the light phase by animals in the control condition. Pre-satiated animals given an infusion or i.p injection of saline vehicle ate a small amount of chow but consumed it rapidly and, predominantly, early in the test session. As the proportion of time spent feeding decreased, so the animals progressed through the satiety sequence engaging in active behaviours followed by grooming and finally a period of inactivity. The transition from ingestive behaviour to inactivity occurred between approximately 20 to 25 minutes into the test session. The distribution of the mean proportion of behaviours over time was

also very similar to that previously observed using wet mash rather than chow (Clifton and Cooper, 1996).

In experiment 3.1 intra-Acb baclofen, the $GABA_B$ agonist, enhanced intake of chow in a dose dependent manner in pre-satiated animals by increasing the proportion of time spent feeding at the start of the session. The proportion of ingestive behaviour remained high across the 30 minute test period at the highest dose. Active behaviour was significantly lower at the start of the session at all doses but, as ingestive behaviour tailed off, so active behaviour increased as would be expected in a normal BSS. Grooming was unaffected by drug treatment at any dose but the increase in the proportion of ingestive behaviour was reflected in a consequent absence of inactive behaviour over this timescale. This consequently meant that there was no transition from feeding to inactivity over the 30 minute test. The possibility that this represents a temporal shift in the BSS due to effects on motivation rather than via non-specific drug effects will be discussed in detail below.

In contrast, in experiment 3.2, the $GABA_A$ agonist muscimol increased intake (to a similar degree at all the doses tested) but also significantly changed the pattern of expression of all other behavioural categories in the BSS. It would aoppear that increased feeding behaviour was expressed at the expense of the rest of the behavioural repertoire throughout the 30 minute test period. In other words, activity and grooming were significantly lower across the session and inactive behaviour was conspicuous by its almost total absence.

DAMGO had no significant effect on any of the non-feeding behaviours. DAMGO prolonged feeding by enhancing the proportion of time spent in ingestive behaviour in the latter part of the BSS test session which, in turn, decreased the incidence of inactive behaviour expected at this stage (but not significantly so). The delayed peak in the proportion of ingestive behaviour resulted in there being no transition from feeding to inactive behaviour within the 30 minute test. The pattern of behaviour observed with DAMGO is clearly different to that observed with either of the GABA agonists.

Fasting for 4 or 8 hours increased feeding across the session, significantly so at the onset of recording, whilst it concurrently decreased active behaviour. The pattern looked similar to that recorded with the two lower doses of baclofen. Also, as with

baclofen, grooming remained unaffected but the amount of inactive behaviour appearing at the end of the session was reduced following fasting (but not significantly so). The increased proportion of feeding and decreased proportion of inactive behaviour was reflected in a delay in the transition from feeding to inactivity.

Peripheral administration of the BZ bretazenil, which acts as an agonist at the $GABA_A$ receptor in a variety of brain regions, increased feeding in a dose dependent manner, which was expressed as a very high proportion of feeding behaviour in the first five minutes. The increase in the proportion of ingestive behaviour was only significantly higher during the first half of the session. In contrast to the effects of baclofen or fasting, active behaviour was reduced initially and an increase in activity to the equivalent seen in the vehicle condition occurred much later in the session. Grooming was also significantly reduced at the end of the session. When a mash meal was used instead of chow, bretazenil induced a very large proportion of feeding at the start of the session, as with the chow meal, but this rapidly gave way to inactive behaviour at the expense of the rest of the behavioural repertoire. This apparent 'meal type' induced disruption of the BSS will be further discussed below.

The effects of GABA agonists on the BSS

In experiment 3.1, animals given baclofen spent more time feeding in each time bin but this was only significant at the start of the session at the lower doses. The relative proportion increase was greater per time bin with each dose. At the two lowest doses, the overall pattern was characterised by a gradual decrease of ingestive behaviour over time which was similar to that observed with the control group. At the highest dose the proportion of ingestive behaviour remained higher than vehicle levels throughout the test period but still decreased slightly over time. The increase in the proportion of feeding with each dose echoes the dose dependent variation in total intake.

Activity levels were lower with baclofen treatment at the start of the test session but this most likely reflects the increased proportion of time spent feeding rather than a direct drug effect on activity (Ishii et al., 2003a). This assertion is supported by the observation that, broadly, there was no significant difference in activity levels between the drug or vehicle treatments in the last 15 minutes of the test session (although a slight elevation is clear from the figures and there was a significant difference in the last 5

minutes at the 220ρmols dose). Grooming behaviour was unchanged by the drug treatment, which fits in with the postulation that an initial increase in the proportion of feeding behaviour due to $GABA_B$ mediated mechanisms does not concomitantly directly affect other species-specific motor behaviours.

Inactive behaviour was conspicuous by its absence across the test period but, because more time was spent feeding in the drug condition, the point at which the transition to inactive behaviour occurs may not have been reached over a 30 minute session. With the lack of aberrant changes in the patterns of active and grooming behaviour it is suggested that the behaviour recorded could reflect a temporal shift in the BSS to the right (i.e. a delay). This conclusion is supported by observations in the laboratory that, when subjects were given access to chow for longer periods (i.e. 60 minutes), all animals did eventually settle down in a resting posture or even go to sleep.

It should be mentioned here that the group of animals used for this experiment (and for Exp. 3.3 BSS with DAMGO, below) were implanted with guide cannulae using the original set of co-ordinates based on those used by Ward et al. (2000); (AP), + 1.2mm, mediolateral (ML), ± 1.5mm relative to bregma and dorsoventral (DV), -5.8mm relative to skull surface. This put some of the infusions very close to the core region (as Fig. 3.3 above shows). Those animals that had purely core infusions on both sides were excluded from the final analysis. There were some infusions however that did potentially fall at the boundary between the two regions but the data from these animals was still used. The data reported were further analysed using the categories “shell only”, “boundary” and “core only”. No significant interaction between drug and infusion site was found. This tallies well with previous work in this laboratory that indicates that there is no relationship between individual baclofen infusion site and degree of feeding enhancement in this strain (Ward et al., 2000).

It is also interesting to note that the animals treated with baclofen increasingly engage in some additional behaviour including what appeared to be consumption of sawdust or faeces and licking the floor for crumbs, particularly at the highest dose. This was recorded as part of the ‘Ingest’ category. Despite this additional behaviour the incidence of active behaviours and grooming were not disrupted and this may account for the apparently prolonged meal at the highest dose.

Ward et al. (2000) concluded that baclofen probably does not induce a motor stereotypy for chewing because no responses were directed towards wooden blocks shaped like chow pellets. If the additional behaviours observed here were not an expression of stereotypy then they could be directed at getting every last scrap of food which certainly appeared to be what the animals were doing. In addition, coprophagy is a normal species-specific behaviour; rats will consume 50-65% of faeces in the home cage (Barnes et al., 1957) and it is possible that this too would increase as a part of an enhancement of the normal feeding process although it has not been reported before.

Muscimol infused into the Acb in experiment 3.2 also robustly increased intake of chow. In contrast to baclofen however, the $GABA_A$ agonist appeared to disrupt the normal BSS even at the lowest dose of 220ρmols. The proportion of time spent engaged in ingestive behaviour was significantly higher throughout the test session at all doses of muscimol and active behaviours were consequently much lower than those observed with the vehicle treated group for the first 20 minutes of the session. Grooming behaviour was virtually absent at all doses of muscimol, as was inactive behaviour. It must be noted that, although the feeding is so voracious, it is possible that grooming and inactive behaviours would appear if the session were longer. Unfortunately none of the experiments carried out using muscimol extended beyond a 30 minute observation period so no comment can be made as to the pattern of the BSS over 60 minutes.

It is interesting to note that, although the proportion of time spent engaged in feeding behaviour with muscimol takes up a disproportionate amount of the 30 minute period, the animals did not consume much more than the baclofen treated animals (e.g. total mean intake at 440ρmols baclofen = 4.31g ± 0.34 and at the molar equivalent dose of muscimol = 5.44g ± 0.45). This could be due to the fact that the ingest category includes handling of the food and animals that received muscimol were in contact with the food much of the time although not necessarily eating. Additionally animals forced so much food into their mouths that they would chew and gag for long periods before collecting the next pellet, a phenomenon previously reported (e.g. Kelley et al., 2005b).

One potential reason for the different profiles of behaviour observed with baclofen and muscimol that should be considered when interpreting these results could be the different nutritional status of the animals prior to testing. The baclofen animals were fed

ad lib up until 1 hour before testing whereas the muscimol group were on a restricted diet but were pre-fed 1 hour prior to testing. It is, consequently, possible that there were differences in the relative motivational state of the animals.

Food restriction has been widely demonstrated to modify the neurochemistry of the motivation control circuits in the brain and the levels of gut hormones that act as neurotransmitters. However, perhaps surprisingly, there appears to be no data on the effects of deprivation on endogenous GABA levels or GABA receptor expression despite their established role in feeding. Of course the behaviour expressed in the BSS with muscimol could be due to the interaction between $GABA_A$ receptor stimulation and modified function or expression of a multitude of neurotransmitters and/or their receptors elsewhere in the motivation control circuit. The implications of this are complex and will be discussed later in the thesis when the behavioural effects of intra-Acb baclofen and muscimol are further elucidated.

Despite evidence that the effects of acute and chronic food restriction on the expression and activity of receptors in the brain is very important in understanding the control of appetite the consequent acute effects of re-feeding are not clear. In particular there is minimal data available about the differences in the striatal gene expression for endogenous feeding related neurotransmitters (or their receptors) in the brains of animals depending on food motivational conditions prior to testing. At the level of the Acb the only data available refers to fluctuations in opioids.

It would appear that preproenkephalin (PPE – the precursor to enkephalin) activity is reduced in animals that have recently eaten regardless of whether they have been food restricted or fed *ad lib* prior to a chow meal (Will et al., 2007). However Will et al. (2007) also found that, in the medial Acb region PPE was down regulated to a lesser degree by short-term satiation with chow pellets when the animals were food restricted. This would only be relevant however if the effects of GABA receptor stimulation were due to an interaction with the opiate control of feeding. This possibility will also be discussed later in the thesis. At this stage it can be stated that, without pharmacological intervention, the food restricted but re-fed animals in experiment 3.2 expressed a ‘normal’ BSS in response to chow (compared to ‘typical’ BSS, Fig. 3.1 in introduction). Furthermore it has been previously demonstrated that, at a behavioural level, the effects

of prior deprivation are attenuated following satiation at the time of testing (Wong and Traupmann, 1973, Wong and Traupmann, 1975).

It is acknowledged that if a lower dose of muscimol had been used an increase in the proportion of ingestive behaviour without the apparent disruption of the rest of the BSS may have been seen. The main argument against this possibility is that other investigators were unable to find a significant effect on intake at this dose whilst the molecular equivalent dose of baclofen did cause an increase in total intake (Stratford and Kelley, 1997b). This could only be confirmed by repeating this experiment with a wider dose range and *ad libitum* fed animals.

The effects of an opioid agonist on the BSS

In experiment 3.3 the effects of one dose of DAMGO were investigated and the pattern of feeding was quite different from that seen with the GABA receptor agonists. Although the proportion of feeding was elevated throughout the 30 minute session this was never to the levels observed with either baclofen or muscimol. The smaller increase in intake with DAMGO in each time bin compared to that with the GABA agonists could be due to the possibility that the dose used was not an equivalent in terms of its stimulatory effects on feeding. The mean total intake with DAMGO however was 4.63g ± 0.96 which is very close to the amount consumed in the GABA experiments. In contrast to muscimol and baclofen, which elevated the proportion of feeding at the start of the session, DAMGO did not significantly elevate intake relative to vehicle during the first 15 minutes but proportions remained high in the last 15 minutes as feeding dropped off in the vehicle group. Also, in contrast to the muscimol group, all other behaviours were left intact suggesting this reflects the normal progression of the BSS with a delayed onset of satiety causing a shift to the right.

Halford et al. (1998) do point out that some drugs that cause mild sedation will reduce the frequency of behaviour but extend the duration and this could be posited as an explanation for these findings. Some of the animals infused with DAMGO showed clear signs of sedation but were eliminated from the final analysis. For the others, the proportion of feeding behaviour at the start of the DAMGO induced BSS was still higher than that elicited by vehicle infusions (if not significantly so) so the pattern seen in Fig. 3.12 and 3.13 is not believed to be due to sedative effects.

Hanlon et al. (2004) returned animals to the home cage following DAMGO infusions prior to behavioural testing because "the maximum effect of DAMGO on food intake occurs 30–60 min post-infusion". However this was in response to a highly palatable sucrose solution (Zhang and Kelley, 1997). Earlier work in the same laboratory showed that the effects of DAMGO on consumption of laboratory chow had a delayed onset with a peak in intake between 1-2 hours (Bakshi and Kelley, 1993a). It was not suggest that the delay is due to sedative effects but most likely an initial suppression of locomotion (Zhang and Kelley, 1997), which was reported many years ago (Babbini & Davis, 1972). The results reported here for DAMGO could indicate that the process of satiety is delayed and would be consistent with a delayed peak in feeding behaviour with this drug at 1-2 hours post infusion. Further studies to confirm the conclusion that the BSS described reflects a behaviourally selective effect on feeding could be carried out using a lower dose of DAMGO or imposing a delay post-infusion before testing.

It is also possible that the results are consistent with the evidence that opioids have an effect on palatability of the meal (Kelley et al., 2002, Woolley et al., 2006, Pecina and Berridge, 2000). The BSS was somewhat delayed in a similar manner to the effects of adding saccharin to test food which causes a "modest delay" in the progression of the BSS caused by what was termed "atypical" feeding late in the session (Ishii et al., 2003b). In simple terms this could be viewed as the animal continuing to enjoy the food beyond the point at which they would normally reach sensory specific satiety. Indeed Ishii et al. (2003a) suggest that a pattern characterised by prolonged feeding but no intense increase in the initial response may indicate an effect on satiety rather than "hunger motivation". Certainly our results would indicate that intra-Acb DAMGO does not produce a BSS resembling that recorded after a period of fasting nor does it look anything like that recorded with baclofen or muscimol.

The effects of fasting on the BSS

Not surprisingly, in experiment 3.4, withholding food from the rats before testing enhanced the amount of food consumed but, interestingly, there was no significant difference in the total amount consumed after a 4 or 8 hour fasting period suggesting a ceiling effect of acute induction of hunger over this timescale. Ishii et al. (2003a) point out that it has been widely demonstrated that short periods of fasting <24 hour increase the amount ingested in a nonlinear fashion. Here, the proportion of ingestive behaviour

was increased in each 5 minute time bin, significantly so early on in the session, and the meal was slightly prolonged with a delay in the transition from feeding to inactivity. There was a consequent reduction in activity at the start of the BSS and a slight elevation 30 minutes into the session following 4 hours of food deprivation. Grooming was unaffected but resting behaviour was decreased (although, in this case, not significantly so). The reduction in inactive behaviour was probably due to the increase in time spent feeding. This data represents a BSS temporally shifted to the right and reflects the effects of an increase in hunger motivation or "appetite".

There is good agreement between these data and the general pattern observed by Ishii et al. (2003a) although their animals had been habituated to a 1 hour test period and hence their behaviour may have been shaped by the expectation of a longer period of access. This group reported that fasting increased the total duration of feeding whilst concurrently reducing active behaviour, delayed peak grooming and shifted the transition to inactive behaviour to the right (Ishii et al. 2003). They do not report the pattern of active behaviours later in the session preferring only to give absolute values for total duration and frequency.

It is well known that fasting causes increases in activity as well as in total food consumption (Finger, 1951, Cornish and Mrosovsky, 1965, Finger and Mook, 1971) and this may explain the slight increase in activity part way through the session. In the presence of food, mechanisms that control the urge to eat could override the more general effects on locomotion but once the animal is satiated, increases in activity may be expressed. It is interesting that a similar increase was also seen with the 220ρmols dose of baclofen. The BSS recorded at the two lower doses of baclofen looks similar to the BSS with 4 and 8 hours of fasting. The only difference is the greater reduction in inactive behaviour with the GABA agonist but it may support the conclusion that the baclofen BSS is temporally shifted without a direct affect on non-feeding behaviours.

The effects of a peripheral benzodiazepine on the BSS

Bretazenil administered systemically in experiment 3.5 significantly increased intake of chow, an effect that has not been demonstrated before, and there was no apparent disruption in the sequence at either dose. The amount of time spent eating in each time bin and the length of the meal was reflected in a reduction of other active components

of behaviour. Active behaviour was concurrently reduced during the first 15 minutes while feeding behaviour was significantly higher. There was a smaller proportion of grooming at both doses in the last 5 minutes of the session but, since both doses shifted the BSS to the right by ~5 minutes this could be an artefact of this delay. This finding was surprising given that it has previously been suggested that the BSS is actually disrupted by bretazenil because it appears to speed up the transition from feeding to resting behaviour at the expense of intervening behaviours (Clifton and Cooper, 1996). As a result the experiment was repeated using a mash meal.

The effects of a peripheral benzodiazepine and meal type on the BSS

In experiment 3.6 systemic bretazenil increased intake of palatable mash although pre-feeding prevented levels reaching the extremes reported previously (Clifton and Cooper, 1996). The animals then showed a rapid transition to inactivity, failing to engage in a normal level of active behaviour post feeding or to groom as much as they did under vehicle treatment thus the disruption in the BSS previously reported was replicated. These results suggest that it is the large volume of the mash meal consumed (intake at 1 mg/kg bretazenil: mash=19.0 ±0.3g whereas chow=4.3 ± 0.3g) that may lead to higher levels of resting rather than a disruption of the BSS due to direct increases in somnolence.

The observation that resting may increase because of the volume of meal consumed is in agreement with a recent shift in the approach to understanding the effects of feeding on sleep in humans. In the late 1990s there was some interest in the existence of gastrointestinal effects on post-prandial somnolence, which appeared to be independent of hunger and necessary for the onset of sleepiness (Harnish et al., 1998). More recently it has been suggested that gut neurohormones (e.g. orexin) and autonomic responses to feeding may directly affect areas of the brain responsible for the control of sleep which overlap with those controlling hunger (Bazar et al., 2004). Irrespective of the possible explanation for the difference in the satiety sequence with a BZ and chow or mash what is most important here is that, with chow, there appears to be a normal but temporally shifted BSS. The BSS expressed during bretazenil induced hyperphagia in response to chow is actually fairly similar to that seen with baclofen but, surprisingly, is completely different from that induced by DAMGO despite the fact that both drugs are thought to increase intake via effects on palatability.

The apparent interaction between the type of meal used for assessing the BSS with bretazenil and the shape of the sequence produced could have important implications for interpreting all of the data presented here. John Blundell's laboratory, probably the most prolific users of the BSS as a biobehavioural assay, favour the use of a highly palatable wet mash diet and have also started routinely testing animals in the dark phase of a reversed light/dark cycle so as to start with high baseline intake (Halford et al., 1998, Ishii et al., 2003b, Ishii et al., 2003a, Tallett et al., 2008) because this "obviates the need for prior food deprivation" (Ishii et al., 2003b). Looking in detail at their method (Halford and Blundell, 1996) it would appear that often they also effectively fast the animals by removing food 4 hours before testing.

This research group from Liverpool University argue elsewhere (Ishii et al. 2003a) that short fasts of between 2–4 hours only minimally increase free feeding (Bare and Cicala, 1960) but in the same paper they state that a 3 hour fast "clearly produced a prolongation of feeding behaviour prior to peak grooming and the onset of resting". In effect, therefore, they are starting with animals that are predisposed to express a modified BSS characterised by rapid onset of intense feeding behaviour in response to highly palatable diet and prolongation of the meal due to removal of food from the home cage prior to testing. The key here is that their model has only been used for testing anorectic drugs that reduce intake and, therefore, they prefer to start with a high baseline level of intake. It should be noted that food in the home cage was not removed 4 hours prior to testing when they tested the effects of pre-satiation or food deprivation to produce reference profiles with which to compare drug effects (Ishii et al. 2003a).

In contrast we have opted for test conditions in which animals are pre-satiated so as to start with low baseline intake, fed standard laboratory chow which is not as motivating as mash and tested during the light phase of the light dark cycle. There were a number of reasons for this decision. First of all it was felt that there might be some ceiling effect when using mash. For example there could be a physical limitation on how much could be consumed. Starting with satiated animals and giving them a food that they would not normally over consume leaves room for the large increases usually observed with GABA agonists infused into the Acb. Researchers from Blundell's laboratory do themselves suggest that "ca. 22 g mash may approximate the upper limit of consumption under present test conditions". Some of our drug treated animals

consumed more than double the amount of chow than they did in the control condition (see results section). In experiment 3.6 animals in the control condition ate approximately 15g of mash which did not leave much scope for the robust increases in intake expected following infusion of GABA agonists into the Acb.

Another reason to use chow instead of mash was because studies that have demonstrated an orexigenic effect of intra-Acb GABA receptor stimulation have done so primarily using standard laboratory chow. It seems most appropriate to use the same food in the characterisation of the BSS. Furthermore, adding the element of high palatability may cause a meal type/drug interaction, as indeed is the case with opioids where the effects are more robust when sweet or fatty foods are provided (Bodnar, 2007). Although no interaction between GABA receptor stimulation and meal type has been observed in terms of total intake of different macronutrients with muscimol (Basso and Kelley, 1999) no studies have focused on the microstructural changes that might occur in the pattern of consumption with GABA receptor agonism.

Testing during the light phase rather than the dark phase could result in interactions between drug and time of testing due to neurochemical fluctuations associated with circadian rhythms. However, once again, consistency with previous studies on the effects of GABA agonists on intake was of primary importance for these experiments. A review of all the studies published by Kelley's laboratory showed that testing was always carried out during the light phase. Tallett et al. (2008) do report a slightly lower amount of resting behaviour in animals tested in the light phase rather than the dark phase in their laboratory but the overall pattern of the BSS was not changed.

It must be pointed out that there is, predictably, a degree of inter-individual variability in the progression of the BSS and that the expected shape of a curve representing the BSS will never represent the specific behaviour of any one individual animal. As Halford et al. (1998) state "the BSS is a clearly identifiable stochastic progression of behavioural trends over time (not a strict deterministic sequence)". Nevertheless the credibility of our approach is supported by the production of a recognisable BSS in the control condition and the good agreement between our fasting data and that reported by Ishii et al. (2003a) who also tested during the light phase but used a mash meal rather than chow. Despite the much lower overall volumes of chow consumed (compared to

mash) the BSS for our pre-satiated, vehicle treated animals was very similar in the basic profile of each curve to those reported by Blundell's laboratory. Ishii et al. (2003a) also report similar total intake of chow pellets (1.86g ± 0.46) to those presented in this chapter over an identical test session to that used to obtain their mash data but do not indicate what the BSS looked like.

General methodological issues

As mentioned in the introduction to this chapter there is some debate about the method used to record data for the BSS. Halford et al. (1998) point out that a MTS procedure underestimates the frequency of behaviour and only produces 10 observations for each animal per 5 minute time bin making "standard parametric analysis difficult". However, in experiments 3.1 to 3.3 (central drug infusions) a total of 360 observations or 60 for each animal in each time bin were generated improving the resolution. In those experiments where a cohort of 12 animals were observed (3.4 to 3.6) only 10 observations were recorded for each time bin but in both cases the data does, none the less, agree well with that published by laboratories using a continuous observation method (as discussed above).

Halford et al. (1998) also introduces the concept of "event over state observer bias" whereby an observer is more likely to code an active behaviour or "event" (e.g. rearing) when there is a transition occurring between this and a "state" behaviour (e.g. resting) when deciding what the animal is doing. Experience indicates that this is indeed a possible pitfall, however an awareness of this potential source of error, plenty of practice by the observer to speed up decision making and recognition of behaviours, minimises the effect of this bias even if it does not totally eliminate it. It is not believed to be a confounding factor in the results reported in this Chapter. The results from these experiments also indicate that the effects of different drug or physiological manipulations that induce hyperphagia can be readily distinguished using this experimental paradigm i.e. analysis of the BSS expressed by pre-satiated rats fed a chow meal during the light phase recorded using a MTS method. This raises some questions as to the methodological approach used by other laboratories that favour high baseline intake to test anorectic effects of drugs.

General discussion

All in all the pattern of the BSS induced by the two lowest doses of baclofen probably represents a behaviourally selective temporal shift and the similarity to the BSS induced by fasting may also suggest that baclofen induced shifts in the BSS are due to an effect on the "hunger motivated" or appetitive phase of ingestion. Animals that were fasted in experiment 3.4 only started to transition from ingestive to inactive behaviours at ~30 minutes (see Fig. 3.16), the point at which the baclofen test was ended which could explain the lack of a clear transition from feeding to inactivity with baclofen.

As Ishii et al. (2003) point out, "if drug-induced changes in the temporal characteristics of the BSS truly reflect an action on the normal physiological mechanisms of appetite regulation, then these changes should resemble (at least in part) those seen in response to variation in nutritional status", as they do here. The results reported here support the hypotheses that the hyperphagic action of baclofen is of physiological and functional significance (Stratford, 2007). It would also indicate that stimulation of the $GABA_B$ receptor might reflect selective effects on appetite or satiety but probably not through an increase in palatability estimation.

A lack of effect of $GABA_B$ receptor agonists on palatability but an effect on appetite or satiety is consistent with the theory that there are two classes of Acb neurons that contribute to neural processing immediately before and during consumption via, 1) inhibition related to initiation (appetite) and maintenance of consummatory behaviours and 2) excitations that encode reinforcer palatability (Taha and Fields, 2005b). This is in turn consistent with data published by Ward et al. (2000) that showed that intra-Acb baclofen decreased licking bout size in response to a palatable solution whereas increased palatability estimation is reflected in increased bout size (Clifton, 2000).

These results also raise questions as to how and why the pattern of behaviours induced by $GABA_B$ receptor manipulation is different from those exhibited with drug action at the $GABA_A$ receptor, which may well have significantly disrupted the normal sequence of satiety. One possible clue may be found in the apparent attention bias highlighted in the results section. During the BSS test phase it was noted that animals under the influence of centrally applied muscimol appeared to be 'stuck' in their behavioural expression of increased ingestion and were seemingly oblivious to the observer whereas

the baclofen group were much more aware of the surroundings and would break off feeding to observe what was going on in the test environment. This would seem to indicate that the application of the $GABA_A$ agonist does indeed lock the animals into a specific motor pattern expressed as a perseveration of the consummatory response in line with Kelley's hypothesis.

Recently it has been suggested that the striatum, including the Acb, contains central pattern generators (CPGs) (Grillner, 2006, Grillner et al., 2005) and "possesses the connectivity and intrinsic mechanisms to orchestrate pattern generation" (Carrillo-Reid et al., 2008). Carrillo-Reid et al., (2008) recently demonstrated that in the dorsal striatum blockade of fast glutamatergic transmission prevented subsequent correlated firing but blockade of GABAergic transmission locked the neuronal microcircuits into a single dominant state that eliminated assembly diversity. If this turns out to hold true for the very similar cellular assemblages present in the ventral striatum then this could go some way to explaining the ability of GABA at the two receptor subtypes to differentially effect downstream outputs via MSNs. MSNs from the Acb project to the hypothalamus, which contains a series of nuclei, termed the behavioural control column (Swanson, 2000), that in turn recruit and modulate motor pattern generators in the hindbrain and spinal chord. The circuit could therefore be locked into stimulation of a subset of pattern generators responsible for 'feeding' with $GABA_A$ receptor stimulation.

The $GABA_B$ induced behaviour presented here, dominance of one behaviour (feeding) but ability to express other behaviours, is more akin with the prevention of correlated firing observed with blockade of fast glutamatergic transmission than with locking of neuronal microcircuits into a single dominant state. This would be consistent with baclofen's actions at heteroreceptors on glutamatergic inputs. This would suggest that the route by which the MSN output is inhibited (presynaptic $GABA_B$ receptors or postsynaptic $GABA_A$ receptors) is critical in determining subsequent behaviour exhibited and global inhibition of the shell does not reflect the endogenous role of GABA at different receptor subtypes. This concept will be discussed in more detail in the final chapter, Chapter 7.

So to summarise, the results presented in this chapter show that intra-Acb baclofen releases intense feeding sequences but the broad behavioural organisation associated

with the onset of satiety is maintained whereas muscimol disrupts the progression of the satiety sequence. The hyperphagic action of intra-Acb baclofen is consistent with an enhanced motivation to feed observed following short periods of fasting. The temporal shift in the BSS with baclofen does not look like that observed with intra-Acb opioid agonists posited to delay satiation by disrupting the process of sensory specific satiety. Neither does it mimic the effects of enhanced palatability caused by a peripheral BZ.

The data are not consistent with the view that intra-Acb baclofen simply leads to the indirect release of downstream motor components of feeding behaviour (Kelley et al., 2005b, Kelley et al., 2005a) but the effects of muscimol could well be due to the predominance of specific feeding related motor behaviours expressed at the expense of the remainder of the behavioural repertoire. This is the first demonstration that stimulation of $GABA_B$ receptors in the Acb has an effect on the motivational control of feeding.

Questions raised

The most important questions raised here are about the possible differentiation between the effects of $GABA_A$ and $GABA_B$ agonists. If GABA at the $GABA_B$ receptor affects motivation how will it affect an animal's willingness to work for a food reward and the various stages of ingestion? These questions will be approached in the next two chapters where the results from experiments using an operant schedule to test the affects of baclofen (Chapter 4) and muscimol (Chapter 5) will be presented. The nature of the potential behavioural differences between intra-Acb infusions of these different agonists will be further investigated in both chapters where the results from the analysis of videos of behaviour elicited by the operant schedules will be presented. As a starting point in attempting to establish whether the differential effects of $GABA_A$ and $GABA_B$ agonists in the Acb occur at a structural macrocircuit level neuronal activation in feeding related circuitry marked by Fos like immunoreactivity will be compared in Chapter 6.

Chapter 4

$GABA_B$ receptor stimulation in the accumbens: effects on instrumental responding by pre-fed rats in a second order operant schedule

Introduction

In the previous chapter it was suggested, on the basis of Kelley's hypothetical explanation for the role of intra-Acb GABA in feeding, that disinhibition of a fragment of motor behaviours required for ingestion could interfere with other feeding related behaviours. On this basis it was predicted that the BSS would be significantly disrupted by both intra-Acb baclofen and muscimol. In contrast the experiments reported in Chapter 3 demonstrated that the $GABA_B$ agonist baclofen caused an increase in feeding behaviour without the loss of other behavioural components that make up the BSS when pre-fed rats were given free access to laboratory chow. The BSS was temporally shifted to the right (the onset of satiety was delayed) in a manner consistent with the effects of hunger following periods of fasting.

One of the key questions raised by these results was therefore, if intra-Acb $GABA_B$ receptor stimulation affects direct intake of freely available food in a manner consistent with the effects of hunger, would this be reflected in an increase in motivation to perform more complex, goal-directed behaviours such as operant responding for food, as would be the case with a state of deprivation? The effects of intra-Acb muscimol on operant responding for food has been investigated using a PR schedule and a test of the rate of acquisition of a food rewarded operant response (Hanlon et al., 2004, Zhang et al., 2003). It was concluded that accumbens $GABA_A$ receptor stimulation does not affect food motivated operant responding (Kelley et al., 2005b, Kelley et al., 2005a).

No direct comparison of the actions in the accumbens of $GABA_A$ agonists, $GABA_B$ agonists and the manipulation of endogenous GABA levels on motivational processes have been reported. The finding that the BSS recorded for baclofen differed from that recorded with muscimol indicates that baclofen might also have a different effect on operant responding for food. This possibility will be explored further in the experiments reported in this chapter.

The lack of comparable operant studies with muscimol and baclofen to date appears to be due to an assumption being made that the end-point, inhibition of MSN output, is the same whether this occurs via direct stimulation of $GABA_A$ receptors located on the output neurons or by stimulation of $GABA_B$ receptors on glutamate nerve terminals (Kelley et al., 2005b). Presumably this original assumption was based on a pivotal study that demonstrated that the predominant effect of baclofen in the Acb was to inhibit glutamate release presynaptically (Uchimura and North, 1991). The upshot of this has been that, for the experiments that lead to the consequent formulation of Kelley's working hypothesis to explain the role of GABA sensitive neurons in the Acb in ingestive behaviour, only the effects of muscimol were further explored.

Previous experiments with muscimol focused predominantly on discovering the effects of $GABA_A$ receptor stimulation on motivation for food reward through neurobiologically regulated effects on the value of the food. The basic premise with both the PR schedule and in the acquisition of lever pressing was that hunger makes food reward more desirable and hence animals will learn more efficiently what needs to be done to obtain food and will work harder to gain access. Since intra-Acb muscimol did not mimic these effects of hunger GABA was deemed incapable of mediating motivational processes in isolation. It is possible however that this conclusion was reached because of the nature of the tests applied rather than because GABA in the Acb does not have a specific role in motivation.

The PR schedule has been used extensively to study the effects of modifying reward strength (Hodos, 1961) either by manipulating internal state or by directly adjusting the desirability of both food (e.g. palatability) and addictive drugs (e.g. concentration) (Richardson and Roberts, 1996). However, when using this schedule to explore the effects of muscimol, the authors themselves ((Zhang et al., 2003) point out that a change in break-point does not distinguish effects on motor effort directed at incentive stimuli (i.e. pressing the lever) from effects on the perceived rewarding value of the reinforcer. In other words, these investigators did not use schedules that could specifically discriminate between the neuronal processes involved in appetitive food seeking behaviours and consummatory food taking behaviours. Hence it would not be possible to directly evaluate the effects of intra-Acb GABA receptor agonists on these two (allegedly) dissociable mechanisms using a PR schedule. Furthermore, the nature of

the schedules used previously means that the animals' motivation to press is primarily tested following the ingestion of the food reward. It was not possible therefore to exclude confounding factors, such as the process of satiety, competing with the strength of drug effects on appetitive and consummatory behaviour.

There is good reason to believe that measuring the salience of cues that drive appetitive responding independently from those that drive consummatory behaviours is important. Kelley et al., (2005b) point out that the large and instant increase in feeding seen with intra-Acb $GABA_A$ or $GABA_B$ receptor agonism (or manipulation of endogenous GABA levels) looks much like the orexigenic effects of electrical stimulation of the lateral hypothalamus (ESLH) (Berridge and Valenstein, 1991) or infusion of a glutamate receptor agonist into the LH (Stanley et al., 1996, Duva et al., 2001). It has been suggested that stimulation of the LH causes an increase in feeding via effects on incentive salience (Berridge and Valenstein, 1991).

There are indirect connections between the Acb and the LH via the VP and a unique direct connection between the AcbSh and the LH. Infusions of baclofen and muscimol both activate this area (Heimer et al., 1991b, Groenewegen et al., 1993, Stratford and Kelley, 1997a, Stratford and Kelley, 1999, Stratford, 2005). By inference then a pathway involving GABA receptors that could disinhibit the LH via receptor manipulation at the level of the Acb might also increase incentive salience attribution. As a result it was important to choose a schedule for which effects on incentive salience as well as on reward value could be measured.

Berridge and Valenstein (1991) also suggest that different mechanisms might control feeding when it is induced by mild or severe deprivation and that ESLH might recruit components of neural circuitry that are only involved in the former state. Given the suggestion that the Acb can exert both direct and indirect control over the LH (Stratford, 2007), the lack of effects of the $GABA_A$ agonists in the schedules tested thus far (Kelley et al., 2005b) could be due to the possibility that the schedules were not sensitive enough to provide the behavioural resolution needed to distinguish effects across a spectrum of motivational 'severity'. The schedule chosen for the experiments reported here had therefore to provide more sensitivity and detail for an instrumental response in terms of, for example, effects on reaction times.

It is also relevant that there has been some lack of consistency in the approach to testing potential dose related effects of GABA receptor stimulation in the past. For example, a study to regionally map and characterise macronutrient and taste preference following bilateral muscimol infusion into the accumbens (discussed in Chapter 1) utilised two doses; 40 and 100ng (350 and 880ρmols)(Basso and Kelley, 1999). In the PR study a dose range of 40, 100 and 200ng (350, 880 and 1750ρmols) of muscimol were infused in total bilaterally (Zhang et al., 2003). The ability of muscimol to potentiate the acquisition of pressing for a food reward (Hanlon et al., 2004) was only tested at a dose of 200ng (1750ρmols), which did not have any effect. Hanlon et al. (2004) themselves point out that there may have been different effects on acquisition of instrumental responding if more doses of muscimol were tested.

Given that, at least at 100ng (880ρmols) of muscimol or above, there are potentially confounding motor effects that could disrupt instrumental responding (Scheel-Kruger et al., 1977b, Scheel-Kruger et al., 1977a) the results from the experiments above may not demonstrate a lack of effect of the drug on motivation. As a consequence, the $GABA_B$ agonist used in this chapter was tested at doses previously demonstrated to be behaviourally active in terms of intake but low enough not to cause overt myorelaxant effects during testing (See results, Chapter 3). In the following chapter the same approach will be taken when further testing the effects of the $GABA_A$ agonist muscimol on operant responding.

The experiments highlighted above i.e. the effects of intra-Acb GABA agonism on responding on a PR schedule responding on a PR schedule (Zhang, 2003) and on conditioning (Hanlon et al., 2004) therefore raise three key issues relevant to the choice of studies carried out for this thesis; 1) only the effects of a $GABA_A$ agonist on motivated behaviours has been tested previously 2) motivational effects were always measured in response to the expected incentive value of the reward and the postingestional consequences of consuming it in close temporal association to each other and 3) the doses of GABA agonist used could have disrupted motor behaviours and masked motivational effects.

In this Chapter therefore a group of experiments will be reported for which an alternative schedule that allows the independent measurement of food-seeking behaviour versus food taking behaviour was used. This schedule also needed to provide an opportunity to measure intra-Acb GABA receptor agonist effects on food seeking (appetitive responding) in the absence of concomitant affective responses to the reward and without potential postingestional processes. A second order operant schedule was chosen to a) study goal-directed food motivated behaviours in pre-satiated animals, b) separate out the stages of feeding behaviour i.e. appetitive food seeking behaviour and maintenance of consummatory behaviour and c) minimise postingestional effects leading to satiety, a second order operant schedule was chosen.

With this type of schedule both reward seeking and reward taking behaviour can be characterised and compared by carrying out a separate analysis of the behaviour that occurs prior to and following the first reinforcer presentation (Everitt and Robbins, 2000, Arroyo et al., 1998). Such a schedule allows separate analysis of responses for reward vs. responses affected by access to the reward (providing a measure of the reinforcing efficacy of the reward) but also of the impact of reward-associated cues on responding (Everitt and Robbins, 2000).

Second order operant schedules were designed following modifications to chained conditioned reinforcement schedules developed in the 1950's to study motivation for food (Kelleher, 1966). Intermittent reinforcement of a number of identical units of responding with a conditioned reinforcer maintains operant behaviour in the absence of the primary reinforcer for long periods (Kelleher and Goldberg, 1977). Consequent studies of both fixed interval and fixed ratio versions of second orders schedules confirmed that only very intermittent presentation of food supported schedule maintained constant rates of responding (Goldberg and Tang, 1977, Kelleher and Goldberg, 1977, Goldberg et al., 1981) whilst a selective association between a CS and the primary reinforcer could be established (Goldberg et al., 1975). Everritt and Robbins (2002) point out that FR(FR) schedules result in strong A-O associations and could also amplify the role of the CS in supporting responding. With the latter approach, the willingness of the animal to work for a reward can be initially studied without the confounding effects of satiety on motivation because the CS can robustly support responding.

The use of second order schedules to double dissociate neural mechanisms underlying behaviours driven by the primary reinforcer from those related to motivational, reward seeking behaviours (partially driven by conditioned stimuli that have acquired salience by their association with the reward) have been demonstrated for appetitive instrumental responding for sexual reinforcement (Everitt and Stacey, 1987, Everitt et al., 1987, Everitt et al., 1989), self-administration of drugs (for reviews see (Everitt and Robbins, 2000, Schindler et al., 2002) and ethanol reinforcement (Samson et al., 2000). Second order schedules have been used to assess effects of natural reinforcer magnitude in pigeons (Lee and Gollub, 1971, Stubbs, 1971) primates (Kelleher and Goldberg, 1977, Goldberg et al., 1981) and rats (Everitt et al., 1987, Everitt et al., 1989)

The second order schedule employed in this thesis was based on a version designed to assess both appetitive and consummatory responses within the same trial (Thornton-Jones et al., 2005). The schedule exploited the ability of the conditioned stimulus (CS), in this case a light, to maintain instrumental responding for an extended period of time whilst allowing minimal access to the reinforcer (Schindler et al. 2002). The most widely employed schedule type involves FI units during which FR units must be performed to gain access to the CS and, if criterion is reached during the FI, the reward (Schindler et al., 2002, Everitt and Robbins, 2000).

The schedule used here is described in detail in Chapter 2 (page 73). It has previously been employed to characterise the effects of various pharmacological manipulations known to reduce ingestion (Thornton-Jones et al., 2005, Greenhalgh, 2007, Greenhalgh et al., 2008). The schedule employs units of fixed ratio (FR) responding with a further fixed ratio of ratios superimposed upon this. An initial 5 minute fixed interval (FI 5), during which FR responding for the acquisition of the light CS (FI(FR5: S) without subsequent food reward, provides a measure of food-seeking behaviour (‘the appetitive’ phase). This is then followed by 25 minutes when the animal must perform five presses for the light CS and turn on the light five times, described by the notation FR5(FR5: S), before the food is delivered. This constitutes the consummatory phase. Although there will be some components of appetitive behaviour during the consummatory phase, the first 5 minutes is considered to be purely appetitive since the animals gain no access to the food reward. The entire schedule can thus be denoted FI5(FR5: S), FR5(FR5: S) according to the nomenclature developed by Kelleher (1966).

The 2nd order schedule is also suitable for testing responding in animals that have been pre-satiated prior to experimental treatment. It has long been known that pre-feeding reduces instrumental behaviour for food (Toates, 1981, Toates, 1986), even in highly trained rats (Dickinson and Balleine, 1994) but cues that signal food while animals are food deprived can still initiate a meal and potentiate eating when those animals are satiated (Weingarten, 1983, Weingarten, 1984, Weingarten and Martin, 1989). Pre-fed rats would therefore be expected to exhibit a low level of operant responding that could be increased by a pharmacological manipulation that affected motivation.

The presence of *some* responding even in pre-satiated animals would allow a direct comparison between the pattern of responding due to the motivational effects of the CS and any change in motivation following intra-Acb GABA receptor stimulation. Thornton et al. (2005) reported that pre-feeding animals kept on a restricted diet and maintained at 85% of free-feeding weight prior to testing on this second order schedule resulted in an overall reduction in reinforced lever pressing but had no significant effect on non-reinforced or incorrect lever presses.

The data presented in Chapter 3 suggested that the introduction of baclofen could cause changes in responding specifically in the appetitive phase, the consummatory phase or both. Nevertheless, the current model proposed for the mode of action of GABA agonists in the AcbSh would suggest that if the effects of $GABA_A$ and $GABA_B$ agonist on motivated behaviour are the same there should be no effect on instrumental responding (Baldo and Kelley, 2007, Kelley et al., 2005b, Kelley et al., 2005a). Consequently a positive pharmacological experimental control was also run. In this case a compound that has both a well documented effect on ingestive behaviour and associated effects on operant responding for a food reward was required. Two possible candidates were considered.

It has been previously demonstrated that the reinforcing value or incentive salience attributed to the CS can be robustly increased by intra-Acb infusions of the indirect dopamine agonist, d-amphetamine, which potentiates instrumental responding for the CS (Phillips et al., 1994, Taylor and Robbins, 1984) however there is no concomitant increases in free feeding. In contrast a role for peripheral opioids in modulating

responding to freely available food and in performing operant responses for unconditioned food reinforcers has been well established. It would appear therefore that an opioid agonist would be the more appropriate positive control although the specific role of intra-Acb opioids (rather than peripherally administered agonists) in operant responding is not entirely clear.

Although results from earlier studies using a variety of opioid agonists are mixed (Kelley and Domesick, 1982, Cunningham and Kelley, 1992b, Cunningham and Kelley, 1992a) it has been shown that DAMGO increases responding on a CR paired lever (Phillips et al., 1994). Zhang et al. (2003) demonstrated that DAMGO infused into the accumbens significantly increased both the rate of lever pressing and the breakpoint in a PR schedule for sucrose pellet reward. The authors suggested that "enhancement of positive hedonic properties of food" could be directly translated into a high level of motivation (and hence into increases in food seeking behaviour) (Zhang et al., 2003). For this set of studies therefore the μ-opioid agonist DAMGO (used in Chapter 3) was used as the positive control.

The experiments reported in this chapter will examine the effect of 1) a low dose range baclofen; 110, 220 and 440 ρmols/μl^{-1}, 2) a higher dose range baclofen; 220 and 660 ρmols/μl^{-1} or 3) DAMGO at 0.025ng/μl^{-1}. Effects will be analysed in terms of the magnitude and accuracy of responding on two operant levers with respect to the programmed schedule. The efficacy of the dose ranges chosen on intake of freely available food will be confirmed by measuring total intake of chow in the same experimental groups using the same doses following the operant schedule test phase.

These experiments will contribute to our understanding of the specific effects of intra-Acb $GABA_B$ receptor stimulation revealed in Chapter 3 and will also address four of the key aims of the thesis. In experiment 4.1 the effects of an identical range of doses of baclofen for which the BSS was characterised will be recorded for 5 minutes of purely appetitive responding for a CS and for 25 minutes of mixed appetitive/consummatory behaviour on the second order schedule. The effect on total intake of freely available chow of infusions of the highest dose of baclofen from the range will also be assessed in the same cohort of animals.

The aim of experiment 4.1 will be to test whether baclofen a) potentiates appetitive responding for food-paired stimuli in the absence of ingestive behaviour and the consequences of ingestion and b) potentiates responding when the primary reinforcer becomes intermittently available. This will further test the hypothesis put forward by Kelley and colleagues that GABA receptor stimulation in the Acb causes increased intake because of the consequent release of downstream motor pattern generators. Detailed temporal analysis of responding across the session, the relationship between CS presentations and pressing and between pellet delivery and pressing will allow an interpretation of the results in terms of motivation theory and the neurobiology underlying any effects.

Experiment 4.2 will involve intra-Acb infusions of one dose of the μ-opioid agonist DAMGO. Although it is not clear if this pharmacological manipulation will have any effect during the appetitive phase of the schedule it is predicted to at least increase responding during the consummatory phase. Because of this possible dichotomy in effects on appetitive vs. consummatory responses the use of DAMGO could help to clarify whether or not the schedule is sensitive to both aspects of motivation for reward.

A comparison between the temporal analysis for DAMGO, which is known to effect intake via effects on palatability, and the results from the baclofen manipulation may provide insights into the behavioural mechanisms underlying the latter results. This will also help to elucidate whether the effects of GABA receptor stimulation are specific to different phases of ingestion e.g. anticipatory responding, consummatory behaviour and satiety mediated by the Acb. The effect on total intake of freely available chow of infusions of the same dose will also be assessed in the same cohort of animals.

Finally in experiment 4.3 the effects of baclofen in the second order schedule will be further explored across a broader dose range in view of the results reported in experiment 4.1. This will include the analysis of the effects of baclofen on behaviours other than lever pressing using videos of the test sessions. In this experiment a full dose range, free-feeding intake study will be carried out following completion of the second order schedule testing phase using the same counterbalanced design to allow a direct comparison between any effects on operant responding and on intake. Given that the effects of hunger and satiety have already been reported for the schedule (Greenhalgh,

2007, Thornton-Jones et al., 2005) it will help to put the role of GABA in the context of naturally elicited feeding and motivational control in general. Results reported in this chapter and in Chapter 5, where the effects of muscimol will be further investigated using the second order schedule, will further contribute to the characterisation and comparison of the effects of GABA receptor subtype stimulation on feeding related behaviours.

To summarise, previous research indicates that, although intra-Acb $GABA_A$ agonist infusion increases intake of freely available food, it does not potentiate the acquisition of a stimulus-food reward association nor does it increase operant responding in a PR schedule. However, the effects of $GABA_B$ agonists on acquisition or performance of operant responses has not been investigated. Furthermore, the effects of muscimol were tested across a narrow dose range that encompasses concentrations that could cause motor disruption. Finally the schedules used previously tested appetitive responding in close temporal association with consummatory responses. This meant that the effects of GABA receptor stimulation on a specific subset of the mechanisms underlying food motivation could not be distinguished. Neither could the potential confounding effects of post-ingestional processes and satiety mechanisms be excluded when interpreting the results. The three experiments reported in this chapter will:

1) Test the effects of a range of doses of a $GABA_B$ agonist on instrumental behaviour in the second order schedule (Experiment 4.1 and 4.3). Does the $GABA_B$ agonist baclofen affect operant responding?
2) Characterise the magnitude and temporal pattern of responses made following intra-Acb $GABA_B$ infusion during both appetitive and consummatory operant phases (Experiment 4.1 and 4.3). Does the $GABA_B$ agonist baclofen cause any divergence in responding depending on the phase?
3) Test the effects of a range of doses of a $GABA_B$ agonist on behaviours other than lever pressing expressed during an operant schedule (Experiment 4.3). Does the $GABA_B$ agonist baclofen have any effects on general activity, other indices of motivated behaviour or on adjunctive behaviours not picked up by measuring lever pressing alone?
4) Test the effects of a μ-opioid agonist infused into the same location on operant responding in the second order schedule (Experiment 4.2). Does the μ-opioid

agonist DAMGO, at a dose that increases free intake, affect operant responding on this specific type of schedule?

5) Characterise the magnitude and temporal pattern of responses made following intra-Acb μ-opioid agonist infusion during both appetitive and consummatory operant phases (Experiment 4.1 and 4.3). Does the μ-opioid agonist DAMGO cause any divergence in responding depending on the phase?
6) Compare the effects of a $GABA_B$ agonist to those of a μ-opioid agonist in the Acb on the magnitude and temporal pattern of responses made during appetitive and consummatory operant phases (Comparison of data from experiments 4.1, 4.2 and 4.3). Does the $GABA_B$ agonist baclofen control operant responding for food via similar mechanisms known to underlie μ-opioid agonist effects on intake?

The overarching question posed in this chapter is:
Does intra-Acb administration of the $GABA_B$ agonist baclofen potentiate any aspect of complex goal-directed motivated behaviour on a second order schedule in a manner consistent with selective effects on appetitive and/or consummatory behaviours?

Experiments presented in this chapter

Experiment 4.1
The effects of bilateral intra-Acb infusions of a range of doses of baclofen in pre-fed rats trained to respond on a second order operant schedule.

Experiment 4.2
The effects of bilateral intra-Acb infusions of DAMGO in pre-fed rats trained to respond on a second order operant schedule.

Experiment 4.3
The effects of bilateral intra-Acb infusions of a higher range of doses of baclofen in pre-fed rats trained to respond on a second order operant schedule, on associated behaviours and on free intake.

Materials and methods

Animals

Drug naïve animals (n=12) were used for Experiment 4.1 (2nd order with baclofen). This same group of animals were then run through the test again for Experiment 4.2 (2nd order with DAMGO). A second drug naïve group of animals (n=12) was used in Experiment 4.3 (2nd order with higher dose range baclofen). Subjects were bought in weighing between 150-200g. This meant that, on a restricted diet and over the longer habituation and training period associated with the schedule, animals had reached approximately the same size as those used in chapter 3 when surgery was undertaken. This ensured that their position in the stereotaxic equipment was not significantly different as can be the case with larger animals that have developed greater musculature and/or fat deposits in the cheeks. In all cases the animals were housed in pairs during training on the 2nd order schedule. They were then split and housed singly in Perspex cages for a minimum of 7 days prior to surgery and for the remainder of each experiment.

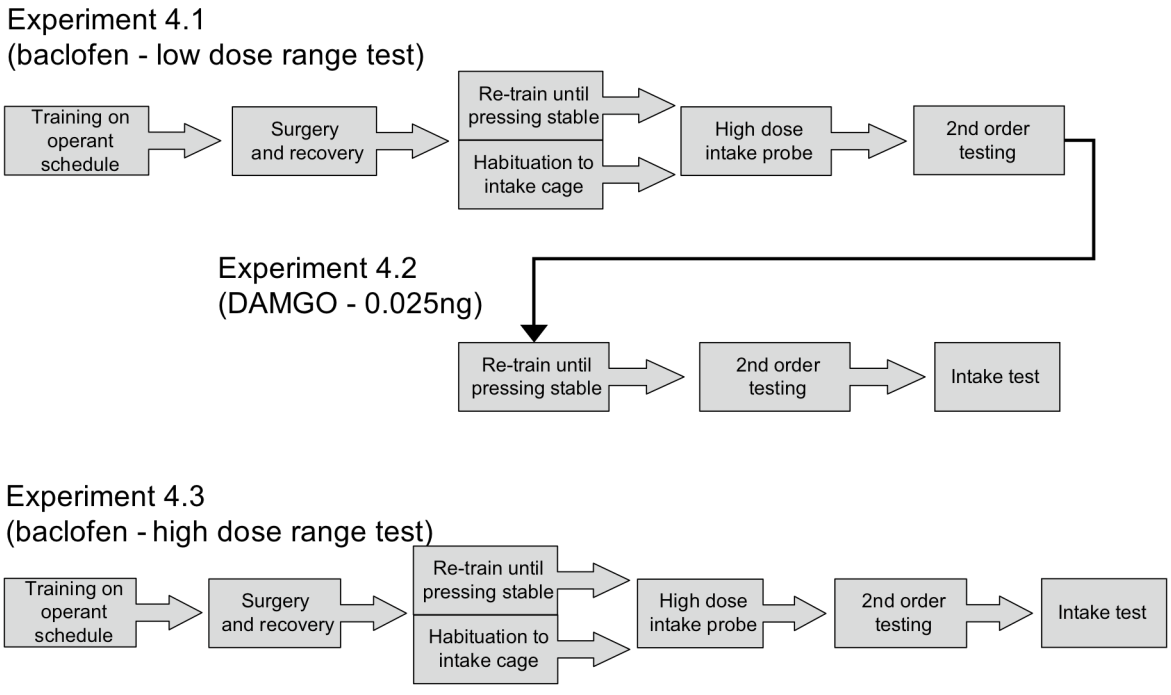


Figure. 4.1. Diagram to show which groups of animals were used for more than one test procedure within experiments 4.1, 4.2 and 4.3 and the timeline for each.

Apparatus and habituation

The apparatus, habituation and training procedure for the 2nd order schedule is described in detail in Chapter 2. All animals were food deprived for a minimum of 7 days prior to magazine training and returned to an *ad lib* diet for a minimum of 4 days prior to surgery. Post surgery and recovery, animals were put back on a restricted feeding regime, re-trained and then given sham infusions as described in Chapter 2 (page 67).

Post surgery, animals were also habituated to the test cages for the free intake test sessions. Habituation to eating chow pellets in a non-food deprived state (to match the motivational state of animals tested in Chapter 3) required that the rats be pre-fed prior to being transferred to the intake test cages (see habituation procedure described in Chapter 2 (page 69). To recap, food was removed from the home cage one hour before habituation and replaced with 5 fresh pellets to which rats had access for 30 minutes before being transferred to the tests cages for a 30 minute acclimation period. As a result it was necessary to weigh how much was eaten during the pre-feeding period and in the intake test cages and to adjust the amount they would subsequently be fed for the day to maintain their weight at 85% of free feeding weight.

Animals were also pre-fed in the home cage prior to infusions for the 2nd order test session. In this case food was removed from the home cage one hour before habituation and replaced with 5 fresh pellets to which rats had access for 30 minutes and then they remained in the home cage for a further 30 minutes leading up to the infusion. All of these experiments were carried out using a within subject, counterbalanced, Latin square design such that each animal served as its own control. Animals were always re-exposed to the schedule in a food deprived state the day before a test session, irrespective of how many days had elapsed between tests.

Specific procedural details – central drug administration

On test days only two animals were infused at a time to compensate for the time taken for each infusion. The programme controlling the operant boxes allowed each box to be started individually. This meant that testing of each animal could be temporally staggered and there were 4 operant boxes. Infusions for the next batch of 4 animals to be tested began a few minutes before the previous batch was due to come out and the

whole cohort could be run over a period of approximately 4 hours (between 12.00 and 16.00). There was a minimum of 48 hours between infusions.

Experiment 4.1

The range of doses of baclofen to be tested in the 2nd order schedule was the same as the range used in Chapter 3, experiment 3.1 i.e. 110, 220 and 440 ρmols/μl^{-1} or vehicle (0.9% sterile saline – used throughout) infused immediately before transfer to the operant boxes. Initially a counterbalanced intake probe using the highest dose of 440ρmols vs. vehicle was run to ensure that the placements were behaviourally active in terms of increased feeding in pre-fed rats (for method for intake test see Chapter 2, page 69). This was followed by the test run on the 2nd order schedule (as detailed in Chapter 2, page 75) using the whole dose range of baclofen. These animals were given >2 days drug free. This experiment was followed by a second run with DAMGO (Exp. 4.2).

Experiment 4.2

In experiment 4.2 only one dose of 0.025ng/μl^{-1} of DAMGO and vehicle were tested. This dose was chosen because the higher dose of 0.25 ng/μl^{-1} used in the BSS study reported in Chapter 3, experiment 3.3 caused sedative effects in some of the animals. Following 2nd order test days the animals were run through a counterbalanced intake test with chow using the same dose of DAMGO and vehicle.

Experiment 4.3

In response to the results for experiment 4.1 (see below) a broader range of baclofen doses was tested. As a result an intermediate dose between the highest (440ρmols) used in experiment 4.1 and 880ρmols used by Stratford and Kelley (1997) was chosen. The final range tested here was therefore 220, 660 ρmols/μl^{-1} baclofen or vehicle. Initially a counterbalanced intake probe using the highest dose of 660ρmols vs. vehicle was run to ensure that the placements were behaviourally active in terms of increased feeding in pre-fed rats (for intake test method see Chapter 2, page 69). This was followed by the test run on the 2nd order schedule (as detailed in Chapter 2, page 75). The 2nd order test was followed by a counterbalanced dose response test of intake of chow in pre-fed animals using the same range of doses. Finally duration and frequency data for distinct

categories of behaviours exhibited during responding were extracted from videos of the 2nd order operant test sessions (see below for details of video analysis).

Data analysis

The final analysis groups were decided on the basis of histological verification of infusion sites as before. Unix Shell scripts were written, by Prof. Pete Clifton and myself, to extract all of the following information from the raw output recorded at the time of testing.

First of all, total reinforced, non-reinforced and incorrect presses data for each treatment group were consolidated from the raw output and compared using a repeated measures, mixed design ANOVA with dose and press category as factors. To recap, presses on the correct lever made while the CS was on could not contribute to the next ratio (and hence to delivery of food) and hence were defined as 'non-reinforced'. Incorrect presses were those made on the second lever that had no programmed consequences. Next data for each treatment group for the mean cumulative reinforced lever presses were extracted and plotted to illustrate the way in which treatments affected the gross temporal progression of responding on the schedule.

Rates of responding were calculated for the two different phases; the appetitive phase (first 5 minutes) and the consummatory phase (remaining 25 minutes) derived on the basis of the total number of presses per minute made during each phase. Comparisons of rate could then be statistically compared using a within subjects, repeated measures ANOVA with phase and dose as factors.

Next 5 minute timebin reinforced presses (and non-reinforced in experiment 4.1 only) were consolidated from the raw output. By splitting the pressing behaviour into time-bins it was possible to look at the temporal structure of the test session in more detail. The mean total presses in each time bin and the relative changes in these totals over time were compared between doses using a repeated measures, mixed design ANOVA with dose, and time-bin as factors.

The total number of pellets delivered was extracted to give a measure of intake within the operant schedule compared to intake when pre-fed animals were given free access to

chow. Pellets received were analysed with a within subjects, repeated measures ANOVA with dose as factor. In all cases where post hoc comparisons were carried out Dunnett's test was used to compare drug effects with vehicle control or the Bonferroni test was used if it was of interest to compare effects between all treatments.

Next the mean cumulative proportion of inter-lever press intervals (ILIs) of discrete durations of between 0 – 30 seconds was parsed from the data. Of the possible transitions that these intervals spanned i.e. from the first press of an FR5 to the second, from the second to the third etc. the period of interest was the progression from the 5^{th} press that elicited the CS and the press that followed this. By looking at how long this ILI was in relation to the 8s period of the CS presentation it was possible to see if animals were responding to the contingency requirement of the schedule and if the drug treatments disrupted this. Therefore only data for the 5^{th} to 1^{st} ILI were further extracted and characterised. These data were split into the cumulative ILIs during the first 5 minutes of unrewarded pressing and the subsequent 25 minutes when reward was available. Due to the nature of the data output generated for the ILIs it was only analysed as far as providing descriptive statistics.

A script was also used to calculate the interval between the delivery of pellets and the next time the animals made a reinforced lever press. This interval was termed the inter pellet-lever interval (IPLI). Because there was a unequal number of IPLIs for each animal for each dose the mean length of all IPLIs between the vehicle and drug treatment group were compared in SPSS using Levene's test for equality of variances and, dependant on the outcome, followed by the appropriate one-way ANOVA or one-tailed t-test. The one-way ANOVA was followed by planned post-hoc analyses. Hochberg's GT2 was specified (which uses a harmonic mean to exclude bias from data with unequal sample sizes) and the results for the comparisons were verified using the Games-Howell procedure (Field, 2005).

Where relevant the effects on total intake was analysed using a within subjects, repeated measures ANOVA with dose as factor. When reporting all of the above data in the results section post hoc analysis using Dunnett's test will be referred to as paired comparisons but where the Bonferroni test was used to compare effects between drug doses or Games-Howell procedure (as above) were used these will be specified.

Data extraction from videos

In experiment 4.3 other behaviours associated with responding in the operant box on the 2nd order schedule were recorded from the videos of each test session. Unix Shell scripts were used to extract the total durations and frequency of each behaviour and to parse the data into 5 minute time bins for more detailed temporal analysis. The means for total duration and frequency of behaviours in each treatment group were compared using a repeated measures ANOVA with dose and behavioural category as factors. The timebin data were analysed using the same ANOVA design but including dose, behavioural category and time-bin as factors. Timebin data were plotted as line plots to illustrate the change in the durations and frequencies of each individual behaviour across the session.

It was then possible to calculate the rate of ingestion of pellets from the duration of eating behaviour recorded for each animal and the total number of pellets consumed. The mean rate of feeding for the treatment groups was then compared using a repeated measures ANOVA with dose as factor. The same approach was taken for calculating the overall rate of lever pressing across the session from the time spent at the reinforced lever and the total number of presses made on this lever (reinforced + non-reinforced).

Whilst recording occurrences of rearing behaviour from the videos a separate tally was kept for rears made specifically to the cue light and/ or the sound of the pellet delivery hopper turning. The mean proportion of rears to the CS vs. the total number of rears made was then compared between treatment groups using a repeated measures ANOVA with dose as factor.

Next the relationship between lever pressing and number of nose-pokes into the magazine was investigated. The total number of reinforced lever presses vs. total duration and frequency of nose-pokes was calculated and the means compared using a repeated measures ANOVA with dose as factor.

Preliminary observations suggested that there might be dose related effects on grooming behaviour so the presence or absence of a normal grooming sequence characterised by a rule-driven (syntactic) chain with a stereotyped order of paw, head and body movements (Berridge and Whishaw, 1992, Aldridge et al., 2004) was recorded. Any other changes in the pattern of behaviour were noted while coding the 8 categories.

Results

Experiment 4.1: **The effects of bilateral intra-Acb infusions of baclofen at 110, 220 & 440ρmols/μl^{-1} in pre-fed rats on free intake and responding on a second order operant schedule for food.**

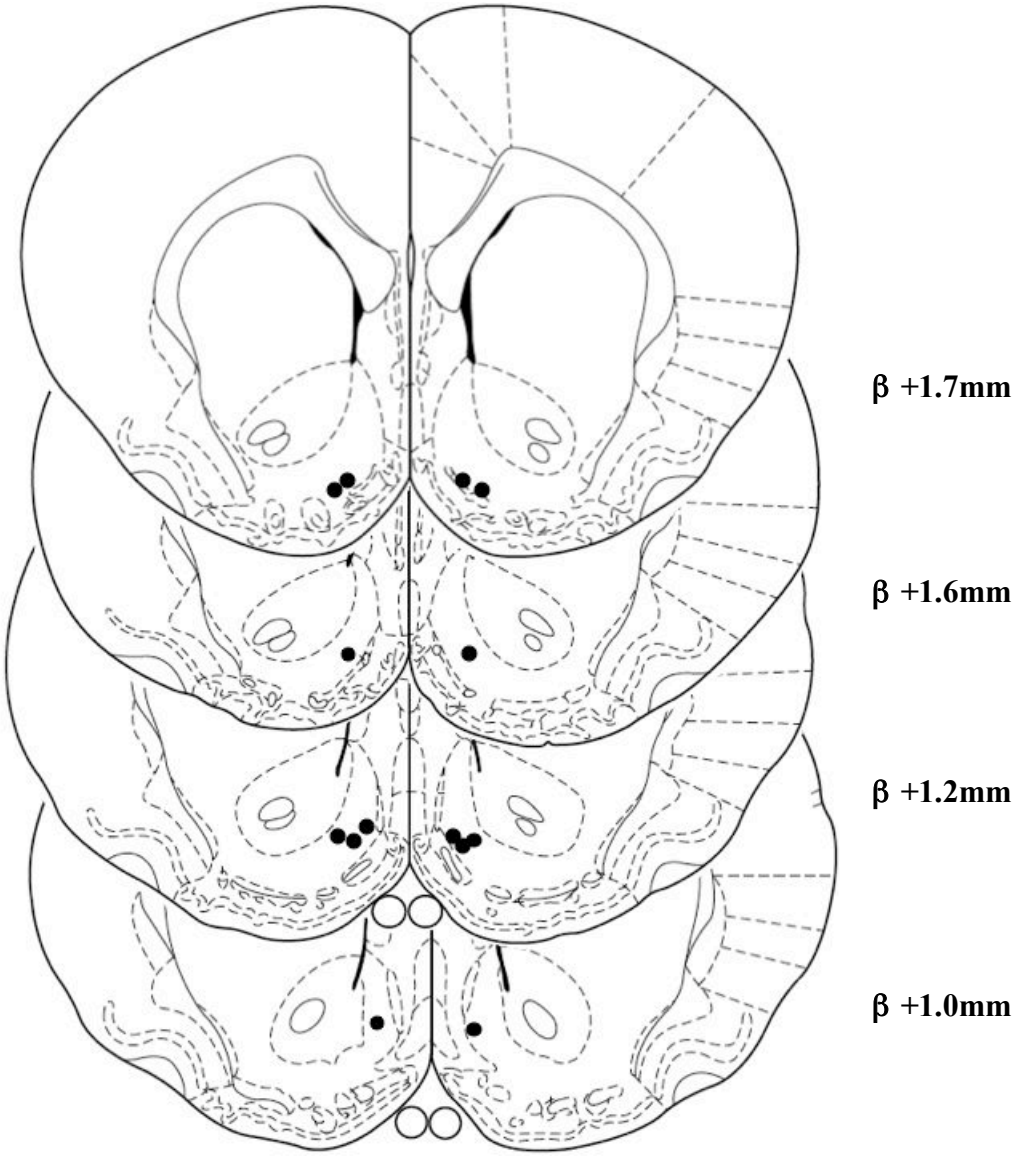


Figure 4.2. Injection sites plotted on drawings taken from Paxinos and Watson (1998); sections are anterior relative to bregma (β). Bilateral target coordinates (n=7 of original n=12 with acceptable placements) were (AP), + 1.4mm, mediolateral (ML), ± 0.9mm relative to bregma and dorsoventral (DV), -7.8mm relative to skull surface.

Prior to testing animals in the operant chambers, responsiveness to baclofen in pre-fed animals was verified at a dose of 440ρmols baclofen. Two of the original cohort of n=12 animals were noted to have consumed less chow at this dose than with vehicle treatment. These were among the animals excluded from the final analysis on the basis of the histology. A total of n=7 animals were verified as having acceptable placements, as defined in Chapter 2, and were included in the final analysis. A schematic illustration of Acb infusion site placements is given in Fig. 4.2. These animals consumed significantly more chow with baclofen treatment than with vehicle over a 30 minute period [Fig. 4.3: $F(1,6)=84.46$, $p<0.001$].

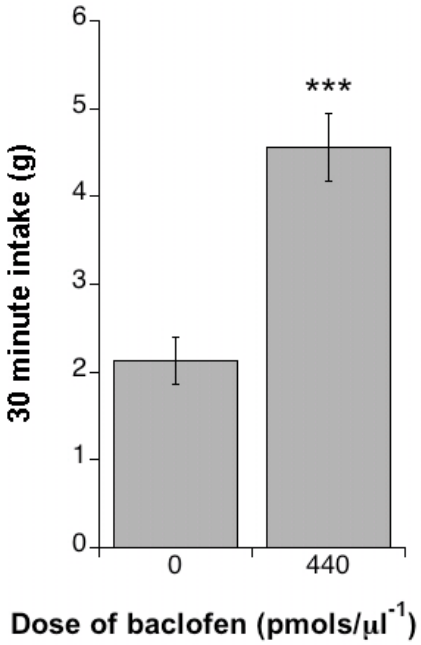


Figure 4.3. The effects of intra-Acb bilateral infusions of saline or baclofen in pre-fed rats (n=7) given access to laboratory chow over a 30 minute test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★★★ p<0.001.

In the operant test session, rats that had been pre-fed (access to 5g of chow for 30 minutes) prior to infusions of a range of doses of baclofen nevertheless demonstrated a significant increase in the total number of reinforced lever presses for food [Fig. 4.4: $F(3,18) =10.95$, $p<0.001$]. Planned post-hoc analysis using the Bonferroni test revealed that the 220ρmols significantly increased reinforced responding relative to vehicle ($p<0.01$) and to both other doses of baclofen ($p<0.05$ in both cases). The 440ρmols dose also significantly increased reinforced responding relative to vehicle ($p<0.05$). There

was no significant increase in errors due to drug i.e. there was no increase in presses on the 'incorrect' lever but there was a significant increase in non-reinforced presses [Fig. 4.4: $F(3,18) = 4.03$, $p=0.024$]. Paired comparisons revealed that the total number of non-reinforced presses was only significantly increased relative to vehicle levels at 220ρmols. When the total number of non-reinforced presses was expressed as a proportion of total presses on the reinforced lever (i.e. non-reinforced / non-reinforced + reinforced) there was no significant difference between the doses i.e. the increase in non-reinforced lever presses was proportional to the increase in reinforced lever presses.

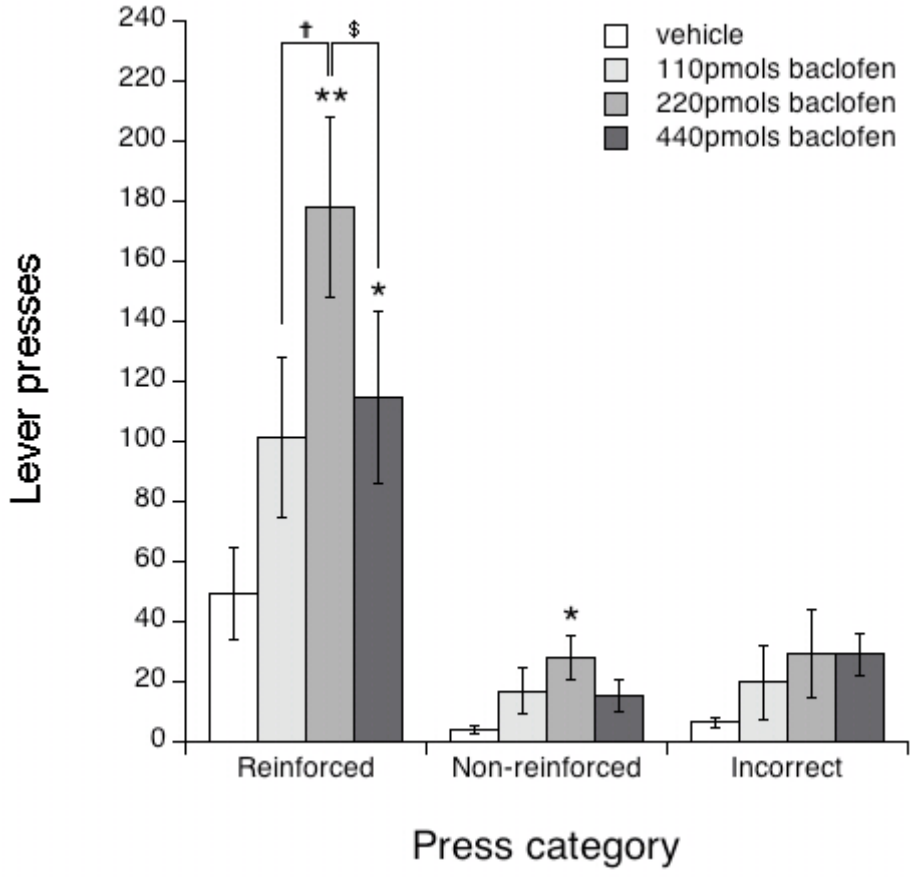


Figure 4.4. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=7) on total lever presses in a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ $p<0.05$, ★★ $p<0.01$. Significant differences versus the next higher drug dose are denoted by † $p<0.05$. Significant differences versus the preceding lower drug dose are denoted by $ $p<0.05$.

There was no significant effect of previous drug treatment on the total number of reinforced lever presses made by food deprived rats on the training days immediately following testing. Furthermore there was no significant difference between pressing on these training days and the levels of pressing prior to the start of testing on the 2nd order schedule.

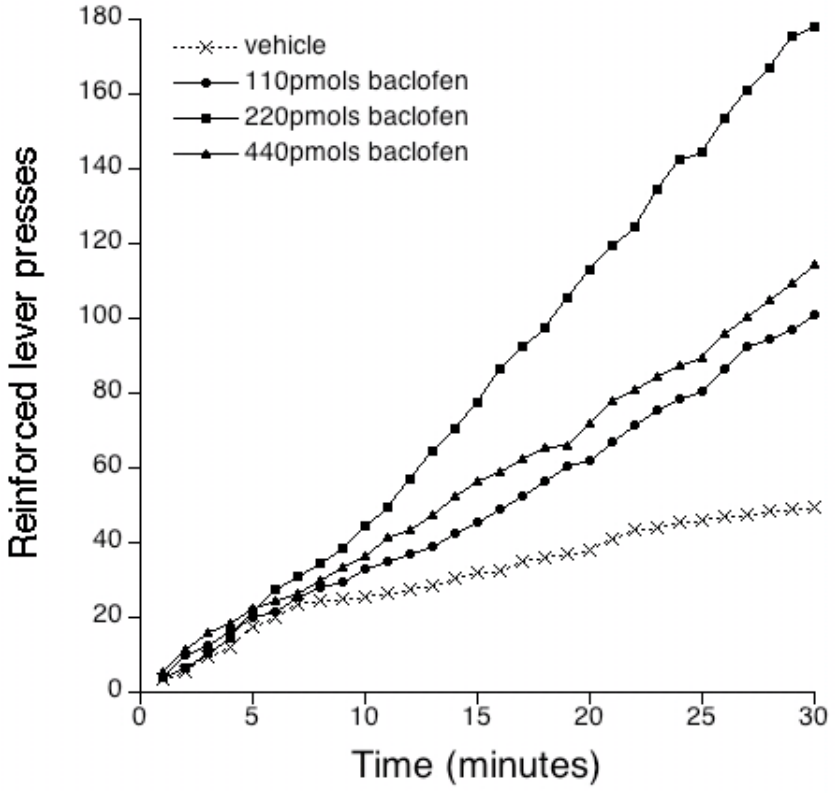


Figure 4.5. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=7) on cumulative reinforced lever presses over a 30 minute second order operant test session.

The plots of cumulative reinforced lever presses over time shown in Fig. 4.5 above suggest a linear increase in total lever presses with baclofen treatments as the session progressed. In contrast, rats under the vehicle condition appeared to decrease their response rate after the first 5 minute appetitive phase. The rates of lever pressing, expressed as reinforced presses per minute, across the first 5 minutes (appetitive) and subsequent 25 minutes (consummatory) of the schedule are shown for each treatment in Table 4.1.

With vehicle, 110ρmols and 440ρmols of baclofen, the rate of pressing in the late phase was lower than in the early phase but not significantly so. With the 220ρmols treatment the rate of pressing during the late phase was significantly greater than during the early phase [$F(1,6)=21.26$, $p = 0.004$]. A comparison of the rates of pressing between each treatment during the early phase indicated that there was no significant difference. During the subsequent 25 minutes there was a main effect of drug, increasing the rate of pressing above vehicle levels [$F(3,18)=15.37$, $p <0.001$]. Multiple post hoc comparisons made using the Bonferroni test revealed that, at 220ρmols the rate was greater than with vehicle ($p < 0.01$), 110ρmols dose ($p < 0.01$) or 440ρmols ($p < 0.05$). At the 440ρmols dose, the rate of pressing was significantly greater than with vehicle ($p < 0.05$).

Table 4.1. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=7) on the rate of reinforced lever pressing during the first 5 minutes (appetitive phase) and last 25 minutes (consummatory phase) of a second order operant test session. Significant differences between phases for each treatment are indicated by the '*p*' values reported in the last column. Significant differences between treatments for each phase for drug vs. vehicle are denoted by ★ p<0.05, ★★ p<0.01. Significant differences relative to lower drug doses are denoted by $$ p<0.01. Significant differences relative to higher drug doses are denoted by † p<0.05.

Treatment	Phase	Rate presses/min	Difference between phases *p* =
Vehicle	Appetitive	3.49 ± 0.99	NS
	Consummatory	1.29 ± 0.57	
110pmols baclofen	Appetitive	4.00 ± 1.05	NS
	Consummatory	3.25 ± 0.91	
220pmols baclofen	Appetitive	4.29 ± 1.01	0.004
	Consummatory	6.27 ± 1.01 ★★/$$/†	
440pmols baclofen	Appetitive	4.46 ± 0.97	NS
	Consummatory	3.69 ± 1.03 ★	

When the data were split into 5 minute bins no significant main effect of time on reinforced lever pressing was revealed but there was a significant interaction between drug and time (see Fig. 4.6). Further analysis, restricting the ANOVA to individual time bins, revealed that the animals given drug pressed significantly more frequently on the reinforced lever from 5 minutes onwards. Multiple comparisons made using the Bonferroni test showed that only the 220ρmols dose significantly increased pressing above vehicle levels between 5 and 30 minutes (5–15 minutes, $p<0.05$ and 15–30 minutes, $p<0.01$). Between 15 and 20 minutes pressing with 220ρmols was also significantly higher than with either of the other two drug doses (110ρmols, $p<0.05$; 440ρmols, $p<0.01$).

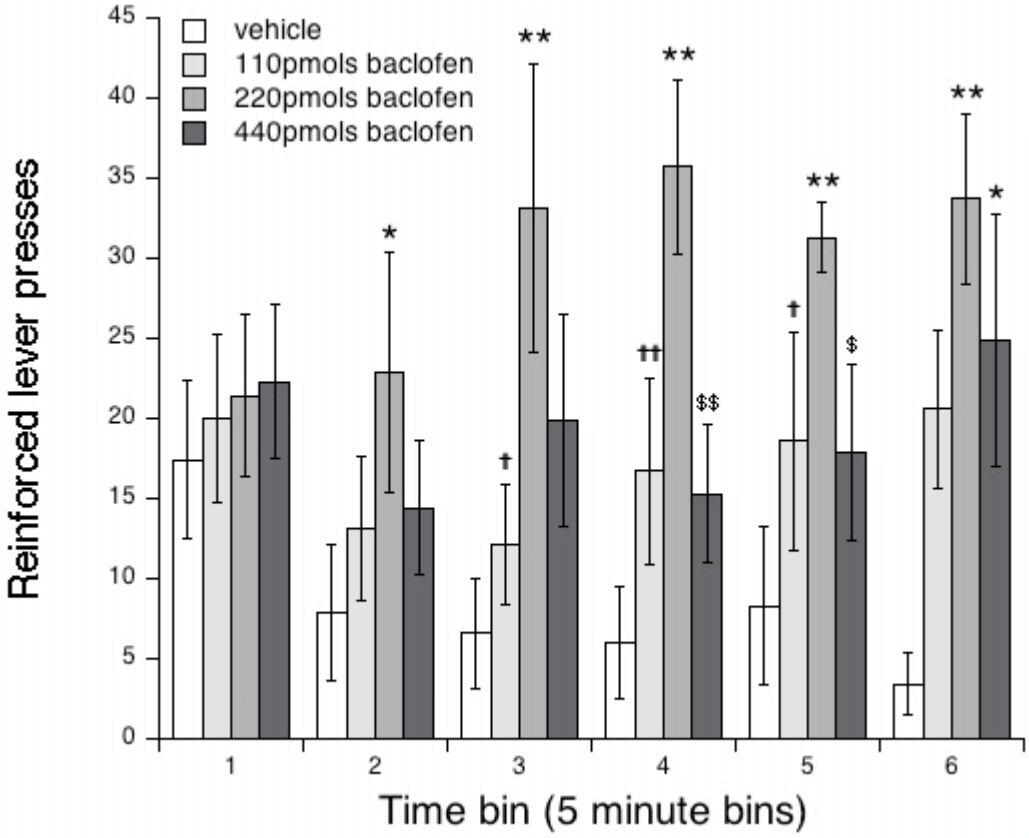


Figure 4.6. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=7) on reinforced lever presses across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ p<0.05, ★★ p<0.01. Significant differences versus the next higher drug dose are denoted by † p<0.05, †† p<0.01. Significant differences versus the preceding lower drug dose are denoted by $ p<0.05, $$ p<0.01.

The effect of drug on non-reinforced lever pressing was revealed to be due to a significant main effect of dose [$F(3,18)=4.03$, $p = 0.024$]. Paired comparisons revealed that only the 220ρmols dose significantly increased non-reinforced presses and only between 15-25 minutes ($p<0.05$). The significantly higher level of non-reinforced pressing coincided with the peak in reinforced presses on this lever (Fig. 4.7 compared to Fig. 4.6).

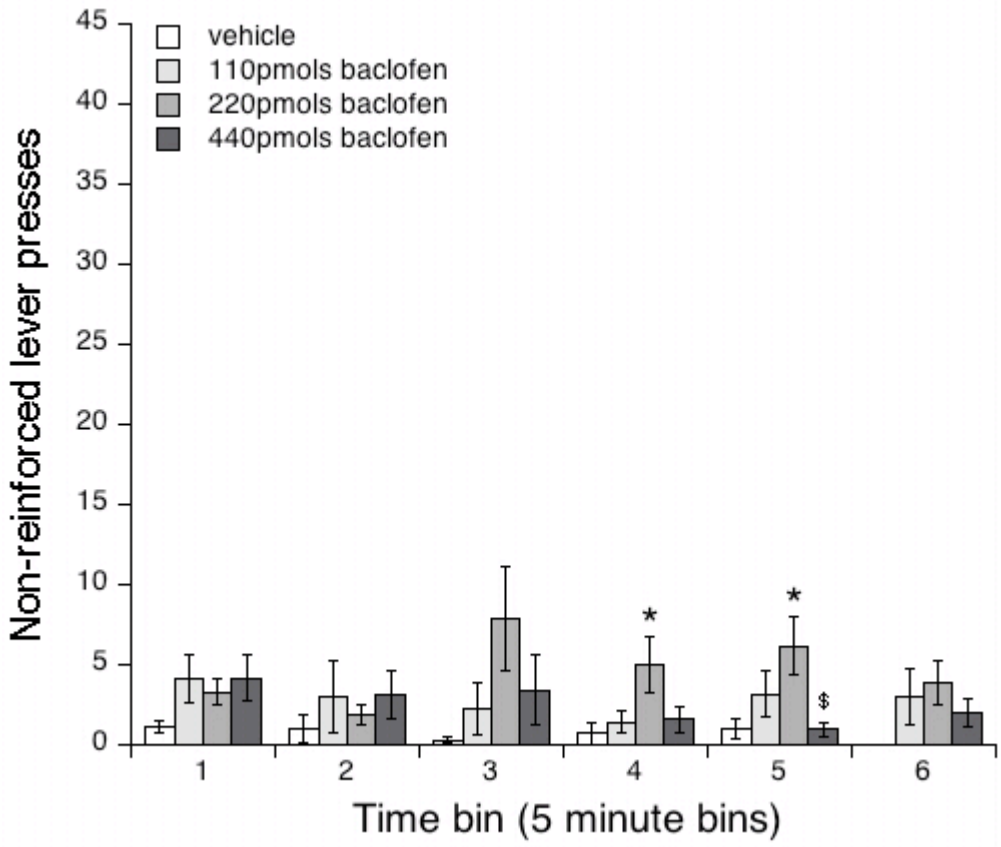


Figure 4.7. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=7) on non-reinforced lever presses across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ $p<0.05$. Significant differences versus the preceding lower drug dose are denoted by $ $p<0.05$.

The cumulative mean proportions of the length of inter lever press intervals (ILIs) between the 5th press of an FR5 response and the 1st press of the next FR5 across the first 5 minutes of the 2nd order schedule are depicted in Fig. 4.8 below. The distributions of ILIs for the remaining 25 minutes of the test session are shown in Fig. 4.9. In the first 5 minutes of the session, when no lever presses could be rewarded with the primary reinforcer, animals did not press during the CS with vehicle but did do so with all drug treatments. At 110ρmols an average of 20% of ILIs were within the first 8 seconds i.e. when the cue light was still on. An average of 34% ILIs at 440ρmols but only 5% for the 220ρmols dose were made during the CS. There was no apparent trend in the maximum amount of time animals waited to press.

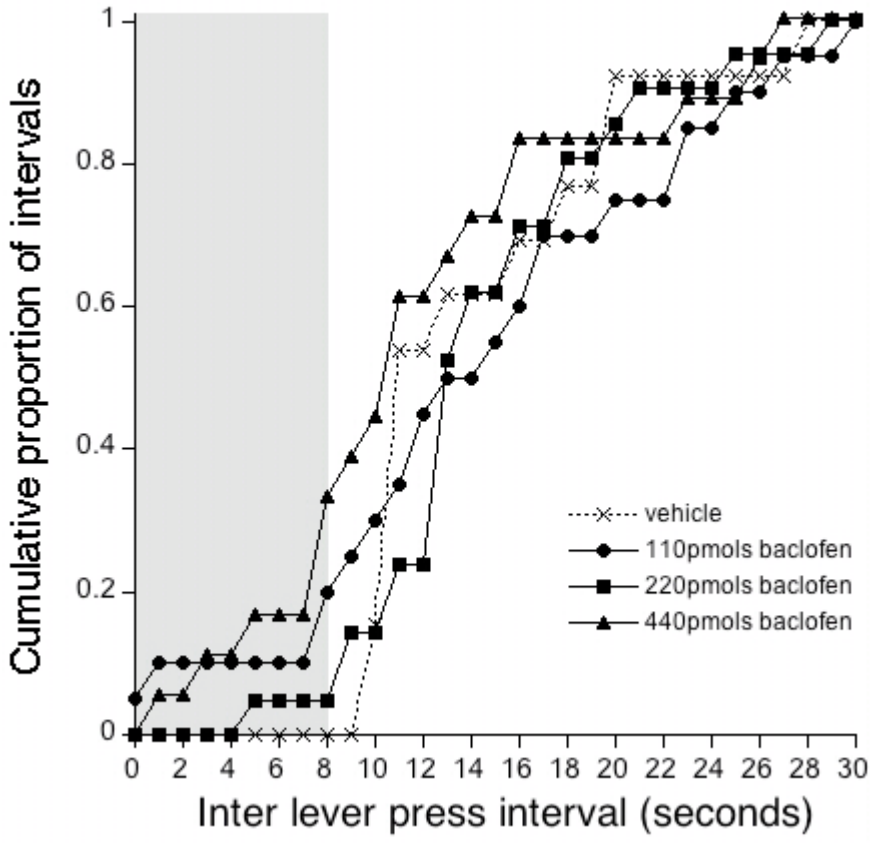


Figure 4.8. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=7) on cumulative ILIs between the 5th and 1st presses of the FR5 requirement on the reinforced lever. Data represents responding across the first 5 minute unrewarded phase of a second order operant test session. The shaded phase represents the duration of the CS (8 seconds). The CS indicated that criterion had been met and that concomitant presses would not be reinforced.

For the remaining 25 minutes of the session some animals pressed during the CS with all treatment conditions but, with the higher two doses of baclofen, some of the animals did not wait at all to start pressing. With 220ρmols of baclofen the inter lever latencies were shifted the to the left relative to vehicle.

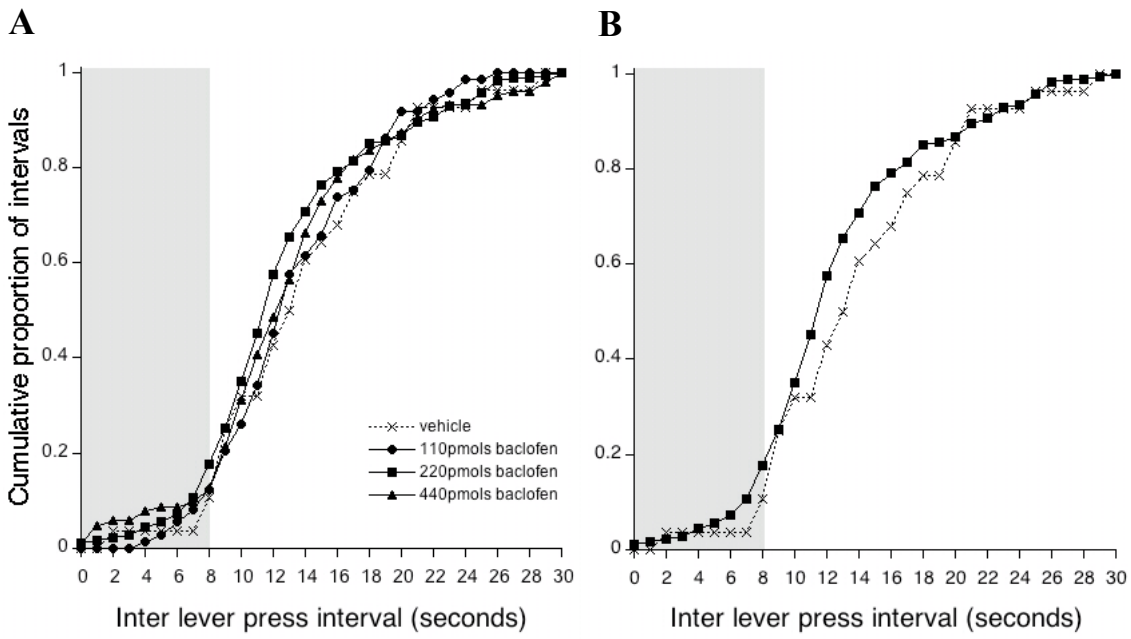


Figure 4.9. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=7) on cumulative inter lever press intervals between the 5th and 1st presses of the FR5 requirement on the reinforced lever. Data represents responding across the last 25 minute rewarded phase of a second order operant test session at A) all doses tested and B) with just vehicle or 220pmols. The shaded phase represents the duration of the CS (8 seconds). The CS indicated that criterion had been met and that concomitant presses would not be reinforced.

There was a significant increase in the number of pellets delivered with drug treatment relative to vehicle [Fig. 4.10, $F(3,18)=15.56$, $p<0.01$]. Paired comparisons revealed that the increase in the number of reinforced presses with the 220ρmols dose of baclofen resulted in a significant increase in the total number of pellets delivered ($p<0.01$). The highest dose of 440ρmols also significantly increased the number of pellets delivered relative to vehicle ($p<0.05$). Not all of the animals at all of the doses pressed enough times to receive pellets over the 30 minute period. As a result, any calculation of a mean latency to pellet delivery for each treatment group would have had to have been based on a different numbers of subjects. Because of this it was not possible to carry out a meaningful statistical analysis on latency to first pellet delivery.

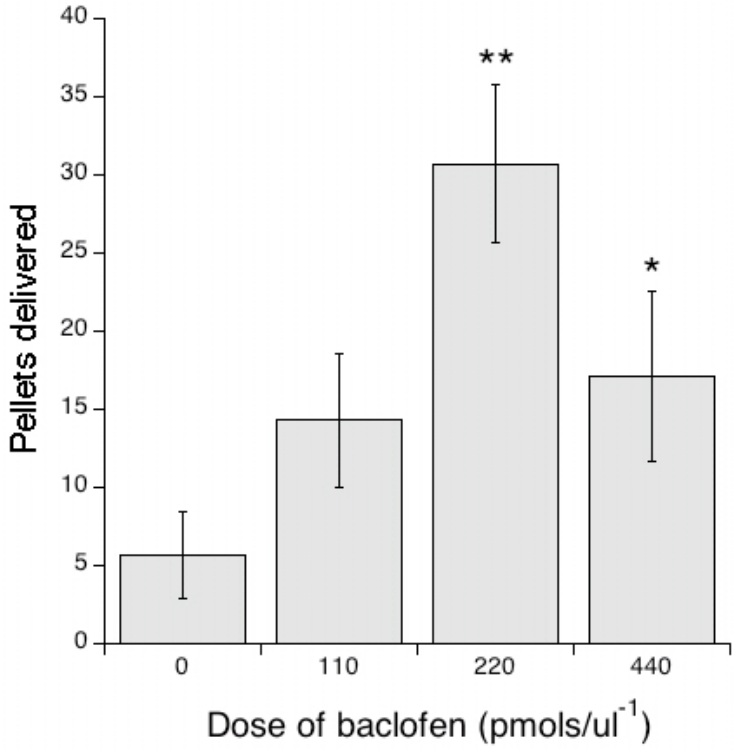


Figure 4.10. The effects of intra-Acb bilateral infusions of saline or a range of doses of baclofen in pre-fed rats (n=7) on total number of pellets delivered due to responding on the reinforced lever over a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ $p<0.05$ and ★★ $p<0.01$.

For those animals that pressed frequently enough to receive a food reward the average amount of time consequently taken to start pressing for reward again, the inter pellet-lever latency (IPLI) is depicted in Fig. 4.11. Levene's test confirmed that there was equal variance between treatment groups. There was a significant main effect of drug on the duration of the IPLIs [$F(3,86)=4.74$, $p=0.004$] i.e. one or more of the doses decreased the latency to press post pellet delivery. Planned post hoc analysis using the Games-Howell procedure revealed that there was no significant difference in the duration of IPLIs between vehicle and the 110ρmols or 440ρmols doses. At 220ρmols, animals returned to pressing following pellet delivery significantly faster than with vehicle ($p=0.018$) or 110ρmols of baclofen. Observation of the behaviour of the animals from videos of the test sessions indicated that the pellets were consumed before the animals, under the influence of 220ρmols baclofen, went back to pressing.

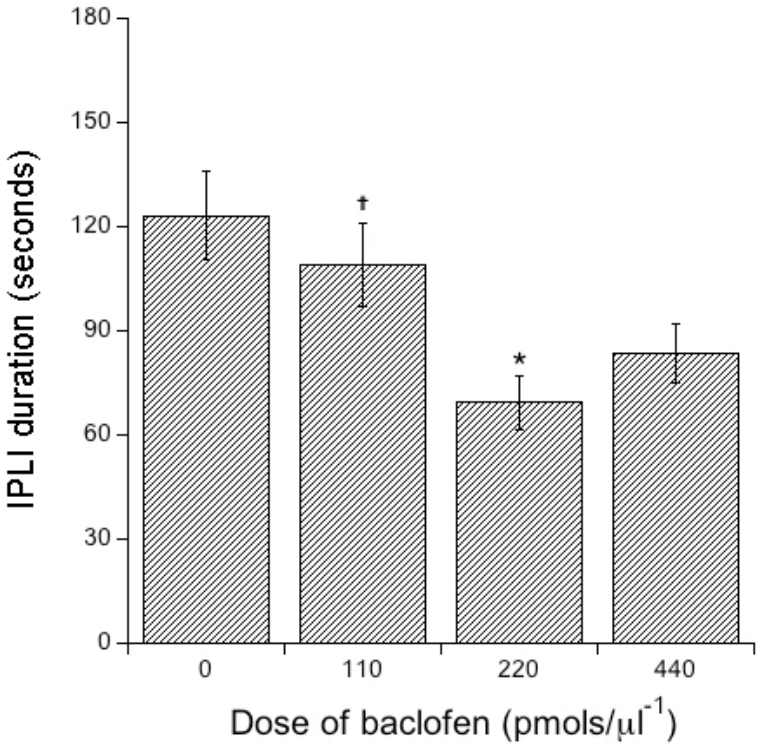


Figure 4.11. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=7) on the total duration of intervals between pellet delivery and next reinforced press (IPLIs). Data represents responding across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ $p<0.05$. Significant differences versus next higher drug dose are denoted by † $p<0.05$.

Summary of results for experiment 4.1

In experiment 4.1 n=7 of n=12 subjects were included in the final analysis. The 440ρmols dose of baclofen significantly increased intake of freely available chow prior to testing on the 2nd order operant schedule.

Both 220ρmols and a higher 440ρmols dose significantly increased the total number of reinforced presses across the 30 minute operant session. The 220ρmols dose also increased the total number of non-reinforced presses for 10 minutes of the session but this was proportional to the overall increase in responses on the reinforced lever and only significant at the peak of reinforced pressing.

Analysis of reinforced lever pressing across 5 minute time bins revealed that 220ρmols baclofen significantly increased the total number of reinforced lever presses during the first 10 minutes of the operant test session which encompassed 5 minutes when no primary reinforcer was available. The total presses per bin and the rate of lever pressing with 220ρmols also increased relative to vehicle and other drug doses once reward became available during the latter 25 minutes of the session.

Over the first 5 minutes of the session baclofen increased the proportion of pressing on the reinforced lever that occurred during CS presentations at all doses although this appeared to be due more to presses being made prematurely i.e. before the CS terminated rather than animals failing to stop pressing when the CS first came on. This effect was not apparent over the remainder of the test session.

The increase in reinforced lever pressing at 220ρmols and 440ρmols resulted in the delivery of significantly more pellets than with vehicle. The 220ρmols dose of baclofen might have decreased the latency to first pellet delivery but the total number of animals that received pellets with vehicle was too low to carry out a statistical verification of this effect. The 220ρmols dose did however significantly decrease the length of the interval between pellet delivery and the next reinforced lever press that was made.

Experiment 4.2: The effects of bilateral intra-Acb infusions of DAMGO at 0.025μg/μl^{-1} in pre-fed rats on free intake and responding on a second order operant schedule for food.

Refer to Fig. 4.2, experiment 4.1 for a schematic illustration of Acb infusion site placements. In the 2nd order schedule there was no significant effect of 0.025μg DAMGO infused into the Acb of pre-fed animals on the total number of reinforced, non-reinforced or incorrect lever presses (Fig. 4.12A). There was a significant main effect of time on reinforced lever pressing [F(5,30)=3.01, p=0.026] reflecting a decrease in the number of presses across each 5 minute time bin as the session progressed (Fig. 4.12B). There was no interaction between drug and time.

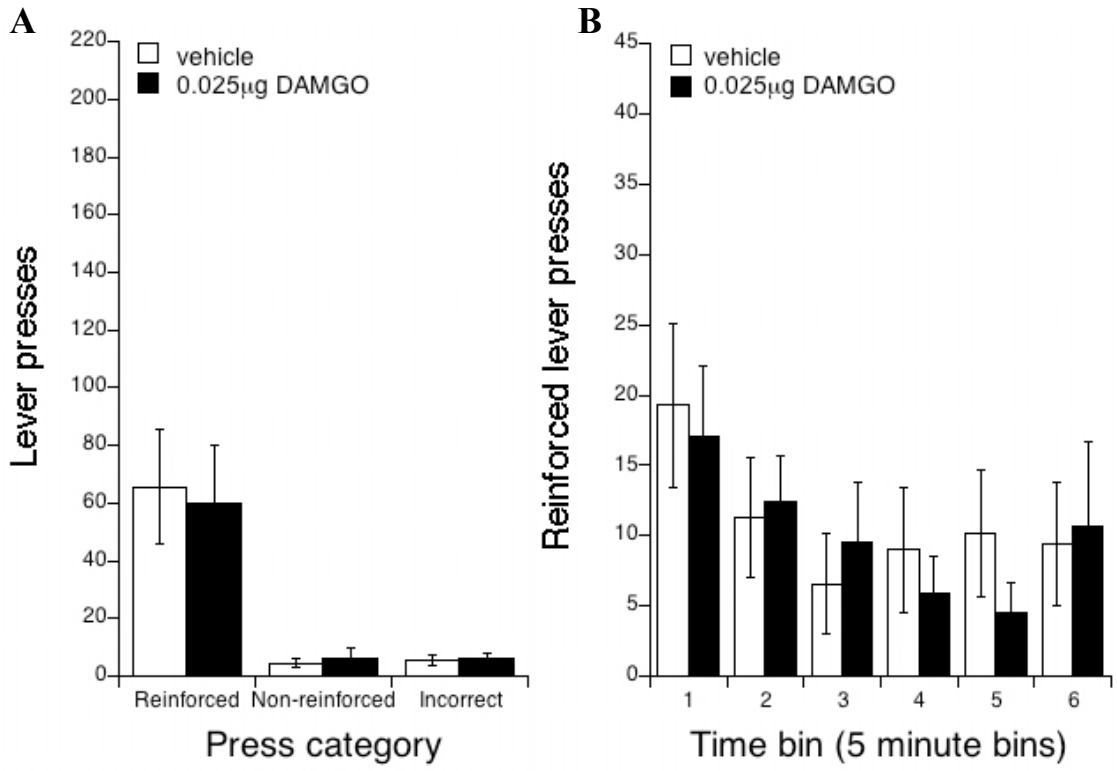


Figure 4.12. The effects of intra-Acb bilateral infusions of saline or DAMGO in pre-fed rats (n=7) on A) total lever presses and B) reinforced lever presses across a 30 minute second order operant test session. Error bars represent ±SEM.

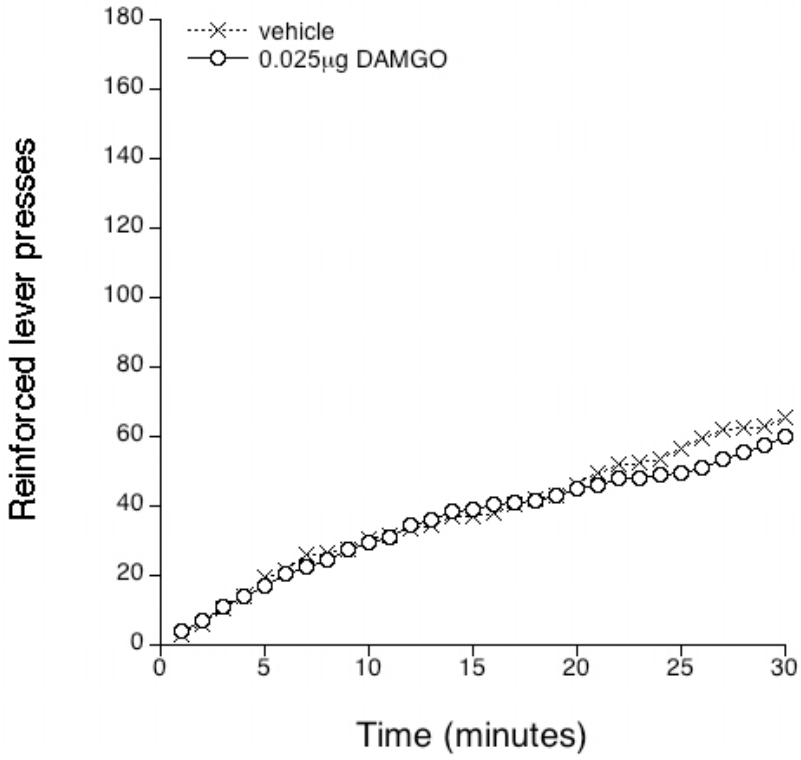


Figure 4.13. The effects of intra-Acb bilateral infusions of saline or DAMGO in pre-fed rats (n=7) on cumulative reinforced lever presses over a 30 minute second order operant test session.

Table 4.2. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=7) on the rate of reinforced lever pressing during the first 5 minutes (appetitive phase) and last 25 minutes (consummatory phase) of a second order operant test session. Significant differences between phases for each treatment are indicated by the '*p*' values reported in the last column.

Treatment	Phase	Rate presses/min	Difference between phases *p* =
Vehicle	Appetitive	3.86 ± 1.17	NS
	Consummatory	1.86 ± 0.72	
DAMGO	Appetitive	3.40 ± 1.02	NS
	Consummatory	1.73 ± 0.61	

Fig. 4.13 illustrates the distribution of cumulative reinforced lever presses across the 30 minute 2nd order operant session with intra-Acb infusions of vehicle or DAMGO. The figure suggests there was no effect of drug. The rates of lever pressing, expressed as reinforced presses per minute, across the first 5 minutes (appetitive) and subsequent 25 minutes (consummatory) of the schedule are shown for each treatment in Table 4.2. There was a main effect of phase that the rate was lower during the final 25 minutes than the first 5 for both treatments [$F(1,6)=7.97$, $p=0.03$] but no main effect of drug or an interaction.

The cumulative proportions of the length of ILIs between the 5th press of an FR5 response and the 1st press of the next FR5 during the first 5 minutes of the 2nd order schedule (unrewarded) are depicted in Fig. 4.14. The distributions of ILIs for the remaining 25 minutes of the test session are shown in Fig. 4.15. There was no apparent effect of DAMGO compared to vehicle.

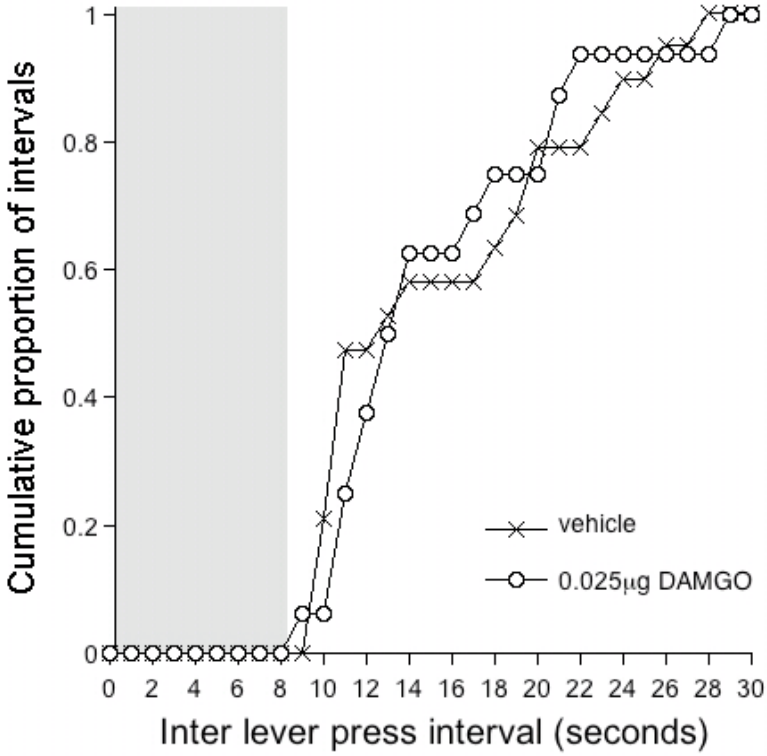


Figure 4.14. The effects of intra-Acb bilateral infusions of saline or DAMGO in pre-fed rats (n=7) on cumulative ILIs between the 5th and 1st presses of the FR5 requirement on the reinforced lever. Data represents responding across the first 5 minute unrewarded phase of a second order operant test session. The shaded phase represents the duration of the CS (8 seconds). The CS indicated that criterion had been met and that concomitant presses would not be reinforced.

For the remaining 25 minutes of the session some animals pressed during the CS with vehicle and DAMGO. However with DAMGO 13.1% of presses were made 4 seconds before the CS was due to terminate compared to only 2.7% with vehicle.

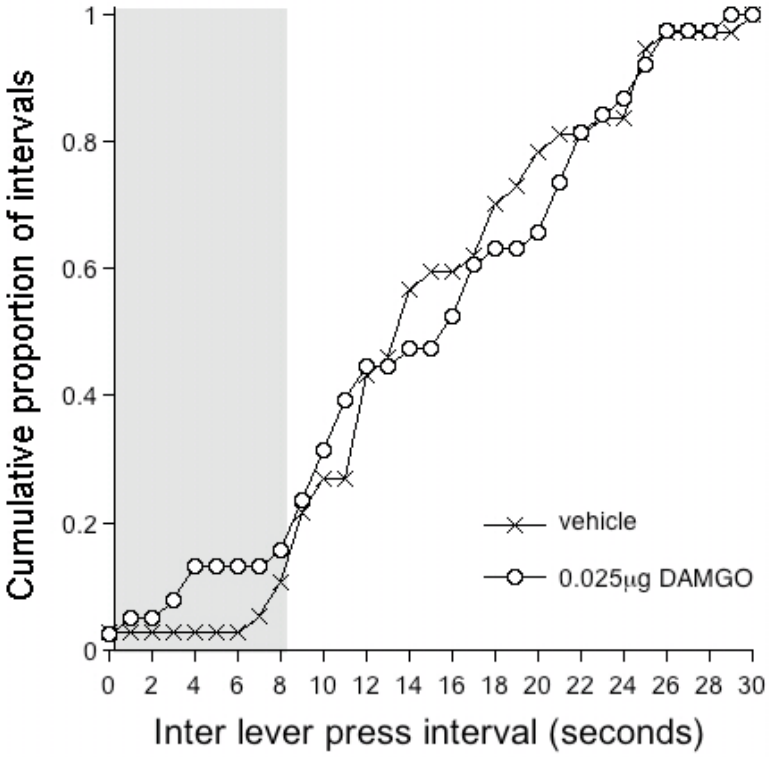


Figure 4.15. The effects of intra-Acb bilateral infusions of saline or DAMGO in pre-fed rats (n=7) on cumulative inter lever press intervals between the 5th and 1st presses of the FR5 requirement on the reinforced lever. Data represents responding across the last 25 minute rewarded phase of a second order operant test session. The shaded phase represents the duration of the CS (8 seconds). The CS indicated that criterion had been met and that concomitant presses would not be reinforced.

A few days after testing on the 2nd order schedule the same animals pre-fed with chow before treatment with 0.025μg DAMGO significantly increased their consumption of freely available chow relative to vehicle over 30 minutes [Fig. 4.16; $F(2,10) = 31.63$, $p<0.001$].

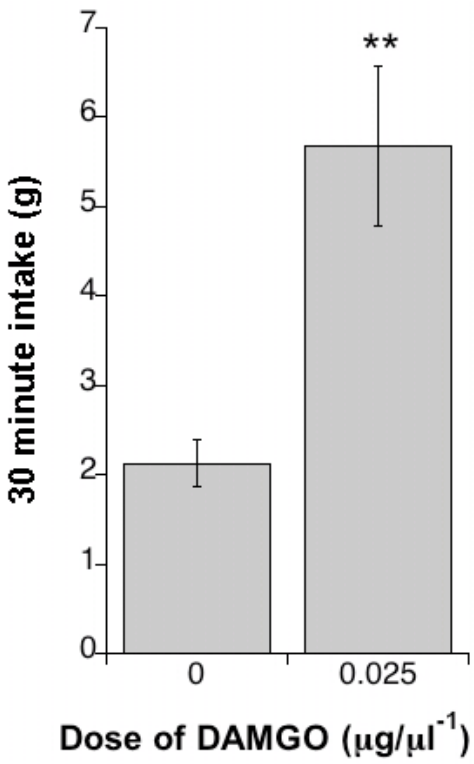


Figure 4.16. The effects of intra-Acb bilateral infusions of saline or DAMGO in pre-fed rats (n=7) given access to laboratory chow over a 30 minute test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★★ p<0.01.

Summary of results for experiment 4.2

DAMGO at a dose of 0.025μg had no significant effect on the total number of reinforced, non-reinforced or incorrect presses or the rate of reinforced lever pressing relative to vehicle across the session. Analysis of the microstructure of reinforced pressing across 5 minute time bins revealed that there was no significant effect at any stage during the 30 minute test session. During the first 5 minutes, when pressing could not be rewarded with the primary reinforcer, DAMGO had no significant effect on the duration of inter lever intervals. Over the remaining 25 minutes of the test session nearly 5 times as many lever presses were made ≤4 seconds into the CS presentation than with vehicle. This dose of DAMGO significantly increased intake of freely available chow.

***Experiment 4.3*: The effects of bilateral intra-Acb infusions of baclofen at 220 and 660ρmols/μl^{-1} in pre-fed rats on free intake and responding on a second order operant schedule for food.**

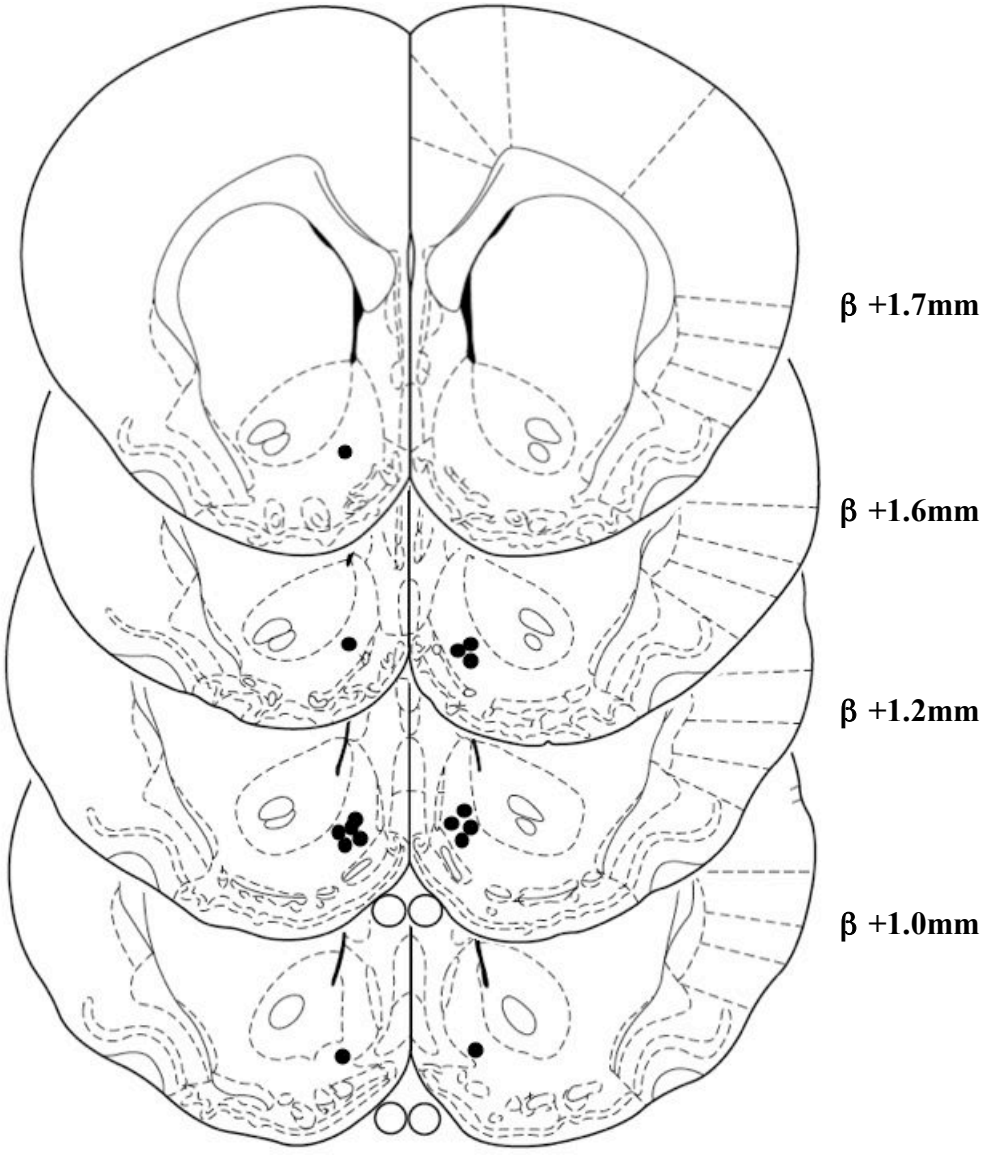


Figure 4.17. Injection sites plotted on drawings taken from Paxinos and Watson (1998); sections are anterior relative to bregma (β). Bilateral target coordinates (n=8 of original n=12 subjects with acceptable placements) were (AP), + 1.4mm, mediolateral (ML), ± 0.9mm relative to bregma and dorsoventral (DV), -7.8mm relative to skull surface.

Prior to testing animals in the operant chambers, responsiveness to baclofen was verified in pre-fed animals at 660ρmols/μl^{-1} baclofen. Mild myorelaxant effects were noted in some of the animals at the start of the test session but these animals were subsequently excluded on the basis of histology. One animal had blocked cannulae and also had to be excluded from the study at this stage. A total of n=8 of the original n=12 animals were verified as having acceptable placements, as defined in Chapter 2, and were included for the final analysis. A schematic illustration of Acb infusion site placements is given in Fig. 4.17. These animals consumed significantly more chow with 660ρmols/μl^{-1} baclofen than with vehicle over a 30 minute period [Fig. 4.18: $F(1,7)=30.69$, $p<0.001$].

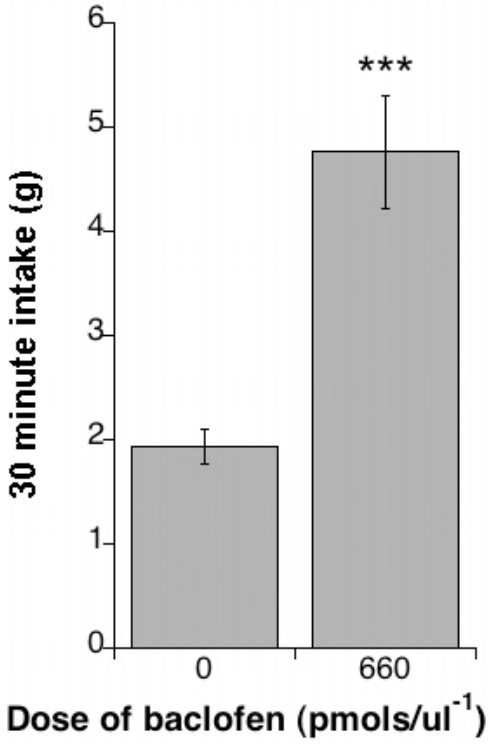


Figure 4.18. The effects of intra-Acb bilateral infusions of saline or baclofen in pre-fed rats (n=8) given access to laboratory chow over a 30 minute test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★★★ p<0.001.

In the operant test sessions, rats that had been pre-fed prior to infusions of a range of doses of baclofen significantly increased total reinforced lever presses due to drug [Fig. 4.19: $F(2,14)=12.83$, $p<0.001$]. Planned post-hoc analysis using the Bonferroni test revealed that only the 220ρmols dose increased responding relative to vehicle ($p<0.01$) and relative to the higher 660ρmols dose ($p<0.01$). There was no significant increase in errors or in non-reinforced presses (see Fig. 4.19). When the total number of non-reinforced presses was expressed as a proportion of total presses on the reinforced lever (i.e. non-reinforced / non-reinforced + reinforced) there was actually a significantly lower proportion relative to vehicle at both 220ρmols ($p<0.05$) and 660ρmols ($p<0.01$).

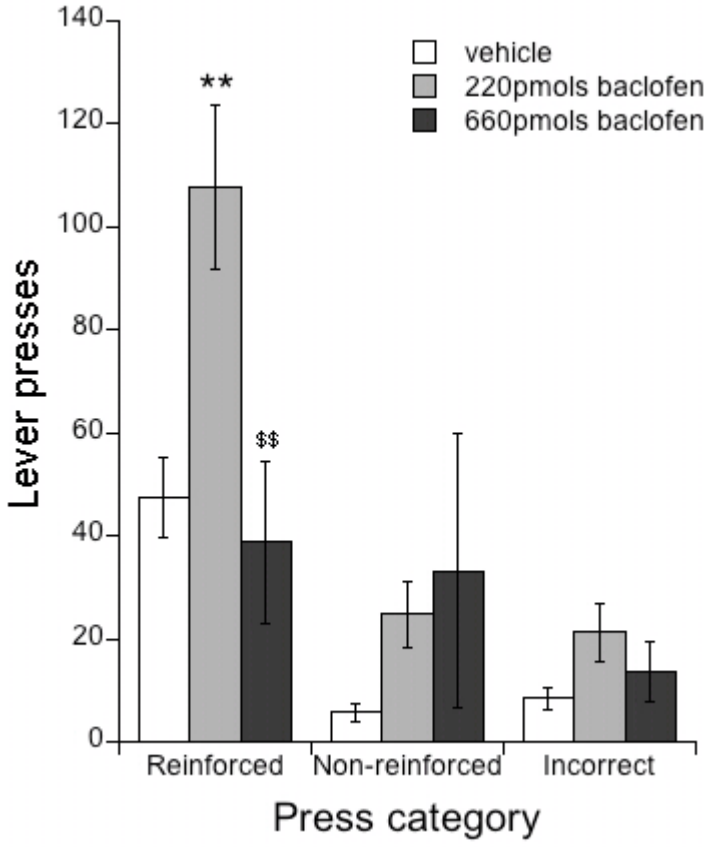


Figure 4.19. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on total lever presses in a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ⋆⋆ $p<0.01$. Significant differences versus the preceding lower drug dose are denoted by $$ $p<0.01$.

There was also no significant effect of previous drug treatment on the total number of reinforced lever presses made by food deprived rats on the training days immediately following drug treatment. Furthermore there was no significant difference between pressing on these training days and levels of pressing prior to the start of testing.

Plots of cumulative reinforced lever presses, shown in Fig. 4.20 below, suggest a possible linear increase in total lever presses as the session progresses with the intermediate 220ρmols baclofen treatment. Rats under the highest dose treatment appear to decrease their response rate after the appetitive phase.

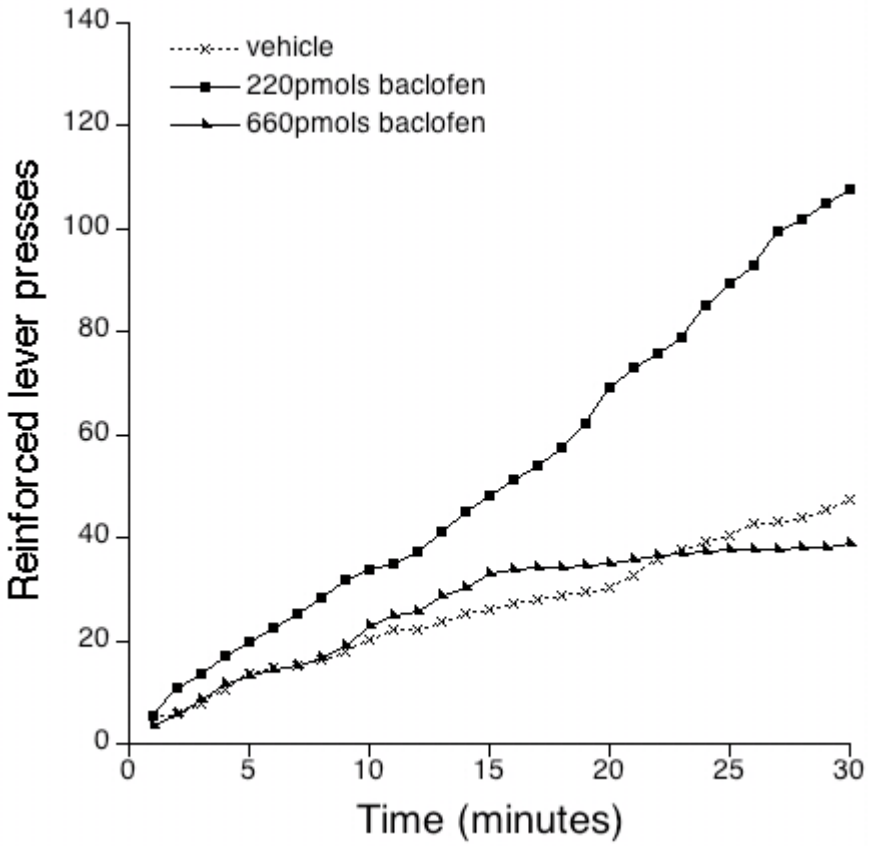


Figure 4.20. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on cumulative reinforced lever presses over a 30 minute second order operant test session.

The rate of lever pressing, expressed as reinforced presses per minute, across the first 5 minutes and subsequent 25 minutes of the schedule is shown for each treatment in Table 4.3. With both vehicle [F((1,7)=10.12, p =0.015] and 660ρmols of baclofen [F(1,7)=7.99, p = 0.025], the rate of pressing in the late phase is significantly lower than in the early phase. The rate of pressing during the late phase with the 220ρmols treatment is not significantly different from the early phase.

Comparing the rates of pressing between treatments during the early phase (first 5 minutes) indicates that there is a main effect of drug [F(2,14)=5.5, p = 0.017] due to a significant increase above vehicle and 660ρmols levels at the 220ρmols dose, revealed using the Bonferroni test ($p < 0.05$ in both cases). During the subsequent 25 minutes there was also a main effect of drug on the rate of pressing [F(2,14)=8.80, p = 0.003]. Multiple comparisons made using the Bonferroni test revealed that the 220ρmols dose significantly increased the rate of pressing above vehicle levels ($p < 0.01$) and the rate with 660ρmols ($p < 0.01$).

Table 4.3. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on the rate of reinforced lever pressing during the first 5 minutes (appetitive phase) and last 25 minutes (consummatory phase) of a second order operant test session. Significant differences between phases for each treatment are indicated by the '*p*' values reported in the last column. Significant differences between treatments for each phase for drug vs. vehicle are denoted by ★ p<0.05, ★★ p<0.01. Significant differences relative to higher drug dose are denoted by † p<0.05, †† p<0.01.

Treatment	Phase	Rate presses/min	Difference between phases *p* =
Vehicle	Appetitive	2.73 ± 0.49	0.015
	Consummatory	1.35 ± 0.25	
220pmols baclofen	Appetitive	4.60 ± 0.35★/†	NS
	Consummatory	3.74 ± 0.51★★/††	
660pmols baclofen	Appetitive	2.68 ± 0.68	0.025
	Consummatory	1.02 ± 0.54	

When the data were split up into 5 minute bins a significant increase in reinforced presses due to drug was found [$F(2,14)=20.39$, $p<0.001$] as well as a significant effect of time [$F(5,35)=5.38$, $p<0.001$] but there was no interaction due to the broadly similar pattern in the levels of lever pressing across time-bins for each dose (see Fig. 4.21). Nevertheless, post hoc analysis using the Bonferroni test revealed that the intermediate dose of 220ρmols of baclofen significantly increased reinforced lever pressing relative to both other treatments in the first 5 minutes ($p<0.05$) and then again between 15 and 20 minutes ($p<0.001$). At 20-25 minutes pressing was higher at 220ρmols than at 660ρmols ($p<0.05$). A transient increase in pressing with vehicle at this time point meant that the level of pressing at 220ρmols was not significantly higher. By 25 to 30 minutes pressing at 220ρmols was again significantly higher than with vehicle and 660ρmols ($p<0.01$).

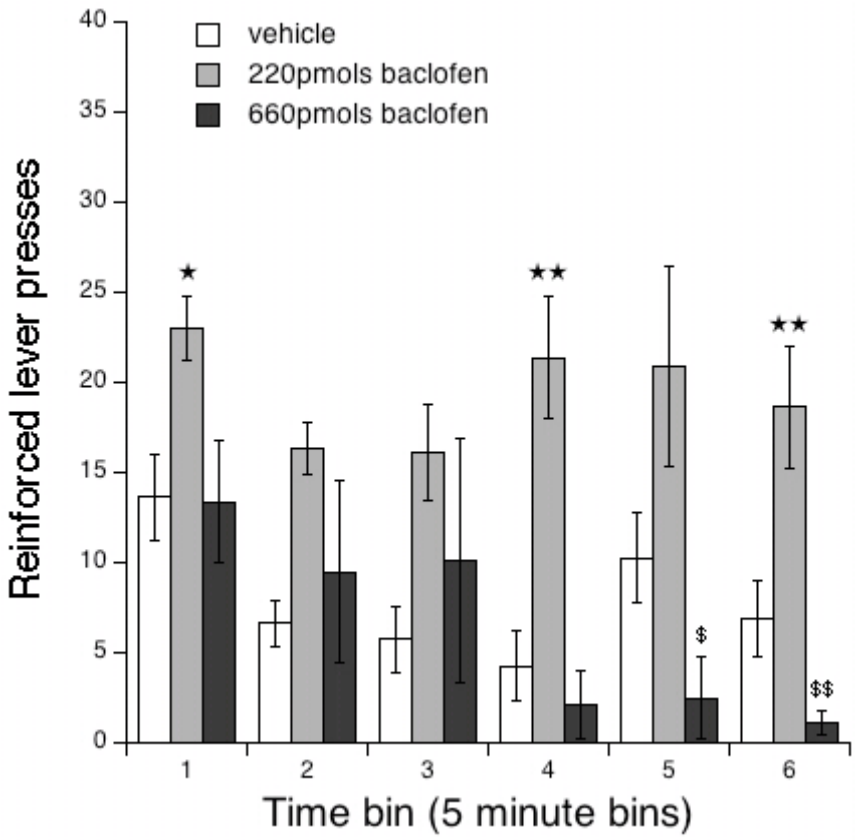


Figure 4.21. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on reinforced lever presses across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ $p<0.05$, ★★ $p<0.01$. Significant differences versus the preceding lower drug dose are denoted by $ $p<0.05$, $$ $p<0.01$.

The cumulative proportions of the duration of ILIs between the 5th press of an FR5 response and the 1st press of the next FR5 for the first 5 minutes of the 2nd order schedule (unrewarded) are depicted in Fig. 4.22. The distributions of ILIs for the remaining 25 minutes of the test session are shown in Fig. 4.23. In the first 5 minutes of the session, when lever presses could not be rewarded with the primary reinforcer, animals under the influence of vehicle or the high dose of 660ρmols baclofen started to press, at the earliest, 1 second before the cue light went off. In contrast, at 220ρmols, 7.7% of presses were made only 3 seconds after the light came on.

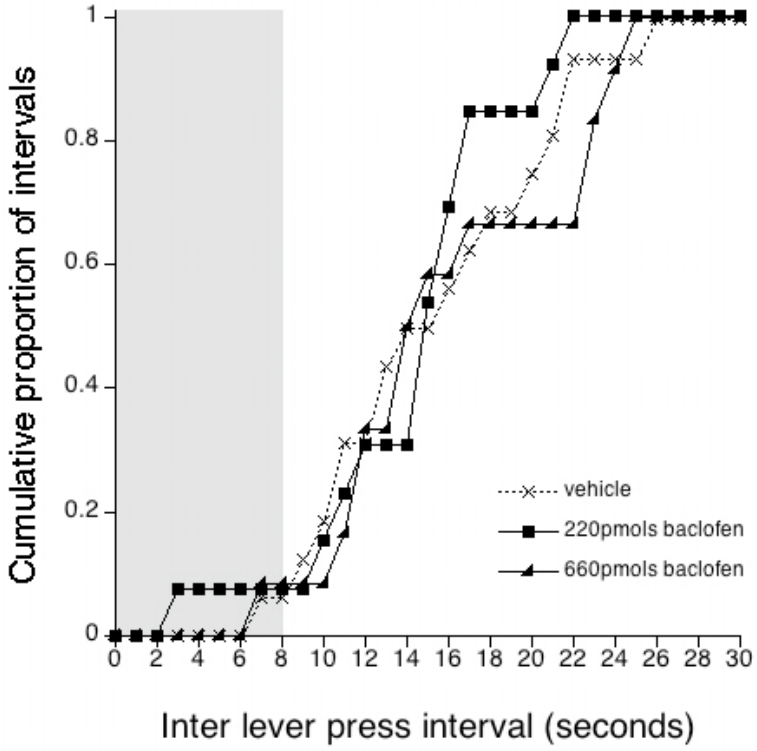


Figure 4.22. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on cumulative inter lever press intervals (ILIs) between the 5th and 1st presses of the FR5 requirement on the reinforced lever. Data represents responding across the first 5 minute unrewarded phase of a second order operant test session. The shaded phase represents the duration of the CS (8 seconds). The CS indicated that criterion had been met and that concomitant presses would not be reinforced.

For the remaining 25 minutes of the session (Fig. 4.23) those presses that occurred within 8 seconds of a completed FR5 were infrequent for vehicle with only an average of 3.4%. At the 220ρmols dose 20% of presses were made during the CS but half of these only occurred 1 second before the termination of the CS. At 660ρmols an average of 14.3% of presses were made as soon as the CS was presented and more than half of the first presses in an FR5 were made during the CS (57.1%). Presses made once the CS had gone off were closer together with both doses of baclofen with more than 80% of all ILIs less than 18 seconds long compared to 80% of ILIs less than 25 seconds long with vehicle.

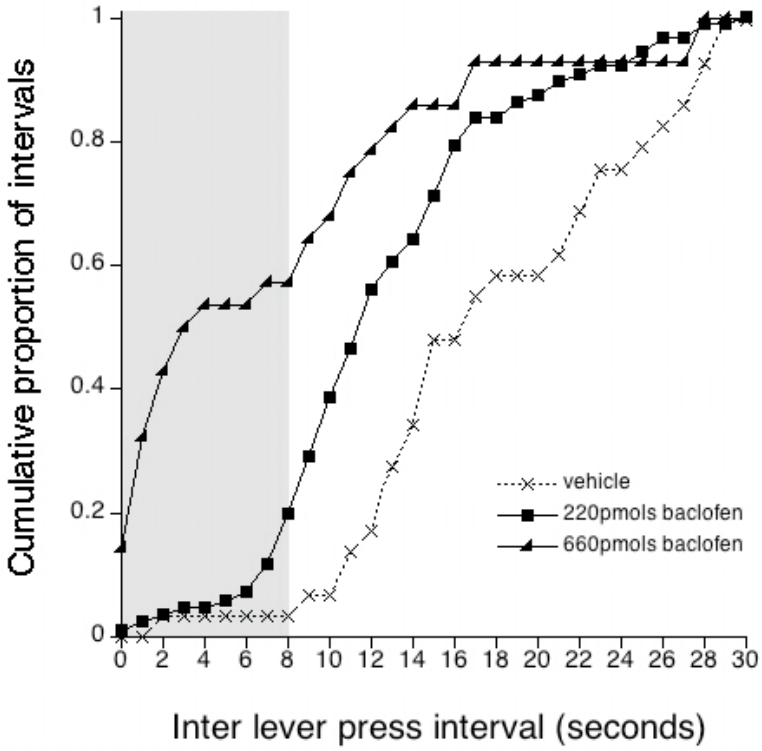


Figure 4.23. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on cumulative inter lever press intervals between the 5th and 1st presses of the FR5 requirement on the reinforced lever. Data represents responding across the last 25 minute rewarded phase of a second order operant test session. The shaded phase represents the duration of the CS (8 seconds). The CS indicated that criterion had been met and that concomitant presses would not be reinforced.

There was a significant increase in the number of pellets delivered due to drug treatment [F(2,14)=8.49, p=0.004]. Paired comparisons confirmed that the increase in the number of reinforced presses with the 220ρmols dose of baclofen resulted in a significant increase relative to vehicle in the total number of pellets that were consequently delivered at this dose (p<0.01). Not all of the animals at all of the doses pressed enough times to receive pellets over the 30 minute period. As a result, the calculation of the mean latency to first pellet delivery for each treatment group would have to have been based on different numbers of subjects so it was not possible to carry out any meaningful statistical analysis.

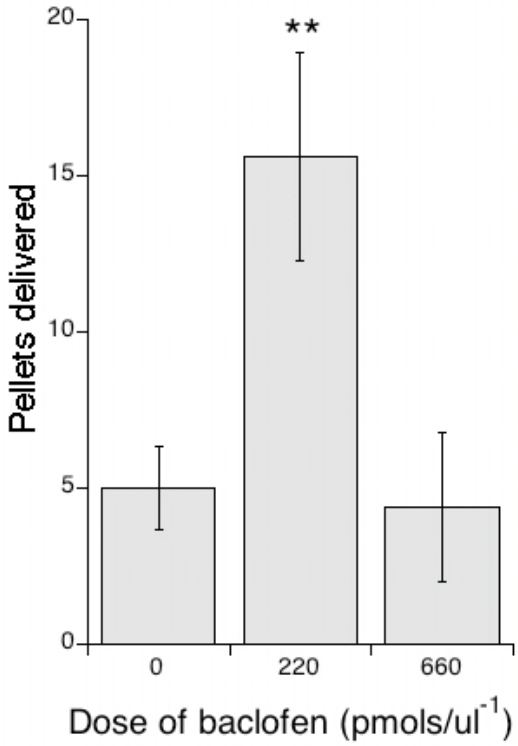


Figure 4.24. The effects of intra-Acb bilateral infusions of saline or a range of doses of baclofen in pre-fed rats (n=8) on total number of pellets delivered due to responding on the reinforced lever over a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★★ p<0.01.

For those animals that pressed frequently enough to receive a food reward the average duration of IPLIs is depicted in Fig. 4.25. Only 3 out of 8 animals at the highest dose pressed enough to receive pellets so these data were not taken to be representative of the group and were consequently excluded. As a result statistical analysis was only carried out to compare the effects of vehicle treatment with 220ρmols of baclofen for the n=8 subjects. Levene's test confirmed that there was equal variance between treatment groups. A one-tailed t-test revealed a significant decrease in the mean duration of the IPLIs with drug [t(30)=3.33, p=0.001].

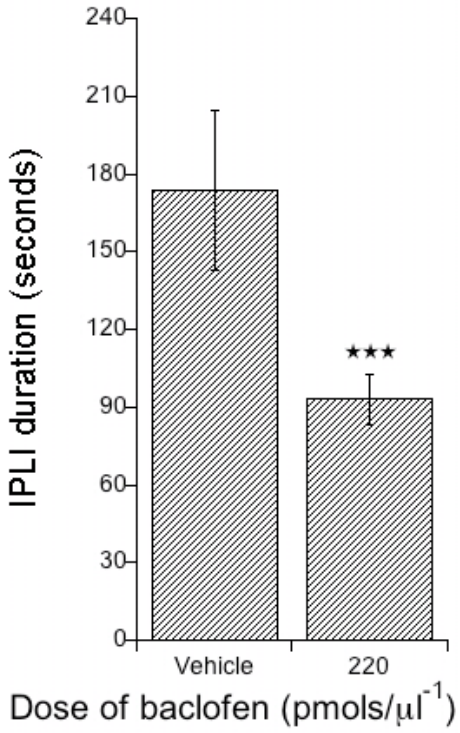


Figure 4.25. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on the total duration of intervals between pellet delivery and next reinforced press (IPLIs). Data represents responding across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★★★ p<0.001.

A few days after testing on the 2nd order schedule the same animals pre-fed with standard laboratory chow prior to treatment with 220ρmols and 660ρmols of baclofen exhibited a significant increase in consumption of chow over a subsequent 30 minute test (Fig. 4.26). They then continued to consume chow over a further 30 minute period. Paired comparisons revealed that both drug doses significantly increased intake relative to vehicle at both times points ($p<0.01$). There was no significant difference in the amount of food consumed with 660ρmols at this stage compared to that consumed the first time the animals were given free access to chow (prior to the 2nd order testing phase).

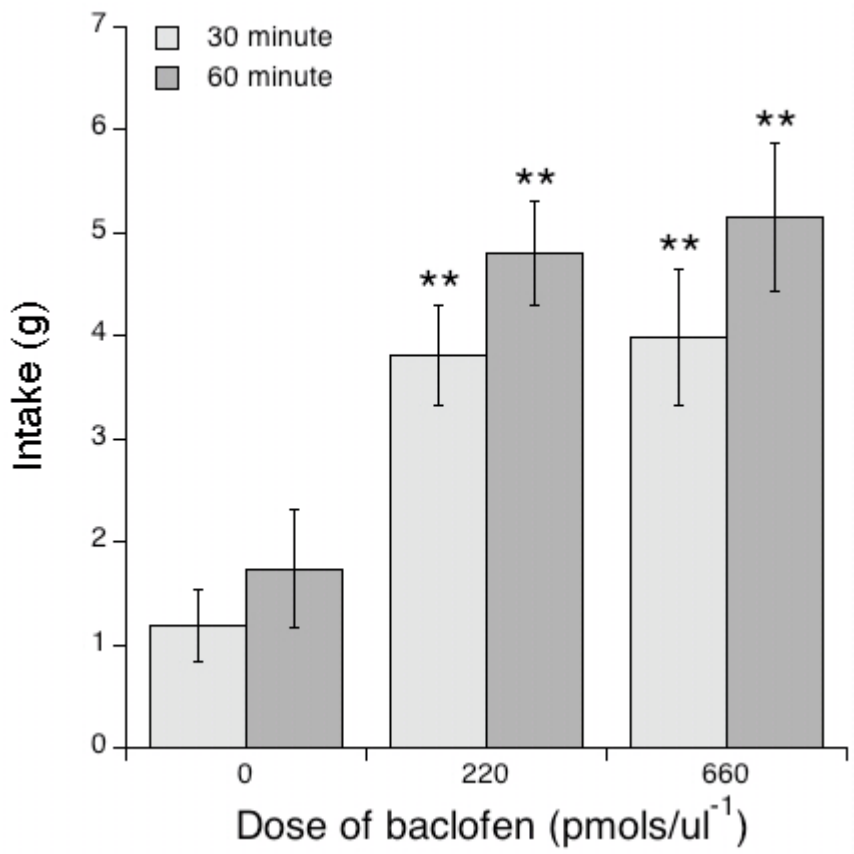


Figure 4.25. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on the total duration of intervals between pellet delivery and next reinforced press (IPLIs). Error bars represent ±SEM. Significant differences from vehicle are denoted by ★★p<0.01.

Associated behaviours

Analysis of the videos of testing in the operant boxes for experiment 4.3 revealed a significant interaction between dose of baclofen and total duration of the 8 behaviours recorded [Fig. 4.27: $F(16,112)=5.66$, $p<0.001$]. The same was true for the frequencies of these behaviours across the session [Fig. 4.28: $F(16,112)=2.15$, $p=0.011$]. The pattern of the effects of drug on duration and frequency did not always correspond (see Fig. 4.27 & 4.28). The relationship between duration and frequency is described for each behavioural category in the following sections. The amount of the behaviour that was coded as off screen was low and there was no significant difference in duration or frequency at any dose. Consequently behaviours that could have occurred off screen (and therefore have been underestimated) i.e. oral stereotypy, active, rear, groom or ingest were not adjusted.

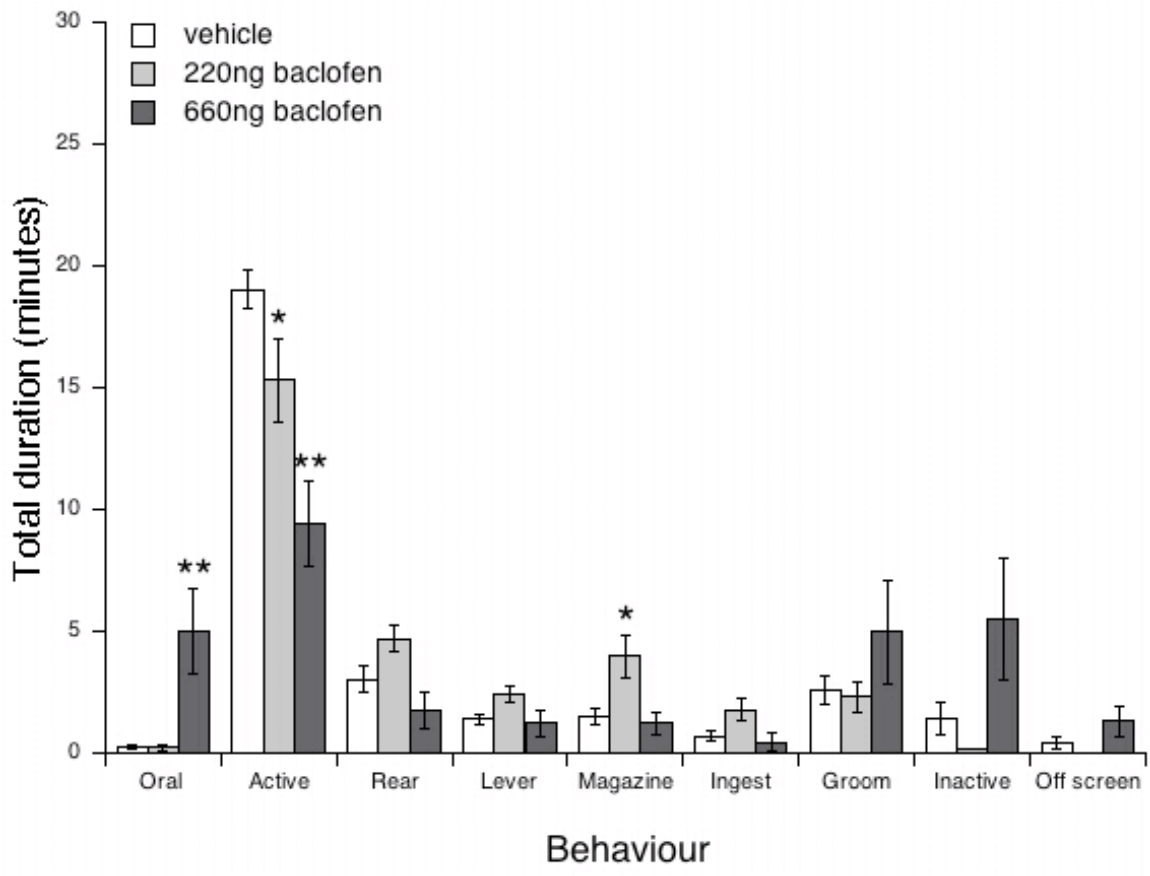


Figure 4.27. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on the total duration of 8 behaviours recorded across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ $p<0.05$, ★★ $p<0.01$.

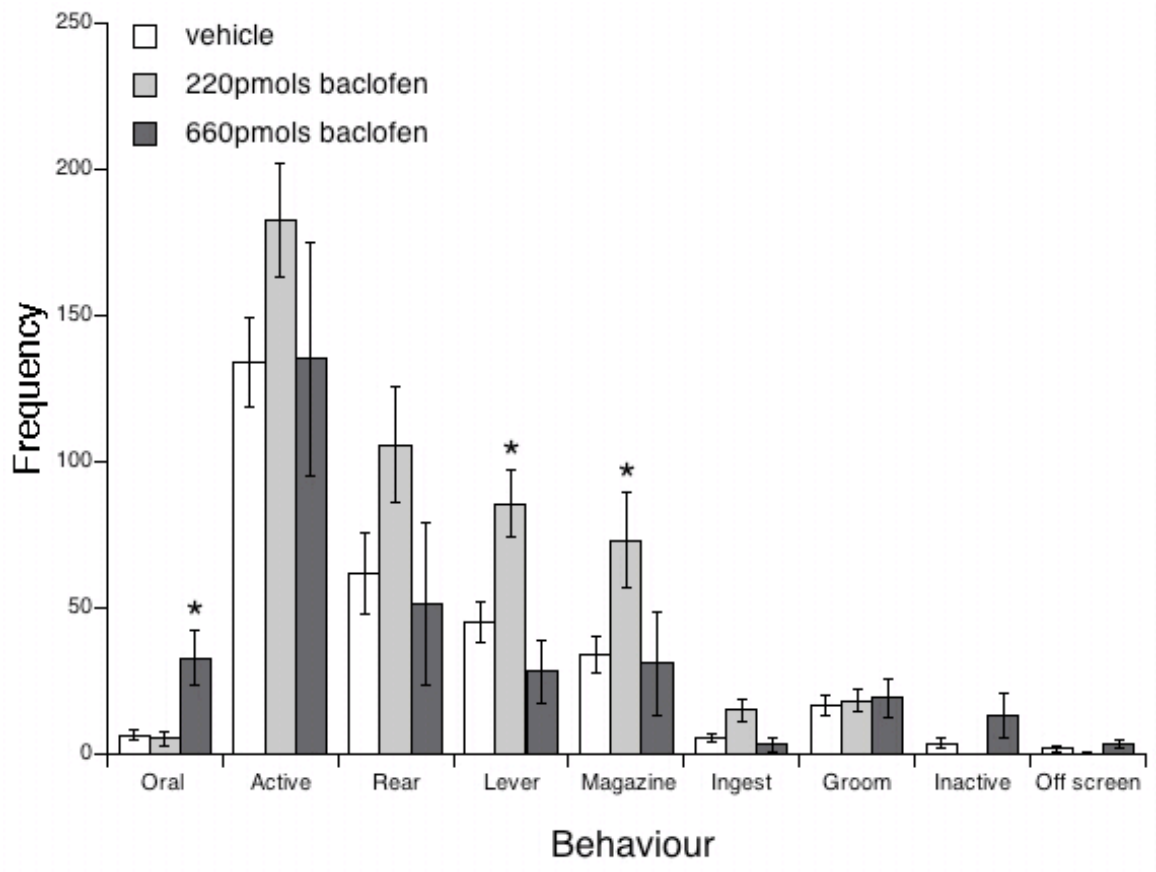


Figure 4.28. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on the total frequency of 8 behaviours recorded across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ $p<0.05$.

Oral stereotypy

The total duration [Fig. 4.27; $F(2,14)=7.35$, $p=0.007$] and frequency [Fig. 4.28; $F(2,14)=7.2$, $p=0.007$] of behaviours categorised as oral stereotypy was significantly increased by drug over the 30 minute session. Paired comparisons revealed that this was due to a significant increase at 660ρmols relative to vehicle levels in duration ($p<0.01$) and frequency ($p<0.05$). When the duration and frequency data were further split into time bins there was no interaction between drug effects and time across the session (Fig. 4.29). Observations from the videos indicated that oral stereotypy was predominantly expressed as licking and chewing of the mesh flooring. At the high dose this was sometimes directed towards the magazine or to the levers (possibly over-inflating the press counts).

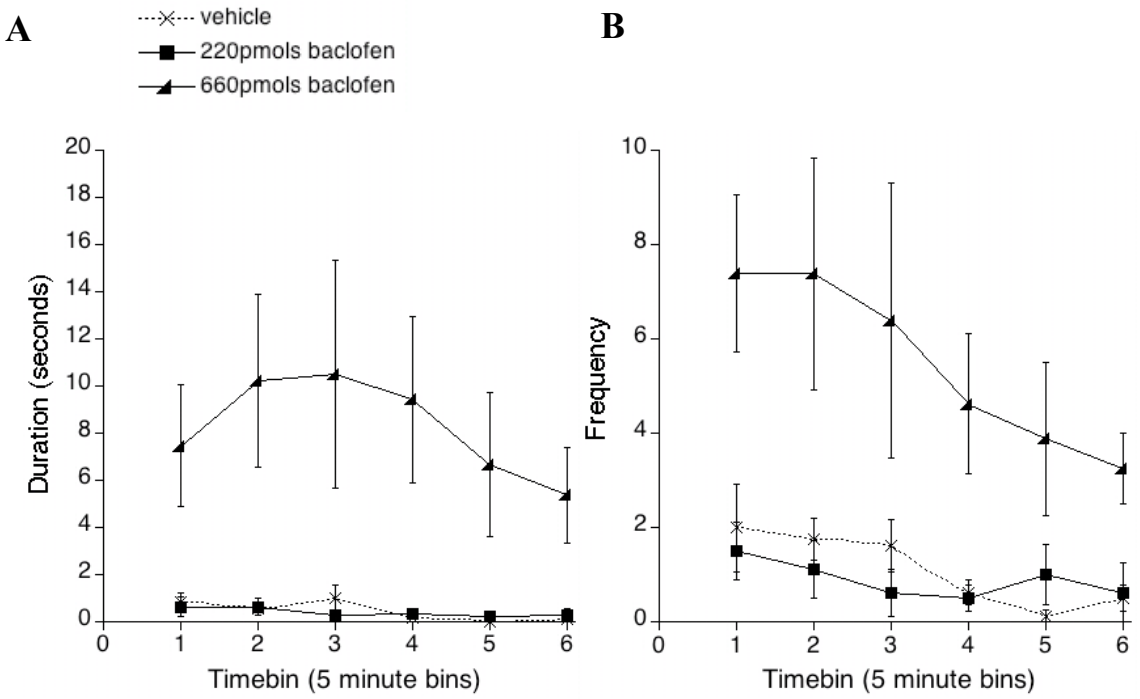


Figure 4.29. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on A) duration and B) frequency of ORAL STEREOTYPY across a 30 minute second order operant test session. Error bars represent ±SEM.

Activity

The total duration of active behaviours were significantly and dose dependently reduced by baclofen [Fig. 4.28; $F(2,14)=10.88$, $p<0.001$] but there was no significant effect on the frequency. Paired comparisons showed that duration at both drug doses was lower than with vehicle ($p<0.05$ at 220ρmols, $p<0.01$ at 660ρmols). When duration data were split into 5 minute bins an interaction between drug and time was revealed [Fig. 4.30A: $F(10,70)=3.01$. $p=0.003$]. At 220ρmols activity was significantly lower relative to vehicle between 10 and 15 minutes ($p<0.05$). At 660ρmols activity was significantly lower relative to vehicle between 5 and 25 minutes ($p<0.01$). There was no interaction between time and drug for frequency (Fig. 4.30B). It was noted that behaviour coded as 'active' varied considerably from rapid jumping to 'active standing'. In the latter case animals remained on all fours (thus the behaviour could not be coded as inactive) but stayed relatively motionless for long periods. At 660ρmols some of the behaviour coded as active involved animals moving around the cage whilst lying down using 'swimming' motions of the four limbs.

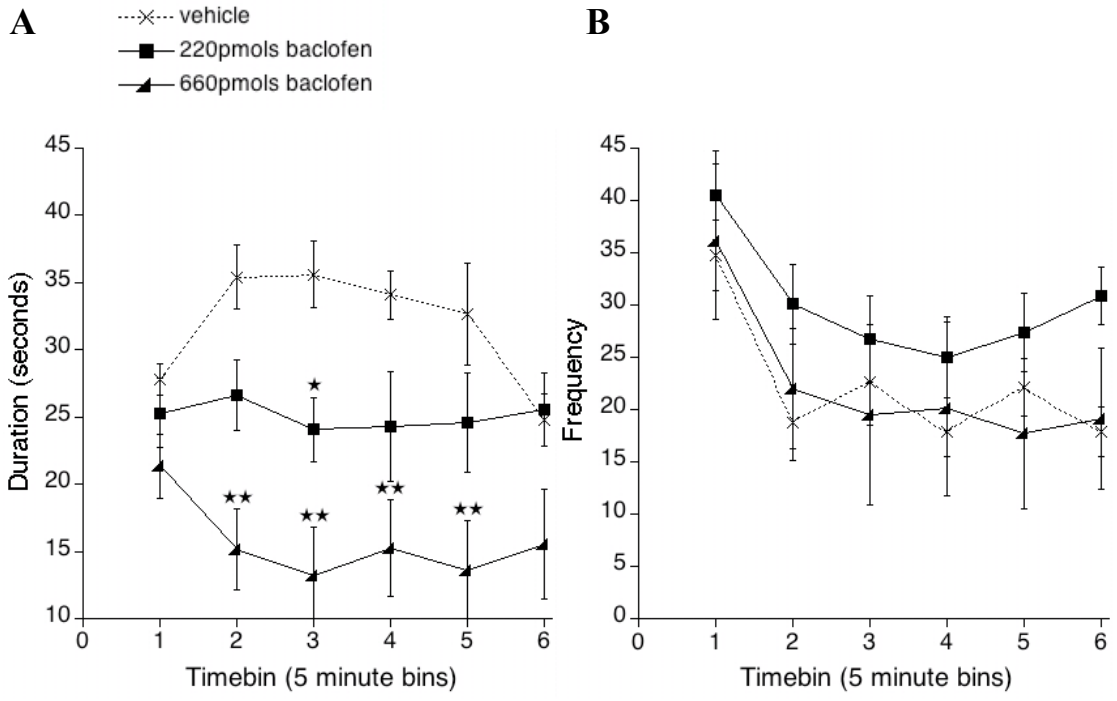


Figure 4.30. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on A) duration and B) frequency of ACTIVE behaviours across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ p<0.05 and ★★ p<0.01.

Rearing

Although baclofen had a significant main effect on the total duration of rearing [F(2,14)=4.76, p=0.026] post hoc analysis using the Bonferroni test revealed that this was only due to significantly less time spent rearing with 660ρmols relative to the 220ρmols drug dose (p<0.05) (See Fig. 4.27). There were no significant effectss on total frequency. Analysis of the total number of rears to the CS indicated that there was a significant main effect of baclofen [F(2,14)=6.45, p=0.01]. Paired comparisons revealed that only 220ρmols significantly increased the total number of rears to the CS relative to vehicle (See Fig. 4.31A, p<0.05). There was no significant difference in the number of rears that were directed at the CS as a proportion of total rears made with each dose (see Fig. 4.31B).

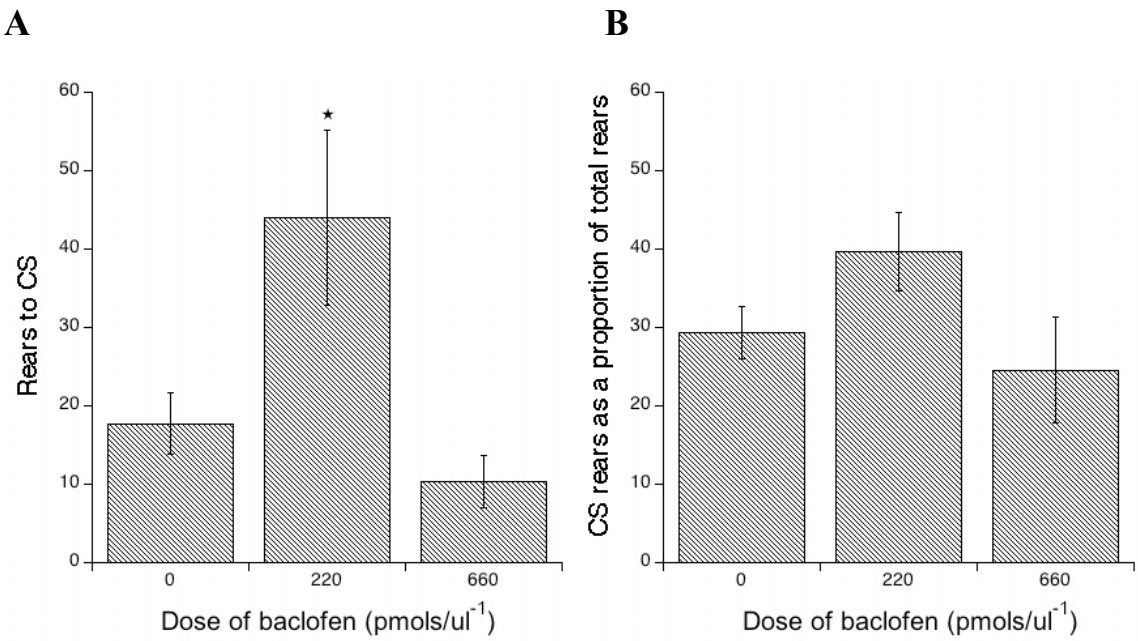


Figure 4.31. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on A) the total number of rears to the CS and B) the proportion of the totals rears made that were directed towards the CS across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences are denoted by ★ p<0.05.

Splitting the durations of all rearing behaviour into 5 minute time bins revealed that there was a significant effect of time on rearing [Fig. 4.32A; $F(5,35)=20.58$, $p<0.001$] but there was no interaction between drug and time because, in all three treatments, the duration of rearing gradually decreased across the session. There was no interaction between time and drug for frequency (Fig. 4.32B).

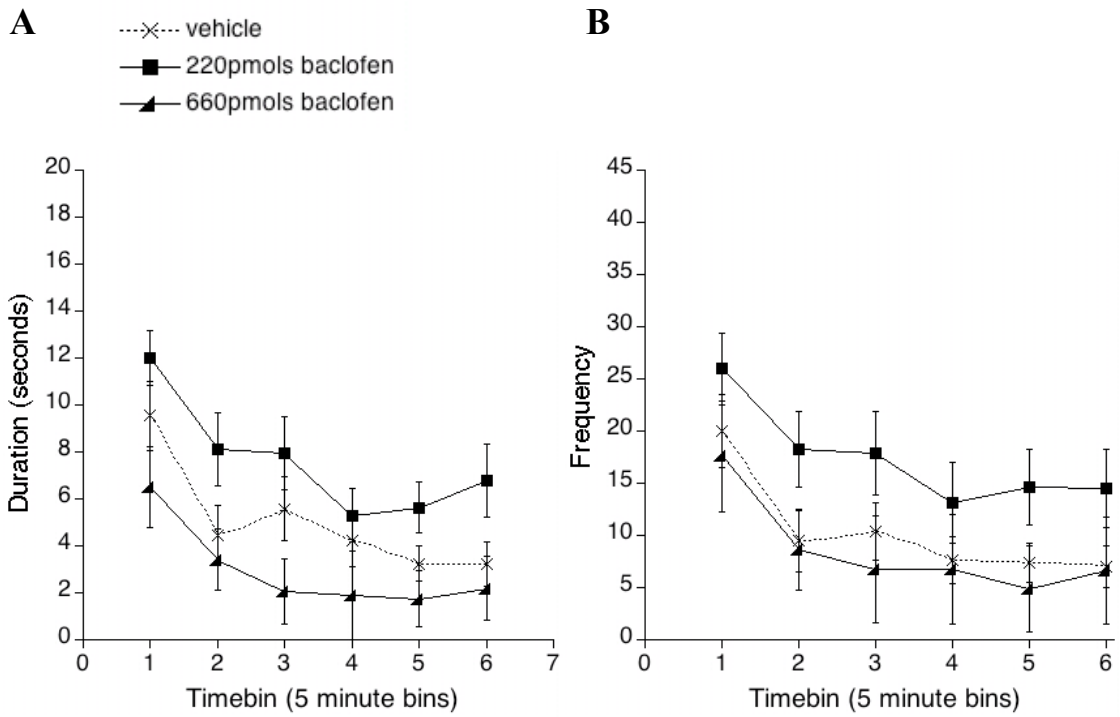


Figure 4.32. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on A) duration and B) frequency of REARING across a 30 minute second order operant test session. Error bars represent ±SEM.

Lever

Baclofen had no significant effect on the length of time spent interacting with the levers but there was a significant dose related increase in the frequency of visits to the levers [Fig. 4.28; $F(2,14)=9.4$, $p=0.003$]. Paired comparisons revealed only baclofen at 220ρmols significantly increased frequency relative to vehicle ($p<0.05$). Splitting duration data into time bins did not reveal any effects of time or interactions between time and drug (see Fig 4.33A). When the frequency data were split into time bins there was a significant effect of time on the number of visits to the levers [$F(5,35)=12.71$, $p<0.001$] but no interaction between drug and time (see Fig. 4.33B).

The duration of lever interactions encompasses a number of different types of lever pressing. Observations from the videos indicated that, whilst in contact with the lever, some animals made clear pressing motions whilst others appeared to hold it down for a prolonged period. Furthermore, some animals used their noses rather than their paws.

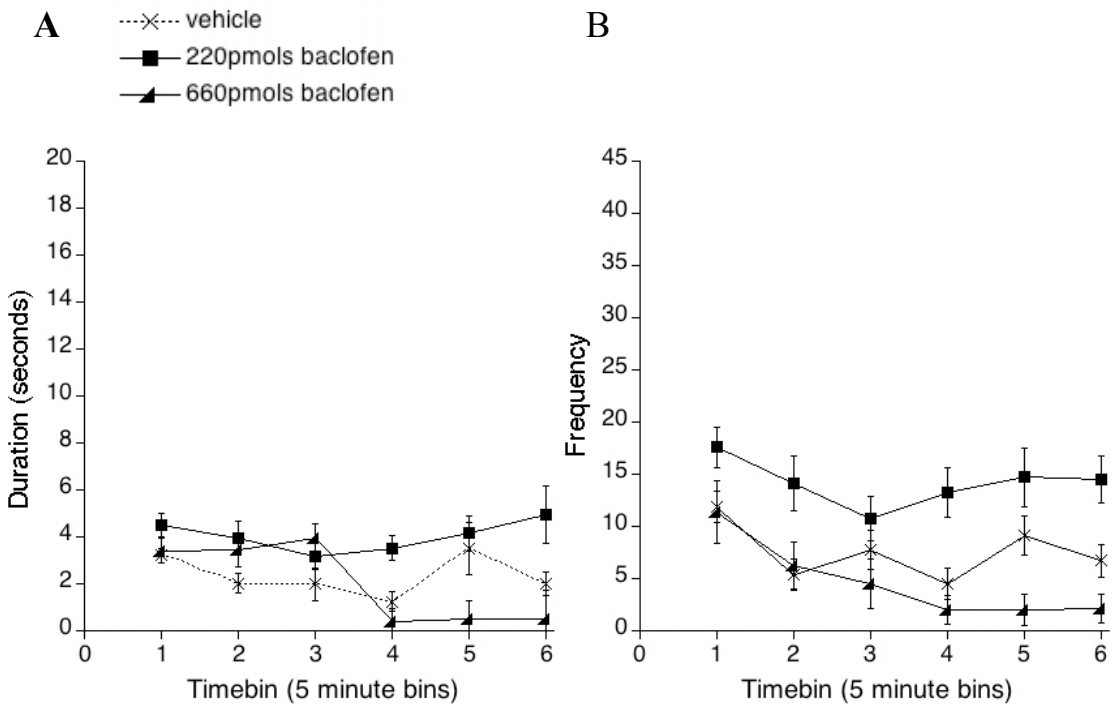


Figure 4.33. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on A) duration and B) frequency of LEVER PRESSING across a 30 minute second order operant test session. Error bars represent ±SEM.

Headpokes into magazine

Baclofen had a significant and dose dependant effect on both the duration [Fig. 4.27; $F(2,14)=7.21$, $p=0.007$] and frequency of head pokes into the magazine [Fig. 4.28; $F(2,14)=4.38$, $p=0.033$]. Both the duration ($p<0.05$) and frequency ($p<0.05$) of headpoking into the magazine were significantly higher at 220ρmols than with vehicle.

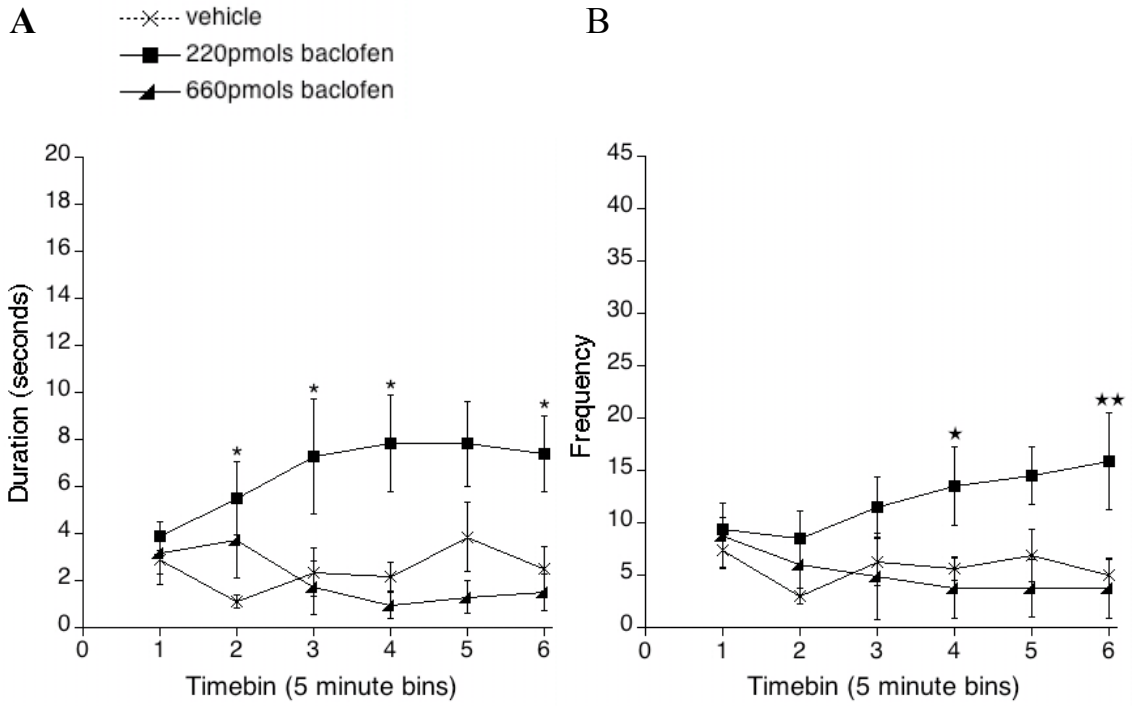


Figure 4.34. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on A) duration and B) frequency of headpokes into the MAGAZINE across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ $p<0.05$ and ★★ $p<0.01$.

Further splitting the durations and frequency of responses into time bins revealed an interaction between drug and time in both cases; [duration; Fig. 4.34A; $F(10,70)=2.32$, $p=0.02$] and [frequency; Fig. 4.34B; $F(10,70)=2.33$, $p=0.02$]. Paired comparisons showed that there was an increase in the duration at the 220ρmols dose compared to vehicle between 5-20 and 25-30 minutes (5-15, $p<0.05$; 15-20, $p<0.01$; 25-30, $p<0.05$). The effect of baclofen on frequency of headpokes into the magazine was shown to be due to a significant increase at 220ρmols relative to vehicle between 15 and 20 minutes ($p<0.05$) and 25 to 30 minutes ($p<0.01$).

When the total duration or frequency of headpokes into the magazine was compared to the total number of reinforced lever presses at each dose there was no significant differences in the duration of interaction with the magazine per press. When the duration per press data were broken down and compared across time bins for vehicle and the 220ρmols baclofen there was a relationship between the phase in the session and the amount of time subjects interacted with the magazine (Fig. 4.35). In both cases the duration of headpokes per press rose across the last 25 minutes of the session with a peak occurring just prior to the mean time point at which pellets were first delivered.

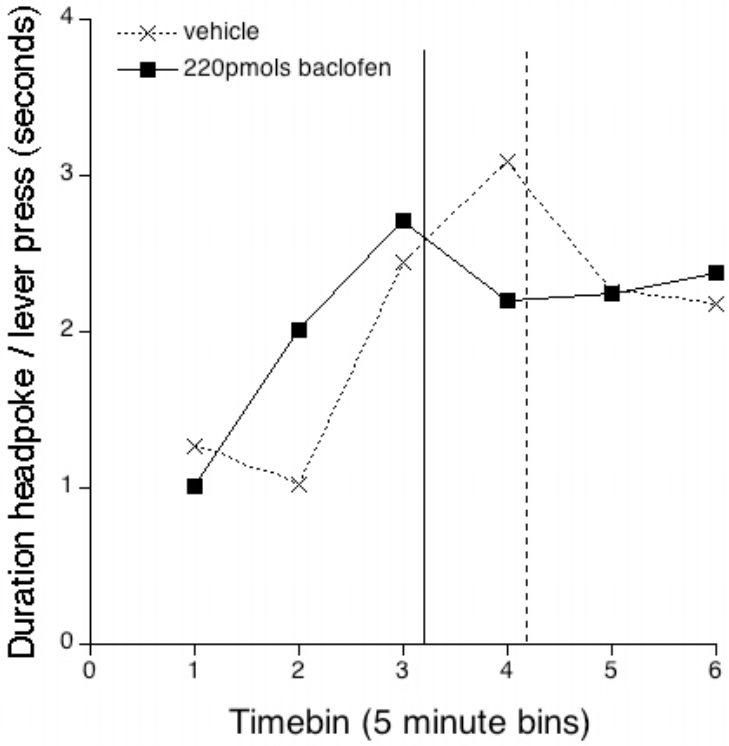


Figure 4.35. The effects of intra-Acb bilateral infusions of saline or 220ρmols baclofen in pre-fed rats (n=8) on the duration of headpokes into the magazine per reinforced lever press for 5 minute time bins across a 30 minute second order operant test session. The vertical lines indicate the mean time of first pellet delivery under baclofen or vehicle treatment.

Observations recorded whilst coding the videos revealed that 'duration' of headpokes into the magazine masked the variability in strategy employed by individuals. Some animals put their head in and waited whilst others repeatedly removed and replaced their head in the hopper whilst apparently checking the cue light. Some headpoked then reared to the CS.

Ingest

There was no significant effect of drug on the duration of ingestive behaviour. Baclofen had a significant main effect on frequency [F(2,14)=4.31, p=0.035] but post hoc analysis using the Bonferroni test revealed this to be due only to a significant difference between the 220ρmols dose relative to 660ρmols ($p<0.05$). Timebin analysis revealed that, with both the duration [F(10,70)=2.33, p=0.02] and frequency [[F(10,70)=2.74, p=0.007] of ingestive behaviours, there was a significant interaction between the effects of drug and time (Fig. 4.36). Paired comparisons revealed a significant increase in the duration of ingestion with the 220ρmols dose relative to vehicle between 20 and 25 minutes (Fig. 4.36A; $p<0.01$). Frequency was also significantly increased with 220ρmols relative to vehicle between 20-25 minutes (Fig. 4.36B; $p<0.01$).

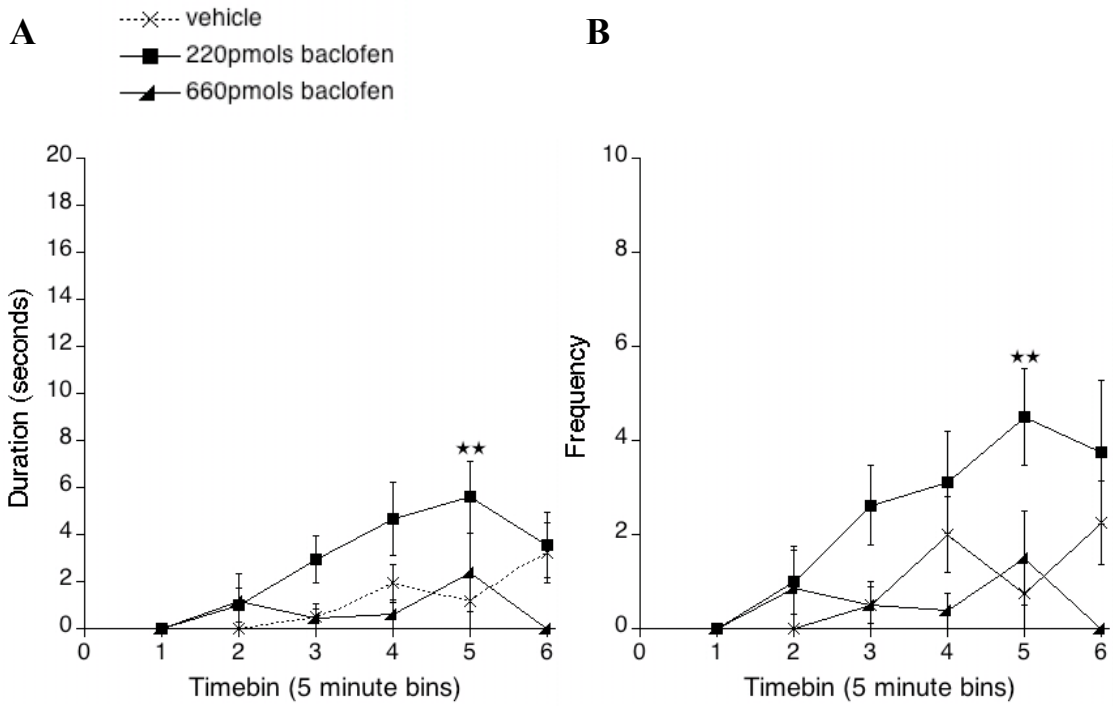


Figure 4.36. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on A) duration and B) frequency of pellet INGESTION across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★★ p<0.01.

The time taken to eat each meal of 5 pellets (calculated from the total duration of ingestion versus pellets consumed by each individual) is shown in Fig. 4.37. Not all animals in the cohort pressed enough times to achieve a food reward at every dose so statistical comparisons could not be made.

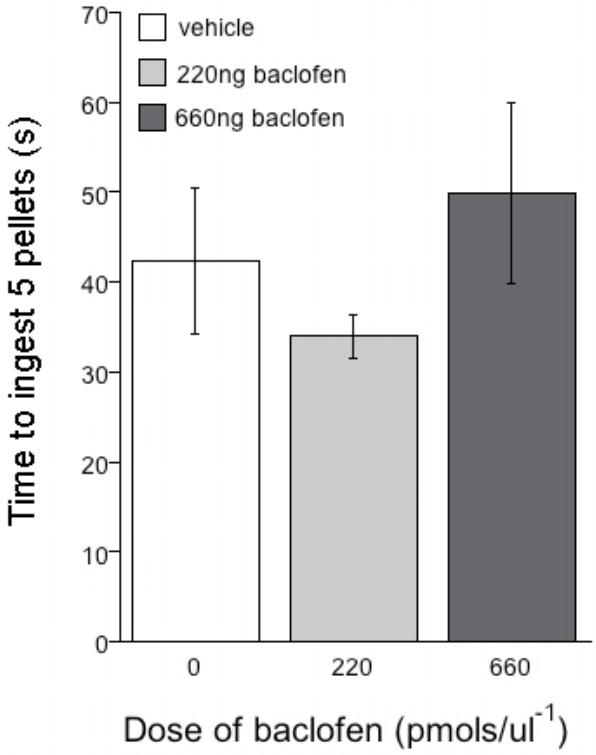


Figure 4.37. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on the average time to ingest 5 pellets across a 30 minute second order operant test session. Error bars represent ±SEM.

It was noted whilst watching the videos that there was very little variability in ingestive behaviour. All pellets were eaten very close to the hopper and there was rapid retrieval of the next pellet once the preceding one had been consumed. There were also relatively few meals of 5 pellets rewarded to the animals for the amount of effort expended with only three rats pressing enough to receive reward with 660ρmols of baclofen.

Groom

There was no significant effect of baclofen on either the total duration or frequency of grooming (see Fig. 4.27 and Fig. 4.28) however timebin analysis revealed a significant interaction between baclofen and time on the duration [Fig. 4.38: $F(10,70)=2.35$, $p=0.019$]. Paired comparisons revealed a significant increase with 660ρmols relative to vehicle between 15 and 20 minutes ($p<0.05$). There was no interaction between drug and time for frequency of grooming behaviour (see Fig. 4.38A)

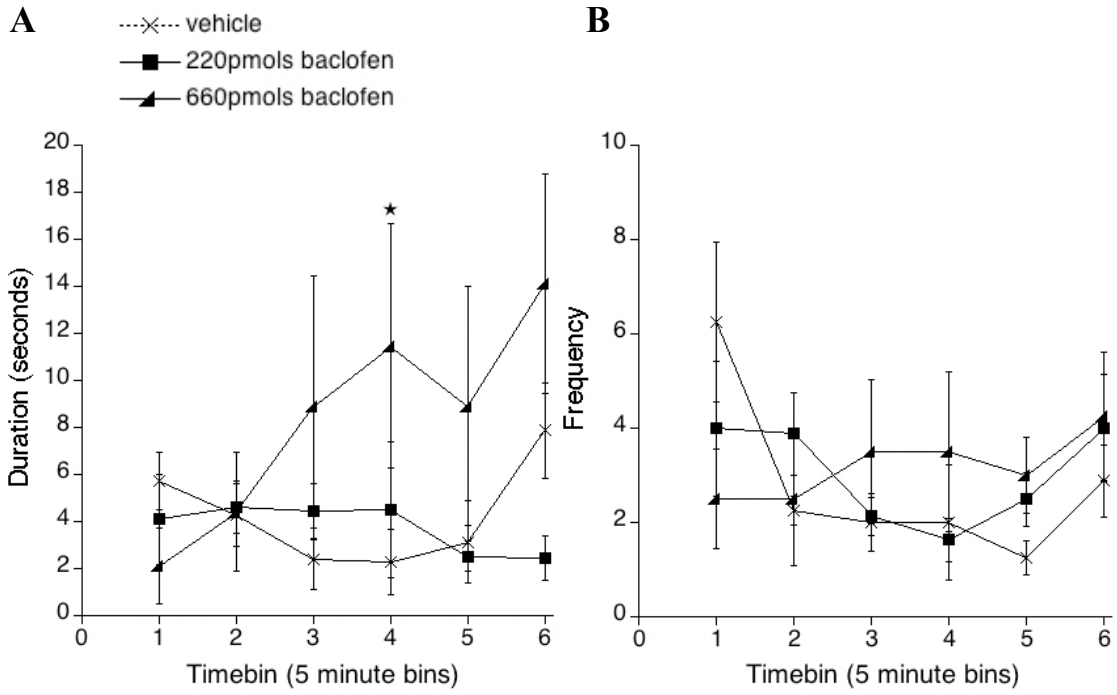


Figure 4.38. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on A) duration and B) frequency of GROOMING across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ $p<0.05$.

Whilst coding the behaviour in the videos there was a particular type of 'grooming' behaviour that stood out. At the 660ρmols dose, none of the animals exhibited a normal grooming sequence characterised by a rule-driven (syntactic) chain with a stereotyped order of paw, head and body movements (Berridge and Whishaw, 1992, Aldridge et al., 2004). Instead 6 out of 8 animals predominantly groomed either the tail or genitals. In particular tail grooming occurred over long periods without interruption. Animals looked as if they had become fixated on the tip of the tail specifically. Genital and tail grooming was accompanied by a great deal of what appeared to be rolling around or falling over. This pattern of behaviour was not seen during BSS experiments, Chapter 3.

Of the 2 animals that did not display this group of behaviours, one was almost constantly on his belly indicative of a strong myorelaxant effect. The other animal appeared to be at the opposite end of the scale behaving more like a rat under the lower 220ρmols dose (performing reinforced presses and rearing more than the rest of the cohort at this dose). In contrast animals under the other doses of drug or with vehicle all exhibited this syntactic chain during the test session and also tended to groom after pellet consumption and a short period of activity (a bit like a mini BSS).

Inactive

There was no significant effect of baclofen on the duration or frequency of inactive behaviour (See Fig. 4.27 and 4.28) nor was there any interaction between drug and time. Looking at the data split into time bins in Fig. 4.39 there was, nevertheless, more inactive behaviour expressed at the highest dose of 660ρmols across the session.

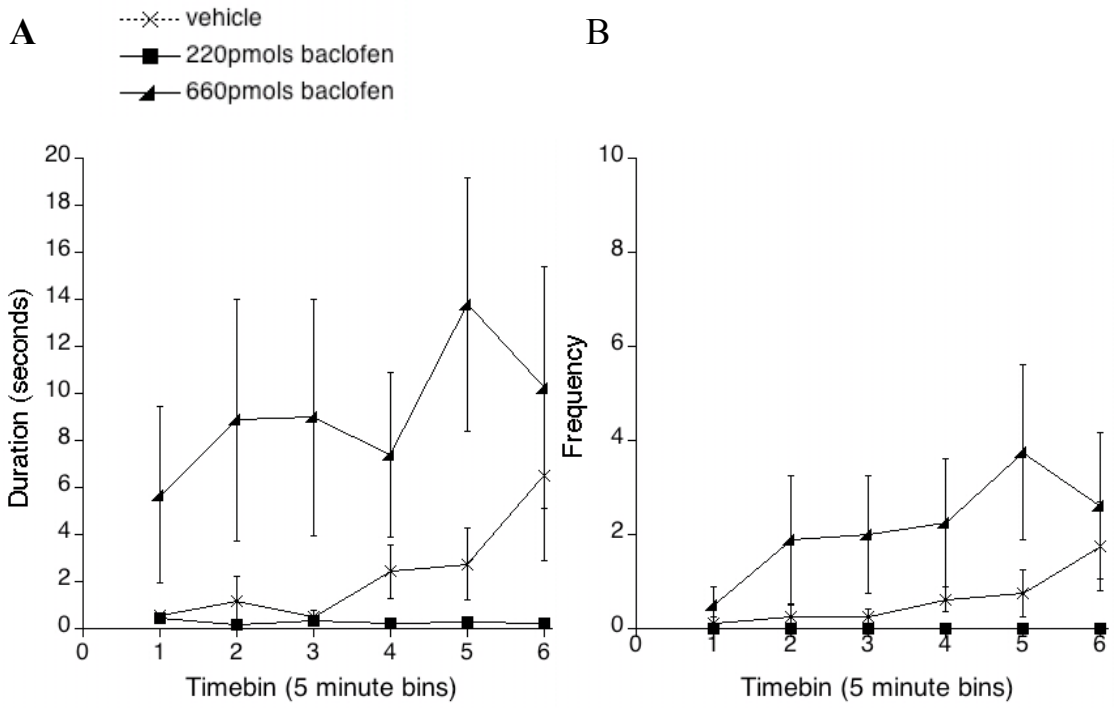


Figure 4.39. The effects of intra-Acb bilateral infusions of saline and a range of doses of baclofen in pre-fed rats (n=8) on A) duration and B) frequency of INACTIVE across a 30 minute second order operant test session. Error bars represent ±SEM.

Watching the videos it became evident that there may have been a myorelaxant effect at the 660ρmols with some animals frequently adopting a particularly languid position with limbs splayed and head resting on the mesh rather than on the forepaws (as was seen during inactive behaviour with vehicle or 220ρmols of baclofen).

Summary of results for experiment 4.3

In experiment 4.3 n=8 subjects were included in the final analysis. With these animals 660ρmols of baclofen significantly increased intake of freely available chow prior to testing on the 2nd order operant schedule. Both 220ρmols and 660ρmols also significantly increased total chow intake relative to vehicle treatment a few days following testing on the operant schedule. There was no significant difference between the amounts of chow consumed with 660ρmols prior to and following multiple infusions for the operant test.

The 220ρmols dose of baclofen significantly increased the total number of reinforced presses across the 30 minute operant session, total reinforced presses in the first 5 minutes only and the rate of reinforced pressing during this 5 minute appetitive phase. The rate of pressing with 220ρmols did not increase significantly once reward became available during the latter 25 minutes. There was a significant increase in total presses in the 5 minute bin after the mean time point at which the first batch of pellets was delivered. In contrast there was no significant effect of 660ρmols baclofen on either the total number of presses or the rate of pressing across the phases. With vehicle and 660ρmols baclofen, pressing rate during the latter 25 minutes was significantly lower than during the first 5 minutes. There was no significant effect of either dose of baclofen on total non-reinforced or incorrect lever presses but the proportion of non-reinforced presses to total presses made on that lever was lower with both drug doses. The increase in reinforced lever pressing with 220ρmols resulted in the delivery of significantly more pellets.

At 220ρmols, baclofen appeared to have little effect on the proportion of presses that were made during CS presentations. With the 660ρmols dose however >50% of presses were made during the CS i.e. when presses could not be rewarded. Nevertheless, given that presses that occurred during the CS were classified as non-reinforced presses this shift in the pattern of behaviour was not significant. For those presses that were made once the CS was terminated baclofen dose dependently decreased the interval between lever presses shifting the cumulative ILIs curve to the left, particularly at 660ρmols.

The increase in reinforced lever pressing at 220ρmols resulted in the delivery of significantly more pellets. This dose of baclofen might have decreased the latency to first pellet delivery but the total number of animals that received pellets was too low to carry out a statistical analysis. The 220ρmols dose did significantly decrease the length of interval between pellet delivery and the next reinforced press compared to vehicle.

Of the 8 other behaviours that were recorded from the videos of the test sessions baclofen significantly modified the total durations of oral stereotypy (increased at 660ρmols), activity (shorter durations at 220ρmols and 660ρmols) and headpokes into the magazine (increased at 220ρmols) compared to durations with vehicle. When the total frequencies for these three were compared only oral stereotypy (increased at 660ρmols) and headpokes into the magazine (increased at 220ρmols) were affected by baclofen. There was an increase in frequency of interactions with the lever at 220ρmols.

Analysis of each behaviour split into 5 minute time bins revealed that, with 220ρmols of baclofen, the duration of activity was significantly reduced relative vehicle between 10-15 minutes. At 220ρmols activity was replaced by more frequent visits to the lever across the session (although there was no significant interaction between time and dose for specific bins) and an increase in the duration and frequency of headpokes into the magazine as the session progressed. Head poking peaked in the time bins preceding an increase in the duration and frequency of bouts of ingestion between 20 to 25 minutes and just before the mean time point of first pellet delivery. An increase in the total duration of rears, although not significant, must also have contributed to the overall reduction in other activities at 220ρmols. Significantly more rears were made to the CS at this dose but the increase was proportional to the increase in rearing as a whole.

At 660ρmols the duration of bouts of activity dropped after the first 5 minutes and was even lower than with the 220ρmols dose throughout the rest of the session. Instead, at this dose, animals were significantly engaged in frequent and long bouts of oral stereotypy. There also appeared to be an increase in grooming (although abnormal grooming patterns were exhibited) and in inactive behaviour as oral stereotypy decreased over time but a large degree of inter-individual variability meant that this was not significant across time bins.

Discussion

The experiments reported in this chapter were designed to investigate the acute effects, across a range of doses, of intra-Acb $GABA_B$ receptor stimulation on ingestion versus performance of food motivated operant responses in pre-fed rats. In this case a 2nd order schedule was used to record operant responding for a previously food paired CS during an 'appetitive' phase and to the CS and intermittent primary reinforcer deliveries during a 'consummatory' phase. The effects were compared with those of intra-Acb DAMGO, a μ-opioid agonist that has been previously demonstrated to increase operant responding. In the final experiment the effects of baclofen on 8 mutually exclusive categories of behaviour expressed during responding on the 2nd order schedule were analysed from videos of the sessions. Intake of freely available chow was subsequently measured using an identical counterbalanced range of doses in the same cohort of animals.

Reinforced lever presses with baclofen

In experiment 4.1 220ρmols and 440ρmols of baclofen significantly increased total reinforced lever presses and the total number of pellets delivered over 30 minutes. In experiment 4.3, it was confirmed that 220ρmols significantly increased total reinforced lever presses and number of pellets delivered. A higher dose of 660ρmols did not significantly alter reinforced lever pressing. None of the doses of baclofen tested had any effect on incorrect presses on the second lever.

Phases of responding with baclofen

In experiment 4.1 there was no significant effect of baclofen on the total reinforced presses in the first 5 minutes but there was a significant increase at 220ρmols within 10 minutes (prior to first pellet delivery). There was no significant effect of drug on rate of responding in the first 5 minutes but a significant increase across the final 25 minutes at 220ρmols and 440ρmols. The rate across the final 25 minutes was also significantly higher with 220ρmols than with the other drug doses. In experiment 4.3, during the first 5 minutes and across the last 25 minutes, the 220ρmols dose significantly increased total reinforced lever presses and the rate of lever pressing relative to vehicle and 660ρmols baclofen. During this final phase the rate of pressing was significantly lower than during the first 5 minutes with vehicle and 660ρmols of baclofen. In experiment

4.1 the mean latency to first pellet delivery fell between the second and third 5 minute time bins. Pressing in the third timebin was consequently higher than in the second suggesting an invigoration of responding following food reward. The same relationship held true in experiment 4.3 but mean pellet delivery fell between the third and fourth time bins with a subsequent increase in pressing in the fourth bin.

Other behaviour durations and frequency with baclofen

In experiment 4.3 the main changes in behaviour seen on the videos at the 220ρmols dose were specific and significant increases in reward related behaviours. This included an increase in the frequency of interactions with the lever, rears to the CS, duration and frequency of headpokes into the magazine and in the frequency of bouts of pellet ingestion. These increases were associated with a concomitant decrease in the duration of other active behaviours. When the data for the 220ρmols dose were parsed into 5 minute time bins it became evident that, as the session progressed, animals spent more time headpoking into the magazine per reinforced lever press as the delivery of pellets became imminent. This was accompanied by a decrease in the duration and frequency of rearing across successive time bins followed by an increase in time spent ingesting.

In contrast, 660ρmols significantly decreased activity and rearing whilst causing an increase in oral stereotypy. Analysis of behaviour split into time bins demonstrated an increase in the duration but not the frequency of oral stereotypy from 5 minutes onwards. By 20 minutes the duration and frequency of oral stereotypy began to fall as grooming behaviour increased. There was a small increase in inactive behaviour but individual variability masked any significance. Observations recorded from the videos indicated that, at this dose, some animals appeared to be suffering myorelaxant effects.

Free food intake with baclofen and DAMGO

In experiment 4.1 a dose of 440ρmols baclofen significantly increased intake of freely available chow prior to the animals being run through the 2nd order testing phase. These same animals still increased their intake of chow when they were tested later with DAMGO having previously been infused on multiple occasions with both baclofen and DAMGO during the 2nd order testing phase. In experiment 4.3 a dose of 660ρmols baclofen increased intake prior to 2nd order testing and both 220 and 660ρmols baclofen

also increased free feeding after the 2nd order testing. There was no significant difference in the amount of food consumed prior to and post 2nd order testing with the 660ρmols dose.

Operant responding with DAMGO

In experiment 4.2, a dose of 0.025μg DAMGO had no significant effect on the rate of pressing, the total number of reinforced presses or on the pattern of pressing across the session when it was broken down into 5 minute time bins. There was also no significant effect of this dose of DAMGO on the proportion of presses made during CS presentations across the first 5 minutes of the schedule. For the remaining 25 minutes, when the primary reinforcer became available, some presses were made prematurely i.e. before the CS terminated. This dose of DAMGO did, however, significantly increase intake of freely available chow following testing on the operant schedule.

Preliminary interpretation of key results summarised above

It would appear that, firstly, an optimum dose of baclofen infused into the Acb can increase operant responding for a 2nd order reinforcer in the absence of the primary reinforcer i.e. appetitive responding, without causing a breakdown in the relationship between the schedule requirements (response accuracy) and associated behaviours. In both experiments 4.1 and 4.3 the most effective dose of 220ρmols increased total reinforced presses and the rate of pressing but did not perseverate responding on the lever once criterion had been met. A small proportion of presses at this dose were made prematurely (before the CS had terminated), particularly during the appetitive phase. In experiment 4.1 these non-reinforced presses were only significant across a short period of the session and the increase was proportional to the total increase in pressing on that lever. There was no significant increase in premature, non-reinforced responses in experiment 4.3.

Secondly the most effective 220ρmols dose of baclofen increased the total amount of reinforced responding relative to vehicle levels during the 'consummatory' phase. The number of reinforced lever presses made in each time bin decreased across the session but increased shortly after the mean time point at which animals received their first batch of pellets. Nevertheless, animals under the influence of baclofen pressed significantly more than with vehicle both prior to and following food reward and the

overall rate of pressing across the consummatory phase was increased relative to vehicle levels.

Finally 220ρmols significantly modified motivated behaviours including lever pressing, rearing to the CS and headpoking into the magazine without causing any disruption in other behaviours such as non-cue oriented rearing or grooming. In contrast the higher dose of 660ρmols caused the emergence of oral stereotypy, abnormal grooming patterns and decreased activity accompanied by a possible breakdown in response accuracy. With a large percentage of presses occurring during the CS at this high dose and a lack of effect on the rate or total number of reinforced presses these results suggest a disruption of the normal pattern of schedule induced responding seen with vehicle.

Thus, not only does baclofen potentiate operant responding in a food motivated schedule at doses that also increase free intake of chow but it does so in a dose dependant manner that could reflect motivational effects on both appetitive and consummatory aspects of ingestive behaviour. In contrast DAMGO, infused in a cohort of animals previously shown to be responsive to baclofen, has no significant effect on appetitive or consummatory operant responding on this type of schedule at a dose that subsequently increased free intake of chow.

The effect of baclofen on reinforced lever pressing suggests a classic inverted U-shaped dose response function over the dose range tested (0 - 660ρmols). In contrast, data from both experiments 3.1 (Chapter 3, Fig. 3.4) and experiment 4.3 suggest that there was a positive linear relationship between dose of baclofen and total intake of freely available chow (see Fig. 4.40).

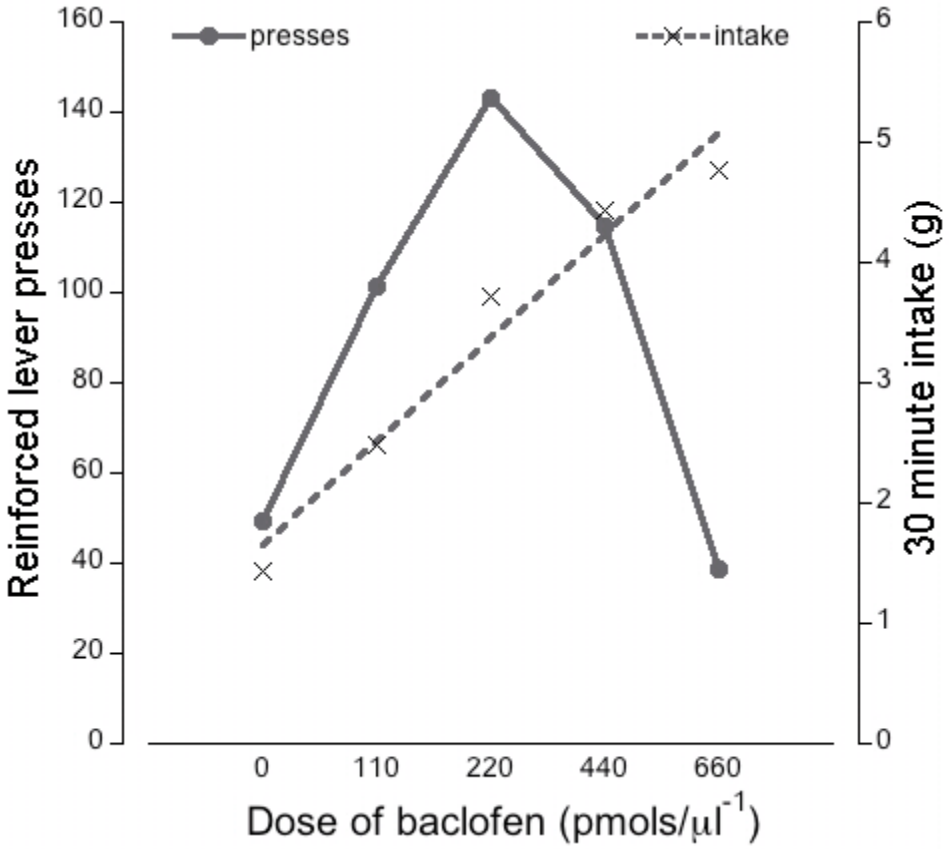


Figure 4.40. Representative data for total intake of freely available chow over 30 minutes (based on means from all intake experiments reported in Chapter 3 and Chapter 4) compared to total reinforced lever pressing in a 30 minute 2nd order operant test session (based on mean pressing data from experiments 4.1 and 4.3).

Possible explanations for baclofen induced increase in lever pressing

It could be argued that the increase in lever pressing following infusions of baclofen into the Acb was not due to effects on food associated motivational processes *per se* but on, for example, locomotor activity, inhibitory control over cue-evoked responding or on attentional processes. All of these processes have previously been demonstrated to be influenced either by destruction or temporary inactivation of the Acb and these issues will be further dealt with in context below. Taking into account the combined findings from the analysis of pressing data broken down into; 1) the two phases, 2) 5 minute time bins and 3) intervals between presses as well as the behavioural measures taken from the videos potential explanations for a significant effect relating to procedural and methodological issues will be discussed:

1) Spread of baclofen to other critical brain regions in the motive circuit
2) Location of infusions relative to 'hot spots' or behavioural gradients in AcbSh

3) The nature of the behaviour induced by a 2nd order schedule.

The following alternative explanations for the results will be also be discussed:

4) Psychomotor activation
5) Reduction of response inhibitory control
6) Improved attention (increased vigilance to cues or decreased latent inhibition)
7) Increased hedonic impact of primary reward
8) Increased transfer of hedonic properties to the neutral cue
9) Increased attribution of salience to the cue in the absence of hedonic effects

Finally a putative mechanisms for the effects reported will be discussed with reference to both the chemical and structural processes that might be involved and the implications of this hypothesis for work in further chapters will be highlighted.

1) Spread of baclofen to other brain regions

Before a hypothesised mechanism to explain the results is suggested potential explanations based on methodological issues need to be taken into account. As highlighted in Chapter 3 it could be argued that there was some potential spread of baclofen or DAMGO infusions into either the boundary between the core and shell or into more ventral structures such as the ventral pallidum (VP). Infusion of a $GABA_A$ antagonist into the VP has been shown to increase intake in satiated animals (Stratford et al., 1999) and to increase positive taste reactivity following conditioned taste aversion (Inui et al., 2007). The $GABA_A$ agonist muscimol in the VP decreases instrumental responding on an FR5 schedule for food (Farrar et al., 2008).

Extensive studies of the role of the VP in mediating 'liking' reactions demonstrate that activity in this region selectively tracks hedonic values of tastes and there is strong evidence for neural coding of natural sensory "pleasures" (Tindell et al., 2006). Thus infusions of baclofen into medial portions of the VP might be expected to cause a decrease in intake, a decrease in responding on the reinforced lever and, perhaps, augmentation of these effects during the consummatory phase compared to the appetitive phase. This is clearly not the case so it can be concluded that it is unlikely the results reported are due to infusions spreading into regions ventral to the AcbSh.

The divergent roles of the core and shell in cue driven behaviours is considered in this discussion but it would appear that the results are also unlikely to be due to direct inhibition of neurons in the core. If the core were involved then a somewhat different behavioural pattern would be expected. For example lesions of the AcbC significantly reduce Pavlovian approach behaviour and animals also fail to discriminate between CS+ and CS- (Parkinson et al., 1999, Cardinal et al., 2002b). Lesions of the AcbC also cause significant elevations in premature responses in the 5CSRTT (Christakou et al., 2004) and have also been shown to cause hyperactivity (Parkinson et al., 1999). Probably most important however is the fact that infusions of GABA agonists into the core have been shown to have little effect on free intake (Kelley and Maldonado-Irizarry, 1995, Stratford and Kelley, 1997b).

2) Location of infusions within the AcbSh

The second explanation relates to the location of the infusions. By chance the mean AP co-ordinate for the group of animals deemed to have acceptable placements in experiment 4.1 was more rostral (approximately β+1.5mm) than in experiment 4.3 (approximately β+1.3mm). In two micro-injection mapping studies to assess the effects of AMPA/kainate receptor blockade or GABA receptor stimulation in different locations of the AcbSh it was shown that a more caudal infusion was most effective in terms of free food intake (Kelley and Swanson, 1997, Basso and Kelley, 1999) and Stratford an Kelley (1997) found an infusion at an AP coordinate of β+1.4mm to be most effective.

Reynolds and Berridge (2002) reported that muscimol infused into the AcbSh caused increases in positive hedonic taste reactivity more rostrally but that the robust increase in intake was positively correlated with suppression of this effect further back. Although the distances seem very small this shift occurred between β+1.6mm and β+1.2mm. Thus the coordinates for experiment 4.1 fall closer to the region where hedonic responses were increased but effects on intake were lower and the coordinates for experiment 4.3 fall within the region where there was a positive correlation between hedonic suppression and increased intake.

This lends some support to the general assertion that I will elaborate on in the rest of this discussion that a hedonic explanation for the results with baclofen is unlikely but a salience attribution hypothesis could explain the data. Extensive mapping studies using opioids suggest that there are "hot spots" within the AcbSh for the effects of neurotransmitter function in terms of intake and hedonic effects (Zhang and Kelley, 2000, Pecina and Berridge, 2000, Pecina and Berridge, 2005, Pecina et al., 2006). It is also possible that this phenomenon could be important in the control of cue saliency attribution. It has been demonstrated that there are significant gradients in the levels of DA activity in the AcbSh (Wickens et al., 2007) which, as will also be discussed below, is involved in the response of the Acb to cue presentations (see electrophysiological evidence).

3) Nature of behaviour induced by the schedule

Another potential issue is the observation that there are some differences between the results reported in experiments 4.1 and 4.3. Specifically 1) while there were significant increases in responding during the first 5 minutes in experiment 4.3 this effect was not evident until 10 minutes into the session in experiment 4.1, 2) that animals pressed less overall and took longer to reach criterion and hence pellet delivery in experiment 4.3 and 3) that there was a pronounced effect of baclofen on the latency to respond post CS in experiment 4.3 but this was milder in experiment 4.1 (see length of ILIs).

Two potential explanations for these differences are suggested. First of all it is well known that responding under a fixed ratio schedule does not produce the highly structured pattern of responding that one might expect with, for example, an FI schedule. As Everitt and Robbins (2002) point out animals could take 5 or 50 minutes to make the same number of responses and the FR(FR) type 2^{nd} order schedule does not exert strong temporal control over responding. Secondly, unlike the method based on that designed by Kelleher (1966) where the FI period of appetitive responding is indicated by the presence of a distinctive light cue, the animals in this schedule had no explicit knowledge of when the 5 minute appetitive phase ended. Thus differences in the timing of peak appetitive responding or the total number of presses between experiments are probably irrelevant.

4) Psychomotor activation

The simplest explanation for the results with 220ρmols could be that baclofen at this dose simply indiscriminately increased spontaneous activity (including active behaviours directed towards the levers) during this schedule. Nevertheless there are some straightforward reasons to reject this hypothesis. First of all there was no generalised effect on total pressing *per se*. Increased motor activation, for example, would be expected to significantly increase pressing on both the reinforced and incorrect levers but, even at the highest dose that disrupted schedule control over reinforced pressing, the animals did not increase their responses on the incorrect lever.

The second key point here is that, if anything, the behavioural data from the videos indicated that activity was slightly reduced at the dose that was most effective at increasing lever pressing. Nevertheless this was only significant over a short period that coincided with significantly increased engagement in other behaviours (e.g. lever pressing, rearing, head poking into the magazine) suggesting a lack of any direct psychomotor effects. This is supported by the fact that it has been previously demonstrated that transient 'inactivation' of the AcbSh using baclofen doses ranging from 88 – 876ρmols has no significant effect on locomotor activity in a standard cage (Stratford and Kelley, 1997b) and permanent inactivation using excitotoxic lesions does not affect motor activity in an open field test (Maldonadoirizarry and Kelley, 1995). At higher doses baclofen infused into the Acb causes locomotor depression due to myorelaxation (Stefanski et al., 1990, Jelen et al., 1994), which would disrupt pressing.

5) Response inhibitory control

An increase in operant responding at 220ρmols could be argued to be an indication of a lack of behavioural inhibition over prepotent behaviour, which constitutes a form of impulsivity (Evenden, 1999). Response inhibition can be expressed as control over impulsive action or impulsive choice (which requires action restraint) or over compulsive action (which requires action cancellation) (Robinson et al., 2009, Evenden, 1999, Dalley et al., 2008, Schachar et al., 2007).

Although potentially distinct roles for the AcbC and AcbSh have begun to emerge it is likely that their involvement is co-modulatory (Murphy et al., 2008) and, in the majority of cases, only manipulation of AcbC function has any significant impact on various

measures of impulsive action and impulsive choice (Cole, 1989, Cardinal, 2001, Pothuizen, 2005, Acheson, 2006). However, just because a distinct role for the AcbSh has not yet been identified, it does not mean that it does not exist. Afferents to the shell could also influence the core by "channelling information via cascading reciprocal and non-reciprocal feedback and feedforward loops" (Murphy et al., 2008). The 2nd order schedule used here might offer animals opportunities to exhibit impulsive actions, impulsive choices or compulsive actions and each will be dealt with in turn.

Impulsive action

Impulsive actions are manifested as inappropriate behaviours either in terms of the timing or target of operant responses and could be measured on this schedule as premature responses on the reinforced lever or increased presses on the incorrect lever. The latter case can be excluded immediately since there were no significant effectss at any drug dose on incorrect lever pressing. In both experiments with baclofen, presses that occurred during the CS under the influence of drug did occur prematurely i.e. before the CS was terminated (ILI <8s, see Fig 4.8, 4.9, 4.22 and 4.23). Nevertheless there was no significant increase in the total number of these non-reinforced presses at 110, 440 or 660ρmols. At the 220ρmols dose there was an increase in non-reinforced presses in experiment 4.1 but not in experiment 4.3. The increase in experiment 4.1 was proportional to the total increase in reinforced pressing and coincided with time bins in which reinforced pressing was at a peak.

Given the lack of significance of the total number of premature (non-reinforced) presses across the majority of the test session in experiment 4.1 and no repetition of the effect in experiment 4.3 this cannot be taken as a strong indication of impaired impulse control. This is consistent with the view that the AcbSh is not the predominant locus of impulsive action control (see Chapter 1, introduction).

Compulsive action

The schedule might also be considered to encompass elements of a go-no-go task whereby the absence of a cue is the "go" signal and the presence of the cue is the "no-go" signal that terminates responding. Alternatively it could mirror aspects of a stop signal reaction time task whereby animals have to stop an already initiated chain of responses when the cue (stop signal) comes on. Vehicle treated animals in all three

experiments consistently withheld responding during the CS suggesting that the cue did indeed set in motion processes that inhibited ongoing prepotent responses. In this case if baclofen caused an inability to withhold perseverative responses during the CS this could indicate an effect of the drug on compulsive action.

As explained above, although animals under the influence of 220ρmols did make a proportion of presses during the CS these were not usually a continuation of pressing as the CS came on (ILI of 0, see Fig 4.8, 4.9, 4.22 and 4.23) but premature responses just before it was due to terminate. Not only did the rats stop pressing when the CS came on but, observations of their behaviour in the videos showed that they consequently made a combination of headpokes and/or rears to the CS while they were waiting. Some animals even clearly looked up to check the CS whilst headpoking.

Impulsive choice

The large amount of pressing required for delivery of primary reward may be perceived as a delay by the animals or could affect the perceived probability of reward delivery. In this case the animals might make a choice about how to allocate their effort across the schedule. The use of an FR schedule plus an unrewarded phase, the end of which is not signalled, means that the timing of the delay to and the probability of reward might appear somewhat random to the subjects. Thus the schedule could be particularly conducive to impulsive choice behaviours usually seen on delay or probability discounting tasks. Evidence presented in the introduction to this thesis in delay or probability suggests that impulsivity can be expressed in terms of choice to press more at a particular stage in a schedule (rather than a lack of control over incorrect or unrewarded responses).

However impulsivity on a delay-discounting task is measured as a tendency to choose small immediate reward, which, unless the CS itself is perceived as inherently rewarding (see further discussion later) is not an option on this task. Equally if the task was very unpredictable they might chose small guaranteed reward over large improbable reward if acting impulsively with baclofen. Again this could only be manifested as a choice to respond more for the CS, which would be more predictable than getting pellets. Experimental evidence in the literature suggests that this would be a core mediated effect (Cardinal, 2001). Thus an effect of baclofen on impulsive choice

would depend on changes in the value of the CS and it would require an explanation involving AcbSh mediation of an AcbC mediated process.

It has been postulated that the shell may be capable of indirectly modulating some aspects of impulsivity by affecting core and dorsal striatum function via a circuit through the SN (Jongen-Relo et al., 2002). It has since emerged that there are also direct connections between the core and shell sub-regions (van Dongen et al., 2005) so an interaction between the two and, hence, a shell mediated effect on impulsive choice cannot be ruled out. Nevertheless it is not believed that effects on impulsive choice could fully account for the results at 220ρmols.

6) Attentional effects

In considering the issue of 'attention' in this discussion the term is used not in reference to explicit behaviours such as eye movements and orienting behaviours but with respect to an implicit role for attentional processes in the way in which animals learn about their environment and adapt those associations during schedule responding. Increased vigilance to discrete programmed cues within the schedule or decreased latent inhibition (LI) to contextual cues in the environment associated with food might serve to increase reinforced lever pressing by making the experience more arousing and/or salient.

There is an established role for the Acb in attentional processes but, as with impulsivity, the contribution of AcbC and AcbSh regions is probably distinct. Jongen-Relo et al., (2002) conclude that inactivation of the AcbC disrupts attentional processes related to discrete cues engaged during PPI whereas inactivation of the AcbSh attenuates LI. It is therefore more likely that any effects of baclofen would be due to inactivation of the AcbSh and hence decreased LI rather than increased vigilance to discrete cues.

As was noted in Chapter 3 that there did appear to be some basis for considering effects on attention. With muscimol animals appeared not to be paying attention to external cues such as movement of the observer, noises from other animals or rearing of another animal in the cages beside them. In contrast, with baclofen, animals were generally more vigilant and did clearly respond to such events. Baclofen infused into the shell region might not significantly disrupt responding to discrete cues, as appears to be the

case with muscimol, but could have attenuated previously established LI over cues other than the CS.

Animals were pre-exposed to the box on multiple magazine training days prior to the first instrumental conditioning session and hence may have learned to ignore contextual cues such as the sound of the fans etc. These contextual cues experienced in the training environment (and hence during the delivery of food reward when hungry) could have become influential on responding if LI was attenuated. This explanation is very hard to confirm or discount just on the basis of the results reported here without further tests but it does not necessarily supersede a more direct motivational explanation and may contribute to other effects of baclofen.

7) Hedonic effects on primary reward value

It is generally thought that the Acb modulates motivated behaviours elicited by both primary reinforcers and by cues associated with these reinforcers (Robbins et al., 1989) (Everitt et al., 1999, Cardinal et al., 2002a, Holland and Petrovich, 2005). It is possible therefore that an increase in reinforced pressing at 220ρmols could be mediated by a perceived increase in the hedonic value of the food reward (primary reinforcer) at this dose. However it must be taken into account that in experiment 4.3 there was a significant increase in responding in the first 5 minutes at the 220ρmols dose when animals could not access the primary reward. There is also the problem that DAMGO, was expected to increase operant responding by increasing palatability of the reinforcer, did not increase pressing during either phase. These factors will be addressed in the following discussion of the possible effects of baclofen on primary reward value.

Appetitive and consummatory phase with DAMGO

As discussed in the introduction to this chapter, DAMGO probably does not affect appetitive behaviours in the absence of the primary reinforcer. This finding was confirmed in experiment 4.2 where DAMGO had no significant effect on operant responding during the appetitive phase of the schedule in contrast to the significant effect of baclofen. However DAMGO also had no significant effect on pressing during the consummatory phase, despite the fact that the dose used has previously been demonstrated to increase operant responding for food reward on a PR schedule (Zhang et al., 2003).

This lack of effect cannot be explained by any delay in the action of DAMGO because there was no sign of a biphasic inhibitory-stimulatory effect (Meyer et al., 1994) on operant responding at the dose used in experiment 4.2. The peak levels of correct responding on the reinforced lever occurred within the first 10 minutes post infusion which was the same pattern seen with the vehicle treated animals. Free intake of chow was also significantly increased in the 30 minute period following DAMGO infusions.

The most likely explanation for a lack of effect probably relates to the type of schedule used. It has previously been reported that the mixed μ and δ-agonist morphine infused directly into the Acb increases responding for a conditioned reinforcer or, conversely, has no effect (Kelley and Domesick, 1982, Cunningham and Kelley, 1992b, Cunningham and Kelley, 1992a). The mixed μ and δ-agonist [D-Ala2-Met5]-enkephalin (DALA), the μ-agonist DAMGO and the δ-agonist [D-Pen2,5]-Enkephalin (DPEN) were initially shown to have no significant effect on responding to a CR (Cunningham and Kelley, 1992b) but, in a second study DPEN, significantly increased responding (Cunningham and Kelley, 1992a). Elsewhere it has been shown that DALA, DAMGO and DPEN all increase responding on a CR paired lever (Phillips et al., 1994). On the PR schedule used by Zhang et al. (2003), a small work requirement at the start meant that the animals experienced the food reward under the influence of DAMGO as soon as they made an operant response. It has been demonstrated that close contiguity between the timing of DAMGO infusions and food consumption is critical for reversing sensory specific satiety in pre-fed animals and that the effect is short term and temporary (Woolley et al., 2007). What probably happened is that, on the 2nd order schedule, pre-fed animals under the influence of DAMGO simply did not get to experience the primary reward before they lost interest in pressing.

Appetitive phase with baclofen

Any hedonic effect of baclofen on pressing pre-reward delivery in the schedule used here would have to be explained in terms of a previous experience of baclofen's effects on reward value. It is possible that experiencing the ingestional consequences of consuming chow under the influence of baclofen during the intake probe test in each experiment resulted in a subsequent revaluation of the expected rewarding properties of the pellets because of their similarity to the chow. This hypothetical transferral of

inflated value to the pellets could then be responsible for an increase in reinforced pressing across the appetitive phase.

Balleine (1992), however, argues that learning processes link the hedonic value of the reward to the motivational state in which it was experienced which consequently restricts the conditions under which the new incentive value will modify instrumental actions. If this is the case then only a combination of experiencing the chow reward under the influence of baclofen prior to testing and experiencing the same baclofen induced state during testing with pellets would mediate operant responding. Since increased responding only occurred at the 220ρmols dose but the animals increased their consumption of chow at 440ρmols (Exp. 4.1) and or 660ρmols in (Exp. 4.3) this explanation is not adequate to account for the results

It is also significant that animals that experienced chow under the influence of DAMGO did not subsequently respond to the chance to access pellets as if they were more rewarding when given the same dose in the operant session. This finding suggests that there is no link between the hedonic value of the chow consumed and the pellets used in the operant session. It seems highly unlikely therefore that increased responding during the appetitive phase could be explained by hedonic effects of baclofen on primary reward value.

Consummatory phase

In the experiments presented, detailed analysis of the pattern of lever pressing across the 30 minute session, combined with temporal analysis of associated behaviours indicated that there could have been an effect of intra-Acb baclofen on the motivating value of the reward post pellet delivery. Animals returned to lever pressing following ingestion of pellets significantly faster under the influence of the most effective dose of baclofen than with vehicle and the total number of lever presses increased post pellet delivery. It was also shown that, during the consummatory phase, baclofen shifted the latency of lever presses post CS presentation to the left. In experiment 4.1, the rate of pressing across this phase was significantly higher than during the appetitive phase. In experiment 4.3 the rate of pressing across this phase was higher than with vehicle or the higher drug dose.

A shift in the association between action and outcome (A-O) could be responsible for some of these behaviours in line with an incentive theory such as that originated by Tolman in the late 1940s and revisited by Dickinson and Balleine (e.g. see Dickinson and Balleine, 1994). The premise of this argument would be that intra-Acb baclofen modified the incentive value of the reward and that, following the operant action, the more rewarding consummatory experience consequently increased the drive to perform the action again and to do so sooner. This hypothetical process has been referred to as "*incentive learning*" (Balleine and Dickinson, 1992, Dickinson, 1994, Dickinson, 1995). Post-training reinforcer revaluation procedures (Rozeboom, 1958) have become, as Holland (2008) points out, a gold standard for measuring the presence or absence of instrumental associations and the degree to which the association changes following alterations of the value of the reward.

Dickinson (1994) also argues however that changes in the hedonic value of a reward (liking) alone are not enough to initiate ingestion (and therefore food seeking) and only the establishment of a learned association between the new value and contiguous cues will support increased operant responding. Thus only repeated exposure over time to the consumption of pellets under the influence of baclofen might modify responding on the 2nd order schedule (Dickinson and Balleine, 1994). It is unlikely that the very low number of primary reward presentations would support the development of a strong association between increased value and contiguous cues within the consummatory phase of one session. Furthermore the counterbalanced design meant that animals did not experience the same baclofen induced state across repeated test sessions.

All the evidence for the effects of incentive learning on A-O revaluation has come from changes recorded between test sessions and there is no evidence that this is a process than can be measured within one operant test session. It has also been suggested that revaluation effects are actually quite small in absolute terms given, for example, that animals will still make numerous operant responses even following devaluation (Holland, 2008). Even more compelling is recent evidence that operant responding based on expectancy of a particular reward following revaluation may depend very little on what is actually presented (Galarce et al., 2007). Furthermore, in this study, a lack of any carry over of effect onto pressing between test days would tend to argue against an

increased hedonic value of the pellet reward subserving a learned increase in operant responding.

If the animals have not learnt to press more for a hedonically more valuable cue would an increase in hedonic value still have an acute effect on pressing immediately post ingestion? In a study designed to directly assess the impact of length of training it was shown that, following long periods of exposure to a schedule, instrumental responses were predominantly governed by current motivational state and not by prior manipulation of reward value through incentive learning (Dickinson et al., 1995). However, it is widely believed that when an animal is well trained (or 'over' trained), as they are on the 2nd order schedule, their operant responses become habitual and are insensitive to presentations of the reward regardless of current motivational state (e.g. see Holland, 2004). This is evident even in the earliest studies designed to explore the impact of varying presentations of food or drug reward and reward related cues on a 2nd order schedule (Kelleher and Goldberg, 1977, Goldberg et al., 1981)

Thornton-Jones et al., (2005) reported that pre-feeding animals with chow prior to testing in this second order schedule resulted in an overall reduction in reinforced pressing but their data also indicated a significant increase in pressing on this lever once pellets became available. Pre-feeding suppressed responding during the 5 minute appetitive phase but the availability of food in the consummatory phase invigorated responding to the extent that there was no difference between pressing in hungry or pre-fed animals (Thornton-Jones et al., 2005). This means that our 2nd order schedule is sensitive to both current motivational state and to primary reward presentation. This is consistent with Everitt and Robins' (2002) suggestion that an FR(FR) type 2nd order schedule does not engender habitual responding as robustly as an FI(FR) type.

Thornton-Jones et al. (2005) suggested that increases in pressing after primary reward presentation could reflect an increase in feeding due to the initial activation of positive feedback mechanisms that promote feeding, in line with studies carried out in the 1970s (Wiepkema, 1971). They also suggested that, alternatively, the pellets might differ enough from the chow consumed during pre-feeding that they effectively re-invigorated feeding in animals that had reached sensory specific satiety for chow. This explanation was in line with numerous studies investigating the effects of presenting diets that differ

from those that have been recently ingested (Clifton et al., 1987, Treit et al., 1983). Most importantly in the context of the studies reported here this indicates that, despite devaluation of food reward by pre-feeding with chow to satiation, the pellets were still subsequently rewarding.

It was not clear from the data presented in experiment 4.1 that this re-invigoration phenomenon occurred following the delivery of pellets to the pre-satiated, vehicle treated group. The total number of lever presses was lower than those reported for pre-satiated animals by Thornton-Jones et al., (2005). Only four rats in the vehicle treatment pressed enough to receive pellets and, as a result, there is a great deal of variability in the total number of presses made by individuals across the time bin immediately following mean first pellet delivery time. It does not therefore adequately represent post pellet pressing with vehicle. In experiment 4.3, however, there is a clear increase in pressing during the timebin immediately following mean pellet delivery time for animals in the vehicle condition. It is concluded therefore that the invigoration of responding by pellets during the consummatory phase, either via positive feedback mechanisms or by overriding sensory specific satiety, was replicated in these studies.

Increases in rate of pressing post pellet delivery might mean that schedule controlled responding in these experiments could well be sensitive to acute effects of baclofen on reward value during the consummatory phase. However if this were the case two separate mechanisms, both modulated by $GABA_B$ receptors in the AcbSh would need to be present to increase appetitive responding and to increase consummatory responding. A more parsimonious theory would encompass a single mechanism that could subserve both effects. Furthermore there are alternative explanations for some of the potential evidence of increased responding due to hedonic effects mentioned above. For example, although animals returned to lever pressing post pellet delivery more quickly with the optimum dose of baclofen this could have been because they consumed the pellets more rapidly rather than because they valued the next batch of pellets more highly.

Also it should be noted that with the 440ρmols dose that also increased the total number of reinforced presses, animals did not return to lever pressing more rapidly post pellet delivery, there was no concomitant increase in the rate of pressing during the consummatory phase and no significant increase in pressing in time bins following

pellet delivery. Thus an operant effect of baclofen was recorded without associated evidence of increased reward value. Finally, although lever pressing was increased post pellet delivery with 220ρmols in experiment 4.3, the relative frequency and duration of headpokes per press i.e. consummatory behaviour, was not increased. In other words animals were pressing more but were not trying harder to gain access to the pellets.

It is suggested therefore that $GABA_B$ receptor stimulation in the Acb must have affected appetitive responding via acute effects on a mechanism other than increased reward value and that the further increase in pressing post pellet delivery was primarily due to the same acute effects superimposed on positive feedback processes or on satiety mechanisms previously reported (Thornton-Jones, 2004). This is consistent with the view that intra-Acb $GABA_A$ stimulation does not increase the hedonic impact of food (Kelley et al., 2005b, Kelley et al., 2005a, Baldo and Kelley, 2007) although it does not automatically follow that $GABA_B$ stimulation also fails to impact hedonic value.

8) Hedonic effects associated with the CS

One possible caveat to the argument above against hedonic effects of baclofen in the 2nd order schedule is that there is evidence that the CS itself might acquire hedonic properties from its association with the reward and that the AcbSh encodes this information (Kerfoot et al., 2007).

Early studies showed that cues associated with sucrose or quinine could stimulate the same orofacial taste reactivity responses with unflavoured water (Delamater et al., 1986). It was suggested however that the cues simply initiated a prepotent behavioural pattern, including orofacial responses, learnt as part of the initial S-R acquisition (Berridge and Schulkin, 1989). Berridge and Schulkin (1989) later showed however that, even when the animals had learnt aversive orofacial responses to a neutral flavour-cue paired with an aversive concentration of sodium (S-R learning), they exhibited appetitive taste reactivity responses to the cue alone when sodium appetite was pharmacologically induced. Thus Berridge and Schulkin (1989) showed that positive taste reactivity responses could be transferred to, and controlled by a CS without explicit pairing with the revalued reinforcer.

It has been suggested that the spontaneous appearance of hedonic responses to cues post reward revaluation (despite the fact that the cues have not been explicitly linked to the new value) may depend on the degree to which the animal has been trained (Holland, 2005). Thus in the early stages of development of a CS-US association the CS activates a large variety of processes usually activated by the US, including perception of the hedonic properties of the reward in its absence, but that extended training narrows the range of systems involved (Holland, 2008).

Holland's (2008) hypothesis was confirmed in a recent study carried out by the same laboratory, which showed that taste reactivity to a CS occurred after limited training but was absent after extended training (Holland et al., 2008). It is unlikely therefore that the results reported here are associated with any hedonic value or 'liking' attributed to the cue because the extended training on the 2nd order schedule would likely have eliminated this effect.

9) Increased attribution of salience to the cue

In the context of motivational theories such as those discussed in the introduction to this thesis (e.g. Toates, 1981, Toates, 1986, Bindra, 1969, Bindra, 1974b, Bindra, 1978) saliency attribution is an intrinsic part of the learning processes that leads to cue driven instrumental actions. The *saliency attribution* hypothesis suggests that there is a process or processes whereby animals actively assign "salience and attractiveness to visual, auditory, tactile, or olfactory stimuli that are themselves intrinsically neutral" (Berridge and Valenstein, 1991). Berridge and Valenstein (1991) hypothesise that some manipulations of brain function can bypass the need for an association to be made between a neutral stimulus and the hedonic effects of a reward for cues to become salient and independently exert control over reward seeking behaviour.

In attempting to explain the apparent paradox that ESLH both rewards instrumental behaviour (self administration) and initiates behaviour (food seeking in satiated rats) but does not enhance hedonic responses, Berridge and Valenstein (1991) suggested that it only increases the incentive salience of stimuli. Since both Kelley and Stratford have commented on the similarity between behaviours following ESLH and intra-Acb GABA receptor stimulation (e.g. see Stratford, 2007, Stratford and Kelley, 1997b,

Kelley, 1999a), it would make sense that manipulating the circuit at the Acb level could also have an effect on the salience of cues, perhaps via the LH.

In the experiments reported here there was an increase observed in the number of reinforced presses prior to any interaction with the primary reinforcer. This could have been due to a direct result of increased cue saliency and hence increased 'wanting' without any effects on reward value. As is evident from the preceding discussion, other explanations cannot be completely discounted for this result reported on its own but it is suggested that this, combined with a decrease in reaction time to the cues across the session and an increase in approach responses to the cue, all point to increased cue salience. The relevance of decreases in various indices of reaction time and of increases in approach behaviours will be discussed first and then evidence that an increase in operant responding can indicate an increase in cue salience will be considered.

Changes in reaction time to the CS

Behavioural activation in operant schedules has already been discussed in terms of impulsivity and attentional processes however another index of potentiated operant responding is reaction time (RT) (Cole and Robbins, 1989). In experiment 4.1 and 4.3, baclofen at the most effective dose caused an increase in the rate of pressing across the session. In experiment 4.3 there was also a pronounced shift to the left in the latency to lever press once the CS was terminated. Calculation of the latency to resume pressing following pellet delivery in this experiment revealed a significantly shorter mean interval duration with the most effective dose of baclofen. All of these effects could indicate a decrease in RT to cues with baclofen.

In a RT task that requires conditioned lever release the salience of available cues is associated with the value of the reward previously associated with those cues (Amalric and Koob, 1987). The mechanisms that control the speed of the animal's behavioural output include information processing systems to evaluate stimulus saliency and response selection processes. The total time to react can be described as a choice reaction time (CRT) (Blokland, 1998b). When an animal performs cue-potentiated operant responses in the absence of primary rewards the CRT represents the speed of motor initiation and depends in part on the strength of the cue to activate behavioural output. When there is no breakdown in response inhibitory control (no premature

responses) or response accuracy (e.g. responding on the wrong lever) the CRT can be taken as the predominant process determining response rates.

Rats have been shown to significantly decrease their CRT to cues predictive of larger rewards (Brown and Bowman, 1995, Hauber et al., 2000) and Hauber (2000) suggests that it is the expectancy of reward value that guides the speed of instrumental responses. In the 2nd order operant schedule the time taken to begin pressing again as the CS is terminated could be partially determined by the salience of the CS. Unlike the lever release based RT test designed by Amalric and Koob (1987) however, there could have been some possible confounding effects on the speed of the RT if the animals were not engaged in performing the task (e.g. grooming or exploring) at the specific time that termination of the cue indicated a response should be made. Also, during the consummatory phase the RT to respond to the cue will sometimes include the time taken to eat the pellets. Nevertheless since there were decreases in indices of CRT it may still be a useful indicator of the strength of cue saliency.

The effect of manipulating activity of the striatum on RT has predominantly focused on the role of DA in this structure. Non selective DA receptor stimulation decreases RTs but decreases the rate of correct responding and increases impulsivity whilst the opposite is true with DA antagonists (for a review see (Blokland, 1998b). AMPA/kainate antagonists infused into the Acb have been shown to decrease reaction times to cues predictive of reward without disrupting the previously learned instrumental association with reward magnitude or impairing performance accuracy (Giertler et al., 2003). NMDA antagonism increases RT, blocks the decrease associated with DA and increases impulsivity (Blokland, 1998b, Hauber et al., 2000). The blockade of striatal muscarinic receptors does not affect RT but disrupts control over the pattern of responding (Blokland, 1998a).

It would appear therefore that increased cue saliency decreases RT to perform operant responses, the Acb modulates this process and there is evidence that GABA could have an effect via glutamatergic transmission. Furthermore, glutamatergic influenced RT time is modulated via AMPA/kainate receptors but not NMDA receptors, which is also true for the increase in free intake due to glutamate receptor blockade (Maldonadoirizarry et al., 1995). Although this is a simple and compelling argument,

difficulties arise in trying to decipher the role of the Acb in stimulus evaluation and response selection because these processes could be dependant on the way in which the animals have acquired the task in the first place (Pavlovian or instrumental associations). This issue will be discussed in more detail in Chapter 7 (General discussion)

Changes in approach behaviours

It is also clear from the analysis of behaviours from the video that the CS elicited approach responses because the animals reared to the light and/or the area just above it (from which the sound of the food delivery mechanism emanated). These rears did not appear to be coincidental or random as they occurred in close contiguity with lever pressing and magazine head entries. The target area of this category of rears was also very specific. Other rears were usually made to the corners of the ceiling or the animals simply stood up in the open part of the box. CS rearing also increased in proportion to the total number of reinforced lever presses, the majority of which were associated with presentations of the CS not the primary reinforcer.

The rears to the CS suggest that, while the animals were explicitly trained on a 2nd order schedule, they had concomitantly developed an approach response. In other words a subset of their behavioural repertoire in the box had been subject to autoshaping (Brown and Jenkins, 1968). It is generally accepted that autoshaping is under the control of Pavlovian not instrumental contingencies and this has been confirmed in the rat (Bussey et al., 1997). It is currently believed that processes that subserve the ability of cues to control behavioural output in different schedules e.g. CRF, PIT or Pavlovian approach are mutually exclusive (Holland and Petrovich, 2005, Galarce et al., 2007).

Although aspects of Pavlovian approach directed at the lever could not be dissociated from instrumental responding the incidental autoshaping of approach behaviours to the CS allows a measure of any potential disruption of this aspect of behaviour. It might appear on the face of it that baclofen caused a significant increase in approach behaviour to the CS. However, on closer inspection, the number of rears to the CS increased in proportion to the increase in lever presses and to CS presentations that occurred as a result. In other words Pavlovian approaches to the CS were not directly potentiated but the CS was approached more because it appeared more.

One school of thought suggests that behaviours directed towards a CS associated with positive reinforcement potentiate operant responding by increasing the likelihood that the animal will find itself in the proximity of the response location (Krank et al., 2008). If this were the case then an increase in cue salience might be expected to elicit more approach behaviour. However, conditioned reinforcement with food reward has recently been shown to be independent of appetitive Pavlovian approach responses to the lever because potentiation of instrumental behaviour could be induced even when the location of the CS was incongruent (Di Ciano and Everitt, 2004). Thus an alternative school of thought suggests that cue-induced increases in instrumental responding are independent of approach responses (Holland and Petrovich, 2005). Moreover a disproportionate increase in approach behaviours to the cue has been demonstrated to disrupt operant responding (Krank, 2003). Thus a lack of a proportional increase in approach responses to the CS is not incompatible with an increase in the salience of the cue driving instrumental behaviours.

Other evidence of increased cue-reinforced operant responding from the literature

A variety of studies have been published that implicate the Acb in cue potentiated behaviours. For example it was shown that intra-Acb infusions of amphetamine enhanced the control of CRfs over responding for water and that, conversely, dopaminergic lesions of the Acb blocked this potentiating effect (Taylor and Robbins, 1984, Taylor and Robbins, 1986, Robbins et al., 1989). Intra-AcbSh amphetamine infusions have been clearly shown to significantly increase cue-potentiated pressing without any concomitant increase in 'liking' of the primary reinforcer (Wyvell and Berridge, 2000). In another series of experiments Everitt and colleagues showed that the ventral striatum was involved predominantly in appetitive sexual behaviours but not in consummatory processes and that, at least to some degree, this was dependent on the association with cues that predict sexual reinforcement (Everitt et al., 1989).

Other early studies of the role of the Acb in cue potentiated responding for food suggested that the structure as a whole was involved in operant behaviours potentiated by CRfs (Robbins et al., 1990). An interesting study using excitotoxic lesions showed that food carrying-to-leave behaviour (hoarding), a species-specific spontaneous expression of motivated behaviour related to cues other than the primary reinforcing value of the food, was blocked by loss of Acb neurons (Whishaw and Kornelsen, 1993).

Other behavioural evidence that the Acb is necessary for cue salience attribution?

Cue-induced reinstatement tests are similar to tests of PIT and, like transfer, are known to depend on the ventral striatum (McFarland and Kalivas, 2001, Kalivas and McFarland, 2003, Floresco et al., 2008). The effects of baclofen on reinforced responding discussed in this chapter are supported by a recent report that 'inactivation' of the AcbSh using a cocktail of baclofen and muscimol resulted in significant potentiation of responding during cue induced reinstatement of food seeking behaviour while AcbC inactivation decreased responding (Floresco et al., 2008). These authors concluded that "the shell is involved in updating stimulus-reinforcer contingencies upon changes in the motivational relevance of conditioned stimuli", a hypothesis consistent with our results.

The finding that there can be potentiation of cue-induced reinstatement following infusions of GABA agonists into the Acb may appear counter-intuitive given that a current area of great interest in addiction is the apparent ability of GABA agonists such as baclofen to block reinstatement of cocaine, heroin, alcohol and nicotine seeking (e.g. see Filip and Frankowska, 2007, Spano et al., 2007, Maccioni et al., 2008, Fattore et al., 2009). However baclofen/muscimol inactivation of the AcbC blocks reinstatement whereas the shell does not have any effect on cue-induced reinstatement of drug-seeking or ethanol-seeking behaviour (Fuchs et al., 2004, McFarland and Kalivas, 2001) (Chaudhri et al., 2008).

Filip and Frankoska (2007) also reported that blockade of cue-induced drug seeking behaviour by baclofen was more effective than for cue-induced food seeking. Floresco et al., (2008) suggest that one explanation for a AcbSh mediated potentiation of reinstatement for food but not drug related cues could be that differences in the circuits that mediate instrumental responding induced by drug related and food related cues at the level of the amygdala (McLaughlin and Floresco, 2007). Interestingly it has recently been suggested that context induced reinstatement may be more dependent on the AcbSh (Crombag et al., 2008, Fuchs et al., 2008) which could also have implications for the role of baclofen in increasing free feeding.

Electrophysiological evidence of a role for Acb in saliency attribution to stimuli?

The suggestion that intra-AcbSh baclofen could affect cue saliency is also supported by electrophysiological studies of Acb neuronal activity in behaving rats that demonstrate

phasic firing consistent with signals related to expectation and delivery of reward and to stimuli associated with reward. During instrumental responding for natural rewards (water, nutritive solution or food) sub-populations of Acb neurons fire in a specific phasic sequence (pre-response excitation, short post-response inhibition, prolonged post-response excitation) time locked to the temporal organisation of approach to the lever, response initiation, execution, completion and reward acquisition (Carelli and Deadwyler, 1994). This pattern does not simply represent the encoding of motor control (Schultz et al., 1992, Lavoie and Mizumori, 1994, Carelli and Deadwyler, 1997, Carelli and Deadwyler, 1994, Carelli et al., 2000, Hollander et al., 2002, Roop et al., 2002).

Phasic sequences of temporally organised neuronal firing in the Acb is also reported to be determined by the behavioural state of the animals and the context in which testing takes place, is tightly stimulus bound and is conditioned in responsive populations of neurons (Carelli and Deadwyler, 1997, Carelli et al., 2000, Carelli, 2002, Carelli and Wightman, 2004). Patterned neuronal activity is attenuated during extinction of instrumental responses and rapid recovery during reinstatement is only seen in the post-reinforcement active cell populations that probably respond to the A-O contingency (Hollander et al., 2002). Conditioned stimuli paired with natural reward (sucrose) evoke rapid DA release, transient increases in DA and the onset of the phasic firing sequence is coincident, DA fluctuations occur over the same time interval and with the same temporal synchrony as the previously described pattern in neuronal activity and neurons that do not exhibit fluctuations in DA do not show patterned phasic firing (Roitman et al., 2004, Carelli, 2004, Cheer et al., 2007).

The sequence of the phasic firing pattern is not specifically related to the type of reinforcer (e.g. water or cocaine) but separate populations of neurons may be recruited by drug vs. natural rewards (Carelli and Deadwyler, 1994, Carelli and Deadwyler, 1997, Carelli et al., 2000). Separate populations of neurons also appear to specifically encode aversive vs. appetitive stimuli (Roitman et al., 2005) and fire according to the expected outcome that the stimuli represents (Wilson and Bowman, 2005). However these separate populations are evenly distribute throughout the core and shell of the Acb (Carelli and Wondolowski, 2006, Wightman et al., 2007).

This activity is greater when cues are better predictors of reward than when they are less predictive and when the animals makes a response than when none is made (Nicola et al., 2004). It has also been shown that neural activity in the ventral striatum reflects the proximity of upcoming rewards in ratio schedules of reinforcement (Bowman et al., 1996). Interestingly it is also possible to dissociate between the pattern of activity induced by the cue and the primary reinforcer in a 2nd order operant schedule although the authors suggest partially overlapping mechanisms in the Acb process the information (Wilson and Bowman, 2004b).

The evidence from the experiments reported in this chapter of an increase in operant responding prior to primary reward delivery, decreased reaction times to the CS combined with the multiple lines of evidence for a role for the Acb in cue-reinforced responding, cue induced reinstatement and from electrophysiological data is believed to offer strong support for a saliency attribution hypothesis to explain the effects of baclofen. This will be further discussed below.

GABA mediated effects on saliency attribution at the neurotransmitter level

On the basis of the discussion thus far it is postulated that the behavioural effects of baclofen reported in this chapter cannot automatically be attributed to inactivation of MSN outputs although the original premise of the numerous elegant experiments carried out by Kelley's laboratory using GABA agonists was to do precisely that (Kelley, 1999b). Kelley found that blockade of AMPA/kainate (but not NMDA) receptors or stimulation of GABA receptors elicited robust free feeding (Maldonadoirizarry et al., 1995, Kelley and Swanson, 1997, Stratford et al., 1998, Stratford, 1997).

However it has been shown that transient inactivation of the Acb using lidocaine (Giertler et al., 2004) or permanent inactivation using excitotoxins (Brown and Bowman, 1995) has no effect on cue potentiation of operant responding whereas blockade of AMPA/kainate receptors significantly decreases RTs to discriminative cues. Conversely, transient inactivation of the Acb using TTX or a cocktail of high dose glutamate antagonists to totally block the activity of MSNs has been demonstrated to cause an increase in the latency to respond to discriminative cues predicting sucrose reward whilst increasing responses to a non-rewarded stimulus, un-cued lever presses

and presses on an inactive lever (Yun et al., 2004a). It has been hypothesised that, when output of the Acb is completely inhibited, it can be bypassed and alternative circuits subsequently recruited in the control of behavioural output (Giertler et al., 2004).

It has been suggested elsewhere that the large array of changes in behaviour following inactivation of the Acb via lesioning that have been published could be caused by the disruption of inputs originating in afferent structures rather than reflecting specific functions of the Acb *per se* (Goto and Grace, 2008). As Jongen-relo et al., (1991) point out, the specific method used to inactivate the Acb may also dictate the resultant behavioural effects given that, for example, electrolytic lesions destroy both cell bodies and fibre tracts that pass through the structure between other brain regions.

It has also been reported that the shell and core subregions may be differentially sensitive to the type of excitotoxins used to destroy cell bodies (Parkinson et al., 1999). Furthermore it has been demonstrated that the standard staining procedure used to visualise the extent of the lesions (staining for Nissl substance) probably results in an underestimation of lesion size and regional overlap (Jongen-Relo et al., 2002). This could mean that behaviours attributed to the Acb could actually be modulated by other structures that are also lesioned at the same time. This, along with some of the conflicting evidence for the role of the Acb in a variety of behaviours discussed previously are taken as evidence that an 'inhibition' explanation is inadequate.

It does not follow either that the unique effects reported here can be explained in terms of inhibition of Acb output because they occur at significantly lower doses of baclofen than have been previously used by other laboratories and are abolished at higher doses. Berridge and Wishaw (1992) point out that changes in natural sequential patterns of behaviour do not depend on learning and memory in the way that schedule controlled instrumental responses do. As such they are a good indicator of manipulations of neural function that effect behavioural sequencing of motor output. Given that Kelley's theory for the role of GABA in the Acb suggests an action at the level of output to the LH and consequent motor patter generation, this is particularly relevant.

It would appear that higher doses of baclofen disrupt the initiation and/or completion of a normal grooming syntactic chain. This dose is molecularly equivalent to a dose in the middle of the range of muscimol doses previously tested in operant schedules (see

introduction to this Chapter). An effect at this high dose of baclofen on grooming is consistent with the effects of excitotoxic lesions of the striatum, which prevents full expression of the normal sequence (Berridge and Fentress, 1987). It would seem therefore that, at the higher dose, baclofen caused behavioural deficits consistent with inactivation of striatal output but the lack of such effects at lower doses indicates that some other mechanism subserves the effects on operant responding.

Thus it is suggested that there is no strong basis for concluding that the behavioural effects of baclofen can be fully explained by a postulated blockade of glutamatergic transmission within the Acb (Kelley et al., 2005b). It is hypothesised that the effects of low doses of baclofen on cue-induced instrumental responding could be due to interactions with other neurotransmitter systems within the AcbSh subserving motivational processes in the Acb. At low doses the pattern of interaction with local circuits results in increased cue salience that can drive instrumental responding but at higher doses inactivation of the AcbSh output might still restrict the consequent repertoire of motor patterns that can be expressed. There would be a crossover point at which this restriction does not reduce operant responding but it does increase free intake by narrowing behavioural selection to those involved in ingestion. Beyond this point instrumental responding is disrupted either by a breakdown in syntactic behavioural chains that control lever pressing or by myorelaxant effects (or both) but, where less complex behaviours are required, free feeding is still dose dependently increased via restricted release of specific feeding related motor patterns.

GABA mediated effects on saliency attribution at the structural level

Holland and Petrovich (2005) argue that cue-potentiated feeding in food-sated rats is mediated by a macrocircuit that includes the BLA and LH but probably not the CeA and Acb but they do not completely discount a role for the connection between the Acb and LH. Recent studies suggest that the LH is necessary for context induced reinstatement of reward seeking behaviour (Marchant et al., 2009, Hamlin et al., 2009). Habitual responding for an incentive represented by the CS could depend on serial processing between the BLA, the AcbC and the ascending intrastriatal circuitry that links this system to the dorsal striatum (Belin et al., 2009). However these circuits are involved specifically in Pavlovian associations and it has been argued that the processes that subserve Pavlovian and instrumental responding are dissociable (e.g. for a review see

(Balleine, 2005). However transfer of cue salience through Pavlovian processes could involve either a CeA/AcbC circuit or a BLA/AcbSh circuit depending on how the schedule is manipulating their behavioural output. This is only a very brief suggestion of potential circuits involved because evidence presented in the following chapters (Chapter 5 and Chapter 6) will contribute to understanding the circuits which may be influenced by the action of baclofen in the AcbSh.

Summary

It has been demonstrated in this chapter that, contrary to predictions based on Kelley's hypothesis, intra-AcbSh baclofen significantly increased instrumental responding for a food paired cue in a dose related manner without disrupting response accuracy or causing non-specific increases in Pavlovian approach behaviours. This increase occurred in the absence of the primary reinforcer and thus represented appetitive responding. This increase could be explained by a loss of behavioural inhibition over prepotent operant responding but this is more likely to be an AcbC mediated mechanism. Alternatively the effect could be due to an attenuation of AcbSh mediated latent inhibition of exteroceptive cues. It is unlikely that the animals experienced an increase in the value of the primary reward under the influence of baclofen and this would not explain increased responding during the appetitive phase but it is highly likely that the saliency of reward related cues (discrete CS or contextual) was potentiated. In contrast the highest dose of baclofen tested disrupted schedule control over operant responding, induced significant oral stereotypies and caused pronounced disruption of grooming motor patterns.

It is postulated that low doses of baclofen control the saliency of reward related cues via interactions with other neurotransmitter systems at the local level, not via total inactivation of MSN output. At higher doses increases in free feeding without concomitant effects on instrumental responding could still be explained by total inactivation of the AcbSh and hence release of downstream feeding related motor patterns. These issues will be further discussed in light of the findings to be reported in Chapters 5 and 6.

Questions raised

Much of the theoretical discussion here is probably as much associated with the way in which the Acb is involved in learning about the availability of food and the cues that predict reward as it is in the expression of appetitive and consummatory behaviours post acquisition. However this is because the acquisition process dictates which cortico-striatal circuits are consequently activated when the animal responds to cues in the future. However the AcbSh and GABA transmission within this region clearly has a significant role in the behavioural output described in this chapter.

The key questions raised by these results are 1) will low doses of muscimol infused into the AcbSh increase operant responding for a food paired cue? 2) What neuronal circuitry is recruited during intra-AcbSh baclofen induced increases in operant responding? 3) If the effect is not due to inhibition of MSN output what other mechanism could subserve this effect? The first will be addressed in the next chapter, Chapter 5, where instrumental responding will be tested again on the 2nd order schedule using muscimol. In Chapter 6 the presence of the immediate early gene *c-fos* will be used to map regional activation following intra-AcbSh infusions of baclofen or muscimol. The final question will be addressed in light of the results from Chapter's 5 and 6 and what is known about the functional anatomy of the AcbSh in the General Discussion, Chapter 7.

Chapter 5

$GABA_A$ receptor stimulation in the accumbens: effects on instrumental responding by pre-fed rats in a second order operant schedule

Introduction

The studies presented in Chapters 4 and 5 have demonstrated that intra-AcbSh infusions of baclofen and muscimol have distinguishable effects on the BSS in response to freely available chow. The effects of baclofen on the BSS resembled the effects of food deprivation. Muscimol increased feeding at the expense of the rest of the behavioural repertoire in the BSS and it did not resemble the BSS following fasting. Neither treatment produced a BSS that looked like that associated with manipulations thought to affect reward value (intra-Acb opioid agonist or systemic benzodiazepine).

Studies presented in Chapter 4 demonstrated that intra-AcbSh baclofen increases both appetitive and consummatory responses without affecting response accuracy on a 2nd order operant schedule in animals that have been pre-satiated. This effect was dose dependent and the pattern of responding suggested a classic inverted U-shaped dose response function over the range tested (0, 110, 220, 440, 660ρmols). Furthermore, at the most effective dose tested (220ρmols) baclofen modified several other indices of behaviour including increasing headpokes into the magazine as reward became imminent, decreasing the latency to return to lever pressing following reward delivery and increasing rearing behaviour including rears directed specifically at the CS. At the highest dose the oral stereotypical behaviour and abnormal grooming behaviour emerged.

These results indicate that, contrary to current theory to explain the effects of GABA in the Acb on feeding (Kelley et al., 2005m), the effects of intra-AcbSh baclofen and muscimol on motivated behaviour may not be equivalent but the possibility remains that muscimol could also increase operant responding on the 2nd order schedule. This possibility remains because 1) not all the evidence for a lack of motivational effects of muscimol has come from an equimolar dose range as low as that shown to be effective with baclofen and 2) because the schedules used may have negated the possibility of

any independent measure of motivational effects because of satiety mechanisms associated with immediate access to reward (Zhang et al., 2003).

The experiments from which this evidence comes are described in detail in the introduction to this thesis but, to recap some key findings, in a PR schedule intra-AcbSh muscimol at doses of 350, 880 and 1750ρmols failed to increase the rate of lever pressing, accuracy of pressing on the reinforced lever or the break point (Zhang et al., 2003). The authors speculated that, because DAMGO and amphetamine did increase the breakpoint, neither increases in palatability or increases in incentive salience could increase the willingness of animals to work on this schedule. They concluded therefore that muscimol must have had no effect on motivation driven by wanting (incentive salience) or liking (palatability). However, as discussed in Chapter 4, a change in break-point does not distinguish effects on motor effort directed at incentive stimuli (i.e., pressing the lever) from effects on the perceived rewarding value of the reinforcer (Hanlon et al., 2004).

Muscimol at a dose of 1750ρmols also failed to potentiate the acquisition of a lever pressing response for food reinforcement (Hanlon et al., 2004). In this case it was suggested that the potentiation of acquisition of lever pressing for food required both increases in hedonic affect and in incentive salience at the same time. Again, given that muscimol appeared to have no effect on either of these proposed mechanisms, whether in combination or not, and did not enhance instrumental learning it was concluded that there was a dissociation between the effects of GABA on ingestion and on other behaviours "typically associated with an appetitive CMS" (Kelley et al., 2005b).

Given that it was hypothesised in the previous chapter that baclofen may exert effects on the incentive attributed to the stimuli it is also important to test the possibility that the same is true for muscimol on an appropriate schedule at equimolar doses. Also, as was pointed out in Chapter 4, at 100ng (880ρmols) muscimol or above, there are potentially confounding motor effects that could disrupt instrumental responding (Scheel-Kruger et al., 1977b, Scheel-Kruger et al., 1977a). Muscimol across a low dose range will therefore be tested on the same 2nd order schedule as was used in Chapter 4. However it is predicted that, even at lower doses, muscimol will not increase operant responding.

Stratford (2007) points out that $GABA_A$ agonists will block activity in the vast majority of synapses within the AcbSh because the $GABA_A$ receptor is so widely distributed postsynaptically. This means that AcbSh output is blocked at a level one step removed from integration at the level of local interneurons and thus $GABA_A$ agonists are less likely to modulate the function of other receptor types as the $GABA_B$ agonist could. The data from Chapter 3 indicates that intra-AcbSh infusions of muscimol does not allow the full expression of the BSS and the results were consistent with the idea that muscimol inactivates this region rather than modulates local control over motivational processes. Because of this baclofen will be used in experiment 5.1 to confirm that the infusions sites are behaviourally active in terms of increased instrumental responding.

The experiments reported in this chapter will examine the effect of 1) one dose of baclofen; 220 ρmols/μl^{-1} versus a low dose range of muscimol; 110, 220, 440ρmols/μl^{-1} and 2) a higher dose range muscimol; 220 and 660ρmols/μl^{-1}. Effects will be analysed in terms of the magnitude and accuracy of responding on two operant levers with respect to the programmed schedule. The efficacy of the dose ranges chosen on intake of freely available food will be confirmed by measuring total intake of chow in the same experimental groups using the same doses following the operant test phase.

These experiments will contribute to our understanding of the specific effects of intra-Acb $GABA_A$ receptor stimulation revealed in Chapter 3 and will also address three of the key aims of the thesis. In experiment 5.1 the effects of the optimum dose of baclofen tested in Chapter 4 will be compared with an equimolar range of doses of muscimol to those of baclofen used in experiment 4.1 will be recorded for 5 minutes of purely appetitive responding for a CS and for 25 minutes of mixed appetitive/consummatory behaviour on the second order schedule. The effect on total intake of freely available chow of infusions of the highest dose of muscimol from the range will also be assessed in the same cohort of animals.

In experiment 5.1 the effects of muscimol in the second order schedule will be further explored across a broader dose range equimolar to those doses of baclofen tested in experiment 4.3. This will include the analysis of the effects of muscimol on behaviours other than lever pressing using videos of the test sessions. In this experiment a full dose range, free-feeding intake study will be carried out following completion of the second

order schedule testing phase using the same counterbalanced design to allow a direct comparison between any effects on operant responding and on intake.

As was pointed out in Chapter 4 the effects of hunger and satiety have already been reported for the schedule (Greenhalgh, 2007, Thornton-Jones et al., 2005) and these experiments will add further to the picture that is building up of the role of GABA in the context of naturally elicited feeding and motivational control in general. Results reported in this chapter will further contribute to the characterisation and comparison of the effects of GABA receptor subtype stimulation on feeding related behaviours in the context of the current hypothesised mechanism subserving these effects.

To summarise, previous results reported in this thesis indicate that intra-AcbSh $GABA_B$ receptor agonist infusion increases intake of freely available food without disrupting the BSS and increases both appetitive and consummatory operant responding for food on a 2^{nd} order schedule. In contrast results reported in this thesis indicate that intra-AcbSh $GABA_A$ muscimol induced free feeding disrupts the BSS and it has been reported elsewhere that muscimol has no obvious effect on motivational processes subserving operant responding. However, the effects of muscimol on operant responding were tested at higher equimolar doses than the baclofen dose shown to be most effective in increasing operant responding in Chapter 4 and the range encompasses concentrations that could cause motor disruption. Additionally the effects of muscimol have not been previously tested on a schedule that allows distinctions to be made between effects on stimulus saliency and reward value in the absence of potentially confounding post-ingestional processes.

The two experiments reported in this chapter will:

1) Test the effects of a range of doses of a $GABA_A$ agonist compared to the effects of an optimum dose of a $GABA_B$ agonist on instrumental behaviour in the second order schedule (Experiment 5.1 and 5.2). Does the $GABA_A$ agonist muscimol affect operant responding when infused into the same region of the Acb where baclofen increases it?

2) Characterise the magnitude and temporal pattern of responses made following intra-Acb $GABA_A$ infusion during both appetitive and consummatory operant phases (Experiment 5.1 and 5.2). Does the $GABA_A$ agonist muscimol cause any divergence in responding depending on the phase?

3) Test the effects of a range of doses of a $GABA_A$ agonist on behaviours other than lever pressing expressed during an operant schedule (Experiment 5.2). Does the $GABA_A$ agonist muscimol have any effects on general activity, other indices of motivated behaviour or on adjunctive behaviours not picked up by measuring lever pressing alone?

The overarching question posed in this chapter is:

Does intra-Acb administration of the $GABA_A$ agonist muscimol potentiate any aspect of complex goal-directed motivated behaviour on a second order schedule in a manner consistent with selective effects on appetitive and/or consummatory behaviours?

Experiments presented in this chapter

Experiment 5.1
The effects of bilateral intra-Acb infusions of 220ρmols baclofen compared to a range of doses of the $GABA_A$ agonist muscimol on responding on a 2^{nd} order operant schedule.

Experiment 5.2
The effects of bilateral intra-Acb infusions of a higher range of doses of the $GABA_A$ agonist muscimol on responding on a 2^{nd} order operant schedule, on associated behaviours and on free intake.

Materials and methods

Animals

One drug naïve group of animals (n=12) was used for Experiment 5.1 (2^{nd} order with baclofen and muscimol) and a second drug naïve group of animals (n=12) was used in Experiment 5.2 (2^{nd} order with high dose range baclofen). As in Chapter 4 subjects were bought in weighing 150-200g. In all cases the animals were housed in pairs during

training on the 2nd order schedule. They were then housed singly in Perspex cages for a minimum of 7 days prior to surgery and for the remainder of each experiment.

Apparatus and habituation

All apparatus and habituation procedures were identical to those used in Chapter 4 and the reader is referred to the relevant details on page 149.

Specific procedural details – central drug administration

On test days only two animals were infused at a time to compensate for the time taken for each infusion. The programme controlling the operant boxes allowed each box to be started individually. This meant that testing of each animal could be temporally staggered and there were 4 operant boxes. Infusions for the next batch of 4 animals to be tested began a few minutes before the previous batch was due to come out and the whole cohort could be run over a period of approximately 4 hours (between 12.00 and 16.00). There was a minimum of 48 hours between infusions.

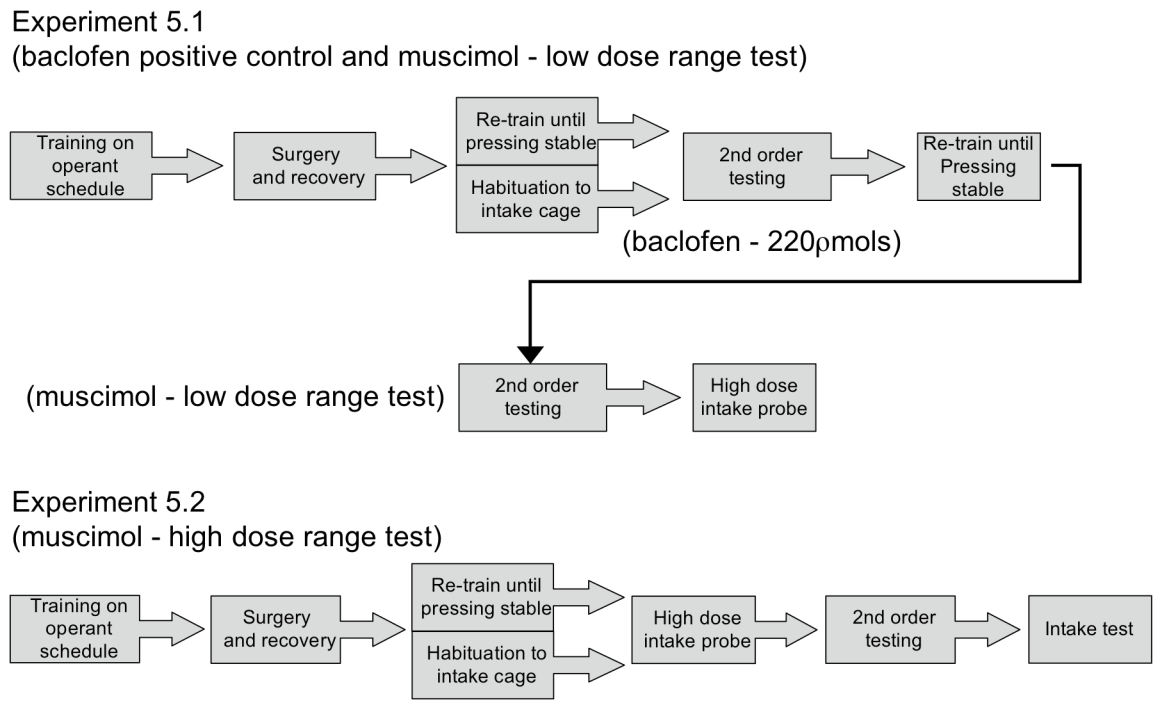


Figure. 5.1. Diagram to show which groups of animals were used for more than one test procedure within experiments 5.1 and 5.2 and the timeline for each.

Experiment 5.1

Initially a counterbalanced test was carried out on the 2nd order operant schedule (method as detailed in Chapter 2, page 75) using baclofen at 220ρmols/μl^{-1} or vehicle (0.9% sterile saline – used throughout). These animals were given a few days drug free and re-exposed to the schedule in this state (see Fig 5.1). This was followed by the test run on the 2nd order schedule using a dose range of muscimol equimolar to doses of baclofen used in Chapter 4, experiment 4.1 i.e. 110, 220 and 440 ρmols/μl^{-1} infused immediately before transfer to the operant boxes. Following testing on the second order schedule the same animals were tested with an intake probe using the highest dose of 440ρmols vs. vehicle to ensure that the placements were behaviourally active in terms of increased feeding in pre-fed rats (for method for intake test see Chapter 2, page 69).

Experiment 5.2

The range of muscimol doses tested here was equimolar to those doses of baclofen used in Chapter 4, experiment 4.3 i.e. 220, 660 ρmols/μl^{-1} baclofen or vehicle. Initially a counterbalanced intake probe using the highest dose of 660ρmols vs. vehicle was run to ensure that the placements were behaviourally active in terms of increased feeding in pre-fed rats (for intake method see Chapter 2, page 69). This was followed by the test run on the 2nd order schedule (as detailed in Chapter 2, page 75). The 2nd order test was followed by a counterbalanced dose response test of intake of chow in pre-fed animals using the same range of doses. Finally duration and frequency data transcribed for distinct categories of behaviours exhibited during responding were extracted from videos of the 2nd order operant test sessions.

Data analysis

The final analysis groups were decided on the basis of histological verification of infusion sites as before. Data from the 2nd order operant tests were extracted and analysed exactly as described for the experiments carried out in Chapter 4 (page 151).

Data extraction from videos

In experiment 5.2 other behaviours associated with responding in the operant box on the 2nd order schedule were recorded from the videos of each test session. Unix Shell scripts were used to extract the total durations and frequency of each behaviour and to parse the data into 5 minute time bins for more detailed temporal analysis. The means for total duration and frequency of behaviours in each treatment group were compared using a repeated measures ANOVA with dose and behavioural category as factors. The timebin data were analysed using the same ANOVA design but including dose, behavioural category and time-bin as factors. Timebin data were plotted as line plots to illustrate the change in the durations and frequencies of each individual behaviour across the session.

Whilst recording occurrences of rearing behaviour from the videos a separate tally was kept for rears made specifically to the cue light and/or the sound of the pellet delivery hopper turning. The mean proportion of rears to the CS vs. the total number of rears made was then compared between treatment groups using a repeated measures ANOVA with dose as factor.

Because it was noted in Chapter 4 that baclofen had a dose related effect on grooming behaviour such that animals at the highest dose never exhibited a normal sequence characterised by a rule-driven (syntactic) chain (Berridge and Whishaw, 1992, Aldridge et al., 2004) the presence or absence of this was also recorded for these experiments. Any other changes in the pattern of behaviour were noted while coding the 8 categories.

Results

Experiment 5.1: **The effects of bilateral intra-Acb infusions of baclofen at 220 mols/μl^{-1} or muscimol at 110, 220 & 440ρmols/μl^{-1} in pre-fed rats on responding on a second order operant schedule for food.**

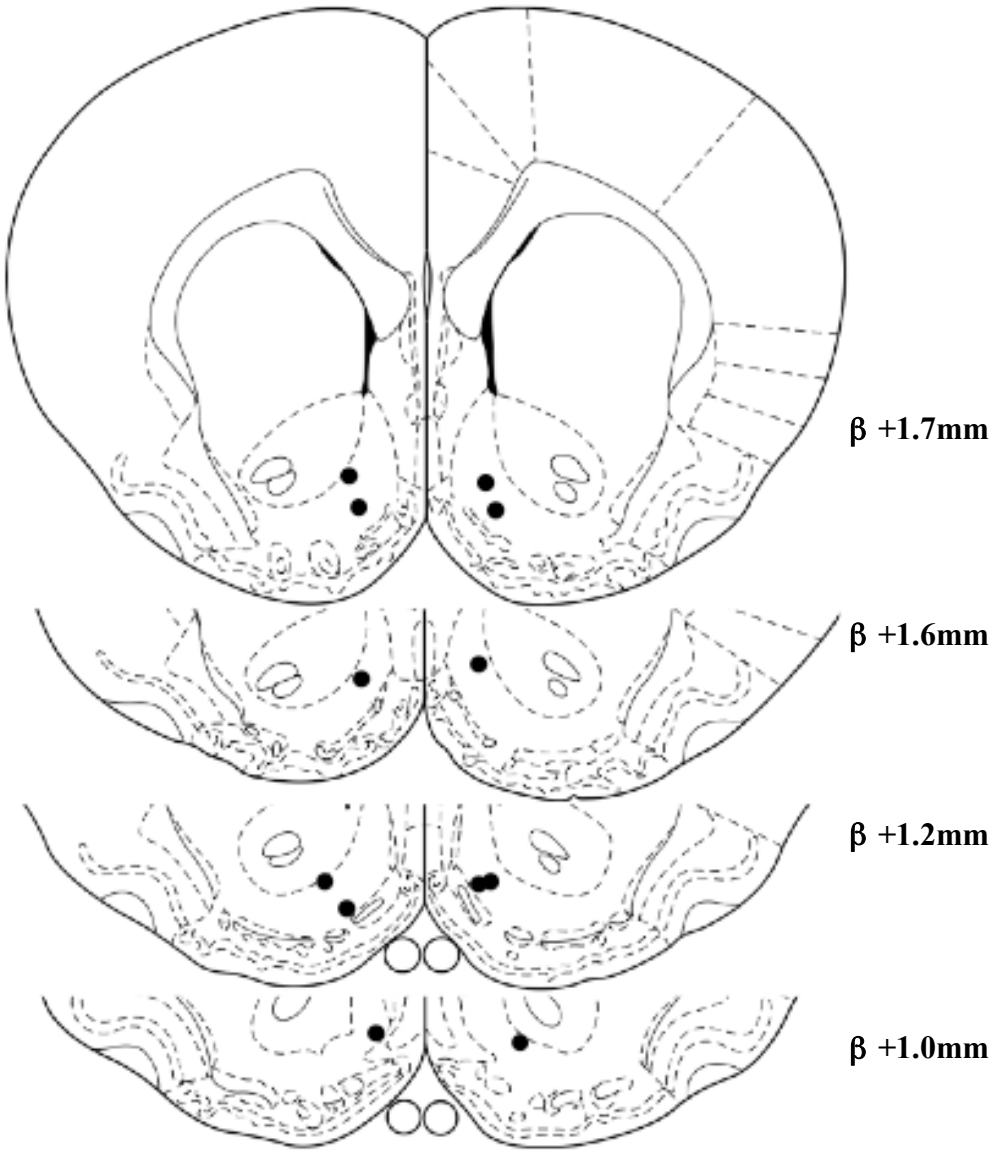


Figure 5.2. Injection sites plotted on drawings taken from Paxinos and Watson (1998); sections are anterior relative to bregma (β). Bilateral target coordinates (n=6) were (AP), + 1.4mm, mediolateral (ML), ± 0.9mm relative to bregma and dorsoventral (DV), -7.8mm relative to skull surface.

Prior to testing animals with muscimol the effectiveness of baclofen at increasing reinforced pressing was verified at a dose of 220ρmols. Bilateral infusions of baclofen only increased pressing in 7 out of 12 subjects. Histological examination revealed that these n=7 of the original n=12 animals had placements that fell within the acceptable boundaries defined in previous chapters but one was excluded because 1 or more infusions had fallen outside the target site at some point (See Fig 5.2). The remaining 5 animals that had placements outside the target site were those that were not responsive to baclofen in terms of total intake.

A total of n=6 animals were therefore verified as having acceptable placements and were included in the final analysis. A schematic illustration of Acb infusion site placements is given in Fig. 5.2. These animals pressed significantly more on the reinforced lever with 220ρmols baclofen than with vehicle [Fig. 5.3A: $F(1,5)=7.34$, $p=0.042$]. Muscimol had no significant effect on reinforced presses at any dose tested (Fig. 5.3B). Neither baclofen nor muscimol had a significant effect on total non-reinforced or incorrect lever presses.

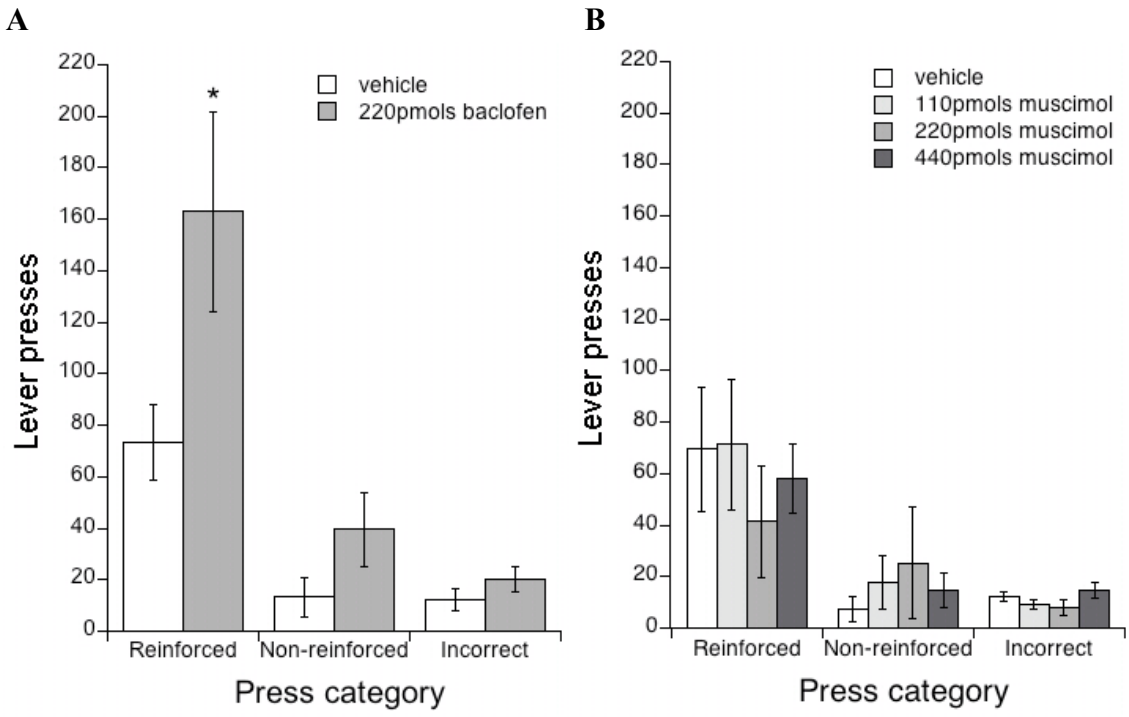


Figure 5.3. The effects of intra-Acb bilateral infusions of saline or A) 220ρmols of baclofen and B) 110, 220 or 440ρmols of muscimol in pre-fed rats (n=6) on total lever presses in a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ $p<0.05$.

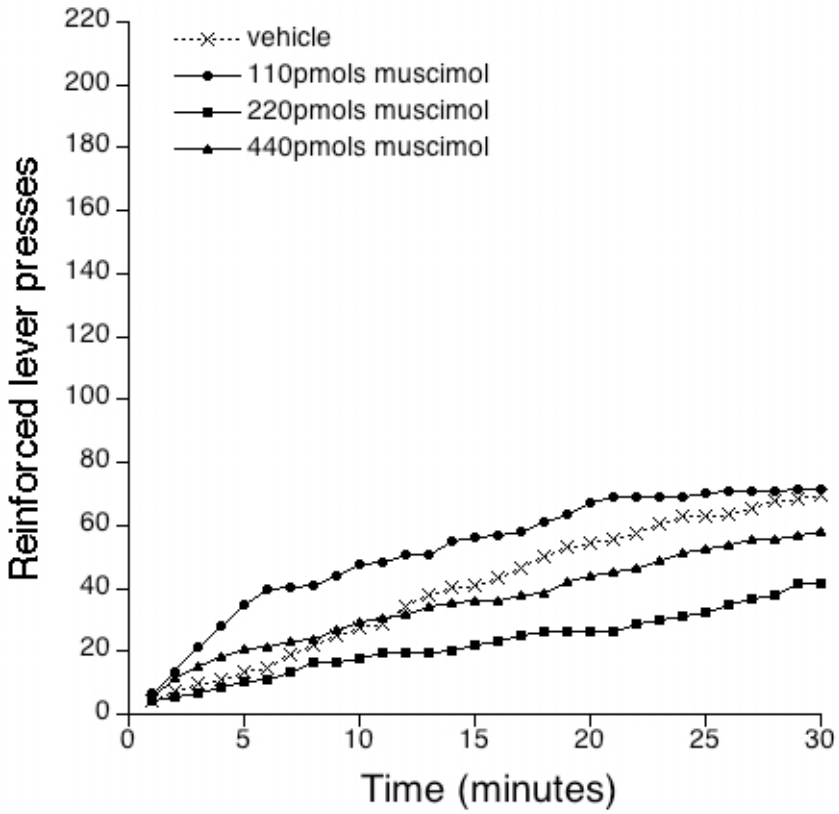


Figure 5.4. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=6) on cumulative reinforced lever presses over a 30 minute second order operant test session.

Fig. 5.4 illustrates the distribution of cumulative reinforced lever presses across the 30 minute 2nd order operant session with intra-Acb infusions of vehicle or muscimol. The rate of lever pressing, expressed as reinforced presses per minute, across the first 5 minutes and subsequent 25 minutes of the schedule was calculated and the early vs. late phase rates were compared within each dose (see Table 5.1). There was no main effect of drug dose but a main effect of time phase [F(1,5)=7.02, p=045] but no interaction between the two. A comparison of rates of pressing between treatments during the early phase or the subsequent 25 minutes indicated that there was no significant difference.

When the data were split up into 5 minute bins (see Fig. 5.5) there was no main effect of drug on total pressing in each bin but a main effect of time [F(2,25)=4.49, p=0.005] and a significant interaction between the effects of drug and time [F(15,75=1.94, p=0.032]. Paired comparisons did not reveal any significant effects of dose of muscimol when restricted to individual time bins. The interaction probably reflects increased responding in both the 110 and 440ρmols treatment groups in the first 5 minutes.

Table 5.1. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=6) on the rate of reinforced lever pressing during the first 5 minutes (appetitive phase) and last 25 minutes (consummatory phase) of a second order operant test session. There were no significant differences.

Treatment	Phase	Rate presses/min
Vehicle	Appetitive	2.63 ± 0.93
	Consummatory	2.25 ± 0.83
110pmols muscimol	Appetitive	6.97 ± 2.96
	Consummatory	1.46 ± 0.62
220pmols muscimol	Appetitive	2.07 ± 0.69
	Consummatory	1.24 ± 0.77
440pmols muscimol	Appetitive	4.17 ± 0.90
	Consummatory	1.49 ± 0.45

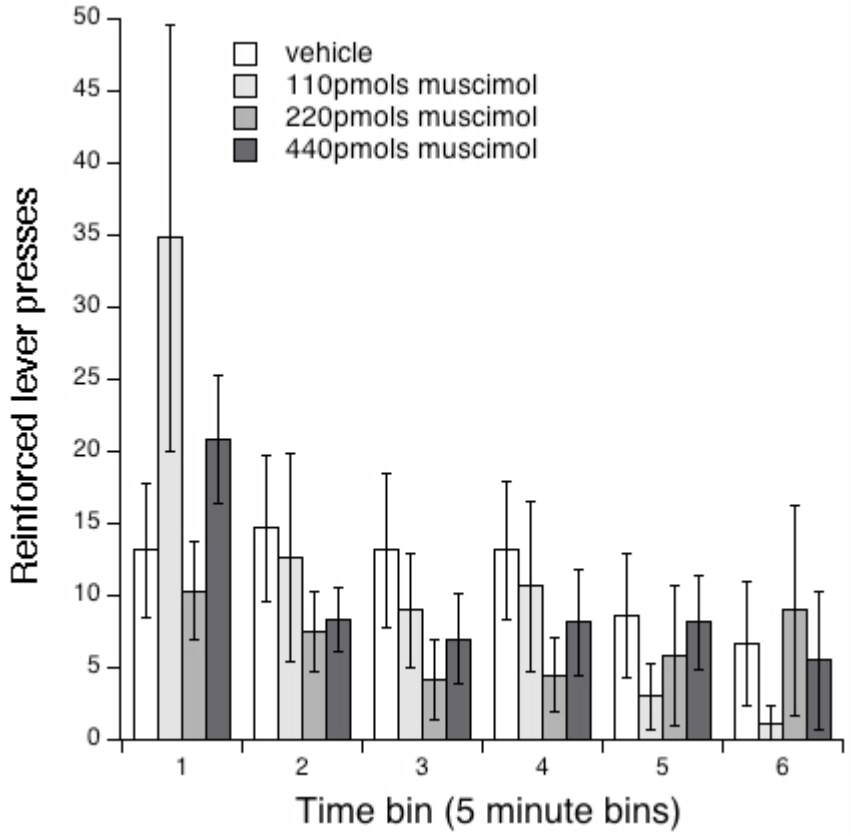


Figure 5.5. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=6) on reinforced lever presses across a 30 minute second order operant test session. Error bars represent ±SEM.

The cumulative mean proportions of the length of inter lever press intervals (ILIs) between the 5th press of an FR5 response and the 1st press of the next FR5 across the first 5 minutes of the 2nd order schedule (unrewarded) are depicted in Fig. 5.6. The distributions of ILIs for the remaining 25 minutes of the test session are shown in Fig. 5.7. In the first 5 minutes of the session, when no lever presses could be rewarded with the primary reinforcer, animals did not press at all during the first 5 seconds CS with vehicle, 110 and 220ρmols but at the highest dose 10% of presses were made within 1 second of the CS coming on.

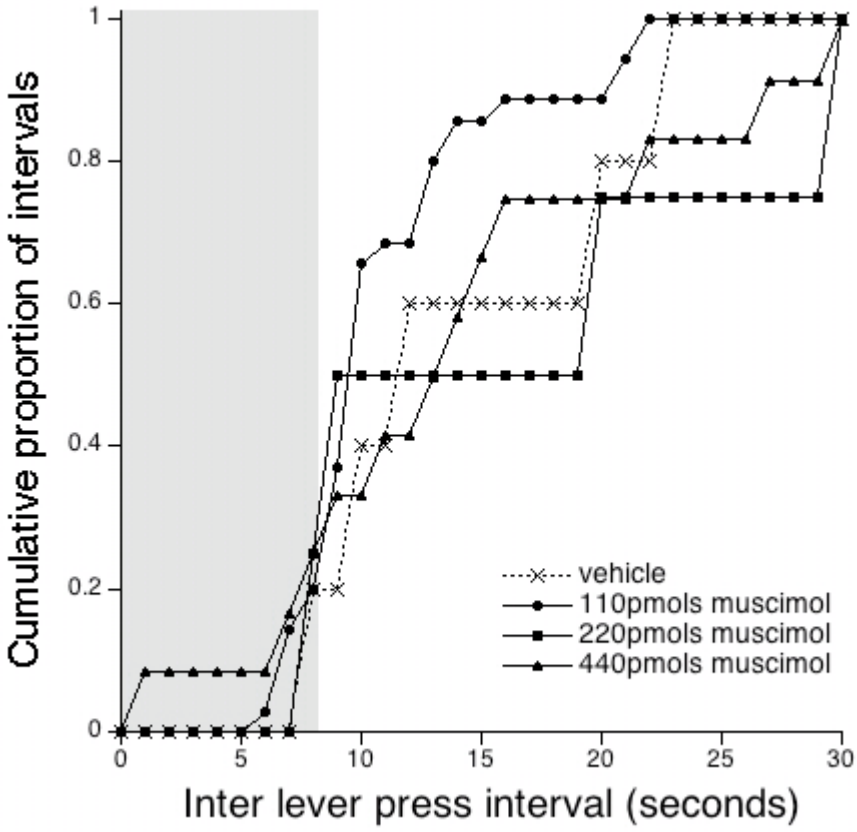


Figure 5.6. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=6) on cumulative ILIs between the 5th and 1st presses of the FR5 requirement on the reinforced lever. Data represents responding across the first 5 minute unrewarded phase of a second order operant test session. The shaded phase represents the duration of the CS (8 seconds).

For the remaining 25 minutes of the session some animals made the first press of the next ratio during the CS with all treatment conditions. With vehicle 18% of presses were made while the CS was still on. At the 110ρmols dose 39.4% of presses were made while the CS was still on, at 220ρmols this was 28.6% and at 440ρmols 15.9%. Within a second of the CS terminating 68% of presses had been made at 110ρmols compared to 32% with vehicle, 38.1% with 220ρmols and 26.4% with 440ρmols.

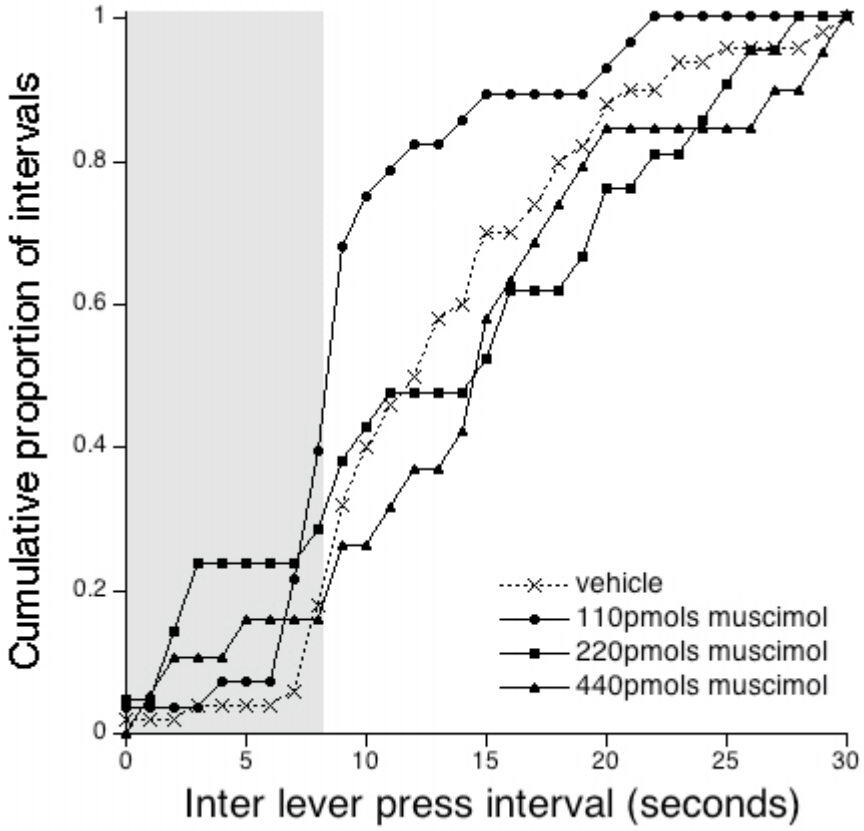


Figure 5.7. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=6) on cumulative inter lever press intervals between the 5th and 1st presses of the FR5 requirement on the reinforced lever. Data represents responding across the last 25 minute rewarded phase of a second order operant test session. The shaded phase represents the duration of the CS (8 seconds).

A few days after testing on the 2nd order schedule the same animals, pre-fed with standard laboratory chow prior to treatment with 440ρmols muscimol, exhibited a significant increase in consumption of chow over a subsequent 30 minute test period [Fig 5.8; F(1,5)=18.38, p=0.008].

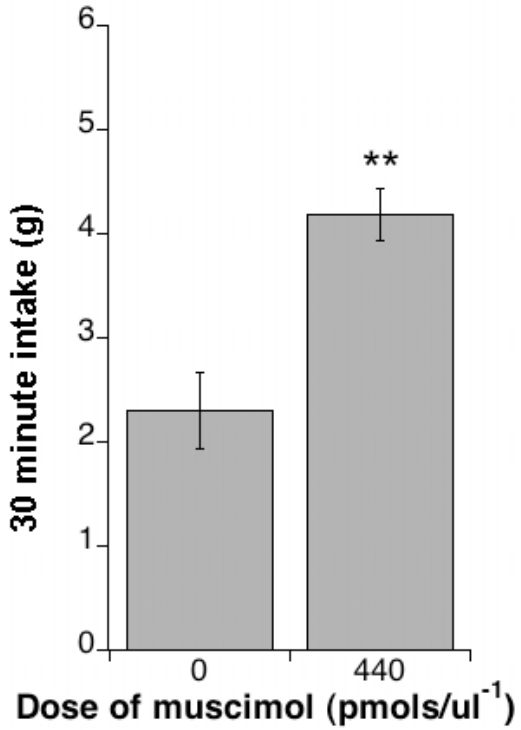


Figure 5.8. The effects of intra-Acb bilateral infusions of saline or muscimol in pre-fed rats (n=6) given access to laboratory chow over a 30 minute test. Error bars represent ±SEM. Significant differences from vehicle are denoted ★★p<0.01.

Summary of results for experiment 5.1

In experiment 5.1 n=6 subjects were included in the final analysis. The 220ρmols dose of baclofen significantly increased reinforced pressing on the 2nd order operant schedule but muscimol, at doses of 110, 220 and 440ρmols, had no significant effect on the total number of reinforced presses across the session or per 5 minute timebin. Muscimol also had no significant effect on the rate of pressing either during the first 5 minutes when reward was unavailable or during the following 25 minutes of the test session. At 110ρmols the drug appeared to increase the proportion of presses that were made while the CS was on and within 1 second of it termination. Muscimol at a dose of 440ρmols did increase free food intake in pre-fed animals over a 30 minute period.

Experiment 5.2: **The effects of bilateral intra-Acb infusions of muscimol at 220, 440 and 660ρmols/μl^{-1} in pre-fed rats on free intake versus responding on a second order operant schedule for food.**

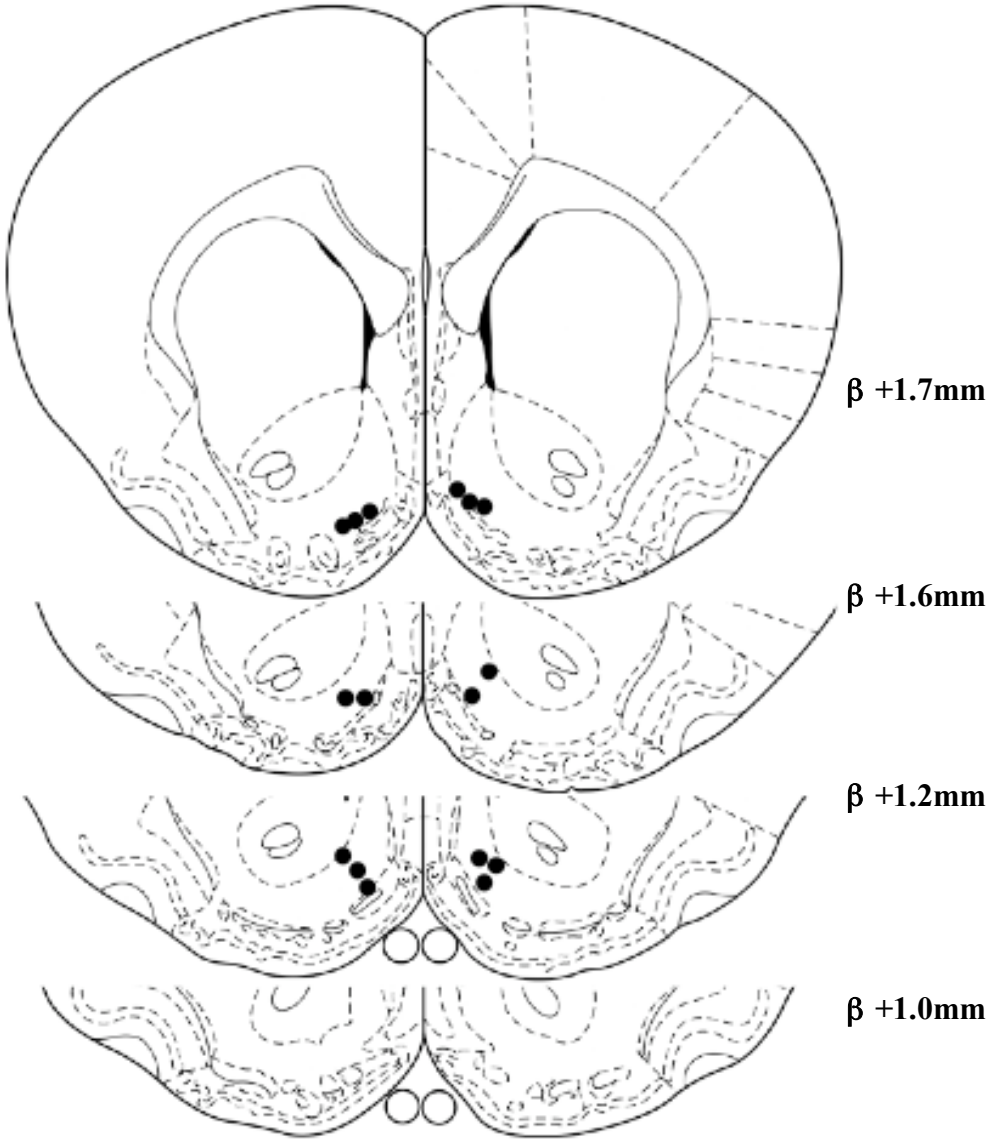


Figure 5.9. Injection sites plotted on drawings taken from Paxinos and Watson (1998); sections are anterior relative to bregma (β). Bilateral target coordinates (n=8 of original n=12 subjects with acceptable placements) were (AP), + 1.4mm, mediolateral (ML), ± 0.9mm relative to bregma and dorsoventral (DV), -7.8mm relative to skull surface.

Prior to testing animals in the operant chambers, responsiveness to muscimol in terms of free intake in pre-fed animals was verified at a dose of 660ρmols/μl^{-1}. A total of n=8 animals out of the original n=12 were later verified as having acceptable placements on the basis of the histology and were included in the final analysis. A schematic illustration of Acb infusion site placements is given in Fig. 5.9. These 8 animals consumed significantly more chow with muscimol treatment than with vehicle over a 30 minute period [Fig. 5.10: $F(1,7)=34.46$, $p<0.001$]. Some animals appeared to be mildly sedated but were still able to feed. There was no obvious correlation between infusion site and sedative effects. Muscimol had no significant effect on total reinforced presses at any dose tested (Fig. 5.11) nor did it affect the total number of non-reinforced or incorrect lever presses.

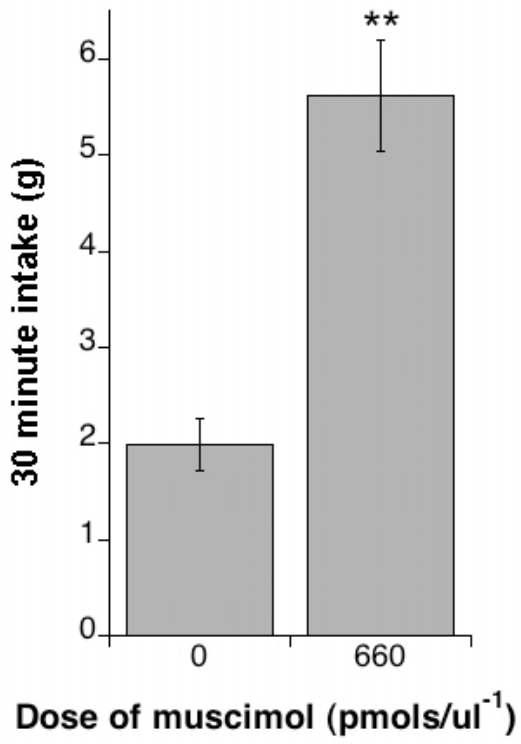


Figure 5.10. The effects of intra-Acb bilateral infusions of saline or muscimol in pre-fed rats (n=8) given access to laboratory chow over a 30 minute test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★★ p<0.001.

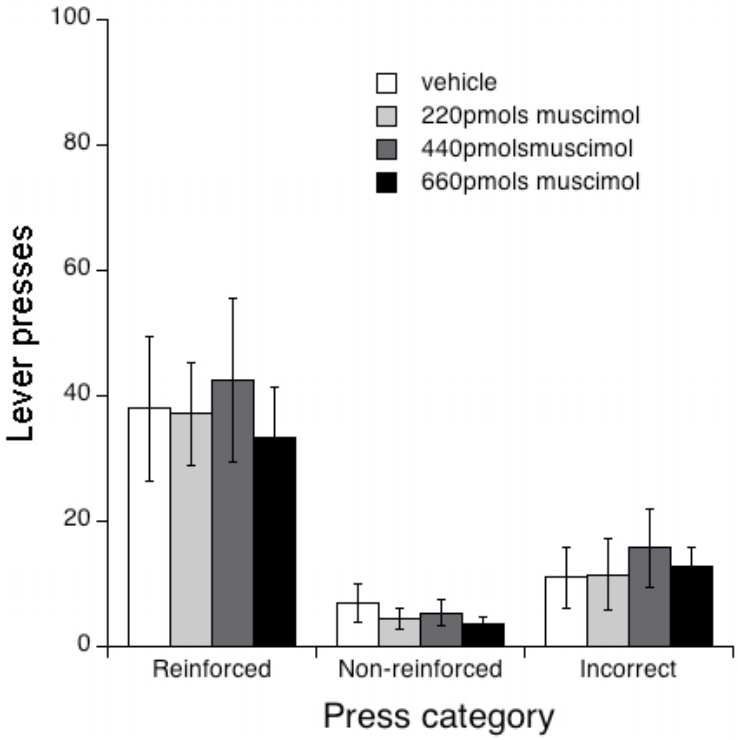


Figure 5.11. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on total lever presses in a 30 minute second order operant test session. Error bars represent ±SEM.

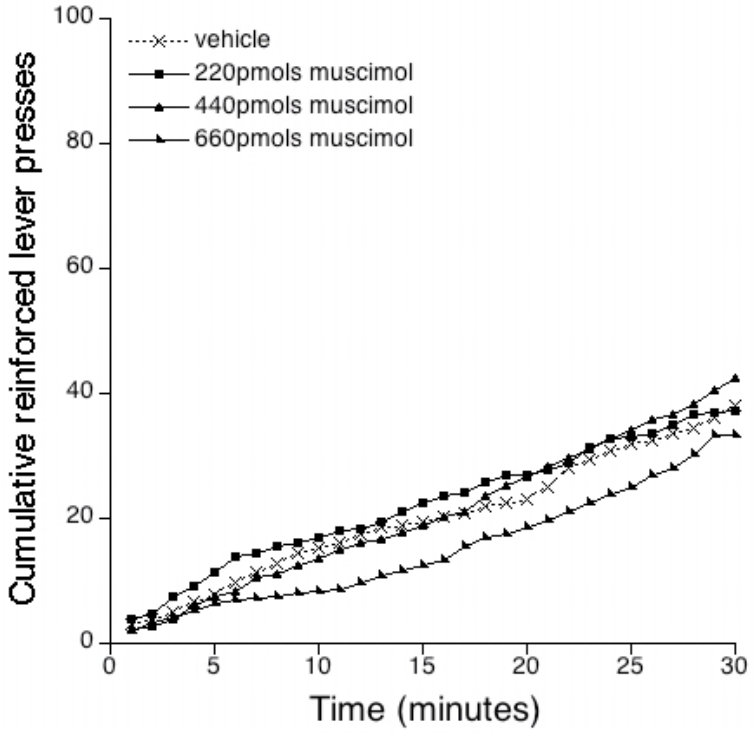


Figure 5.12. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on cumulative reinforced lever presses over a 30 minute second order operant test session.

Fig. 5.12 illustrates the distribution of cumulative reinforced lever presses across the 30 minute 2nd order operant session with intra-Acb infusions of vehicle or muscimol. The rate of lever pressing, expressed as reinforced presses per minute, across the first 5 minutes and subsequent 25 minutes of the schedule was calculated and the early vs. late phase rates were compared for each dose (see Table 5.2). There was no significant main effect of drug dose, of time phase or any interaction between the two. A comparison of the rates of pressing between treatments during the early phase indicated that there was no significant difference. During the subsequent 25 minutes there was again no significant difference between doses.

Table 5.2. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on the rate of reinforced lever pressing during the first 5 minutes (appetitive phase) and last 25 minutes (consummatory phase) of a second order operant test session. There were no significant differences.

Treatment	Phase	Rate presses/min
Vehicle	Appetitive	1.55 ± 0.41
	Consummatory	1.21 ± 0.41
220pmols muscimol	Appetitive	2.28 ± 0.33
	Consummatory	1.03 ± 0.29
440pmols muscimol	Appetitive	1.48 ± 0.40
	Consummatory	1.41 ± 0.49
660pmols muscimol	Appetitive	1.28 ± 0.22
	Consummatory	1.08 ± 0.32

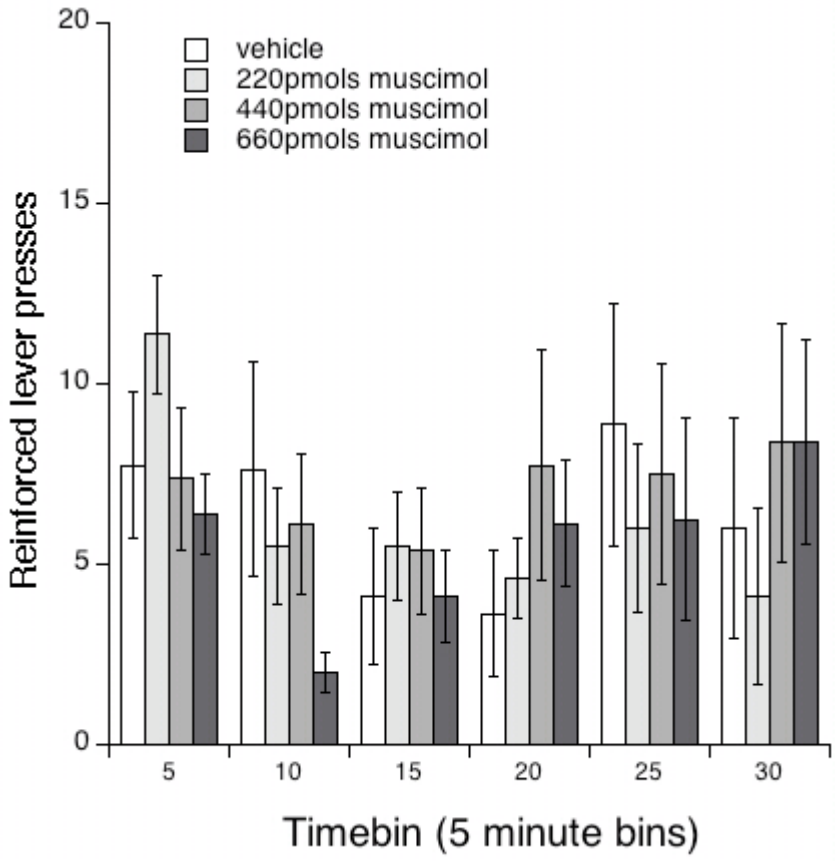


Figure 5.13. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on reinforced lever presses across a 30 minute second order operant test session. Error bars represent ±SEM.

When the data were split up into 5 minute bins there were no main effects of drug or time on total reinforced lever presses per bin. There was also no interaction between the dose of drug administered and time across the session (see Fig. 5.13).

The cumulative mean proportions of the length of inter lever press intervals (ILIs) between the 5th press of an FR5 response and the 1st press of the next FR5 across the first 5 minutes of the 2nd order schedule (unrewarded) are depicted in Fig. 5.14. The distributions of ILIs for the remaining 25 minutes of the test session are shown in Fig. 5.15. In the first 5 minutes of the session, when no lever presses could be rewarded with the primary reinforcer, animals did not press at all during the first 5 seconds CS with vehicle or any dose of muscimol.

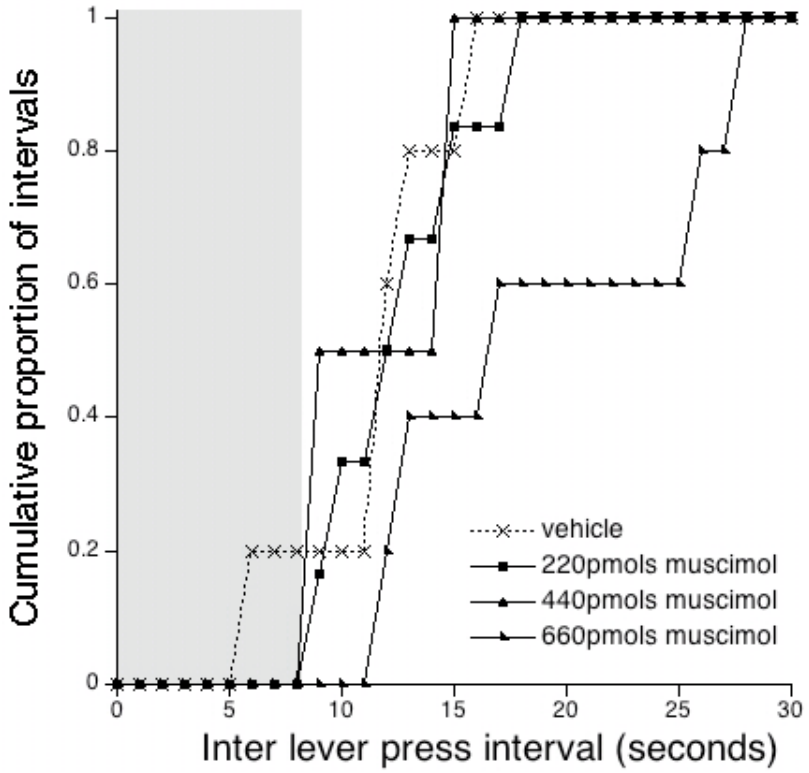


Figure 5.14. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on cumulative ILIs between the 5th and 1st presses of the FR5 requirement on the reinforced lever. Data represents responding across the first 5 minute unrewarded phase of a second order operant test session. The shaded phase represents the duration of the CS (8 seconds).

For the remaining 25 minutes of the session some animals made the first press of the next ratio during the CS with all treatment conditions. With vehicle a mean of 18% of presses were made while the CS was still on. While the CS was still on 13%, 13%, 17% and 18% of presses were made with vehicle, 220, 440 and 660ρmols of muscimol respectively. Within a second of the CS terminating a mean of 17% of presses with vehicle were made while 27%, 34% and 30% were made with 220, 440 and 660ρmols. A few days after testing on the 2nd order schedule the same animals, pre-fed with standard laboratory chow prior to treatment with 220, 440 and 660ρmols of muscimol, exhibited a significant increase in consumption of chow over a subsequent 30 minute test (Fig 5.16; [$F(3,21)=13.76$, $p<0.001$]). Paired comparisons revealed that all three doses significantly increased intake relative to vehicle ($p<0.01$ in all three cases). There was no difference in intake with 660ρmols prior to and post 2nd order testing. At the highest dose mild sedative effects were again noted in some animals but not across the entire 30 minute intake test session.

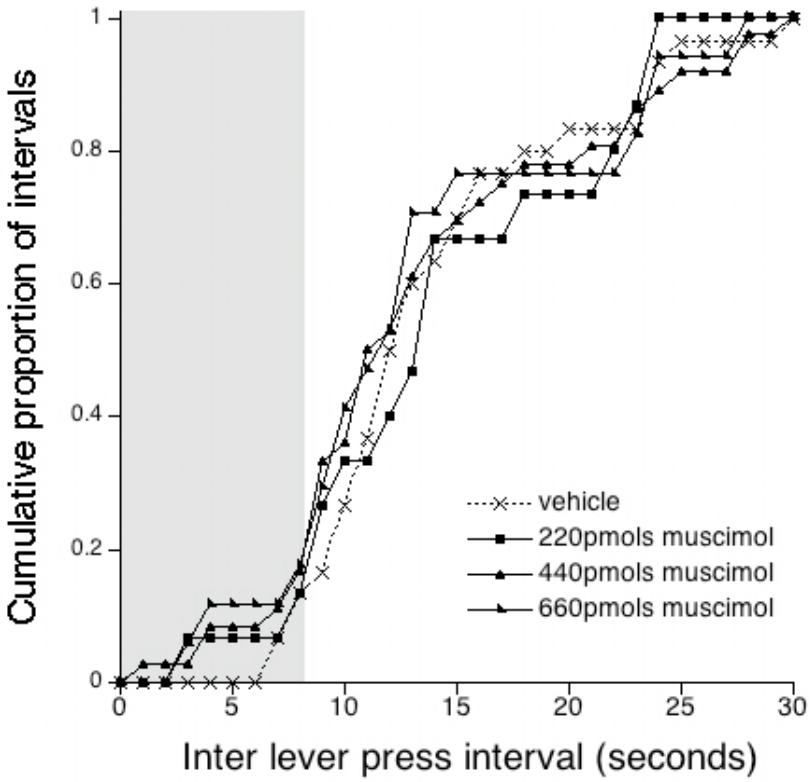


Figure 5.15. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on cumulative inter lever press intervals between the 5th & 1st presses of the FR5 requirement on the reinforced lever. Data represents responding across the last 25 minute rewarded phase of a second order operant test session. The shaded phase represents the duration of the CS (8 sec).

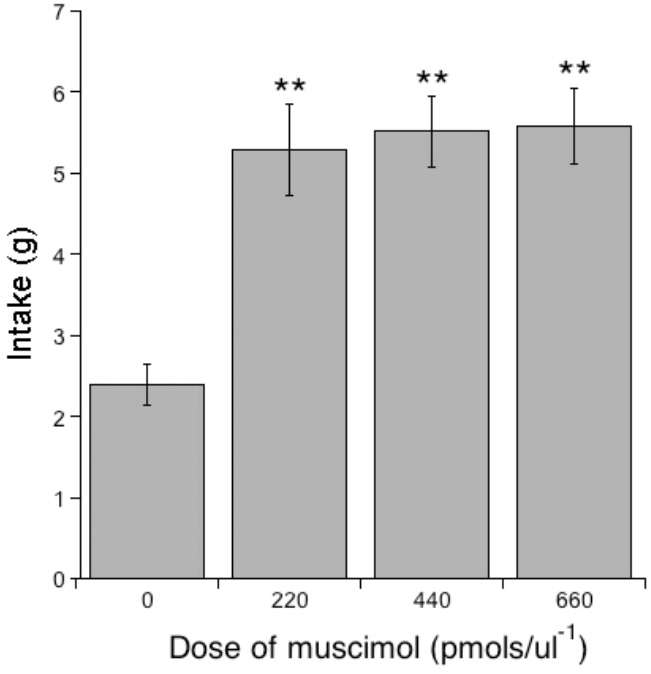


Figure 5.16. The effects of intra-Acb bilateral infusions of saline or a range of doses of muscimol in pre-fed rats (n=8) given access to laboratory chow over a 30 minute test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★★p<0.01.

Associated behaviours

Analysis of the videos of testing in the operant boxes revealed a strong tendency towards an interaction between dose of muscimol and the total duration of the 8 behaviours recorded [Fig. 5.17; $F(24,168)=1.55$, $p=0.059$]. There was a significant interaction between drug and behavioural category for the frequency of the eight behaviours across the session [Fig. 5.18; $F(24,168)=1.6$, $p=0.045$]. The pattern of the effects of drug on duration and frequency of individual behaviours did not always correspond and are described in detail later (see Fig. 5.17 & 5.18). The amount of behaviour that was coded as off screen was low, as was the case in Chapter 4 (Fig. 4.27 and Fig. 4.28). There was no significant difference in duration or frequency of off screen data recorded at any dose.

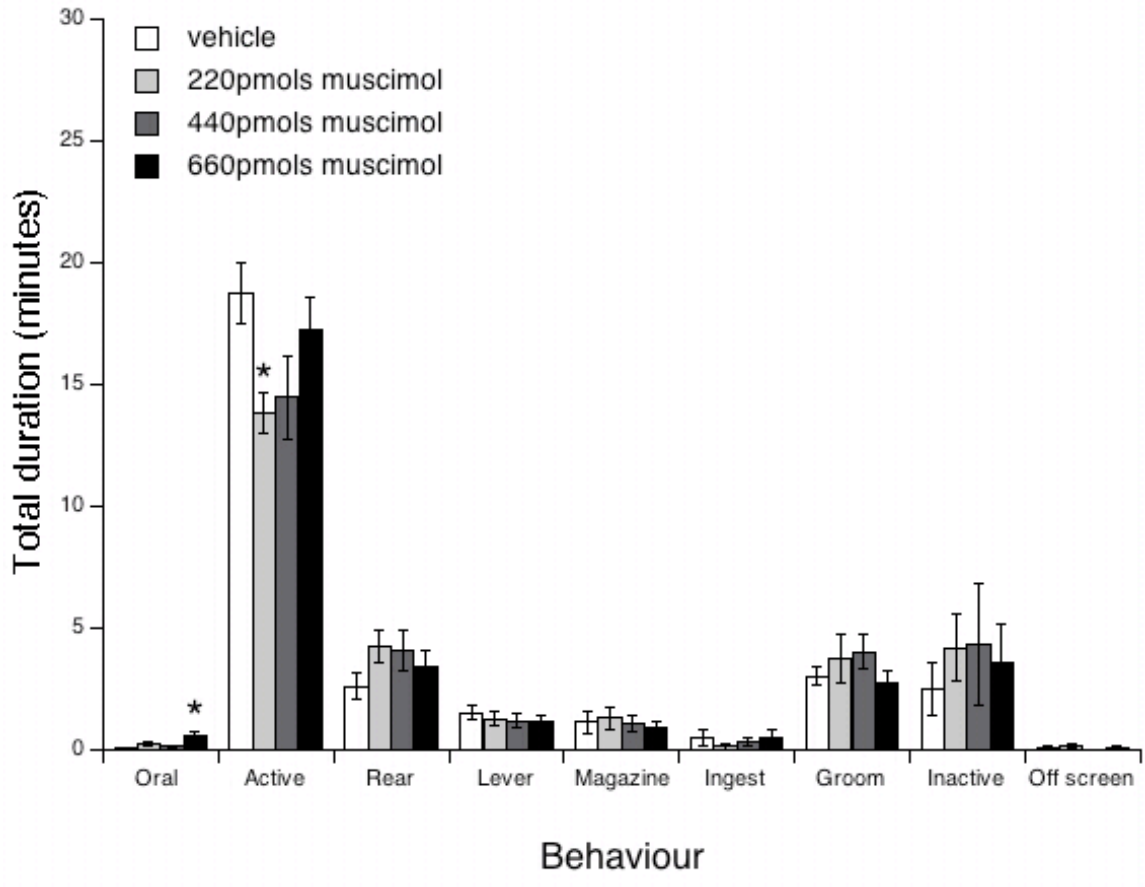


Figure 5.17. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on the total duration of 8 behaviours recorded across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ $p<0.05$.

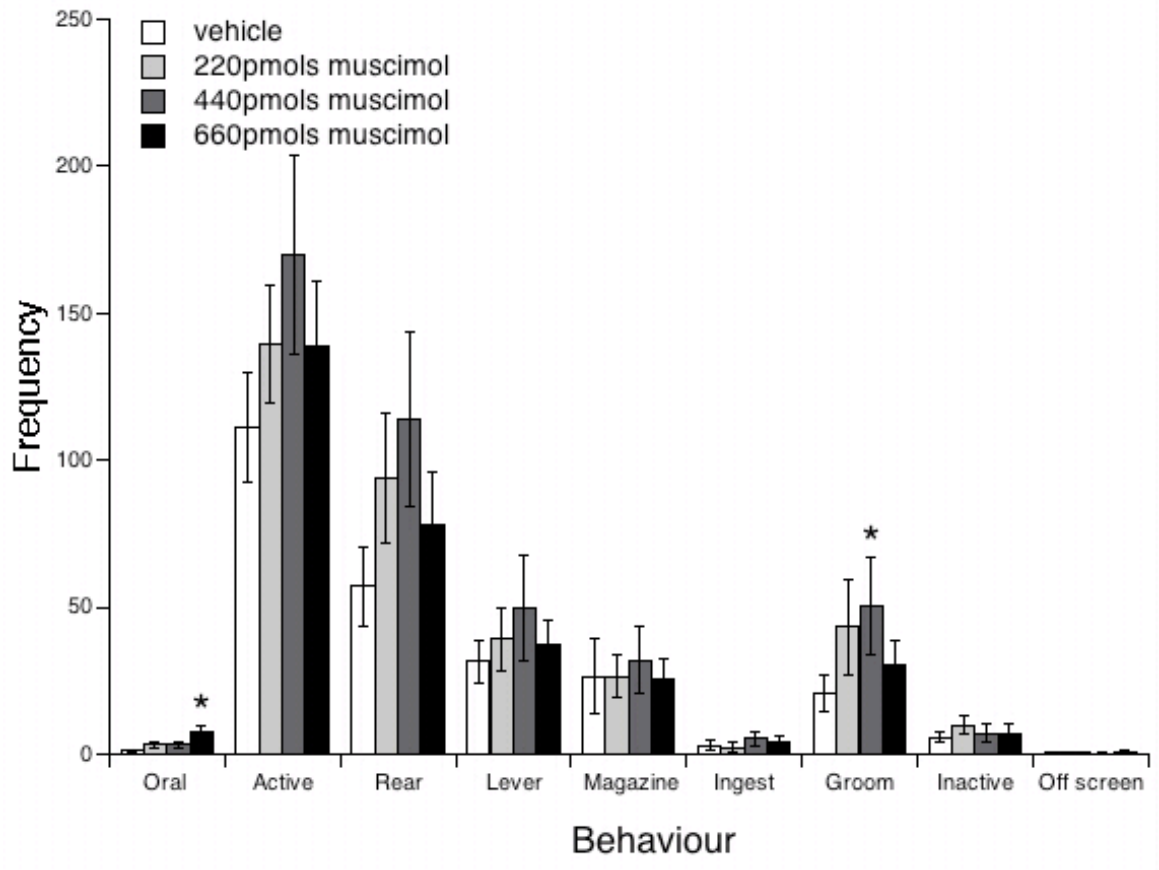


Figure 5.18. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on the total frequency of 8 behaviours recorded across a 30 minute second order operant test session. Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ p<0.05.

Oral stereotypy

The total duration [Fig. 5.17; $F(3,21)=4.00$, $p=0.021$] and frequency [Fig 5.18; $F(3,21)=4.03$, $p=0.021$] of behaviours categorised as 'oral stereotypy' was significantly increased by drug but post-hoc analysis revealed that this effect occurred only at 660ρmols ($p<0.05$ in both cases). When the duration data were split into 5 minute time bins there was a significant main effect of drug [$F(3,21)=4.0$, $p=0.021$] and main effect of time bin [$F(5,35)=4.66$, $p=0.002$] but there was no interaction (See fig. 5.19A). There was also a significant main effect of drug [$F(3,21)=4.03$, $p=0.021$] and of time bin [$F(5,35)=3.68$, $p=0.009$] but no interaction for frequency (See Fig. 5.19B). Oral stereotypical behaviours represented less than a minute of behaviour across the entire session, even at the highest dose. It was noted during the video analysis that oral stereotypical behaviour only occurred in very short bouts rather than being prolonged sessions of floor licking as was seen with baclofen. Furthermore it was directed just as much at the levers, hopper and walls at to the floor.

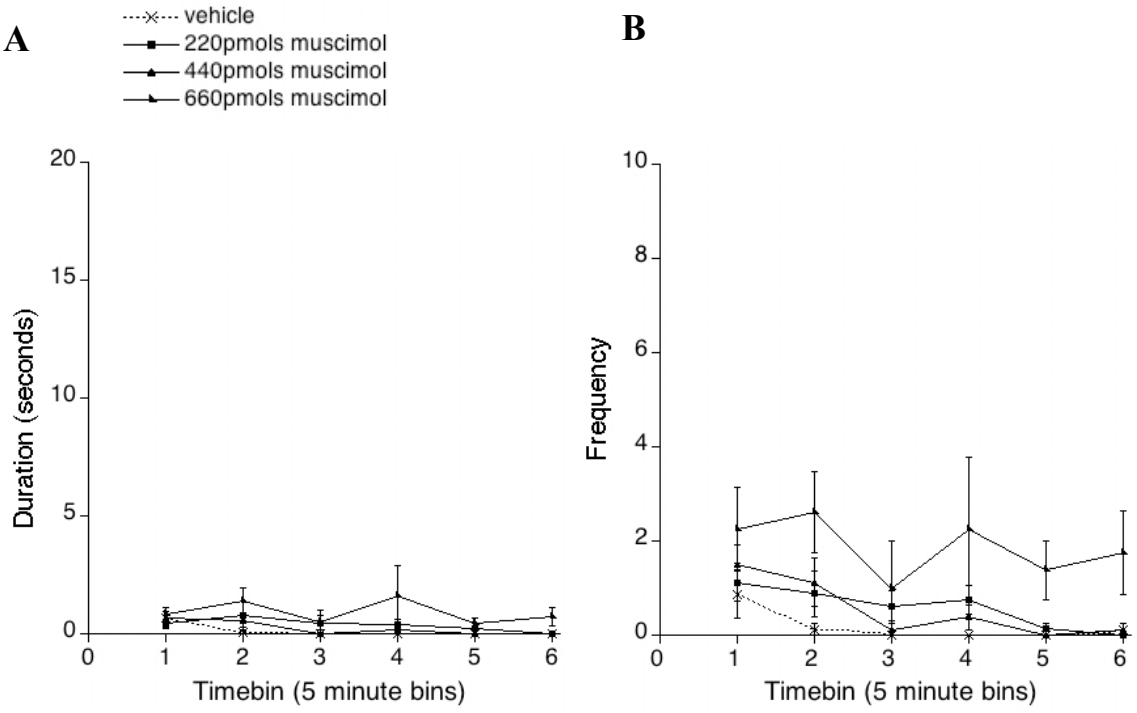


Figure 5.19. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on A) duration and B) frequency of ORAL STEREOTYPY across a 30 minute second order operant test session. Error bars represent ±SEM.

Activity

There was a significant effect of muscimol on the total duration of active behaviour [Fig. 5.17; $F(3,21)=3.21$, $p=0.044$]. Post hoc analysis revealed that this was due to a significant decrease in the duration of activity at 220ρmols (see Fig. 5.17 $p<0.05$). There was no significant effect of muscimol on the total frequency of bouts of active behaviour (See Fig. 5.18). When the data were split into 5 minute time bins a significant main effect of drug on the duration of active behaviour [$F(3,21)=3.2$, $p=0.044$] was seen but there was no main effect of time bin or any interaction (See Fig. 5.20A). There was no main effect of drug on the frequency of periods of active behaviour, there was a main effect of time bin [$F(5,35)=16.33$, $p<0.001$] but no interaction (See Fig. 5.20B).

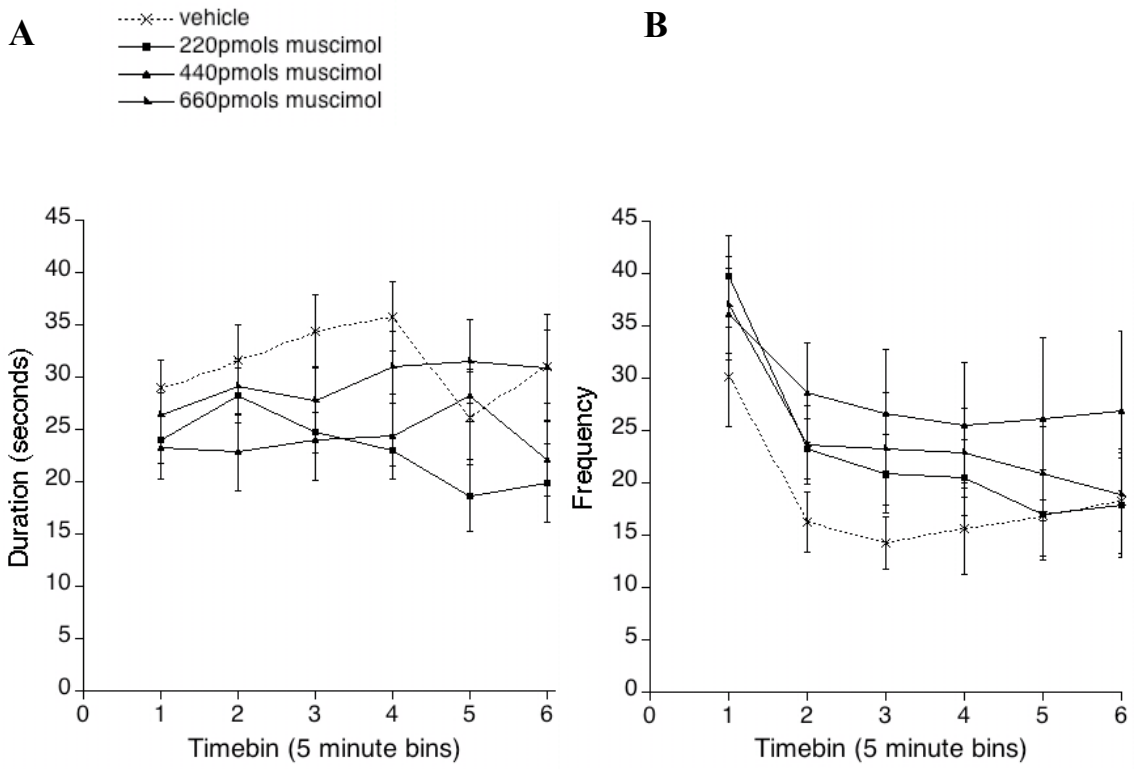


Figure 5.20. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on A) duration and B) frequency of ACTIVE behaviours across a 30 minute second order operant test session. Error bars represent ±SEM.

Rearing

Muscimol had no significant effect on the total duration or frequency of rearing (See Fig. 5.17 and 5.18). When the data were split into 5 minute time bins (not shown) there was a significant main effect of time on the duration [$F(5,35)=16.17$, $p<0.001$] and frequency [$F(5,35)=11.64$, $p<0.001$] of rears in each bin but no interaction with drug treatment. Analysis of the separate record of the number of rears to the CS indicated that there was no significant effect of drug (See Fig. 5.21A) and there was no significant difference in rears directed at the CS as a proportion of total rears with each dose (Fig. 5.21B).

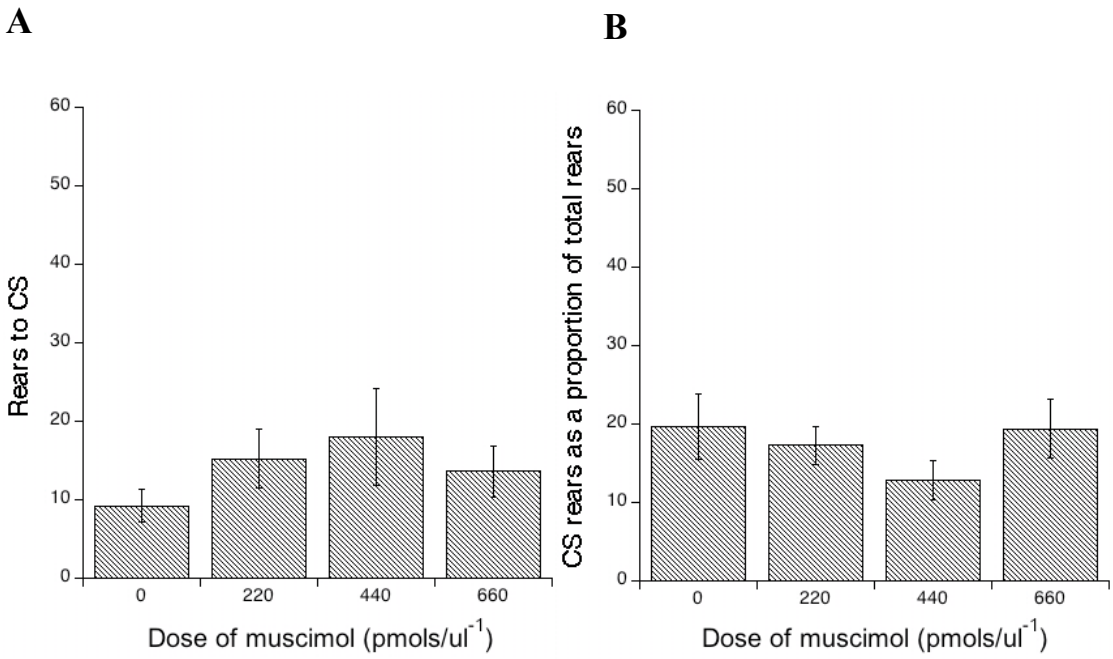


Figure 5.21. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on A) the total number of rears to the CS and B) the proportion of the totals rears made that were directed towards the CS across a 30 minute second order operant test session. Error bars represent ±SEM.

Lever

There was no main effect of drug on the total duration or frequency of interactions with the lever and splitting the data into time bin did not reveal an effect of drug, time or any interaction between the two on the duration or frequency per 5 minute bin.

Headpokes into magazine

The total duration and frequency of headpokes into the magazine was not changed by muscimol and there was no significant effect of drug or time on this behaviour across 5 minute bins.

Ingest

The total duration and frequency of ingestion was not changed with muscimol at any dose and when the data were split into 5 minute bins there was no significant effect of drug in any bin. There was a significant main effect of time for both duration [$F(5,35)=2.41$, $p=0.056$] and frequency[$F(5,35)=4.73$, $p=0.002$) but no interaction with drug dose.

Groom

The total duration of grooming behaviour was not affected by drug (see Fig. 5.17) but the total frequency was significantly increased [Fig. 5.18; $F(3,21)=3.71$, $p=0.028$]. Paired comparisons revealed this to be due to an increase in the frequency of bouts of grooming at 440ρmols ($p<0.05$) (See Fig. 5.18). When the data were split into 5 minute bins there was no main effect of drug on the duration, a significant effect of time [$F(5,35)=3.48$, $p=0.012$] but no interaction (See fig. 5.22A). For frequency there was a significant main effect of drug [$F(3,21)=3.67$, $p=0.029$] and of time bin [$F(5,35)=5.47$, $p<0.001$] but no interaction (Fig 5.22B). It was noted that animals at all drug doses would start a normal syntactic chain of grooming but often terminate it before it was complete at higher doses. Normal grooming behaviour was fragmented. The grooming stereotypies observed with baclofen (e.g. fixation on tail) were not seen.

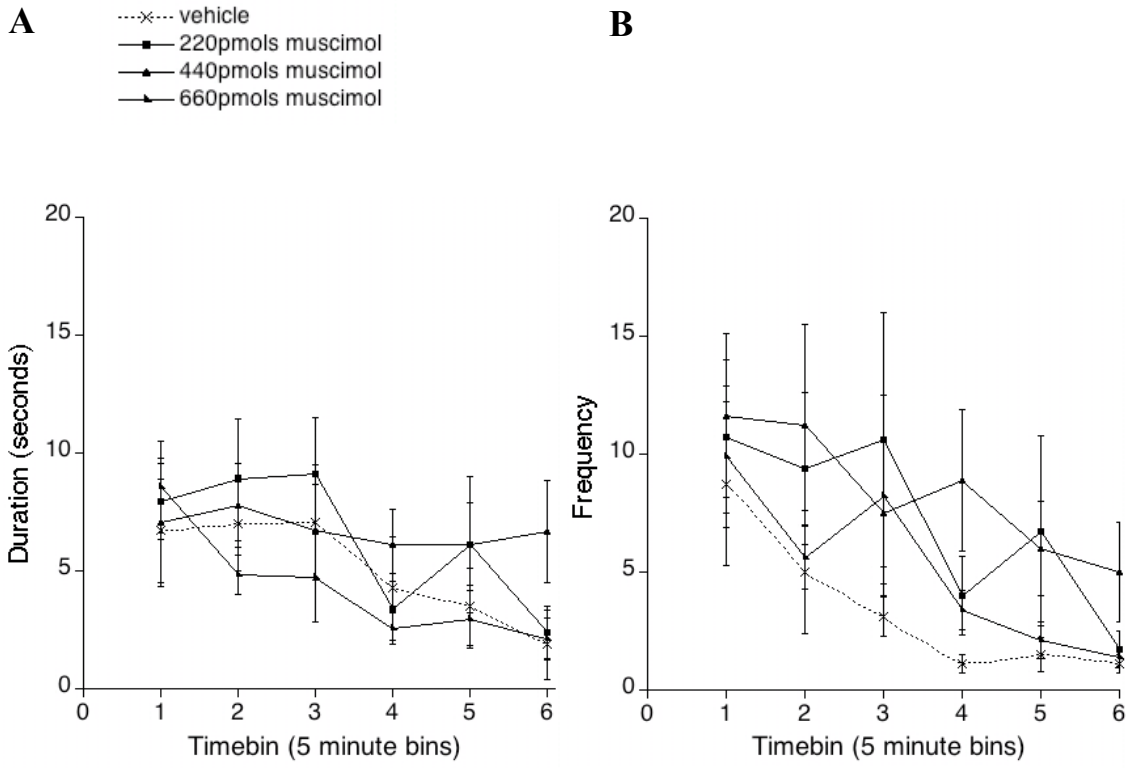


Figure 5.22. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on A) duration and B) frequency of GROOMING behaviour across a 30 minute second order operant test session. Error bars represent ±SEM.

Inactive

There was no main effect of drug on the total duration (see fig. 5.17) or frequency (see Fig. 5.18) of inactive behaviour but a significant effect of time for both duration [Fig. 5.23A; $F(5,35)=2.41$, $p=0.056$] and frequency [Fig. 5.23B; $F(5,35)=3.24$, $p=0.017$] when data were split into 5 minute bins. It was noted from observations of the videos that while under the influence of 660ρmols some animals appeared mildly sedated once the initial arousal associated with being put into the boxes had dissipated. With both 440 and 660ρmols animals would intermittently lie down throughout the session. In contrast inactive behaviour with vehicle and 200ρmols occurred near the end of the session and appeared to occur because animals had lost interest in doing anything else.

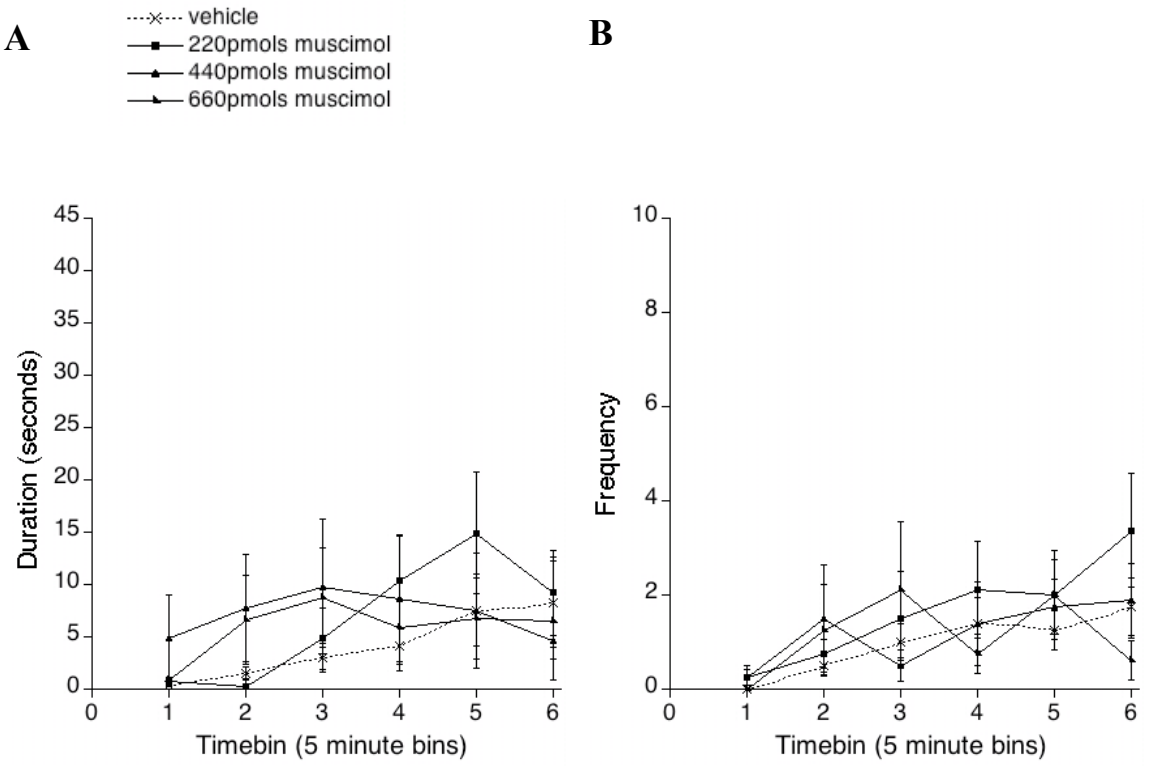


Figure 5.23. The effects of intra-Acb bilateral infusions of saline and a range of doses of muscimol in pre-fed rats (n=8) on A) duration and B) frequency of INACTIVE behaviour across a 30 minute second order operant test session. Error bars represent ±SEM.

Summary of results for experiment 5.2

In this experiment n=8 subjects were included in the final analysis. With these animals 660ρmols of baclofen significantly increased intake of freely available chow prior to testing on the 2nd order operant schedule. Subsequently 110, 220 and 660ρmols also significantly increased total chow intake relative to vehicle treatment a few days following testing on the operant schedule. There was no significant difference between the amount of chow consumed with 660ρmols prior to and following multiple infusions for the operant test. There was also no significant difference between amounts of chow eaten with each dose. Muscimol, at doses of 220, 440 and 660ρmols, had no significant effect on the total number of reinforced presses or per 5 minute timebin. Muscimol also had no significant effect on the rate of pressing either during the first 5 minutes when reward was unavailable or during the following 25 minutes of the test session. Of the 8 other behaviours that were recorded from the videos of the test sessions muscimol significantly increased the total duration and frequency of bouts of oral stereotypy at 660ρmols, decreased total duration of active behaviour at 220ρmols and increased total frequency of bouts of grooming at 440ρmols. Observations from the videos suggested that behavioural patterns had become fragmented with only partial grooming sequences expressed and infrequent short bouts of oral stereotypy, particularly at the highest dose.

Discussion

This chapter primarily addressed the key question raised in Chapter 4; will low doses of muscimol infused into the AcbSh increase operant responding for a food paired cue in a second order schedule? Using the same approach as in Chapter 4, the two experiments reported here were designed to investigate the acute effects, across a range of doses, of intra-Acb $GABA_A$ receptor stimulation on ingestion versus performance of food motivated operant responses in pre-fed rats. In the first experiment responding with muscimol was directly compared in the same animals with intra-Acb baclofen at the 220ρmols dose that was previously demonstrated to increase operant responding in Chapter 4. In the second experiment in addition to effects of muscimol on operant responding the effects on 8 mutually exclusive categories of behaviour expressed during responding on the 2nd order schedule were analysed from videos of the sessions. Intake of freely available chow was subsequently measured using an identical counterbalanced range of doses in the same cohort of animals.

Reinforced lever presses with baclofen and muscimol

As was expected, in experiment 5.1 baclofen at a dose of 220ρmols significantly increased reinforced lever pressing but in contrast muscimol had no significant effect on total reinforced presses at any of the doses tested (110, 220, 440ρmols). In experiment 5.2 the lack of effect of muscimol at these doses was confirmed and in addition it was shown that a higher dose of 660ρmols had no significant effect on reinforced responding. Baclofen and muscimol had no significant effect on non-reinforced or incorrect lever presses.

Phases of responding with muscimol

When the data were split into early (first 5 minutes) and late (last 25 minutes) phases there was no significant difference between phases for each dose or between doses for each phase in experiment 5.1 or 5.2. Further splitting the data into 5 minute time bins did not reveal any significant dose effect on total reinforced pressing in each time bin.

Other behaviour durations and frequency with muscimol

With 660ρmols muscimol there was a significant increase in both the duration and frequency of oral stereotypical behaviours however this only represented less than 1 minutes worth of behaviour across the entire 30 minute session. It was also noted from the videos that the pattern of oral stereotypical behaviour was different from that seen with baclofen. With muscimol animals only expressed short intermittent bouts of chewing/licking directed at the hopper, levers and walls as much as it was to the floor of the boxes. At 220ρmols there was a significant decrease in total activity across the session, which was seen as a gradual decrease over time. At 440ρmols there was a significant increase in the frequency but not in the duration of bouts of grooming.

Free food intake with muscimol

In experiment 5.1 440ρmols muscimol increased intake of freely available chow when animals were tested after the 2nd order test phase. In experiment 5.2 a dose of 660ρmols baclofen increased intake prior to 2nd order testing and doses of 220, 440 and 660ρmols baclofen also increased free feeding after the 2nd order testing. There was no significant difference in total intake prior to and post 2nd order testing with the 660ρmols dose.

Preliminary interpretation of key results summarised above

The results reported in this chapter suggest that muscimol across the dose range tested does not have any effect on responding for food on a second order schedule. The results with baclofen in experiment 5.1 confirmed that infusions were reaching an area of the AcbSh that was behaviourally active in terms of increase in reinforced pressing. Muscimol infused in the same cohorts of animals post 2nd order testing caused a significant increase in feeding. The extended dose range in experiment 5.2 was infused at coordinates that were very similar to those in experiment 5.1 so it is unlikely that a placement issue was responsible for the lack of effect on operant responding. Again the muscimol infused post 2nd order testing caused a significant increase in intake.

Because muscimol also had very little impact on the other behaviours that were recorded it is suggested that these results confirm that the changes in behaviour reported in Chapter 4 with baclofen are related to stimulation of $GABA_B$ receptors within local neurotransmitter circuits rather than occurring as a result of inactivation of Acb output. It is further suggested that activation of $GABA_A$ receptors does inactivate Acb output and cause a linear increase in ingestive behaviour through release of feeding related motor behaviours, a mechanism that does not support an increase in more complex food seeking behaviours. In contrast activation of $GABA_B$ receptors modulates local neurotransmitter function subserving motivational processes e.g. saliency attribution.

Possible explanations for muscimol increase in intake but not operant responding

The results of the experiments reported in this chapter are consistent with the theory put forward by Kelley et al. (2005a, 2005b) that activation of GABA receptors, in this case specifically $GABA_A$ receptors, results in inhibition of Acb output and hence disinhibition of downstream control over fragments of ingestive motor patterns. As such there is little more to add to the explanation of the effects of intra-AcbSh muscimol on feeding. However taking this proposed mechanism into account there are some issues that do need to be further explained. First of all muscimol at the doses tested did not appear to increase feeding in a linear manner as has been previously reported (e.g. Stratford and Kelley, 1997b, Basso and Kelley, 1999). Secondly analysis of the videos of behaviour did demonstrate some effects of muscimol on activity, grooming and oral stereotypy during operant responding.

Muscimol effects on free intake

Baclofen and muscimol are reportedly equipotent in causing feeding in satiated rats (Stratford and Kelley, 1997b) but, while baclofen caused a linear increase in intake of chow in experiments 3.1 (Chapter 3) and 4.3 (Chapter 4) in experiment 5.2 there was no difference between the amount of food consumed with each dose of muscimol. In experiment 3.2 (Chapter 3) the 220 – 660ρmols dose range of muscimol was tested in the BSS for chow and here too there was no difference between the doses. Intake was not tested at the lowest dose of 110ρmols dose in this set of studies. Elsewhere a positive linear relationship between dose of muscimol and intake has been repeatedly observed (Stratford and Kelley, 1997b, Basso and Kelley, 1999). Nevertheless it is fairly straightforward to explain the apparent lack of a linear increase in free intake with increased doses of muscimol.

It has been mentioned in Chapter 4 and in this chapter that there were signs of myorelaxation with baclofen at 660ρmols and sedation at 660ρmols muscimol. This was also noted during various pilot studies and it was concluded that this strain of rats (Lister Hooded rats from Harlan, UK) was actually much more sensitive to both baclofen in terms of myorelaxant effects and muscimol in terms of sedation than has been reported for other strains. For example Stratford and Kelley (1997b) did not record any locomotor effects up to and including a dose of 876ρmols muscimol in male Sprague Dawley rats (Harlan, Indianapolis, USA). There was no obvious correlation between the exact location of infusions and the degree of sedation (or lack of it) noted and putting the animals into the operant boxes appeared to override these effects to some degree. The sedative effects appeared to be much more pronounced in the free intake test sessions than during the operant test sessions. It is suggested that the more complex task and the more salient testing environment in the operant boxes causes a level of arousal in the animals that partially overrides the sedative effects.

At 660ρmols there is also evidence that behavioural control patterns breakdown with both baclofen and muscimol and in this case they may both be acting via inactivation of Acb output. With baclofen a change in behavioural control patterns was manifested at 660ρmols as a disappearance of normal syntactic grooming chains, their replacement with a stereotyped alternative grooming pattern and the emergence of oral stereotypy

directed at the mesh flooring. However it should be noted that in the BSS study with baclofen the total duration of grooming was not affected but animals did lick the floor/sawdust (experiment 3.1, Chapter 3). With muscimol as the dose increased animals would start to engage in a normal grooming sequence but terminate it prematurely and, at 660ρmols some possible oral stereotypies emerged although they were really just fragments of oral behaviour with no distinct target. In the BSS study with muscimol the duration of grooming behaviour was significantly reduced (experiment 3.2, Chapter 3). It is concluded therefore that the lack of linear increase in feeding between 220 and 660ρmols muscimol is due to a combination on sedative effects (that are partially overridden in the operant boxes) and a breakdown of control over chains of behaviour such as grooming that interferes with ingestive motor patterns.

Muscimol effects on activity, grooming and oral stereotypy

The only potential caveat to the argument that the effects of muscimol and baclofen are subserved by different mechanisms is that, at 660ρmols, both baclofen and muscimol caused the emergence of oral stereotypies during the schedule. Interestingly the total number of presses with the 220ρmols dose was higher (not significantly so) than with vehicle or the other drug doses in the first 5 minutes which is consistent with Zhang et al.'s (2003) finding that muscimol caused "slightly elevated" responding early in the session. It is possible that this is due to the possibility that muscimol might not be totally selective for $GABA_A$ receptors and could also stimulate $GABA_B$ receptors (Yamauchi et al., 2000, Xiao et al., 2007, Morl et al., 2003). It is suggested therefore that the small effect of muscimol on oral stereotypy manifested as very short, less focused or unidirectional bouts than those seen with baclofen may be due to binding to the $GABA_B$ receptor rather than due to any $GABA_A$ mediated mechanism. However an alternative explanation based on the evidence to be reported in Chapter 6 will be discussed in the final chapter, Chapter 7.

It is difficult to find any obvious functional reason why muscimol at 220ρmols would decrease activity. There is certainly no evidence in the literature that this would be the case. Looking at the inactive behavioural category there was an increase as the session progressed with this dose but the effect was not significant. It was noted however that not many of the animals pressed enough to get pellets and became increasingly

disinterested in doing anything in the operant box as the session progressed. The slight decrease in activity could be due to what is effectively boredom, perhaps exacerbated by the lack of opportunity to express ingestive motor patterns caused by inactivation of AcbSh output. Although the latter mechanism would also hold true at the 440 and 660ρmols dose it has already been noted that disruption of specific motor behavioural patterns related to grooming and oral stereotypy increased with increasing dose and behaviour became fragmented. As a result there were more transitions between these fragments that were coded as 'active'.

It is proposed that three mutually exclusive but potentially interacting mechanisms are responsible for the results reported in Chapters 3, 4 and 5. First of all stimulation of $GABA_B$ receptors at low doses of baclofen engages motivational processes such as saliency attribution but at higher doses the net result of stimulation of $GABA_B$ receptors is inactivation of Acb output. This is the same mechanism that is responsible for free intake via stimulation of $GABA_A$ receptors but could disrupt operant responding. At the highest doses of baclofen and muscimol myorelaxant and sedative effects respectively also begin to block free intake. A schematic illustration of the contrasting effects of baclofen and muscimol on feeding versus operant responding is shown in Fig. 5.24 and the interaction of the three mechanisms is explained in more detail in the next section.

Hypothesised mechanisms to explain baclofen vs. muscimol effects

Numbered stages in Fig. 5.24 indicate the proposed shift between hypothesised mechanisms with muscimol or baclofen depending on dose such that:

With baclofen

1) increased cue saliency at low doses of baclofen increases operant responding in a linear manner up to a maximum dose somewhere in the region of 220ρmols, 2) release of fragments of motor responding at doses of baclofen above 220ρmols begin to interfere with saliency effects on operant responding, 3) myorelaxation at high doses of baclofen disrupt operant responding, 4) increased cue saliency at low doses of baclofen could also be responsible for increasing free intake in a linear manner but 5) release of fragments of ingestion related motor responding focus the animals behaviour into predominantly consummatory responses increasing it further as dose increases and 6) free feeding is disrupted by myorelaxation effects at the highest end of the dose range.

With muscimol

7) Release of ingestion related fragments of motor responding at low doses of muscimol does not prevent operant responding but 8) at higher doses sedative effects disrupt it whilst 9) release of ingestion related fragments of motor responding could increase free feeding in a linear manner up to 10) higher doses which cause sedative effects that make it difficult to express feeding behaviour.

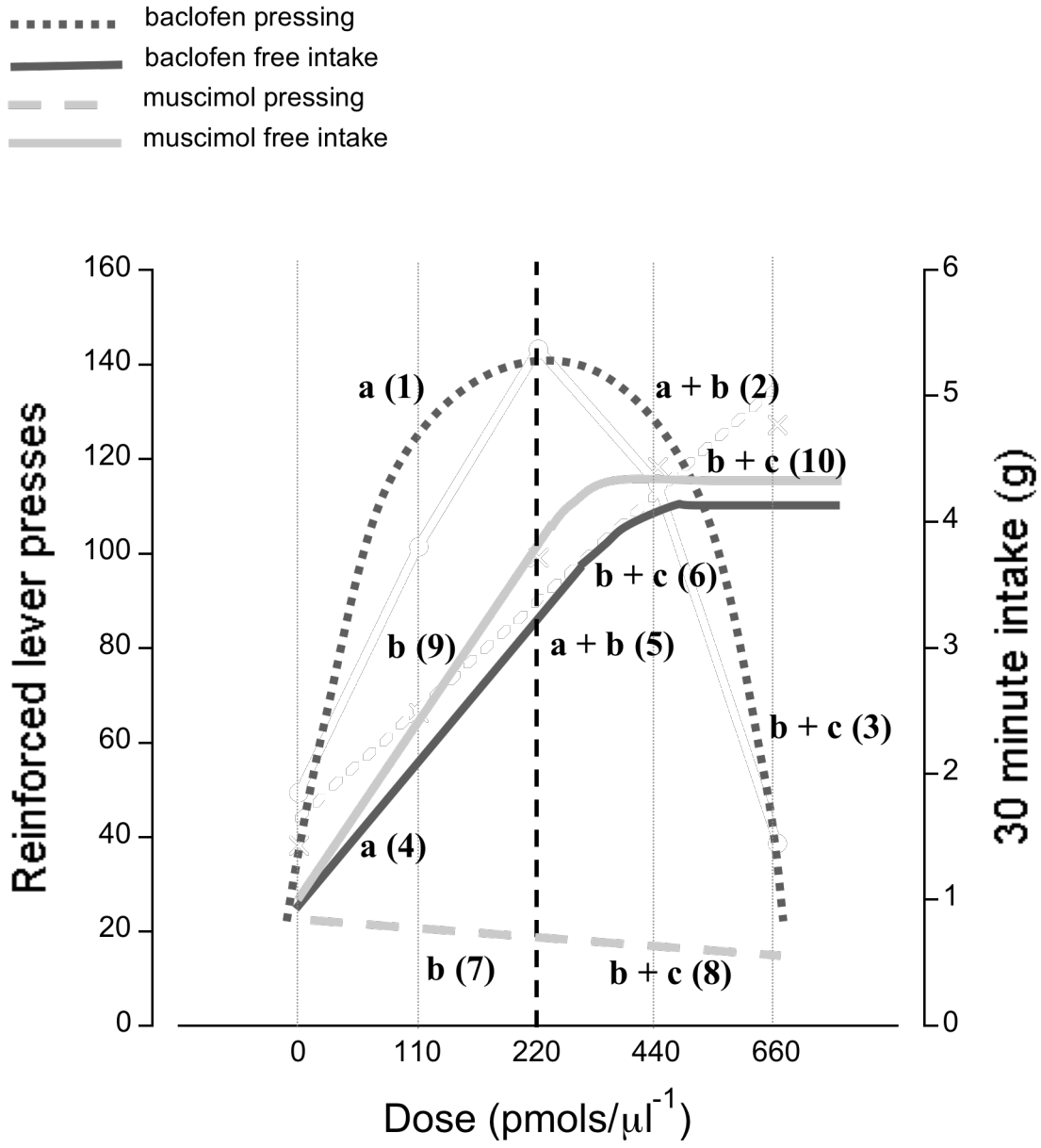


Fig. 5.24. Schematic illustration of the effects of baclofen and muscimol across the dose range tested on free intake vs. responding on the 2nd order schedule. The proposed mechanisms that subserve this pattern are a) increased cue saliency (baclofen only), b) release of a subset of ingestion related motor patterns (baclofen and muscimol) and c) myorelaxation (baclofen) or sedation (muscimol). Detailed explanations for the numbered combinations across the dose range are given in the main text.

Summary

It has been demonstrated in this chapter that, as would be predicted from Kelley's hypothesis, intra-Acb muscimol does not increase operant responding at doses that increase free feeding. At higher doses there appears to be a breakdown in behavioural control patterns over grooming and oral stereotypy (although the latter effect may be due to activation of $GABA_B$ receptors by muscimol). It is postulated that, while low doses of baclofen could increase operant responding via effects on cue saliency subserved by changes in local neurotransmitter circuits, muscimol exerts its effects via inactivation of Acb output and hence disinhibition of the LH. It is possible that the proposed mechanisms above and the interaction between mechanisms depending on dose effects on $GABA_A$ or $GABA_B$ receptors could well be subserved by different patterns of activation within brain circuitry involved in motivational processes and the control over ingestive behaviours. This provides a particularly compelling reason to investigate the effects of baclofen and muscimol on neuronal activation in other regions of the brain associated with motivation and reward. Some tentative explanations of the effects of baclofen at both the neurotransmitter and structural level have been discussed already in Chapter 4. Given that the results reported in this chapter have lead to the hypothesis that different mechanisms subserve baclofen and muscimol effects on ingestive behaviour, at least at low doses, any further functional explanations will be considered in light of the findings to be reported in the next Chapter.

Questions raised

The key questions raised by these results are 1) given that baclofen increases operant responding but muscimol does not is there any evidence of different brain circuits subserving their effects? And 2) if there are differences in the structures involved in the effects of baclofen and muscimol what could be going on at the level of the GABA receptors local to the AcbSh. As noted in Chapter 4 the first question will be addressed in Chapter 6 by using the presence of the immediate early gene *c-fos* to map regional activation following infusions of baclofen or muscimol. The potential mechanisms subserving the effects reported in Chapters 3, 4 and 5 will be discussed in full in light of the result from Chapter 6 in the General Discussion, Chapter 7.

Chapter 6

$GABA_A$ and $GABA_B$ receptor stimulation in the accumbens: comparison of regional brain activation using Fos like immunoreactivity as a marker

Introduction

The results reported so far in Chapters 3,4 and 5 strongly suggest that, across the dose range tested, equimolar intra-Acb infusions of baclofen or muscimol could control feeding behaviour via different mechanisms. Baclofen increased intake of freely available chow at all the doses tested and delayed the onset of satiety without disrupting associated behaviours that constitute the BSS. Baclofen at 220ρmols increased operant responding on a 2nd order schedule during appetitive and consummatory phases without affecting response accuracy. There was a linear relationship between dose and total intake of chow whereas there was a classic inverted U-shaped dose response function for reinforced operant responding. The 220ρmols dose of baclofen also increased headpokes into the magazine in anticipation of pellet delivery and increased rears to the CS. The highest dose of baclofen, 660ρmols, did not affect the duration of grooming in the BSS but disrupted the normal pattern of expression of grooming in the 2nd order schedule. In the latter case this dose also elicited grooming and oral stereotypies.

In contrast while muscimol increased intake of freely available chow and delayed the onset of satiety in the BSS it also significantly decreased the expression of all associated behaviours. While the relationship between dose of muscimol and total chow intake was very similar to that seen with baclofen this $GABA_A$ agonist failed to increase operant responding on the 2nd order schedule. Muscimol did not have any effect on response accuracy. At the highest dose tested, 660ρmols, muscimol reduced the total amount of grooming during the BSS and fragmented grooming behaviour into short bouts during the 2nd order schedule. This high dose also produced a significant amount of short bouts of oral behaviour that did not look like the stereotypy noted with baclofen.

There has been very little evidence published to date to suggest that baclofen and muscimol infused into the Acb could increase feeding via different mechanisms. In his

2007 review of the role of the AcbSh in feeding Stratford suggests that no significant differences had been reported for the effects of either AMPA/kainate receptor antagonists, GABA receptor agonists or in manipulating endogenous GABA levels (Stratford, 2007). In the same year a paper was published that demonstrated that baclofen but not muscimol significantly increased the total amount of time animals spent feeding within a 60 minute period despite the observation that similar amounts of food were consumed (Lopes et al., 2007). Investigators in our laboratory found that intra-AcbSh baclofen did not increase intake of a palatable nutrient solution (Ward et al., 2000) as muscimol reportedly does (Basso and Kelley, 1999, Stratford and Wirtshafter, 2007) although it did change the bout structure of the liquid meal.

While these two observations are hardly compelling alone it is important to remember that, as was highlighted in Chapters 4 and 5, the effects of intra-AcbSh baclofen on feeding have only been previously explored in terms of total intake. Baclofen had not been tested in any other kind of feeding related schedule. By applying some well established paradigms to explore AcbSh GABA mediated feeding I believe that two major differences have emerged 1) muscimol but not baclofen disrupts the normal progression of feeding by increasing intake at the expense of the rest of the behavioural repertoire and 2) baclofen increases unconditioned consummatory responses and complex appetitive instrumental responses both in anticipation of and during access to food.

While one can only speculate what is going on in terms of inter-Acb GABA receptor subtype mediated mechanisms locally that determine output signals from this region it is highly likely that the different classes of behaviour recorded (consummatory vs. instrumental) are subserved by different neuronal macrocircuits. In Chapter 5, I hypothesised that there were indeed different mechanisms mediated by different receptor subtype and that, whether we know what they are or not, we can look for different patterns of activation within brain circuitry involved in motivational processes and the control over ingestive behaviours.

Although anatomical investigations have indicated a variety of downstream components of circuits that could be modulated by activity in the Acb (discussed in Chapter 1) such data does not indicate a functional circuit that subserves any particular class of

behaviour. Functional circuits can be identified with some confidence by comparing data on the ability of the Acb to exert physiological changes, modulate gene expression, influence electrophysiological responses and effect glucose utilisation in each anatomically connected region during a particular behaviour. A functional link can then be further tested by directly manipulating neural activity in apparent downstream sites during Acb manipulations and monitoring the consequences. One way to identify areas of the brain that constitute functional components of the circuits that subserve the behavioural effects of AcbSh manipulations is to look for increases in neuronal activity.

The most widely used method to specifically visualise neuronal activation is the induction of cellular immediate–early genes (IEGs) such as *c-fos,* which are rapidly activated by a variety of stimuli and can be labelled with single cell resolution (e.g. see Sheng and Greenberg, 1990, Hoffman et al., 1993). In the 1980s it was demonstrated that the transduction cascades set in motion by excitatory neurotransmission resulted in the induction of many IEGs including *c-fos* (Greenberg et al., 1986, Sheng and Greenberg, 1990, Morgan and Curran, 1986).

Gene expression following transduction cascades in neurons is mediated by neurotransmitters, electrical membrane activity and neurotrophic growth factors (Sheng and Greenberg, 1990, Hoffman and Lyo, 2002). Furthermore subsets of the 100s of IEGs identified to date are uniquely activated by only one of these pathways (e.g. Dijkmans et al., 2009, Liu et al., 2003). Nevertheless it was demonstrated early on that *c-fos* transcription occurs regardless of the specific route by which a critical influx of Ca^{2+} is triggered (Morgan and Curran, 1986, Greenberg et al., 1986, Sheng et al., 1988) and hence it has come to be viewed as a marker of general neuronal activity following acute stimulation. The low constitutive levels of *c-fos* observed would suggest that it is not a marker of activity *per se* (e.g. Melzer and Steiner, 1997, Cirelli and Tononi, 2000).

The IEG *c-fos* encodes for the protein c-Fos or Fos (Morgan and Curran, 1986). The Fos protein can be localised using simple chromagenic immunocytochemistry. The basic principle of this method is to bind a primary antibody that has specific affinity for the Fos protein and then attach a biotinylated secondary antibody to this that can be chromagenically labelled. In the method used in this chapter a standard peroxidase

avidin-biotin complex (ABC) bound to the biotin on the secondary antibody is used to pick up diaminobenzidine (DAB) stain. This process is illustrated in Fig. 6.1. DAB stained Fos protein is restricted to the nucleus of cells within the CNS (Morgan and Curran, 1991).

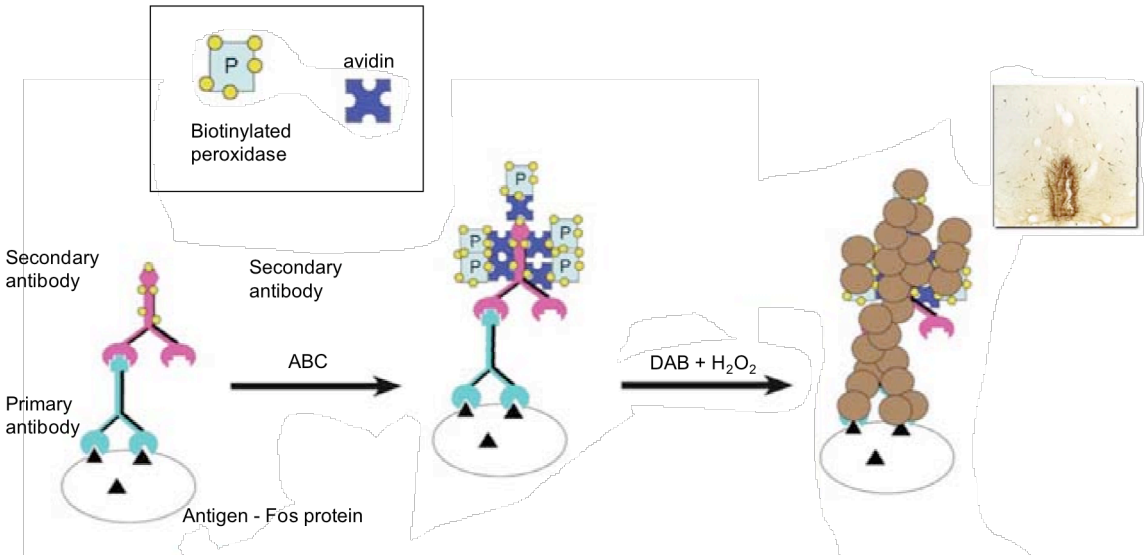


Figure 6.1. Schematic illustration of the principal stages in the immunocytochemical method to visualise the protein Fos in neurons. A primary antibody bound to the antigen of interest reacts with a biotinylated secondary antibody. An avidin / biotinylated peroxidase complex (ABC) will bind to the biotin on the secondary antibody. When incubated with DAB the coloured insoluble product is deposited at sites in proximity to the enzymes (Modified from figures in Hoffman et al., 2008).

The primary antibody for Fos also shows some affinity for other Fos related proteins (FRAs) (Herrera and Robertson, 1996) but the difference in the timing of induction of these (e.g. see Young et al., 1991, Morgan and Curran, 1989) minimises their contribution to marking of "active" neurons. However it is possible that basal levels of staining in 'control' conditions may include binding to the FRAs because they are expressed for longer periods than Fos (i.e. could have been previously activated) and may accumulate long-term (Pennypacker et al., 1995). Thus the use of the Fos protein as a quantifiable marker of *c-fos* expression can be complicated by the potential dual labelling of both Fos and FRAs activated prior to the specific stimulation of interest or related to the behavioural history of the subject. Because of this, neurons with labelled nuclei are sometimes described as exhibiting Fos-like immunoreactivity (FLI), a convention that will be adopted in describing the results in this chapter.

Because baseline levels of *c-fos* expression are low or even undetectable in quiescent cells (Sheng and Greenberg, 1990, Herdegen et al., 1995) inhibition of active brain regions is not easy to pick up. However some regions of the brain do show constitutive expression of *c-fos* (Chan et al., 1993) that could be repressed by inhibitory neurotransmission. Since the particular interest in this thesis lies in the short term acute effects of infusing GABA receptor subtype agonists into the Acb on initiating behaviour the use of an IEG such as *c-fos* as a marker is believed to be both appropriate and adequate for the purpose of highlighting areas of the brain that are activated. FLI has already been demonstrated to be a useful tool in confirming the general patterns of activation within the feeding motivation circuits modulated by the Acb and by specific neurotransmitter activation therein.

Activation in a number of regions of the brain has been reported following both bilateral and unilateral infusions of muscimol into the AcbSh. Stratford (2005) argues however that following bilateral infusions it is not possible to be sure that activation occurs as a result primarily of changes in AcbSh output rather than, for example, as a consequence of secondary systemic effects. Following unilateral infusions unilateral, lateralised activation is a good indicator that there is a direct effect of the AcbSh manipulation on activity in the region in question. Table 6.1, summarises the regions of the brain that are reportedly activated by intra-AcbSh muscimol infusions.

It must be noted that, with the exception of Yoshida et al. (1997) and Stratford (2005), other authors have focused primarily on activation in various nuclei of the hypothalamus and have not systematically identified other regions of the brain activated by intra-AcbSh muscimol infusion. In addition only Stratford (2005) has undertaken quantitative assessment and statistical analysis of the significance of increases in FLI in a number of brain regions. For the majority of regions listed in Table 6.1, it has merely been observed that FLI appears to be greater than in control conditions. In general the pattern of activity in various nuclei throughout the brain corresponds to the primary targets of AcbSh efferents discussed in Chapter 1 including the VP, LH, VTA and SN. Of the regions listed both Yoshida et al. (1997) and Stratford (2005) report primarily ipsilateral induction of activity only in the VP, LH, VTA and SNc.

In the experiments reported in this chapter therefore it was concluded that FLI in the VP, LH, VTA and SNc should be counted following unilateral infusions of baclofen or muscimol. Given that the LH extends quite a distance rostrocaudally, Stratford and Kelley (1999), Zheng et al., (2003), Baldo et al., (2004) and Stratford (2005) all investigated the amount of FLI at a number of different levels throughout the region. FLI in the LH was therefore counted at the four levels defined by Stratford (2005). This meant that it was not possible to process enough sections at the same time to include sections through the level of the SN defined by Stratford (2005) as being the point at which FLI counts were highest so this area was not analysed.

Given the evidence presented in Table 6.1, counts were also made in the Arc, which contains numerous feeding related peptides, and in the PVN which is also critically involved in the control of ingestion (for a review of the role of these regions in feeding (see e.g. Nauta, 1961, Williams et al., 2000, Gao and Horvath, 2007, Williams et al., 2001). Finally it was hypothesised in Chapters 4 and 5 that the effects of baclofen of instrumental responding could relate to changes in incentive motivation e.g. via the saliency of the reward or reward related cues. Given that the amygdala is critically involved in incentive processing (e.g. for a review see Balleine and Killcross, 2006), the Acb indirectly innervates both the CeA and the BLA (See Chapter 1, page 33) and the CeA at least was activated following muscimol infusion (Stratford, 2005) FLI was also counted in these two nuclei of the amygdala complex.

To summarise, evidence presented in Chapters 3, 4 and 5 suggests that there could be differences in the mechanisms by which activation of $GABA_B$ and $GABA_A$ receptors in the AcbSh modulate ingestion and food motivated behaviours. Different mechanisms and consequent patterns of behavioural expression could be subserved by different functional macrocircuits of which the AcbSh is a critical node. One way to identify functional links in such circuits is to identify which areas of the brain are activated following infusions of baclofen or muscimol into the AcbSh using *c-fos* expression as a marker of neuronal activity. Unilateral infusions make it possible to identify direct uncrossed functional links between the AcbSh and regions of the brain activated ipsilaterally. On the basis of evidence from previous studies using muscimol, increases in the amount of FLI will be used as an index of *c-fos* expression to identify activation in the VP, four levels of the LH, the Arc, the PVN, the VTA and in the CeA and BLA.

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Table 6.1. Brain regions activated following intra-AcbSh muscimol application indicated by increases in the number of neurons positive for Fos-like immunoreactivity (FLI). Yoshida et al., (1997) did not quantify any of the increases observed but state that they were ipsilateral to the side that received muscimol. Stratford and Kelley (1999), Zheng et al., (2003) and Baldo et al., (2004) only quantified FLI in the LH. Stratford (2005) quantified FLI in ten brain regions. Accordingly blue ticks represent observed increases not tested statistically. Black ticks represent data that was tested statistically and significant differences are indicated by ★. One tick represents unilateral activation, one large tick and one small tick represents primarily unilateral activation. Two large ticks represents bilateral activation.

Experiments presented in this chapter

Experiment 6.1

The effects of unilateral intra-Acb infusions of baclofen on regional *c-fos* expression.

Experiment 6.2

The effects of unilateral intra-Acb infusions of muscimol on regional *c-fos* expression.

Materials and methods

Animals

Prior to looking at the effects of intra-Acb GABA receptor subtype agonists on Fos induction all subjects had been tested to establish behavioural profiles of these pharmacological manipulations. The animals used for the baclofen study, experiment 6.1, came from experiment 4.3 following testing on the second order operant schedule and for free intake (n=5). Animals used for the muscimol study, experiment 6.2, came from experiment 5.2 following testing on the second order operant schedule and for free intake (n=5). Animals were given a minimum of 7 days post testing with no exposure to either the operant boxes or the intake test environment. They were still maintained on a restricted feeding schedule and fed at the same time of day as before. Fos induction took place during the light cycle across the same period as previous tests had been carried out i.e. between 12.00 and 16.00hrs.

Procedure for Fos induction – central drug administration

Transcription of *c-fos* is rapid and transient starting within 5 minutes and continuing for only 15 to 20 minutes while peak levels of accumulated mRNA occur at 30 – 45 minutes following stimulation (Morgan and Curran, 1991). The protein encoded by *c-fos*, called c-Fos or Fos, starts to appear 30 - 45 minutes post-stimulation and reaches a peak at about 2 hours but the gene is rapidly deactivated (Morgan and Curran, 1986) and the protein broken down, returning to basal levels within 4 hours (Zangenehpour and Chaudhuri, 2002). This time course determined the timing of the experiments carried out to induce Fos.

On the day before testing soiled bedding and sawdust was replaced following feeding to ensure that there was no food hoarded in the home cage. Animals were randomly

allocated to receive unilateral infusions of the appropriate GABA agonist into the right or left cannulae. An identical infusion was made into the second cannulae using vehicle. The unilaterally infused animals were used as their own control on the basis of the arguments put forward by Stratford (2005, 2007) discussed briefly above and explained in more detail on the discussion at the end of the chapter. In both experiments 6.1 and 6.2 animals were given this final infusion following exactly the same procedures described for testing in Chapters 3, 4 and 5. Immediately post infusion animals were replaced in the home cage where they had no access to food.

In experiment 6.1 and 6.2 molar equivalent doses of baclofen and muscimol were used. The dose of baclofen that had previously been demonstrated to significantly and most effectively potentiate instrumental responding whilst also causing an increase in chow intake was chosen i.e. 220ρmols. The molar equivalent dose of muscimol that had also been previously demonstrated to significantly increase chow intake (but that had no effect on operant responding) was used (See Chapter 5). Infusions were temporally staggered to allow time for perfusions. A 90 minute period was allowed for Fos induction and after this period animals were heavily and terminally sedated using pentobarbital. They were then transcardially perfused using filtered phosphate buffered saline (PBS) followed by paraformaldehyde (PFA). Brains were further fixed for 24 hours in PFA and cryoprotected in 30% phosphate buffered sucrose (>48 hours) before being stored at -80^{o}C as described in Chapter 2, page 67.

Tissue preparation for cFos immunocytochemistry

The accuracy of the placements was checked histologically prior to putting sections through the immunohistochemical procedure. First of all brains were brought up to -20^{o}C on a freezing microtome and 60μm coronal sections were taken through the cannulae tracks and any gliosis that extended rostrocaudally. These sections were mounted for anatomy whilst the brains were returned to storage at -80^{o}C. Once the final group for the behavioural analysis for Chapters 4 and 5 had been decided on the basis of the histology a subset of animals were chosen for cFos regional mapping. This group was chosen on the basis of how close the infusion sites were to the target co-ordinates and also on the basis that a minimal amount of visible gliosis was present. A total of n=5 animals from the baclofen group and n=5 animals from the muscimol group went on to the next stage.

These 10 brains were sectioned coronally at 40μm on a freezing microtome and the tissue floated in 0.9% physiological saline. Every 6th section from relevant areas of the brain (see below) were mounted on gelatinized slides and, following alcohol dehydration, run through a standard Nissl staining procedure using Thionin. These were later used to identify landmarks for structural identification of areas of Fos positive nuclei present on the remaining sections. The block of sections started from the point at which the anterior commissure crosses the midline of the brain at approximately β -0.25mm. The most caudal sections were cut at approximately β -5.8mm (see Fig. 6.2).

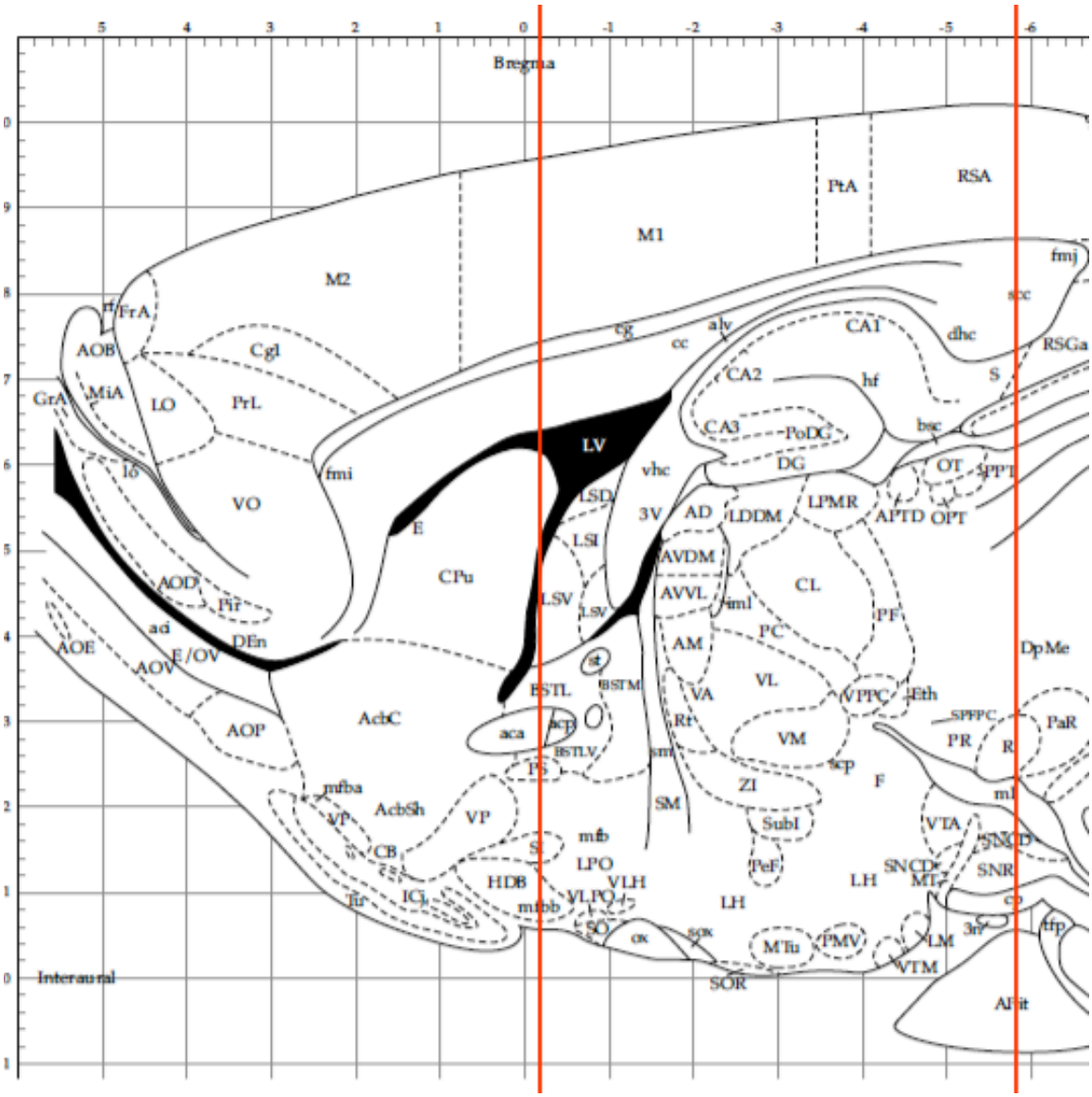


Figure 6.2. Saggital section at 1.4mm from the midline of the brain (ML co-ordinate at which infusions were made showing the extent of brains sectioned for mapping of regional cFos expression (From Paxinos and Watson, 1998).

Method for immunohistochemical staining

The procedures used here are based on a protocol described by Elmquist et al., (1996) and adapted in this laboratory for use with free floating rat brain sections on the basis of a protocol currently used by one of our collaborators (Kalinichev et al., 2000). The resultant protocol does not differ significantly from that described on "Data Sheet PC38" provided by Merck Chemicals Ltd. (UK) with the primary antibody. The final protocol used had previously been demonstrated in this laboratory to produce low background staining but a high immunocytochemical signal in the nuclei of neurons in rat brain slices (unpublished data). In each run at least two brains worth of sections were processed and stained simultaneously. Thus each run included brains from experiment 6.1 (baclofen) and 6.2 (muscimol).

Following removal of sections for anatomy alternate sections from those remaining were transferred into Tris buffered PBS/0.3% Triton X-100 solution, pH 7.4 (immuno buffer). Sets of three sections in sequence were placed into 1ml of immuno buffer in individual wells of a 25 well disposable plastic Petri dish (Bibby Sterilin Ltd., UK). Three more sections were taken randomly from the beginning, middle and the end of the run and placed in the final well to be used as negative controls for non-specific binding. Unless otherwise stated all washing and incubation stages described below took place at room temperature on an orbital shaker.

Sections were first washed in immuno buffer for 15 minutes. The immuno buffer was pipetted off and the tissue was then incubated for 30 minutes in 1% hydrogen peroxide to remove endogenous peroxidases. This was followed by two 10 minute washes in immuno buffer. Next, non-specific antibody binding was blocked by incubating the sections for 1 hour in 2% goat serum (Sigma, UK) diluted in immuno buffer. The brain sections were then incubated for approximately 48 hours at 4^{o}C to 2mls per well of the primary (1^{o}) antibody (a 1:20,000 dilution of Anti-c-Fos (Ab-5) Rabbit pAb in immuno buffer, Calbiochem supplied by Merck Chemicals Ltd., UK). The 1^{o} antibody was omitted at this stage for the negative control sections in the last well.

On removal from the fridge sections were brought up to room temperature and then taken through three 10 minute washes in immuno buffer to remove all traces of excess 1^{o} antibody. They were then incubated for 2 hours in 0.8ml per well of the secondary

(2^o) antibody (a 1:20,000 dilution of biotinylated goat anti-rabbit IgG (H+L) in immuno buffer, Vector Laboratories, USA). During this incubation period a horseradish peroxidase avidin-biotin complex (ABC) reagent from an Elite standard peroxidase ABC kit (Vector Laboratories, USA) was made up using 12 drops each of reagents A and B diluted in 30mls of immuno buffer and left to stand for at least 30 minutes prior to use.

Unbound 2^o antibody was removed with three 10 minute washes in immuno buffer. A graduated pipette was used to add 0.6mls of ABC reagent to each well and sections were incubated for 2 hours. The sections were taken through a further three 10 minute washes using a Tris-0.84M HCl buffer (Tris-HCl), pH 7.4. For the final reaction diaminobenzidine (DAB; 0.035%) was made up using a DAB Kit for peroxidase (Vector Laboratories, USA) following the manufacturers instructions. The nickel-ammonium sulphate solution was excluded thus giving a brown coloured product not black/grey. 0.6ml of the DAB solution was pipetted into each well and the sections incubated for 10 minutes. Care was taken to make sure all the sections were submerged and the speed of the shaker was turned up to ensure that sections were well exposed to the DAB.

The reaction was terminated by washing the sections for 10 minutes with Tris-HCl buffer. Finally the sections were given a last wash in 0.9% physiological saline from which they were mounted on gelatinised slides. Slides were air dried and the sections fixed in formaldehyde vapour for 1 hour. Sections were cleared in Histoclear (R.A. Lamb, UK) then cover slipped with Histomount (R.A. Lamb, UK). Once the mounting agent had fully set, slides were cleaned and polished with IMS.

Light microscopy and image analysis

For the purposes of evaluating Fos localisation and to allow counting of fos positive nuclei, tissue sections were viewed with a Zeiss Akioskop 2 plus microscope and images of sections were captured using an AxioCam HRc digital camera (Carl Zeiss, UK) using AxioVision 3.1 software (Imaging Associates, Bicester, UK). Once the FLI stained sections had been matched up to the anatomy sections the relevant brain areas

were identified and marked up. Individual coronal sections representing target regions were matched between animals.

Criteria used to identify and define brain areas to be analysed

Areas of interest and their respective coordinates were predominantly chosen on the basis of the AP coordinates for representative sections used by Stratford (2005) from the Paxinos and Watson (1998) 4th edition rat brain atlas. In this case LH counts were made in sections through the brain at β - 1.6, β - 2.6, β - 3.6 and β - 4.3 (See Fig. 6.3). Counts for the PVN and Arc were made at β - 1.88 and β - 2.8 respectively (See Fig. 6.3). Counts for the CeA were made in sections at β - 3.14 (See Fig. 6.3).

Where co-ordinates for the area of interest had not been specified by Stratford (2005) for the VP, BLA and VTA these were chosen on the basis of the highest level of staining recorded within available sections through the relevant structure for each animal. These were matched within the same series of three sections for that region between animals. Only the region of the VP caudal to the point at which the anterior commissure transacted the midline could be analysed because more rostral sections were damaged by the guide cannulae or exhibited gliosis around the cannulae tract. Nevertheless clear areas of Fos immunoreactivity were distinguishable at approximately β - 0.26mm (See Fig. 6.3).

In the case of the BLA the sections chosen were slightly posterior to those where counts were made for the CeA within - 0.16mm of the coordinates for the CeN i.e. at β - 3.30mm (See Fig. 6.3). The sections used for counts of FLI in the VTA were partially dependent on how far back those sections that fitted into one run of the labelling procedure went. However consistent areas of increased FLI were seen at β - 5.30mm where an obvious region of VTA cells in located (See Fig. 6.3).

For those areas of the brain that could be clearly delineated visually on the basis of anatomical markers, a template was created from the relevant Nissl stained slide that could be superimposed onto the Fos stained sections. One template was created for each brain area based on the shape and size of the structure in the rat that exhibited the most extensive Fos staining in that structure (See Fig. 6.3 for outlines). No nuclei were

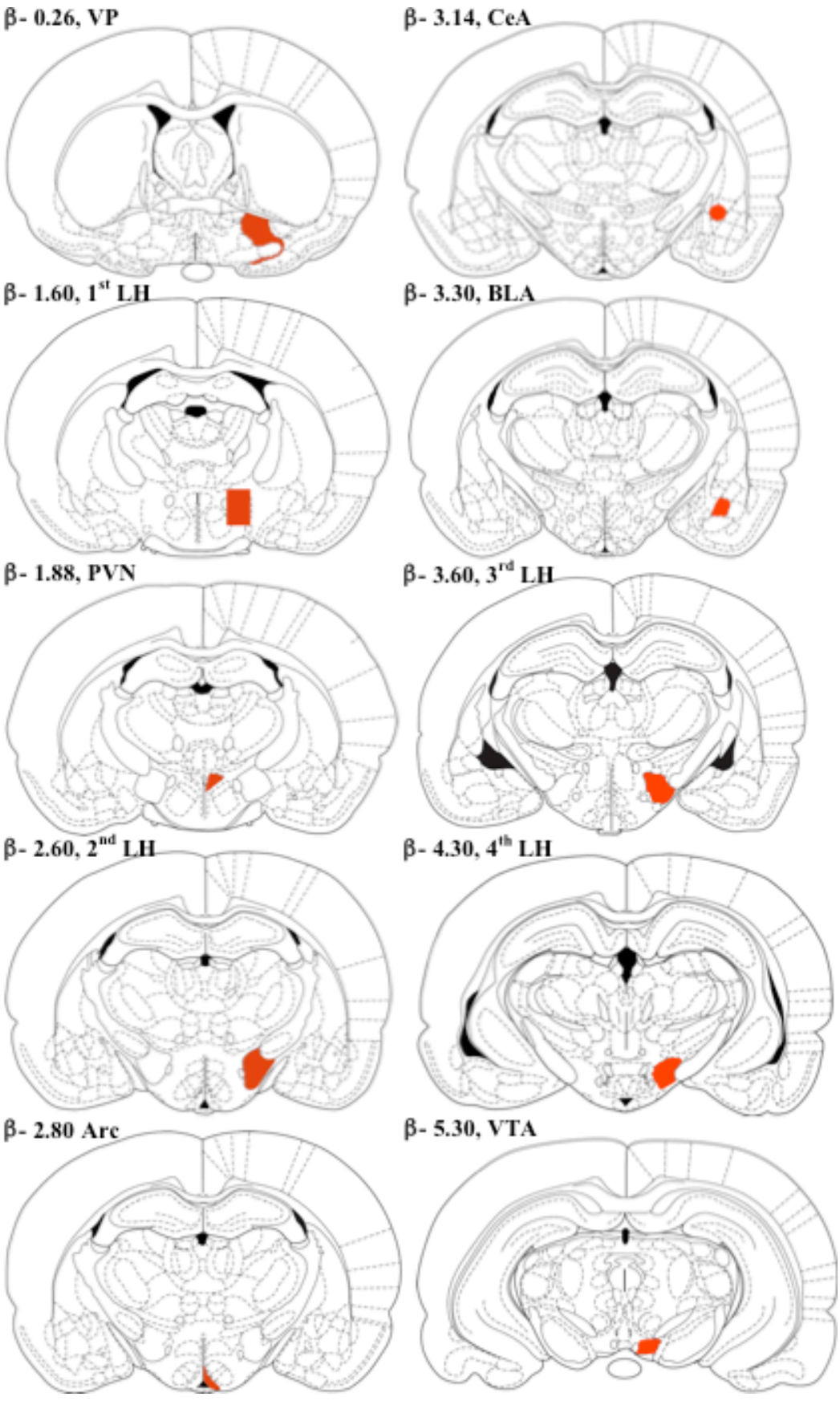
missed in the brains with the highest levels of activation due to some falling outside the boundary of a template and all were encompassed within the template when levels were low.

Where a specific structure could not be delineated clearly a rectangular template was created representing a sample within the area of interest. This was only used for the rostral most level of the LH and the boundaries and size of the rectangular template in this case were based on those defined by Baldo et al., (2004). Following the approach employed by (Baldo et al., 2004) the division between medial and lateral hypothalamus was based on the position of the fornix. Using ImageJ software the cross section of the fornix was measured at its widest point. A dorsoventrally orientated line was drawn bisecting the hypothalamus through the midpoint of the fornix and the rectangular template was placed using this line as its medial boundary (See Fig. 6.3).

Method used for counting FLI positive cells

Counting was automated using ImageJ 1.4 for Mac which is an open source, image processing and analysis Java programme developed by the Research Services Branch of the National Institute of Mental Health, a division of the National Institutes for Health (USA). Photomicrographs of areas of Fos induction were transferred into ImageJ in .tif format and converted from RGB colour to 8-bit greyscale. A template for the relevant brain area was pasted onto the image and rotated to for the structure in question. The remainder of the image outside this template was cleared and the section to be counted saved as a new image.

Figure 6.3. (Next page) Illustrations from Paxinos and Watson (1998) of brain sections at stereotaxic coordinates at which ten regions of the brain were located in which neurons expressing FLI were counted following intra-AcbSh GABA receptor agonist infusions. The red shading represents the approximate shape of the templates used to delineate each region for the purposes of counting labelled nuclei. Counts were made bilaterally both in the side that was infused with muscimol and the side infused with saline.


β- 0.26, VP
β- 3.14, CeA
β- 1.60, 1st LH
β- 3.30, BLA
β- 1.88, PVN
β- 3.60, 3rd LH
β- 2.60, 2nd LH
β- 4.30, 4th LH
β- 2.80 Arc
β- 5.30, VTA

Appropriate functions in ImageJ were used to clear up background noise and, if necessary, to adjust the brightness and contrast to highlight those cells that would have been identified as labelled in a count by hand. Next the thresholds for the density of staining to be included as "labelled" were set. Initially thresholds were set by eye so that the software whilst minimising overlap highlighted the maximum number of fos positive cells. For each brain area to be counted an outline was drawn around the smallest cell and largest cells that appeared to be Fos positive and the area of these in pixels was recorded.

A boundary for inclusion based on area in pixels was then calculated at each magnification to encompass the smallest and largest cells. A preliminary count was then made. The mean size of the cells counted was checked and compared to the previous measures for representative cells at that magnification. If this did not match to within 0.0001 units the threshold was adjusted accordingly and the count re-run. Once the mean size of cells for each brain area for each animal were in agreement the final counts were made and the output transferred to excel.

A subgroup of sections from each region of the brain was also counted by hand to check the accuracy of the automated counting. This confirmed that there was never any more than a 1% difference between automated counts and hand counts made by eye. This agreed well with an extensive pilot study carried out in this laboratory to verify the use of the ImageJ software to quantify fos positive nuclei at various magnifications. In this large-scale comparison it was found that the mean manual counts were within 1.5% of the automated counts (Horwood 2007, unpublished data).

Data analysis

Total mean numbers of nuclei exhibiting FLI were compared for each drug using a within subjects, repeated measures ANOVA with treatment (drug or saline) and brain area as factors. Where there was a significant interaction between treatment and area the ANOVA was then restricted to individual levels of the brain. A comparison was also made between drug treatments using a repeated measures, mixed design ANOVA using drug type, treatment (drug or saline) and brain area as factors.

Results

Experiment 6.1: **The effects of unilateral intra-Acb infusions of baclofen on regional c-fos expression.**

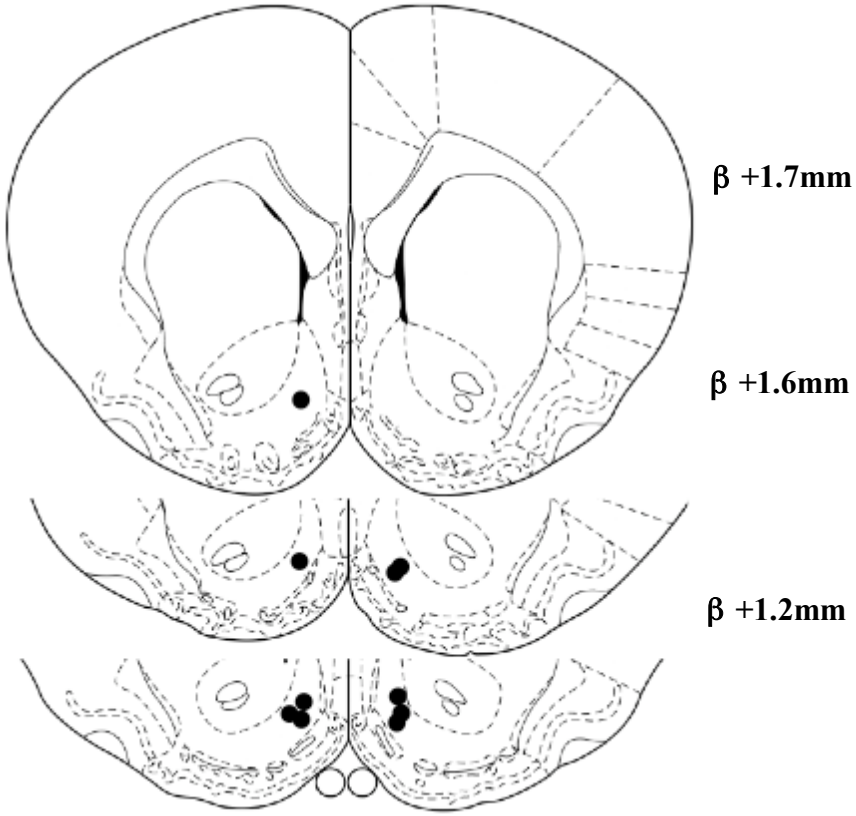


Figure 6.4. Injection sites plotted on drawings taken from Paxinos and Watson (1998); sections are anterior relative to bregma (β). Placements for n=5 of original n=8 animals from experiment 4.3, Chapter 4. Bilateral target coordinates were (AP), + 1.4mm, mediolateral (ML), ± 0.9mm relative to bregma and dorsoventral (DV), -7.8mm relative to skull surface.

The location of infusions in the group of n=5 animals used are shown in Fig. 6.4. Unilateral infusions of 220pmols baclofen into the AcbSh of n=5 rats (of n=8 used in Experiment 4.3, Chapter 4) resulted in a significant main effect of drug [$F(1,4)=433.72$, $p<0.001$] on the mean number of FLI cells which was increased in the side ipsilateral to the infusion compared to the contralateral saline infused side across the areas investigated. There was also a main effect of area [$F(9,36)=15.93$, $p>0/001$] and an interaction between drug and area [$F(9,36)=27.94$, $p<0.001$].

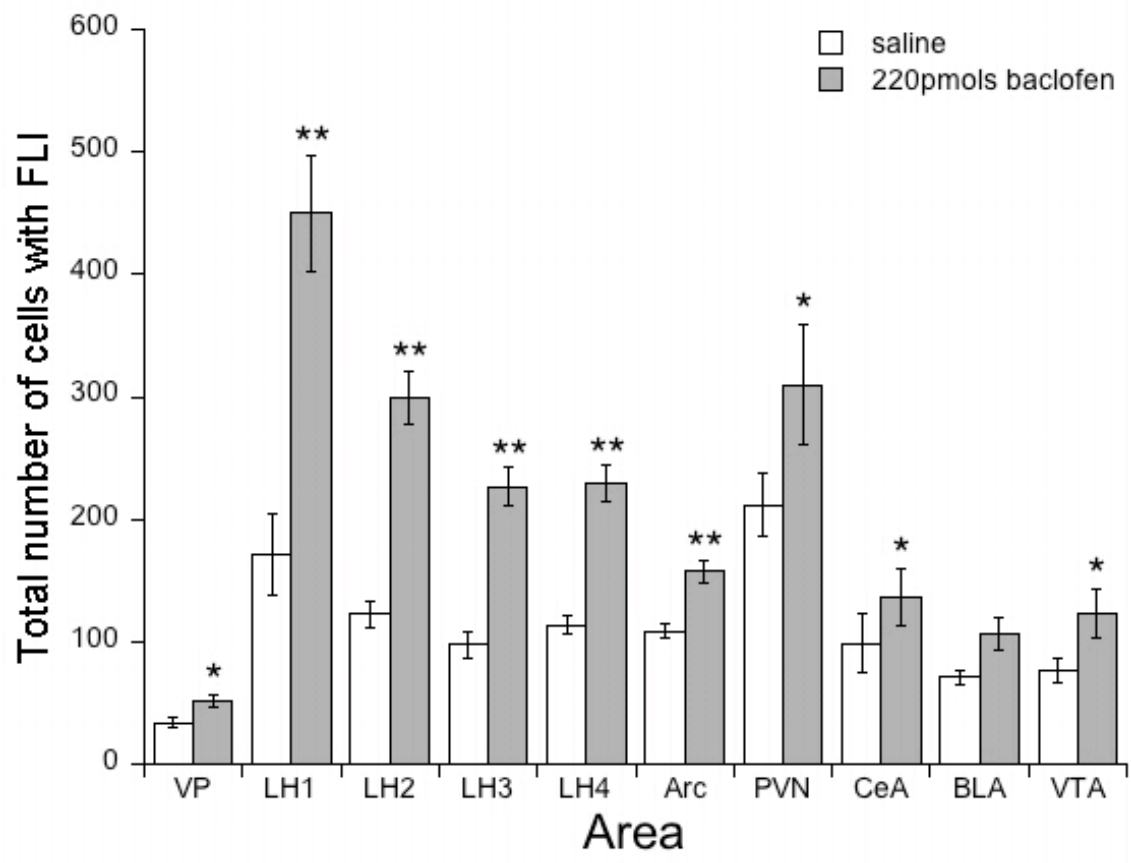


Figure 6.5. The number of cells with FLI in the side of the brain infused with 220pmols baclofen or in the side infused with saline in ten different regions (n=5). Error bars represent ±SEM. Significant differences from vehicle are denoted by ⋆ p<0.05 and ⋆⋆ p<0.01.

When the analysis was restricted to individual areas of the brain this dose of baclofen significantly increased the mean number of FLI cells throughout the rostrocaudal extent of the LH investigated in the side ipsilateral to the drug infusion compared to the saline infused side (See Fig. 6.5). The mean FLI count was significantly increased in the first LH level [$F(1,4)=233.62$, $p<0.001$], in the second LH level [$F(1,4)=84.26$, $p<0.001$], in the third LH level including the perifornical nucleus [$F(1,4)=94.14$, $p<0.001$] and in the most caudal fourth level of the LH [$F(1,4)=43.92$, $p=0.003$]. Representative sections are shown in Fig 6.6.

The mean FLI count was also significantly increased in the side ipsilateral to the drug infusion compared to the saline infused side in the PVN [Fig. 6.5; $F(1,4)=15.2$, $p=0.018$], Arc [Fig. 6.5; $F(1,4)=74.37$, $p<0.001$], VP [Fig. 6.5; $F(1,4)=17.74$, $p=0.014$], VTA [Fig. 6.5; $F(1,4)=10.59$, $p=0.031$] and in the CeA but not the BLA [Fig. 6.5; $F(1,4)=16.75$, $p=0.015$]. Representative sections are shown in Figs 6.7, 6.8 and 6.9.

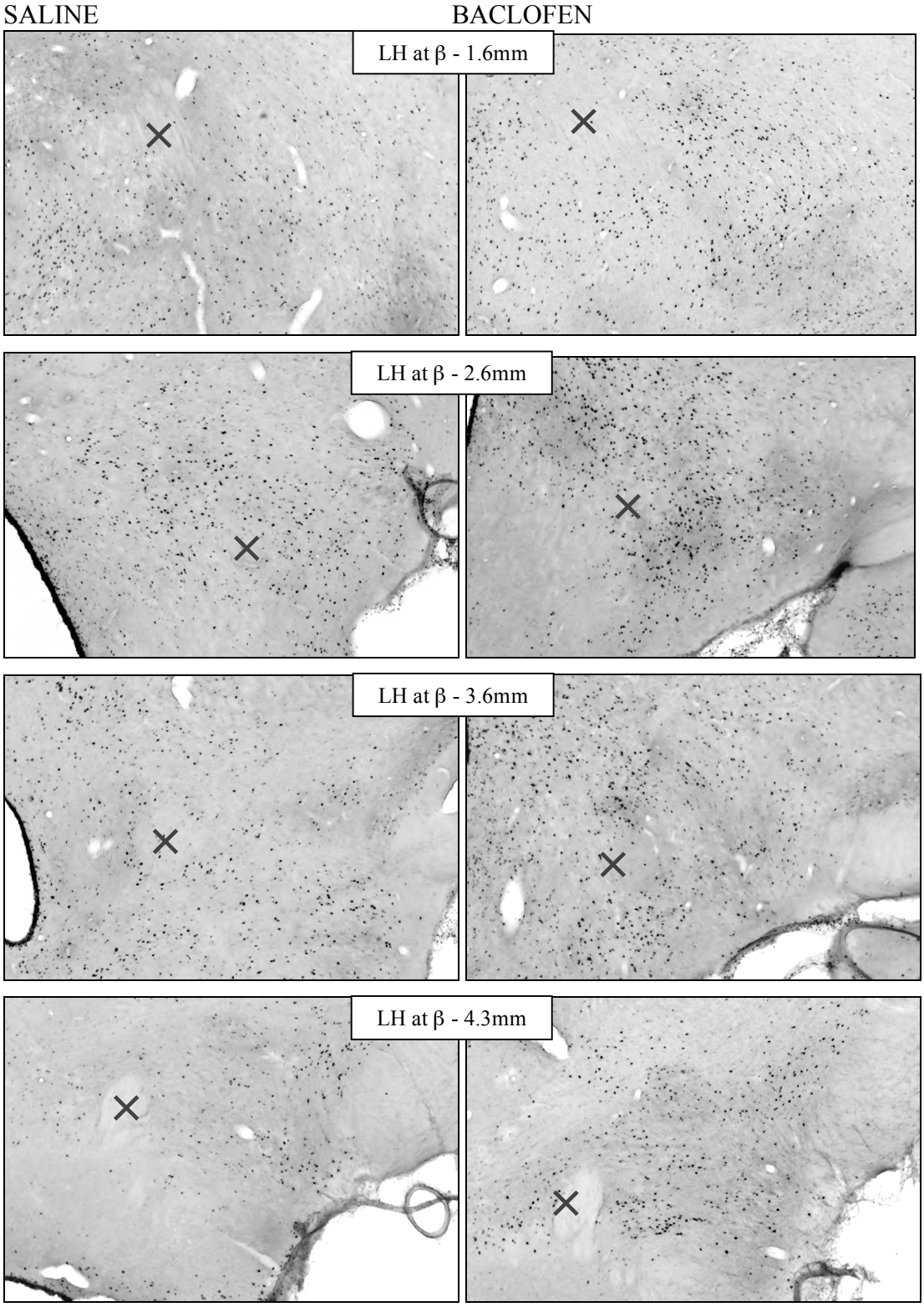


Figure 6.6. Photomicrograph of representative sections showing FLI in cells in four levels of LH in the side of the brain infused with saline (left column) and the opposite side infused with 220pmols baclofen (right column). The fornix is marked with a cross and the areas to the right are classed as LH. In each case the image is shown with medial structures to the left and lateral to the right regardless of which side of the brain it came from. Pictures were taken at a magnification of 50x. Images have been resized and computer enhanced for the purposes of clarity .

SALINE BACLOFEN

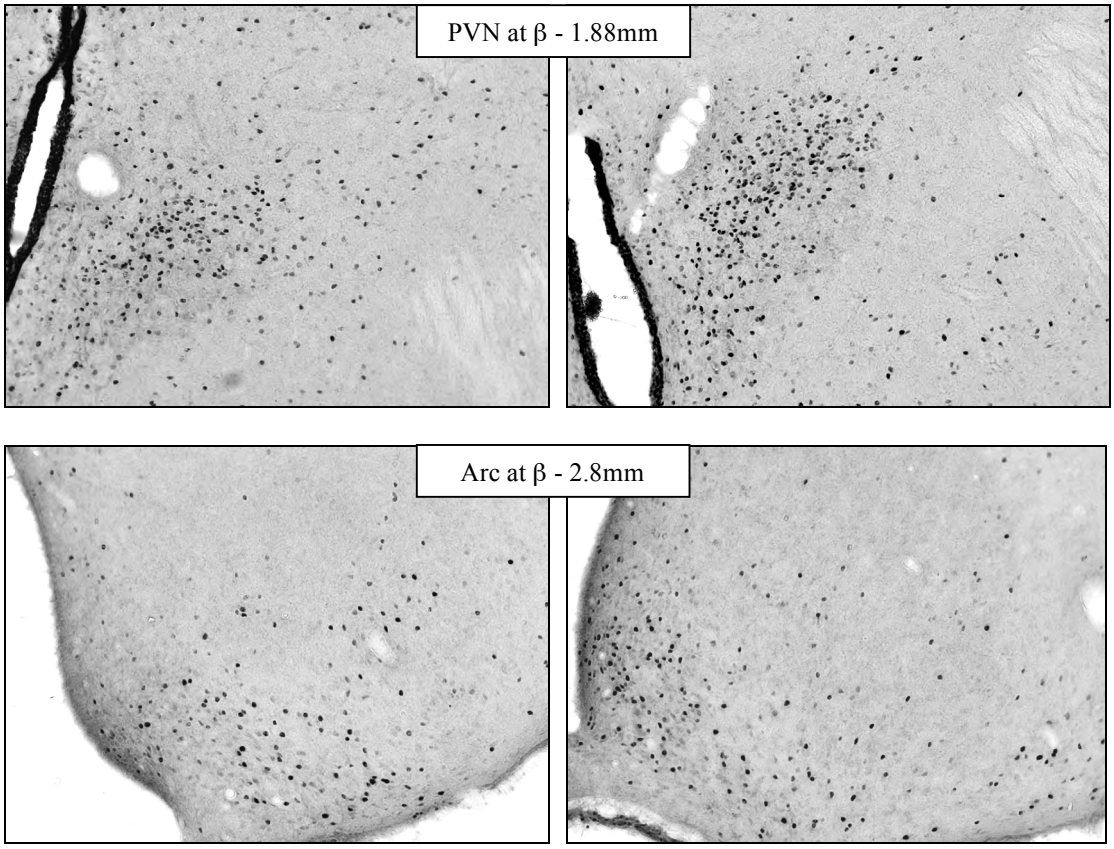


Figure 6.7. Photomicrograph of representative sections showing FLI in cells in the PVN and Arc in the side of the brain infused with saline (left column) and the opposite side infused with 220pmols baclofen (right column). In each case the image is shown with medial structures to the left and lateral to the right regardless of which side of the brain it came from. Pictures of both the PVN and Arc were taken at a magnification of 100x. Images have been resized and computer enhanced for the purposes of clarity .

SALINE BACLOFEN

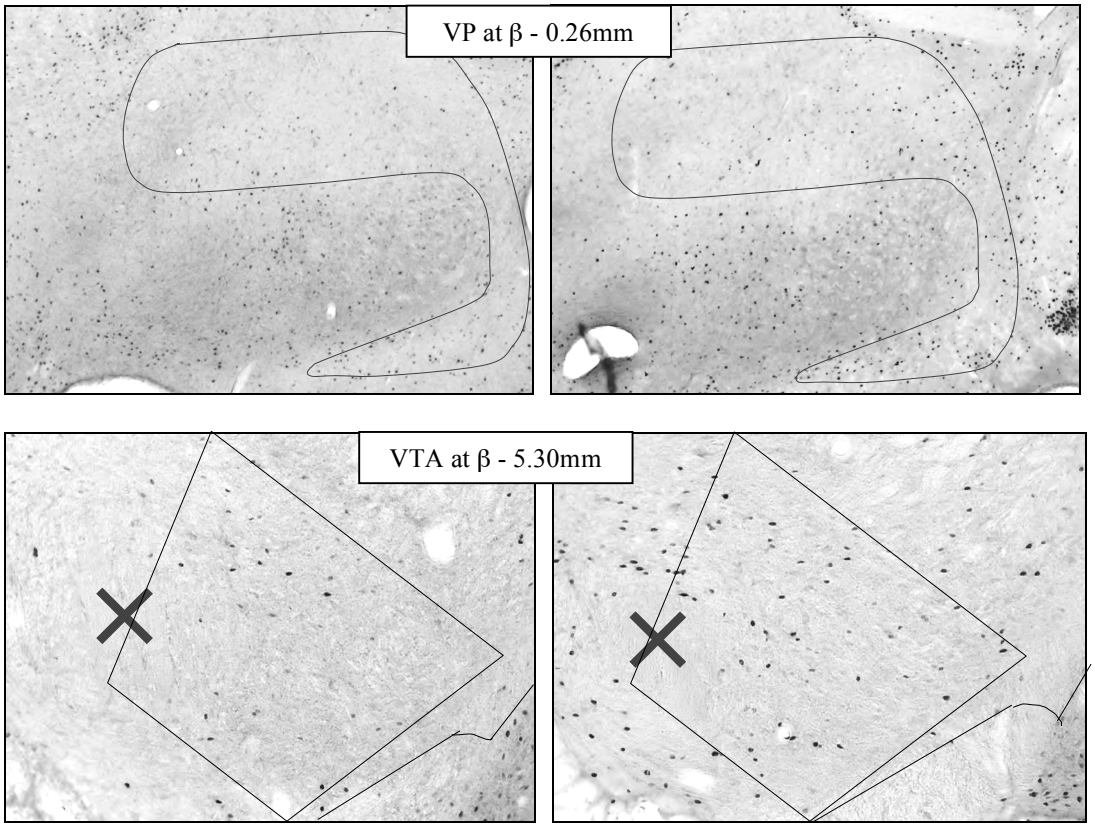


Figure 6.8. Photomicrograph of representative sections showing FLI in cells in the VP and VTA in the side of the brain infused with saline (left column) and the opposite side infused with 220pmols baclofen (right column). The outline of the area classed as the VP is shown which excludes the basal region of the substantia innominata medially and the magnocellular preoptic nucleus that crosses the VP ventrally. The VTA was classed as the region bounded medially by the fasciculus retroflexus (marked with a cross) and laterally by the mammillary peduncle/ medial terminal nucleus of the accessory optic tract (structures to the right of the line). The approximate area in which VTA counts were made is outlined. In each case the image is shown with medial structures to the left and lateral to the right regardless of which side of the brain it came from. Pictures of the VP were taken at a magnification of 50x and of the VTA at 100x. Images have been resized and computer enhanced for the purposes of clarity .

SALINE BACLOFEN

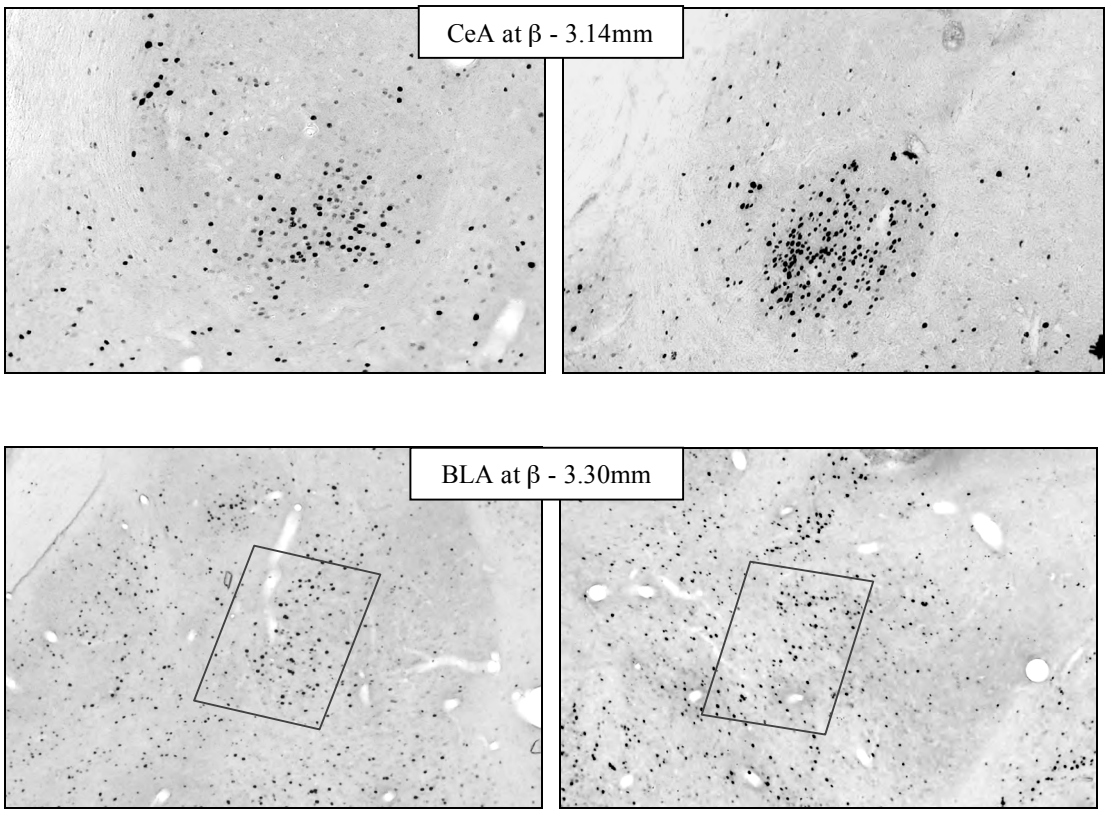


Figure 6.9. Photomicrograph of representative sections showing FLI in cells in two nuclei of the amygdala in the side of the brain infused with saline (left column) and the opposite side infused with 220pmols baclofen (right column). While the outline of the CeA is clearly visible the area that circumscribes the BLA is marked with a box. In each case the image is shown with medial structures to the left and lateral to the right regardless of which side of the brain it came from. Pictures of the CeA were taken at a magnification of 100x and of the BLA at 50x. Images have been resized and computer enhanced for the purposes of clarity .

Experiment 6.2: **The effects of unilateral intra-Acb infusions of muscimol on regional c-fos expression.**

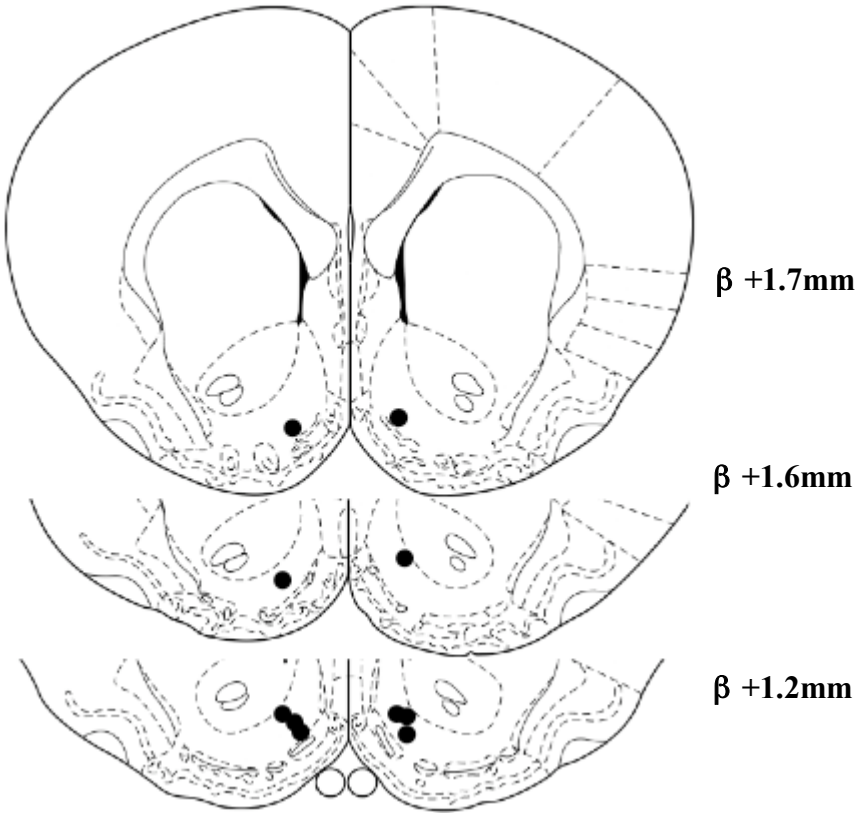


Figure 6.10. Injection sites plotted on drawings taken from Paxinos and Watson (1998); sections are anterior relative to bregma (β). Placements for n=5 of original n=8 animals from experiment 5.2, Chapter 5. Bilateral target coordinates were (AP), + 1.4mm, mediolateral (ML), ± 0.9mm relative to bregma and dorsoventral (DV), -7.8mm relative to skull surface.

The location of infusions in the group of n=5 animals used are shown in Fig. 6.10. Unilateral infusions of 220pmols muscimol into the AcbSh of n=5 rats (of n=8 used in Experiment 5.2, Chapter 5) resulted in a significant main effect of drug [$F(1,4)=99.18$, $p>0.001$] on the mean number of FLI cells which was increased in the side ipsilateral to the infusion compared to the contralateral saline infused side across the areas investigated. There was also a main effect of area [$F(9,36)=36.8$, $p>0.001$] and an interaction between drug and area [$F(9,36)=24.4$, $p>0.001$].

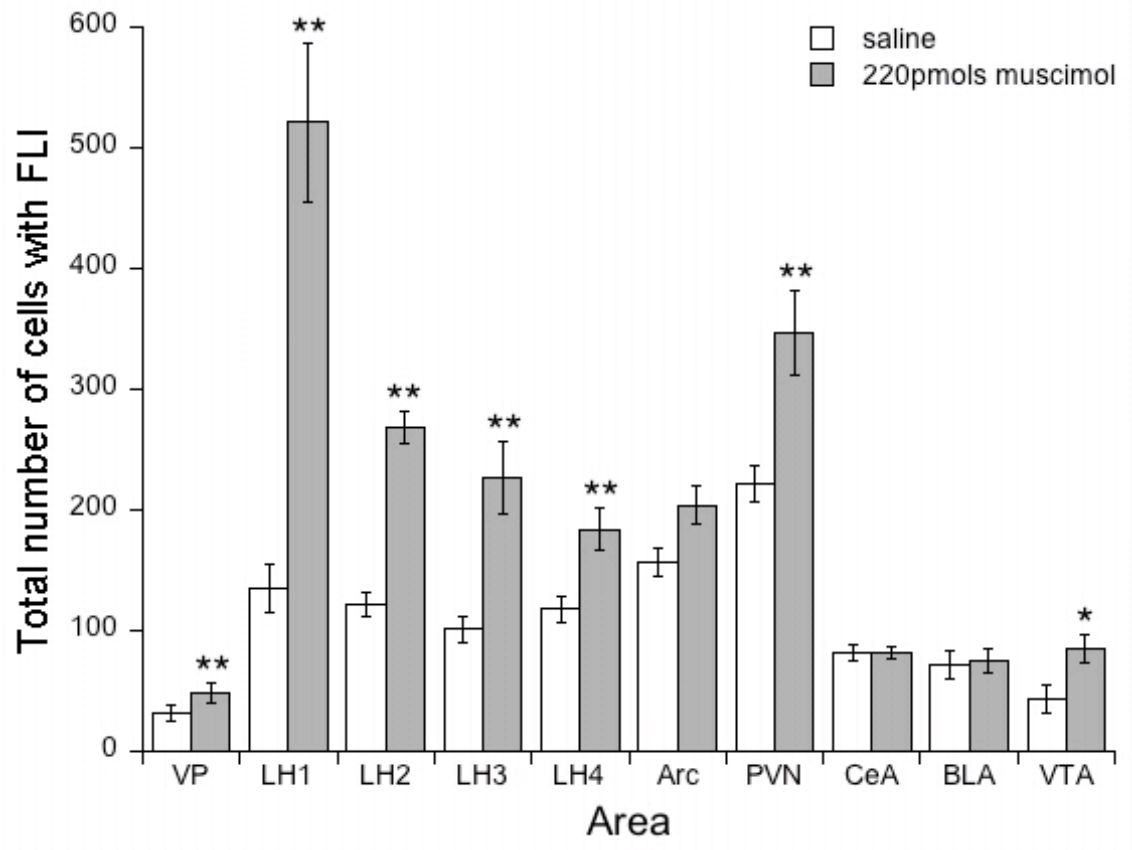


Figure 6.11. The number of cells with FLI in the side of the brain infused with 220pmols muscimol or in the side infused with saline in ten different regions (n=5). Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ p<0.05 and ★★ p<0.01.

When the analysis was restricted to individual areas of the brain this dose of muscimol significantly increased the mean number of FLI cells throughout the rostrocaudal extent of the LH in the side ipsilateral to the drug infusion compared to the saline infused side (See Fig. 6.11). The mean FLI count was significantly increased in the first LH level [F(1,4)=43.94, p=0.003], in the second LH level [F(1,4)=47.67, p=0.002], in the third LH level including the perifornical nucleus [F(1,4)=34.19, p=0.004] and in the fourth level of the LH [F(1,4)=10.24, p=0.033]. Representative sections are shown in Fig 6.12.

The mean FLI count was also significantly increased in the side ipsilateral to the drug infusion compared to the saline infused side in the PVN [Fig. 6.11; F(1,4)=30.14, p=0.005], VP [Fig 6.11.; [F(1,4)=53.78, p=0.002] and in the VTA [Fig. 6.11; [F(1,4)=17.3, p=0.014]. There was no significant difference between sides in the Arc, CeA or BLA. Representative sections are shown in Figs 6.13, 6.14 and 6.16.

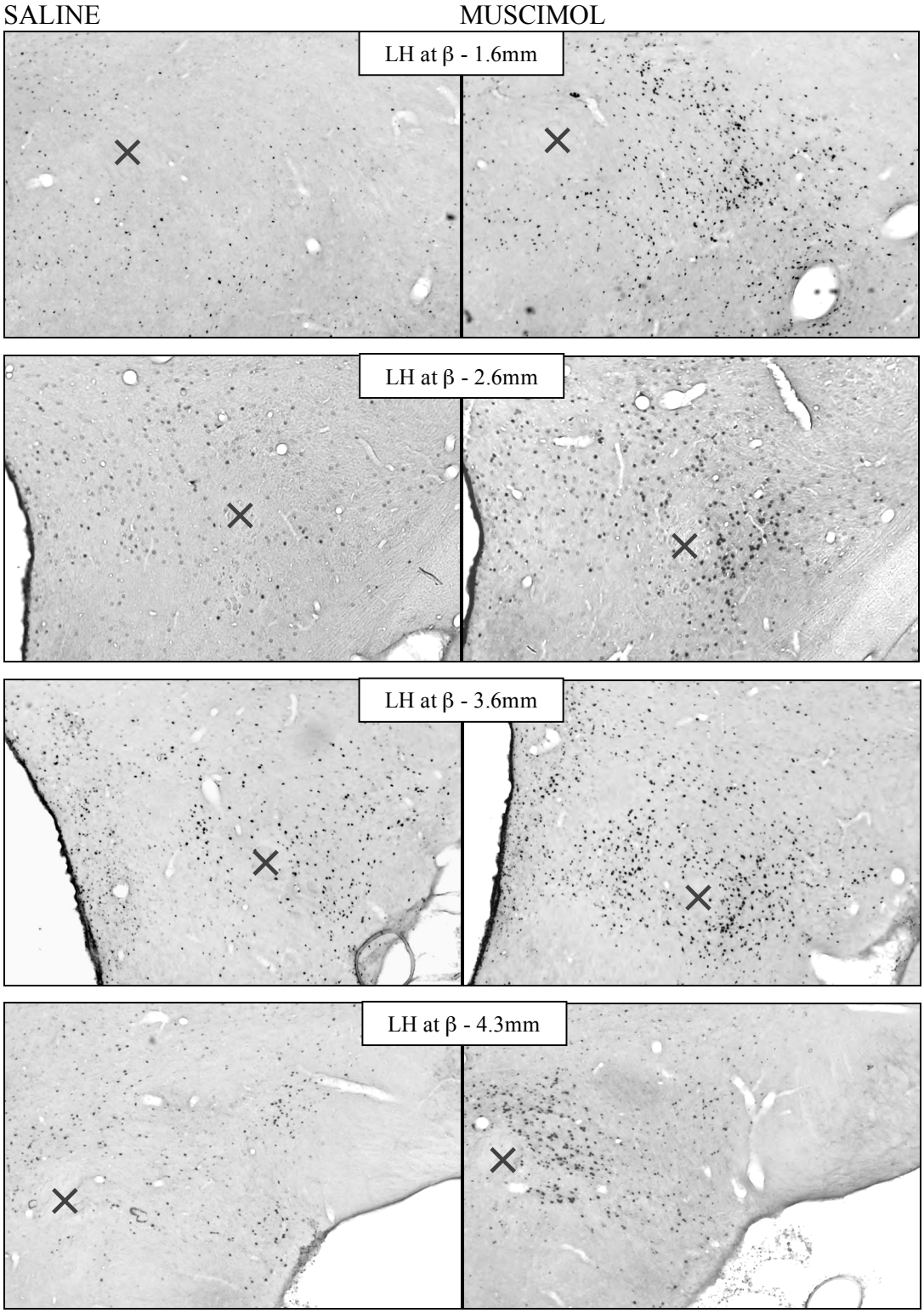


Figure 6.12. Photomicrograph of representative sections showing FLI in cells in four levels of LH in the side of the brain infused with saline (left column) and the opposite side infused with 220pmols muscimol (right column). The fornix is marked with a cross and the areas to the right are classed as LH. In each case the image is shown with medial structures to the left and lateral to the right regardless of which side of the brain it came from. Pictures were taken at a magnification of 50x. Images have been resized and computer enhanced for the purposes of clarity .

SALINE MUSCIMOL

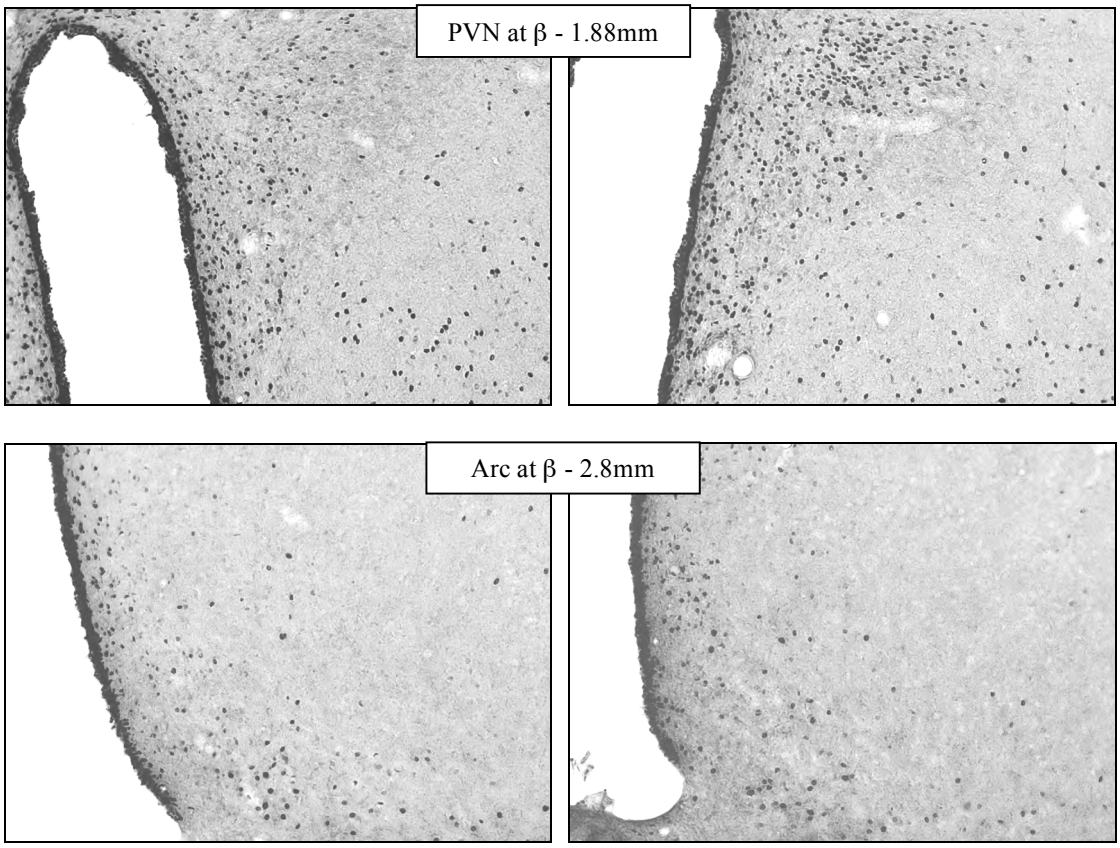


Figure 6.13. Photomicrograph of representative sections showing FLI in cells in the PVN and Arc in the side of the brain infused with saline (left column) and the opposite side infused with 220pmols muscimol (right column). In each case the image is shown with medial structures to the left and lateral to the right regardless of which side of the brain it came from. Pictures of both the PVN and Arc were taken at a magnification of 100x. Images have been resized and computer enhanced for the purposes of clarity .

SALINE MUSCIMOL

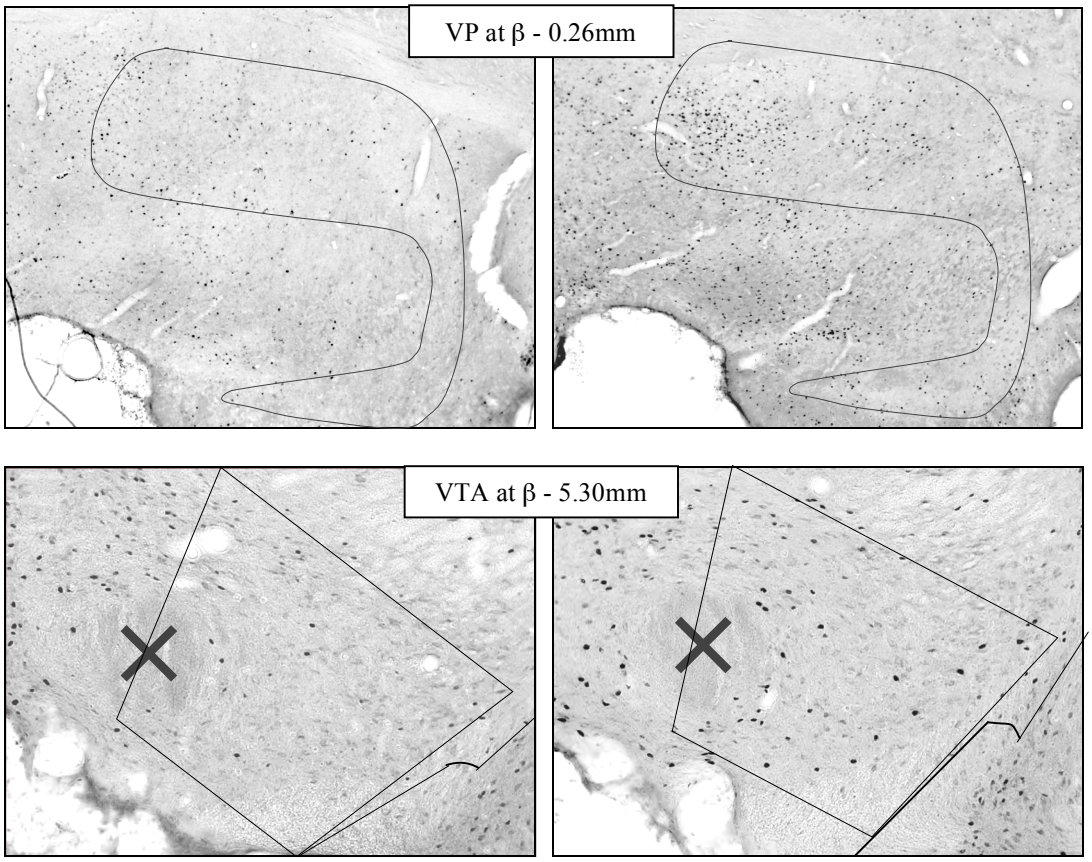


Figure 6.14. Photomicrograph of representative sections showing FLI in cells in the VP and VTA in the side of the brain infused with saline (left column) and the opposite side infused with 220pmols muscimol (right column). The outline of the area classed as the VP is shown which excludes the basal region of the substantia innominata medially and the magnocellular preoptic nucleus that crosses the VP ventrally. The VTA was classed as the region bounded medially by the fasciculus retroflexus (marked with a cross) and laterally by the mammillary peduncle/ medial terminal nucleus of the accessory optic tract (structures to the right of the line). In each case the image is shown with medial structures to the left and lateral to the right regardless of which side of the brain it came from. Pictures of the VP were taken at a magnification of 50x and of the VTA at 100x. Images have been resized and computer enhanced for the purposes of clarity .

SALINE MUSCIMOL

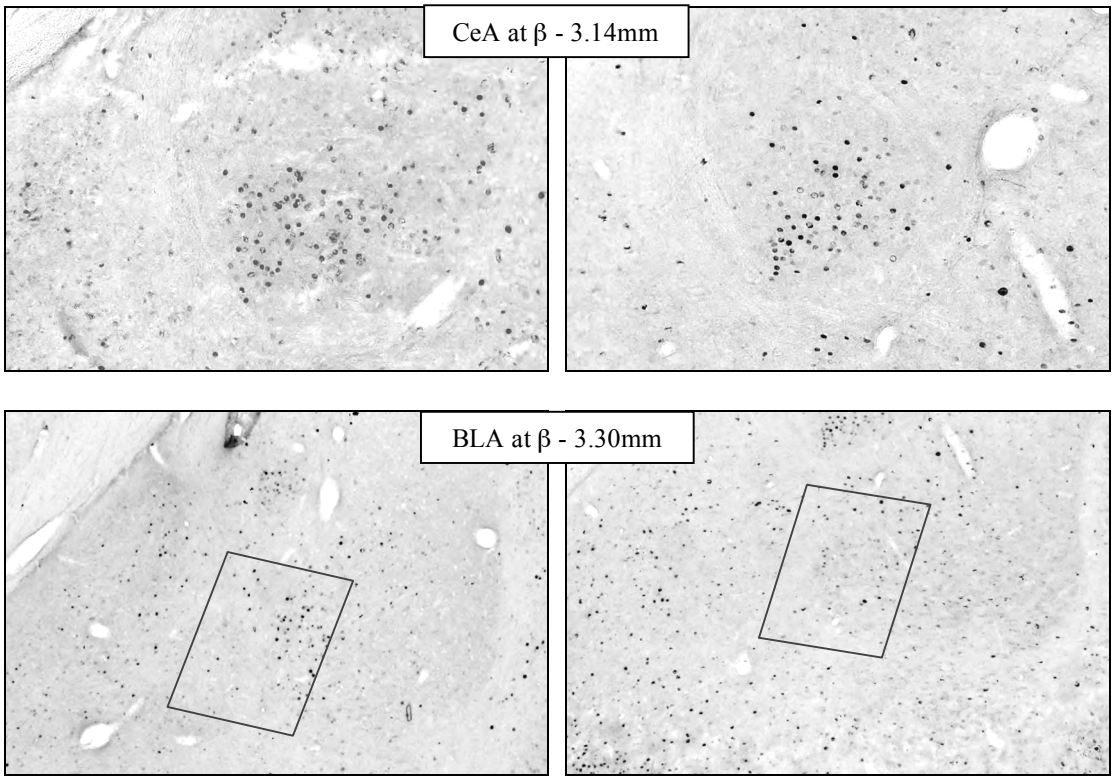


Figure 6.15. Photomicrograph of representative sections showing FLI in cells in two nuclei of the amygdala in the side of the brain infused with saline (left column) and the opposite side infused with 220pmols baclofen (right column). While the outline of the CeA is clearly visible the area that circumscribes the BLA is marked with a box. In each case the image is shown with medial structures to the left and lateral to the right regardless of which side of the brain it came from. Pictures of the CeA were taken at a magnification of 100x and of the BLA at 50x. Images have been resized and computer enhanced for the purposes of clarity .

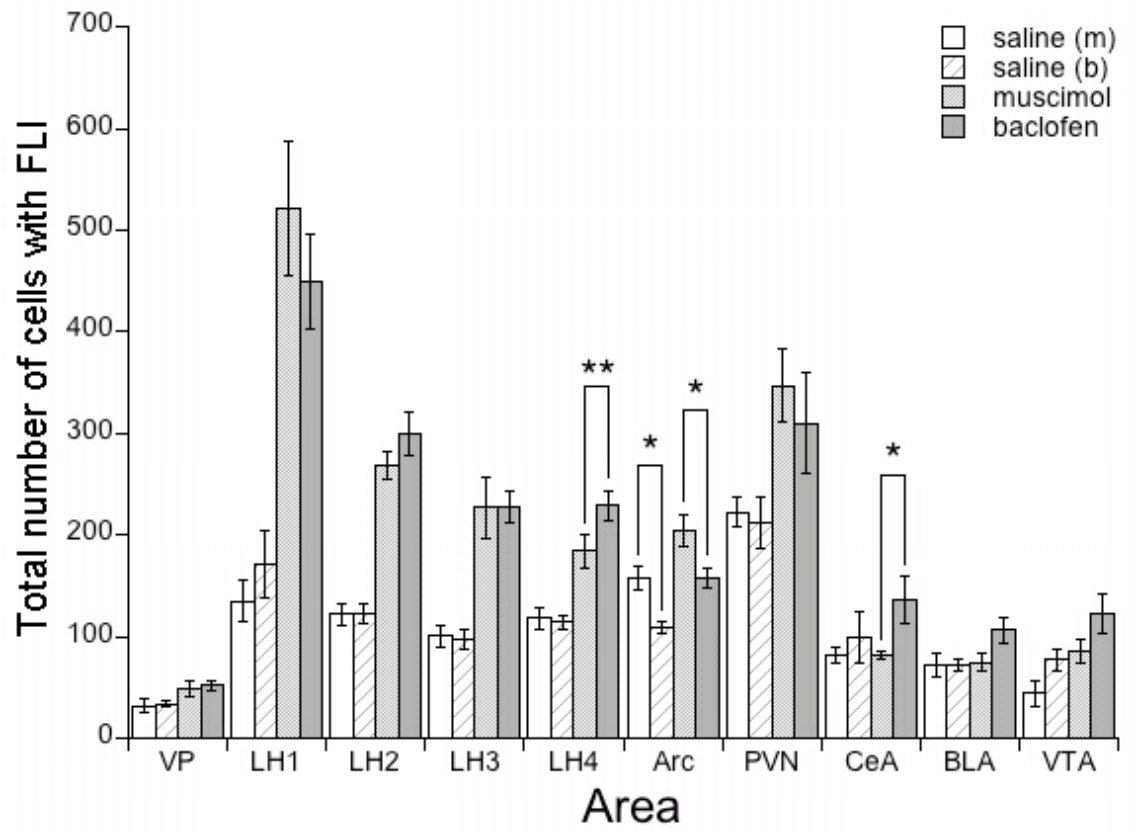


Figure 6.16. Comparison between the total number of cells with FLI in the side of the brain infused with 220pmols baclofen or muscimol or between the opposite sides infused with saline in ten different regions (n=10). Error bars represent ±SEM. Significant differences from vehicle are denoted by ★ p<0.05 and ★★ p<0.01.

Following infusion of muscimol or baclofen sections from both experiments were run through the staining procedure simultaneously to allow direct comparison of the number of labelled neurons. Comparing the effects of baclofen and muscimol on the total number of FLI cells in the various regions of the brain investigated (See Fig. 6.16). revealed that baclofen increased FLI counts significantly more than muscimol in the most caudal fourth level of the LH and in the CeA. There was no difference in the number of FLI cells in the contralateral saline infused sides in either case. In contrast muscimol increased FLI in the Arc significantly more than baclofen did. Furthermore in the side of the Arc contralateral to the baclofen infusion the number of FLI cells was significantly lower than in the side contralateral to the muscimol infusion.

Discussion

The experiments reported in this chapter were designed to investigate the induction of *c-fos* expression in brain structures associated with feeding and food motivated behaviours following unilateral AcbSh infusions of baclofen or muscimol. The presence of FLI in cells was indicated by chromagenicaly labelling the Fos protein using an immunocytochemical method. In both experiments 6.1 and 6.2 two groups of n=5 animals previously demonstrated to respond to drug infusions into the AcbSh were used. In both experiments the number of FLI cells in ten regions of the brain was counted after counterbalanced unilateral infusions of a 220ρmols dose of drug on one side and saline into the contralateral side of the AcbSh. While I have referred to FLI in cells throughout the results I will assume from now on that this was most likely indicative specifically of Fos synthesis.

Unilateral infusions of baclofen at a dose of 220ρmols significantly increased Fos synthesis ipsilaterally to the infusion relative to activity in the saline infused side throughout the rostrocaudal extent of the LH, in the Arc, PVN, VP, VTA, CeA but not in the BLA. In contrast muscimol increased ipsilateral Fos synthesis in all levels of the LH, PVN, VP, VTA but not in the Arc, CeA or BLA. When the level of activity was compared between areas of the brain ipsilateral to the infusions of baclofen or muscimol it was shown that baclofen increased Fos synthesis significantly more in the caudal region of the LH than muscimol did. In addition baclofen caused a significant ipsilateral increase in Fos synthesis in the CeA relative to the levels seen ipsilateral to the muscimol infusion.

When levels of Fos in the Arc were compared animals that had received unilateral infusions of muscimol had higher levels of synthesis in the Arc both ipsilateral and contralateral to the drug infusions compared to ipsilateral and contralateral levels following baclofen infusions. Since increased Fos synthesis is most likely a marker of increased neuronal activation (Sheng and Greenberg, 1990) it is concluded that neurons in the regions with significantly increased Fos synthesis probably increase their firing rate following infusion of GABA agonists into the AcbSh.

In the remainder of this discussion I will first deal with some potential methodological issues that could contribute to the explanation of the results reported in experiments 6.1 and 6.2. These issues include:

1) The possible effects of prior history of drug infusions into the AcbSh
2) Induction of Fos synthesis in regions close to the infusion site.
3) The implications of assessing neuronal activity following unilateral compared to bilateral infusions of drug.
4) What does unilateral activation in regions of the brain mean and what can be concluded when animals are used as their own controls?

It is most likely that the regions of the brain activated by infusions of baclofen or muscimol into the AcbSh form functional macrocircuits but to understand how these might be arranged it is useful to first consider both the anatomical and functional evidence for the role of each region in feeding and food motivated behaviour. The second part of the discussion will therefore consider each area investigated separately before an attempt is made to build up a picture of the functional associations between regions investigated.

The pattern of neuronal activation in these experiments was purely a consequence of activation of GABA receptor subtypes in the AcbSh and does not indicate what patterns of activity might be seen in animals given the consequent opportunity to express behaviour in response to food or food related cues. The pattern of activation with baclofen and muscimol will be compared with evidence in the literature for the pattern of activation associated with hunger and with feeding.

1) Effects of prior history of drug infusions into the AcbSh

Fos protein synthesis was measured as FLI in cells following a number of prior infusions of baclofen or muscimol. Stratford (2005) suggests that it is possible that the results could be complicated by "expectation or conditioning effects". Because the specific behavioural effects of GABA agonist infusions appear to vary across quite minor shifts along a rostrocaudal gradient it was believed to be important to investigate *c-fos* expression following infusions that are known to be behaviourally active in the individuals used. It was therefore deemed more important to use animals that had been behaviourally tested than to use naïve animals to avoid possible experience related complications.

Very similar patterns of neuronal activation have been reported in the brains of both drug naïve animals (Stratford, 2005) and those that had previously experienced the opportunity to feed following intra-AcbSh muscimol infusions prior to the final infusion when food was unavailable (Stratford and Kelley, 1999). It would appear therefore that expectation at least may not be important although this could depend on the amount of experience the animals have of drug followed by food. From the data presented in Chapters 4 and 5 there was no significant difference in the amount of freely available chow consumed following the first infusion of 660ρmols baclofen or muscimol in naïve animals and the infusion given at the end of the study with the 660ρmols. Although this does not exclude more subtle effects on learnt associations it certainly indicates that the animals were no more or less sensitive to the feeding inducing effect of the drug at the end of the experiment.

It is also potentially relevant that animals that were used for experiment 6.1 had a different profile of prior experience to those used in experiment 6.2 which could complicate the interpretation of direct comparisons between the groups. In experiment 4.3 using baclofen the animals went through a free feeding test with a dose of 660ρmols, the 2nd order test following infusions of vehicle, 220 or 660ρmols and a free feeding test with vehicle, 220 and 660ρmols. In experiment 5.2 with muscimol animals went through a free feeding test with a dose of 660ρmols, the 2nd order test following infusions of vehicle, 220, 440 or 660ρmols and a free feeding test with vehicle, 220, 440 and 660ρmols. Thus the muscimol group experienced more infusions and more doses. However the doses were counterbalanced so it would be unlikely that the animals had been conditioned to have any particular expectation of effect following a specific drug dose and the extra dose given to the muscimol group would not change this argument. As mentioned above multiple infusions did not appear to have any effect on the efficacy of the drug pre and post 2nd order testing.

2) Induction of Fos synthesis in regions close to the infusion site

Stratford and Kelley (1999) suggest that infusions in the brain can cause Fos synthesis in the neurons surrounding the injection site and it is possible that activation of neurons within the VP occurred because of its close proximity to the cannulae tract. However in the experiments reported here Fos counts in the VP were deliberately taken in sections

that were caudal to both the cannulae tracts and associated areas of gliosis identified in the Nissl stained anatomy sections. It is suggested therefore that it is highly unlikely that any significant increase in Fos synthesis would be due to the process of infusion itself. None of the other brain regions investigated were close enough to the infusion site for this to be an issue.

3) Assessing Fos synthesis following unilateral or bilateral infusion of drugs

Activation in brain regions following unilateral infusions rather than bilateral infusions of muscimol has only been reported in one study (Stratford, 2005). Stratford (2005, 2007) suggests that, when infusions are made bilaterally, it is not possible to be sure that the downstream effect is unique to a direct projection or due to indirect pathways or even secondary stimulus bound changes. In contrast a unilateral infusion that causes unilateral activation is better representative of direct functional as well as temporal links in activity. Lateralised Fos induction strongly suggests that the region in question is "part of an uninterrupted neural circuit involving the AcbSh" (Stratford, 2005).

Stratford (2005) points out that projections from the AcbSh are almost exclusively ipsilateral and he suggests that bilateral activation is more likely to occur as a result of secondary systemic changes following AcbSh GABA agonist infusion. He does acknowledge however that activation of regions that are a number of synapses removed from the AcbSh could themselves give rise to bilateral projections to areas that are bilaterally activated.

4) What does increased activity following unilateral infusions mean?

Stratford (2005) suggests that there are three possible patterns of activity that can be recorded following unilateral infusions of a drug. First of all this can result in exclusive activation in regions of the brain ipsilateral to the infusion. To be sure that activation is exclusively ipsilateral the level of activity in the saline injected side would need to be the same as in that recorded if no drug was injected in either side. The experiments reported in this chapter did not include a saline only control group thus it is not possible to be sure that the level of activation ipsilateral to the saline infusion is the equivalent of basal levels. It is not possible therefore to be sure that any of the increases seen ipsilateral to the drug infusion in these experiments are exclusively ipsilateral.

However where such an effect has previously been reported following muscimol infusions it might be possible to infer a similar conclusion if the numbers are very similar, particularly if the region in question usually exhibits low constitutive levels of Fos expression. Stratford (2005) concludes that the only region of the brain in which activation was exclusively ipsilateral to the drug infusion was the LH. The counts from experiment 6.2 are compared with the counts reported by Stratford (2005) following either unilateral saline and drug infusion or unilateral saline infusion alone in Table 6.2. It is interesting to note that there appears to be an effect of dose because, although the pattern of activation in experiment 6.2 is very similar to that reported by Stratford (2005) the counts are all much lower.

Table 6.2. Comparison of number of neurons expressing Fos like immunoreactivity (FLI) following unilateral infusions of muscimol and saline (n=9) and unilateral infusions of saline only (n=6) in experiments reported by Stratford (2005) with numbers following unilateral infusions of muscimol and saline (n=5) in Experiment 6.2. The values given are mean counts of cells expressing FLI ± SEM.

	Stratford (2005) saline and 880pmols muscimol		Stratford (2005) unilateral saline only		Experiment 6.2 saline and 220pmols muscimol	
	drug	**saline**	**nothing**	**saline**	**drug**	**saline**
LH1	857 ± 52.4	170 ± 35.2	151 ± 25.5	148 ± 19.3	521 ± 66.5	135 ± 20.6
LH2	775 ± 64.0	157 ± 38.5	62 ± 19.7	88 ± 17.6	269 ± 13.4	122 ± 10.5
LH3	707 ± 41.0	113 ± 28.5	58 ± 12.4	73 ± 10.8	227 ± 29.8	101 ± 10.5
LH4	445 ± 22.1	114 ± 27.6	66 ± 16.0	85 ± 13.2	184 ± 17.4	118 ± 11.3

Stratford (2005) reported that there was no significant difference between counts of cells expressing FLI between the saline infused side in animals that received muscimol or no infusion contralaterally. It is clear from Table 6.2 that counts of cells expressing FLI in the side of the brain infused with saline in Experiment 6.2 are well within the range reported by Stratford (2005) and hence it is highly unlikely that there was any effect of muscimol on contralateral cells counts in the LH. It is fairly safe therefore to conclude that unilateral muscimol infused into the AcbSh in Experiment 6.2 caused exclusively ipsilateral activation of LH neurons. Furthermore, because there was no significant difference between levels of Fos synthesis in the LH in saline infused sides of the brain in Experiments 6.1 (baclofen) and 6.2 (muscimol) it can also be concluded

that intra-AcbSh baclofen induced exclusively ipsilateral activation of the LH. Exclusively ipsilateral activation of brain regions following manipulations of AcbSh output are highly likely to indicate an uncrossed lateralised pathway connecting regions.

The second pattern of activity that can be measured following unilateral infusions of drug is "primarily ipsilateral" activity (Stratford, 2005). Stratford (2005) reported that for all regions other than the LH there was some degree of activation in the side contralateral to the drug infusion. Primarily ipsilateral activation can be identified when there is a significant difference between activity ipsilateral and contralateral to the drug infusion but the level of activity in the saline infused side is also significantly elevated above basal levels in unilateral saline control animals. Without having this latter control group it is not possible to measure effects of drug in the contralateral side but it is possible to conclude that any significant ipsilateral induction of activity in areas other than the LH is at least primarily ipsilateral if the two sides are different. Small increases contralateral to drug infusion could be due to a small number of crossed efferents from the AcbSh within monosynaptic pathways or, more likely, bilateral efferents from intervening brain regions several synapses removed from the AcbSh.

The final pattern of activity is bilateral activation following unilateral drug infusion. Without a unilateral saline control group it is not possible to say whether, when levels of Fos synthesis in regions of the brain are the same ipsilateral and contralateral to the drug infusion, they are not activated or both equally increased above basal levels. For example there was no significant difference between levels of activity in the two sides of the Arc following muscimol infusion but activity in both sides was higher than following baclofen infusions so the Arc could be bilaterally activated with muscimol. As explained above bilateral activation could occur either via secondary systemic changes or via a polysynaptic and potentially crossed pathway. Either way however such activation cannot be directly attributed to an interaction between the AcbSh and the region in question.

With respect to the findings reported in this chapter therefore it can be concluded that a) both baclofen and muscimol probably caused increases exclusively ipsilateral to the drug infusion in the LH, b) that other areas that appeared to be activated ipsilaterally i.e. the Arc, PVN, VP, VTA and CeA with baclofen and the PVN, VP and VTA with

muscimol may also have been characterised by a small amount of activation contralateral to the infusion and c) a lack of divergence between activity in the two sides of a brain region could theoretically reflect bilateral activation but this would not be due to a direct input from the AcbSh.

Increased neuronal activity in hypothalamic nuclei

Lateral hypothalamus

These studies demonstrate that both intra-AcbSh infusions of baclofen and muscimol increase Fos synthesis in neurons throughout the rostrocaudal extent of the LH. It has been reported by a number of different authors that bilateral and unilateral intra-AcbSh infusions of muscimol increases *c-fos* expression in the LH (Stratford and Kelley, 1997a, Yoshida et al., 1997, Zheng et al., 2003, Stratford and Kelley, 1999, Stratford, 2005, Baldo et al., 2004). The effect of baclofen has not been previously investigated.

Bilateral muscimol infusions reportedly increase Fos synthesis predominantly in the rostral LH (Stratford and Kelley, 1997a). The densest patches of Fos synthesis following both bilateral and unilateral muscimol infusions were seen in the proximity of the perifornical area of the LH (Stratford and Kelley, 1999, Stratford, 2005). A role of the LH in feeding is well established (see discussion in Chapter 1, Introduction). Both electrical or chemical excitation of neurons in the LH induce voracious feeding in satiated animals (e.g. Stanley et al., 1993, Miller, 1963, Valenstein and Campbell, 1966). Increases in firing of cells in the LH in response to food and feeding has also been reported (e.g. Anand et al., 1964, Rolls et al., 1976). While activation of various nuclei in the hypothalamus is clearly an important part of the mechanism via which the AcbSh regulates feeding, particularly because the AcbSh is the only striatal region that projects to the LH, hypothalamic mechanisms are predominantly associated with homeostatic control of ingestion (Schwartz et al., 2000) which would not explain increases in appetitive responding following baclofen infusions.

It was hypothesised by Stratford and Kelley (1999) that blocking GABA receptors in the region of the perifornical in the LH would mimic the effects of inhibiting firing of GABAergic projection neurons from the AcbSh however GABA receptor antagonist infusions into the LH did not significantly increase feeding (Stratford and Kelley, 1997a, Stratford and Kelley, 1999). Injection of an NMDA antagonist that has

previously been shown to attenuate deprivation induced feeding (Stanley et al., 1996) also blocked AcbSh muscimol induced feeding suggesting that activity within the LH in mediated by glutamatergic connections (Stratford and Kelley, 1997a, Stratford and Kelley, 1999).

As Stratford and Kelley (1999) point out it is not possible to ascertain from increases in Fos synthesis whether the AcbSh influences neurons in the LH directly through monosynaptic connections or via a polysynaptic pathway. A polysynaptic pathway could involve one or more additional brain regions between the AcbSh and LH or indeed could be controlled by intervening interneurons between the primary efferents of the AcbSh and the primary output neurons of the LH. The VP is anatomically interposed between the AcbSh and the LH and it has been suggested that the AcbSh activates the LH indirectly via a polysynaptic pathway involving this region (see below).

However it has since been demonstrated that AcbSh GABAergic projections probably do not form synapses with primary feeding related neurons in the LH (Sano and Yokoi, 2007). These authors showed that GABAergic Acb efferents terminate in a dense patch in the LH anterior to the region in which MCH and orexin containing neurons were detected (Sano and Yokoi, 2007). They also showed that there were predominantly glutamatergic interneurons in the same region where the MSNs terminated and suggested that MSNs synapse onto these glutamatergic interneuron, which in turn projected to more caudal regions of the LH where MCH and orexin containing neurons were found.

Thus Acb projection neurons probably do not directly convey signals to the primary LH neurons but instead signals are relayed via excitatory interneurons that stimulate neurons in more posterior regions when disinhibited by inhibiting GABA afferents (Sano and Yokoi, 2007). This model is consistent with the reports mentioned above that show that both deprivation induced and intra-AcbSh muscimol induced feeding is attenuated by glutamate rather than GABA antagonists in the LH. Sano and Yokoi (2007) also suggest that it could be the MCH neurons in the more posterior levels of the LH that are activated and that these in turn send inhibitory projections to the AcbSh that further increase activity in the LH.

Zheng et al (2003) showed that the percentage of Fos synthesis in orexin neurons in the perifornical area of the LH was significantly increased by AcbSh muscimol infusions and that there was a net increase in Fos synthesis across the LH. However these authors reported that there was no Fos synthesis at all in MCH neurons in the LH. The increase in activity specifically in orexin but not MCH neurons has been confirmed elsewhere although numerous unidentified neuronal subtypes in the LH were also activated (Baldo et al., 2004). It is also important to remember that these counts were all made in the absence of access to food and it is not known if this population of MCH neurons would be activated during muscimol or baclofen induced feeding.

Baldo and Kelley (2004) suggested that while mild hyperphagia is induced by central administration of orexins (e.g. Sakurai et al., 1998, Rodgers et al., 2000) these peptides also affect waking and arousal and cause increases in activity in many brain regions that could account for the levels seen during feeding (Espana et al., 2001, Espana et al., 2002). In addition a wide range of pharmacologically arousing manipulations and stressors also activate these neurons so it is difficult to interpret the functional significance of activation in orexin neurons following intra-AcbSh muscimol infusions (Baldo et al., 2004).

It has been hypothesised that hypothalamic orexin neurons "may be an important cellular and molecular link in the integration of sleep and energy homeostasis" (Willie et al., 2001). They are anatomically interposed between the AcbSh and feeding related neurons in the Arc VMH and neurons of the PVN (Date et al., 1999, Trivedi et al., 1998, Marcus et al., 2001). Baldo and Kelley (2004) pointed out that arousal effects associated with a novel stimuli appeared to be mediated by a medial population of orexin neurons in the LH whilst intra-AcbSh muscimol increased activity preferentially in a lateral population suggesting that different subpopulations of orexin neurons could be differentially involved in mediating arousal versus food motivation.

Activation of LH orexin neurons has since been shown to increase preference for cues associated with reward and this activation also reinstates reward seeking post extinction (Harris et al., 2005). It has been shown recently that inactivation of neurons in the LH prevents context induced reinstatement of food seeking and that neurons in the ventral AcbSh that project to the LH were activated during reinstatement (Marchant et al.,

2009). Furthermore is has been suggested that LH orexin containing neurons constitute a key input to the VTA for the modulation of behaviour elicited by reward associated stimuli (Aston-Jones et al., 2009).

Both muscimol and baclofen increased Fos synthesis throughout the LH but baclofen caused a greater increase in the most caudal region investigated than muscimol did. If indeed the Acb MSN terminal region within the LH falls within a small, anterior portion of the LH then it is difficult to imagine how the indirect activation of posterior LH neurons via glutamatergic interneurons could differ along a rostrocaudal gradient. However it is possible that specific groups of MSNs could terminate within distinct clusters of interneurons responsible for activating different regions of the LH to determine distinct behavioural outputs. Whether or not such a mechanism has any functional implication depends on whether there are rostrocaudal gradients in the types of neurons found throughout the LH and/or in the topographical organisation of projections to other brain regions from the LH.

Because of the complexity of distinguishing between LH neurons and neurons of the MFB that are interspersed between them it is very difficult to define specific regions within this area of the hypothalamus on either cytoarchitectural or neurochemical grounds. However the LH can be broadly divided into anterior, tuberal and mammillary levels (Simerly, 2004). At this major level of division there are unique connections with other brain regions from each region (Saper et al., 1979). Within the LH neurons expressing MCH mRNA extend from β -1.1 to the most caudally detectable cells at β -4.1 (in the tuberal and mammillary LH) whereas orexin mRNA expressing cells are only found between β -1.4 to β -3.8 (tuberal LH) (Broberger et al., 1998). Both cell populations form clusters in the perifornical area but at different rostrocaudal levels within the LH (Broberger et al., 1998).

Both orexin and MCH positive neurons originating in the LH project widely to most regions in the brain (Bittencourt et al., 1992, Peyron et al., 1998). Orexin neurons innervate all of the regions that were activated by intra-AcbSh baclofen/muscimol infusions but send particularly dense projections to the Arc, slightly less dense projections to the PVN, VP, VTA and CeA but avoid many of the nuclei in the brainstem involved in motor functions (Peyron et al., 1998). MCH neurons send

projections of similar densities to the same regions including the Arc, PVN, substantia innominata (that contains the VP), VTA, CeA but also to the BLA and similarly avoid nuclei in the brainstem involved in motor functions (Bittencourt et al., 1992). Both types of LH neurons do however innervate brainstem structures that influence motor behavioural output (Peyron et al., 1998, Bittencourt et al., 1992).

It would seem that robust activation with baclofen of neurons in the caudal limit of the LH at β -4.3 could specifically recruit neurons that do not express MCH or orexin and muscimol recruits a smaller number of this caudal population of neurons than baclofen. However given the proximity of the sections to the most caudal location of MCH neurons it is also possible that baclofen but not muscimol stimulates activity in MCH neuronal populations of LH. It is clearly important therefore to determine both the neurochemical profile and terminal fields of neurons activated by baclofen in this posterior region of the LH.

It is certainly conceivable that differential patterns of activation in the LH with baclofen and muscimol could mediate different effects on feeding related behaviour via connections from this region to other hypothalamic nuclei or beyond. It is also possible that baclofen activates different populations of neuronal subtypes that might result in different patterns of activation in other regions of the brain. Depending on the profile of orexin, MCH and other neuronal subtypes activated by baclofen and muscimol these manipulations could act via indirect release of motor pattern generators as hypothesised by Kelley et al. (2005b) but equally could mediate control of conditioned responding that is dependent on intact LH function and orexin neuron activity.

Arcuate nucleus

In the experiments reported in this chapter there was no significant difference between the amount of Fos synthesis in the drug or saline injected side following intra-AcbSh muscimol infusions. However there was a significant increase in the Arc ipsilateral to baclofen infusions. When levels of activity were compared between drug treatments it emerged that, on the side contralateral to the drug infusion, there was a significantly higher level of Fos synthesis with muscimol than with baclofen. The level of activation was also higher in the side ipsilateral to muscimol infusion than in the side ipsilateral to

baclofen. This could be interpreted as a bilateral increase in activity with muscimol and a smaller, unilateral increase with baclofen.

Significant bilateral increases in Fos synthesis in the Arc have been reported following bilateral intra-AcbSh muscimol infusions (Baldo et al., 2004). Stratford (2005) reported that there appeared to be primarily ipsilateral Fos synthesis in the Arc following unilateral muscimol infusions but the effect was less robust than in other regions and no statistical verification of this observation was undertaken. There are no direct connections between the AcbSh and the Arc but it is innervated by both orexin and MCH neurons from the LH and sends reciprocal connections including peptidergic efferents (e.g. NPY) to regions containing both neuronal subtypes in the LH (Broberger et al., 1998, Elias et al., 1998). The Arc also sends NPY positive projections to the PVN (Chronwall, 1985). It is not clear how bilateral activation following muscimol infusion could occur unless the LH sends crossed projections to the Arc or activates it via polysynaptic pathways including other intervening brain regions. However it is possible that if baclofen activates a population of MCH neurons in the LH that are not activated by muscimol as was hypothesised above this would explain the unilateral activation in the Arc to which they are directly connected.

Zheng et al (2003) reported that, following bilateral intra-AcbSh infusions of muscimol, fewer POMC/CART immunoreactive neurons were positive for Fos synthesis in the ventrolateral aspect of the Arc but there were more cells showing Fos synthesis in the dorsomedial aspect of the Arc where the majority of NPY neurons were found. They did not report what the effect of muscimol was on the net total number of FLI cells in the whole of the Arc compared to saline infusions. It is possible that activation of NPY neurons which co-express GABA might itself be the cause of the reduction in activity in POMC/CART neurons to which the former project (Cowley et al., 2001). NPY levels in the Arc rise at the onset of the natural feeding cycle in rats and decrease following consumption of food and particularly carbohydrates (Akabayashi et al., 1994, Beck et al., 1990, Chang et al., 2005, Jhanwar-Uniyal et al., 1990, Wang et al., 1999). Feeding increases activity in POMC/CART neurons in the Arc (Johnstone et al., 2006).

Although no attempt was made to distinguish between the ventrolateral and dorsomedial portions of the Arc in these experiments a visual inspection of the sections

indicates that there was clearly more FLI cells in the dorsomedial portion ipsilateral to baclofen and muscimol infusions (See Fig. 6.7. and Fig. 6.13). Baldo and Kelley (2004) reported a significant increase in activity in Arc neurons following bilateral infusions of muscimol into the AcbSh. Both inhibition of POMC/CART immunoreactive neurons and activation of NPY neurons would result in net increases in feeding behaviour but it is not clear which other facets of behaviour (e.g. consummatory versus appetitive responses) would/could be mediated by the balance between the two systems. It has been reported however that intracerebroventricular infusions of NPY antagonists suppressed increases in free intake elicited by intra-AcbSh muscimol (Stratford and Wirtshafter, 2004).

Paraventricular nucleus

Both unilateral baclofen and muscimol caused significant increases in Fos synthesis ipsilateral to the infusions in the PVN in Experiments 6.1 and 6.2 and there was no difference in the magnitude of this effect between treatments. Significant bilateral increases in Fos synthesis in this area has been reported following bilateral intra-AcbSh infusions of muscimol (Zheng et al., 2003) and significant increases ipsilateral to drug following unilateral muscimol infusions have also been shown (Stratford, 2005). Stratford (2005) points out that there is no direct connection between the AcbSh and the PVN but that there must be an uncrossed polysynaptic neural pathway, perhaps via the LH. Zheng et al., (2003) reported that, while muscimol induced significant Fos synthesis in neurons of the PVN, there was no significant change in activity in oxytocin or CART neurons in the region.

The apparent lack of an effect of intra-AcbSh muscimol infusions on activity in neurons expressing anorexigenic peptides in the PVN suggests increased feeding is not caused by suppressing the release of satiety factors. However it is possible that these neurons were quiescent while animals did not have access to food and suppression might be seen during feeding when anorexigenic peptide release should be increased. It is also possible that increases in activity in NPY neurons in the Arc increases release of NPY in the PVN. NPY injected into the PVN stimulates food intake in satiated animals (Stanley and Leibowitz, 1985, Elmquist et al., 1996, Williams et al., 2001). The PVN is a key region activated by various acute and chronic stressors (e.g. see Melia et al., 1994) but it is always equally activated on both sides of the brain suggesting the robust

ipsilateral increases reported here are not due to general increases in arousal or stress associated with the procedure.

Section summary

While intra-AcbSh infusions of both baclofen and muscimol significantly increase activity in the LH the former appears to recruit more neurons in the caudal region. These neurons activated by baclofen might be MCH neurons, which could in turn cause the ipsilateral increases in activity in the Arc, to which they project. There was no significant ipsilateral increase in activity in the Arc with muscimol but higher levels in this region on both sides relative to activity following baclofen infusions suggest the Arc may have been bilaterally activated. If this was due to activation of LH neurons that project to the Arc they would have to be part of a crossed pathway or have affects on an intervening brain region. While both baclofen and muscimol increased activity ipsilaterally in the PVN muscimol reportedly has no effect on activity of anorexigenic peptide neurons in the region. Increased activity in the Arc could cause an increase in NPY in the PVN that would stimulate feeding. Overall it would appear that the effects of baclofen and muscimol on feeding might be mediated by a complex interaction between orexigenic and anorexigenic neurons in multiple hypothalamic nuclei. Different patterns of Fos expression in the LH and Arc indicate that the organisation of these interactions might differ following intra-AcbSh baclofen and muscimol infusions.

Increased neuronal activity in other primary AcbSh efferent target regions

Both unilateral intra-AcbSh baclofen and muscimol caused significant increases in Fos synthesis ipsilateral to the infusions in the VP and the VTA and there was no difference in the magnitude of these increases between treatments. Although none of the papers that report the effects of intra-AcbSh muscimol infusions on activity in these regions have tested the results statistically it has been observed that bilateral infusions increase activity bilaterally in these regions (Stratford and Kelley, 1999) and unilateral muscimol increases activity ipsilaterally (Yoshida et al., 1997, Stratford, 2005). The AcbSh connects monosynaptically with both these brain regions (See Chapter 1).

The VP is involved in the regulation of ingestive behaviour (Johnson et al., 1996, Stratford et al., 1999) and it has recently been suggested that it primarily encodes the hedonic value of reinforcers (for a review see (Smith et al., 2009). Disinhibiting a

population of cells in the VP could be one of the events necessary for the initiation of feeding via the AcbSh because the $GABA_A$ antagonist bicuculline in the VP suppresses the inhibitory effects of AcbSh efferents and causes a robust dose-dependent increase in feeding (Stratford, 2007). Although unpublished, Stratford reports that there is evidence that fibre-sparing excitotoxic lesions of the VP significantly but not completely (~70%) reduced the magnitude of the intra-AcbSh muscimol induced feeding (Stratford, 2007). Stratford and Wirtshafter (unpublished data discussed in Stratford, 2007) suggest that an element of muscimol induced feeding is mediated via direct connections to the LH.

In the same year it was reported that 1) increases in 'liking' by opioid stimulation in either the AcbSh or VP hedonic hotspots increased Fos activation in the other hotspot and 2) opioid agonist induced hedonic taste reactivity (liking) enhancement in either hotspot was blocked by simultaneous antagonist infusions in the other hot spot suggesting that the two regions act in unison as a single opioidergic circuit that subserves the hedonic impact of food rewards (Smith and Berridge, 2007). However while intra-VP naloxone (a μ-opioid antagonist) blocked hedonic orofacial responses caused by opioid stimulation in the AcbSh it did not affect the increase in food intake (Smith and Berridge, 2007).

These authors suggest that the wanting element of AcbSh opioid induced intake is modulated by an independent route that bypasses the VP, possibly the direct AcbSh-LH connection (Smith and Berridge, 2007). Thus the VP is a necessary component, along with the AcbSh and LH, in a circuit subserving the full and natural expression of feeding related behaviours but is not essential for increases in intake alone. This is consistent with the report that intra-AcbSh muscimol at least can increase orofacial responses to food but infusions that most robustly increase intake do not correlate with hedonic increases that might be mediated via the VP (Reynolds and Berridge, 2002).

It is possible that $GABA_A$ receptor stimulation releases a greater proportion of inhibitory control of the LH via direct monosynaptic connections than intra-AcbSh opioid stimulation. There is no data available to indicate what the effect of intra-AcbSh baclofen might be on hedonic responses to food. However, given that there was no significant difference in activity in the VP between drug treatments in the experiments reported here (See Fig. 6.16) muscimol and baclofen probably both have a similar

impact on the VP mediated element of the feeding response unless different classes of neurons in this region are activated.

The AcbSh sends monosynaptic efferents to the VTA (Heimer et al., 1991b), which is one of the major sites from which AcbSh dopaminergic inputs originate (Swanson, 1982). It has not yet been shown which neurons in the VTA the AcbSh efferents synapse onto. However increased activation of the VTA could result in increases in DA efflux in target regions in the forebrain including in the Acb. Reverse dialysis of muscimol increased firing of mesolimbic DA neurons, particularly in the A10 group including neurons in the VTA, which resulted in an increase in DA release and metabolism in the Acb (Yoshida et al., 1997). Dopaminergic input from the VTA to the Acb is necessary for the control of appetitive behaviours by neurons that are responsive to cues predicting reward (Yun et al., 2004b). DA in the Acb has been demonstrated to increase the conditioned incentive salience of food reward (Wyvell and Berridge, 2000).

Section summary

Intra-AcbSh infusions of both baclofen and muscimol caused ipsilateral increases in activity in the VP and in the VTA. While the VP might be an essential node in the circuit subserving the full expression of AcbSh GABA mediated feeding it would appear that blocking activity in this region does not abolish intake suggesting a direct connection to the LH is also involved. The VP has been implicated in an AcbSh mediated circuit subserving the hedonic control of intake but non-hedonic increases in feeding bypass this region. The VTA in contrast is responsible for increases in DA in the AcbSh, which in turn increases the incentive salience of reward and is necessary for cue induced responding. Measures of activity in these two regions alone without double labelling to identify which populations of neurons are activated does not indicate divergence in the effects of stimulating $GABA_A$ or $GABA_B$ receptors at a macrocircuit level. It may be relevant however that increased activity in the VTA could cause increased DA efflux in other forebrain regions such as the amygdala.

Increased neuronal activity in the central nucleus of the amygdala

Neither baclofen nor muscimol had any significant effect on activity in the BLA relative to vehicle treatment however baclofen significantly increased activity in the CeA on the side ipsilateral to the drug infusion. The only report that mentions an effect of muscimol

on activity in the CeA demonstrated that Fos synthesis was significantly increased bilaterally following unilateral infusions suggesting this was due to a secondary systemic effect (Stratford, 2005). The CeA receives direct input from the AcbSh (Heimer et al., 1991b) but the CeA only influences the AcbSh via indirect pathways that include midbrain dopaminergic neurons (See Chapter 1, Introduction). As mentioned above orexin and MCH neurons in the area of the LH that is activated by both intra-AcbSh GABA receptor agonists also project directly to the CeA and increased activity in the VTA can increase DA release in the amygdala (Yoshida et al., 1997)..

It is well established that the CeA is involved in the control of feeding (See Chapter 1). Food deprivation has been shown to increase Fos synthesis in both the CeA and BLA (Moscarello et al., 2009). There is also a reciprocal relationship between opioid mediated effects on feeding in the AcbSh and CeA (Kim et al., 2004). Temporary inactivation of the CeA but not the BLA using lidocaine attenuates increases in intake in response to palatable food by disrupting the expression of feeding motor responses (Ahn and Phillips, 2003). Inactivation of the CeA but not the BLA using muscimol dose dependently blocks the expression of AcbSh muscimol induced feeding suggesting this region is necessary for the expression of consummatory behaviour (Baldo et al., 2005).

It has also been suggested that the CeA encodes the incentive salience or affective properties of stimuli associated with "biologically significant events" (Balleine and Killcross, 2006). Cardinal et al., (2003) state that "the CeA acts as a 'controller of the brainstem', using its widespread projections to the hypothalamus, midbrain reticular formation and brainstem to orchestrate behavioural, autonomic, and neuroendocrine responses". The CeA also probably has a modulatory role in attention and arousal through its projections to the reticular formation (Cardinal et al., 2003). Thus it is possible that activation of the CeA following intra-AcbSh baclofen infusions could increase intake via effects on incentive salience or wanting. This would also potentially support increases in instrumental responding as was suggested in Chapters 4 and 5. This will be discussed in more detail in the general discussion in the final chapter but the CeA could be the critical point at which $GABA_B$ mediated and $GABA_A$ mediated feeding circuits differ.

Fos activation following food deprivation and in response to food

It was suggested in Chapter 3 that infusions of baclofen into the AcbSh elicited a behavioural profile, in terms of the BSS, similar to that elicited by hunger and it is interesting to consider whether a similar pattern of Fos synthesis is seen following food deprivation as is reported here following intra-AcbSh baclofen. Food deprivation has been shown to induce Fos synthesis in a multitude of brain regions including the AcbC, AcbSh, medial preoptic area, dorsomedial caudate nucleus, lateral septum, BLA, Arc, dorsomedial hypothalamus, LH and lateral habenula (LHb) but no increase in the VP, PVN, CeA (Carr et al., 1998). These authors suggest that this widespread increase in activity could be due to the effects of stress associated with food restriction rather than hunger *per se* because the animals had not been fed for between 19-21 hours.

Carr et al., (1998) also acknowledge however that, while the animals were culled at a time of day when Fos synthesis associated with circadian cycle would be low in *ad lib* fed animals, the cohort used had been conditioned to expect food during the light cycle and Fos synthesis could have been increased because they were expecting a meal. Johnstone et al., 2006 showed that animals that were anticipating feeding during a restricted period but had not received food had increased levels of Fos synthesis (relative to levels in animals not expecting food) in the VMH, LH, dorsomedial portion of the Arc, PVN and LHb but levels were similar in the "posterior" hypothalamus. These authors do not report any data pertaining to levels of Fos synthesis in the VP, amygdala or striatum (Johnstone et al., 2006).

Increases in activity following intra-AcbSh muscimol infusion have been reported for all of the areas activated by food restriction/expectation bar the dorsal and ventral striatum which could not be assessed because of the location of the guide cannulae (See Table 6.1). It is interesting however that food restriction does not activate the VP, PVN and CeA while expectation recruits the PVN but does not activate the posterior hypothalamus suggesting that intra-AcbSh baclofen and muscimol might both recruit additional circuits not active in hungry animals or necessary for the initiation of feeding.

Summary

The results from experiments reported in this chapter indicate that intra-AcbSh infusions of baclofen and muscimol activate neurons in a number of hypothalamic

nuclei but that the pattern of activation differs between treatments. While the current hypothesis to explain the effects of intra-AcbSh muscimol on food intake suggest that disinhibition of neurons in the LH releases patterns of ingestive motor behaviour (Kelley et al., 2005b) it would appear that both baclofen and muscimol could recruit populations of orexin neurons essential for modulating the effects of external stimuli on feeding. However baclofen also recruits a circuit that involves the CeA suggesting that this manipulation alone actually modulates a functional circuit known to be involved in the response to stimuli and in the affective value of these stimuli. It would not appear that circuits essential for the hedonic effects of food and food associated cues is important for baclofen or muscimol effects on feeding but circuits subserving wanting that include the VTA and CeA are important for the baclofen effect on instrumental responding. The recruitment by both intra-AcbSh baclofen and muscimol infusions of areas of the brain not apparently involved in circuits activated by hunger and food expectation *per se* further supports this assertion.

Questions raised

While activation of some neurons in the LH could contribute to the expression of conditioned responses the evidence presented in this thesis suggests that intra-AcbSh muscimol infusions do not affect instrumental responding. Further studies need to be carried out using double labelling techniques to determine the neurochemical subtype of neurons that are activated following intra-AcbSh baclofen and muscimol, particularly in the LH and other hypothalamic nuclei. Also, while Kelley et al., (2005b) hypothesise that disinhibition of the LH results in release of downstream motor components there is no functional evidence for this. It remains to be determined whether the subtly different patterns of activation in hypothalamus regions with baclofen and muscimol will have different effects on motor pattern generation if they have any direct effect at all. The evidence presented in this chapter that $GABA_A$ and $GABA_B$ receptors activate different pathways strongly suggests that a different pattern or magnitude of changes in the activity of AcbSh MSN output is mediated by stimulation of the two receptor subtypes. This leaves us with the question of why the pattern of output would be different. Possible local mechanisms to explain these differences will be discussed in the final chapter.

Chapter 7

General Discussion

Overview

A specific role for GABA in the AcbSh in the control of feeding behaviour was first demonstrated over a decade ago (Stratford and Kelley, 1997b). In the following decade a number of papers were published reporting the role of a variety of neurotransmitters and neuromodulators in this region in modulating feeding and food motivated behaviour. However the nature of the feeding response elicited by intra-AcbSh infusions of the $GABA_A$ agonist muscimol and the $GABA_B$ agonist baclofen has not been extensively investigated in the intervening years. In particular the role of baclofen has only been tested in terms of the effects on total intake of food, latency to begin feeding and meal duration (Lopes et al., 2007, Stratford and Kelley, 1997b) although Ward et al., (2000) demonstrated that increased food intake was not due to non-specific increases in oral behaviours or increased palatability.

Intra-AcbSh muscimol potentiates orofacial expression of hedonic responses to food but increases in feeding appear to be independent of positive taste reactivity (Reynolds and Berridge, 2001). Muscimol can elicit both positive and aversive responses along a rostrocaudal gradient in the AcbSh and supports the development of place preference/avoidance (Reynolds and Berridge, 2002) although preference could be due to the reported anxiolytic effects of muscimol (Lopes et al., 2007). Muscimol in the AcbSh does not increase instrumental responding on a PR schedule and fails to facilitate the acquisition of a Pavlovian association or increase the hedonic value of food as hunger does (Zhang et al., 2003, Hanlon et al., 2004, Basso and Kelley, 1999). A functional link between the AcbSh and the LH subserves GABA receptor agonist induced increases in food intake (Stratford and Kelley, 1997a, Stratford and Kelley, 1999, Stratford, 2005, Marchant et al., 2009). Kelley and colleagues (2005b) hypothesised that inhibition of AcbSh MSN output disinhibits neurons in the LH motor/autonomic control columns which in turn releases a subset of motor pattern generators in the brainstem that organise ambulation towards and ingestive responses to food but does not support expression of more complex goal-directed behaviours.

The main aim of this thesis was to further explore the behavioural impact of activating GABA receptors subtypes in the AcbSh on both food intake and food seeking behaviours. The behavioural paradigms chosen provided measures that dissociated between consummatory responses to food and instrumental responding for food reinforcement. Comparisons were made with both physiological and pharmacological manipulations known to increase these behaviours via specific mechanisms. The behavioural paradigms chosen could also provide some insight into whether intra-AcbSh GABA receptor agonists increase feeding via effects on appetite or satiety.

To reiterate the results briefly; baclofen at all doses tested increased intake of freely available food, particularly at the start of the meal and delayed the onset of satiety whilst the gross pattern of the BSS remained intact. The pattern of behaviour resembled that seen following food deprivation but not that associated with AcbSh μ-opioid receptor agonist infusions, or systemic bretazenil. Baclofen at an optimum low dose increased instrumental responding during appetitive and consummatory phases of a 2nd order operant schedule suggesting that it initiates food seeking rather than attenuating satiety signals. At a high dose baclofen disrupted the expression of grooming behaviour and elicited grooming and oral stereotypies during the operant schedule and also induced non-feeding oral behaviours during the BSS test.

Muscimol also increased feeding at all doses tested but at the expense of the rest of the behavioural repertoire that constitutes the BSS. Feeding behaviour was elevated throughout the test session. The pattern of behaviour did not resemble those elicited by baclofen, hunger, AcbSh μ-opioid receptor agonist infusions or systemic bretazenil. Muscimol had no significant effect on instrumental responding but at a high dose disrupted the natural expression of grooming behaviour and elicited adjunctive oral behaviour.

The dose of baclofen that increased instrumental responding activated neurons in many of the same AcbSh efferent target regions that were also activated by an equimolar dose of muscimol. In addition intra-AcbSh baclofen activated a subpopulation of neurons in the caudal most region of the LH and in the Arc and CeA, areas not activated by muscimol.

These results suggested that neither baclofen nor muscimol inhibit satiety mechanisms but instead were consistent with effects on the initiation and maintenance of feeding behaviour. It was hypothesised that, at low doses, baclofen in the AcbSh increases intake and appetitive instrumental responding for food via increases in the incentive salience (but not the hedonic value) of reward or reward predictive cues that can initiate behaviour while muscimol initiates unconditioned motor responses to food but does not affect motivation *per se*. The hypothesised divergence in mechanisms subserving the increased feeding elicited by intra-AcbSh GABA receptor subtype agonists is supported by the observation that baclofen and muscimol infused into the AcbSh activate components of different neuronal pathways within feeding related macrocircuits.

These results raise a number of questions about the mechanisms that might subserve the reported effects of intra-AcbSh baclofen and muscimol:

1) Divergence between the effects of intra-AcbSh baclofen and muscimol at the behavioural and anatomical level must be subserved by differences in the pattern and/or magnitude of activity of the AcbSh MSNs, but how would this arise?
2) Which functional macrocircuits might be activated and how do these contribute to the expression of different behaviours following stimulation of different GABA receptor subtypes in the AcbSh?
3) Increased intake and increases in operant responding can occur as a result of increases in hedonic value of the reward or reward associated cues (liking), increases in incentive salience of cues (wanting), decreases/blockade of satiety mechanisms, changes in attentional processes or because of a decrease in response inhibitory control but which of these mechanism might be critical to the results reported here?
4) Why are different effects on behaviour seen across the dose range of baclofen tested and, to a lesser extent, across the dose range of muscimol tested and is this related to the macrocircuits that subserve the effects?
5) Although the evidence for a role for GABA in AcbSh mediated feeding is compelling is there any evidence that GABA in the AcbSh is part of a biologically relevant feeding control mechanism?
6) How does all of the above contribute to our understanding of the processes involved in the control of motivated behaviour in general at the level of the Acb?

Differences at the level of GABA receptors - could $GABA_A$ or $GABA_B$ receptors differentially control activity of AcbSh MSNs?

Kelley's hypothesis to explain the role of GABA in the AcbSh in feeding is dependent on the assertion that both $GABA_A$ and $GABA_B$ receptor agonists inhibit firing of GABAergic output neurons (2005b). Kelley et al., (2005b) suggest that baclofen causes functional inhibition of MSNs because stimulation of $GABA_B$ receptors blocks glutamatergic inputs to the MSNs. By contrast muscimol stimulates $GABA_A$ receptors on the MSNs directly inhibiting firing rates. It is implicit within Kelley's hypothesis that global inactivation of AcbSh inhibitory output neurons increases food intake although Stratford (2007) explicitly spells this out. It is possible however that there are differences in the effects of stimulating G-protein coupled receptors ($GABA_B$) and ionotropic receptors ($GABA_A$) and the location and sensitivity of these receptors may ultimately determine the pattern and proportion of MSNs inhibited. Such mechanisms are particularly important given that the integrative role of the Acb in motivated behaviour requires that a multitude of different types of input that converge on this region must be filtered to produce control of different types of behavioural output.

GABA receptor types and location

Ionotropic $GABA_A$ receptors in the Acb are found postsynaptically on MSNs and on GABAergic interneurons (Galvan et al., 2006) at GABAergic synapses which arise from inputs from interneurons, local axon collaterals and from minor GABAergic innervation from the VP and VTA (Churchill and Kalivas, 1994, Van Bockstaele, 1995, Meredith, 1999, Taverna et al., 2004). $GABA_A$ receptors are also found postsynaptically at non-GABAergic synapses (Fujiyama et al., 2000) that could be dopaminergic or cholinergic but not apparently in glutamatergic synapses (Galvan et al., 2006). Activation of $GABA_A$ receptors results in rapid phasic induction of inhibitory postsynaptic potentials (Mody, 1994).

G-protein coupled $GABA_B$ receptors are located presynaptically and postsynaptically (Kerr and Ong, 1995). The majority of postsynaptic $GABA_B$ receptors are extrasynaptic but a small number are found in GABAergic synapses and at the edge of glutamatergic synapses (Galvan et al., 2006). Postsynaptic $GABA_B$ receptors are thought to modulate slower hyperpolarisations (Otis et al., 1993, Mody et al., 1994). Presynaptic $GABA_B$ receptors function as both auto- and heteroreceptors (Bettler et al., 2004). Autoreceptors

in GABAergic terminals modulate GABA release (Nisenbaum et al., 1992, Bowery, 1993, Takahashi et al., 1998, Waldmeier et al., 2008). Heterorecptors are found at glutamatergic and possibly dopaminergic terminals and modulate release of neurotransmitter (Charara et al., 2000, Calabresi et al., 2000, Smith et al., 2001, Lacey et al., 2005). Stimulation of G-protein coupled $GABA_B$ receptors may also have other secondary effects on activity of Acb neurons via signal transduction cascades (Calver et al., 2002, Kerr and Ong, 1995).

Postulated effects of stimulating $GABA_B$ receptors

Baclofen at μM doses reduces glutamate release, which in turn reduces excitation in MSNs but there does not appear to be any postsynaptic effect of baclofen in the striatum (although there is in other brain regions) (Nisenbaum et al., 1993). Baclofen also reduces the small inhibition elicited by excitatory input to MSNs by blocking GABA release at autoreceptors from axon collaterals that synapse onto other MSNs(Nisenbaum et al., 1993). However, whether autoreceptors are located on the MSN terminals of the axon collaterals that innervate other MSNs (feedback inhibition) or on the GABAergic interneurons that are also stimulated by glutamatergic inputs (feedforward inhibition) is not clear (Nisenbaum et al., 1993).

It has been widely reported that $GABA_B$ autoreceptors are activated by much lower concentrations of baclofen than are required to activate $GABA_B$ postsynaptic receptors or presynaptic heteroreceptors on glutamatergic terminals (Calver et al., 2002). However Uchimura and North (1991) reported that in the Acb the half maximal effective concentration (EC_{50}) of baclofen required for inhibition of MSN activity by GABA release is 2μM while the EC_{50} for glutamatergic excitation was 0.6μM. This suggests that $GABA_B$ receptor mediated release of GABA in the Acb *in vitro* is three times less sensitive to baclofen than glutamate release and hence the main effect of lower doses of baclofen (within a μM concentration range) would be inhibition of MSNs via attenuation of excitatory input (Uchimura and North, 1991).

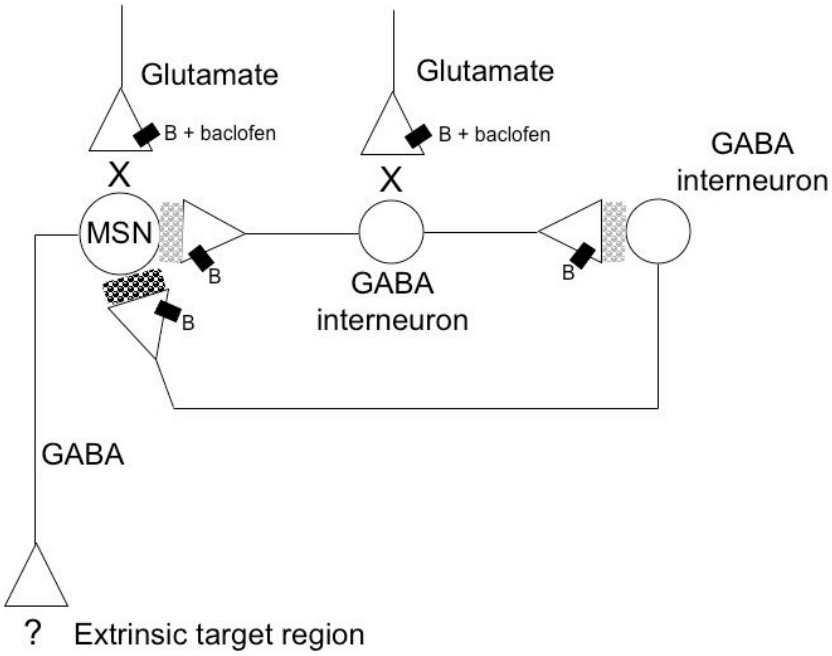
The EC_{50} for baclofen effects on glutamatergic transmission in the Acb is a thousand fold greater than the highest dose of baclofen used in the experiments reported in this thesis and it is difficult to predict what effects such low doses of baclofen would have *in vivo* on auto vs. heteroreceptor activation. However it seems highly likely that, at such

low doses the ability of baclofen to block glutamatergic transmission in different synapses will depend ultimately on the net effect of interactions between multiple glutamatergic, dopaminergic, serotonergic, cholinergic and GABAergic inputs to the AcbSh MSNS (see Chapter 1, page 31). In addition it is not known to what extent excitatory afferents to the Acb synapse onto interneurons and the effect that stimulating heteroreceptors in these synapses will have on MSN output.

At the simplest level it is postulated that blocking excitatory input to a subset of GABAergic interneurons that receive glutamatergic afferents would reduce their inhibitory influence on MSNs and interneurons to which they project. Some local neurons could therefore become simultaneously more active as the excitation of others is reduced. Excitatory input to MSNs from glutamatergic afferents would be blocked, inhibitory inputs from some GABAergic interneurons would be removed but inhibitory input from others could be increased. Although it has been postulated that $GABA_B$ receptors are located presynaptically on DA afferents, it has been shown that baclofen does not affect the release of DA *in vivo* (Santiago et al., 1993). At a behavioural level baclofen does not block turning behaviour elicited by unilateral DA receptor agonist infusions into the AcbSh suggesting that $GABA_B$ receptors do not contribute significantly to the modulation of DA input to the Acb (Akiyama et al., 2004). This means that baclofen will not prevent dopaminergic inputs from influencing MSN output. Blockade of glutamatergic input could actually increase the impact of DA modulation of activity in the AcbSh. Which MSNs do and don't fire will then depend on the net influence of blocking neurotransmitter release from heterogeneously distributed excitatory afferents, releasing or increasing inhibitory intrinsic inputs from different interneurons and favouring the impact of DA afferents.

At higher doses of baclofen the contribution of interneurons to the activity of MSNs will be blocked by blocking GABA release via autoreceptors. Instead stimulation of the postsynaptic receptor will inhibit the MSN. This means that, at low doses of baclofen, functional pathways intrinsic to the AcbSh could contribute to the net output depending on the pattern of innervation of MSNs by glutamatergic afferents and subclasses of interneurons but the interneuron contribution will be cancelled out at higher doses (See Fig. 7.1). This could explain the dose response effects of baclofen on instrumental responding reported in Chapter 4.

A) Low dose of baclofen



B) High dose of baclofen

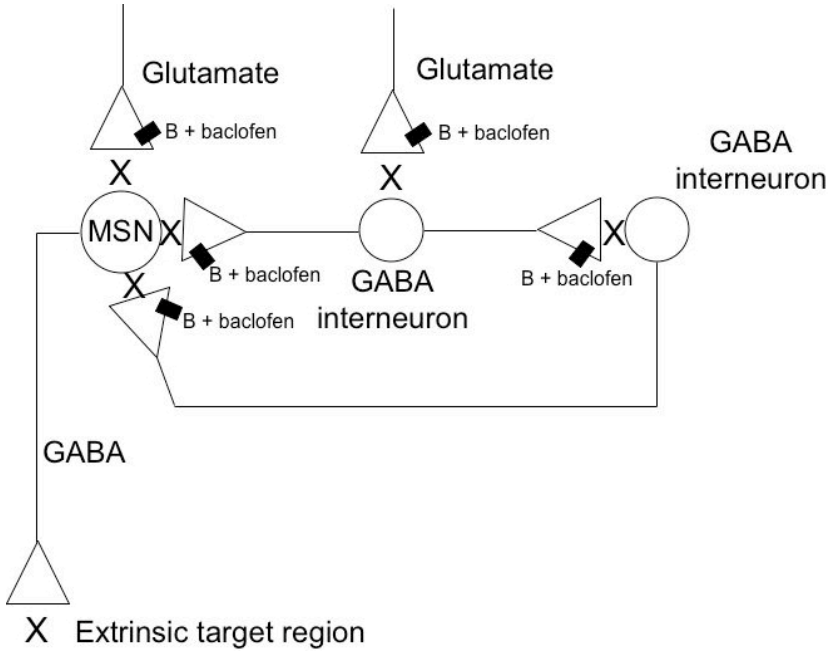


Figure 7.1. Schematic illustration to show the postulated effects of applying a low concentration vs. a high concentration of baclofen to the AcbSh. ▮ B = presynaptic $GABA_B$ receptor. ✕ = no release of neurotransmitter. At low doses (A) GABAergic transmission is not affected but glutamatergic transmission is blocked by stimulation of heteroreceptors and the output of the MSN is determined by the net effect of inhibitory and excitatory inputs. At high doses (B) both GABAergic and glutamatergic transmission is blocked and the output of the MSN is probably determined solely by occupation of a postsynaptic $GABA_B$ receptor by baclofen (not shown for the sake of clarity) which will inhibit MSN firing.

Postulated effects of stimulating $GABA_A$ receptors

Because muscimol binds with a much higher affinity to the $GABA_A$ receptor than endogenous GABA does (Krogsgaard-Larsen and Johnston, 1978) and because $GABA_A$ receptors are so widely distributed in the Acb (Galvan et al., 2006), the net effect of infusions of muscimol into this region could well be complete inhibition of MSN output as is generally believed. Although excitatory inputs to MSNs and interneurons will not be affected their influence will be negated by high levels of GABA receptor activation postsynaptically on MSNs.

Although it has been shown that intra-AcbSh muscimol increase DA efflux in the Acb via disinhibition of mesolimbic DA neurons (Yoshida et al., 1997) the hypothesised presence of postsynaptic $GABA_A$ receptors in dopaminergic synapses (Fujiyama et al., 2000) could modulate the impact of increased DA. At a behavioural level muscimol can block turning behaviour elicited by unilateral DA receptor agonist infusions into the AcbSh (Akiyama et al., 2004). This is consistent with the suggestion that $GABA_A$ receptors are found postsynaptically at DA synapses.

Section summary

Broadly speaking it is postulated that baclofen at low doses might only inhibit a subpopulation of MSNs by decreasing excitatory input and modulating the contribution of DA afferents and local interneurons. Which MSNs are ultimately inhibited will determine which macrocircuits are activated and which classes of behaviour elicited. In contrast stimulation of $GABA_A$ receptors with muscimol will result in near global inhibition of MSN output by bypassing all of the input neurons and interfering with DA transmission. Infusions of muscimol could effectively override the normal functional control of feeding via other neurotransmitter systems. The postulated effects of muscimol on MSN activity, but not of baclofen, are consistent with a lack of motivational effects of GABA stimulation in the Acb reported in this thesis and by other investigators (Kelley et al., 2005b).

Differences at the level of local signalling systems – could stimulation of $GABA_A$ or $GABA_B$ receptors differentially control activity of AcbSh MSNs and hence control of feeding by modulating activity of other local neurotransmitters?

As Kelley et al., (2005b) point out, while the effects of individual neurotransmitters and the individual firing of separate classes of neurons in the Acb produce apparently diverse changes in motivated behaviour, signalling pathways within the Acb are heavily interdependent. It is important to take into account the possibility that the different effects of AcbSh $GABA_A$ and $GABA_B$ receptor stimulation on food motivated behaviour reported in this thesis could arise because of different interactions with other neurotransmitter systems within the Acb at the level of the interneurons and axon collaterals from MSNs. However little is known about how the feeding effects of stimulation/ blockade of various receptors in the Acb is mediated intra-striatally. Two major sources of neurotransmitter in the AcbSh are the local axon collaterals of cholinergic interneurons which release ACh within the AcbSh (Zhou et al., 2002) and local axon collaterals from both enkephalinergic and dynorphinergic MSNs which are responsible for local opioid release.

Cholinergic and opioidergic interneurons

While the role of opioids in the AcbSh in feeding is well established (e.g. for a review see Levine and Billington, 2004) the role of ACh is less well understood but stimulation of both $GABA_A$ and $GABA_B$ receptors in the Acb could modulate the activity of both resulting in a complex net effect on feeding. Hoebel and colleagues (Hoebel et al., 2007) have suggested that increased levels of ACh following activation of cholinergic interneurons could mediate satiety mechanisms in downstream targets because 1) ACh levels in the Acb peak post maximal food intake (Mark et al., 1992), 2) pharmacologically induced hypophagia is associated with increased Acb ACh release (Mark et al., 1995) and 3) stimulation of feeding via hypothalamic mechanisms reduces Acb ACh release (Rada et al., 1998, Rada et al., 1999).

Kelley and colleagues (Kelley et al., 2005b) have hypothesised that cholinergic interneurons could play a role in potentiating food seeking or consumption, possibly via interactions with opioids in the AcbSh because 1) blocking ACh release in the AcbSh reduces performance of an operant response for food and acutely reduces free intake of

food (Pratt and Kelley, 2004), 2) blocking ACh release in the Acb reduces opioid induced food intake (Kelley et al., 2005b), 3) blocking ACh release in the AcbSh reduces 24 hour food intake and reduces AcbSh opioid gene expression (Pratt and Kelley, 2005) and 4) increased AcbSh opioid gene expression could be associated with "energy state signalling" that encourages food intake via hedonic mechanisms (Kelley et al., 2005a). Thus they argue that tonic activity in cholinergic interneurons may be important for motivational mechanisms via interactions with opioidergic interneurons while stimulation of ACh release could also contribute to satiety mechanisms in line with the hypothesis put forward by Hoebel and colleagues (Kelley et al., 2005b).

In *in vitro* studies it has been shown that stimulation of cholinergic interneurons via activation of afferents to the Acb increases inhibition of MSNs, which suppresses the inhibitory output of the Acb (de Rover et al., 2002), which in turn would disinhibit downstream feeding related circuitry. In addition since there is no direct evidence that ACh in the AcbSh reduces feeding via effects on satiety, instead probably quite the opposite, it would appear to be more likely that cholinergic interneurons are involved in the potentiating of food seeking or consummatory responses, as suggested by Kelley and colleagues. The increase in ACh in the Acb during feeding could therefore be part of a positive feedback mechanism that involves the hypothalamus which potentiates the AcbSh opioidergic hedonic system. The reinforcing role of ACh in the Acb is supported by the observation that rats will self administer an ACh agonist into the AcbSh (Ikemoto et al., 1998) and the involvement of cholinergic interneurons in the synchronised response of the Acb to reward related cues (Reynolds and Wickens, 2004).

Interestingly it has been demonstrated elsewhere that baclofen and muscimol have different effects on both intra-AcbSh opioid and ACh agonist induced behaviours. While it has been reported that both baclofen and muscimol reduce extracellular concentrations of ACh (Rada et al., 1993) other authors have suggested that only activation of $GABA_A$ receptors with muscimol tonically regulates release of ACh while stimulation of $GABA_B$ receptors has a minimal effect (Anderson et al., 1993). At the behavioural level however it has been reported that baclofen but not muscimol blocks turning behaviour elicited by unilateral ACh receptor agonist infusions (Akiyama et al., 2004). It has been reported that cholinergic interneurons are connected to each other in

part via GABAergic interneurons and stimulation in a small population of cholinergic interneurons causes $GABA_A$ receptor mediated inhibition of the rest of the population (Sullivan et al., 2008). It is difficult to envision how these conflicting reports can be reconciled without further investigation, but it would appear that baclofen and muscimol in the Acb could well interact differently with cholinergic feeding and reward modulatory pathways.

It has also been reported that there is a reciprocal relationship between opioid and GABA signalling in the AcbSh modulation of feeding that depends on whether $GABA_A$ or $GABA_B$ receptors are activated. While blockade of δ-, κ- and μ-opioid receptors using a non-selective opioid antagonist or selective antagonists attenuates muscimol induced feeding (Khaimova et al., 2004, Znamensky et al., 2001) only δ- and κ-antagonists attenuate baclofen induced feeding (Khaimova et al., 2004). Conversely feeding induced by a μ-opioid receptor agonist in the AcbSh is increased by blockade of $GABA_A$ receptors but decreased by blockade of $GABA_B$ receptors (Znamensky et al., 2001).

Once again it is difficult to envision how these different interactions occur and their implications for the mechanisms that subserve muscimol or baclofen induced feeding without further investigation. For example, what would be the effect of infusing both GABA and opioid agonists at the same time? It was noted in Chapter 3 that, based on evidence from Will et al., (2007), animals that were on a food restricted diet could have higher levels of striatal enkephalin expression even when fed to satiety that may or may not contribute to the BSS recorded following muscimol infusions. It is possible however that, given the evidence that blockade of $GABA_A$ receptors potentiates μ-opioid receptor agonist induced feeding (Znamensky et al., 2001) the two effects are not mutually compatible. It seems unlikely that the muscimol induced BSS reported in Chapter 3 included a hedonic component modulated by opioid signalling in the AcbSh.

Section summary

The effects of stimulating $GABA_B$ or $GABA_A$ receptors on intake and responding for food reward do not occur in isolation. It is possible that both baclofen and muscimol infused into the AcbSh modulate the interaction of inhibitory amino acid signalling

systems and other neurotransmitter systems. It has been shown that the relationship between $GABA_A$ or $GABA_B$ receptors and ACh or opioid systems differs although the nature of the interaction is hard to decipher without more evidence. The ACh system has been implicated in the control of satiety mechanisms but the evidence is circumstantial. In contrast, there is direct evidence for the role of AcbSh Ach signalling in response to reward and possible the hedonic value of food via its interactions with the opioid signalling system. Given that the BSS with baclofen did not resemble that seen with intra-AcbSh opioid stimulation it seems likely that this manipulation might eliminate the impact of hedonic modulation of feeding via opioid/Ach mechanisms.

Differences at the level of neuronal macrocircuits - could the regions of the brain activated by AcbSh receptor subtype stimulation be part of functionally dissociable macrocircuits?

While differences in the BSS recorded following intra-AcbSh baclofen or muscimol infusions provided evidence that the effects of stimulation of different GABA receptor subtypes could be dissociable it does not really give any clues as to what macrocircuits could be involved. However the observation that baclofen, but not muscimol, increased instrumental responding during both the appetitive and consummatory phase of a 2^{nd} order schedule does provide some insight into which circuits could subserve the effect. This evidence will be discussed in the next section. However, the direct anatomical evidence from the experiments reported in Chapter 6 that used FLI as a marker of increased activity showed that a distinct profile of LH activation was elicited by intra-AcbSh baclofen infusions compared to muscimol infusions. In addition the CeA was uniquely activated by baclofen.

The direct anatomical evidence for potential differences in the effects of $GABA_A$ and $GABA_B$ receptor stimulation on activity in the LH, Arc and PVN was discussed in some detail in Chapter 6 but the wider functional implications of the different patterns of activation were only briefly considered. The key issue that is raised by these results is how they fit into the model of GABA mediated feeding proposed by Kelley et al., (2005b) who suggested that the unique projections from the AcbSh to the LH access "feeding-selective nodes of the behavioural control column". The "behavioural control column" (BCC) was proposed by Swanson (2000) to be interposed between these brain regions and the hierarchically arranged motor system.

The BCC consists of a core ventromedial group of hypothalamic nuclei and other midbrain nuclei divided into a rostral portion critical for circuits that regulate ingestion, reproduction and defensive behaviours, and a caudal portion essential for circuits subserving exploratory and foraging behaviour (Swanson, 2000). The rostral portion includes the medial preoptic nucleus, the Arc, the PVN, the ventromedial nucleus, the tuberal nucleus and the premammillary nuclei and is further subdivided into regions involved specifically in ingestive behaviour which is exemplified by the PVN while the remainder of the nuclei subserve reproduction and defence (Swanson, 2000). The caudal segment encompasses the mammillary body, VTA and SNr (Swanson, 2000). The motor system that the BCC accesses can be subdivided into the somatomotor, autonomic visceral and neuroendocrine secretomotor systems, and Swanson (2000) suggests that the hypothalamus can coordinate responses in all three via connections to motor initiator regions which in turn project to motor generator regions.

The results reported in Chapter 6 indicate that both muscimol and baclofen infused into the AcbSh activate feeding related circuits via nodes in the ingestion (in particular the PVN) and exploratory/foraging related (VTA) portions of the BCC. It is difficult on the basis of these results alone then to determine how each receptor agonist would elicit different types of behavioural organisation (consummatory vs. appetitive responses). However Swanson (2000) points out that ventromedial hypothalamic nodes in the rostral portion of the BCC are "interconnected in a massive, highly differentiated way" and, as was discussed in Chapter 6, it could be the differing interactions between neurons in the LH, Arc and PVN that determine the behavioural output following intra-AcbSh muscimol or baclofen infusions.

The BCC is also a functional link between the cerebral hemispheres and voluntary control of behaviour and Swanson (2000) suggests that the PVN receives cognitive and behavioural state input, relayed via the BNST and ventrolateral septal nucleus which receive input from the PFC, hippocampus and amygdala. The CeA is suggested to be specialised for relaying cortical information to the autonomic motor system (Swanson and Petrovich, 1998). Given that the CeA was activated by baclofen, but not muscimol, it is likely that feeding induced by $GABA_B$ receptor stimulation in the AcbSh is mediated by at least two distinct pathways, one direct to the BCC and one indirect involving connections between the CeA and the BCC.

The functional macrocircuit that links the AcbSh with feeding responsive nodes in the LH is probably a cortico-striato-thalamo-cortical loop while the nodes themselves send projections both to the motor system and to the thalamus (Swanson, 2000). The functional macrocircuit that links the AcbSh and CeA includes indirect projections via the PB, the BNST, the ventrolateral septal nucleus and the NTS (Fudge and Haber, 2000, Fudge et al., 2002, Risold et al., 1997). While activity in these regions was not analysed in the experiments reported in Chapter 6, activity in the lateral septum, thalamus, BNST and NTS has been reported following unilateral muscimol infusions into the AcbSh (Stratford, 2005). Some of the implications of activating these macrocircuits will be discussed later in this chapter.

Section summary

Evidence at the anatomical level presented in this thesis suggests that regions of the brain activated by intra-AcbSh GABA receptor subtype agonists probably participate in different functional macrocircuits. These macrocircuits could gain access to the motor control system via different nodes within the BCC described by Swanson (2000). To fully characterise the functional macrocircuits subserving baclofen and muscimol induced feeding it will be necessary to analyse activity in other brain regions. Swanson (2000) also points out that it is not yet clear how behaviours subserved by different subsystems that involve direct inputs to the motor systems as well as inputs elsewhere in the hierarchically organised motor control system (e.g. to nodes in the BSS) could be integrated and prioritised (Swanson, 2000)

Differences at the level of behavioural output - could different behavioural control mechanisms be subserved by intra-AcbSh GABA receptor subtype stimulation?

Discussion in the previous section provides some evidence at the anatomical level that behaviour elicited by intra-AcbSh infusions of baclofen and muscimol could be subserved by different putative macrocircuits. It is also possible to construct a fuller picture of these macrocircuits by considering what is known about regions of the brain that subserve the types of behaviour reported specifically for the effects of baclofen but not muscimol i.e. increased appetitive and consummatory responses in a 2nd order schedule. To this end the evidence for the contribution of different processes that could

subserve the behaviour recorded with baclofen will first be reviewed and then the brain regions that could be involved in these processes will be discussed.

Cue salience (wanting) or hedonic value (liking)?

Berridge and colleagues have put forward a strong case for the role of both incentive salience/appetitive motivation and hedonic value/palatability, which are termed 'wanting' and 'liking' respectively, in the expression of motivated behaviours (for a review see (Berridge, 2004, Berridge, 2009). While the activation of the VP by both intra-AcbSh infusions of muscimol and baclofen reported in Chapter 6 could indicate a hedonic component (liking) to the GABA mediated feeding response there is no compelling behavioural evidence in the experiments reported here or in the literature that this is the case. Previous studies show that muscimol does not increase the hedonic value (palatability) of ingesta and positive taste reactivity does not correlate with increases in intake (Basso and Kelley, 1999, Reynolds and Berridge, 2002). Secondly baclofen does not increase palatability of a liquid meal as indicated by a lack of the expected changes in the bout structure (Ward et al., 2000).

In this thesis it has been demonstrated that, while baclofen produces a BSS resembling that seen following food deprivation (which can increase both the incentive salience and hedonic value of food), the pattern of behaviour is not consistent with increases in palatability alone (induced by intra-AcbSh infusions of the μ-opioid DAMGO). The BSS with muscimol did not produce a pattern consistent either with combined increases in incentive salience/hedonic value associated with hunger or with increases in palatability alone.

In the 2nd order schedule muscimol had no significant effect on instrumental responding in agreement with previous reports that it does not increase responding on a PR schedule (Zhang et al., 2003), and this lack of effect was evident across an extended dose range compared to that tested in the PR schedule. It would appear that, in the case of intra-AcbSh muscimol infusion, neither increases in wanting or liking can explain the restricted effect on consummatory behaviour. As was concluded in Chapter 5, it is highly likely that as Kelley's hypothesis (2005b) suggests, artificial stimulation of postsynaptic $GABA_A$ receptors in the AcbSh biases the animals towards expression of consummatory responses subserved by increases in subsets of feeding related motor

patterns via changes in activity in the hypothalamus. In contrast intra-AcbSh baclofen increased instrumental responding during both the appetitive and consummatory phases of the 2nd order schedule which suggests some motivational component to the effect. From this point forward the discussion will focus solely on the effects of baclofen.

It was concluded in Chapter 4 that the effects of baclofen could not have been as a result of hedonic increases in primary reward value (or of the CS itself). First of all, at the optimum 220ρmols dose, instrumental responding initially increased before the animals gained access to food. Secondly high rates of responding once food became available cannot be due to hedonic effects because this would require prior experience of the A-O contingency (Dickinson and Balleine, 1994).

In addition total instrumental responding across the session and the rate of responding during the appetitive phase was increased at a higher 440ρmols dose of baclofen that did not increase responding during the consummatory phase suggesting that the instrumental effects of baclofen were independent of the A-O association. In addition the experience of consuming pellets under the influence of baclofen did not cause animals to re-value their reinforcing properties since because response rates between test days did not change as a function of prior drug experience. This did not reflect insensitivity of the animals to the hedonic value of the reward *per se* because, when they received pellets their pressing increased above the rates supported by the CS alone consistent with the suggestion by Thornton-Jones at al., (2005) that positive feedback mechanisms related to feeding can potentiate responding on this schedule.

It was hypothesised that the effects of low doses of baclofen on intake and instrumental responding could be due to increased cue saliency both in terms of the discrete cue for which animals responded in the 2nd order schedule and possibly to contextual cues associated with the test environment in the free feeding test. This was based on the assertion that increased instrumental responding is a measure of increased wanting and can occur without increases in liking of the reinforcer (Wyvell and Berridge, 2000). In addition there was some evidence that baclofen decreased the latency to begin pressing again post pellet delivery suggesting decreased reaction times that are associated with the increased salience of cues predictive of higher reward magnitude (Giertler et al., 2003, Blokland, 1998b).

There was also a shift to the left in the latency to press the lever post cue presentation, the majority of which were not associated with pellet delivery, suggesting animals were quicker to perform the instrumental response irrespective of primary reward presentation. Evidence was presented in Chapter 4 that neurons in the Acb respond to cues that predict reward (e.g. Nicola et al., 2004), DA transmission fluctuates in response to cues (Roitman et al., 2004, Carelli, 2004, Cheer et al., 2007). Increased DA levels in the AcbSh significantly increases cue-potentiated instrumental responding (Wyvell and Berridge, 2000). It has been argued that increased DA transmission increases incentive salience (Berridge, 2007b)

Structures subserving motivational control of appetitive responding with baclofen

The potential role for the AcbSh in basic associations between cues and reward, in instrumental learning and the interaction between the two was briefly discussed in Chapter 1. However, as was pointed out in Chapter 4, it would appear that the processes (and hence underlying macrocircuits) that subserve the ability of cues to control behavioural output in different schedules e.g. CRF, PIT or Pavlovian approach are mutually exclusive (Holland and Petrovich, 2005, Galarce et al., 2007, Balleine, 2005).

While it has been argued that Pavlovian conditioned cue reinforcement of consummatory responses to food is dependent on circuits that involve the BLA and LH, but not the AcbSh and CeA (Holland and Petrovich, 2005), it has been clearly demonstrated that the expression of Pavlovian approach behaviour and cue-potentiated consumption is independent from the expression of conditioned instrumental responding (Di Ciano and Everitt, 2004) (Holland and Petrovich, 2005). Furthermore Holland and Petrovich (2005) acknowledge that the processes that mediate reward, approach and consumption in Pavlovian responding are probably completely separate from those that mediate these behaviours in instrumental behaviours.

It is difficult to decipher exactly how animals experience both the process of acquisition and consequent performance of the 2nd order schedule with regards to these different forms of Pavlovian and instrumental processes. As has been pointed out by other authors “the functional role of the second-order stimulus within second-order schedules is complex” (Wilson and Bowman, 2004a). The results in all three experiments reported in Chapter 4 showed that, in vehicle infused animals, the light cue presented in the 2nd

order schedule was able to maintain a low level of instrumental responding despite the fact that they were pre-fed, suggesting that the CS functioned as a conditioned reinforcer. However Balleine (2005) suggests that, in a 2nd order paradigm, the CS acts both as a conditioned reinforcer, which involves incentive properties associated with the CS itself, and also acts as a more general signal that reinforces and maintains S-R associations.

Parkinson et al., (1999) demonstrated that, in a conditioned reinforcement paradigm the potentiating effects of amphetamine were critically dependent on the intact function of the AcbSh. In a similar experiment it was shown that potentiation of conditioned reinforcement by infusions of amphetamine into the Acb was blocked by lesions of the CeA (Robledo et al., 1996). The direct anatomical evidence from Chapter 6 that intra-AcbSh infusions of baclofen increased activity in the VTA and the CeA would be consistent with an increase in DA in the AcbSh (Wallace et al., 1992). In addition it was argued earlier in this discussion that baclofen but not muscimol infused into the AcbSh could increase the relative contribution of the DA to behaviour.

In a series of PIT experiments it has been demonstrated that the influence of cues over instrumental responding depends on whether the animals are responding on an outcome specific or general form of the schedule (e.g. see Holland and Gallagher, 2003, Corbit and Balleine, 2005). While outcome specific PIT appears to rely on signalling in the BLA (but not the CeA) and on the integrity of the AcbSh (Blundell et al., 2001, Corbit et al., 2001, Corbit and Balleine, 2005) generalised PIT procedures rely on the CeA (but not the BLA) and on the integrity of the AcbC (Holland and Gallagher, 2003, Hall et al., 2001, Corbit and Balleine, 2005). In the generalised form of PIT animals probably associate the action, pressing on a single lever, with reward but not necessarily with the specific properties or value of that reward (Balleine and Killcross, 2006). These authors suggest that the BLA is involved in the formation of specific associations between cues and the incentive value of rewards whereas the CeA is more involved in the development of preparatory responses on the basis of general affective or incentive properties.

In an extension of this argument they suggest that the BLA is involved in processing the value of a cue in a 2nd order paradigm as an instrumental incentive but the CeA

modulates its salience as a reinforcement signal (Balleine and Killcross, 2006). Given that intra-AcbSh infusions of baclofen specifically increase activity in the CeA it seems likely that an incentive explanation for increased appetitive responding is plausible. Thus it could be argued that although the AcbSh might not be directly involved in cue potentiated circuit that would specifically increase the general motivating properties and excitatory effects of stimuli is activated and would be responsible for the increase in goal directed responding.

Although the evidence from studies of PIT suggest that the AcbC but not the AcbSh might be involved in the attribution of incentive salience to cues in the 2nd order schedule it must be remembered that these studies involved lesions of these regions. It is possible that when the integrity of the AcbSh remains intact following baclofen infusion it is able to influence cue related processes mediated by the AcbC and associated circuits via both the direct and indirect connections between these regions discussed in Chapter 1.

Brain substrates subserving disruptions in motor behaviour at high doses of baclofen of muscimol

One point that has not been dealt with yet was the observation that high doses of baclofen and muscimol disrupted the pattern of motor responses in the 2nd order operant schedule. Baclofen produced a breakdown of the normal grooming pattern but elicited grooming and oral stereotypies whereas muscimol fragmented grooming behaviour and elicited fragmented oral behaviour. It is suggested here however that these effects are not incompatible with activity in the proposed circuitry that subserves consummatory and/or appetitive behaviour at lower drug doses.

It has been demonstrated that blocking striatal output disrupts the expression of syntactic chains of motor responses (Berridge and Fentress, 1987). One possible explanation for this is that inactivation of AcbSh output removes the inhibitory influence of the direct Acb projections to mesolimbic DA neurons in, for example, the VTA. It has been argued that increases in DA transmission via manipulation of VTA function enhances the salience of multiple stimuli and behaviour becomes fragmented as animals try to respond to each (Badiani et al., 1995). It has also been shown that psychostimulants (which increase DA transmission in the brain) can elicit motor

stereotypies and that these probably arise as a result of an imbalance in the activity of heterogeneous compartments in the striatum (Canales and Graybiel, 2000). Thus fragmented behaviour with muscimol could be a direct result of increased DA transmission from VTA afferents in the AcbSh while oral stereotypies arise because baclofen modulates the contribution of increased DA to different local circuit activity in the AcbSh.

Section summary

The macrocircuit subserving the behavioural effects of intra-AcbSh muscimol infusions is probably a simple loop involving the direct projections of AcbSh MSNs to the LH which artificially biases animals towards consummatory responses by engaging a specific subset of motor pattern generators in line with Kelley's hypothesis. In contrast selective stimulation of $GABA_B$ receptors in the AcbSh using baclofen engages 1) a macrocircuit that produces consummatory responses involving the LH, 2) another circuit that involves the CeA which is involved in salience associated with the CS acting as a general motivator of response and driving voluntary instrumental responding and 3) subcircuits that result in increased DA release in the AcbSh via connections between the CeA and VTA which could also increase the salience of the cue acting as a reinforcer either directly or via its connections to the AcbC. At high concentrations both agonists cause disruption of behavioural output because of excessive levels of DA transmission in the same circuits but the behavioural impact depends on which GABA receptor subtypes in the AcbSh are stimulated.

Is endogenous AcbSh GABA involved in feeding?

The evidence discussed above suggests that the use of a $GABA_A$ antagonist to inhibit AcbSh output might not re-produce the effects of endogenous GABA because it bypasses multiple inputs to the MSNs that contribute to motivational control. However stimulation of $GABA_B$ presynaptic receptors modulates the relative contribution of the various inputs to MSNs, which might be more in line with the effects of endogenous GABA. Consistent with this hypothesis intra-AcbSh infusions of baclofen produced a BSS that resembled to that produced following food deprivation and increased instrumental responding for food reward consistent with the effects of hunger which potentiates instrumental responding. It was also shown here that, in agreement with previous studies, stimulation of GABA receptors in the AcbSh activates regions of the

brain that have well established roles in feeding related neural circuitry and are similarly activated by hunger and anticipation of feeding (Carr et al., 1998, Stratford, 2005, Johnstone et al., 2006)

Elsewhere it has reported that increasing endogenous levels of GABA by blocking the activity of the GABA-transaminase inhibitor, gamma-vinyl-GABA induces a large increase in feeding (Stratford and Kelley, 1997b). The onset of consummatory responses to food is characterised by inhibition of a population of neurons in the Acb (Taha and Fields, 2005b, Taha and Fields, 2006). Microdialysis studies show that glutamate decreases in the AcbSh during feeding consistent with the possibility that endogenous GABA binds to presynaptic $GABA_B$ heteroreceptors (Rada et al., 1997, Rada et al., 2003). As mentioned previously, it has been reported that feeding initiated by food deprivation and feeding initiated by intra-AcbSh muscimol infusions can both be attenuated by blocking activity in the LH (Stratford and Kelley, 1999, Stanley et al., 1996). Intracerebroventricular administration of NPY antagonists also attenuates feeding induced by both food deprivation and intra-AcbSh muscimol infusions (Stratford and Wirtshafter, 2004) (Chamorro et al., 2002).

Section summary

Evidence presented in this thesis suggests that $GABA_B$ but not $GABA_A$ receptor stimulation could mimic some of the effects of endogenously released GABA in the AcbSh. The observation that this manipulation produces behavioural patterns (delayed BSS and increased instrumental responding) consistent with feeding induced by food deprivation suggests the manipulation is similar to natural modulators of motivational control. When all of this data is taken together with evidence from other studies in the literature it provides a strong body of evidence that suggests the AcbSh and GABA signalling in this region is a critical region in circuits that control the normal, physiological (and possibly cognitive/ voluntary) control of food motivated behaviour.

A modified model to explain the role of inhibitory processes in the AcbSh in motivational control systems.

Kelley et al., (2005b) acknowledge that “artificially enhancing GABA tone...bypasses the control of feeding behaviour exerted by brain regions sending highly processed,

glutamate-coded sensory and motivational information to the ventral striatum". Furthermore inhibiting the AcbSh via indiscriminate postsynaptic binding of muscimol short circuits feeding interruption signals (Kelley et al., 2005b). When tonic inhibition of feeding nodes in the behavioural control column are artificially released by pharmacological blockade of AcbSh output the animals behaviour becomes biased towards consummatory behaviour (Kelley et al., 2005b).

In formulating a model to explain the role of endogenous GABA in the AcbSh in modulating food motivated behaviour Kelley et al., (2005b) suggested that feeding is initiated by extensive glutamatergic stimulation of the entire striatum which would initiate local GABA release from MSN axon collaterals, which inhibits AcbSh output control of downstream feeding related circuits. However an animal needs to be able to switch from feeding to other behaviours, for example when under threat, and Kelley and colleagues suggest that the AcbSh acts as a "sensory sentinel" limiting or gating excitatory interruption signals that would stop feeding but allowing particularly strong focal glutamatergic input to this region to override this behaviour. They go on to state that, while the restricted region of the AcbSh acts a sensory sentinel it only has access to a limited portion of the BCC and even less to voluntary motor control systems. Thus it is rest of the striatum (AcbC and dorsal striatum) that organises more complex environmentally organised motor output.

Taken together, these ideas and observations suggest that the extent to which GABA signalling in the AcbSh limits or gates input depends on the degree to which endogenous GABA recruits presynaptic $GABA_B$ receptors or postsynaptic $GABA_A$ receptors. $GABA_B$ receptors have a high affinity for GABA (Jones et al., 1998) whereas $GABA_A$ receptors have a much lower affinity for GABA and also desensitize more rapidly (Macdonald and Olsen, 1994). Thus when endogenous GABA in the Acb is increased it will more likely recruit $GABA_B$ receptors before $GABA_A$ receptors. Widespread glutamatergic input to the entire striatum will increase GABA release but, within normal physiological parameters e.g. energy deficit that drives hunger, could predominantly activate presynaptic $GABA_B$ receptors. This could serve to initiate consummatory responses but also modulate the relative impact of DA inputs that energise responding and contribute to more complex food seeking strategies based on the salience of cues that predict food availability. Thus, while consummatory responses

are mediated by inhibitory neurotransmission in this region, this does not exclude a role for the AcbSh in the organisation of more complex behaviours. The AcbSh can access voluntary behavioural control circuits via indirect pathways involving the LH, CeA and reciprocal connections between the AcbSh and AcbC and the associated increases in DA transmission drives appetitive responding as a result of increases in cue saliency. Increases in hedonic value of reward will be driven predominantly by physiological activation of LH circuits which in turn directly recruits the opioid signalling pathway in the AcbSh and all of these processes will be in direct competition with the cognitive control of responding for food via the glutamatergic interruption signals that Kelley et al., (2005b) refer to.

Future directions

As was pointed out at the start of this discussion the nature of the feeding response elicited by intra-AcbSh infusions of the $GABA_A$ agonist muscimol and the $GABA_B$ agonist baclofen has not been extensively explored. The results reported in this thesis suggest that there is good reason to believe that stimulation of $GABA_B$ receptors more appropriately reveals the effects of endogenous GABA in the AcbSh than blocking $GABA_A$ receptors which bypasses much of the normal circuitry involved in the control of motivation.

It will be important to determine if intra-AcbSh baclofen will also have different effects to those reported for muscimol in paradigms that have already been used to test the role of AcbSh GABA in feeding. For example it remains to be determined if baclofen can increase instrumental responding for reward on a PR schedule or if it can potentiate the acquisition of an instrumental response for food reward. It will also be interesting to find out whether baclofen has any effect on taste reactivity, whether there is a similar positive/ aversive rostrocaudal gradient as that reported for intra-AcbSh muscimol infusions and if it can support the development of place preference and avoidance. It would also be interesting to explore whether baclofen or muscimol have any effects on long term intake in, for example, a meal patterning experiment.

In addition it will be interesting to use different behavioural paradigms to determine whether intra-AcbSh infusions of baclofen increase food intake and food motivated instrumental responding via effects on motor pattern generators, on cue salience or on

hedonic value of the reinforcer. To this end I suggest that the effects of intra-AcbSh baclofen could be tested in a conditioned incentive paradigm whereby the cue is presented in the absence of reinforcement. It might also be interesting to set up experiments where the impact of discrete and contextual cues is manipulated or test the animals in familiar or novel environments that could effect the total amount consumed by the animals. The effects of intra-AcbSh baclofen infusions on macronutrient selection should also be further explored. The effects of baclofen and muscimol in the AcbSh on PIT, conditioned reinforcement and Pavlovian cue-reinforced responding could also be investigated.

Finally given the tantalising evidence reported here that intra-AcbSh baclofen activates regions of the brain not activated by muscimol it will be important to further explore the levels of activity in other regions of the brain such as the SN, thalamus, lateral habenula, nucleus of the solitary tract and the bed nucleus of the stria terminalis using Fos as a marker. In particular there is a need for double labelling studies to clarify whether, in the regions where both baclofen and muscimol exert effects, different populations of neurons are activated. It would also be important to determine what areas are activated during both baclofen induced consummatory behaviour and instrumental behaviour. To determine the functional links between these regions it would then be interesting to find out if blockade of activity in any of these regions attenuates or changes the baclofen induced feeding response. For example would blockade of activity in the CeA decrease feeding and/or decrease the effects of baclofen on instrumental responding for food?

Implications of the results reported in this thesis

The results reported in this thesis point to a broader and more complex role of the AcbSh in the control of motivated behaviour than one limited to an involvement in the release of predetermined behavioural patterns and unconditioned responses to goals. It is possible that the nucleus AcbSh modulates cognitive processing systems responsible for the assessment of the salience of environmental stimuli, and that it is involved in circuits that determine both wanting and liking of reinforcers. The AcbSh may also be a critical node in circuits subserving motivational processes through its ability to both directly and indirectly influence the activity of the AcbC and the dorsal striatum.

The need to understand how the AcbSh contributes to the control of motivated behaviours is particularly compelling given its role in addiction to drugs of abuse (Leshner and Koob, 1999, Koob, 2000, Berridge, 2004, Cardinal and Everitt, 2004, Carelli and Wightman, 2004, Everitt et al., 2008) as well in the pathological functioning of feeding related circuits in eating disorders and the development of obesity (Shapira et al., 2005, Kelley, 2004, Kelley et al., 2005b, Kelley et al., 2005a, Hetherington and Ranson, 1939, Berridge, 2007a, Stratford, 2007). Furthermore the Acb is implicated in a number of affective disorders in humans (e.g. Pizzagalli et al., 2009, Sarter et al., 2005, Munte et al., 2007, Menzies et al., 2008). The parallel between research in the areas of addiction and obesity are becoming increasingly apparent and many investigators believe that drugs of abuse 'hijack' systems subserving motivation for biologically relevant stimuli such as food (Volkow et al., 2008).

There are also broader implications of this work. There has been a recent move towards the evaluation of the Acb as a therapeutic target using deep brain stimulation to treat, for example, obsessive compulsive disorders (Okun et al., 2007), depression (Aouizerate et al., 2009), obesity (Halpern et al., 2008) and addiction (Kuhn et al., 2009). A better understanding of the role of the AcbSh subregion will be essential for developing appropriate strategies for therapies that target this brain region that will not have undesirable effects on non-pathological aspects of patient behaviour.

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