Genetic and pharmacological investigation of α4-containing $GABA_A$ receptors in conditioned behaviours influenced by cocaine

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

Signature ……..

University of Sussex

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Genetic and pharmacological investigation of α4-containing $GABA_A$ receptors in conditioned behaviours influenced by cocaine

α4-subunit containing $GABA_A$ receptors (α4-$GABA_A$Rs) are often found co-assembled with δ-subunits in extrasynaptic locations on nucleus accumbens (NAc) medium spiny neurons (MSNs), were they mediate a tonic form of inhibition thought to control the excitability of the neuron. This thesis combines genetic and pharmacological techniques to explore the role of α4-$GABA_A$Rs in locomotor and reward-conditioned behaviours.

Activation of α4-$GABA_A$Rs by systemic or intra-accumbal administration of THIP, a $GABA_A$R agonist with a preference for δ-subunits, was able to reduce cocaine-potentiated locomotor activity in wildtype but not $GABA_A$R α4-subunit knockout mice. Similarly, the ability of repeated cocaine to induce behavioural sensitisation was unaffected in $GABA_A$R α4-subunit knockout mice, but systemic THIP was able to reduce the sensitised increase in locomotor activity in wildtype but not knockout mice. α4-$GABA_A$Rs are also able to modulate behavioural responses to reward-conditioned stimuli and their enhancement by cocaine. Deletion of $GABA_A$R α4-subunits from dopamine D1-expressing neurons facilitated cocaine-CPP, and activation of α4-$GABA_A$Rs on NAc D1-MSNs was able to attenuate cocaine-enhancement of cocaine CPP. Conversely, deletion of $GABA_A$R α4-subunits from dopamine D2-expressing neurons increased CRf responding, and activation of α4-$GABA_A$Rs on NAc D2-MSNs was able to attenuate cocaine-potentiation of CRf responding. These data also indicate that there is a dissociation in the NAc MSNs mediating cocaine-CPP and CRf responding. This may be explained by the different glutamatergic inputs needed to provide information about conditioned cues important for these behaviours.

The data presented within this thesis indicate that α4-$GABA_A$R-mediated inhibition of D1- and D2-expressing neurons plays an important physiological role in controlling behavioural responses to conditioned cues. Furthermore, NAc α4βδ $GABA_A$Rs may provide a potential therapeutic target by which to limit the enhancement of locomotor and conditioned-behaviours by cocaine.

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List of Abbreviations

α2-$GABA_AR$	=	α2-containing $GABA_A$ receptor
α4-$GABA_AR$	=	α4-containing $GABA_A$ receptor
ACh	=	Acetylcholine
AMG	=	Amygdala
AMPA	=	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BAC	=	Bacterial Artificial Chromosome
BDZ	=	Benzodiazepine
BG	=	Basal Ganglia
BLA	=	Basolateral Amygdala
CDP	=	Chlordiazepoxide
CeA	=	Central Nucleus of the Amygdala
Cl^-	=	Chloride
CNS	=	Central Nervous System
CPA	=	Conditioned Place Avoidance
CPP	=	Conditioned Place Preference
CRf	=	Conditioned Reinforcement
CS	=	Conditioned Stimulus
DREADD	=	Designer Receptor Exclusively Activated by a Designer Drug
eGFP	=	Enhanced Green Fluorescent Protein
EPSP	=	Excitatory Postsynaptic Potential
GABA	=	γ-aminobutyric acid
$GABA_AR$	=	$GABA_A$ receptor
GFP	=	Green Fluorescent Protein
GPe	=	Globus Pallidus externa
GPi	=	Globus Pallidus interna
GVG	=	Gamma-Vinyl GABA
HIP	=	Hippocampus
LTP	=	Long-Term Potentiation
mPFC	=	Medial Prefrontal Cortex
MSN	=	Medium Spiny Neuron
NAc	=	Nucleus Accumbens
NL2	=	Neuroligin2

NMDA	=	N-methyl-D-aspartic acid
PFC	=	Prefrontal Cortex
PIT	=	Pavlovian to Instrumental Transfer
PPTg	=	Pedunculopontine Tegmental Nucleus
qRT-PCR	=	Quantitative Reverse Transcriptase Polymerase Chain Reaction
Ro 15-4513	=	Ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-1,4-benzodiazepine-3-carboxylate
SNc	=	Substantia Nigra pars compacta
SNr	=	Substantia Nigra pars Reticulata
SNP	=	Single Nucleotide Polymorphism
STN	=	Subthalamic Nucleus
THIP	=	4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol
TM	=	Transmembrane
VI	=	Variable Interval
VP	=	Ventral Pallidum
VTA	=	Ventral Tegmental Area
WT	=	Wildtype

Chapter 1

Introduction

1.1. The nature of drug abuse and addiction

Recent world health organisation (WHO) statistics estimate that between 3.4-6.6% of the global adult population used an illicit drug in the five years leading up to, and including 2010 (World Health Organization, 2012). Within the UK alone approximately 5.6 million adults (8.9% of the national population) used an illicit drug in 2012 (UK Home Office, 2012). Of these users, some 10-13% continue to be problem users with drug dependence and/or use disorders (British Medical Association, 2012). While the use of many illicit drugs has fallen in recent years, global cocaine use has remained stable, with between 13.2-19.5 million users worldwide (World Health Organization, 2012), Indeed, within the UK, approximately 2.2% of adults (1.4 million people) are reported to have used cocaine within 2012 (UK Home Office, 2012). With the cost of Class A drug-related crime estimated to be approximately £100billion since 1998, with an extra £10billion in health costs, it is clear that substance abuse and addiction to drugs of abuse, including cocaine, are considerable economic and social problems (British Medical Association, 2012). Thus there is an explicit need for comprehensive research into the cellular, molecular, genetic and behavioural etiology of addiction to drugs of abuse, in order to provide efficacious treatments.

1.2. Why study $GABA_A$ receptors in the context of addiction?

γ-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter within the mammalian central nervous system and thus is critically involved in the regulation of neuronal excitability. $GABA_A$ receptors ($GABA_ARs$) are widely distributed throughout the brain and are extremely heterogeneous, with individual subunit compositions conferring a broad range of physiological properties and functional roles. It is now clear that $GABA_ARs$ may play an important role in mediating the rewarding and motivational properties of addictive drugs. Indeed, $GABA_ARs$ are reported to influence the effects of many abused drugs, including; psychostimulants, alcohol, benzodiazepines and

barbiturates. Consequently, compounds altering the activity of specific $GABA_AR$ subtypes may also prove efficacious in the treatment of addiction to drugs of abuse.

This thesis will explore the role of α4-containing $GABA_ARs$ (α4-$GABA_ARs$) in behaviours associated with addiction to drugs of abuse. Furthermore, the role of α4-$GABA_ARs$ in controlling cocaine influences on addiction-related behaviours will also be explored. This introduction will begin by describing reward-associated neuronal circuitry and properties of $GABA_ARs$. Finally, $GABA_AR$-associated control of addiction-related behaviours will be discussed.

1.3. Reward circuitry and GABAergic components

1.3.1. The Basal Ganglia

The basal ganglia (BG) are a collection of interconnected subcortical nuclei, including the striatum, globus pallidus externa (GPe) and interna (GPi), substantia nigra pars compacta (SNc) and pars reticulata (SNr), and subthalamic nucleus (STN). Regions within the BG are anatomically linked to the cerebral cortex and thalamo-cortical motor system via a series of parallel, but largely structurally and functionally distinct cortico-subcortical circuits (Alexander and Crutcher, 1990; Haber, 2003). This topology is proposed to dynamically and adaptively mediate the flow of information from the frontal cortex to the motor system, resulting in facilitation or inhibition of competing actions (Mink, 1996; Nicola, 2006). Thus, the BG are critical for the coordination of cognitive, motor and emotional functions, and their dysfunction underlies a multitude of neuropathologies (Alexander and Crutcher, 1990; Haber, 2003; Cohen and Frank, 2009).

1.3.1.1. Basic circuit anatomy of the Basal Ganglia

Classically, BG loops are identified according to the presumed role of the main cortical projection areas, and have been subdivided as; motor, oculomotor, limbic, associative, and orbitofrontal circuits (Alexander et al., 1986; Alexander and Crutcher, 1990; DeLong and Wichmann, 2007). Alternatively, these circuits have also been functionally categorized into the; motor, visual, executive, and motivational loops (Seger, 2008;

Seger and Spiering, 2011). Each of these loops share certain topological features common to all cortico-subcortical circuits. Specific regions of the cortex send excitatory glutamatergic projections to the input structures of the BG, including the striatum and STN (Alexander and Crutcher, 1990). From here, the BG ouput nuclei, including the GPi, SNr and ventral pallidum exert a tonic GABA-mediated inhibitory control over their target nuclei in the thalamus. Finally, the thalamus then sends excitatory glutamatergic projections back to the cortex, thus completing the 'loop'.

1.3.1.2. Basal Ganglia Pathways

Within each circuit, the influence of neuronal afferents coding for specific actions or tasks are modulated with the support of three different pathways, passing from the cortex to the thalamus, known as the 'direct', 'indirect' and 'hyperdirect' pathways.

The *direct* pathway, often referred to as the "Go" pathway, originates in dopamine D1-receptor expressing striatonigral neurons co-expressing the peptides substance-P and dynorphin (Vincent et al., 1982; Christensson-Nylander et al., 1986). Neurons in the *direct* pathway form monosynaptic inhibitory connections with SNr/GPi neurons, suppressing inhibition of the thalamus, and ultimately disinhibiting selected behaviours (Chevalier et al., 1985; Chevalier and Deniau, 1990). Contrary to the *direct* pathway, the *indirect* pathway, termed the "NoGo" pathway, originates in dopamine D2-receptor expressing striatopallidal neurons co-expressing enkephalin (Beckstead and Kersey, 1985; Gerfen et al., 1990). Neurons within the *indirect* pathway project to the GPe and onto the SNr/GPi complex via a polysynaptic disinhibitory connection, and an indirect GPe-STN-GPi connection, ultimately inhibiting the thalamus and suppressing selected behaviours (Albin et al., 1995; Cohen and Frank, 2009).

More recently the importance of the STN has been highlighted, with the discovery of the cortico-subthalamo-pallidal "hyperdirect" pathway, in which cortical afferents bypasses the striatum altogether, projecting directly to the STN (Nambu et al., 2002). This pathway has been termed the "Global NoGo" pathway as the STN sends diffuse excitatory projections to many GPi neurons, producing a global rather than selective suppression of responses (Frank, 2006; Cohen and Frank, 2009). It has been proposed that this pathway is especially important for premature response inhibition, and termination of initiated behaviours (Aron and Poldrack, 2006; Frank, 2006).

Figure 1.1.

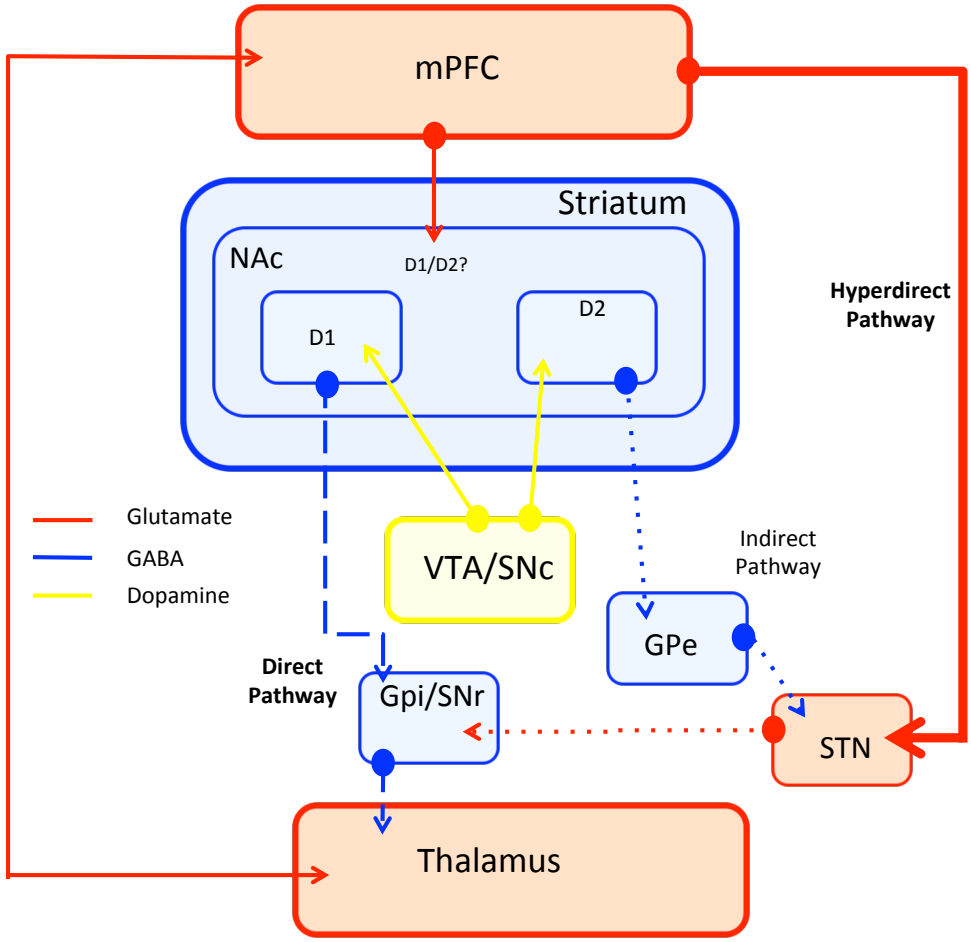


Fig.1.1. A simplified model of striatal *direct*, *indirect* and *hyperdirect* pathways. Striatonigral D1 *direct* pathway neurons inhibit the GPi/SNr and release inhibition of thalamic activity, promoting behaviour. Whereas, striatonigral D2 *indirect* pathway neurons inhibit the GPe, disinhibiting the STN and exciting the GPi/SNr, which ultimately inhibits the thalamus and thus suppresses behaviour. The cortico-subthalamo-pallidal *hyperdirect* pathway also suppresses behaviour by exciting the STN, which then excites the GPi/SNr resulting in an inhibition of the thalamus. The balance between these opposing projections is likely to be regulated by both dopaminergic and GABAergic signaling within the striatum. mPFC, medial prefrontal cortex; NAc, nucleus accumbens; VTA, ventral tegmental area; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; GPe, external globus pallidus; GPi, internal globus pallidus; STN, subthalamic nucleus.

1.3.1.3. The Striatum

Effective BG action selection is contingent upon the precise modulation of neuronal excitability within each of the BG nuclei, a role thought to be principally mediated by neurons within the largest component and primary afferent structure of the BG, the striatum.

1.3.1.3.1. Striatal Medium Spiny Neurons (MSNs)

The vast majority of neurons within the striatum are GABAergic projection medium spiny neurons (MSNs), accounting for approximately 95% of the total neuronal population (Wilson, 1993). MSNs are the target of glutamatergic inputs from the cortex, ventral hippocampus, amygdala and thalamus synapsing at the spines, as well as midbrain dopaminergic projections received at the dendrites and spine necks (Smith et al., 1994). Striatopallidal MSNs are a major target for topographical sensorimotor corticostriatal projections, afferents from neighbouring regions of the cortex projecting to neighbouring regions of the striatum (Berretta et al., 1997; Wright et al., 1999).

Classically, MSNs have been characterised electrophysiologically by their hyperpolarized resting membrane potential and low input resistance (Kita et al., 1984). More recently the development of bacterial artificial chromosome (BAC) transgenic mice expressing enhanced green fluorescent protein (eGFP) under the control of promoters for D_1 and D_2 receptors has allowed for the investigation of distinct physiological differences between striatonigral and striatopallidal MSNs. Whole-cell patch-clamp recordings revealed D_2-expressing indirect-pathway MSNs to exhibit larger EPSPs and greater repetitive spiking than D_1-expressing direct-pathway MSNs (Kreitzer and Malenka, 2007). This physiological dichotomy may in part be explained by anatomical differences between D_1- and D_2 MSNs; D_1 MSNs have a considerably greater dendritic surface area than that of D_2 MSNs (Gertler *et al.*, 2008). However this difference was not attributable to greater branching or length of dendrites, but to a greater total number of primary dendrites on D_1 MSNs.

Early *in vivo* recordings also revealed striatal MSNs to demonstrate irregular burst firing, accompanied by a shift between two preferred subthreshold membrane potential states (Wilson and Kawaguchi, 1996). Membrane potentials alternate between a resting hyperpolarized ‘down’ state (-90 to -70 mV), and a less hyperpolarized ‘up’ state (-60 to

-40 mV). Irregular spike discharge and spontaneous burst firing are observed only during the up state during which MSNs are only a few millivolts (3-5 mV) below spike threshold (Wilson and Groves, 1981). Thus the transition from the down state to the up state is proposed to be critical for spike firing in MSNs.

The two-state behaviour of MSNs arises from both their intrinsic membrane properties, and phasic changes in the excitatory inputs they receive (Wilson and Kawaguchi, 1996). During the down state the input resistance of MSNs is low (10-30 MOhms), creating a stable membrane potential that is relatively insensitive to small synaptic inputs. This inward rectification is created by the high expression of hyperpolarization-activated KIR2 potassium channels, which move the membrane potential closer to the potassium reversal potential and therefore limit membrane depolarization (Nisenbaum and Wilson, 1995). However, in the presence of a sufficiently strong synchronous depolarizing input, MSNs can shift to an outwardly rectifying up state (Blackwell *et al.*, 2003). This up state is dependent upon sustained excitatory input and is modulated by the influence of depolarization-activated potassium channels, largely in the Kv1 family, which maintain the membrane potential within a relatively narrow range marginally below the spike threshold (Shen *et al.*, 2004). Given the action of these intrinsically rectifying ion channels it has been questioned what kind of synaptic input can trigger spiking in the up state? One possibility is that brief depolarisations following rapidly changing synaptic currents provide a window during which large sudden inputs can trigger a spike before voltage-sensitive channels are recruited to oppose their action (Wilson, 1995). Another possibility is that GABAergic activity may synchronise with large excitatory inputs to enable spikes to be triggered.

GABAergic activity has been revealed to be involved in the generation of the up state in striatal MSNs (Kita, 1996). Although GABAergic inputs to MSNs have classically been considered inhibitory, activation of $GABA_A$Rs has been demonstrated to produce excitatory effects under certain physiological conditions (Cherubini et al., 1991; Gulledge and Stuart, 2003). The reversal potential of $GABA_A$-mediated synaptic responses in MSNs lies within the range for activation by outwardly rectifying up state currents (Misgeld *et al.*, 1982). It has been shown that when the up state in MSNs is below the reversal potential of $GABA_A$ inputs, inhibition from fast-spiking interneurons results in depolarisation of MSNs (Plenz and Kitai, 1998), further adding to the

excitatory cortical inputs directly onto the MSNs.

1.3.1.3.2. Striatal Interneurons

The remaining 5% of striatal neurons consist of aspiny interneurons and can be characterised anatomically and histochemically as large cholinergic interneurons, and medium-sized GABAergic interneurons, divided into *(a)* parvalbumin-, *(b)* somatostatin-, neuropeptide Y- and nitric oxide synthase-, and (c) calretinin-expressing interneurons (Kawaguchi *et al.*, 1995). In comparison with MSNs, receptor phenotype and functional role of these striatal interneurons remain relatively unclear.

The best best-explored interneurons, cholinergic interneurons, are characterised morphologically by their large (20-50um diameter) cell body and widespread dendritic and axonal fields (Wilson et al., 1990; Kawaguchi et al., 1995). Electrophysiologically, *in vivo* recordings reveal cholinergic interneurons to exhibit slow irregular but tonic spontaneous activity (2-10Hz), a depolarised resting potential and long-duration action potentials (Wilson et al., 1990; Bennett et al., 2000). In addition to excitatory innervation from the thalamus and cortex, cholinergic interneurons receive inhibitory GABAergic inputs from MSNs (Bolam et al., 1986; Chang, 1988; Brown et al., 2012). Although few in number (1-2% of striatal cells), these interneurons exert a powerful influence over the striatum, integrating synaptic inputs over an extensive area, and projecting to multiple MSNs, and to a lesser extent, fast-spiking parvalbumin-expressing interneurons (Bolam et al., 1984; Chang and Kita, 1992). More recently it has been argued that cholinergic interneurons are heavily involved in reward-based learning and act as key mediators of dopamine-dependent striatal plasticity (Wang et al., 2006). Midbrain dopamine neurons and tonically active striatal cholinergic interneurons are known to act cooperatively, dynamically modifying their activity to signal reward-related events (Cragg, 2006). A pause in the tonic activity of cholinergic interneurons in response to salient cues is proposed to serve as a 'temporal window', allowing phasic dopaminergic activity to be distinguished from previously gated tonic dopamine states (Morris et al., 2004). This complex partnership makes it is possible for dopamine to signal *what* to learn, and cholinergic interneurons to signal *when* to learn. This is further supported by recent evidence that GABA projection neurons from the ventral tegmental area (VTA) synapse almost exclusively on NAc cholinergic interneurons, inhibiting their activity to promote stimulus-outcome learning (Brown *et al.*, 2012).

GABAergic interneurons have also been shown to express distinct physiological properties. A population of roughly 1% of all striatal neurons are distinguished histochemically by the selective expression of the calcium-binding protein parvalbumin (Berke, 2011). Electrophysiologically, these interneurons express fast-firing and short duration action potentials with a short-spike after-hyperpolarization, and thus have been termed fast-spiking (FS) interneurons (Kawaguchi et al., 1995). FS interneurons receive excitatory inputs from the cortex and thalamus, and inhibitory inputs from other interneurons and a subpopulation of neurons within the globus pallidus (Chang and Kita, 1992; Bevan et al., 1998; Sidibé and Smith, 1999; Ramanathan et al., 2002). Unlike MSNs, which receive very few synapses from many different afferents, FS interneurons receive multiple inputs from individual afferent fibres (Bennett and Bolam, 1994). FS interneurons are functionally coupled via both chemical synapses and gap junctions on their dendrites (Kita et al., 1990). The resulting matrix is predicted to synchronize FS interneuron activity and coordinate inhibitory projections onto MSNs (Fukuda, 2009). The presence of $GABA_A$-mediated synaptic contacts onto numerous MSN somata and dendrites allows FS interneurons to inhibit the generation of action potentials (Bennett and Bolam, 1994; Kubota and Kawaguchi, 2000). Each FS interneuron projects to as many as 135-541 MSNs, with each MSN receiving input from approximately 4-27 FS interneurons (Koós and Tepper, 1999). The complete role of these interneurons is not yet clear, although given their rich inhibitory connectivity with MSNs it is proposed that parvalbumin-containing FS interneurons contribute significantly to regulation of MSN activity (Berke, 2011).

The second group of GABAergic interneuron are those expressing somatostatin, neuropeptide Y, and nitric oxide synthase (Kawaguchi et al., 1995). These interneurons exhibit unique electrophysiological properties, demonstrating low-threshold and persistent plateau depolarizations, high input resistance, and relatively depolarized resting potentials, and thus have been termed low-threshold spiking (LTS) interneurons (Kawaguchi, 1993). Morphologically these LTS interneurons are characterized by fewer dendritic branches and less dense, more extensive axonal arborisation in comparison to FS and cholinergic interneurons (Kawaguchi, 1993). Similar to FS interneurons, LTS interneurons receive innervation from both the cortex and the thalamus, and project to MSNs (Vuillet *et al.*, 1989). However, LTS interneurons have also been shown to receive synaptic inputs from nigrostiatal dopaminergic afferents (Kubota *et al.*, 1988).

Interestingly a third population of striatal GABAergic interneurons has been revealed to express similar morphological and electrophysiological properties to those expressing somatostatin, neuropeptide Y, and nitric oxide synthase, and thus are thought to be a subtype of LTS interneurons. These interneurons express calretinin, a calcium binding protein, and are found predominantly within the rostral-medial region of the caudate putamen, where they are proposed to act as calcium buffers (Baimbridge et al., 1992; Résibois and Rogers, 1992). Although exhibiting inputs from cortical afferents similar to those of other LTS interneurons, calretinin-expressing interneurons do not receive any innervation from the thalamus (Sidibé and Smith, 1999). Although it is likely that LTS neurons also act to dynamically modulate the activity of the MSNs to which they project, their physiological role is largely yet to be elucidated.

1.3.1.3.3. Striatal Architecture

Classically, the striatum has been divided into two subregions, dorsal and ventral, on the basis of specific cortical, thalamic and dopaminergic afferents (Heimer and Wilson, 1975; McGeorge and Faull, 1989). Broadly, the dorsal striatum comprises the caudate nucleus and putamen, whereas the ventral striatum incorporates the nucleus accumbens (NAc) core and shell, olfactory tubercle, and the ventromedial portions of the caudate and putamen. However, it is difficult to clearly define anatomical boundaries between these subregions, and an alternative ventromedial-dorsolateral functional zonation has also been proposed (O'Doherty et al., 2004; Voorn et al., 2004; Atallah et al., 2007).

In accordance with a functional delineation, cortical, thalamic and amygdaloid inputs into the striatum are primarily arranged in a dorsolateral-to-ventromedial fashion. Premotor and motor cortical areas, the mediodorsal, ventroanterior and ventrolateral thalamus, and the central nucleus of the amygdala (CeA), largely project to the caudate nucleus and putamen, supporting the involvement of the dorsal striatum in sensorimotor control and motor planning (Kemp and Powell, 1970; Aldridge et al., 1980; McFarland and Haber, 2000). Conversely, axon collaterals from the orbital and medial prefrontal cortex, midline and medial intralaminar nuclei, and basolateral amygdala (BLA), are received within the ventral striatum, notably the NAc, and are thought to be critical in the development of reward-based learning and goal-directed behaviours (Kunishio and Haber, 1994; Everitt et al., 1999; Haber, 2003). A further input is from ventral hippocampus, which may play an important role in context (Crombag et al., 2008).

Thus, although cytoarchitecturally overlapping, the substantial convergence of afferents into distinct domains, has lead to the dorsal and ventral striatum being widely functionally distinguished within the literature as the caudate-putamen complex and NAc, respectively (McFarland and Haber, 2000; Haber, 2003).

1.3.1.3.4. The Nucleus Accumbens (NAc)

The NAc, comprising the subregions core and shell, is known to be a critical structure for mediating the rewarding and motivational properties of addictive drugs (Wise, 1998; Everitt et al., 2001). Inputs from the VTA, prefrontal cortex (PFC) and BLA converge within the NAc, where goal-seeking actions are selected in response to reward-predictive stimuli (Wise, 1998; Hikosaka et al., 2006; Nicola, 2006). A common facet of many drugs of abuse is their ability to increase dopamine release within the NAc, especially within the shell subregion (Wise, 1987; Di Chiara and Imperato, 1988; Wise, 1988; Pettit and Justice, 1989). Electrophysiological evidence has revealed NAc neurons to increase in sensitivity to dopamine following repeated cocaine exposure (Henry and White, 1991). This neural sensitisation of the NAc and other mesolimbic dopamine systems is known to be extremely robust, with behavioural effects of sensitisation, including potentiated locomotor activity following amphetamine, persisting undiminished for over a year, and possibly longer (Robinson and Berridge, 1993). Moreover it has been argued that repeated drug exposure also leads to sensitisation to the incentive motivational properties of drugs (Robinson and Berridge, 1993). In keeping with these ideas, pre-sensitisation with amphetamine, cocaine, morphine or ethanol has been shown to facilitate the later acquisition of self-administration or conditioned place preference (CPP) produced by the same, or a different drug (Lett, 1989; Horger et al., 1990; Piazza et al., 1990; Mendrek et al., 1998; Hoshaw and Lewis, 2001; Camarini and Hodge, 2004).

The incentive-sensitisation theory proposed by Robinson and Berridge (1993), suggests that the neural substrates mediating ‘wanting’, the attribution of incentive salience, and ‘liking’, the hedonic experience, of a drug are dissociable. Following repeated exposure to the drug, ‘wanting’ but not ‘liking’ becomes sensitized (Berridge and Robinson, 1995). Through associative learning, drug-associated stimuli gain incentive salience and can in themselves trigger ‘wanting’, even in the face of reduced ‘liking’. This notion of a dissociation between ‘wanting’ and ‘liking’ is supported by an anatomical separation

between the reinforcing effects of drugs, and more general aspects of natural reward. Lesions of the NAc shell were sufficient to block cocaine- and amphetamine-CPP (Sellings and Clarke, 2003; Sellings et al., 2006), and accordingly, intra-NAc shell administration of cocaine facilitated CPP, with core infusions having no effect (Liao *et al.*, 2000). Similarly, intra-NAc shell infusions of amphetamine facilitated the ability of a Pavlovian reward cue to trigger increased instrumental responding for a sucrose reward, without increasing the positive hedonic reaction to the sucrose (Wyvell and Berridge, 2000). Interestingly, lesions of the NAc shell attenuated of the induction of, but not expression of behavioural sensitisation to cocaine (Todtenkopf et al., 2002a; 2002b), and intra-NAc shell but not core infusions of cocaine or amphetamine were sufficient to induce sensitisation (Pierce and Kalivas, 1995; Filip and Siwanowicz, 2001). More recently, it has been revealed that this functional delineation may not be as simple as first appeared, with the both the NAc shell and core being implicated in drug-seeking, however the former controlling behaviour by spatial/contextual information, and the later controlling behaviour by discrete cues (Bossert et al., 2007; Ito et al., 2008; Ito and Hayen, 2011). The intricacies of these processes and the influence of information from hippocampal and amygdala projections to the core and shell have yet to be elucidated.

The neural mechanisms of action selection within the NAc are still largely unclear, but a recent hypothesis posits that neuronal ensembles within the NAc may represent different stimulus-action associations, which when activated by conditioned stimuli compete with each other for control of behaviour (Nicola, 2006). This is supported by histochemical evidence that following repeated exposure to cocaine in a specific environmental context, a small population of sparsely distributed accumbal neurons are selectively activated by cocaine only when in the conditioned environment (Crombag *et al.*, 2002; Mattson *et al.*, 2008). Selective inactivation of these neurons with the 'Duan02 inactivation method' was demonstrated to attenuate cocaine-induced locomotor sensitisation in animals receiving cocaine in the drug-paired but not non-paired environment (Koya et al., 2009). Under this model, drug-induced dopamine release in the NAc acts to increase the firing of neurons representing stimulus-action associations likely to lead to maximal reward (Nicola, 2006). Although still unclear, it has been suggested that $GABA_A$Rs within the NAc may also facilitate action selection and discriminative amplification via their ability to mediate lateral inhibition between

NAc MSNs, thus suppressing competing interactions between single projection neurons (Taverna et al., 2004; 2005).

Indeed, a role for NAc $GABA_A$Rs in mediating the rewarding and motivational properties of drugs, and addiction-related behaviours, has begun to be revealed. Microinjections of $GABA_A$ receptor agonists directly into the NAc shell have been demonstrated to increase consumption of sucrose, with no difference in water consumption (Basso and Kelley, 1999). Moreover, GABAergic transmission within local microcircuits of the NAc shell have been shown to mediate motivated behaviours, with rostral shell infusions of the $GABA_A$ agonist muscimol inducing CPP, and caudal shell infusions inducing conditioned place aversion (Reynolds and Berridge, 2001; 2002).

1.3.1.4. The Ventral Tegmental Area (VTA)

The VTA is the origin of the dopaminergic cell bodies of the mesolimbic dopamine system, and projects to the striatum, in particular the NAc, as well as limbic-related areas, including the amygdala, hippocampus and prefrontal cortex (Swanson, 1982; Albanese and Minciacchi, 1983; Ikemoto, 2007). Rodents have been shown to readily self-administer cocaine, morphine, nicotine or ethanol directly into the VTA (Bozarth and Wise, 1981; Corrigall et al., 1994; David et al., 2004; Rodd et al., 2004; 2005), and accordingly, lesions of the VTA disrupted self-administration of cocaine and heroin (Roberts and Koob, 1982; Bozarth and Wise, 1986). Furthermore, it has been proposed that neuroadaptations of the VTA play an important role in the initiation of behavioural sensitisation, while the NAc is involved in the expression of sensitisation (Kalivas and Stewart, 1991; Pierce and Kalivas, 1997; White and Kalivas, 1998). This is supported by evidence that repeated administration of cocaine, amphetamine or dopamine re-uptake inhibitors into the VTA resulted in initiation of behavioural sensitisation (Vezina, 1996; Cornish and Kalivas, 2001).

Finally, the dopaminergic projection from the VTA to the NAc has been demonstrated to be crucial for behavioural and NAc neuronal firing responses to incentive cues (Yun *et al.*, 2004). VTA neurons are known to increase their firing rate in response to a conditioned stimulus previously paired with primary rewards (Schultz, 1997; Fiorillo et al., 2003). Inactivation of the VTA by the $GABA_A$ agonist muscimol abolished the

ability of conditioned cues to increase instrumental responding in a test of Pavlovian to instrumental transfer (PIT), as well as decreasing cocaine-seeking maintained by conditioned reinforcers (Di Ciano and Everitt, 2004; Murschall and Hauber, 2006).

1.3.1.5. The Ventral Pallidum (VP)

Dopaminergic afferents from the VTA and GABAergic projections from the NAc converge within the VP, which in turn projects back to reward-associated structures of the mesolimbic dopamine system and output nuclei, mediating reciprocal information exchange as well as motor output for limbic motivation signals (Mogenson et al., 1980; Mogenson and Yang, 1991; Groenewegen et al., 1993; Churchill and Kalivas, 1994). It is thought that release of VP neurons from the tonic GABAergic inhibitory inputs of the NAc is a key 'downstream' mechanism by which hyperpolarization of the NAc stimulates reward and motivation (Smith et al., 2009). Lesions of the VP, as well as muscimol microinjection-induced VP inactivation, decrease voluntary food and drink consumption, replacing positive hedonic taste reactions with aversive reactions (Cromwell and Berridge, 1993; Shimura et al., 2006). VP lesion or inactivation has further been shown to attenuate Pavlovian incentive learning, reducing instrumental responding for alcohol or cocaine, as well as blocking acquisition and expression of sucrose, amphetamine or morphine CPP (Robledo and Koob, 1993; Harvey et al., 2002; June et al., 2003). VP neurons demonstrate phasic burst firing in response to sucrose rewards, as well as anticipatory firing in response to conditioned cues predicting sucrose reward (Tindell *et al.*, 2004). Recent evidence suggests that VP neurons encode incentive sensitisation, and use separate population- and firing rate activity-patterns to distinguish 'liking' from 'wanting' enhancements by amphetamine and opiates (Smith et al., 2009).

1.3.2. The Prefrontal Cortex (PFC)

Neuroimaging studies reveal the orbitofrontal cortex of addicted subjects to be activated during intoxication, craving, and bingeing, and deactivated during withdrawal (Goldstein and Volkow, 2002). The prefrontal cortex (PFC) provides excitatory glutamatergic projections to multiple sites within the mesolimbic dopamine system, including the VTA and NAc, inducing burst firing of DA neurons (Sesack et al., 1989; Chergui et al., 1993; Carr and Sesack, 2000). Although PFC GABA neurons do not

directly project to reward-associated circuitry in the basal ganglia, they can indirectly modulate NAc and VTA activity by inhibition of PFC glutamatergic afferents to various BG nuclei (Christie *et al.*, 1987; Matsumura *et al.*, 1992).

Intra-PFC microinjections of the $GABA_A$ receptor antagonist bicuculline in primates have been shown to increase impulsivity, associated with addiction (Sawaguchi *et al.*, 1988; 1989). More recently, direct administration of the $GABA_A$ receptor agonist muscimol into the ventral medial PFC potentiated morphine-induced conditioned place preference (CPP), while picrotoxin, a $GABA_A$ receptor antagonist (channel blocker), had the inverse effect (Rozeske et al., 2009). Similarly, the ventral medial PFC projection onto GABAergic MSNs expressing D1-receptors within the NAc shell has been implicated in mediating relapse vulnerability to drugs of abuse (Fuchs et al., 2004; Bossert et al., 2012).

1.3.3. The Amygdala

The amygdala has classically been associated with the modulation of memory consolidation and emotional learning, including appetitive and fear conditioning (Gallagher et al., 1990; Everitt et al., 2000; Wilensky et al., 2000; McGaugh, 2002; Paré, 2003). In particular, the BLA, which projects heavily to the NAc as well as the medial PFC (mPFC) and hippocampus, is proposed to play an important role in mediating affective motivational behaviour (Everitt et al., 2000; Balleine et al., 2003; Cardinal et al., 2003; Balleine and Killcross, 2006).

The BLA sends monosynaptic excitatory glutamate projections to both principle pyramidal and parvalbumin-expressing GABAergic interneurons within the mPFC, inducing either excitatory responses or feed-forward inhibition of mPFC neurons (Pérez-Jaranay and Vives, 1991; Gabbott et al., 2006). In turn, the mPFC projects back to the BLA forming a neural loop proposed to be important for integrating affective information with information of stimulus properties, thus forming stimulus-outcome associations (Quirk *et al.*, 2003). From here, the BLA also sends glutamatergic projections to GABAergic NAc MSNs, critically involved in the modulation of incentive motivational properties of reward-associated stimuli, and the central nucleus of the amygdala (CeN). The CeN is the main source of amygdala output to the

brainstem, hypothalamus and basal forebrain, known to be involved in the mediation of fear and anxiety responses (Davis, 1992; Quirk et al., 2003; Kalin et al., 2004; Stuber et al., 2011), as well as positive incentives (Everitt et al., 1999; 2003; Balleine and Killcross, 2006).

BLA neurons have been demonstrated to phasically fire in response to reward-predictive cues, with synaptic strength of BLA neurons directly correlated to the success of reward-learning (Uwano et al., 1995; Tye and Janak, 2007; Tye et al., 2008). Accordingly, lesions of the BLA attenuate sucrose, cocaine or morphine CPP, as well as reducing cue-induced reinstatement of cocaine self-administration (Everitt et al., 1991; Fuchs et al., 2002; Yun and Fields, 2003; Milekic et al., 2006). Interestingly, rats with BLA lesions are still able to acquire normal conditioned responding to a stimulus paired with a food reward, but fail to adjust their responding to the conditioned stimuli accordingly when the reward is devalued (Hatfield et al., 1996; Balleine et al., 2003). This suggests that the BLA is necessary for encoding or retrieval of the absolute value of an unconditioned stimulus associated with a learnt conditioned stimulus, information needed to guide effective reward-seeking behavioural responses (Cardinal et al., 2003; Everitt et al., 2003).

It also appears that the dopamine transmission within the BLA may modulate stimulus-reward learning. Increased extracellular dopamine is observed in the BLA of rats during, and following performance under a discriminative operant task, as well as in response to cocaine-paired cues (Hori *et al.*, 1993; Weiss *et al.*, 2000). Intra-BLA administration of amphetamine or the dopamine agonist 7-OH-DPAT, have also been shown to enhance appetitive Pavlovian conditioning in a discriminative approach task, and intra-BLA dopamine antagonism is sufficient to attenuate conditioned reinstatement of drug-seeking (Hitchcott et al., 1997a; 1997b; See et al., 2001). More recently, evidence suggests a switch occurs in BLA dopamine receptor control of reward memory, with blockade of BLA D1, but not D2 receptor transmission blocking morphine CPP in drug-naïve rats, and the reverse demonstrated in drug-dependent and animals in drug withdrawal (Lintas et al., 2011).

1.3.4. The Hippocampus

The hippocampus formation underlies the learning of associations between environmental contexts and unconditioned stimuli, known to be a powerful determinants of drug-seeking behaviour and relapse (Selden et al., 1991; Kim and Fanselow, 1992; Shalev et al., 2000; Robbins, 2002). Stimulation of the ventral subiculum of the hippocampus has been demonstrated to induce enduring dopamine (DA) release within the NAc through increased firing of VTA dopaminergic projection neurons (Brudzynski and Gibson, 1997; Legault et al., 2000). Theta-burst hippocampal stimulation also resulted in reinstatement of drug-taking behaviour by contextual cues following extinction of cocaine-self administration in rats, an effect subsequently blocked by pharmacological inactivation of the hippocampus (Vorel et al., 2001; Luo et al., 2011). Similarly, bilateral lesions or pharmacological inactivation of the hippocampus have also been revealed to block both the acquisition and expression of cocaine or morphine CPP (Meyers *et al.*, 2003; 2006; Milekic *et al.*, 2006), abolish the potentiating effect of intra-NAc amphetamine on locomotor activity and responding with conditioned reinforcement (Burns *et al.*, 1993), and impair acquisition of cocaine self-administration (Caine *et al.*, 2001).

Behavioural evidence, gene expression and anatomical projection patterns suggest that the hippocampus can be divided into separate structures (see (Fanselow and Dong, 2010) for review). The dorsal hippocampus is implicated primarily in the cognitive process of learning and memory associated with navigation, exploration, and locomotion (Jung et al., 1994; Moser et al., 1995; Zhang et al., 2004), whereas the ventral hippocampus is the part of the temporal lobe associated with motivational and emotional behavior (Henke, 1990; Ferbinteanu and McDonald, 2001; Kjelstrup et al., 2002).

1.3.5. Summary

The NAc is the main input structure of the basal ganglia and is thought to be a critical structure for action selection and decision-making. Glutamatergic projections from regions including the amygdala, hippocampus and PFC are thought to compete within the NAc for control over behavioural responses to salient stimuli, which are

communicated to basal ganglia output structures by *direct* or *indirect* pathways. Dopamine and GABA are thought to act within the NAc to modulate the influence of reward-related glutamatergic projections. A detailed anatomical model of connectivity between structures involved in the mediation of reward is provided bellow (Fig. 1.2.).

Figure 1.2.

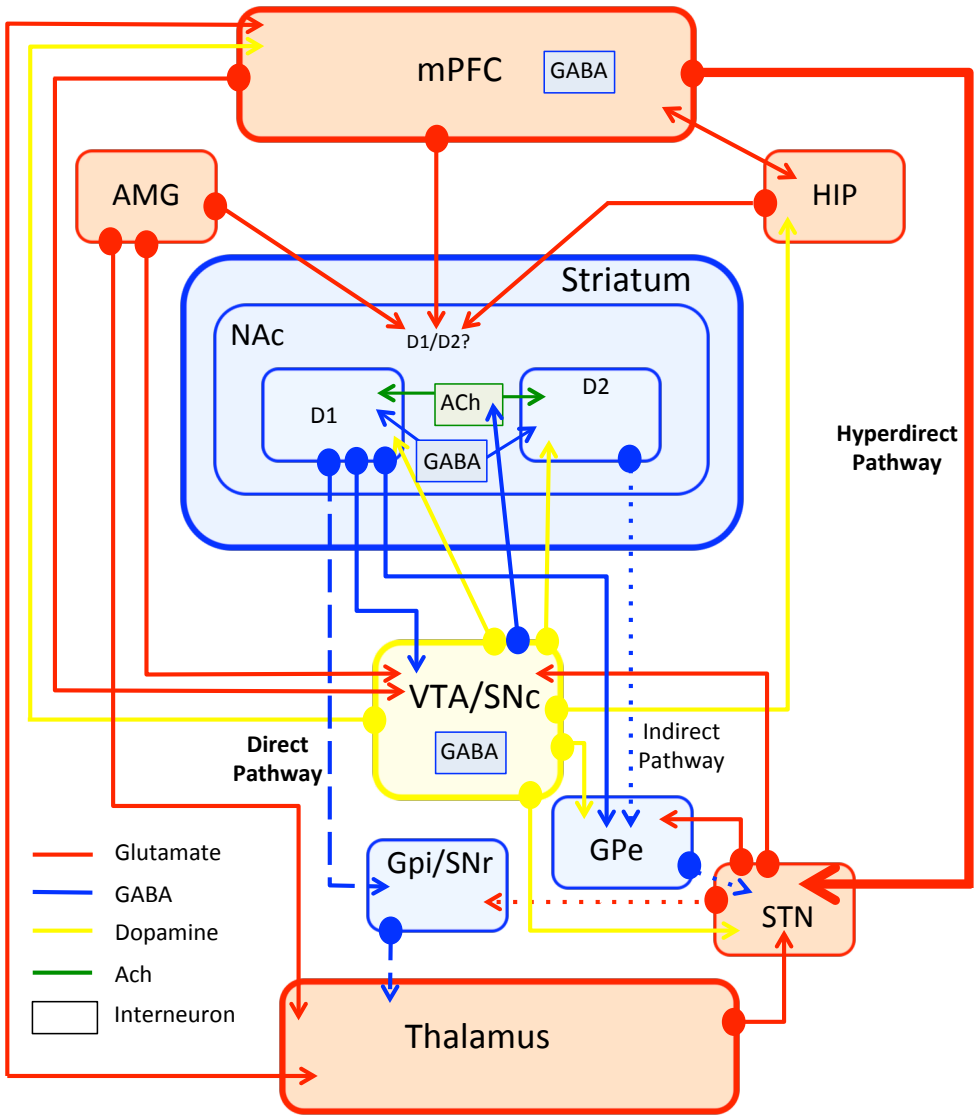


Fig. 1.2. An updated model of reward circuitry. Recent investigations have revealed a rather more complex organization of cortical and subcortical anatomy, complicating the investigation of neuronal pathways involved in mediating reward. mPFC, medial prefrontal cortex; ACh, Acetylcholine; AMG, amygdala; HIP, hippocampus; NAc, nucleus accumbens; VTA, ventral tegmental area; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; GPe, external globus pallidus; GPi, internal globus pallidus; STN, subthalamic nucleus.

1.4. Structure of $GABA_A$Rs and molecular basis of $GABA_A$R signalling.

1.4.1. $GABA_A$ receptors

$GABA_A$Rs are heteropentameric chloride channels belonging to a large super-family of cys-loop ligand-gated ion channels, also including nicotinic acetylcholine receptors, glycine receptors, and the 5-HT3 receptor (Goetz et al., 2007; Connolly, 2008). $GABA_A$R subunits consist of four hydrophobic transmembrane domains (TM1–4) of about 20 amino acids, with TM2 believed to line the pore of the channel (Jacob et al., 2008; Macdonald and Botzolakis, 2009). The large extracellular amino terminus is the site of GABA binding (between the alpha and beta subunits), and also contains binding sites for psychoactive drugs, such as benzodiazepines (Bz) (between alpha and gamma) and barbiturates (Ba) (between the alpha and beta) (Johnston, 2005). Each receptor subunit also contains a large intracellular domain between TM3 and TM4 that is the site for various protein interactions and post-translational modifications that modulate receptor activity (Macdonald and Botzolakis, 2009). There are currently known to be 18 $GABA_A$R subunits, which can be divided by sequence homology into seven subunit categories: α (1–6), β (1–3), γ (1–3), δ, ε, θ, π and ρ (1-3) (Jacob et al., 2008). However despite the potential for vast numbers of individual receptor isoforms, mammalian CNS $GABA_A$R stoichiometry typically consists of two α subunits, two β subunits and one γ (or one δ) subunit (Whiting et al., 1995; Sieghart, 2006). Selective assembly of $GABA_A$R isoforms occurs within the endoplasmic reticulum. This allows $GABA_A$Rs of different composition to be selectively expressed and targeted to specific subcellular localities, where receptors of different composition reveal functional differences in their physiological and pharmacological properties.

Figure 1.3.

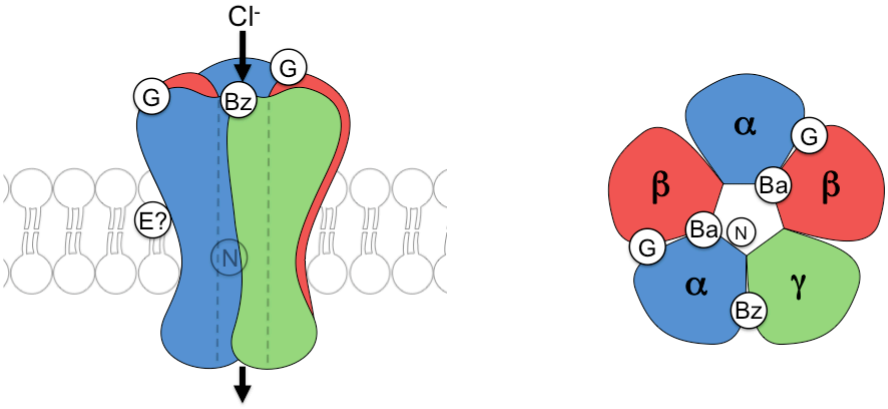


Fig 1.3. Heteropentameric composition of a $GABA_A$ receptor including binding sites for GABA (G), benzodiazepines (Bz), barbituates (Ba), neurosteroids (N), and a disputed binding site for ethanol (E). $GABA_A$ receptors typically consist of two α-subunits (α1-6), two β-subunits (β1-3) and either a γ- or δ-subunit, around a central chloride (Cl^-) channel.

1.4.2. Synaptic vs Perisynaptic/Extrasynaptic $GABA_A$ receptors

Classically, $GABA_A$ receptors have been reported to mediate inhibition in the adult mammalian CNS via fast 'phasic' transmission of activity occurring within the synapse. However, within the last two decades a growing body of evidence has identified $GABA_A$ receptors located extrasynaptically, either perisynaptically or distant from synapses. These extrasynaptic $GABA_A$Rs respond to low levels of ambient or spillover GABA to generate a 'tonic' form of inhibition (Wei et al., 2003; Farrant and Nusser, 2005; Brickley and Mody, 2012). Typically, synaptic phasic $GABA_A$Rs comprise α1, α2, α3 or α5, with β2/3 and γ2 subunits, whereas tonic extrasynaptic $GABA_A$Rs have largely been found to be comprised of α4 or α6, coupled with β2/3 and δ subunits, although there is some evidence of α1 and γ within extrasynaptic $GABA_A$Rs (Barnard et al., 1998; Nusser et al., 1998; Crestani et al., 2002; Mortensen and Smart, 2006).

The γ2 subunit is thought to be crucial to the targeting and anchoring of specific $GABA_A$Rs to GABAergic postsynaptic densities via complex interactions with scaffolding proteins gephyrin, collybistin and neuroligin-2 (Essrich et al., 1998; Sudhof,

2008; Poulopoulos et al., 2009). Targeted deletion of γ2 results in reduced synaptic $GABA_AR$ clustering (Essrich et al., 1998). More recent evidence suggests that in perisomatic postsynaptic densities, stabilisation of $GABA_ARs$ and neuroligin-2, but not gephyrin may be mediated by the dystrophin-glycoprotein complex (Panzanelli et al., 2011). However, γ2 has also been found to couple with α5 and α6 within extrasynaptic $GABA_ARs$ in hippocampal and cerebellar granule cells, respectively (confirmed by lack of colocalisation with gephyrin), suggesting that specific subunit compositions may nullify the synaptic anchoring properties of the γ2 subunit (Crestani et al., 2002; Wisden et al., 2002). Interestingly, an alternative mRNA splicing of the γ2 subunit has been reported. Short (γ_{2S}) and long (γ_{2L}) subunits are identical except for the presence of an eight-amino-acid sequence that is present in the larger intracellular loop of the γ_{2L} subunit (Whiting et al., 1990; Wafford et al., 1991). However, analysis of chimeric γ2 subunits indicates that the fourth transmembrane domain (TM4) but not the major cytoplasmic loop domain of the γ2 subunit is essential for clustering of $GABA_A$ receptors at synapses (Alldred et al., 2005). Thus, the γ_{2S} and γ_{2L} variations of the γ2 subunit are not likely to account for differences in the subcellular location of $GABA_ARs$ containing γ2 subunits.

1.4.3. Sensitivity to GABA and other endogenous/exogenous drugs

Gating of the $GABA_AR$ chloride ion channel is mediated via a GABA binding site at the interface between the α and the β subunits, where the binding of two GABA molecules induces channel opening (Baumann *et al.*, 2003). However, given the diversity in subcellular $GABA_AR$ localization it is clear that exposure to GABA will vary between $GABA_AR$ populations, and the $GABA_AR$ subtypes vary, accordingly, in their biophysical properties, including GABA sensitivity and specificity to endogenous and exogenous molecules. In recombinant $GABA_ARs$ comprising αβ3γ2 combinations, GABA sensitivity is largely determined by a domain of four amino acids in the extracellular N-terminal region of α subunits (Böhme *et al.*, 2004). Studies manipulating the α subunit reveal EC_{50} values of the GABA-induced chloride current to vary between <1 to >50 μM, with a rank order α6>α1>α2>α4>α5>α3 (Böhme et al., 2004; Minier and Sigel, 2004). However, sensitivity to GABA is increased in extrasynaptically located α4βδ extrasynaptic $GABA_ARs$ compared to synaptic α4βγ2 $GABA_ARs$ (Yeung et al., 2003; Mortensen et al., 2010). Along with a higher affinity to GABA and slower

desensitisation in comparison to their synaptic counterparts, α4β3δ extrasynaptic $GABA_A$Rs are also differentially sensitive to a number of allosteric modulators and neurosteroids. Gaboxadol® (THIP; 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol), acts as a high-efficacy superagonist at δ-containing extrasynaptic $GABA_A$Rs due to its ability to increase the frequency and duration of channel opening, but has only partial agonist activity on αβγ-type synaptic receptors (Ebert *et al.*, 1994; Mortensen *et al.*, 2004). Similarly, a higher potency agonist activity of muscimol is seen at α4β3δ extrasynaptic $GABA_A$Rs compared to synaptic α4β3γ2 and α1β3γ2 $GABA_A$Rs, though this difference may be caused by reduced desensitisation (Mortensen et al., 2010).

1.4.4. Expression of $GABA_A$R subunits throughout the brain

Immunocytochemical and RNA analysis has revealed $GABA_A$ receptor subunit isoforms to each exhibit a unique distribution within the brain (Wisden et al., 1992; Laurie et al., 1992a; 1992b; Pirker et al., 2000).

α1 and α2 subunits are found extensively throughout the brain, with expression of α1 the most abundant (Wisden et al., 1992; Pirker et al., 2000). Expression of the α3 subunit isoform is localised to the cerebral cortex, olfactory bulb and brain stem nuclei (Persohn et al., 1992; Wisden et al., 1992; Pirker et al., 2000). The α4 subunit is distributed throughout the thalamus, nucleus accumbens, hippocampus, neocortex and caudate-putamen (Wisden et al., 1992; Pirker et al., 2000; Schwarzer et al., 2001). α5 subunits are highly expressed within the hippocampus, with further moderate expression within the rodent hypothalamus, neocortex and olfactory bulb (Persohn et al., 1992; Laurie et al., 1992a). More recently, rodent and human data indicates that there may also be α5-containing $GABA_A$Rs within the striatum (Ade et al., 2008; Mendez et al., 2013). Finally, expression of the α6 subunit is confined to the cerebellar granule cells, hippocampal pyramidal neurons, and cochlear nucleus granule cells (Wisden et al., 1992; 2002).

All three β subunits are found throughout the brain, with distribution patterns often overlapping (Pirker et al., 2000). β1 subunits are expressed heavily within the hippocampus and olfactory bulb, and to a lesser extent in the cerebral cortex, cerebellum, superior colliculus and substantia nigra (Persohn et al., 1992; Wisden et al.,

1992). The wide distribution of β2 subunits often correlates with α1 subunit expression and is strongly expressed in the pallidum and thalamus (Wisden et al., 1992; Moreno et al., 1994; Pirker et al., 2000). Finally, expression of β3 subunits, which correlates highly with that of α2 subunits, is strongest in the striatum (Miralles *et al.*, 1999; Pirker *et al.*, 2000).

γ1 subunit expression shows minimal expression throughout the brain, with the highest distribution found in the bed nucleus of the stria terminalis (Pirker et al., 2000). Conversely, γ2 subunits are expressed almost ubiquitously in the brain (Wisden et al., 1992; Pirker et al., 2000). The γ3 subunit is diffusely distributed throughout the brain at very low concentrations (Pirker et al., 2000). Expression of δ subunits is highest in the cerebellar granule cells, with further distribution found in the thalamus, striatum, hippocampal dentate granule cells and neocortex (Persohn et al., 1992; Wisden et al., 1992; Pirker et al., 2000; Schwarzer et al., 2001). δ subunits are proposed to be expressed exclusively in extrasynaptic $GABA_A$Rs, where they partner with α6 in the cerebellum, and α4 within the forebrain (Jones *et al.*, 1997; Peng *et al.*, 2002). Expression of ρ subunits is restricted to cerebellum, colliculi and retina (Boue-Grabot et al., 2002; Alakuijala et al., 2005). The θ and ε subunits show remarkably overlapping expression within the brain and typically form receptors with α3 subunits (Bonnert et al., 1999; Pape et al., 2009). Although distributed throughout the brain, strongest expression of these subunits is found in the dorsal raphe and the locus coeruleus (Pape et al., 2009). As yet there is no evidence of π subunit expression within the mammalian CNS, but it is known to be highly expressed within the uterus (Hedblom and Kirkness, 1997; Quezada et al., 2006).

1.4.5. Factors influencing expression of $GABA_A$ receptor subunits and channel kinetics

Further to this, there are known to be over 20 intracellular proteins that act at various sites along the large TM3-TM4 intracellular loops of $GABA_A$Rs to influence surface expression of receptors (Uusi-Oukari and Korpi, 2010). One such interacting protein is $GABA_A$ receptor-associated protein (GABARAP). This microtubule-binding protein has been proposed to act as a linker between $GABA_A$Rs and the cytoskeleton (Wang et al., 1999; Wang and Olsen, 2000). Immunofluorescent staining and green fluorescent protein (GFP)-tagged receptor subunits revealed GABARAP to promote clustering of

$GABA_A$Rs (Chen *et al.*, 2000). The inclusion of the γ2 subunit in $GABA_A$Rs, and the tubulin-binding domain in GABARAP are critical for receptor clustering, suggesting that GABARAP may facilitate formation of postsynaptic receptor clusters by linking the intracellular domain of γ2 subunits (Chen et al., 2000). When recorded in L929 cells expressing α1, β1, and γ2 subunits, alongside GABARAP, GABA-activated recombinant single channel conductance was significantly increased (Chen et al., 2000; Everitt, 2004). It hypothesised that these changes in channel kinetics may occur as a result of protein-protein interactions between intracellular domains of $GABA_A$Rs clustered by GABARAP, inducing cooperative opening and closing of channels (Everitt, 2004).

1.4.6. Summary

The large number of $GABA_A$Rs isoforms confers these receptors with a diverse range of physiological and pharmacological properties. Inclusion of specific subunits allows them to be selectively expressed and targeted to specific subcellular localities, including within synapses, and also perisynaptically and extrasynaptically. Specifically, synaptic $GABA_A$Rs are thought to mediate a phasic inhibitory control over neurons, while extrasynaptic $GABA_A$Rs demonstrate a higher affinity to GABA and thus respond to ambient GABA and spillover GABA from synapses to mediate a tonic form of inhibition.

1.5. Psychostimulants

1.5.1. The neurobiology of psychostimulant abuse

Psychostimulants are psychoactive drugs, which produce a variety of physiological effects within the central and peripheral nervous systems, ultimately increasing psychomotor activity. Critical to the euphoric and motivational properties of psychostimulants, as well as many other drugs and rewarding stimuli, is the ability to increase dopamine transmission within the NAc (Harris and Baldessarini, 1973; Wise and Bozarth, 1987; Di Chiara and Imperato, 1988). Ordinarily, these transporters bind to dopamine released during neuronal signalling and act as a recycling mechanism by delivering dopamine back into storage vesicle within the presynaptic neuron. However, psychostimulants, including cocaine, bind to dopamine transporters, forming a complex that blocks the transporter's function. This results in an accumulation of dopamine within the synaptic cleft, producing a prolonged stimulation of postsynaptic dopamine receptors that is thought to alter the influence of excitatory neuronal inputs.

Repeated exposure to psychostimulants results in neuroadaptations of the systems in which they produce their effects (Nestler, 2001). These changes are known to underlie the sensitisation of drug effects seen following repeated intermittent use, including the sensitisation of incentive motivational properties (Robinson and Berridge, 1993; 2000). The intermediate-term effects of psychostimulant administration include alterations to the amount of dopamine transporters and dopamine receptors present on the surface of neurons (White and Kalivas, 1998; Nestler, 2005). As well as a decrease in the density of glutamate but not GABA immunolabeling within the NAc, possibly indicating an increase in excitatory synaptic activity (Meshul et al., 1998). Furthermore, psychostimulant alter gene expression, including that of the transcription factor protein ΔFosB, thought to act as an important molecular “switch” in the transition from drug abuse to addiction (Nestler et al., 2001; Nestler, 2005). Longer-term psychostimulant use results in changes in the physical structure of neurons, including altered dendritic branching in the NAc (Nestler, 2001; Robinson and Berridge, 2001).

1.5.2. Conditioning in psychostimulant abuse

In humans, addiction to psychostimulants is characterised by drug craving and high rates of relapse during abstinence, often thought to be triggered by re-exposure to drug-associated cues (Stewart et al., 1984; Robinson and Berridge, 1993; Everitt et al., 2001; Crombag et al., 2008). Indeed, exposure to cocaine-associated cues is able to trigger increased physical arousal and craving for cocaine in abstinent cocaine users (Avants et al., 1995). Furthermore, exposure to cocaine-related stimuli results in an increase in striatal dopamine release (Volkow et al., 2006), and increased electrophysiological brain responses, as measured by event-related brain potentials (Franken et al., 2008). It is hypothesised that firing of NAc MSNs in response to reward-associated cues may encode both the predictive value of environmental stimuli and the specific motor behaviors required to respond to them (Nicola et al., 2004).

Cue-induced reward-seeking can be explained by mechanisms of Pavlovian conditioning. Following repeated pairing with an unconditioned rewarding stimulus (US), contextual or discrete cues can acquire the motivational properties of the US and become conditioned stimuli (CS). These associative processes can be modelled in animals using a number of behavioural procedures, including cue-induced reinstatement of drug-seeking, conditioned place preference, conditioned reinforcement and second-order schedules of reinforcement (see reviews (Everitt and Robbins, 2000; Tzschentke, 2007; Crombag et al., 2008)). The use of these paradigms in combination with genetic and pharmacological manipulation of specific molecular targets will likely further our understanding of the psychological and neurobiological mechanisms by which drug-associated stimuli elicit drug-seeking.

1.5.3. Psychostimulant-induced $GABA_AR$ subunit regulation throughout the brain

To date, research has largely focused on drug-induced changes at dopamine and glutamate synapses, including changes in intracellular signal transduction pathways in the mesolimbic dopamine system, and long-term potentiation (LTP) of glutamatergic synapses (see reviews (Kalivas, 2007; Wolf, 2010; Lüscher and Malenka, 2011; Pierce and Wolf, 2013)). However, there is a growing body of evidence that GABAergic

mechanisms also play an important role in mediating the physiological and behavioural effects of psychostimulants.

Early studies exploring GABAergic mechanisms of psychostimulant abuse revealed systemic amphetamine administration was associated with a decline in extracellular GABA concentration within the ventral pallidum (Bourdelais and Kalivas, 1990). Similarly, chronic cocaine administration resulted in a selective attenuation of $GABA_AR$ function within the striatum (Peris, 1996). However, other studies have found no evidence of changes in either quantity or function of striatal $GABA_ARs$ (Jung and Peris, 2001). More recently, a genetic screen revealed chronic cocaine to cause a robust up-regulation of α4 subunit gene expression selectively in D1-MSNs (Heiman et al., 2008). Acutely, cocaine was associated with decreased striatal α1 subunits when measured 1 hour post-administration (Yamaguchi et al., 2000). However, when measured in cocaine self-administration, at both 1 day (acute) and 20 days (chronic), α1 subunit mRNAs where up-regulated, with down-regulation noted for α4, α6, β2, γ2, and δ subunits (Backes and Hemby, 2003).

In line with the genetic studies linking the GABRA2 gene with cocaine addiction (Dixon et al, 2010; Enoch et al, 2010), molecular investigation has also emphasized a pertinent role for α2-containing $GABA_ARs$. Quantitative immunohistochemistry of $GABA_AR$ subunit proteins following chronic cocaine administration revealed a significant decrease in α2 subunits within the hippocampal dentate gyrus and CA1 regions (Lilly and Tietz, 2000). Moreover, following methamphetamine-sensitisation a decrease in $GABA_AR$ α2 is reported within the NAc shell and core (Zhang et al, 2006). Similarly, reversal of cocaine-induced behavioural sensitisation by pergolide/ondansetron treatment normalises $GABA_AR$ α2 expression within the NAc (Chen et al, 2007).

1.5.3. α2-containing $GABA_ARs$ in the NAc mediate cocaine effects on reward-conditioned behaviours

As well as mediating the primary rewarding effects of drugs of abuse, the NAc is implicated in the ability of reward-paired environmental cues to motivate drug-seeking

behaviour (Everitt and Robbins, 2005). It has been proposed that conditioned cues may influence behaviour by activating stimulus-action associations represented in individual NAc MSNs (Nicola *et al.*, 2004). Moreover, GABAergic inhibition of neurons competing for control over basal ganglia output nuclei, by GABAergic interneurons and collateral connections between neighbouring MSNs, has been suggested to facilitate NAc-mediated action selection (Nicola, 2006). These findings raise the possibility that variations in the subunit composition of $GABA_A$Rs may act to differentially modulate the activity of NAc MSNs, and alter behavioural responses to psychostimulants drugs and reward-conditioned cues.

Indeed, targeted deletion of the $GABA_A$R α2 subunit, known to be highly represented within the NAc, blocked the ability of cocaine to induce locomotor sensitisation (Morris et al., 2008). This phenomenon reflects the amplification of behavioural responses to psychostimulants, as well as other drugs of abuse, following repeated intermittent administration (de Wit and Stewart, 1981; Robinson and Becker, 1986). Similarly, selective activation of α2 receptors within the NAc using intracranial infusions of the $GABA_A$R agonist Ro 15-4513, was sufficient to induce behavioural sensitisation in α2(H101R) mutant mice, in which the mutation results in a change in efficacy of Ro 15-4513 from a negative allosteric action to a positive allosteric action (Dixon et al., 2010). Interestingly, the ability of BZs to facilitate the locomotor activating properties of cocaine was abolished in α2(H101R) mice, suggesting that α2 mediation of cocaine-potentiated locomotor activity occurs downstream of facilitated NAc dopamine release.

More recently, deletion of the α2 subunit did not modulate instrumental responding for a reward-conditioned cue, in a test of conditioned reinforcement, but was able to block cocaine potentiation of responding (Dixon et al, 2010). Thus, while the α2 subunit seems not to play a role in the formation of associations between environmental events and rewards, it is important for the ability of cocaine to facilitate cue-induced behaviours associated with reward. As yet, limited evidence suggests that this effect is attributable to α2-containing receptors located on accumbens MSNs.

1.5.4. $GABA_A$ receptors and psychostimulant self-administration

$GABA_ARs$ within the VTA are predominantly located on a subpopulation of GABAergic neurons which provide tonic inhibitory inputs onto neighbouring DA neurons, as well as projecting to various other brain regions involved in mediating reward (Johnston & North, 1992; Churchill et al, 1992; Kalivas, 1993). Recent evidence reveals that optogenetic activation of VTA GABA neurons suppresses the release of DA within the NAc (Van Zessen et al, 2012). Moreover, firing of VTA GABA neurons is facilitated during cues that predict appetitive rewards (Cohen et al, 2012). Thus, it is hypothesised that compounds acting at $GABA_ARs$ will alter neurotransmission between GABA and DA neurons within the VTA, as well as projections fibres to the NAc, and may help modulate reward processing (Van Zessen et al, 2012).

Alprazolam and chlordiazepoxide, two allosteric modulators of $GABA_ARs$, decrease intravenous cocaine self-administration in rats (Goeders et al, 1989; 1993). In human studies, pre-treatment with alprazolam has been shown to reduce the subjective behavioural effects of amphetamine (Rush et al, 2004). Similarly, topiramate, which blocks mesolimbic dopamine release via potentiated GABAergic inhibition and inhibited glutamate function, has been shown to reduce cravings and increase abstinence in cocaine dependence in humans (Johnson, 2005; Reis et al., 2008).

Other high-efficacy $GABA_AR$ modulators including pentobarbital and midazolam have also shown efficacy in selectively attenuating cocaine self-administration in mice (Barrett et al, 2005). Interestingly, ligands acting directly at $GABA_ARs$ have been shown to be freely self-administered directly into the VTA, but show limited efficacy in altering psychostimulant self-administration (David et al, 1997; Ikemoto et al, 1997). Muscimol, a direct agonist at $GABA_ARs$, failed to attenuate the abuse-related effects of cocaine (Barrett et al, 2005).

1.5.5. $GABA_AR$ pharmacogenetics of psychostimulant abuse

As outlined above, the GABRA2 gene encoding $GABA_AR$ α2 subunits has consistently been associated with drug dependence, notably alcoholism (Edenberg et al., 2004; Agrawal et al., 2006; Dick et al., 2006; Edenberg and Foroud, 2006; Soyka et al., 2008). It has subsequently been revealed that haplotypes of the GABRA2 gene are also linked with cocaine addiction, with the same haplotypes as those reported in the Edenberg

study also shown to confer vulnerability or protection to cocaine abuse (Dixon et al., 2010). It has further been proposed that GABRA2 haplotypes may interact with experience of childhood trauma to influence risk of cocaine dependence (Enoch et al, 2010). Again, one haplotype predicted addiction, whereas another haplotype, more common in control subjects, was associated with resilience to addiction following childhood trauma.

1.5.6. Summary

Following repeated pairing with rewards, conditioned cues can take on the motivational properties of the reward and trigger reward-seeking behaviour. Psychostimulants are known to enhance these behavioural responses to conditioned cues by increasing dopamine transmission within the NAc, thus augmenting the influence of reward-related glutamatergic inputs from the PFC, hippocampus and amygdala. The action of GABA at $GABA_A$Rs within the NAc is also a powerful mechanism by which competing action representations are modulated. It is thought that different $GABA_A$R isoforms in specific subcellular localities may provide different roles in mediating behavioural responses to direct psychostimulant reward, as well as reward-conditioned cues and their potentiation by psychostimulants.

1.6. A role for α4-GABA$_A$Rs in mediating addiction-related behaviours?

Several laboratories have reported extrasynaptic GABA$_A$Rs, known to mediate a tonic inhibition, to be particularly sensitive to low alcohol concentrations (10-30mM) (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004; Wallner et al., 2006; Santhakumar et al., 2007; Olsen, 2011). Accordingly, α4βδ GABA$_A$R subtypes are the most rapidly regulated in plastic mechanisms triggered by high-dose alcohol or chronic exposure to alcohol in rats (Liang et al., 2007), and in mice lacking the δ-subunit, alcohol failed to potentiate tonic GABA currents within dentate granule cells (Liang et al., 2006). However, a number of laboratories have failed to replicate these findings and suggest instead that ethanol may modulate extrasynaptic GABA$_A$R-mediated tonic currents indirectly via a presynaptic, or yet unidentified mechanism (Borghese *et al.*, 2005; Botta *et al.*, 2007; Korpi *et al.*, 2007). One theory is that the physiological and behavioural effects of alcohol are attributable to the alcohol-induced neurosteroid modulation of GABA$_A$Rs.

Interestingly, α4βδ extrasynaptic GABA$_A$Rs have also been proposed to mediate the rewarding and reinforcing effects of alcohol. Viral knockdown of the α4 and δ subunits within the medial NAc shell, but not ventral or lateral shell, or core, reduced alcohol intake in a two-bottle choice test (Rewal et al., 2009; Nie et al., 2011). Similarly, operant responding for alcohol was reduced following decreased α4 expression in the NAc shell, but not core (Rewal et al., 2011). These data strongly implicate extrasynaptic GABA$_A$Rs within the medial NAc shell in the modulation of alcohol intake. This role of α4 receptors contrasts with the lack of effect of deletion of α5 (Stephens et al., 2005) or α2 subunits (Dixon et al., 2012) on alcohol self-administration, despite targeted deletion of GABA$_A$R α2 subunits increasing the sedative and ataxic effects of alcohol.

At present, very little is known about the possible role of α4-GABA$_A$Rs in mediating the rewarding and reinforcing of drugs of abuse other than alcohol. However, given the importance of α2-GABA$_A$Rs for behavioural sensitisation to cocaine and cocaine-potentiation of conditioned reinforcement, it could be hypothesised that α4-GABA$_A$Rs may also be important for controlling behavioural responses to cocaine. α4-GABA$_A$Rs are widely expressed within the NAc, where they modulate a tonic form of inhibition that acts to control the excitability of MSNs (Maguire et al, submitted). These receptors

may act to alter the response of MSNs to increased NAc dopamine following cocaine, as well as the controlling the influence of excitatory inputs onto MSNs providing information about natural- or cocaine-conditioned cues.

It is possible to test these hypotheses using a number of behavioural paradigms. Firstly, it is important to establish whether activation of α4-$GABA_A$Rs is able to alter baseline and cocaine-potentiated locomotor activity. As well adding to the characterisation of these receptors, investigation of locomotor activity may have implications for further behavioural tests that could be facilitated or attenuated by altered locomotion. Subsequently, it is possible to explore the role of α4-$GABA_A$Rs in natural- and cocaine-conditioned behaviours using well-established behavioural tests, including behavioural sensitisation, conditioned place preference and conditioned reinforcement.

1.7. Strategies for exploring the role for α4-$GABA_A$Rs in mediating addiction-related behaviours

1.7.1. Genetic strategies for targeting α4-$GABA_A$Rs

In the last few decades, technology has evolved that allows the specific modification of the genetic composition of many organisms. Transgenic research has largely been carried out in mice rather than rats due to the technical ease of genetic manipulation of mouse embryos, the larger number of inbred mice strains, and the greater information about mice genetic locus markers. Genetic engineering has allowed researchers to study the role of specific genes in complex behaviours through the use of 'knockout' mouse models, in which a specific gene is inactivated. The phenotype of a knockout mouse provides valuable insight into the normal role of the targeted gene. Such knockout mouse models have been used to study many kinds of genetic disorders and diseases. In this thesis, mice with a deletion of the $GABA_A$R α4-subunit gene (Gabra4) will be used to explore the role of α4-subunits in mediating behaviours associated with addiction to the psychostimulant cocaine.

The main technique used to create genetic knockout mice, gene targeting, involves specific manipulation of a gene in the nuclei of an embryonic stem (ES) cell. One commonly used method of gene targeting takes advantage of the Cre/LoxP system (Fig

1.4.), which can be used for generating constitutive or tissue-specific gene knockout mice (Kos, 2004).

LoxP (locus of X-over P1) is 34-base pair (bp) DNA sequence originally discovered in the bacteriophage P1, that is composed of an asymmetric 8-bp core, determining the directionality, flanked on each side by 13 bp of complementary sequences. To target a particular mouse gene, loxP sites are introduced on either side of a coding region of the gene in vitro (with an antibiotic resistance gene, to allow selection). The manipulated gene is then reintroduced into the mouse genome, through transfection into ES cells. Cells with targeted recombination are selected through their antibiotic resistance and PCR confirmation. These cells are then microinjected into a blastocyst (early stage embryo) and the blastocyst implanted into pseudopregnant female donors, of which the offspring can be bred to develop mice containing the loxP sites. Chimeric offspring are then bred with wildtype mice and offspring screened for the presence of the genetic manipulation. The gene flanked by LoxP sites is referred to as "floxed" (a contraction of the phrase "flanked by LoxP"). LoxP sites are placed on either side of a sequence that is required for correct gene expression, however it is important that the placement of these sites should not adversely affect gene expression in the "floxed" mouse. Thus, in the absence of Cre-recombinase the gene should remains functional. In the Gabra4 "floxed" mice the loxP sites were inserted into non-coding introns, a BamHI site 625 bps 5' to exon 3 and into an EcoRV site 118 bp 3' to exon 3 (see supplementary information of (Chandra et al., 2006))

The Cre-recombinase enzyme (a contraction of the phrase **C**auses **re**combination) is also derived from the bacteriophage P1 and consists of 4 subunits and two domains: The larger carboxyl (C-terminal) domain, and smaller amino (N-terminal) domain. This is also the catalytic site of the enzyme, therefore the Cre-recombinase can be used to catalyze the recombination between two specific DNA recognition sites.

In mutant mice, Cre expression can be targeted to all tissues in order to make a constitutive knockout line, or to specific tissues or cell types to make tissue or cell-specific knockout lines. The constitutive knockout mice are created by crossing the "floxed" mice with a mouse line in which the *cre* transgene is under the control of the adenovirus EIIa promoter that targets expression of Cre recombinase to the early mouse

embryo. The cell-specific Cre-recombinase lines are created by bacterial artificial chromosome (BAC) engineering. BAC engineering is used to insert an intron containing Cre cassettes, followed by a polyadenylation sequence to terminate transcription of the fusion transcript immediately after the recombinase gene, into the BAC vector at the initiating ATG codon in the first coding exon of the targeted gene. These BAC constructs have advantages over the simple promotor-gene style transgenics as they contain much more regulatory sequence, making them resistant to influences of the genome surrounding the site of insertion (King et al., 2003). These DNA constructs can then be microinjected into the nucleus of a single-cell embryo and implanted into a pseudopregnant female donor. Offspring from this mouse can then be bred to create lines of mice expressing Cre recombinase.

When a hemizygote Cre-expressing mouse is bred with a homozygote "floxed" mouse approximately half of the offspring will inherit both the "floxed" gene and the Cre-expressing transgene, and thus a recombination event is triggered, in the cells targeted by the BAC construct. The Cre-recombinase protein binds to the first and last 13bp regions of a loxP site through a transient DNA-protein covalent linkage, forming a dimer. This dimer then binds to a dimer on the other loxP site to form a tetramer. Depending on the orientation of the two repeat sites the recombination event will either lead to the deletion or the inversion of the DNA segment between the two loxP sites. When two direct repeat sites are in the same orientation, Cre cleaves the intermediate DNA segment and the two stands are then rejoined with DNA ligase. However, when the two repeat sites are inverted then the intermediate DNA segment will be inverted and the two loxP sites remain. Although inversion can also lead to gene inactivation, the DNA segment can invert-back and reactivate, and so is not used to develop knockout mouse constructs.

Figure 1.4.

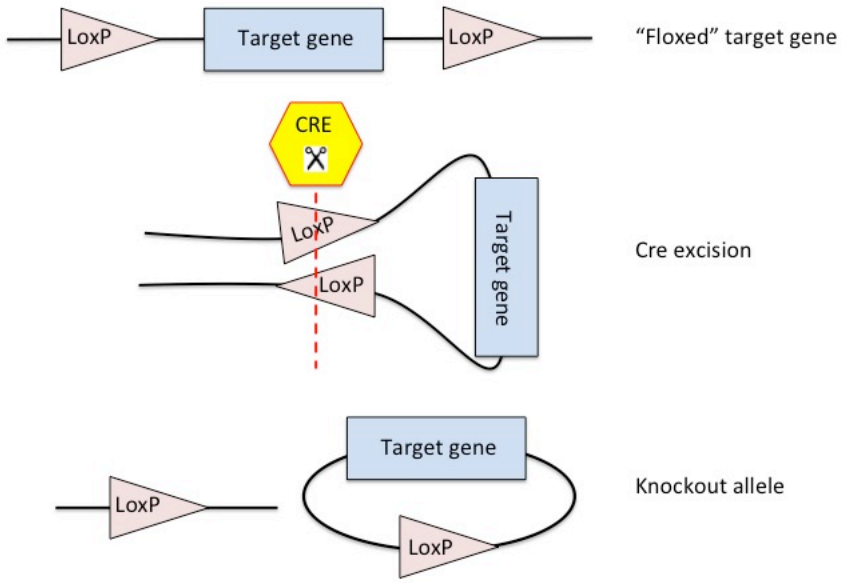


Fig.1.4. Cre/loxP system of transgenic gene knockout. LoxP sites inserted between the target gene are excised by Cre recombinase resulting in a "knockout" allele.

However, there are a number of limitations of using transgenic mice. The site of the transgene insertion into the genome can adversely affect tissue specificity and levels of transgene expression, so a number of founder lines may need to be screened (Davey and MacLean, 2006). Furthermore, deletion of genes encoding for proteins can result in compensatory changes in expression of other proteins. Indeed deletion of the Gabra4 gene encoding for $GABA_AR$ α4-subunits has been reported to result in a reduction of hippocampal $GABA_AR$ δ-subunit protein levels, but an increase in hippocampal α2- and γ2-subunit levels compared to wildtype controls (Suryanarayanan et al., 2011). These changes may occur developmentally as a mechanism to compensate for the normal role of α4-$GABA_ARs$.

In this thesis, the Cre/loxP system will be used to delete the Gabra4 gene throughout the brain, thus creating constitutive $GABA_AR$ α4-subunit knockout mice (described in Chapter 2; 2.2.1.). Additionally, the Gabra4 gene will be deleted selectively from dopamine receptor D1- or D2-expressing neurons, thus creating D1- or D2-expressing

neuron specific $GABA_AR$ α4-subunit knockout mice (described in Chapter 4; 4.2.1.). In order to confirm the gene deletion, constitutive and D1- or D2-expressing neuron specific $GABA_AR$ α4-subunit knockout mice were characterised molecularly using DNA PCR analysis from ear punches (described in Chapter 2; 2.2.2. and Chapter 4; 4.2.2.). Furthermore, in constitutive knockout mice, western blot protein analysis of NAc tissue was conducted (described in Chapter 2; 2.3.1.). To further confirm the deletion of $GABA_AR$ α4-subunits, as well as assess possible compensatory changes in other subunits, quantitative-reverse transcription PCR (qRT-PCR) was used to measure α4-, α2-, γ2- and δ-subunit mRNA expression in the NAc in constitutive and neuron-specific knockout mice (described in Chapter 2; 2.3.2. and Chapter 4; 4.3.1.).

1.7.2. Targeting extrasynaptic α4βδ *$GABA_ARs$ pharmacologically*

As yet, there exists no method to specifically activate α4-$GABA_ARs$ pharmacologically. The $GABA_AR$ agonist muscimol is widely used to elicit GABAergic inhibition, but it activates all $GABA_AR$ subtypes (Krogsgaard-Larsen et al., 1979). Interestingly, muscimol has a greater affinity at α4-, α6- and δ-containing $GABA_ARs$ than α1-containing $GABA_ARs$ (Quirk et al., 1995; Mihalek et al., 1999; Korpi et al., 2002; Chandra et al., 2010). Thus, it may be that extrasynaptic $GABA_ARs$ strongly contribute to the *in vivo* pharmacological effects of muscimol. Nevertheless, the use of muscimol is not viable as a tool for specific activation of α4βδ $GABA_ARs$.

At present, the most commonly employed tool for pharmacological activation of extrasynaptic $GABA_ARs$ is THIP. As previously described, THIP is an agonist with a preference for δ-containing $GABA_ARs$, (Ebert et al., 1994; Mortensen et al., 2010). *In vitro* THIP is able specifically to activate α4βδ $GABA_ARs$ when administered at a concentration of between 1-10μM, but beyond this begins to additionally activate αβγ-type synaptic $GABA_ARs$ (Mortensen et al., 2010). However, THIP has 'superagonist' properties at α4βδ $GABA_ARs$, demonstrating increased efficacy and producing a maximal inhibitory current two-fold greater than that at αβγ-type $GABA_ARs$, and that of a saturating concentration of GABA (Mortensen et al., 2010).

Nevertheless, by using a combined approach of activation of δ-containing $GABA_A$Rs using THIP and genetic deletion of α4-subunits, the role of extrasynaptic α4βδ $GABA_A$Rs in mediating behaviours associated with addiction can be explored.

1.8. Aims and structure of thesis

1.8.1. Chapter 2

Chapter 2 explores the role of $GABA_AR$ α4-subunits in controlling locomotor activity and its potentiation by acute cocaine. Firstly, a cocaine dose response was performed in wildtype and $GABA_AR$ α4-subunit knockout mice to assess whether deletion of these receptors altered the locomotor response to cocaine at various doses. Following this, baseline and cocaine-potentiated activity were measured following activation of α4βδ-$GABA_AR$ activation by various doses systemic THIP in wildtype and $GABA_AR$ α4-subunit knockout mice. The NAc is thought to be an important structure in the mediation of locomotor activity (Costall et al., 1977; Campbell et al., 1997). Therefore, baseline and cocaine-potentiated activity were also measured following intra-accumbal infusion of THIP at various doses in wildtype mice, then repeated at behaviourally active doses in both wildtype and $GABA_AR$ α4-subunit knockout mice. The aim of this chapter is to determine the role of $GABA_AR$ α4-subunits in locomotor activity and its potentiation by acute cocaine. This will also allow consideration for other behaviours that could be affected by changes in locomotor activity, such as conditioned place preference.

1.8.2. Chapter 3

α2-containing $GABA_ARs$ have previously been shown to be crucial for behavioural sensitisation to cocaine (Dixon et al., 2010). The aim of chapter 3 was to investigate whether $GABA_AR$ α4-subunits may also play a role in the control of behavioural sensitisation to cocaine. Wildtype and $GABA_AR$ α4-subunit knockout mice were given repeated, intermittent cocaine at various doses over 10 sessions. Context-specificity of behavioural sensitisation to cocaine was explored by testing for conditioned activity following saline in the drug-paired context, as well as in a novel context. $GABA_AR$ α4-subunits are heavily expressed within the NAc (Pirker et al., 2000), an area known to play a critical role in behavioural sensitisation to cocaine (Everitt and Wolf, 2002). Therefore, the effects of pharmacological activation of α4-$GABA_ARs$ using a systemic challenge injection THIP were investigated in wildtype and $GABA_AR$ α4-subunit knockout mice following behavioural sensitisation to cocaine. This chapter will provide

further incite into the role of $GABA_AR$ subtypes in the mediation of behavioural sensitisation to cocaine. Furthermore, these experiments will provide novel data into a possible role of NAc extrasysnatptic $GABA_ARs$ in mediating behavioural responses to repeated cocaine administration.

1.8.3. Chapter 4

Environmental cues paired with repeated cocaine are known to become associated with the motivational properties of the drug, a phenomenon studied in laboratory animals using the conditioned place preference (CPP) paradigm (Cunningham et al., 2006; Tzschentke, 2007). The aim of chapter 4 is to explore the role of $GABA_AR$ α4-subunits in the acquisition and expression of cocaine-CPP. Following the acquisition of cocaine-CPP in wildtype and $GABA_AR$ α4-subunit knockout mice, the effects of a cocaine challenge on CPP expression were explored. Subsequently, expression of cocaine-CPP and its enhancement by a cocaine challenge were explored following intraperitoneal or intra-accumbal THIP. Finally, the role of striatal pathways in mediating cocaine-CPP were investigated. Following acquisition of cocaine-CPP in dopamine D1-/D2-expressing neuron specific $GABA_AR$ α4-subunit knockout mice and respective wildtypes, the effects of systemic THIP on baseline and cocaine-enhanced cocaine-CPP expression were explored.

1.8.4. Chapter 5

Chapter 5 further explores the role of $GABA_AR$ α4-subunits in mediating behavioural responses to reward-conditioned cues. α2-containing $GABA_ARs$ have previously been shown to be involved in the potentiation of food conditioned reinforcement by cocaine (Dixon et al., 2010). Here, following Pavlovian conditioning, nose-poke responding for a conditioned reinforcer and its potentiation by cocaine at various doses was measured in wildtype and $GABA_AR$ α4-subunit knockout mice. Subsequently, the effects of intra-accumbal THIP on baseline and cocaine-potentiated CRf responding were explored. In order to confirm the importance of $GABA_AR$ α4-subunits within the NAc in controlling CRf responding, baseline and cocaine-potentiated CRf responding was measured following viral knockdown of α4-subunit expression specifically within the NAc. Finally, the role of striatal pathways in mediating CRf responding and it potentiation by

cocaine were investigated. The effects of intra-accumbal THIP on baseline and cocaine-potentiated CRf responding were explored in dopamine D1-/D2-expressing neuron specific $GABA_AR$ α4-subunit knockout mice and respective wildtypes.

Chapter 2

The role of α4-containing $GABA_A$ receptors in baseline and acute cocaine-potentiated locomotor activity

2.1. Introduction

Alongside physiologically essential motor behaviours, including breathing, eating and drinking, locomotor activity is a crucial component of all animals' behavioural repertoire. Usually defined as the movement from one location to another, locomotor activity underlies the ability to explore the surrounding environment, critical for approaching salient stimuli, such as food or sex, and avoiding aversive stimuli, such as predators. As such, stimulus approach and avoidance can be thought of as directed locomotor activity. Novel, or non-habituated, stimuli or environments, where the salience is unknown, are associated with an initial heightened level of exploratory locomotor activity (Butler, 1958). This activity rapidly decreases if the salience is determined to be neutral (Harris, 1943). In rodents, other motor behaviours including rearing and head movements are also recognised as exploratory behaviour. A second form of locomotor activity independent of exploratory behaviour has also been documented. This spontaneous activity occurs in habituated environments, suggesting that animals also display a basal level of locomotor activity (Robbins, 1977; Paulus and Geyer, 1993).

As well as being strongly influenced by environmental conditions, including noise, light and temperature, locomotor activity is affected by administration of a wide range of pharmacological compounds. In general, locomotor activity is enhanced by drugs that facilitate transmission at dopamine synapses and reduced by drugs blocking dopamine receptors or by lesions of dopamine systems (Andén et al., 1970; Kelly et al., 1975; Kelly and Iversen, 1976; Fray et al., 1980). Indeed, psychostimulant drugs, including cocaine, dose-dependently modulate locomotor activity in an inverted U-shaped function (Isaacson et al., 1978). Acute administration of cocaine facilitates locomotor activity up to a point, at which the dose becomes so intense as to interfere with organised locomotor behaviour and induces severe behavioural stereotypy (Randrup and Munkvad, 1967; Bhattacharyya and Pradhan, 1979).

Studies investigating the behavioural outcome of direct injections of dopamine agonists into various regions of the rat forebrain, suggest that locomotor stimulation is primarily mediated by the nucleus accumbens (NAc), and stereotyped behaviours from the dorsal striatum (Costall et al., 1977; Campbell et al., 1997). The NAc is anatomically and neurochemically heterogeneous, with a major subdivision between the medioventral shell and dorsolateral core. Behavioural evidence also suggests a functional compartmentalisation of these areas with intra-accumbal amphetamine microinjection and 6-OHDA lesion studies implicating the NAc shell in mediating the rewarding properties of psychostimulants (Di Chiara et al., 2004; Ikemoto and Wise, 2004), and the NAc core in locomotor and other behavioural activation (Boye et al., 2001; Sellings and Clarke, 2003; Sellings et al., 2006). However, the extent of this functional divide is controversial, with some laboratories reporting that microinjections of amphetamine into the medial shell (Heidbreder and Feldon, 1998) or both shell and core (Ikemoto, 2002) can also facilitate locomotor activity. Furthermore, the majority of these studies were carried out using rats, and it is still unknown whether the NAc core/shell divide exists to the same extent in mouse strains.

It has been theorised that potentiated locomotor responses following intra-accumbal dopamine agonist infusions may be the result of a more general facilitation of approach-investigation behaviour, which is then directed by the environmental conditions (Ikemoto and Panksepp, 1999). Thus, in a neutral environment lacking in interactive stimuli, such as a standard locomotor activity chamber, increased NAc dopamine transmission may simply stimulate exploratory behaviour including locomotion and rearing, whereas in other situations other approach behaviours may be facilitated, such as conditioned activity in an operant task (Taylor and Robbins, 1986; Cador et al., 1991; Kelley and Delfs, 1991). Indeed, projections from limbic structures to regions of the NAc help to guide the initiation of behavioural responses following increased dopamine transmission. In addition to dopaminergic innervation from the VTA, the NAc receives strong glutamatergic inputs from the hippocampus, prefrontal cortex (PFC) and amygdala, and projects to the globus pallidus, making it ideally positioned for the integration of limbic and motor systems (Mogenson et al., 1980; Groenewegen et al., 1996).

The hippocampus plays a crucial role in various cognitive functions, including spatial memory and navigation, and has been suggested to contribute to the initiation of exploratory locomotion (Roberts et al., 1962; Teitelbaum and Milner, 1963; Kleinrok et al., 1980). Electrical stimulation of the ventral hippocampal formation, including the ventral CA1 and subicular areas, which project largely to the medial NAc shell, increases locomotor activity; however, acute inactivation of the same area has no effect (Groenewegen et al., 1987; Bardgett and Henry, 1999). More recently, optogenetic activation of ventral hippocampal axons in the NAc increases, whilst inhibition decreases, cocaine-induced locomotion (Britt et al., 2012). The PFC and basolateral amygdala (BLA), known to play a critical role in the production of goal-orientated behaviours, send projections to both the NAc core and shell. Inactivation of these structures has opposing effects on intra-accumbal amphetamine-induced locomotion, with inhibition of the PFC by lidocaine potentiating, and inactivation of the BLA inhibiting amphetamine-induced hyperlocomotion (Rouillon et al., 2008). However, injection of dopamine into the central nucleus of the amygdala (CeN), which projects largely to the NAc core, has no effect on baseline locomotor activity (Jackson et al., 1975).

Evidence has demonstrated an important role for GABAergic inhibition in mediating locomotor activity. Increased whole-brain GABA levels using systemic administration of drugs blocking GABA breakdown, including amino-oxyacetic acid, di-n-propyl-acetate and γ-acetylenic GABA, attenuates baseline and amphetamine-potentiated locomotor activity (Grimm et al., 1975; Cott and Engel, 1977; Agmo and Giordano, 1985). Similarly, systemic injection of GABA also decreases locomotor activity in mice, rats and rabbits (Śmiałowski et al., 1980). Given that GABAergic MSNs are the primary neural type within the NAc it is likely that manipulations of GABA and $GABA_A$ receptors within this structure may have a significant effect on baseline and psychostimulant-potentiated locomotor activity. Indeed, intra-accumbal injections of GABA elicit a bimodal response in locomotion, with low doses inducing a small increase, and larger doses producing a reduction (Wachtel and Anden, 1978; Jones et al., 1981). Interestingly, elevation of GABA levels in the NAc using the GABA-transaminase inhibitor ethanolamine-O-sulphate abolishes the ability of intra-accumbal dopamine injections to potentiation locomotor activity, but has no significant effect on baseline locomotor activity (Pycock and Horton, 1976b). This may potentially be

explained by differences in the total GABA volume within the NAc following different methods of intra-accumbal GABA manipulation. More recently, compounds allowing researchers to target $GABA_A$ receptors directly have allowed for more accurate control and demonstrate greater concurrence. As predicted from the earlier work, intra-accumbal microinjections of the $GABA_AR$ antagonist picrotoxin enhance baseline and intra-accumbal amphetamine-potentiated locomotor responses (Pycock and Horton, 1979; Jones et al., 1981; Wong et al., 1991). Similarly, muscimol-induced activation of $GABA_A$ receptors in the NAc core but not shell reduce dopamine receptor-mediated motor behaviour in mice (Akiyama et al., 2003; 2004).

Within the VTA, infusions of the $GABA_AR$ agonist muscimol, and $GABA_AR$ antagonist picrotoxin, induce opposing functional effects, which also vary according to the infusion site (Arnt and Scheel-Krüger, 1979). Muscimol infused into the caudal VTA elicits a dopamine-dependent increase in locomotor activity, whilst picrotoxin produces a mild decrease in locomotor activity. However, when injected into the rostral VTA, muscimol decreases, and picrotoxin increases locomotor activity. Furthermore, the locomotor activating effects seen following GABA agonists and antagonists in the caudal and rostral VTA, respectively, are dopamine-dependent (Scheel-Krüger et al., 1980). Dopaminergic afferents from the VTA to the NAc are organised topographically in a rostro-caudal arrangement; thus, GABA, within the VTA, may act to alter the balance between dopamine systems mediating distinct behavioural functions dependent on the various anatomical areas.

A GABAergic projection from the NAc to the ventral globus pallidus is strongly implicated in the control of locomotor activity (Jones and Mogenson, 1980a; Walaas and Fonnum, 1980; Mogenson and Sztorc, 1982). Microinjection of picrotoxin into the ventral globus pallidus enhances locomotor activity in rats (Jones and Mogenson, 1980b). Similarly, increasing globus pallidus GABA levels by infusion of the ethanolamine-O-sulphate (Pycock et al., 1976; Pycock and Horton, 1976a), or microinjection of GABA (Mogenson and Nielsen, 1983), attenuates locomotor activity elicited by intra-accumbal injection of dopamine (Pycock et al., 1976; Pycock and Horton, 1976a). These data support the proposed role of striatopallidal GABAergic projection neurons within the *indirect* pathway (see Chapter 1; Fig.1.1.) in supressing motor behaviour (Kravitz and Kreitzer, 2012; Kravitz et al., 2012).

To conclude, whilst it is clear that dopamine release onto GABAergic MSNs within the NAc is the driving force in the initiation of locomotor activity and the locomotor potentiating effects psychostimulant drugs, it is the $GABA_AR$s within the NAc (with the addition of glutamatergic inputs from other brain regions) that are likely to act to control this dopamine-dependent locomotor activity. However, as yet, very little is understood about the details of accumbal GABAergic transmission, and, in particular, which $GABA_AR$ subtypes may be important in this process. Moreover, the research that has been conducted previously has largely explored the effects of $GABA_AR$ agonists and antagonists targeting synaptically located receptors. However, there is recent evidence that extrasynaptically located $GABA_AR$s may also play a functional role in mediating locomotor activity. Systemic administration of THIP, a $GABA_AR$ agonist with a preference for δ-containing receptors typically found extrasynaptically, inhibits baseline locomotor activity and attenuates enhanced locomotion following intra-accumbal administration of the glutamate agonist 6,7-ADTN (Arnt, 1981; Agmo and Giordano, 1985; Herd et al., 2009; Vashchinkina et al., 2012). The experiments reported here will further investigate the involvement of extrasynaptic α4βδ receptors in mediating baseline and cocaine-potentiated locomotor activity, by examining the effects of pharmacological activation of these receptors with THIP in wildtype and $GABA_AR$ α4-subunit knockout mice.

2.2. Materials and Methods

2.2.1. Animals

Constitutive α4-subunit knockout mice were produced at Sussex University. "Floxed" α4-subunit homozygous mice (strain name; B6.129-*Gabra4*$^{tm1.2Geh}$/J, supplied by The Jackson Laboratory, ME, USA), were crossed with Cre-recombinase expressing hemizygous transgenic mice (strain name; B6.FVB-Tg (Ella-cre)C5379Lmgd/J, supplied by The Jackson Laboratory, ME, USA). Offspring were genotyped and putative Gabra4 heterozygous mice (carrying the CRE transgene (~50% of offspring)) were bred together to generate homozygous knockout, heterozygous (used for breeding) and wildtype littermates (Fig. 2.1.).

Male and female $GABA_A$ α4 wildtype (WT) and knockout ($\alpha4^{-/-}$) mice on a C57BL/6J background, weighing between 20-30g and aged between 2-6 months, were housed in groups of 2-3, or separately for animals undergoing surgery, with food and water available *ad libitum*. A 12hr light/dark cycle was used (lights on at 7:00 A.M.) with holding room temperature maintained at 21±2ºC and humidity 50±5%. All injections, infusions and behavioural testing were performed between 2:00 P.M. and 5:00 P.M. All procedures were conducted in accordance to Animals (Scientific Procedures) Act 1986, following ethical review by the University of Sussex Ethical Review Committee.

Figure 2.1.

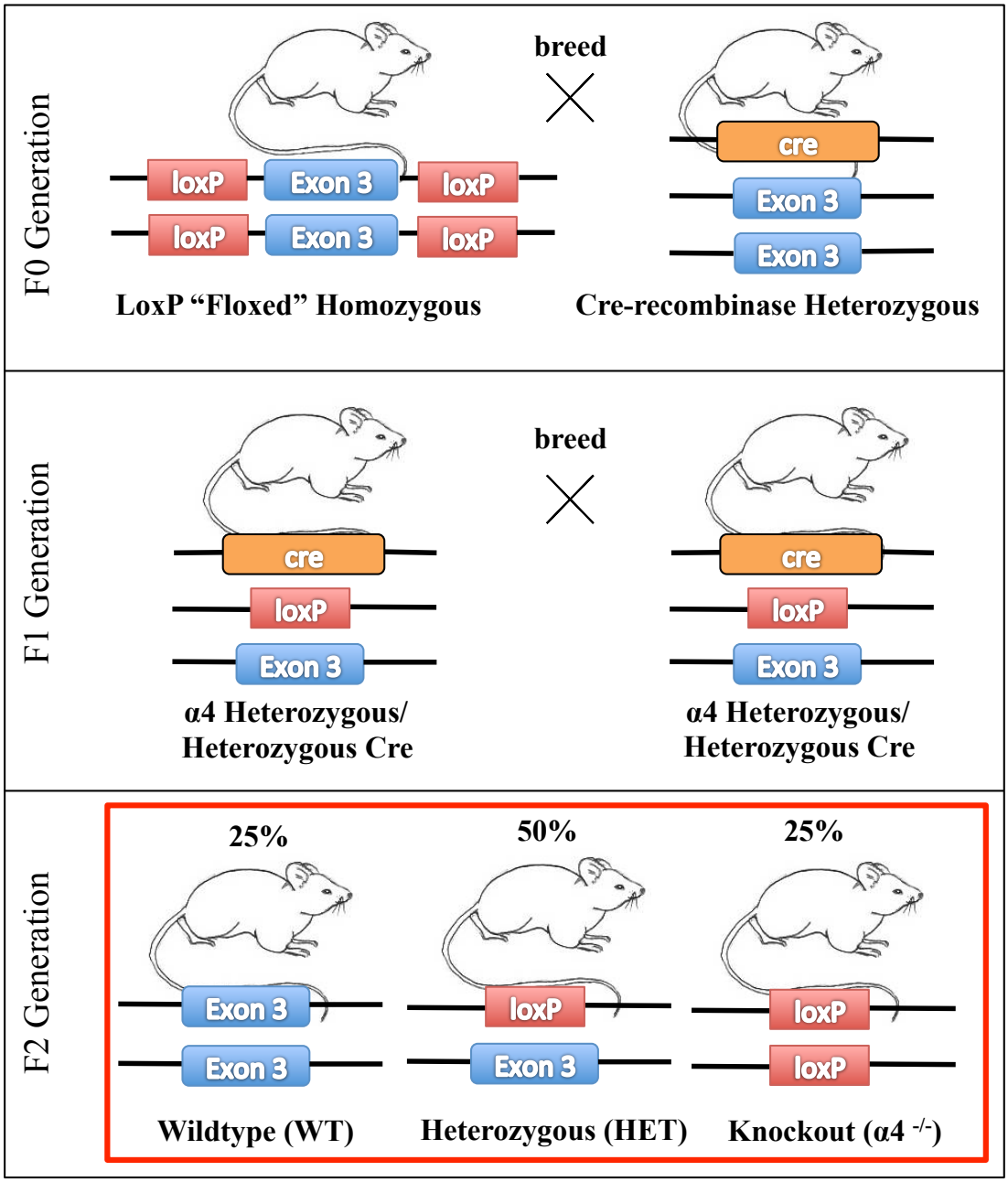


Fig. 2.1. Production of α4 wildtype (WT), heterozygous (HET) and knockout ($\alpha4^{-/-}$) mice. (**F0**) α4-subunit "floxed" homozygous mice were bred with Cre-recombinase expressing heterozygous mice. (**F1**) offspring were heterozygous for the α4 allele (+/-), which were bred to create (**F2**) offspring in approximate ratios, 25% WT, 50% HET, 25% $\alpha4^{-/-}$.

2.2.2. Genotyping

2.2.2.1. DNA Extraction

Mouse ear punches were collected and DNA extracted by digestion in a 20µl solution of a 1mg/ml proteinase K solution (50mg/ml; Roche Products Ltd., UK) and 20mM Tris HCl (Sigma-Aldrich, Dorset, UK) and 10mM EDTA (Sigma-Aldrich, Dorset, UK) lysis buffer. Solutions were overlayed with two drops of purified mineral oil (Sigma-Aldrich, Dorset, UK), then incubated at 55°C for 2 hours, then heated to 95°C for 15 minutes in a thermocycling PCR machine (G-Storm GS1, GRI Ltd., Somerset, UK). Extracted DNA samples were diluted to 100µl with purified PCR water, with gentle mixing.

2.2.2.2. PCR

$GABA_AR$ α4-subunit PCR primer sequences were used from those presented in the supplementary text of (Chandra et al, 2006). Forward and reverse cDNA primers were designed to target and replicate a sequence within the wildtype *gabra4* gene and the shortened gene with a deletion of exon 3 in the α4-subunit knockout mouse. The wildtype primers consisted of a 156bp product (forward primer, AAGATCACCAAGCCAACAGG; reverse primer, TCTTTGGGGAGTTGAGGATG) containing the primary loxP site in the "floxed" mice, and part of the conserved region. The knockout primers consisted of a 241bp product (forward primer, AAGATCACCAAGCCAACAGG; reverse primer, TGCACACTGTAATTCCCATC), which flanked the primary and secondary loxP sites either side of exon 3 of the *gabra4* gene.

For each reaction, 0.5µl of extracted DNA was mixed into a solution of 0.5µl of both forward and reverse primers and 23.5µl of Megamix-Blue (Microzone Ltd., Haywards Heath, UK). Solutions were overlayed with two drops of purified mineral oil, then incubated at 95°C for 5 minutes, followed by 35 cycles of the following; 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, in a thermocycling PCR machine. Finally, PCR samples were held at 72°C for 10 minutes.

2.2.2.3. Gel Electrophoresis and DNA Detection

Following PCR amplification of the targeted DNA, samples were electrophoretically separated on a 1.5% agorose (AGTC Bioproducts Ltd., Leicestershire, UK) gel containing 0.004% ethidium bromide (50mg/ml solution; Sigma-Aldrich, Dorset, UK) in 1% TAE buffer (242g/L tris base (Sigma-Aldrich, Dorset, UK), 57.1ml/L acetic acid (Sigma-Aldrich, Dorset, UK), 0.5M EDTA (14.62g of EDTA (Sigma-Aldrich, Dorset, UK) in 100ml/L dH_2O). Gels placed within a horizontal electrophoresis tank connected to a power supply (BioRad Laboritories Inc., Hercules, CA, USA) were run at 120v for approximately 30 minutes, and then observed under UV light for the presence of the wildtype and knockout (Fig. 2.2.) primers.

Figure 2.2.

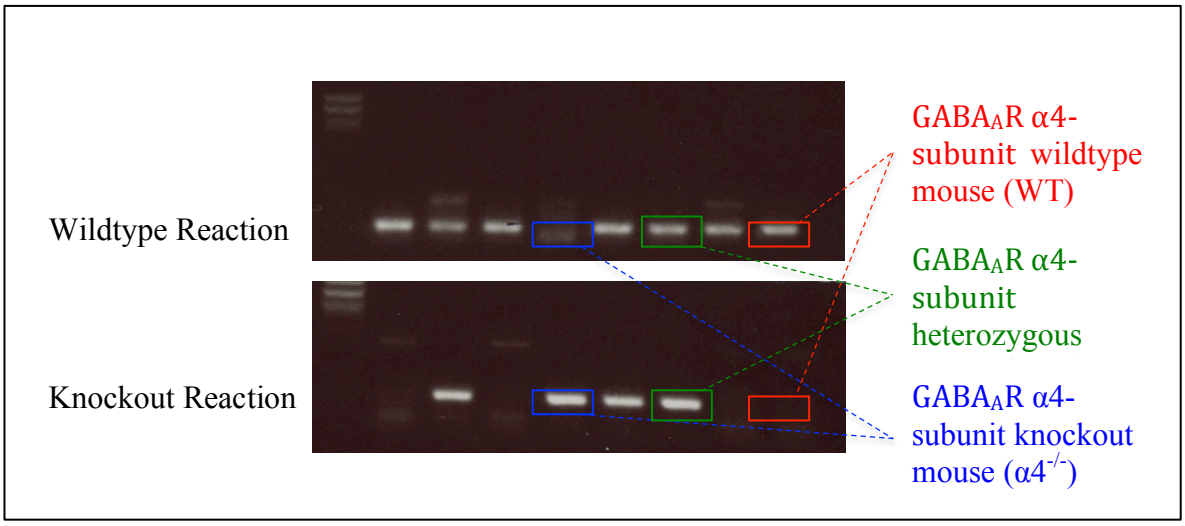


Fig. 2.2. Genotyping of GABA$_A$R α4-subunit wildtype and knockout mice requires two reactions per mouse. The first reaction contains the wildtype primers, and second reaction contains the knockout primers for detection of the *gabra4* gene. The presence of a band in the wildtype reaction, but not the knockout reaction indicates a wildtype mouse. A band in the knockout reaction, but not the wildtype reaction indicates a knockout mouse. A band in both reactions indicates a mouse heterozygous for both the wildtype and knockout gene.

2.2.3. Western Blot protein analysis

2.2.3.1. Preparation of tissue lysates

$GABA_AR$ α4-subunit wildtype, heterozygous and knockout mouse brains were dissected and tissue samples collected from the prefrontal cortex, thalamus and nucleus accumbens using a 1.5mm biopsy punch (Kai Medical Inc., Seki, Japan). Tissue punches were homogenised in 500µl of lysis buffer (10ml solution contains; 860mg sucrose (Sigma-Aldrich, Dorset, UK), 20µl 0.5M Na Vanadate (Sigma-Aldrich, Dorset, UK), 10µl β-mercaptoethanol, 2ml 5X lysis buffer stock (600µl 1M Tris pH 6.8, 0.1g SDS (Sigma-Aldrich, Dorset, UK), 1ml Glycerol (Sigma-Aldrich, Dorset, UK), 400µl purified H_2O), 1 Roche Complete Mini Protease Inhibitor cocktail tablet (Roche Products Ltd., Hertfordshire, UK)).

2.2.3.2. SDS-PAGE electrophoresis and protein transfer

9µl of each protein sample was mixed with 3µl of protein loading dye (10ml solution contains; 1ml 1M Tris, 4ml 10% SDS, 0.002g bromophenol blue (Sigma-Aldrich, Dorset, UK), 2ml glycerol) and separated by SDS-PAGE for 30 minutes at 200V in a 10% acrylamide gel (10% resolving gel, 30ml solution contains; 13.9ml purified H_2O, 8ml 30% acrylamide mix (BioRad Laboritories Inc., Hercules, CA, USA), 7.5ml 1.5M Tris HCl pH 8.8, 0.3ml 10% SDS, 0.3ml 10% ammonium persulphate (Sigma-Aldrich, Dorset, UK), 0.018ml TEMED (BioRad Laboritories Inc., Hercules, CA, USA); 5% stacking gel, 5ml solution contains; 3.4ml purified H_2O, 0.83ml 30% acrylamide mix, 0.63ml 1.5M Tris HCl pH 6.8 with bromophenol blue, 0.05ml 10% SDS, 0.05ml 10% ammonium persulphate, 0.005ml TEMED, using a Mini-PROTEAN Tetra Cell electrophoresis system (BioRad Laboritories Inc., Hercules, CA, USA). Proteins were transferred to a nitrocellulose blotting membrane (BioRad Laboritories Inc., Hercules, CA, USA) using a Trans-Blot Turbo Transfer System (BioRad Laboritories Inc., Hercules, CA, USA).

2.2.3.3. Primary and secondary antibody incubation

Blots were blocked in 15ml of TBS-T (50mM Tris, 150mM NaCl (Sigma-Aldrich, Dorset, UK) and 0.05% Tween 20 (Sigma-Aldrich, Dorset, UK) in 500ml of purified H_20) with 5% milk (Marvel, Wembley, UK) for 60 minutes, then incubated in 15ml of

anti-$GABA_AR$ α4-subunit rabbit anti-mouse polyclonal primary antibody (1:1000; catalogue no. 844-GA4N, Phosphosolutions, CO, USA) in TBS-T with 1% BSA (Sigma-Aldrich, Dorset, UK) overnight at 4°C. Blots were then incubated in 15ml of goat anti-rabbit HRP (horseradish peroxidase) secondary antibody (1:10,000; catalogue no. ab6721, Abcam, Cambridge, UK) in TBS-T for 60 minutes.

2.2.3.4. Protein detection and data analysis

Blots were incubated in 2ml of chemiluminescence substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA) for approximately 5 minutes, then exposed to CL-XPosure photographic film (Thermo Fisher Scientific Inc., Waltham, MA, USA) and developed in a darkroom. Developed films were scanned on an Epson 4990 Photo Scanner (Epson, Nagano, Japan) and quantified using NIH ImageJ (http://rsb.info.nih.gov/ij/). Integrated densities were used, measuring the mean intensity of each selected band. Background correctional values were subtracted from each lane to minimize variability across membranes. Integrated density results were averaged between genotypes and the mean values used to calculate the percentage change from wildtype mice.

2.2.4. Quantitative reverse transcription PCR (qRT-PCR) mRNA analysis

2.2.4.1. Preparation of tissue lysates and phase separation

Constitutive $GABA_A$ α4-subunit wildtype, heterozygous and knockout mice brains were dissected and tissue samples collected from the nucleus accumbens using a 1.5mm biopsy punch (Kai Medical Inc., Seki, Japan). Tissue samples were homogenised in 600µl of Trizol (Life Technologies, CA, USA) and 200µl of RNase-free H_2O (Life Technologies Corp., CA, USA), then mixed with 160µl of chloroform (Thermo Fisher Scientific Inc., Waltham, MA, USA) and phase separated by centrifuging for 15 minutes (12,000g) in pre-spun peqGOLD PhaseTrap A phase lock eppendorf tubes (Peqlab ltd., Erlangen, Germany).

2.2.4.2. RNA precipitation

The aqueous layer of each sample was decanted into an eppendorf tube (Sigma-Aldrich, Dorset, UK), then mixed with 0.5ml of isopropanol (Sigma-Aldrich, Dorset, UK), 50µl of sodium acetate (Sigma-Aldrich, Dorset, UK), and 4µl of glycoblue (Life

Technologies Corp., CA, USA) and incubated at room temperature for 10 minutes. Samples were centrifuged (12,000g) at 4°C for 20 minutes until a RNA pellet formed, the supernatant was discarded and replaced with 1ml of 75% EtOH (Sigma-Aldrich, Dorset, UK) wash then centrifuged (7500g) for 5 minutes at 4°C. The wash was discarded and pellets left to air dry for 30 minutes, then resuspended in 87.5µl of RNase-free H_2O (Sigma-Aldrich, Dorset, UK).

2.2.4.3. RNA cleanup

RNA was extracted using the RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Ltd., West Sussex, UK). To each 87.5µl solution; 10µl of buffer RDD and 2.5µl of DNase I stock solution were added and incubated at room temperature for 10 minutes. Then to this 100µl solution; 350µl of buffer RLT mixed with 3.5µl of β-mercaptoethanol and 250µl of 96-100% EtOH were mixed and immediately transferred to a spin column in a 2ml collection tube, then centrifuged (13,000g) for 15 seconds. Each spin column was transferred to a new collection tube to which 500µl of buffer RPE was added then centrifuged (13,000g) for 15 seconds. Each spin column was again transferred to a new collection tube to which 500µl of 80% EtOH was added then centrifuged (13,000g) for 15 seconds. Finally, each spin column was transferred to a new collection tube and centrifuged (13,000g) for 5 minutes with the lid open. The spin columns were transferred to new 1.5µl eppendorf tubes to which 14µl of RNase-free H_2O was added and centrifuged (13,000g) for 2 minutes. Approximately 12µl of eluted RNA was retrieved.

2.2.4.4. RNA calculation and cDNA production

The amount of RNA was determined using a NanoDrop 2000 micro-volume spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and corrected for between each sample. RNA was added to the appropriate amount of RNase-free H_2O and 2µl of oligo(dT) primer (Life Technologies, CA, USA) to make a total volume of 15µl then incubated at 65°C for 5 minutes in a thermocycling PCR machine. Reactions were snap chilled on ice for 1 minute, after which 4µl of 5Xiscript select react mix (Life Technologies, CA, USA) and 1µl of reverse transcriptase (Life Technologies, CA, USA) were added to each. Finally, reactions were mixed and incubated at 42°C for 60

minutes then 85°C for 5 minutes in a thermocycling PCR machine, to make 20μl of cDNA.

2.2.4.5. qRT-PCR reaction

1μl of each cDNA (at ≤500ng) sample was amplified by PCR in a 25μl reaction mixture; 12.5μl of SYBRGreen mastermix (Sigma-Aldrich, Dorset, UK), 0.6-μl of forward primer (primer sequences were designed using BLAST search with the NCBI tool Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/), presented in Table 2.1.), 0.6-μl of reverse primer and 10.3μl of RNase-free H_2O, using an Mx4000 multiplex quantitative PCR sampler (Stratagene, La Jolla, CA, USA). Sample concentrations were calculated from serial dilution concentration curves, and each reaction was set up in triplicate, including GAPDH and 1μl RNase-free H_2O no template controls.

Table 2.1.

Primer	Forward	Reverse
$GABA_AR$ α4-subunit	*5'-* CGTATTCTGGACAGTTTGCTG GATGGT *-3'* (27)	*5'-* ACGGGCCCAAAGCTGGTGAC AT-*3'* (22)
$GABA_AR$ α2-subunit	*5'-* AAAAGAGGATGGGCTTGGGA- *3'* (20)	*5'-* ACGGGATGTTTTCTGCCTGTA T-*3'* (22)
$GABA_AR$ γ2-subunit	*5'-* GGAGCCTGGAGACATGGGA - *3'* (19)	*5'-* TGAACAAGCAAAAGGCGGTA *-3'* (20)
$GABA_AR$ δ-subunit	*5'-* GGCTCCCCAACCTGGATGGCT *-3'* (21)	*5'-* GGCCACCTCTAGGGCAAGCG - *3'* (20)
GAPDH	*5'-* TGCCCCCATGTTTGTGATG *-3'* (19)	*5'-* TGTGGTCATGAGCCCTTCC *-3'* (19)

Table 2.1. Primer sequences used for qRT-PCR analysis of constitutive and D1/D2-expressing neuron specific $GABA_AR$ α4-subunit knockout mice, heterozygous and relative wildtype controls.

2.2.5. Stereotaxic Surgery

Mice anaesthetised with isoflurane were implanted stereotaxically with bilateral guide cannulae (26 ga., 10mm) aimed at NAc (coordinates AP1.34; L+/− 1.00; DV −3.20, (Paxinos and Franklin, 2001)). Following surgery, mice were singly housed and underwent a one-week recovery/habituation period. A steel infuser (33 ga., 11 mm) connected *via* polyvinyl tubing to a (5 μl) Hamilton Gastight syringe was used to infuse 0.5μl of either saline or THIP (3 mM) bilaterally across 90 seconds and left to settle for 90 seconds before infusers were removed. Location of cannulae was confirmed histologically. One animal was removed from data analysis due to inexact cannulae placements.

2.2.6. Drugs

Cocaine Hydrochloride was obtained from Macfarlan Smith (Edinburgh, UK). THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) was kindly donated by Bjarke Ebert (Lundbeck, Valby, Denmark). Both drugs were dissolved in 0.9% saline, and administered i.p. at an injection volume of 10 ml/kg, and intracranially as described above.

2.2.7. Apparatus

Locomotor activity was measured using 16 annular black Perspex runways, (diameter 24cm, annulate width 6.5cm), placed atop of a clouded Perspex sheet on an elevated frame. A digital camera positioned beneath the sheet captured the silhouettes of the boxes' edges and the mice within them, which was then relayed to a computer to be recorded. A MatLab (MathWorks, Cambridge, UK) video analysis programme and Excel macro converted the video data into a measure of the distance travelled in metres.

2.2.8. Cocaine Dose Response

The locomotor response to acute cocaine at various doses was tested in WT and $\alpha4^{-/-}$ mice. In a Latin-square design, mice were administered with saline, 3, 10, 20 and 30mg/kg cocaine directly prior to placement in the locomotor runways.

2.2.9. Baseline and Cocaine-Potentiated Locomotor Activity

2.2.9.1. Intraperitoneal THIP Dose Response

Prior to testing there were two habituation days, on the first day mice were habituated to the equipment for 60 minutes, on the second day mice received a sham *i.p.* injection of saline prior to being placed in the apparatus. All animals underwent four test days in a Latin square design, during which they were administered saline, 5, 10, 20mg/kg. Saline/THIP Injections were given 20 minutes in advance, followed by a second i.p injection of saline or cocaine (10mg/kg) directly prior to initiating the locomotor test. All locomotor activity was recorded over 60 minutes. Test sessions were spaced 48 hours apart to eliminate the possibility of lingering drug effects from the previous session.

2.2.9.2. Intra-accumbal THIP Dose Response

As previous, mice first received two habituation days, firstly a 60 minute habituation session, then on the second day, a 60 minute habituation session including a sham intracranial infusion (infusers were inserted and left in for 3 min, but nothing was administered) prior to being placed in the apparatus. All animals then underwent four test days in a Latin square design, during which they were administered saline or intra-accumbal THIP at either 0.3, 3, 30, 300 or 3000uM. Intra-accumbal infusions were given immediately prior to an i.p injection of saline or cocaine (10mg/kg). All locomotor activity was recorded over 60 minutes. To reduce possible structural damage following multiple intracranial infusions, each THIP dose was tested in separate groups of mice. Successive sessions were spaced by at least 48h to reduce the possibility of lingering drug effects from the previous session.

2.2.10. Statistical Analysis

2.2.10.1. Western Blot

Developed films were scanned and quantified using NIH ImageJ (http://rsb.info.nih.gov/ij/). Integrated densities were used, measuring the mean intensity of each selected band. Background correctional values were subtracted from each lane to minimize variability across membranes. Integrated density results were averaged between genotypes and the mean values used to calculate the percentage change from wildtype mice.

2.2.10.2. qRT-PCR

Quantitative RNA expression data were collected using the Mx4000 data analysis software (Stratagene, CA, USA), then exported to an Excel worksheet. Reaction triplicates were averaged, and then normalised against the control gene GAPDH to give a measure of the delta CT. The delta CT of the target sample was then normalised against the delta CT of a control sample to give a measure of the delta delta CT. Finally, a mathematical model was used to calculate the fold change of the target gene using the delta-delta CT (see (Pfaffl, 2001)). Statistical analysis of RNA expression of

each receptor subunit was conducted using between-subjects one-way ANOVAs, with genotype as the between-subjects variables, and delta CT as the dependent variable.

2.2.10.3. Cocaine Dose Response

Locomotor activity data for the cocaine dose response study were analysed using a three-way mixed-factors ANOVA with genotype and sex as the between-subjects variables, and meters travelled following each cocaine dose as the within-subjects dependent variable.

2.2.10.4. Intraperitoneal THIP Dose Response

Locomotor activity data for the i.p. THIP dose response study were analysed using a four-way mixed-factors ANOVA with genotype, sex and drug group as the between-subjects variables, and meters travelled following each THIP dose as the within-subjects dependent variable. *Post hoc* analyses were conducted where appropriate using paired t-tests.

2.2.10.5. Intra-accumbal THIP Dose Response

Locomotor activity data for the intra-accumbal THIP dose response study were analysed using a five-way mixed-factors ANOVA with sex and THIP dose as the between-subjects variable, infusion treatment and injection treatment as the within-subjects variables, and meters travelled in each condition as the dependent variable. Subsequently, two separate five-way mixed-factors ANOVA were conducted for each of two THIP doses (3μM and 3mM), with sex and genotype as the between-subjects variables, infusion treatment and injection treatment as the within-subjects variables, and meters travelled in each condition as the dependent variables. *Post hoc* analyses were conducted where appropriate using paired t-tests.

2.3. Results

2.3.1. Western Blot protein analysis

To confirm that the knockout mice lacked the expression of Gabra4, western blot analysis was conducted on tissue samples from the prefrontal cortex (PFC), thalamus and nucleus accumbens (NAc) of WT, HET, and $\alpha4^{-/-}$ mice. An α4-specific antibody (Bencsits, 1999) specifically recognized a ≈67kD protein in WT mice (Fig. 2.5.A.). This protein band is absent in tissue samples from $\alpha4^{-/-}$ mice (Fig. 2.3.). In HET mice Gabra4 expression was reduced in the PFC (52 ±11% reduction compared to WT controls), thalamus (30 ±22% reduction compared to WT controls) and NAc (70 ±12% reduction compared to WT controls) (Fig. 2.3.).

Figure 2.3.

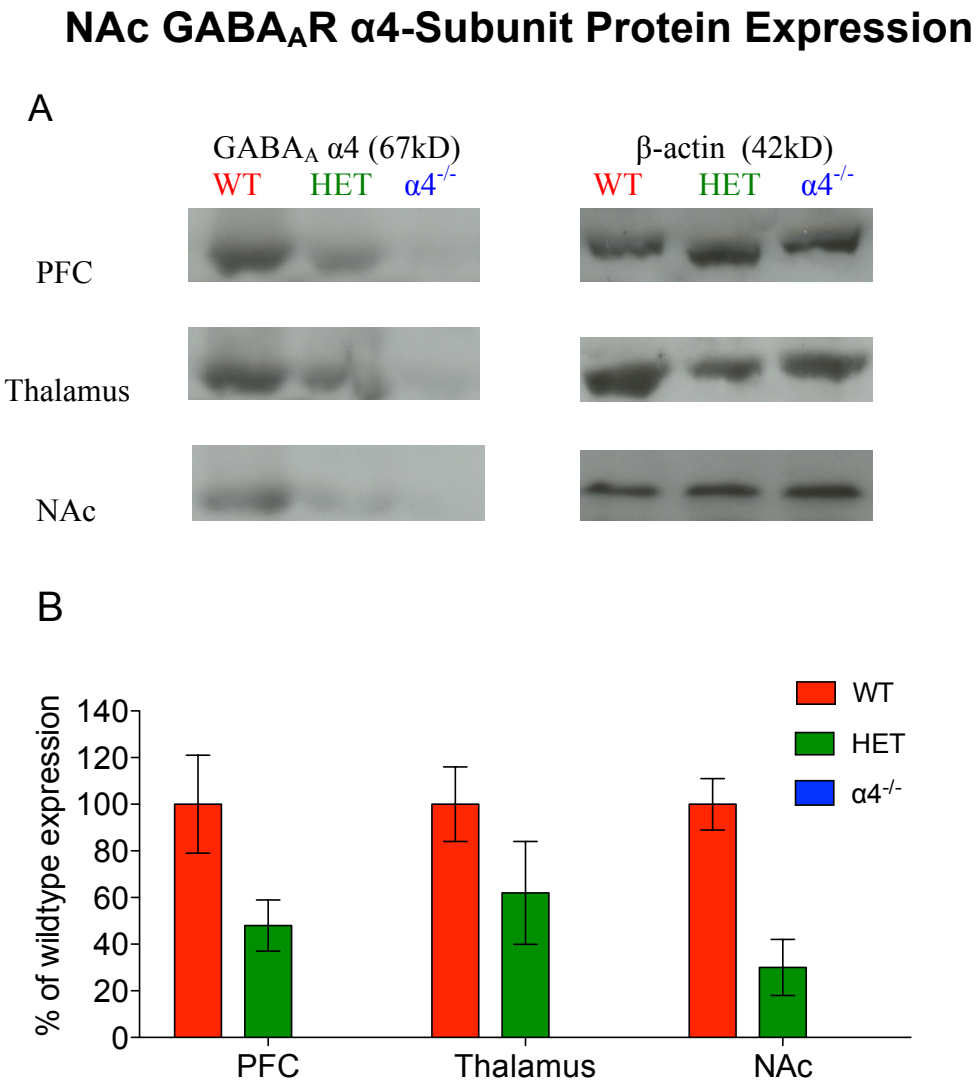


Fig. 2.3. Western Blot analysis of prefrontal cortex (PFC), thalamus and nucleus accumbens (NAc) tissue from GABA$_A$ α4-subunit wildtype (WT, n=3), heterozygous (HET, n=3) and knockout (α4$^{-/-}$, n=3) mouse tissue. (**A**) Representative images of western blot results for GABA$_A$ α4 and β-actin. Blots probed for β-actin show equal loading of samples. (**B**) Percentage change from WTs of the protein Gabra4 in the PFC, thalamus and NAc. The expression of the Gabra4 protein was lacking in all tested brain regions of the α4$^{-/-}$ mice and significantly reduced in HET mice. Error bars represent SEM.

2.3.2. qRT-PCR mRNA analysis in constitutive $GABA_AR$ α4-subunit wildtype, heterozygous and knockout mice

In order to confirm the deletion of $GABA_AR$ α4-subunits, expression levels of α4-subunit mRNA were measured in the NAc of WT, HET and $\alpha4^{-/-}$ mice using qRT-PCR. As previous evidence has also indicated protein levels of α2-, γ2- and δ-subunits to be differentially modulated in hippocampus of $\alpha4^{-/-}$ mice when compared to WT controls, the mRNA expression levels of these subunits were also measured.

qRT-PCR revealed $GABA_AR$ α4-subunit mRNA levels to be reduced in NAc of HET mice and completely absent in $\alpha4^{-/-}$ mice when compared to WT control mice, further confirming the success of the targeted gene knockout (Table 2.2., Fig. 2.4; significant main effect of genotype, $F_{(2,15)} = 4526.05$, $p < 0.001$). Conversely, $GABA_AR$ α2-subunit mRNA levels were increased in the NAc of HET mice and to a greater extent $\alpha4^{-/-}$ mice, when compared to WT controls (Table 2.2., Fig. 2.4; significant main effect of genotype, $F_{(2,15)} = 36.18$, $p < 0.001$). HET and $\alpha4^{-/-}$ mice did not differ significantly from WT controls in the expression of $GABA_AR$ γ2-subunit mRNA in the NAc (Table 2.2, Fig. 2.4; significant main effect of genotype, $F_{(2,15)} = 0.42$, $p = 0.52$, NS). Finally, mRNA expression of $GABA_AR$ δ-subunits was unchanged in HET mice, but reduced in the NAc of $\alpha4^{-/-}$ mice, when compared to WT controls (Table 2.2., Fig. 2.4; significant main effect of genotype, $F_{(2,15)} = 22.18$, $p < 0.001$).

Table 2.2.

Primer	Genotype	Fold change	% Change from WT	Sig.
α4	WT	1 ± 0.18/0.11	0%	
	HET	0.54 ± 0.07/0.05	-46%	$p < 0.001$
	$\alpha4^{-/-}$	0 ± 0/0	-100%	$p < 0.001$
α2	WT	1 ± 0.39/0.17	0%	
	HET	4.87 ± 1.58/0.78	+ 387%	$p < 0.001$
	$\alpha4^{-/-}$	5.07 ± 1.79/0.84	+407%	$p < 0.001$
γ2	WT	1 ± 0.26/0.14	0%	
	HET	1.15 ± 0.20/0.12	+15%	NS
	$\alpha4^{-/-}$	1.11 ± 0.24/0.14	+11%	NS
δ	WT	1 ± 0.24/0.13	0%	
	HET	1.14 ± 0.20/0.11	+14%	NS
	$\alpha4^{-/-}$	0.53 ± 0.20/0.10	-47%	$p < 0.001$

Table 2.2.1. NAc mRNA expression levels of $GABA_AR$ α4-, α2-, γ2- and δ-subunits in HET (n=6) and $\alpha4^{-/-}$ (n=6) mice were compared in triplicate against WT (n=6) controls to give a measure of fold change. Fold change from WTs was tested statistically using Tukey's *post hoc* comparisons.

Figure 2.4.

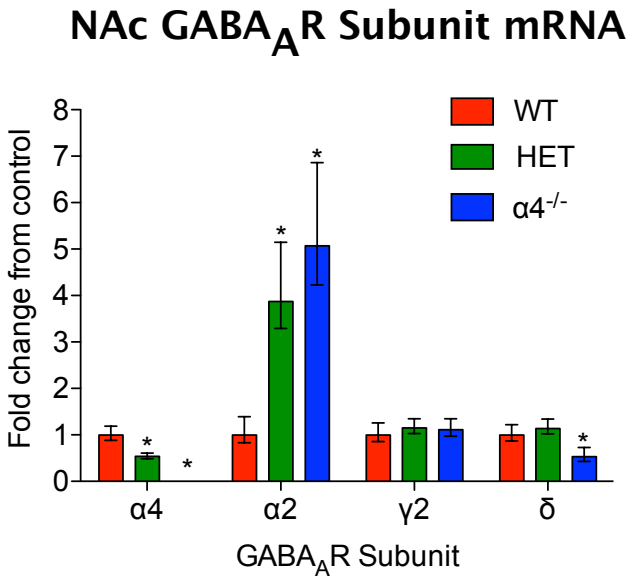


Fig. 2.4. Fold change from WT controls of GABA$_A$R α4, α2-, γ2- and δ-subunit mRNA expression in the NAc of WT (n=6), HET (n=6) and α4$^{-/-}$ (n=6) mice. HET mice show a decrease and α4$^{-/-}$ mice show a complete absence of expression of α4-subunit mRNA when compaired to WT controls. Conversely, HET and α4$^{-/-}$ mice show a large increase in expression of α2-subunit mRNA. Expression of γ2-subunit mRNA was unchanged in HET and α4$^{-/-}$ mice. Finally, expression of δ-subunit mRNA was reduced in α4$^{-/-}$ but not HET mice, when compared to WT controls. Error bars represent SEM. *$p < 0.001$, *post hoc* Tukey's comparisons.

2.3.3. Cocaine Dose Response

Acute administration of cocaine dose-dependently increased locomotor activity equally in WT and $\alpha4^{-/-}$ mice (Fig. 2.5; significant main effect of dose, $F_{(4,48)}= 582.43$, $p < 0.001$; non significant main effect of genotype, $F_{(1,12)}= 0.29$, p = NS; non-significant dose by genotype interaction, $F_{(4,48)}= 0.07$, p = NS). Activity did not differ between sexes (non-significant dose by genotype by sex interaction, $F_{(4,48)}= 0.48$, p = NS).

Figure 2.5.

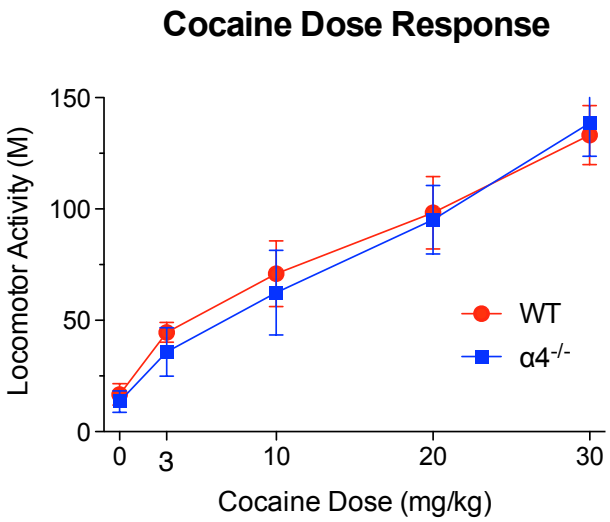


Fig. 2.5. Effect of intraperitoneal acute cocaine on distance travelled over 60 minutes in wildtype and $GABA_A$ α4-subunit knockout mice. Cocaine dose-dependently increased locomotor activity equally in both WT (n=8; males=4, females=4) and $\alpha4^{-/-}$ (n=8; males=4, females=4) mice. Error bars represent SEM.

2.3.4. Baseline and Cocaine-Potentiated Locomotor Activity

2.3.4.1. Intraperitoneal THIP Dose Response

To examine the role of accumbal α4βδ $GABA_A$Rs in cocaine-potentiation of locomotor activity, systemic injections of the δ-$GABA_A$R selective agonist THIP at various doses were paired with systemic injections of saline or cocaine (10mg/kg) in WT and $\alpha4^{-/-}$ mice.

When analysed using the total activity over 60 minutes, intraperitoneal injections of THIP dose-dependently decreased locomotor activity (Fig 2.6; significant main effect of dose, $F_{(3,76)}= 39.12$, $p < 0.001$). Decreased locomotor activity was found to be specific to mice in which THIP injections were paired with i.p. injections of cocaine, but not saline (significant THIP dose by drug interaction, $F_{(3,76)}= 37.05$, $p < 0.001$). There was also a difference between genotypes (significant THIP dose by drug by genotype interaction, $F_{(3,76)}= 3.27$, $p < 0.05$), but not between sexes (non-significant THIP dose by drug by genotype by sex interaction, $F_{(3,76)}= 0.28$, p = NS).

Low (5mg/kg) to medium (10mg/kg) doses of THIP injected *i.p.* with a paired *i.p.* injection of cocaine (10mg/kg), significantly decreased cocaine-enhancement of locomotor activity in WT, but not $\alpha4^{-/-}$ mice (Figure 2.6.). Nevertheless, a high (20mg/kg) dose of THIP decreased cocaine-enhanced locomotor activity equally for both genotypes. Investigation of the timecourse of activity over the 60 minute session revealed a genotype-specific time- and dose-dependent decrease in locomotor activity (significant THIP dose by genotype by time interaction, $F_{(44,1100)}= 5.25$, $p < 0.05$). Activity was greatest in the first 5 minutes, suggesting that this time period may demonstrate the clearest differentiation of THIP dose effects (Figure 2.7.A & C).

When analysed using the first 5 minutes of activity, intraperitoneal injections of THIP dose-dependently decreased locomotor activity in WT, but not $\alpha4^{-/-}$ mice, when paired with i.p. injections of saline or cocaine (Figure 2.7.B & D; data split by injection group, Saline group; significant THIP dose by genotype interaction, $F_{(3,42)}= 3.70$, $p < 0.01$, Cocaine group; significant THIP dose by genotype interaction, $F_{(3,42)}= 15.36$, $p < 0.001$)).

Figure 2.6.

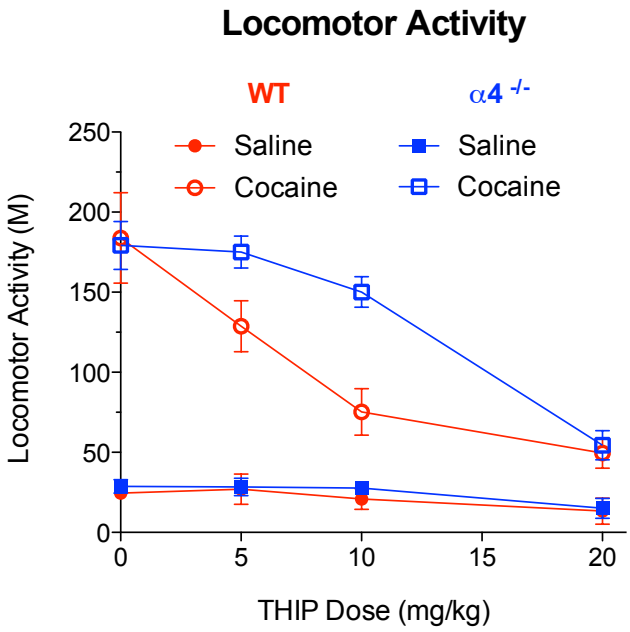


Fig. 2.6. Effects of intraperitoneal THIP on baseline and cocaine- (10mg/kg) potentiated distance travelled over 60 minutes. Intraperitoneal injections of THIP dose-dependently decreased cocaine-enhancement of locomotor activity in WT (n=8; males=4, females=4) but not $\alpha4^{-/-}$ (n=8; males=4, females=4) mice, at low (5mg/kg) to medium (10mg/kg) doses. Error bars represent SEM.

Figure 2.7.

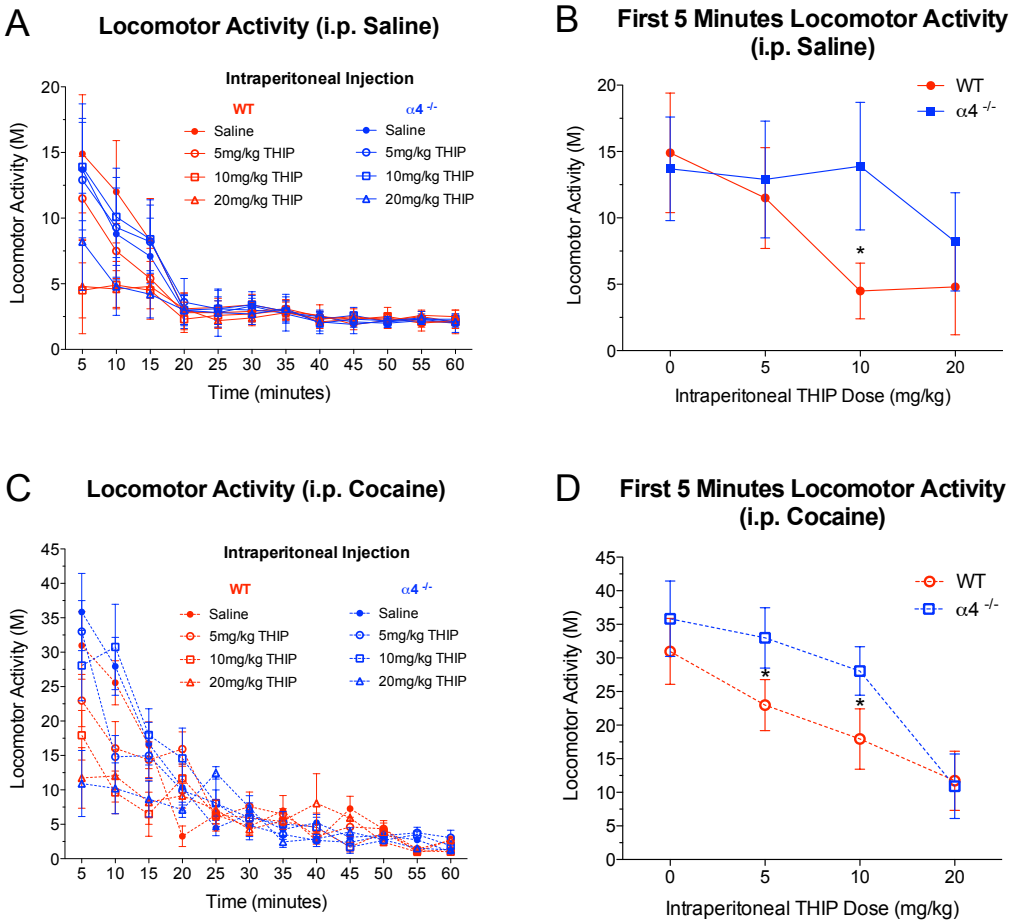


Fig. 2.7. (**A**) Timecourse of activity over 60 minutes following i.p. THIP and i.p. injections of saline. (**B**) Activity over the first five minutes following i.p. THIP and i.p. injections of saline. Locomotor activity was significantly reduced in WT but not $\alpha4^{-/-}$ mice at a dose of 10mg/kg THIP. (**C**) Timecourse of activity over 60 minutes following i.p. THIP and i.p. injections of cocaine (10mg/kg). (**D**) Activity over the first five minutes following i.p. THIP and i.p. injections of cocaine (10mg/kg). Cocaine-potentiated locomotor activity was significantly reduced in WT but not $\alpha4^{-/-}$ mice at THIP doses of 5 and 10mg/kg. Error bars represent SEM.* $p < 0.05$, *post hoc* comparison between genotypes.

2.3.4.2. Intra-accumbal THIP Dose Response

To investigate whether the attenuation of cocaine-potentiated locomotor activity seen with i.p. administration of low-to-medium THIP doses in WT mice was due to a specific action at accumbal α4βδ receptors, THIP at various doses was directly infused into the NAc of WT mice and paired with i.p. injections of saline or cocaine (Fig. 2.8.).

Investigation of the timecourse of activity did not reveal a time-dependent difference between the groups over the 60 minute session (Fig. 2.9.A & B; non-significant THIP dose by infusion by time interaction, $F_{(44,1100)}= 0.92$, p = NS). Therefore, the total activity over 60 minutes was analysed. Intra-accumbal THIP dose-dependently modulated locomotor activity (significant THIP dose by infusion interaction, $F_{(4,93)}= 6.35$, $p < 0.001$). Activity was altered only in mice in which THIP infusions were paired with i.p. injections of cocaine, but not saline (significant THIP dose by infusion by injection interaction, $F_{(1,93)}= 12.01$, $p < 0.001$). There was no difference between sexes (non-significant THIP dose by infusion by injection by sex interaction, $F_{(4,93)}= 0.63$, p = NS).

Doses of intra-accumbal THIP between 0.3μM to 300μM, paired with *i.p.* saline produced no effects on locomotor activity (Fig 2.8.) and did not significantly differ from the control condition (saline infusions with an *i.p.* saline injection), indicating that accumbal THIP at this dose is neither sedative, nor stimulant. However, a 3mM dose of intra-accumbal THIP, paired with *i.p* saline, produced a large increase in locomotor activity (Fig 2.8.). 3μM intra-accumbal THIP paired with i.p. cocaine showed reduced locomotor activity compared with intra-accumbal saline and i.p. cocaine-administered controls (Fig 2.8.). The two active doses, 3μM and 3mM, were followed up in WT and $\alpha4^{-/-}$ mice.

3μM intra-accumbal THIP had no significant effect on baseline locomotor activity, but reduced cocaine-potentiation of locomotor activity in WT but not $\alpha4^{-/-}$ mice (Fig 2.10.A; genotype by infusion by injection interaction, $F_{(1,20)}= 10.49$, $p < 0.001$). Whereas, 3mM intra-accumbal THIP had no significant effect on cocaine potentiated locomotor

activity, but increased baseline locomotor activity in WT but not $\alpha4^{-/-}$ mice (Fig 2.10.B; genotype by infusion by injection interaction, $F_{(1,24)}= 5.24$, $p < 0.01$).

Figure 2.8.

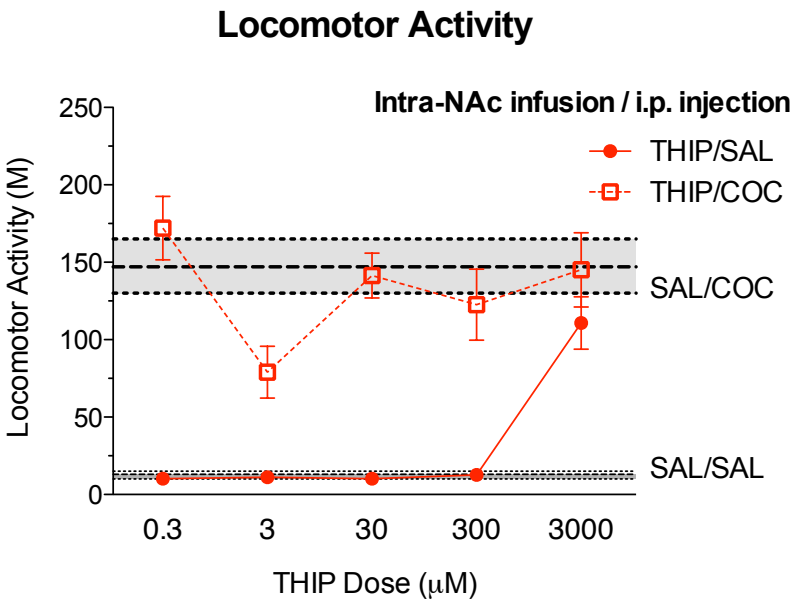


Fig. 2.8. Intra-accumbal THIP dose-response on baseline and cocaine (10mg/kg)-potentiated distance travelled over 60 minutes in WT mice (0.3μM n=14; males=5, females=9, 3μM n=11; males=5, females=6, 30μM n=12; males=6, females=6, 300μM n=11; males=4, females=7, 3000μM n=12; males=6, females=6). Mean locomotor activity of saline infused sessions (n=60; males=26, females=34, collapsed across doses as activity did not significantly vary between experiments) are shown as a dotted line, and the grey highlighting ±SEM. Error bars represent SEM.

Figure 2.9.

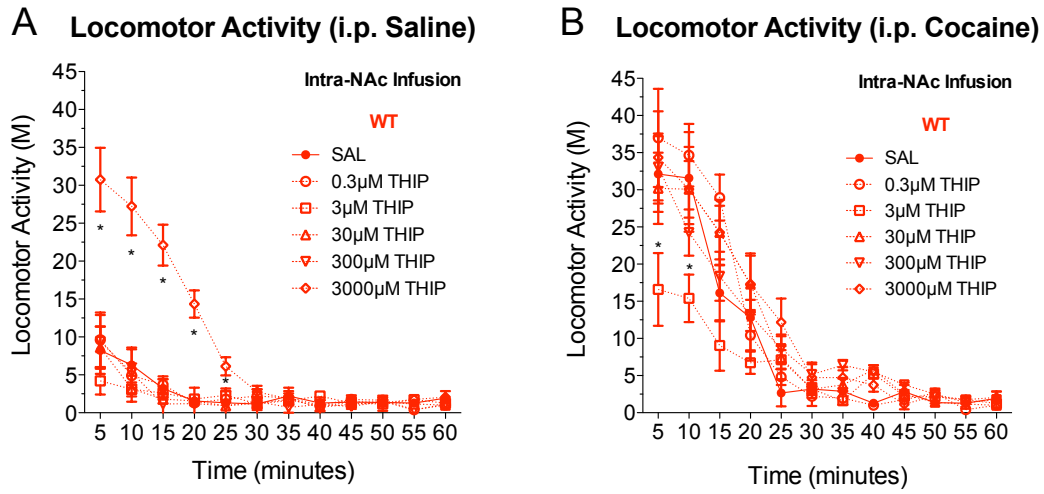


Fig. 2.9. (**A**) Timecourse of activity over 60 minutes following intra-accumbal THIP and i.p. injections of saline. (**B**) Timecourse of activity over 60 minutes following intra-accumbal THIP and i.p. injections of cocaine (10mg/kg). Cocaine-potentiated locomotor activity was significantly reduced at 5 and 10 minutes in mice receiving a THIP dose of 3μM. Error bars represent SEM. * $p < 0.05$, *post hoc* comparison.

Figure 2.10.

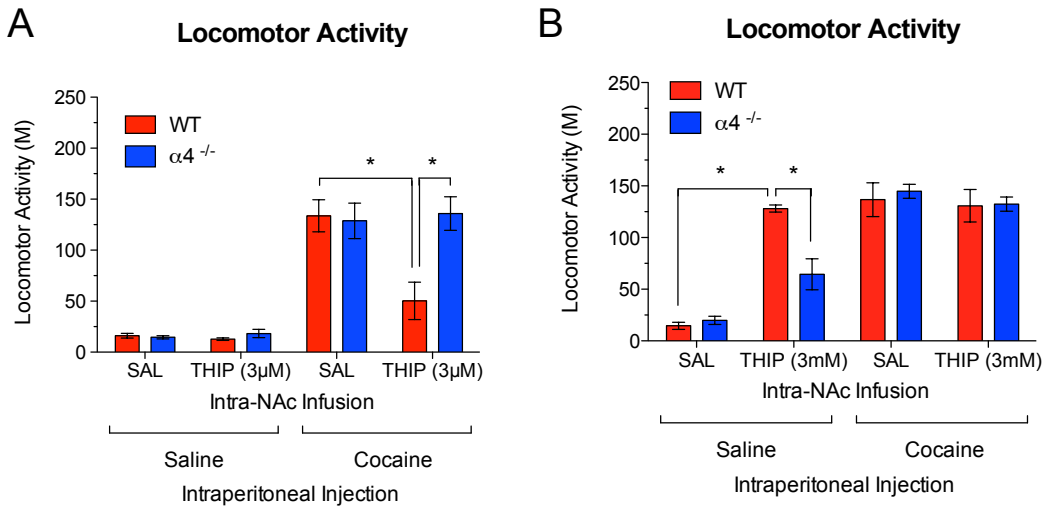


Fig. 2.10. (**A**) Effects of intra-accumbal THIP (3uM) on baseline and i.p. cocaine- (10mg/kg) potentiated distance travelled over 60 minutes. Infusion of THIP decreased cocaine-potentiated locomotor actvity in WT (n=11; males=8, females=3) but not $\alpha4^{-/-}$ (n=11; males=6, females=7) mice. (**B**) Effects of intra-accumbal THIP (3mM) on baseline and i.p. cocaine- (10mg/kg) potentiated distance travelled over 60 minutes. Infusion of THIP increased baseline locomotor activity greater in WT (n=13; males=7, females=6) than $\alpha4^{-/-}$ (n=13; males=6, females=7) mice. Error bars represent SEM. * $p < 0.01$, *post hoc* comparison.

2.4. Discussion

The present data indicate that pharmacological activation of $GABA_A$ α4βδ receptors within the NAc by low doses of THIP attenuates cocaine-potentiation of locomotor activity. Furthermore, activation by high doses of intra-accumbal THIP was able to increase baseline locomotor activity to similar levels as are seen with systemic administration of cocaine (10mg/kg).

Western blot and qRT-PCR analysis of NAc tissue samples confirmed the absence of $GABA_AR$ α4-subunit protein and mRNA expression in $\alpha4^{-/-}$ mice, and a reduction of approximately 50% in HET mice when compared to WT controls. These data confirm that the cre/loxp cleavage of the intended sequence produced a functional effect, blocking the ability of the Gabra4 gene to produce α4-subunit proteins.

The compensatory increase in $GABA_AR$ α2-subunit mRNA expression following deletion of α4-subunits may complicate the investigation of the role of α4-$GABA_ARs$ in mediating addiction-associated behaviours, given the pre-established role of α2-$GABA_ARs$ in mediating a number of cocaine's effects on mouse incentive-learning (Dixon et al., 2010). The Gabra4 and Gabra2 genes, encoding for α4- and α2-subunits, respectively, are positioned next to each other on Chromosome 5. Therefore, a mutation of the Gabra4 gene might be affecting the regulation of the Gabra2 gene, producing an artifactual, rather than a functional up-regulation of α2-subunits. Indeed, the extent to which this increased α2-subunit mRNA translates to functional receptors is disputed. Synaptic currents from $\alpha4^{-/-}$ mice are reported to be more sensitive to potentiation by the sedative-hypnotic agent zolpidem, requiring α1-/α2-, and inactive at α4-$GABA_ARs$, than those from WT mice (Suryanarayanan et al., 2011). However, recent electrophysiological evidence indicates that deletion of α4- or δ-subunits has no impact on the kinetics of the phase currents mediated by synaptic receptors within the NAc (Maguire et al, submitted). Moreover, expression of functional receptors requires the presence of other $GABA_AR$ subunits for the α2-subunits to co-assemble with. In the NAc, synaptic α2βγ2 $GABA_ARs$ are the most widely expressed $GABA_AR$ (Pirker et al., 2000). However, qRT-PCR analysis indicated that there was no change in the expression levels of γ2-subunits in the $\alpha4^{-/-}$ mice. Therefore, it is unlikely that the increase in α2-subunits in the NAc is translated into functional receptors.

In the current studies, deletion of $GABA_AR$ α4-subunits had no effect on baseline locomotor activity. This adds to previous data demonstrating deletion of $GABA_AR$ δ-subunits, often paired with α4-subunits in extrasynaptic α4βδ $GABA_ARs$, to similarly have no influence on baseline locomotion (Herd et al., 2009). Additionally, $GABA_AR$ α4-subunit knockout mice showed no difference from wildtype mice in the direct stimulatory effects of cocaine at various doses, suggesting that these receptors are not normally engaged as a consequence of increased synaptic dopamine.

When targeted pharmacologically using intraperitoneal injections of THIP at various doses, both wildtype and α4-subunit knockout mice did not differ significantly from saline-treated controls at any dose, when measured across a 60 minute session. It could be argued that since locomotor activity scores following systemic saline administration were already extremely low, it was not possible to decrease locomotion further than these baseline levels of spontaneous activity. However, this result is surprising given that previous evidence has repeatedly demonstrated systemic administration of THIP to dose-dependently attenuate locomotor activity (Agmo and Giordano, 1985; Vashchinkina et al., 2012). This decrease at low-to-medium (2-10mg/kg) doses of systemic THIP is absent in $GABA_AR$ δ-subunit knockout mice, suggesting a direct action of THIP at δ-containing extrasynaptic $GABA_ARs$ (Herd et al., 2009). The apparent inconsistency between the findings of previous studies and the current experiment may be explained by the timecourse over which activity was analysed. While previous studies similarly begin analysis between 15-30 minutes post-administration of THIP, activity was analysed over a total of 10 (Agmo and Giordano, 1985) or 20 (Herd et al., 2009) minutes. Indeed, when the current experiment was analysed using only the first 5 minutes of activity, i.p. injections of 10mg/kg THIP significantly reduced baseline locomotor activity in wildtype but not α4-subunit knockout mice (Fig. 2.7.B). Thus the effects of THIP likely decrease following the initial phase of the session and are diluted beyond significance when analysed over the course of 60 minutes beginning 20 minutes post-administration of THIP. The natural decrease in spontaneous activity observed within a 60 minute session may also reduce locomotor activity to a basal level of activity that it could not be further reduced.

A role for α4-$GABA_ARs$ in opposing cocaine was revealed by an intraperitoneal THIP-induced reduction of cocaine-potentiated locomotor activity in wildtype, but not α4-

subunit knockout mice. Systemic THIP reduced cocaine-induced stimulation of locomotor activity over a wide dose range, an effect absent in $\alpha4^{-/-}$ mice except at a high dose, suggesting that cocaine antagonism at low THIP doses is due to activation of α4βδ $GABA_A$Rs. At a high dose, THIP began to decrease cocaine-potentiated locomotor activity in α4-subunit knockout as well as wildtype mice, until both genotypes' activity fell to the levels associated with saline-treated controls. Decreased cocaine-potentiated activity may be attributable to THIP action at δ-containing $GABA_A$Rs coupled with subunits other than α4, such as α6βδ $GABA_A$Rs within the cerebellum (Farrant and Nusser, 2005). Alternatively, it is possible that at high doses THIP may be losing specificity for δ-containing $GABA_A$Rs and additionally activating other $GABA_A$Rs. Indeed, rotorod studies have similarly demonstrated that in the first 30 minutes following injection, i.p. THIP at 10mg/kg was ataxic in wildtype but not δ-subunit knockout mice, but at 30mg/kg was ataxic in both genotypes (Herd et al., 2009).

The decrease in locomotor activity following i.p. THIP is interesting given that α4-subunit knockout mice showed no significant difference to wildtype mice in their locomotor response to injections of saline, or cocaine at various doses. These data suggest that α4-$GABA_A$Rs are not important for the initiation of locomotion, but their activation is able to attenuate baseline and cocaine-potentiated locomotor activity.

This effect was found to be mediated within the NAc as intra-accumbal infusions of THIP at a low dose (3μM) similarly attenuated intraperitoneal cocaine-potentiated locomotor activity, indicating that when NAc α4βδ $GABA_A$Rs are engaged pharmacologically, they are able to functionally oppose cocaine's effects. Cocaine potentiates locomotor activity by prolonging the action of dopamine, released from VTA–NAc projections, on dopamine receptors located on the apical dendrites of the MSNs (Costall et al., 1984). Dopamine may impact upon the sensitivity of MSNs to neighbouring glutamatergic inputs from areas such as frontal cortex, amygdala and hippocampus, thereby influencing the ability of such excitatory drive to generate a signal in the MSN (O'Donnell and Grace, 1995). *In vivo,* MSNs have been suggested to exist in two main states; 1) a "downstate" where the membrane potential is hyperpolarised and the neuron is relatively unexcitable, and 2) an upstate, caused by a synchronous barrage of glutamatergic drive producing a prolonged depolarisation and

consequent action potential discharge. *In vivo* recordings of MSN activity, subjected to local antagonism of the MSN $GABA_A$Rs (by the intracellular $GABA_A$R antagonist picrotoxin), reveals the antagonist to reduce the proportion of silent MSNs, suggesting relief from a resident tonic inhibition (Calhoon & O'Donnell, 2013). When injected directly into the NAc, the predominant effect of THIP is probably postsynaptic upon the MSNs. Although MSNs *in vitro* already exhibit a relatively hyperpolarised resting membrane potential (Maguire et al, submitted), THIP activation of their dendritic and somatic δ-$GABA_A$Rs will further stabilise/promote this hyperpolarised state. This, together with the associated decrease in input resistance, will enhance neural inhibition, consequently reducing the impact of glutamatergic excitation on both dendrites and cell soma. Therefore, *in vivo* intra-accumbal THIP is likely to be promoting a downstate of the MSNs, thereby decreasing the ability of cocaine-enhanced synaptic dopamine to facilitate glutamatergic inputs and to potentiate locomotor activity

Interestingly, high dose (3mM) intra-accumbal THIP was able to potentiate baseline locomotor activity in wildtype, and to a lesser extent in α4-subunit knockout mice, unmasking an additional locomotor-activating role of α4-$GABA_A$Rs. Thus it appears that the sedative effect seen with high doses of intraperitoneal THIP are mediated outside of the accumbens, most likely by activation of extrasynaptic $GABA_A$Rs within the thalamus (Belelli et al., 2005; Wafford and Ebert, 2006). The coincident but significantly smaller locomotor increase in α4-subunit knockout mice at intra-accumbal 3mM THIP suggests that at this dose THIP is also acting at $GABA_A$Rs independent of those containing α4-subunits. It is possible that these may be δ-containing $GABA_A$Rs comprising α-subunits other than α4, such as the α1βδ and α6βδ $GABA_A$Rs reported in hippocampal and neocortical interneurons, or cerebellar granule cells respectively (Brickley and Mody, 2012). However, as yet, there is little evidence to support the existence of such receptors in the NAc (Belelli et al., 2009; Brickley and Mody, 2012). Further investigation will be needed to elucidate which receptors may be contributing to high-dose THIP-induced hyperlocomotion.

Interestingly, in addition to the α4-independent increase in locomotion, wildtype mice also show an α4-dependent increase, demonstrated by a locomotor response two-fold higher than the α4-subunit knockout mice. The reason for this α4 $GABA_A$R mediated increase is similarly still unclear, but a possible explanation may be that at particular

doses intra-accumbal THIP may differentially modulate the activity of dopamine D1- or D2-expressing MSNs through α4-$GABA_AR$-mediated inhibition. It could be speculated that high-dose THIP may have a greater inhibitory response at α4-$GABA_ARs$ on dopamine D2-expressing MSNs, known to contribute to the *indirect* striatal pathway, which when activated attenuates locomotion. This would shift the balance of activity in favour of dopamine D1-expressing MSNs in the *direct* pathway, increasing locomotor activity. However, while most evidence supports the role of D1 receptors in stimulating locomotor activity (Bruhwyler et al., 1991; Mazurski and Beninger, 1991), evidence for a D2 receptor-mediated attenuation of locomotion is inconclusive (Gong et al., 1999; Stuchlik et al., 2007), and may be dose- and time-dependent (Horvitz et al., 2001; Schindler and Carmona, 2002).

To conclude, deletion of $GABA_AR$ α4-subunits had no affect on baseline or acute cocaine-potentiated locomotor activity. However, activation of α4βδ-$GABA_ARs$ using systemic or intra-accumbal THIP was able to reduce cocaine-potentiated locomotor activity. These data indicate that α4-$GABA_ARs$ within the NAc provide an efficacious target for control of the locomotor-activating properties of cocaine.

Chapter 3

The role of α4-containing $GABA_A$ receptors in behavioural sensitisation to cocaine

3.1. Introduction

Behavioural sensitisation describes the enhanced stimulant effects of a drug following repeated, intermittent administration (Tilson and Rech, 1973; Segal and Mandell, 1974; Browne and Segal, 1977; Bailey and Jackson, 1978; Hirabayashi and Alam, 1981; Leith and Kuczenski, 1982; Robinson and Becker, 1986). By analogy, the sensitisation phenomenon has also been proposed for incentive mechanism, and in this context is thought to play a role in the acquisition and maintenance of behaviours that are characteristic of drug addiction, including craving and relapse (Robinson and Berridge, 1993). The enhanced behavioural response has been found to be enduring, persisting up to a year after the final drug exposure, and possibly longer (Paulson et al., 1991; Boileau et al., 2006). It is also known that expression of behavioural sensitisation is enhanced by the learned association of the drug administration with the environmental context (Anagnostaras and Robinson, 1996; Badiani and Robinson, 2004; Crombag and Robinson, 2004). Behavioural sensitisation has been reported following repeated administration of a variety of different drugs, including the psychostimulants; amphetamine (Robinson and Becker, 1986; Cador et al., 1999), cocaine (Post et al., 1987; Kalivas and Stewart, 1991) and methylphenidate (Shuster et al., 1982; McDougall et al., 1999), as well as opiates (Babbini and Davis, 1972; Shuster et al., 1975), nicotine (Benwell and Balfour, 1992; Kita et al., 1992), ethanol (Cunningham and Noble, 1992; Phillips et al., 1997) and natural food rewards (Le Merrer and Stephens, 2006). Given the evidence of cross-sensitisation between drugs of abuse, it has been proposed that these effects may be mediated by common neural mechanisms (Akimoto et al., 1990; Kalivas and Stewart, 1991; Cunningham and Kelley, 1992; Horger et al., 1992). Alternatively, such cross sensitisation (e.g. between food sensitisation and cocaine) may reflect the ability of psychostimulants to facilitate locomotor activation caused by environmental stimuli conditioned to reward (Le Merrer and Stephens, 2006).

Studies exploring the neurobiological basis of cocaine-induced behavioural sensitisation have largely focussed on the mesolimbic dopamine system due to the established role of

this system in mediating the locomotor activating properties of cocaine (Robinson and Berridge, 1993; Pierce and Kalivas, 1997). Most evidence indicates that neural processes underlying the induction and expression of cocaine sensitisation may be anatomically distinct. Dopamine neurons within the VTA have been described to play a critical role in the induction of sensitisation to cocaine and other psychostimulants, while the NAc neurons to which they project are proposed to play a primary role in expression of sensitisation (Robinson et al., 1988; Kalivas and Stewart, 1991; Le Moal and Simon, 1991; Pierce and Kalivas, 1997; White and Kalivas, 1998). Repeated intra-VTA, but not intra-accumbal, injections of psychostimulants potentiate the locomotor stimulant effects of a systemic or intra-accumbal challenge of the same or other such psychostimulants demonstrating the expression of sensitisation (Dougherty and Ellinwood, 1981; DuMars et al., 1988; Kalivas and Weber, 1988; Hooks et al., 1992; Cornish and Kalivas, 2001). During induction of cocaine sensitisation, repeated intermittent cocaine administration is associated with an elevated basal extracellular level of dopamine within the NAc (Di Chiara and Imperato, 1988; Kalivas and Duffy, 1990; Kalivas and Stewart, 1991). This increased basal concentration of dopamine has been found to gradually decline to the level of saline-treated controls following cessation of cocaine treatment (Segal and Kuczenski, 1992; Weiss et al., 1992; Heidbreder et al., 1996; Johnson et al., 2000). Interestingly, after extended periods of withdrawal, expression of behavioural sensitisation is associated with increased dopamine transmission and a sensitised locomotor response higher than that found immediately following cessation (Weiss et al., 1992; King et al., 1993; Heidbreder et al., 1996). Thus, activation of VTA neurons, and the resulting increase in basal NAc dopamine levels, appears to be a critical mechanism for the induction of sensitisation to psychostimulants. Yet, despite the principle contribution of dopamine effects, it is possible they may represent only one aspect in a complex spectrum of changes mediating behavioural sensitisation.

GABAergic MSNs are the major neuronal type within the striatum where GABA serves as a major modulator of nigrostriatal dopamine transmission (Scheel-Krüger, 1986; Gerfen, 1992; Pierce and Kalivas, 1997). It is thought that repeated exposure to cocaine induces changes in GABA systems, resulting in a dysregulation of the neural circuitry mediating behaviour responses to drugs (Koob, 2001). Indeed, whilst GABA binding is unaffected (Jung and Peris, 2001), a decrease in pre- and post-synaptic GABA

transmission (Jung et al., 1999), and function of $GABA_A$Rs is reported in the striatum of cocaine-sensitised rats (Peris, 1996). Conversely, following withdrawal from cocaine sensitisation or repeated cocaine administration in mice, cocaine challenges increase GABA transmission in the medial-PFC (Jayaram and Steketee, 2005) and NAc (Xi et al., 2003). However, the role of GABA in mediating behavioural sensitisation to psychostimulants is complex and is still largely undetermined. Both systemic administration of clonazepam, a benzodiazepine increasing GABAergic transmission (Ito et al., 1997), and systemic and intra-striatal administration of bicuculline, a $GABA_A$R antagonist, block the induction of behavioural sensitisation to amphetamine (Bedingfield et al., 1997). However, it is possible that these observations are artifactual, as while repeated co-administration of the benzodiazepine chlordiazepoxide (CDP) with amphetamine does not block locomotor sensitisation (Stephens et al., 2000), the expression of the sensitized response to amphetamine is seen only when co-administered with CDP, and, in agreement with the previous studies, is absent if amphetamine is given alone, i.e., is state-dependent. GABA-mimetic drugs, which do not bind directly or indirectly at $GABA_A$Rs, but increase GABA transmission through different mechanisms, are also able to modulate behavioural sensitisation to cocaine. Vigabatrin, an irreversible inhibitor of GABA breakdown by GABA transaminase, attenuates the expression of behavioural sensitisation to cocaine, as does gabapentin, a cyclic analogue of GABA that either directly stimulates GABA release or indirectly increases GABA synthesis (Gardner et al., 2002; Filip et al., 2006).

The $GABA_A$R α2-subunit is the predominant alpha subunit within the NAc and thus is likely to play an important role in mediating behavioural responses to cocaine (Schwarzer et al., 2001). Indeed, the Gabra2 gene encoding $GABA_A$R α2-subunits has consistently been linked with drug addiction (Edenberg et al., 2004; Agrawal et al., 2006; Soyka et al., 2008), and different single nucleotide polymorphisms of Gabra2 have been shown to confer vulnerability or protection for cocaine dependence (Dixon et al., 2010; Enoch et al., 2010). Following amphetamine-sensitisation a decrease in $GABA_A$R α2-subunits is reported within the NAc shell and core (Zhang et al., 2006), and reversal of cocaine-sensitisation by combined treatment with the dopamine receptor agonist pergolide and 5-HT_3 receptor antagonist ondansetron is accompanied by normalised NAc $GABA_A$R α2-subunit expression (Chen et al., 2007). Furthermore, targeted deletion of the $GABA_A$R α2-subunit blocks the ability of cocaine to induce

behavioural sensitisation (Dixon et al., 2010), an effect subsequently demonstrated to be mediated within the NAc. Selective activation of α2-containing $GABA_A$Rs within the NAc using intracranial infusions of the atypical benzodiazepine Ro 15-4513, were sufficient to induce behavioural sensitisation in α2(H101R) mutant mice (Dixon et al., 2010). Interestingly, in opposition to previous reports (Bedingfield et al., 1997), benzodiazepine administration was able to facilitate the locomotor activating properties of cocaine, an effect absent in α2(H101R) mice (Morris et al., 2008). These data indicate that $GABA_A$R α2-subunit mediation of cocaine-potentiated locomotor activity occurs downstream of increased NAc dopamine release.

A role of $GABA_A$R α4 subunits in mediating behavioural sensitisation to cocaine has also been suggested. Systemic administration of THIP blocks both the induction and expression of behavioural sensitisation to amphetamine (Karler et al., 1997), though the state-dependency notion has not been tested. Similarly, a single dose of isoflurane, an anaesthetic agent acting as a potent modulator of α4βδ extrasynaptic $GABA_A$Rs, is sufficient to attenuate expression of cocaine-induced behavioural sensitisation in female rats (Jia et al., 2007; Siegal and Dow-Edwards, 2009). In order to further probe the role of $GABA_A$R α4 subunits in mediating behavioural sensitisation to cocaine, the effects of targeted deletion of $GABA_A$R α4 subunits in a mutant mouse strain and pharmacological activation using THIP were examined.

3.2. Materials and Methods

3.2.1. Animals

Male and female $GABA_A$ α4 WT and $α4^{-/-}$ mice on a C57BL/6J background strain, weighing between 20-30g and aged between 2-6 months, were housed in groups of 2-3, with food and water available *ad libitum.* A 12 hr light/dark cycle was used (lights on at 7:00 A.M.) with holding room temperature maintained at 21 ±2ºC and humidity 50 ±5%. All injections and behavioural testing were performed between 2:00 P.M. and 5:00 P.M. All procedures were conducted in accordance to Animals (Scientific Procedures) Act 1986, following ethical review by the University of Sussex Ethical Review Committee.

3.2.2. Drugs

Cocaine Hydrochloride was obtained from Macfarlan Smith (Edinburgh, UK). THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) was kindly donated by Bjarke Ebert (Lundbeck, Valby, Denmark). Both drugs were dissolved in 0.9% saline, and administered i.p. at an injection volume of 10 ml/kg.

3.2.3. Apparatus

Behavioural sensitisation was measured by recording locomotor activity in 16 annular black Perspex runways, (diameter 24cm, annula width 6.5cm), placed atop a clouded Perspex sheet on an elevated frame. A digital camera positioned beneath the sheet captured the silhouettes of the boxes' edges and the mice within them, which was then relayed to a computer to be recorded. A MatLab (MathWorks, Cambridge, UK) video analysis programme and Excel macro converted the video data into a measure of the distance travelled in metres.

Locomotor activity in a novel environment was measured in a rectangular chamber (200 x 450 x 200cm). Five infrared photobeams spaced evenly across the length of the box recorded the activity and location of the mouse and relayed the information to a PC running data collection and analysis software.

3.2.4. Dose Response for Behavioural Sensitisation to Cocaine

Before all sessions mice were allowed to habituate to the equipment for 30 minutes before being returned to their homecage. After 5 minutes, mice were dosed if necessary and entered into the appropriate session. Prior to testing, mice underwent two habituation sessions. On the first day mice were habituated to the equipment for 60 minutes, then, on the second, mice received sham i.p. injections of saline followed by a 60-minute habituation session. Subsequently, mice received repeated, intermittent treatment of : 3, 10 or 20mg/kg cocaine or saline for 10 consecutive daily sessions. Activity was recorded for 60 minutes each session.

3.2.4.1. Conditioned Activity

Mice were placed in the locomotor runways as described above, with all animals receiving 10ml/kg saline injections. Activity was recorded for 60 minutes.

3.2.4.2. Activity in a Novel Environment

To investigate whether any conditioned activity is specific to the context of the locomotor runways, locomotor activity in a novel environment was tested by measurement in rectangular locomotor boxes. All mice were administered 10ml/kg saline injections and activity was recorded for 60 minutes.

3.2.5. Behavioural Sensitisation to Cocaine with Intraperitoneal THIP Challenge

Mice were tested using the same protocol as above with the cocaine group receiving injections of 10mg/kg cocaine. The day after the last session, mice received a challenge i.p. injection of THIP (8mg/kg) 20 minutes prior to start, followed by a second i.p. injection of saline or cocaine (10mg/kg), directly prior to initiation of the locomotor test. Activity was recorded for 60 minutes each session.

3.2.6. Statistical Analysis

3.2.6.1. Dose Response for Behavioural Sensitisation to Cocaine

Dose response for behavioural sensitisation to cocaine was analysed using a four-way mixed-factors ANOVA with genotype, sex, and drug dose as the between-subjects variables, day as the within-subject variable, and meters travelled in each session as the dependent variable. Following this treatment, behavioural sensitisation to cocaine was

confirmed using a four-way mixed-factors ANOVA with genotype, sex, and drug dose as the between-subjects variables, day as the within-subject variable, and meters travelled in sessions 1 and 10 as the dependent variable. *Post hoc* analyses were conducted using paired t-tests and Tukey's tests.

In order to evaluate whether any differences were present in baseline locomotor behaviour before the test sessions, a four-way mixed-factors ANOVA was conducted with genotype, sex, and drug dose as the between-subjects variables, day as the within-subject variable, and meters travelled during each habituation session as the dependent variable. Baseline locomotor behaviour before the test sessions was further investigated using another four-way mixed-factors ANOVA with genotype, sex, and drug dose as the between-subjects variables, day as the within-subject variable, and meters travelled during the session 1 habituation and session 10 habituation as the dependent variable.

3.2.6.2. Conditioned Activity

Conditioned activity following behavioural sensitisation to cocaine was analysed using a three-way independent-factors ANOVA, with genotype, sex and drug dose as the independent variables and meters travelled in the locomotor runways following a saline injection as the dependent variable. *Post hoc* analyses were conducted using paired t-tests.

3.2.6.3. Activity in a Novel Environment

Activity in a novel environment following behavioural sensitisation to cocaine was analysed using a three-way independent-factors ANOVA, with genotype, sex and drug dose as the independent variables and locomotor activity in the rectangular locomotor boxes following a saline injection as the dependent variable.

3.2.6.4. Behavioural Sensitisation to Cocaine with Intraperitoneal THIP Challenge

Behavioural sensitisation to cocaine was analysed using a four-way mixed-factors ANOVA with genotype, sex, and drug treatment as the between-subjects factors, day as the within-subject variable, and meters travelled in sessions 1 and 10 as the dependent variable. Following this, a four-way mixed-factors ANOVA was conducted using genotype, sex and drug treatment as the between-subjects variables, day as the within-

subject variable, and meters travelled in the last session and THIP challenge session as the dependent variables. *Post hoc* analyses were conducted using paired t-tests.

3.3. Results

3.3.1. Dose Response for Behavioural Sensitisation to Cocaine

Repeated, intermittent injections of cocaine induced an increase in locomotor activity over the course of 10 sessions (Fig. 3.1.A; significant main effect of session, $F_{(9,432)}$ =20.05, $p < 0.001$). This increase was dose-dependent and did not occur following repeated, intermittent saline treatment (significant session by drug dose interaction, $F_{(27,432)} = 8.21$, $p < 0.001$). There was no significant difference between genotypes or sexes (non significant session by drug dose by genotype interaction, $F_{(27,432)} = 0.81$, $p = 0.72$, NS; non significant session by drug dose by sex interaction, $F_{(27,432)} = 0.69$, $p = 0.81$, NS; non significant session by drug dose by genotype by sex interaction, $F_{(27,432)} = 1.21$, $p = 0.21$, NS).

Comparison of the difference between session 1 and session 10 confirmed that the behavioural sensitisation to cocaine was similar in both genotypes and sexes (Fig. 3.1.B; significant main effect of session, $F_{(1,48)} = 81.6$, $p < 0.001$, non significant session by drug dose by genotype interaction, $F_{(3,48)} = 1.43$, $p = 0.24$, NS; non significant session by drug dose by sex interaction, $F_{(3,48)} = 0.65$, $p = 0.58$, NS; non significant session by drug dose by genotype by sex interaction, $F_{(3,48)} = 1.26$, $p = 0.29$, NS).

During the course of 10 sessions mice habituated to the runways, as indicated by a decrease in locomotor activity during the 30 min pre-test habituation sessions (Fig. 3.2.A; significant main effect of session, $F_{(9,432)} = 11.34$, $p < 0.01$). This was found to be equivalent in all drug doses, genotypes and sexes (non-significant session by drug dose interaction, $F_{(27,432)} = 0.23$, $p = 0.71$, NS; non-significant session by genotype interaction, $F_{(27,432)} = 0.55$, $p = 0.47$, NS; non-significant session by sex interaction, $F_{(9,432)} = 1.22$, $p = 0.23$, NS; non-significant session by drug dose by genotype by sex interaction, $F_{(27,432)} = 1.05$, $p = 0.33$, NS). This was subsequently confirmed by analysis of habituation sessions 1 and 10 (Fig. 3.2.B; significant main effect of session, $F_{(1,48)} = 64.01$, $p < 0.001$; non-significant session by drug dose interaction, $F_{(3,48)} = 0.72$, $p = 0.49$, NS; non-significant session by genotype interaction, $F_{(1,48)} = 0.63$, $p = 0.55$, NS;

non-significant session by sex interaction, $F_{(1,48)} = 1.66$, $p = 0.20$, NS; non-significant session by drug dose by genotype by sex interaction, $F_{(3,48)} = 1.30$, $p = 0.25$, NS).

Figure 3.1.

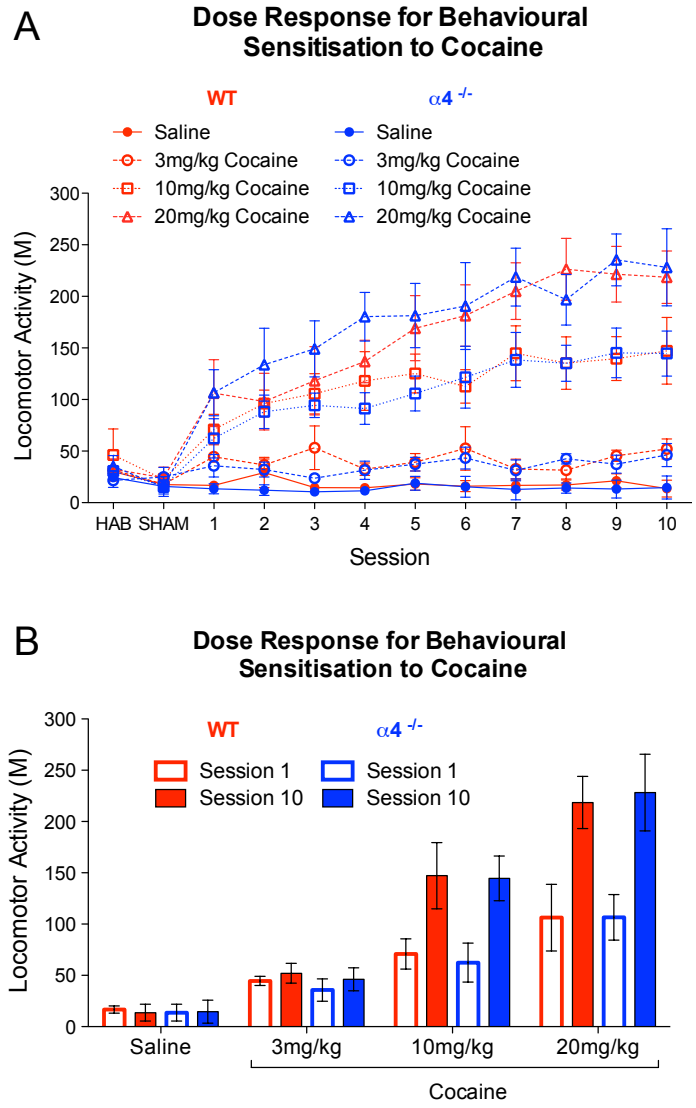


Fig. 3.1. Effects of repeated intermittent cocaine (3, 10 and 20 mg/kg) on locomotor activity in wildtype (saline n=8; males=5, females=3, cocaine 3mg/kg n=8; males=5, females=3, cocaine 10mg/kg n=8; males=4, females=4, cocaine 20mg/kg n=8; males=4, females=4) and $GABA_AR$ α4-subunit knockout (saline n=8; males=5, females=3, cocaine 3mg/kg n=8; males=4, females=4, cocaine 10mg/kg n=8; males=4, females=4, cocaine 20mg/kg n=8; males=5, females=3) mice. (**A**) Locomotor activity was dose-dependent and increased over the course of 10 sessions of repeated, intermittent cocaine administration ($p < 0.001$), equally in both genotypes ($p = 0.72$, NS) and sexes ($p = 0.81$, NS). (**B**) Activity was dose-dependently higher on session 10 compared to session 1 after repeated cocaine administration ($p < 0.001$), independent of genotype ($p = 0.47$, NS) or sex ($p = 0.58$, NS). Error bars represent SEM.

Figure 3.2.

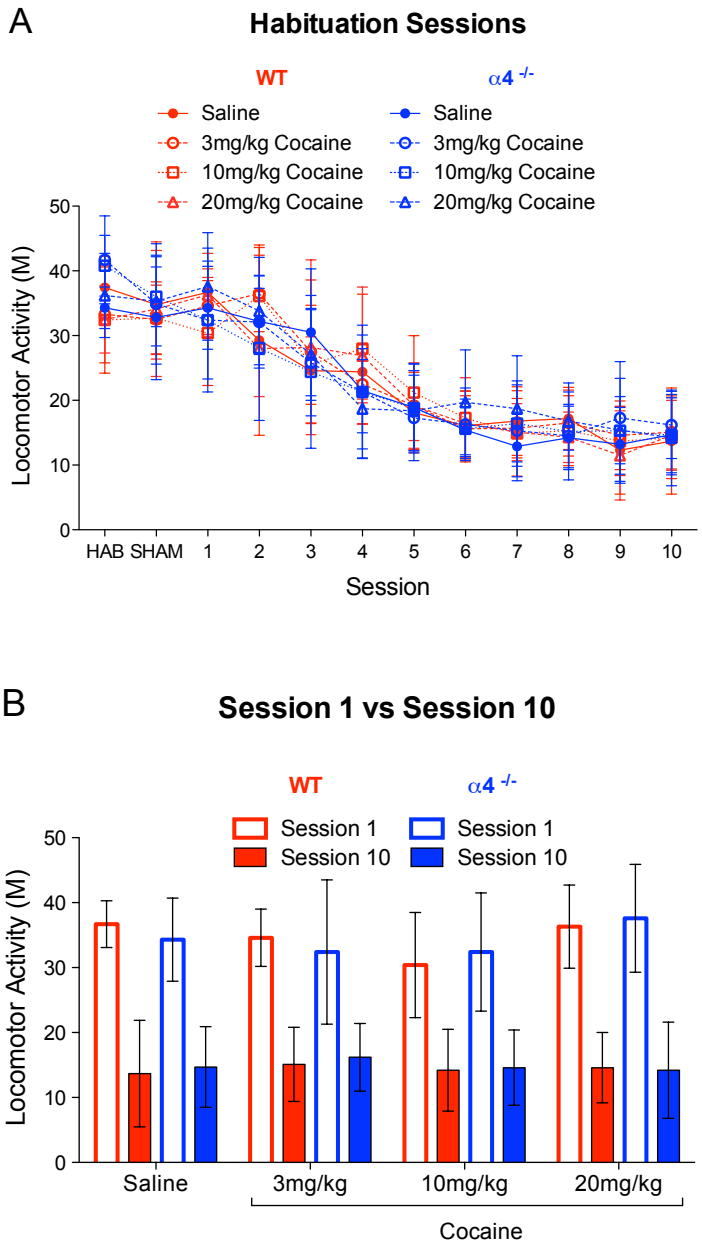


Fig. 3.2. Locomotor activity during habituation sessions prior to each test session (**A**) Activity did not differ according to drug dose ($p = 0.71$, NS), genotype ($p = 0.47$, NS) or sex ($p = 0.23$, NS). (**B**) Comparison of habituation sessions 1 and 10 indicates no effect of drug dose ($p = 0.49$, NS), genotype ($p = 0.55$, NS) or sex ($p = 0.20$, NS). Error bars represent SEM.

3.3.1.1. Conditioned Activity

Following 10 days of repeated saline or cocaine at various doses, mice showed dose-dependent conditioned increases in activity following a saline injection (Fig. 3.3.A; significant main effect of previous drug dose, $F_{(3,48)} = 6.41$, $p < 0.001$). Conditioned activity did not differ according to genotype or sex (non-significant previous drug dose by genotype interaction $F_{(3,48)} = 0.52$, $p = 0.66$, NS; non-significant previous drug dose by sex interaction $F_{(3,48)} = 0.67$, $p = 0.41$, NS; non-significant previous drug dose by genotype by sex interaction $F_{(3,48)} = 0.60$, $p = 0.61$, NS).

Figure 3.3.

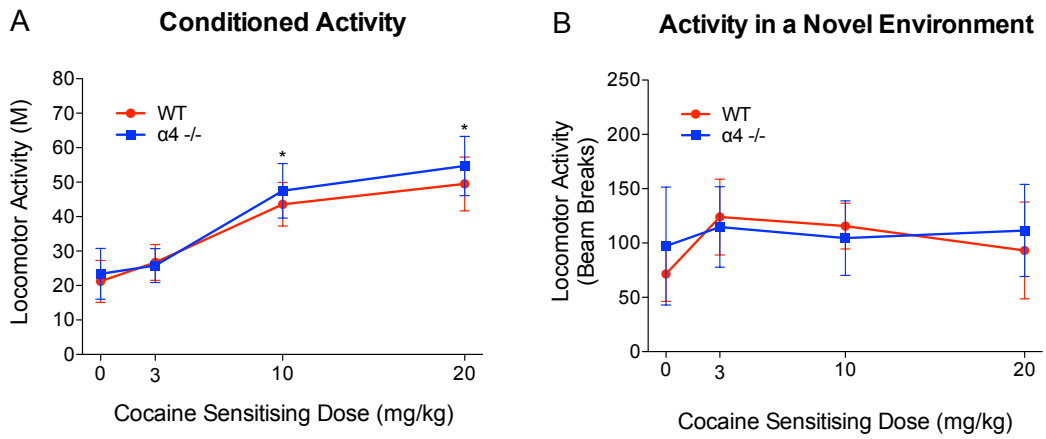


Fig. 3.3. Conditioned Activity (**A**) Conditioned activity following saline administration in the previously saline- or cocaine-paired locomotor runway was significantly increased in mice that had previously received repeated intermittent administration of 10 or 20mg/kg cocaine ($p < 0.001$). There was no difference between genotypes ($p = 0.66$, NS) or sexes ($p = 0.41$, NS) (**B**) Activity in a novel environment following saline administration did not differ with previous drug administration ($p = 0.36$, NS), genotype ($p = 0.15$, NS) or sex ($p = 0.34$, NS). Error bars represent SEM. * $p < 0.01$, *post hoc* comparison.

3.3.1.2. Activity in a Novel Environment

Saline administration within a novel environment following behavioural sensitisation to cocaine did not differ according to previous drug administration, genotype or sex (Fig. 3.3.B; non-significant main effect of drug group, $F_{(3,48)}= 1.08$, $p = 0.36$, NS; non-significant drug group by genotype interaction, $F_{(3,48)}= 1.83$, $p = 0.15$, NS; non-significant drug group by sex interaction, $F_{(3,48)}= 1.13$, $p = 0.34$, NS; non-significant drug group by genotype by sex interaction, $F_{(3,48)}= 0.32$, $p = 0.80$, NS).

3.3.2. Behavioural Sensitisation to Cocaine with Intraperitoneal THIP Challenge

As previously, repeated, intermittent injections of cocaine (10mg/kg), but not saline, induced an increase in locomotor activity over the course of 10 sessions, irrespective of genotype and sex (significant session by drug interaction, $F_{(9,216)} = 5.78$, $p < 0.001$; non significant session by drug by genotype by sex interaction, $F_{(9,216)} = 0.67$, $p = 0.73$, NS).

Following a challenge systemic injection of THIP (8mg/kg) locomotor activity was significantly reduced in wildtype, but not $GABA_AR$ α4-subunit knockout mice, paired with cocaine, but not saline administration, when compared to the sensitised activity on session 10 (Fig. 3.4; significant challenge by drug by genotype interaction, $F_{(1,24)} = 6.86$, $p < 0.01$). There were no difference between sexes (non significant challenge by drug by genotype by sex interaction, $F_{(1,24)} = 0.01$, $p = 0.90$, NS).

Figure 3.4.

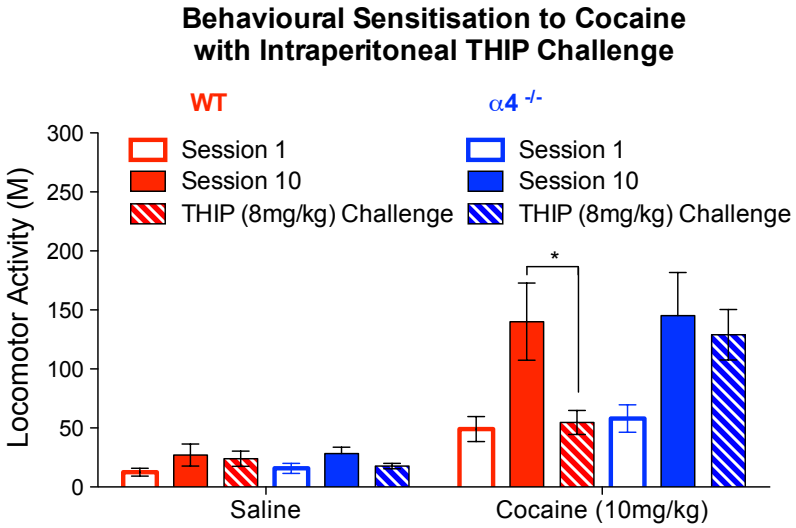


Fig. 3.4. Effects of an intraperitoneal THIP (8mg/kg) challenge on behavioural sensitisation to cocaine (10mg/kg) in WT (saline; n=8; males=5, females=3, cocaine; n=8; males=4, females=4) and $\alpha4^{-/-}$ (saline; n=8; males=4, females=4, cocaine; n=8; males=4, females=4) mice. THIP significantly reduced sensitised locomotor activity in cocaine-, but not saline-paired, wildtype, but not $GABA_AR$ α4-subunit knockout mice ($p < 0.001$). Error bars represent SEM. * $p < 0.01$, *post hoc* comparison.

3.4. Discussion

The current experiments suggest that $GABA_A$ α4βδ receptors are not involved in the induction of behavioural sensitisation to cocaine. Nevertheless, their activation by THIP was able to attenuate sensitised locomotor activity.

In the previous chapter it was demonstrated that $GABA_AR$ α4-subunit knockout mice do not show a significant difference from their wildtype counterparts in their locomotor response to acute cocaine at various doses. Here, these findings were extended to reveal that repeated intermittent cocaine was able to dose-dependently increase locomotor activity equally in both wildtype and $GABA_AR$ α4-subunit knockout mice across the course of 10 sessions, demonstrating locomotor sensitisation. Sensitisation was further confirmed in wildtype and $GABA_AR$ α4-subunit knockout mice by a test of conditioned activity in which mice previously treated with 10 or 20, but not 3mg/kg cocaine showed an increased locomotor response to saline administration compared to those previously receiving repeated saline, when tested in the same environment. However, when a saline challenge was given in a novel environment there was no effect of either genotype or previous drug-treatment, indicating the importance of conditioned environmental cues in the expression of locomotor sensitisation to cocaine. These data also suggest that α4-$GABA_ARs$ are functionally distinct from α2-$GABA_ARs$, which have been reported to be essential for the induction of locomotor sensitisation to cocaine (Morris et al., 2008; Dixon et al., 2010).

The distinct roles of $GABA_AR$ α2- and α4-subunits are not surprising given the difference in location and physiology between receptors comprising these two subunits. In the NAc, $GABA_AR$ α2-subunits are typically found in α2βγ2 $GABA_ARs$ within synapses where they mediate "fast" neuronal inhibition of MSNs, while $GABA_AR$ α4-subunits largely form α4βδ extrasynaptic $GABA_ARs$, which mediate a tonic form of inhibition via their sensitivity to ambient GABA spillover from synapses (Pirker et al., 2000; Wei et al., 2003) It is still unclear how these features of α2-$GABA_ARs$ mediate locomotor sensitisation to cocaine, but it has been hypothesised that their influence may occur downstream of NAc dopaminergic mechanisms; one possibility is that they form the target of MSN axon collaterals that allow lateral inhibition between MSNs competing for behavioural control (Dixon et al., 2010).

Stimuli conditioned to cocaine administration during the sensitisation procedure may control which NAc neurons are subsequently activated by cocaine (Mattson et al., 2008). Information about these stimuli is received within the NAc via glutamatergic afferents from the amygdala, hippocampus and PFC, activating specific populations of MSNs (Pennartz et al., 1994; French and Totterdell, 2003; O'Donnell, 2003). Indeed, histochemical and electrophysiological investigation suggests that a small neuronal ensemble of sparsely distributed NAc MSNs are selectively activated by drug administration in the drug-paired context (Mattson et al., 2008), and that their inactivation can prevent context-specific sensitisation (Koya et al., 2009). However, given the multitude of competing motivational inputs onto NAc MSNs from environmental stimuli, there must exist a method to enhance the activity of selected neurons, while suppressing the activity of surrounding non-selected neurons. One hypothesis is that $GABA_A$Rs may mediate the activity of distributed networks within the NAc by inhibition of neighboring MSNs, allowing the reinforcement of selected un-inhibited ensembles by NAc dopamine transmission (Plenz, 2003; Taverna et al., 2004; Nicola, 2006). The organization of the NAc into networks of MSNs interconnected by GABAergic synapses on axon collaterals is perfectly suited for this lateral inhibition (Czubayko and Plenz, 2002; Taverna et al., 2004). It has been hypothesized that activation of α2-$GABA_A$Rs within the NAc may contribute to this lateral inhibition (Dixon et al., 2010). Thus, in $GABA_A$R α2-subunit knockout mice, the lack of inhibitory control over competing motivational inputs into the NAc may result in the inputs important for producing a sensitised response being overruled. This would explain the lack of behavioural sensitisation to cocaine in these mice (Dixon et al., 2010). However, deletion of $GABA_A$R α4-subunits did not affect the expression of behavioural sensitisation to cocaine, indicating that in this model they are likely not to be involved in mediating GABAergic lateral inhibition of NAc MSNs.

It is conceivable that the lack of effects seen following deletion of $GABA_A$Rs α4-subunits may be the result of compensatory neuronal mechanisms, including a compensatory increase in $GABA_A$R α2-subunits. As described in chapter 2 (Fig 2.3.2.), qRT-PCR analysis revealed a large increase in the expression of α2-subunit RNA within the NAc of $GABA_A$Rs α4-subunit knockout mice compared with wildtype counterparts. Given the above-mentioned role of $GABA_A$Rs α2-subunits in mediating locomotor

sensitisation to cocaine it could be predicted that these physiological changes would alter behavioural responses to repeated cocaine. However, despite the overexpression of $GABA_AR$ α2-subunit RNA, $GABA_AR$ α4-subunit knockout mice do not show a facilitated locomotor sensitisation to cocaine. This may indicate that although $GABA_AR$ α2-subunit RNA transcription is increased in $GABA_AR$ α4-subunit knockout mice, this RNA is not translated into functional $GABA_AR$ α2-subunits. Indeed, electrophysiological evidence indicates that deletion of $GABA_ARs$ α4-subunits has no impact on the kinetics of the phase currents mediated by synaptic receptors within the NAc (Maguire et al, submitted).

Following behavioural sensitisation to cocaine, a systemic THIP challenge reduced sensitised locomotor activity in wildtype, but not $GABA_AR$ α4-subunit knockout mice, to the level of that seen following acute cocaine administration during the first session. These data are consistent with existing evidence that THIP administered directly into the striatum is able to reduce the locomotor response of mice sensitised to amphetamine (Bedingfield et al., 1997), and that boosting GABA transmission, which should also increase the activity of these receptors, opposes the expression of behavioural sensitisation to cocaine (Gardner et al., 2002; Filip et al., 2006). In these animals, a THIP-induced increase in α4βδ $GABA_AR$-mediated tonic inhibition may act to suppress the activity of NAc MSNs, thus reducing locomotor activity. Under this hypothesis, activation of α4βδ $GABA_ARs$ by THIP produces a general decrease in locomotor activity, rather than a blockade of sensitisation. Alternatively, it is possible that increased tonic inhibition of NAc MSNs may attenuate the influence of glutamatergic inputs providing contextual information, thought to be required to produce a sensitised increase in locomotor activity (Mattson et al., 2008). This uncertainty may be elucidated by administration of THIP during a test of conditioned activity in the cocaine-paired environment.

Interestingly, previous evidence suggests that co-administration of systemic THIP is able to block induction of amphetamine-induced behavioural sensitisation (Karler et al., 1997). It is possible that this blockade is mediated by THIP activation of α4βδ-$GABA_ARs$ within the NAc. However, it is difficult to test this hypothesis empirically with intra-accumbal THIP administration due to the complications of tissue damage following multiple intracranial infusions (our sensitisation paradigm requires 10

consecutive days of cocaine injection to see robust sensitisation. This problem may be overcome with an alternative two-injection protocol of sensitisation, in which two administrations of psychostimulants spaced several days apart are sufficient to induce behavioural sensitisation (Valjent et al., 2010). This protocol would allow systemic cocaine injections to be paired with intra-accumbal THIP infusions, and may help to uncover whether NAc α4βδ-$GABA_A$Rs in the NAc are able to modulate the induction of locomotor sensitisation to cocaine.

Finally, although global deletion of $GABA_A$R α4-subunits does not alter behavioural sensitisation to cocaine, it is still unclear what effect deletion of α4-$GABA_A$Rs specifically from D1- or D2-expressing neurons may have. Recent evidence demonstrates that transient disruption of D1-expressing direct pathway neurons or D2-expressing indirect pathway neurons in the striatum using a synthetic inhibitory G_{α_i}-coupled DREADD (designer receptor exclusively activated by a designer drug) receptor is able to impair or facilitate, respectively, behavioural sensitisation to amphetamine (Ferguson et al., 2011). Thus it could be predicted that removal of α4-$GABA_A$R-mediated inhibition from dopamine D1- or D2-expressing neurons would increase or decrease, respectively, behavioural sensitisation to cocaine. If this was the case, then the lack of change in behavioural sensitisation to cocaine following global knockout of $GABA_A$R α4-subunits could be explained by the dissociable effects in dopamine D1- or D2-expressing neurons cancelling each other out and resulting in no overall change. This could be tested using dopamine D1- or D2-expressing neuron specific $GABA_A$R α4-subunit knockout mice.

To conclude, $GABA_A$R α4-subunits do not appear to play a role in the development of behavioural sensitisation to cocaine, as a sensitised response was observed in both wildtype and $GABA_A$R α4-subunit knockout mice following repeated, intermittent cocaine at various doses. However, activation of α4βδ $GABA_A$Rs by systemic THIP was able to attenuate sensitised locomotor activity in wildtype but not $GABA_A$R α4-subunit knockout mice.

Chapter 4

The role of α4-containing $GABA_A$ receptors in conditioned place preference to cocaine

4.1. Introduction

Remembering and actively seeking reward-associated cues are crucial abilities for survival in animals. However, the same learning processes can also lead to the development of maladaptive behaviours, including drug-seeking actions associated with addiction to drugs of abuse. As described in the previous chapter, environments associated with repeated drug experience can become potent modulators of a number of the drug's effects, including their psychomotor properties. Initially neutral environmental cues can also become associated with the motivational properties of rewarding stimuli, including drugs of abuse, a phenomenon routinely studied in laboratory animals using the conditioned place preference (CPP) paradigm (Cunningham et al., 2006; Tzschentke, 2007). CPP describes the preference of one location over another as a result of a previous pairing with a rewarding stimulus. This paradigm has been widely employed as a tool to assess the reward potential of natural and pharmacological stimuli (Bardo and Bevins, 2000).

Although the psychological underpinnings of CPP are not fully understood, the most widely assumed explanation holds that CPP is an incentive-driven behaviour derived from Pavlovian conditioning. During a typical CPP paradigm, two distinct environmental (contextual) cues, are differentially paired with the stimulus of interest, such as a drug, or a second neutral control stimulus, such as saline solution. Following conditioning, animals typically occupy the reward-paired context when allowed to roam freely, demonstrating the development of a conditioned preference. The primary rewarding properties of the drug serve as an unconditioned stimulus (US), that on repeated pairing, allows previously neutral contextual cues to acquire secondary rewarding properties, such that they are able to act as conditioned stimuli (CS) to which the animal is attracted. Under this hypothesis, CPP expression can be described as a form of Pavlovian conditioned approach, comparable to that of sign-tracking behaviour,

in that the animal orients towards the CS (Skinner, 1948). Thus, neural circuits contributing to the development and expression of CPP must be capable of encoding and retrieving memories of the association between contextual cues and the affective state produced by the treatment, as well as initiating directed motor responses.

The NAc acts as an intermediate between limbic and motor systems (Mogenson et al., 1980; Bardo and Bevins, 2000), and is hypothesized to translate information carried by excitatory glutamatergic inputs into relevant reward-seeking behavioural responses during the acquisition and expression of CPP (Skinner, 1948; Cador et al., 1989; Everitt et al., 1991). Lesion studies indicate that pre-conditioning disruption of the NAc shell, but not core, impair acquisition of food place-conditioning (Ferbinteanu and McDonald, 2001; Meyers et al., 2003; 2006; Ito et al., 2008). Similarly, post-conditioning lesions of the NAc shell, but not core, reduce expression of amphetamine-CPP (White and McDonald, 1993; Ferbinteanu and McDonald, 2001; Sellings and Clarke, 2003; Sellings et al., 2006), and infusions of cocaine directly into NAc shell, but not core, are sufficient to produce CPP (Groenewegen et al., 1987; Liao et al., 2000; French and Totterdell, 2003; Di Ciano, 2004; Ambroggi et al., 2008).

The NAc shell receives strong glutamatergic projections from hippocampal 'place' neurons demonstrating location-specific firing and thought to be integral for the processing of associative information embedded within a spatial context (O'Keefe and Dostrovsky, 1971; O'Keefe and Conway, 1978; Britt et al., 2012). Multi-neuron recordings reveal that hippocampal-striatal ensembles involved in the CPP learning experience are reactivated during sleep, and likely contribute to the consolidation and strengthening of associative place-reward information (Lansink et al., 2009). However, the exact contribution of the hippocampus appears complex and may be region-specific. While lesions and muscimol-inhibition of the dorsal hippocampus block acquisition and expression of food- or cocaine-CPP (Ferbinteanu and McDonald, 2001; Meyers et al., 2003; 2006), lesions of the ventral hippocampus lead to an increased preference for a food-paired location, indicating enhanced conditioning (White and McDonald, 1993; Ferbinteanu and McDonald, 2001).

The amygdala complex also sends glutamatergic projections to the NAc shell that are thought to provide information about associations between the affective states produced

by a rewarding stimulus and discrete cues (Groenewegen et al., 1987; French and Totterdell, 2003; Di Ciano, 2004; Ambroggi et al., 2008). Pre-conditioning lesions of the amygdala block acquisition of food- (McDonald and White, 1993), amphetamine- (Hiroi and White, 1991a) and cocaine- (Brown and Fibiger, 1993) CPP. Similarly, pre-training or pre-test inactivation of the amygdala by intracranial infusions of bupivacaine, a local anesthetic drug, block the acquisition and expression of systemic amphetamine-CPP, respectively, however 1-hour post-training infusions have no effect (Hsu et al., 2002). Thus, unlike the hippocampus, the amygdala does not appear to be involved in memory consolidation processes underlying CPP, but is likely to be involved in mediating information about the affective 'state' following rewarding stimuli.

The NAc also receives a dopaminergic projection from the ventral tegmental area (VTA), which triggers dopamine release in response to conditioned incentive cues (White and McDonald, 2002; Roitman et al., 2004; Yun et al., 2004; Day et al., 2007). Accordingly, amphetamine infused directly into the NAc, known to increase dopamine transmission, is sufficient to induce CPP (O'Keefe and Dostrovsky, 1971; O'Keefe and Conway, 1978; Carr and White, 1983; Josselyn and Beninger, 1993; McBride et al., 1999), and systemic amphetamine-CPP is blocked by excitotoxic or 6-hydroxydopamine lesions of the NAc (Spyraki et al., 1982; Olmstead and Franklin, 1996; Lansink et al., 2009). However, it appears that the actions of dopamine D1 and D2 receptor agonists and antagonists on psychostimulant-CPP acquisition and expression are varied (see Table 4.1.).

When given during conditioning, systemic administration of the D1 receptor antagonist SCH23390 blocks acquisition of both amphetamine-CPP (Hiroi and White, 1991b) and cocaine-CPP (Cervo and Samanin, 1995), and intra-accumbal injections of SCH23390 block acquisition of intra-accumbal amphetamine-CPP (Liao, 2008). Similarly, systemic administration of the D1 antagonist SCH23390 during testing blocked expression of amphetamine-CPP and cocaine-CPP (Hiroi and White, 1991b; Cervo and Samanin, 1995). Interestingly, systemic administration of the D1 receptor agonists SKF 38393, SKF 82958, SKF 81297 and ABT-431 during testing also blocked all blocked expression of cocaine-CPP (Graham et al., 2007; Sabioni et al., 2012). Similarly, systemic administration of the D2 antagonist sulpiride given during conditioning blocks

acquisition of amphetamine-CPP (Hiroi and White, 1991b) and intra-accumbal injections of the D2 antagonist raclopride block acquisition of intra-accumbal amphetamine-CPP (Liao, 2008). However, systemic injection of sulpiride does not effect acquisition of cocaine-CPP (Cervo and Samanin, 1995). When given during testing, systemic administration of the D2 agonists quinperole and 7-OH-DPAT both blocked expression of cocaine-CPP (Graham et al., 2007). However, the D2 antagonists flupentixol or metoclopramide have no effect on amphetamine-CPP (Hiroi and White, 1991b). Although it is clear that both D1 and D2 receptors act to modulate acquisition and expression of psychostimulant-CPP, studies employing the use of D1 or D2 agonists and antagonists do not give a clear indication of the exact role of these receptors, possibly due to the crudeness of these compounds (Gerfen and Surmeier, 2011). Contemporary techniques allowing for more direct modulation of the excitability of NAc neurons expressing dopamine D1 or D2 receptors may help to elucidate neuronal mechanisms controlling the acquisition and expression of psychostimulant-CPP.

Indeed, separate striatal pathways have been shown to play an important role in guiding the direction of place conditioning. Reversible neurotransmitter blockade of the dopamine D1-receptor-expressing *direct* pathway attenuates acquisition of food-CPP but does not affect acquisition of electric shock-induced conditioned place avoidance (CPA), while the reverse is observed following blockade of the dopamine D2-receptor-expressing *indirect* pathway (Brown and Fibiger, 1993; Hikida et al., 2010). Similarly, optogenetic activation of D1-expressing NAc MSNs during cocaine-paired chamber conditioning enhanced acquisition of cocaine-CPP, while activation of D2-expressing NAc MSNs in the same conditions attenuated acquisition (Lobo et al., 2010).

Table 4.1.

	Acquisition		**Expression**	
	Systemic	**Intra-accumbal**	**Systemic**	**Intra-accumbal**
D1 agonist	?	?	Block cocaine-CPP (Graham et al., 2007; Sabioni et al., 2012)	?
D2 agonist	?	?	Block cocaine-CPP (Graham et al., 2007)	?
D1 antagonist	Block cocaine-CPP (Cervo and Samanin, 1995) Block amphetamine-CPP (Hiroi and White, 1991b)	Block intra-NAc amphetamine-CPP (Liao, 2008)	No change cocaine CPP (Cervo and Samanin, 1995) Block amphetamine-CPP (Hiroi and White, 1991b)	Block amphetamine-CPP (Hiroi and White, 1991b)
D2 antagonist	No change cocaine-CPP (Cervo and Samanin, 1995) Block amphetamine-CPP (Hiroi and White, 1991b)	Block intra-NAc amphetamine-CPP (Liao, 2008)	No change cocaine-CPP (Cervo and Samanin, 1995) No change amphetamine-CPP (Hiroi and White, 1991b)	No change amphetamine-CPP (Hiroi and White, 1991b)

Table 4.1. The effect of systemic or intra-accumbal administration of dopamine D1 or D2 receptor agonists and antagonists on acquisition and expression of psychostimulant-CPP.

Given that neurons within the NAc are predominantly GABAergic MSNs it is possible that $GABA_A$Rs may be involved in the acquisition of CPP. Benzodiazepines, which act to enhance the effect of GABA at $GABA_A$Rs, have largely been reported to be unable to induce CPP when administered alone. Systemic administration of alprazolam, zolpidem, oxazepam and diazepam all fail to produce CPP (Meririnne et al., 1999; Walker and Ettenberg, 2001; Le Pen et al., 2002; Walker and Ettenberg, 2003; Goeders and Goeders, 2004). However, others find that at higher doses, diazepam administration results in a significant CPP effect (Gray et al., 1999; Papp, 2002). Drugs acting directly at $GABA_A$Rs, including the antagonists bicuculine and picrotoxin, similarly do not produce a place conditioning effect (Chester and Cunningham, 1999; Bossert and Franklin, 2001). However, THIP, a $GABA_A$R agonist with a preference for δ-containing receptors, is able to produce persistent CPA in rodents (Vashchinkina et al., 2012).

Interestingly, when administered directly into specific brain regions, drugs modulating GABAergic inhibition show a different pattern of effects. Indeed, there appears to be a rostrocaudal gradient within the NAc in the ability of $GABA_A$R agonists to induce CPP. Muscimol delivered directly into the rostral NAc shell produces a significant CPP, however, administration into the caudal shell produced a strong CPA (Reynolds and Berridge, 2002). Similar such injections of muscimol or bicuculine into the BLA have been reported not to produce any effects (Zarrindast et al., 2004), although others find significant CPA following intra-BLA bicuculine (Thielen and Shekhar, 2002). Interestingly, in the VTA, administration of a $GABA_A$R agonist and antagonist produce the same result, with muscimol and bicuculine both inducing significant CPP (Laviolette and van der Kooy, 2001).

GABAergic inhibition has also been reported to modulate psychostimulant-CPP. The acquisition and expression of amphetamine- and cocaine-CPP are blocked by systemic diazepam administration (Leri and Franklin, 2000a). Similarly, pre-treatment of GVG, an irreversible GABA-transaminase inhibitor, abolished both the acquisition and expression of cocaine-CPP in rodents, but did not effect CPP for food reward (Dewey et al., 1998). Co-administration of Gastrodia elata Bl, an oriental herb agent known to enhance GABAergic transmission, and post-conditioning treatment with 1*R*,4*S*-4-amino-cyclopent-2-ene-carboxylic acid (ACC), a reversible inhibitor of GABA transaminase, are also reported to attenuate acquisition and expression of cocaine-CPP,

respectively (Ashby et al., 2002; Shin et al., 2011). Evidence that expression of amphetamine-CPP is blocked by intra-NAc administration of diazepam indicates that GABA transmission within the NAc is able to modulate psychostimulant-CPP (Leri and Franklin, 2000b).

As of yet, little is known about the role of individual $GABA_AR$ subtypes in the mediation of cocaine-CPP. Targeted deletion of synaptically located α2-containing $GABA_ARs$ does not affect acquisition of cocaine-CPP (Dixon et al., 2010). However, the possible contribution of extrasynaptic α4βδ $GABA_ARs$, thought to mediate the excitability of NAc MSNs through a tonic form of inhibition, is yet to be revealed. Here, the effects of targeted deletion of $GABA_AR$ α4-subunits are explored in a cocaine-CPP paradigm. Additionally, the effects of THIP, given either systemically or infused directly into the NAc at behaviourally relevant doses (see Chapter 3), were observed in a test of cocaine-CPP. As THIP was previously shown to also modulate behaviours when cocaine was still onboard (see Chapters 3 & 4), in a separate test, THIP was also co-administered with a cocaine challenge. Finally, the contribution of $GABA_AR$ α4-subunits in specific striatal pathways were assessed using dopamine D1- or D2-expressing cell-specific $GABA_AR$ α4-subunit knockout mice and systemic THIP administration using the same experimental design.

4.2. Materials and Methods

4.2.1. Animals

Conditional dopamine D1/D2 expressing neuron specific α4-subunit knockout mice were created by crossing "Floxed" Gabra4 homozygous transgenic mice (strain name; B6.129-*Gabra4*$^{tm1.2Geh}$/J, supplied by The Jackson Laboratory, ME, USA) with either dopamine receptor D1 or D2 neuron specific Cre-recombinase hemizygous transgenic mice (strain name; **α4$^{D1-/-}$** = B6.FVB(Cg)-Tg(Drd1a-cre)EY217Gsat/Mmucd, **α4$^{D2-/-}$** = B6.FVB(Cg)-Tg(Drd2-cre)ER44Gsat/Mmucd, supplied by Mutant Mouse Regional Resource Centers (MMRRC), ME, USA). Breeding was conducted as described in Fig. 4.1.

Male $GABA_AR$ α4 wildtype (WT), knockout ($\alpha4^{-/-}$), and dopamine D1- or D2-expressing neuron specific α4 wildtype ($\alpha4^{D1}$ WT/$\alpha4^{D2}$ WT) and knockout ($\alpha4^{D1-/-}$/$\alpha4^{D2-/-}$) mice on a C57Bl/6J background strain, weighing between 20-30g, were housed in groups of 2-3, or separately for surgery animals, with food and water available *ad libitum*. A 12hr light/dark cycle was used (lights on at 7:00 A.M.) with holding room temperature maintained at 21 ±2ºC and humidity 50 ±5%. All injections, infusions and behavioural testing were performed between 2:00 P.M. and 5:00 P.M. All procedures were conducted in accordance to Animals (Scientific Procedures) Act 1986, following ethical review by the University of Sussex Ethical Review Committee.

Figure 4.1.

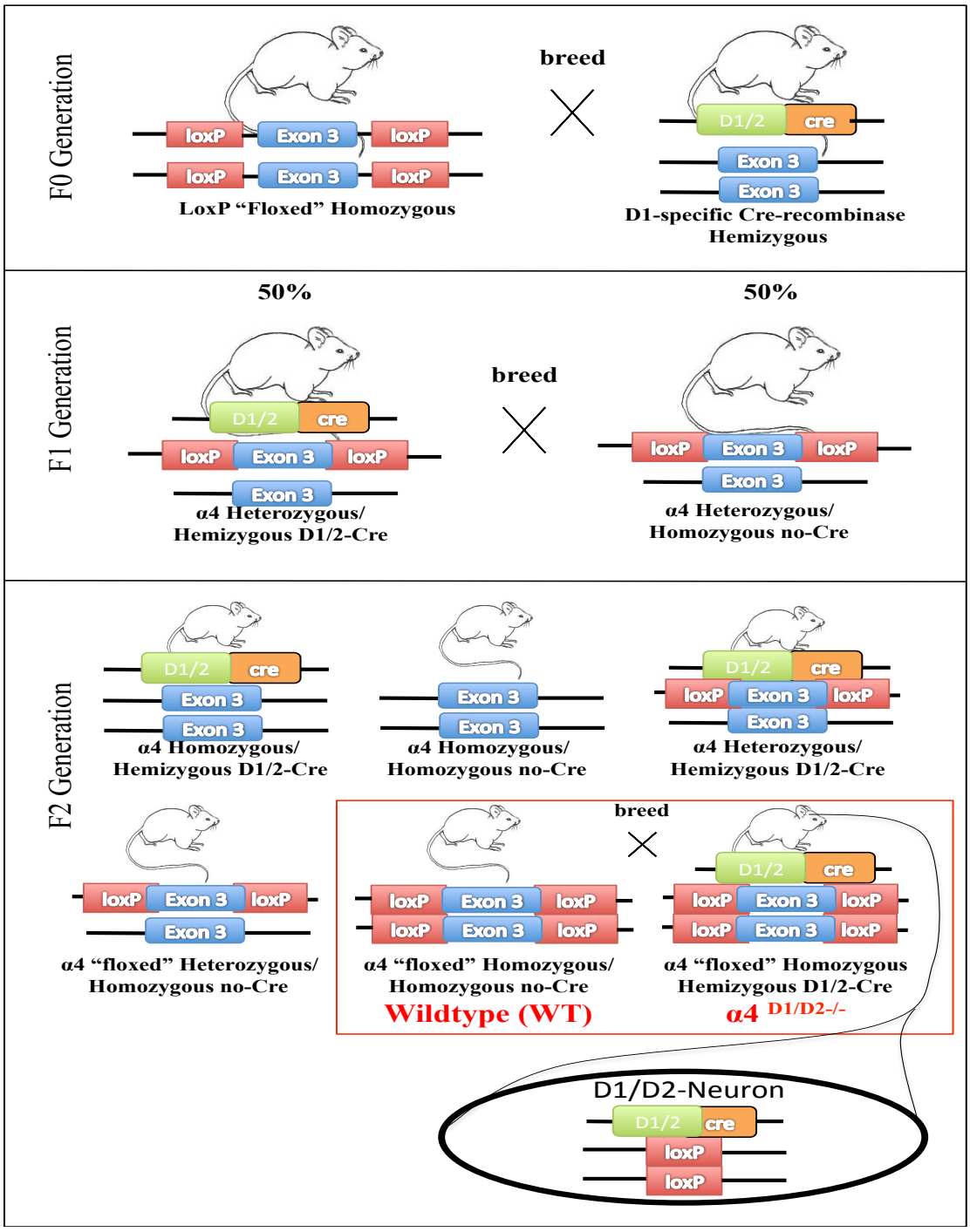


Fig. 4.1. Production of D1 expressing neuron specific α4-subunit wildtype ($\alpha4^{D1}$ WT), and knockout ($\alpha4^{D1-/-}$) mice. (**F0**) α4-subunit "floxed" homozygous mice were bred with D1-expressing neuron specific Cre-recombinase expressing heterozygous mice. (**F1**) offspring heterozygous for the α4 allele (+/-), and heterozygous for D1/D2-CRE or homozygous for no-CRE were bred to create (**F2**) offspring of approximately; heterozygous α4 allele/homozygous no-D1/D2-CRE (25%), heterozygous α4 allele/heterozygous D1/D2-CRE (25.5%), homozygous α4 allele/heterozygous D1/D2-

CRE (12.5%), homozygous α4 allele/homozygous no-D1/D2-CRE (12.5%). Finally, homozygous α4 "floxed" allele/homozygous no-D1/D2-CRE (12.5%) and homozygous α4 "floxed" allele/heterozygous D1/D2-CRE (12.5%) were used to breed the experimental wildtype (50%) and knockout (50%) experimental mice, respectively. The same strategy was used using D2-CRE mice.

4.2.2. Genotyping

Genotyping procedure as described in Chapter 2 (2.2.2.)

4.2.2.1. Conditional dopamine D1-/D2-expressing neuron specific $GABA_AR$ α4-subunit knockout mice PCR

Forward and reverse cDNA primers were designed to target and replicate a sequence contained within the integrated Cre recombinase transgene. The Cre primer consisted of a 102bp product (forward primer; GCGGTCTGGCAGTAAAACTATC, reverse primer; GTGAAACAGCATTGCTGTCACTT).

Figure 4.2.

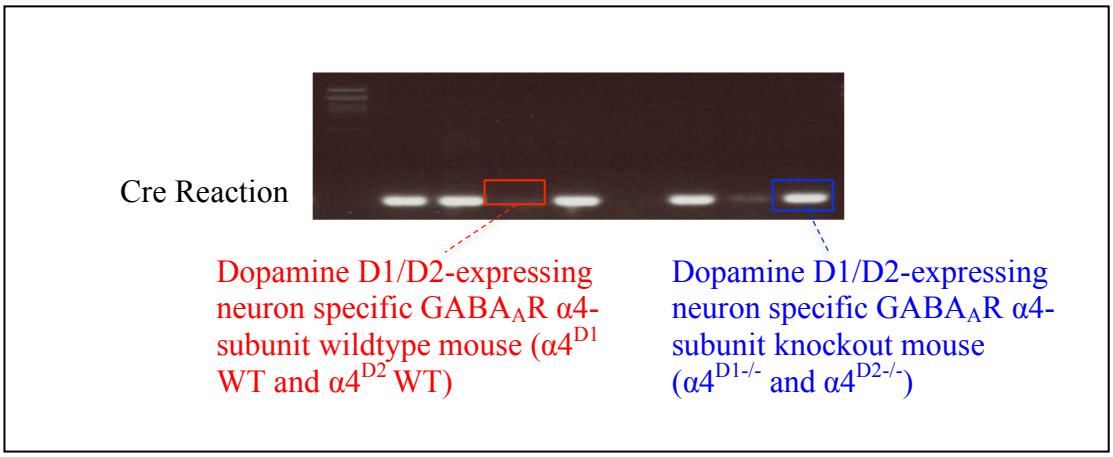


Fig. 4.2. Genotyping of dopamine D1/D2-expressing neuron specific $GABA_A$ α4-subunit wildtype and knockout mice requires a reaction for the detection of Cre. The absence of a Cre band indicates a wildtype mouse, whilst the presence of a Cre band indicates a knockout mouse.

4.2.3. Quantitative reverse transcription PCR (qRT-PCR) mRNA analysis

$GABA_AR$ α4-, α2-, γ2- and δ-subunit mRNA expression was analysed from NAc tissue samples from α4$^{D1-/-}$, α4$^{D2-/-}$ and respective WT control mice (as described in Chapter 2; 2.2.4.).

4.2.4. Stereotaxic Cannulation.

Mice anaesthetised with isoflurane were implanted stereotaxically with bilateral guide cannulae (26 ga., 10mm) aimed at NAcc (coordinates AP1.34; L+/− 1.00; DV −3.20, (Paxinos and Franklin, 2001)). Following surgery, mice were singly housed and

underwent a one-week recovery/habituation period. A steel infuser (33 ga., 11 mm) connected *via* polyvinyl tubing to a (5 μl) Hamilton Gastight syringe was used to infuse 0.5μl of either saline or THIP (3 mM) bilaterally across 90 seconds and left to settle for 90 seconds before infusers were removed. Location of cannulae was confirmed histologically.

4.2.5. Drugs

Cocaine Hydrochloride was obtained from Macfarlan Smith (Edinburgh, UK). THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) was kindly donated by Bjarke Ebert (Lundbeck, Valby, Denmark). Both drugs were dissolved in 0.9% saline, and administered i.p. at an injection volume of 10 ml/kg.

4.2.6. Apparatus

Conditioned place preference was measured using eight three-chamber place conditioning boxes (outer two conditioning chambers measured 200 X 200 X 200 mm and were separated by a central chamber measuring 200 X 50 X 200 mm). One conditioning chamber was white with meshed metal flooring. The other chamber was black and white (each wall was split along the diagonal, with the top and bottom halves colored black and white, respectively) with a smooth clear Perspex floor. The central chamber had grey walls and a smooth clear Perspex floor and could be closed off from the outer chambers during the conditioning phase using clear Perspex doors to prevent movement between chambers. The movement and location of animals was recorded using infra-red beam breaks (Mead et al., 1999).

4.2.7. Conditioned Place Preference to Cocaine

Animals received saline injections in their home cages the day before the first experimental session, following which the experiment was divided into three phases:

Pre-conditioning phase (day 1): Mice were allowed free access to the apparatus for 20 min., during which the time in each chamber was measured in order to exclude the possibility of a chamber bias.

Conditioning phase (days 2-11): Mice were administered cocaine (10 mg/kg, *i.p.*) and were confined to one of the outer chambers for 40 min. On alternate daily sessions the mice were administered saline and confined to the other outer chamber. Mice received a total of 10 pairings during the conditioning phase (5 with cocaine, and 5 with saline). The side of the chamber assigned to cocaine-administration was counterbalanced between mice.

Test Phase (days 12-16): On the first test day mice were allowed free access to the entire chamber for 20 min and time spent in each chamber was recorded for analysis of place conditioning. On the following four test days mice received THIP (i.p. at 8mg/kg administered 20 minutes prior to test, or intra-accumbal infusions at 3uM/3mM directly prior to test), or saline, and an i.p. injection of cocaine (10mg/kg), or saline, directly prior to testing. Drugs were administered in a latin square design and sessions were recorded for 20 min.

4.2.8. Statistical Analysis

4.2.8.1. qRT-PCR

Quantitative RNA expression data were collected using the Mx4000 data analysis software (Stratagene, CA, USA), then exported to an Excel worksheet. Reaction triplicates were averaged, and then normalised against the control gene GAPDH to give a measure of the delta CT. The delta CT of the target sample was then normalised against the delta CT of a control sample to give a measure of the delta delta CT. Finally, a mathematical model was used to calculate the fold change of the target gene using the delta-delta CT (see (Pfaffl, 2001)). Statistical analysis of RNA expression of each receptor subunit was conducted using between-subjects one-way ANOVAs, with genotype as the between-subjects variables, and delta CT as the dependent variable.

4.2.8.2. Conditioned Place Preference to Cocaine in Wildtype and $GABA_AR$ α4-subunit Knockout Mice with Intraperitoneal THIP

Acquisition of CPP to cocaine was assessed by measuring the difference in time spent in the cocaine-paired chamber minus the time spent in the saline-paired chamber from the pre- to the post-conditioning drug-free session. These data were analysed using a three-way mixed-factors ANOVA, with genotype as the between-subjects factor,

conditioning as the within-subjects factor, and time spent in the cocaine-paired chamber minus saline-paired chamber as the dependent variable. Subsequently, the ability of cocaine and i.p. THIP to modulate cocaine-CPP was measured using a five-way mixed factors ANOVA, with genotype as the between-subjects factor, cocaine/saline injection and THIP/saline injection as the within-subjects factors, and time spent in the cocaine-paired chamber minus saline-paired chamber as the dependent variable.

4.2.8.3. Conditioned Place Preference to Cocaine in Wildtype and $GABA_AR$ α4-subunit Knockout Mice with Intra-accumbal THIP

As previous, acquisition of CPP to cocaine data were analysed using a three-way mixed-factors ANOVA, with genotype as the between-subjects factor, conditioning as the within-subjects factor, and time spent in the cocaine-paired chamber minus saline-paired chamber as the dependent variable. Cocaine-CPP data were analysed independently for each THIP dose using five-way mixed-factors ANOVAs, with genotype as the between-subjects factor, cocaine/saline injection and THIP/saline infusion as the within-subjects factors, and time spent in the cocaine-paired chamber minus saline-paired chamber as the dependent variable.

4.2.8.4. Conditioned Place Preference to Cocaine in Wildtype and Dopamine D1/D2-expressing neuron specific $GABA_AR$ α4-subunit Knockout Mice with Intraperitoneal THIP

Acquisition of CPP to cocaine were analysed independently for $\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$ mice and their respective wildtype control mice using three-way mixed-factors ANOVAs, with genotype as the between-subjects factor, conditioning as the within-subjects factor, and time spent in the cocaine-paired chamber minus saline-paired chamber as the dependent variable. The ability of cocaine and i.p. THIP to modulate cocaine-CPP was again analysed independently for each knockout group and it's respective wildtype control mice using five-way mixed factors ANOVAs, with genotype as the between-subjects factor, cocaine/saline injection and THIP/saline injection as the within-subjects factors, and time spent in the cocaine-paired chamber minus saline-paired chamber as the dependent variable.

4.3. Results

4.3.1. qRT-PCR mRNA analysis in dopamine D1-/D2-expresing neuron specific $GABA_AR$ α4-subunit wildtype and knockout mice

In order to confirm the deletion of $GABA_AR$ α4-subunits from D1 or D2-expressing neurons, expression levels of α4-subunit mRNA were measured in the NAc of $\alpha4^{D1-/-}$, $\alpha4^{D2-/-}$ and respective WT controls using qRT-PCR. In addition, NAc mRNA expression levels of α2-, γ2- and δ-subunits were also measured.

$GABA_AR$ α4-subunit mRNA levels were reduced by approximately half of that of respective WT controls in the NAc of $\alpha4^{D1-/-}$ (Table 4.2., Fig. 4.3; significant main effect of genotype, $F_{(1,10)} = 22.05$, $p < 0.001$) and $\alpha4^{D2-/-}$ mice (Table 4.2., Fig. 4.3; significant main effect of genotype, $F_{(1,10)} = 27.12$, $p < 0.001$). Expression of $GABA_AR$ α2-subunit mRNA was increased to a similar degree in the NAc of $\alpha4^{D1-/-}$ (Table 4.2., Fig. 4.3; significant main effect of genotype, $F_{(1,10)} = 55.36$, $p < 0.001$) and $\alpha4^{D2-/-}$ mice (Table 4.2., Fig. 4.3; significant main effect of genotype, $F_{(1,10)} = 42.01$, $p < 0.001$), when compared with respective WT controls. $GABA_AR$ γ2-subunit mRNA levels did not differ from respective WT controls in the NAc of $\alpha4^{D1-/-}$ (Table 4.2., Fig. 4.3; non-significant main effect of genotype, $F_{(1,10)} = 0.65$, $p = 0.45$, NS) and $\alpha4^{D2-/-}$ mice (Table 4.2., Fig. 4.3; non-significant main effect of genotype, $F_{(1,10)} = 1.15$, $p = 0.32$). Finally, expression of $GABA_AR$ δ-subunit mRNA was decreased in the NAc of $\alpha4^{D1-/-}$ (Table 4.2., Fig. 4.3; significant main effect of genotype, $F_{(1,10)} = 51.28$, $p < 0.001$) and $\alpha4^{D2-/-}$ mice (Table 4.2., Fig. 4.3; significant main effect of genotype, $F_{(1,10)} = 32.18$, $p < 0.001$), when compared with respective WT controls.

Table 4.2.

Primer	Genotype	Fold change	% Change from WT	Sig.
α4	$\alpha4^{D1}$ WT	1 ± 0.11/0.06	0%	
	$\alpha4^{D1-/-}$	0.54 ± 0.22/0.12	-46%	$p < 0.001$
	$\alpha4^{D2}$ WT	1 ± 0.15/0.08	0%	
	$\alpha4^{D2-/-}$	0.45 ± 0.18/0.11	-55%	$p < 0.001$
α2	$\alpha4^{D1}$ WT	1 ± 0.07/0.05	0%	
	$\alpha4^{D1-/-}$	6.97 ± 1.76/0.88	+597%	$p < 0.001$
	$\alpha4^{D2}$ WT	1 ± 0.23/0.14	0%	
	$\alpha4^{D2-/-}$	7.23 ± 1.50/0.78	+623%	$p < 0.001$
γ2	$\alpha4^{D1}$ WT	1 ± 0.10/0.05	0%	
	$\alpha4^{D1-/-}$	1.15 ± 0.21/0.11	+15%	NS
	$\alpha4^{D2}$ WT	1 ± 0.23/0.12	0%	
	$\alpha4^{D2-/-}$	1.07 ± 0.25/0.14	+7%	NS
δ	$\alpha4^{D1}$ WT	1 ± 0.12/0.06	0%	
	$\alpha4^{D1-/-}$	0.44 ± 0.26/0.15	-66%	$p < 0.001$
	$\alpha4^{D2}$ WT	1 ± 0.06/0.04	0%	
	$\alpha4^{D2-/-}$	0.47 ± 0.21/0.10	-53%	$p < 0.001$

Table 4.2. NAc mRNA expression levels of $GABA_AR$ α4-, α2-, γ2- and δ-subunits in dopamine D1- or D2-expressing neuron specific α4-subunit knockout ($\alpha4^{D1-/-}$; n=4, $\alpha4^{D2-/-}$; n=4) mice were compared in triplicate against wildtype ($\alpha4^{D1}$ WT; n=4, $\alpha4^{D2}$ WT; n=4) controls in the NAc, to give a measure of fold change. Percentage change from WTs was tested statistically using *post hoc* paired *t* tests.

Figure 4.3.

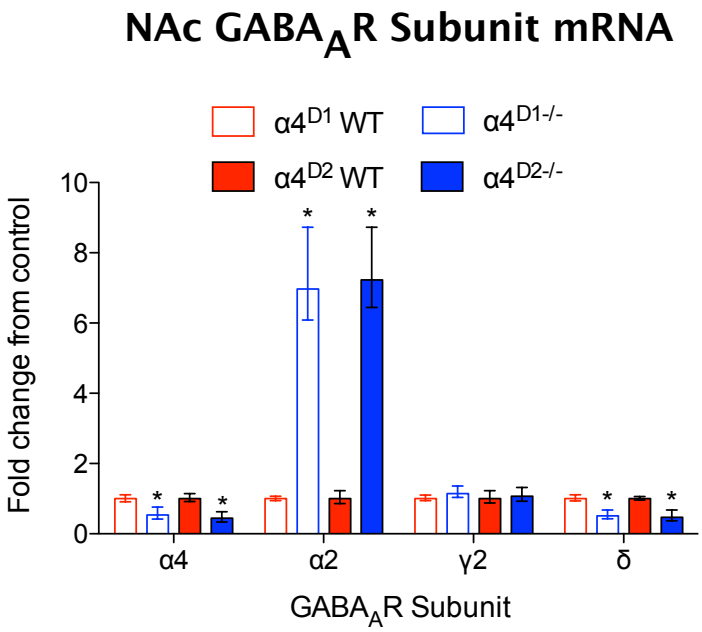


Figure 4.3. Fold change from wildtype controls of $GABA_AR$ α4-subunit mRNA expression in the NAc of wildtype ($\alpha4^{D1}$ WT; n=4 and $\alpha4^{D2}$ WT; n=4) and dopamine D1-/D2-receptor expressing neuron specific α4-subunit knockout ($\alpha4^{D1-/-}$; n=4 and $\alpha4^{D2-/-}$; n=4) mice. $\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$ mice show a decrease in expression of NAc α4-subunit mRNA when compaired to WT controls. Conversely, $\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$ mice show a large increase in expression of α2-subunit mRNA. Expression of γ2-subunit mRNA was unchanged in $\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$ mice. Finally, expression of δ-subunit mRNA was reduced in $\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$ mice, when compared to WT controls. Error bars represent SEM. *$p < 0.001$, *post hoc* paired *t* test.

4.3.2. Conditioned Place Preference to Cocaine in Wildtype and $GABA_AR$ α4-subunit Knockout Mice with Intraperitoneal THIP

A pre-conditioning test found no bias in preference for the two outer chambers (Fig. 4.4.A; non-significant main effect of genotype, $F_{(1,20)} = 0.16$, $p = 0.69$, NS). Subsequently, the ability of cocaine to support the formation of a conditioned place preference was unaltered by a constitutive deletion of the α4 subunit. WT and $\alpha4^{-/-}$ mice demonstrated an equal preference for the cocaine-paired chamber under drug-free (Fig 4.4.A; significant main effect of conditioning, $F_{(1,20)} = 82.49$, $p < 0.001$, non-significant conditioning by genotype interaction, $F_{(1,20)} = 0.56$, $p = 0.45$, NS) and saline (Figure 4.4.B; main effect of conditioning, $F_{(1,20)} = 12.70$, $p < 0.001$, non-significant main effect of genotype, $F_{(1,20)} = 0.23$, $p = 0.63$, NS) conditions.

A challenge injection of cocaine (10mg/kg *i.p.*) given directly prior to the test significantly increased time in the cocaine-paired chamber compared with mice given saline (Fig. 4.4.B; main effect of drug, $F_{(1,20)} = 18.65$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,20)} = 2.27$, $p = 0.83$,NS). To test whether α4βδ $GABA_ARs$ were mediating this effect, systemic THIP (8mg/kg) was administered 20 min prior to CPP testing. There was no effect of THIP alone on time spent in the cocaine-paired chamber (Fig. 4.4.B; non-significant main effect of THIP, $F_{(1,20)} = 3.1$, $p = 0.95$, NS, non-significant THIP by genotype interaction, $F_{(1,20)} = 1.58$, $p = 0.22$, NS), however, THIP significantly reduced cocaine enhancement of cocaine-CPP for WT, but not $\alpha4^{-/-}$ mice (Fig. 4.4.B; significant drug by THIP by genotype interaction, $F_{(1,20)} = 12.40$, $p < 0.01$).

As seen in Chapter 3, locomotor activity measured during the control CPP test session did not differ between WT and $\alpha4^{-/-}$ mice under drug-free (Fig 4.4.C; non-significant main effect of genotype, $F_{(1,20)} = 0.33$, $p = 0.57$, NS) and saline (Fig 4.4.C; non-significant main effect of genotype, $F_{(1,20)} = 0.27$, $p = 0.60$, NS) conditions. A cocaine challenge potentiated activity equally in both genotypes (Fig. 4.4.C; significant main effect of drug, $F_{(1,20)} = 96.28$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,20)} = 0.28$, $p = 0.65$, NS). Intraperitoneal injections of THIP (8mg/kg) did not effect baseline locomotor activity in either WT or $\alpha4^{-/-}$ mice (Fig. 4.4.C; non-significant main effect of THIP, $F_{(1,20)} = 0.40$, $p = 0.53$, NS, non-significant THIP by genotype interaction, $F_{(1,20)} = 0.09$, $p = 0.76$, NS), and although cocaine-potentiated locomotor

activity appeared to be reduced in WT but not $\alpha4^{-/-}$ mice, this result was not found to be significant (Fig. 4.4.C; non-significant drug by THIP by genotype interaction, $F_{(1,20)} = 1.02$, $p = 0.32$, NS).

Figure 4.4.

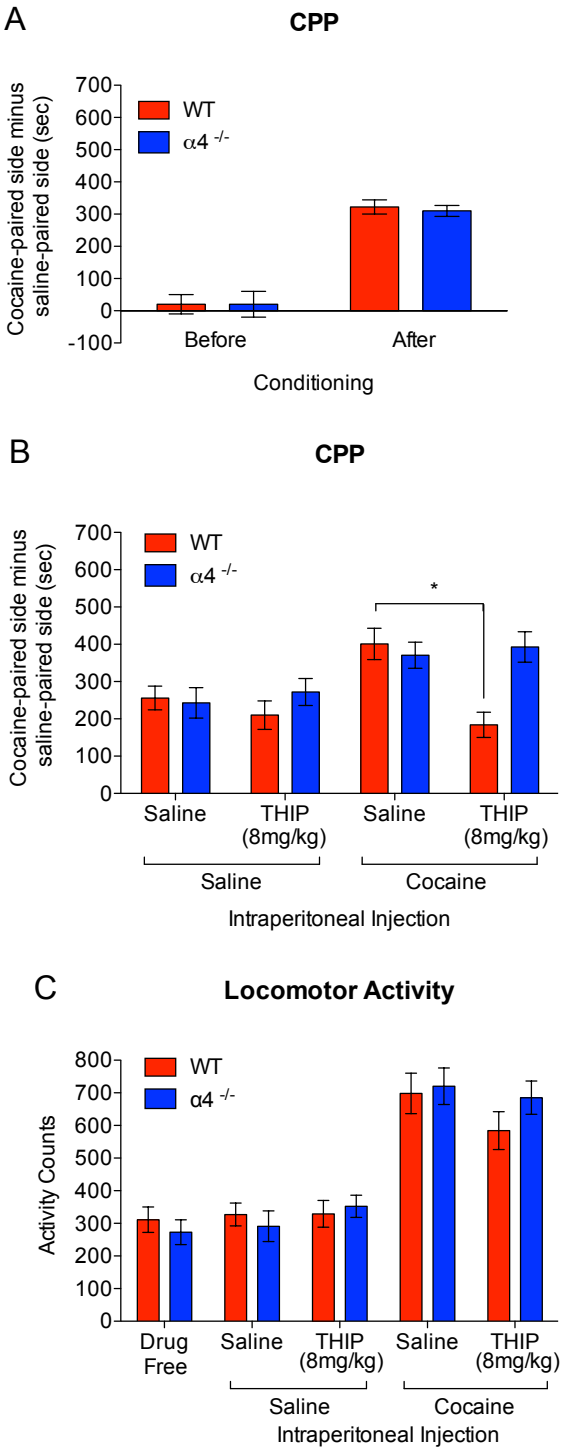


Fig. 4.4. The effect of challenge injections of THIP (8mg/kg)/vehicle and cocaine (10mg/kg)/vehicle on cocaine-CPP in a 20 minute test. (**A**) WT (n=11) and $\alpha4^{-/-}$ (n=11) mice do not show any pre-conditioning preference for either chamber, but following conditioning equally demonstrate a preference for the cocaine-paired chamber in a drug-

free test, indicating significant place conditioning. (**B**) Cocaine induced a significant CPP in both WT and $\alpha4^{-/-}$ mice. A challenge dose of cocaine increased cocaine-CPP equally in WT and $\alpha4^{-/-}$ mice. Systemic THIP had no effect on preference in either genotype, but blocked the cocaine potentiating effect in WT but not $\alpha4^{-/-}$ mice. (**C**) Locomotor activity during CPP. WT and $\alpha4^{-/-}$ mice did not show any differences in activity during CPP, and were both equally potentiated by a cocaine challenge. THIP did not affect baseline or cocaine-potentiated locomotor activity in either genotype. Error bars represent SEM. * $p < 0.01$ *post hoc* comparisons.

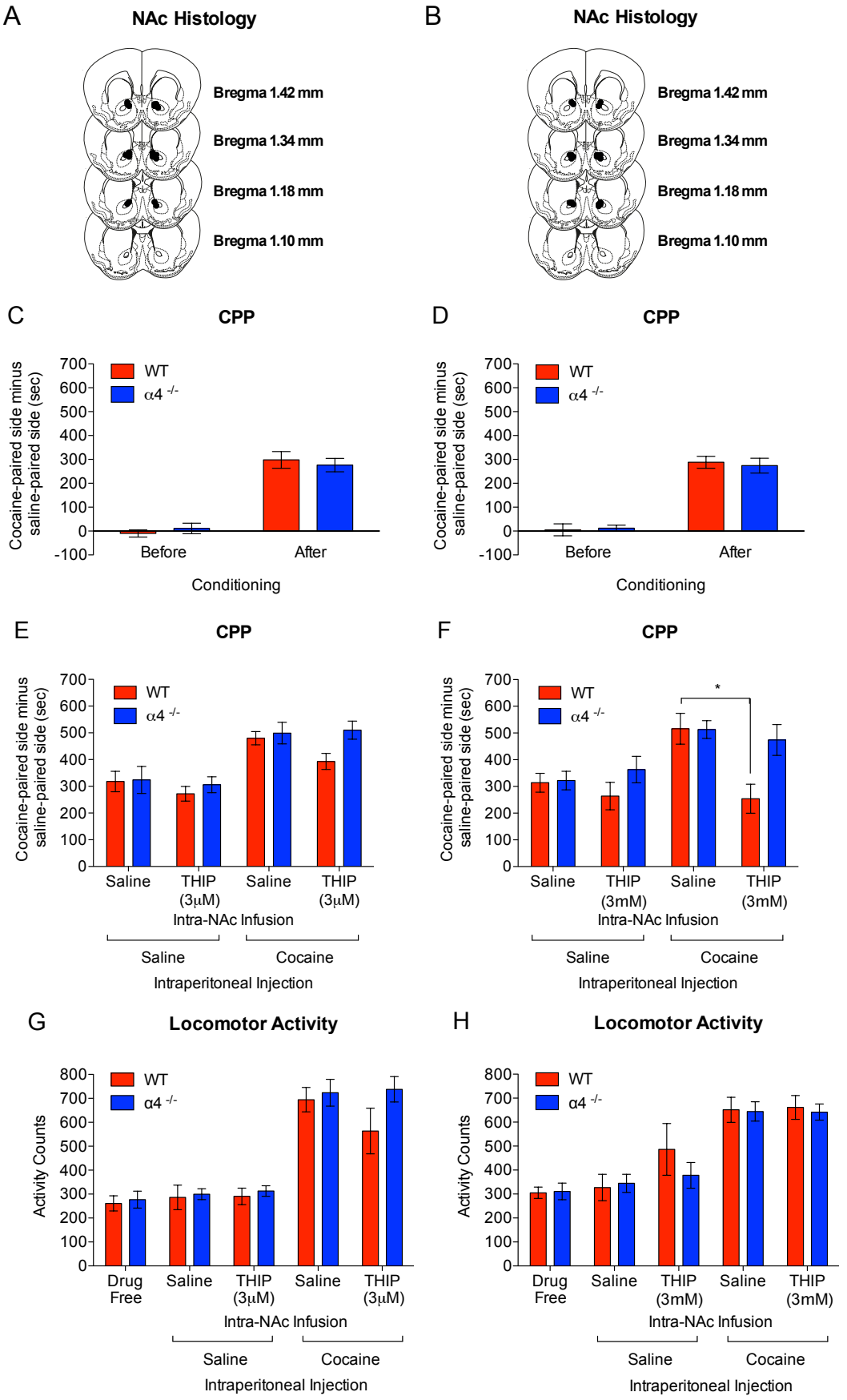
4.3.3. Conditioned Place Preference to Cocaine in Wildtype and $GABA_AR$ α4-subunit Knockout Mice with Intra-accumbal THIP

A pre-conditioning test found no bias in preference for the two outer chambers (Fig. 4.5.C; non-significant main effect of genotype, $F_{(1,14)} = 0.60$, $p = 0.44$, NS, Fig. 4.5.D; non-significant main effect of genotype, $F_{(1,14)} = 0.19$, $p = 0.65$, NS). To test whether accumbal α4βδ $GABA_ARs$ were mediating the THIP-induced decrease in cocaine-potentiated cocaine-CPP, mice were cannulated and tested in the same paradigm. The intra-accumbal THIP doses used were based upon the active doses revealed in the locomotor activity experiments (see Chapter 2), and consisted of a low-dose (3μM) and a high-dose (3mM). As expected, cocaine induced a significant CPP in both WT and $\alpha4^{-/-}$ mice under drug-free (Fig. 4.5.C; main effect of conditioning: $F_{(1,14)} = 55.43$, $p < 0.001$, non-significant conditioning by genotype interaction, $F_{(1,14)} = 0.19$, $p = 0.66$, NS, Fig. 4.5.D; main effect of conditioning: $F_{(1,14)} = 41.53$, $p < 0.001$, non-significant conditioning by genotype interaction, $F_{(1,14)} = 0.63$, $p = 0.43$, NS) and saline (Fig. 4.5.E; significant main effect of conditioning, $F_{(1,14)} = 23.28$, $p < 0.001$, non-significant conditioning by genotype interaction, $F_{(1,14)} = 0.02$, $p = 0.86$, NS, Fig. 4.5.F; significant main effect of conditioning, $F_{(1,14)} = 12.98$, $p < 0.001$, non-significant conditioning by genotype interaction, $F_{(1,14)} = 0.07$, $p = 0.79$, NS) conditions.

Intra-accumbal 3μM or 3mM THIP had no effect on preference in either genotype (Fig.4.5.E; non-significant main effect of THIP infusion, $F_{(1,14)} = 2.39$, $p = 0.14$, NS, non-significant THIP infusion by genotype interaction, $F_{(1,14)} = 1.94$, $p = 0.18$, NS, Fig.4.5.F; non-significant main effect of THIP infusion, $F_{(1,14)} = 3.30$, $p = 0.09$, NS, non-significant THIP infusion by genotype interaction, $F_{(1,14)} = 3.39$, $p = 0.08$, NS). Again, a challenge dose of cocaine enhanced preference in both genotypes (Fig. 4.5.E; significant main effect of drug, $F_{(1,14)} = 29.71$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,14)} = 0.63$, $p = 0.43$, NS, Fig. 4.5.F; significant main effect of drug, $F_{(1,14)} = 15.38$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,14)} = 0.65$, $p = 0.43$, NS). Cocaine challenge enhancement of CPP was blocked by intra-accumbal 3mM but not 3μM THIP in WT, but not in $\alpha4^{-/-}$ mice (Fig. 4.5.E; non-significant drug by THIP infusion by genotype interaction, $F_{(1,14)} = 0.97$, $p = 0.34$, NS, Fig. 4.5.F; significant drug by THIP infusion by genotype interaction, $F_{(1,14)} = 6.12$, $p < 0.05$).

Locomotor activity recorded during the CPP sessions indicated that as previous WT and $\alpha4^{-/-}$ mice did not differ in their activity under drug-free (Fig. 4.5.G; non-significant main effect of genotype, $F_{(1,14)} = 0.24$, $p = 0.67$, NS, Fig. 4.5.H; non-significant main effect of genotype, $F_{(1,14)} = 0.32$, $p = 0.58$, NS) and saline (Fig. 4.5.G; non-significant main effect of genotype, $F_{(1,14)} = 0.17$, $p = 0.77$, NS, Fig. 4.5.H; non-significant main effect of genotype, $F_{(1,14)} = 0.02$, $p = 0.88$, NS) conditions. Similarly, a cocaine challenge potentiated locomotor activity equally in both genotypes (Fig. 4.5.G; significant main effect of drug, $F_{(1,14)} = 161.57$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,14)} = 0.01$, $p = 0.91$, NS, Fig. 4.5.H; significant main effect of drug, $F_{(1,14)} = 68.02$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,14)} = 0.81$, $p = 0.37$, NS). As with intraperitoneal THIP injections, both 3μM and 3mM intra-accumbal THIP alone did not effect locomotor activity in both genotypes (Fig. 4.5.G; non-significant main effect of THIP infusion, $F_{(1,14)} = 0.38$, $p = 0.54$, NS, non-significant THIP infusion by genotype interaction, $F_{(1,14)} = 0.01$, $p = 0.89$, NS, Fig. 4.5.H; non-significant main effect of THIP infusion, $F_{(1,14)} = 3.38$, $p = 0.08$, NS, non-significant THIP infusion by genotype interaction, $F_{(1,14)} = 0.95$, $p = 0.34$, NS). THIP infusions at either dose were similarly unable to effect cocaine-challenge-potentiated locomotor activity in WT or $\alpha4^{-/-}$ mice (Fig. 4.5.G; non-significant drug by THIP infusion by genotype interaction, $F_{(1,14)} = 0.06$, $p = 0.80$, NS, Fig. 4.5.H; non-significant drug by THIP infusion by genotype interaction, $F_{(1,14)} = 0.95$, $p = 0.34$, NS).

Figure 4.5.


A
NAc Histology
Bregma 1.42 mm
Bregma 1.34 mm
Bregma 1.18 mm
Bregma 1.10 mm
B
NAc Histology
Bregma 1.42 mm
Bregma 1.34 mm
Bregma 1.18 mm
Bregma 1.10 mm
C
CPP
Cocaine-paired side minus saline-paired side (sec)
WT
α4 -/-
Before
After
Conditioning
D
CPP
Cocaine-paired side minus saline-paired side (sec)
WT
α4 -/-
Before
After
Conditioning
E
CPP
Cocaine-paired side minus saline-paired side (sec)
WT
α4 -/-
Saline
THIP (3μM)
Saline
THIP (3μM)
Intra-NAc Infusion
Saline
Cocaine
Intraperitoneal Injection
F
CPP
Cocaine-paired side minus saline-paired side (sec)
WT
α4 -/-
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Saline
THIP (3mM)
Saline
THIP (3mM)
Intra-NAc Infusion
Saline
Cocaine
Intraperitoneal Injection
G
Locomotor Activity
Activity Counts
WT
α4 -/-
Drug Free
Saline
THIP (3μM)
Saline
THIP (3μM)
Intra-NAc Infusion
Saline
Cocaine
Intraperitoneal Injection
H
Locomotor Activity
Activity Counts
WT
α4 -/-
Drug Free
Saline
THIP (3mM)
Saline
THIP (3mM)
Intra-NAc Infusion
Saline
Cocaine
Intraperitoneal Injection

Fig 4.5. The effect of 3μM or 3mM intra-accumbal infusions of THIP/vehicle and intraperitoneal injections of cocaine (10mg/kg)/vehicle on cocaine-CPP in a 20 minute test. (**A**) Histology of intra-accumbal saline/THIP (3μM) infusions in WT (n=8) and $\alpha4^{-/-}$ (n=8) mice. (**B**) Histology of intra-accumbal saline/THIP (3mM) infusions in WT (n=8) and $\alpha4^{-/-}$ (n=8) mice. (**C & D**) WT and $\alpha4^{-/-}$ mice do not show any pre-conditioning preference for either chamber, but following conditioning equally demonstrate a preference for the cocaine-paired chamber in a drug-free test. (**E & F**) A cocaine challenge enhanced cocaine-CPP in both genotypes. Intra-accumbal infusion of 3μM or 3mM THIP had no effect on cocaine-CPP in both genotypes. An infusion of 3mM but not 3μM intra-accumbal THIP blocked the ability of a cocaine challenge to enhance cocaine-CPP in WT but not $\alpha4^{-/-}$ mice. (**G & H**) Cocaine potentiated locomotor activity equally in WT and $\alpha4^{-/-}$ mice. Intra-accumbal infusion of 3μM or 3mM THIP had no effect in either genotype on locomotor activity during cocaine-CPP or cocaine-potentiated cocaine-CPP. Error bars represent SEM. * $p < 0.01$ *post hoc* comparisons.

4.3.4. Conditioned Place Preference to Cocaine in Wildtype and Dopamine D1/D2-expressing neuron specific $GABA_AR$ α4-subunit Knockout Mice with Intraperitoneal THIP

$GABA_AR$ α4 subunits are expressed on both dopamine D1- or D2-expressing neurons within the NAc. To investigate possible pathway specific roles of α4 subunits in mediating cocaine-CPP, α4 subunits were knocked out in a cell-specific manner from cells expressing D1 or D2 dopamine receptors respectively.

All genotypes showed no pre-conditioning bias in preference for the two outer chambers (Fig 4.6.A; non significant main effect of genotype $F_{(1,18)} = 0.50$, $p = 0.48$, NS, Fig. 4.6.B; non significant main effect of genotype, $F_{(1,18)} = 1.35$, $p = 0.25$, NS). Subsequently, following place conditioning, WT mice ("floxed α4" mice) in these experiments performed in the same manner as WT mice in the previous experiments (Fig. 4.6.A&B) confirming that the manipulations of the gabra4 gene necessary to make the cell specific knockouts were silent (introduction of LoxP sites (Chandra *et al.*, 2006)). Interestingly, the cell specific knockouts did not perform in the same way as the constitutive knockouts. Mice with the α4 subunit selectively ablated from D1-expressing neurons ($\alpha4^{D1-/-}$) showed enhanced CPP to cocaine (Drug-free conditions, Fig. 4.6.A; significant main effect of conditioning $F_{(1,18)} = 79.71$, $p < 0.001$, significant conditioning by genotype interaction, $F_{(1,18)} = 6.61$, $p < 0.05$, Saline conditions, Fig. 4.6.C; significant main effect of conditioning $F_{(1,18)} = 55.38$, $p < 0.001$, significant conditioning by genotype interaction, $F_{(1,18)} = 9.86$, $p < 0.05$), whereas performance in the mice with the α4 subunit selectively ablated from D2-containing neurons ($\alpha4^{D2-/-}$) was unaffected (Drug-free conditions, Fig. 4.6.B; significant main effect of conditioning, $F_{(1,18)} = 54.35$, $p < 0.001$, non significant conditioning by genotype interaction, $F_{(1,18)} = 0.52$, $p = 0.47$, NS, Saline conditions, Fig. 4.6.D; significant main effect of conditioning, $F_{(1,18)} = 43.73$, $p < 0.001$, non significant conditioning by genotype interaction, $F_{(1,18)} = 1.08$, $p = 0.31$, NS).

A cocaine challenge enhanced cocaine-CPP in WT and $\alpha4^{D1-/-}$ mice (Fig. 4.6.C; main effect of drug, $F_{(1,18)} = 22.44$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,18)} = 1.12$, $p = 0.30$, NS). $\alpha4^{D2-/-}$ mice, on the otherhand showed no cocaine enhancement (Fig. 4.6.D; non-significant main effect of drug, $F_{(1,18)} = 3.85$, $p = 0.06$, NS, significant drug by genotype interaction, $F_{(1,18)} = 18.31$, $p < 0.001$). THIP (8mg/kg

i.p.) alone had no effect on expression of CPP in any genotype (Fig. 4.6.C; non-significant THIP by genotype interaction, $F_{(1,18)} = 0.05$, $p = 0.81$, NS, Fig. 4.6.D; non-significant THIP by genotype interaction, $F_{(1,18)} = 1.12$, $p = 0.30$, NS), however, when co-administered with cocaine, THIP was able to block cocaine-enhanced CPP in WT but not $\alpha4^{D1-/-}$ mice (Fig. 4.6.C; drug by THIP injection by genotype interaction, $F_{(1,18)} = 7.33$, $p < 0.01$). THIP in combination with cocaine had no effect on $\alpha4^{D2-/-}$ mice (Fig. 4.6.D; genotype by drug by THIP injection interaction, $F_{(1,18)} = 9.12$, $p < 0.01$).

Locomotor activity measured during the drug-free and saline CPP tests did not differ between any of the genotypes (Drug-free conditions, Fig 4.6.E; non-significant main effect of genotype, $F_{(1,18)} = 0.81$, $p = 0.37$, NS, Fig. 4.6.F; non-significant main effect of genotype, $F_{(1,18)} = 0.35$, $p = 0.55$, NS, Saline conditions, Fig 4.6.E; non-significant main effect of genotype, $F_{(1,18)} = 0.40$, $p = 0.84$, NS, Fig. 4.6.F; non-significant main effect of genotype, $F_{(1,18)} = 0.27$, $p = 0.60$, NS). A challenge injection of cocaine potentiated activity equally in all genotypes (Fig. 4.6.E; significant main effect of drug, $F_{(1,18)} = 77.56$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,18)} = 2.02$, $p = 0.17$, NS, Fig. 4.6.F: significant main effect of drug, $F_{(1,18)} = 88.50$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,18)} = 0.29$, $p = 0.59$, NS). Intraperitoneal injections of THIP (8mg/kg) did not effect locomotor activity during CPP in either genotype (Fig. 4.6.E; non-significant main effect of THIP, $F_{(1,18)} = 0.01$, $p = 0.89$, NS, non-significant THIP by genotype interaction, $F_{(1,18)} = 0.65$, $p = 0.43$, NS, Fig. 4.6.F; non-significant main effect of THIP, $F_{(1,18)} = 1.43$, $p = 0.24$, NS, non-significant THIP by genotype interaction, $F_{(1,18)} = 0.51$, $p = 0.48$, NS). Cocaine-potentiated locomotor activity was not significantly effected by THIP administration in either WT, $\alpha4^{D1-/-}$ or $\alpha4^{D2-/-}$ mice (Fig. 4.6.E; non-significant drug by THIP by genotype interaction, $F_{(1,18)} = 1.68$, $p = 0.21$, NS, Fig. 4.6.F; non-significant drug by THIP by genotype interaction, $F_{(1,18)} = 0.01$, $p = 0.93$, NS).

Figure 4.6.

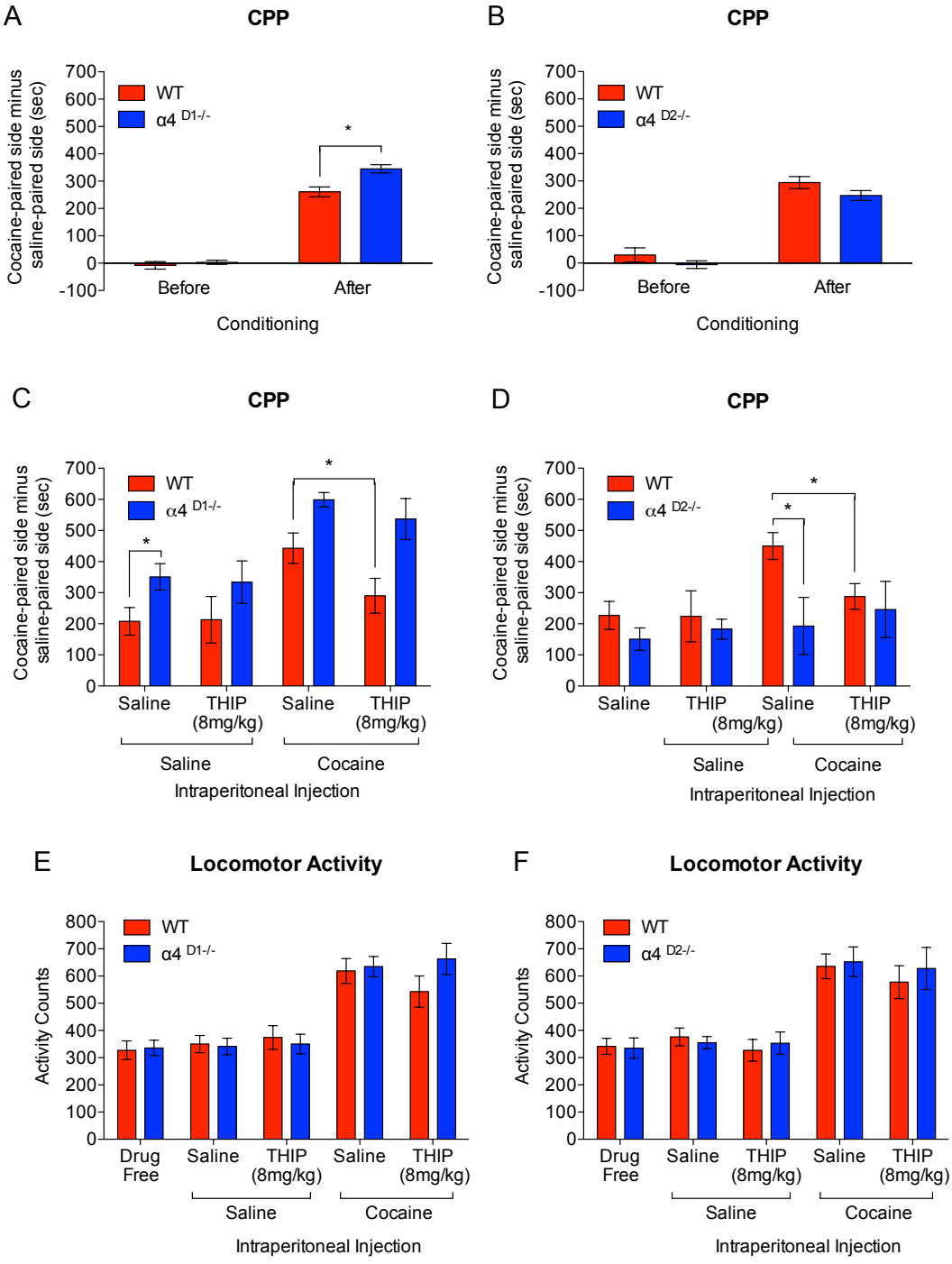


Fig 4.6. The effect of challenge injections of THIP (8mg/kg)/vehicle and cocaine (10mg/kg)/vehicle on cocaine-CPP in a 20 minute test. (**A**) WT (n=10) and $\alpha4^{D1-/-}$ (n=10) mice do not show any pre-conditioning preference for either chamber, but following conditioning $\alpha4^{D1-/-}$ mice show a preference for the cocaine-paired chamber greater than that of their WT counterparts in a drug-free test. (**B**) WT (n=10) and $\alpha4^{D2-/-}$ (n=10) mice do not show any pre-conditioning preference for either chamber, but

following conditioning equally demonstrate a preference for the cocaine-paired chamber in a drug-free test. (**C**) A cocaine challenge enhanced cocaine-CPP equally in both genotypes. Intraperitoneal THIP (8mg/kg) did not effect CPP in either genotype, but blocked cocaine enhancement of CPP in WT, but not $\alpha4^{D1-/-}$ mice. (**D**) Cocaine challenge enhancement of Cocaine-CPP seen in WTs was not present in $\alpha4^{D2-/-}$ mice. Intraperitoneal THIP (8mg/kg) did not effect CPP in either genotype, but blocked cocaine challenge enhancement of CPP in WT mice. (**E**) WT and $\alpha4^{D1-/-}$ mice did not show any differences in locomotor activity during CPP, and activity was equally potentiated in both genotypes by a cocaine challenge. THIP did not affect locomotor activity during CPP, or cocaine-enhanced CPP in either genotype. (**F**) WT and $\alpha4^{D2-/-}$ mice did not show any differences in activity during CPP, and were both equally potentiated by a cocaine challenge. THIP did not effect locomotor activity during CPP, or cocaine-enhanced CPP in either genotype. Error bars represent SEM. * $p < 0.01$ *post hoc* comparisons.

4.4. Discussion

In the current experiments both WT and constitutive $\alpha4^{-/-}$ mice show equivalent levels of cocaine-CPP suggesting $GABA_AR$ α4-subunits are not involved in the acquisition of cocaine-CPP. However, dopamine receptor D1- or D2-expressing MSNs of the *direct* or *indirect* striatal pathways, respectively, can exert opposing actions on certain psychostimulant-induced behaviours, including CPP and locomotor sensitisation (Hikida et al., 2010; Lobo et al., 2010; Beutler et al., 2011; Ferguson et al., 2011). During cocaine-CPP it appears that the balance between these opposing neuronal groups is shifted in favour of activation of the D1-containing *direct* pathway, thus eliciting a preference for the cocaine-paired chamber. The role of α4-subunits on these two pathways may be masked in the constitutive KO by the absence of the α4-containing $GABA_AR$ from all neurons. Therefore, cocaine-CPP was investigated in mice with the α4-subunit ablated from either D1- or D2-expressing neurons. Removal of α4-containing $GABA_AR$-mediated inhibition of D1-expressing cells resulted in an increased cocaine-CPP compared with WT controls, whereas similar removal from D2-expressing cells did not affect cocaine-CPP.

qRT-PCR analysis indicated that expression of α4-subunit mRNA was reduced by approximately 50% in the NAc of $\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$ mice when compared to respective WT control mice. Immunohistochemical evidence indicates that dopamine receptors D1 and D2 are largely segregated on MSNs within the NAc (Gerfen et al., 1990; Surmeier et al., 1996), with α4-subunits expressed equally on each neuronal type (Maguire et al, submitted). Thus, the extent of the reduction in α4-subunit mRNA expression observed in $\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$ mice is as might be expected from a deletion specifically from either dopamine receptor D1- or D2-expressing neurons. In $\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$ mice, expression of α2-subunit mRNA expression was increased, and δ-subunit mRNA reduced in the NAc, when compared to respective WT controls. However, γ2-subunit mRNA expression was unaltered in the NAc in $\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$ mice. These data indicate that the Gabra4 gene deletion was successful in dopamine D- or D2-expressing neurons and that these mice demonstrate similar changes to constitutive knockout mice in that there is an increase in α2-subunit mRNA expression and a decrease in δ-subunit mRNA expression.

Deletion of the α4-subunit specifically from D1-neurons facilitated conditioning, resulting in more time spent in the cocaine-paired chamber during the test phase. Thus, by increasing the excitability of D1-MSNs, through the removal of tonic GABAergic inhibition, mice showed greater CPP to cocaine. This result is in agreement with an optogenetic study demonstrating that activation of D1-MSNs during training enhances cocaine-CPP (Lobo et al., 2010), and supports a model of striatal processing suggesting that activation of the direct pathway is rewarding (Kravitz and Kreitzer, 2012). Given that optogenetic evidence suggests that activation of D2-MSNs attenuates cocaine-CPP (Lobo et al., 2010), it might be predicted that the removal of α4βδ $GABA_AR$-mediated tonic inhibition from D2-MSNs would produce a similar result, however this was not the case. Although $\alpha4^{D2-/-}$ mice appeared to show a reduced preference for the cocaine-paired chamber when compared to WT mice, this result was not significant. Thus it appears that the increased neuronal excitation produced by removal of inhibition from D2-MSNs following deletion of α4-containing $GABA_ARs$ is not sufficient to produce an attenuation of cocaine-CPP such as that reported when D2-MSNs are optogenetically stimulated (Lobo et al., 2010).

It has been reported that activation of α4βδ $GABA_ARs$ is able to attenuate the potentiation of certain behaviours, including locomotor activity (see Chapter 2, Fig.2.6. & Fig.2.8.) and conditioned reinforcement (CRf) responding (see Chapter 5, Fig.5.2.F, Fig.5.5.G. & Fig.5.5.H.) by cocaine. Therefore we tested expression of cocaine-CPP in the presence of a cocaine challenge. Previous studies have demonstrated that preference for a drug-paired environment can be extinguished and subsequently reinstated by drug priming injections (Parker and Mcdonald, 2000). Indeed, drug priming injections have been shown to reinstate CPP for amphetamine (Cruz et al., 2008), cocaine (Mueller and Stewart, 2000), nicotine (Biala and Budzynska, 2006), morphine (Parker and Mcdonald, 2000) and alcohol (Font et al., 2008), following extinction. However, studies exploring the effect of drugs administered during the CPP test have produced mixed findings. Morphine has been shown to enhance expression of morphine-CPP, and naltrexone, an opioid receptor antagonist, enhances expression of naltrexone-CPA (Bespalov et al., 1999). Similarly, methamphetamine enhances expression of methamphetamine-CPP, but only at high doses (Cunningham and Noble, 1992; Shabani et al., 2011). However, ethanol is reported to suppress expression of ethanol-CPP in DBA/2 mice, and have no effect on ethanol-CPP expression in NZB mice (Gremel and Cunningham, 2007). To

our knowledge, the current experiments are the first to show the effects of a cocaine challenge on expression of cocaine-CPP.

Cocaine administered during the test session enhanced cocaine-CPP in both WT and $\alpha4^{-/-}$ mice. This finding may be explained by the interoceptive properties of cocaine increasing the salience of the CPP. Previous evidence has indicated that the sensation caused by the drug stimuli is an important component that is conditioned to the CS during Pavlovian associative learning, and may enhance the retrieval of stimulus-response associations upon exposure to the CS in the drug-paired chamber (Cunningham and Noble, 1992; Bespalov et al., 1999). The ability of α4βδ $GABA_A$Rs to modulate enhancement of CPP expression by a cocaine challenge was further explored through pharmacological activation of these receptors by THIP. The acute effect of cocaine, to increase CPP, is blocked in WT mice by the application of THIP, both systemically and intra-accumbally. This suggests that although α4βδ $GABA_A$Rs do not participate in the cocaine enhancement per se, upon additional activation the increased tonic inhibition opposes that behaviour. The glutamatergic projection from the amygdala to the NAc is thought to provide information about affective states produced by a rewarding stimuli (Groenewegen et al., 1987; French and Totterdell, 2003; Di Ciano, 2004; Ambroggi et al., 2008). It is possible that activation of α4βδ $GABA_A$Rs by THIP may inhibit the arrival or processing of this information within the NAc, thus blocking the state-induced enhancement of cocaine-CPP by a cocaine challenge. This effect of THIP is mediated by α4βδ $GABA_A$Rs on D1-containing neurons as the agonist does not block the cocaine-enhancement in either constitutive $\alpha4^{-/-}$ mice or $\alpha4^{D1-/-}$ mice.

Interpretation of systemic THIP effects are complex as it will activate δ-$GABA_A$Rs expressed elsewhere (e.g. thalamus, hippocampus, cerebellum, ventral tegmental area) and at higher doses may engage additional $GABA_A$R isoforms (Mortensen et al., 2010). However, the effects of systemic THIP were demonstrated to be specific to α4 subunit-containing $GABA_A$Rs as the suppression of cocaine enhanced CPP evident in WT, is absent in $\alpha4^{-/-}$ mice. Furthermore, infusion of THIP directly into the NAc was equally effective in blocking the cocaine enhancement of CPP in WT, but not $\alpha4^{-/-}$ mice, revealing that the principal site of action of systemic THIP in our behavioural studies is α4βδ $GABA_A$Rs of the NAc.

These data indicate that α4βδ-$GABA_A$Rs of D1-MSNs may act as an immediate homeostatic control to prevent excessive neuronal excitation by dopamine, as their activation blocks cocaine enhancement of cocaine-CPP. The actions of dopamine on D1-MSNs are complex, causing both complementary excitatory, but additionally opposing inhibitory effects. D1-receptors stimulate G_s and G_{olf} proteins, which stimulate adenylyl cyclase, increasing intracellular levels of cyclic adenosine monophosphate (cAMP) and activating protein kinase A (PKA). Activation of D1-receptors and PKA increases Cav1 L-type calcium channel currents and decreases somatic potassium channel currents, as well as enhancing AMPA and NMDA receptor function and trafficking (Gerfen and Surmeier, 2011). However, D1 receptor activation also reduces the availability of voltage-gated sodium channels to conduct (Gerfen and Surmeier, 2011). Thus, even at the level of a single D1-MSN, there are multiple homeostatic control mechanisms. Interestingly, recent evidence has also demonstrated that the magnitude of the GABAergic tonic current in D1-MSNs is dynamically increased by D1 receptor activation, achieved either directly by a selective dopamine receptor D1 agonist, or indirectly by amphetamine (Maguire et al, submitted), thereby presumably limiting the excitatory effects of dopamine. The enhanced tonic current is unlikely to be caused indirectly (e.g. by changes of GABA release), but probably occurs within the D1-MSN, as it was prevented by blockade of G-protein coupling by intracellular GDP-βs (Maguire et al, submitted). By contrast, prolonged, but not acute, D2 receptor activation caused a modest decrease of the tonic conductance of D2-MSNs (Maguire et al, submitted). A similar differential effect of dopamine receptor activation on the tonic conductance of D1- and D2-MSNs occurs in the dorsal striatum, reflecting the distinct effects of these G-protein coupled receptors on PKA activity (Janssen et al., 2009). Furthermore, chronic cocaine produces an up-regulation of the Gabra4 gene, encoding for α4-subunits, specifically in D1-MSNs (Heiman et al., 2008). This observation suggests that in addition to providing a short term homeostatic role, when excessively stimulated, expression of these opposing α4βδ-$GABA_A$Rs may be increased, thereby strengthening an intrinsic “brake” on these D1-MSNs.

Interestingly, in $\alpha4^{D2-/-}$ mice, there is no cocaine enhancement of cocaine-CPP. This suggests an important role of tonic inhibition of the indirect pathway in mediating the

cocaine enhancement. As previously described, D2 receptor activation results in a decrease in the D2-MSN tonic GABA current and is likely to increases their excitability (Maguire et al, submitted). The absence of α4βδ-$GABA_A$Rs should further disinhibit D2-MSNs, therefore increasing activity of the *indirect* pathway. Acute cocaine appears to enhance the expression of cocaine-CPP by an action of dopamine at D1-MSNs, therefore given the typically opposing roles of striatal pathways, activation of D2-MSNs might be hypothesised to oppose cocaine enhancement of cocaine-CPP. Indeed, dopamine acting at D2-receptors triggers an intracellular signaling cascade that is ultimately thought to inhibit the cell, and thus would act in partnership with D1-MSN activation to enhance the CPP effect. Thus, absence of α4-$GABA_A$R-mediated inhibition in D2-MSNs may override the inhibition of D2-MSNs normally caused by cocaine-induced dopamine release, resulting in $\alpha4^{D2-/-}$ mice not showing the cocaine enhancement of cocaine-CPP. Deletion of α4-$GABA_A$Rs from D2-MSNs presumably also blocks the effect of cocaine-induced dopamine acting at D1-MSNs to enhance cocaine-CPP. A mechanism for the action of D2 excitation on D1 MSN activity whether might be a direct inhibitory effect of D2-MSN collaterals onto D1-MSNs (Taverna et al., 2004), or via an indirect route. However, the D2-MSN α4βδ-$GABA_A$R effect on cocaine enhancement of preference must occur upstream of the D1-MSN effect as when D1-MSNs cannot be inhibited (in the constitutive KO), the absence of receptors from D2 MSNs does not impact on the phenotype: the $\alpha4^{D1-/-}$ phenotype predominates in the constitutive α4 knockout.

Neither systemic or intra-accumbal THIP alone, affected the expression of cocaine-CPP in WT and constitutive $\alpha4^{-/-}$ mice in the absence of a cocaine challenge. It is possible that the effects of inhibition of both D1- and D2-MSNs in WT mice may cancel each other out and result in no overall change in the expression of cocaine-CPP. However, THIP alone had no effect on preference in the $\alpha4^{D1-/-}$ or $\alpha4^{D2-/-}$ mice. It is still unknown what effect activation of α4βδ-$GABA_A$Rs by THIP may have on the acquisition of cocaine-CPP. It is possible that activation of α4βδ-$GABA_A$Rs by THIP during training may block the conditioning of cocaine place preference.

It has previously been reported that THIP can reduce baseline and cocaine-potentiated

locomotor activity when given i.p. or intra-accumbally (see Chapter 2, Fig.2.6. & Fig.2.8. and Herd et al., 2009; Vashchinkina et al., 2012. This may confound the interpretation of the reported CPP effects as sedation may inhibit the ability of the animal to freely move between chambers, thus increasing or reducing the apparent 'place preference'. Indeed, some studies have reported locomotor activity can be inversely proportional to CPP magnitude (Gremel and Cunningham, 2007), and so a small sedative effect of THIP might be expected to increase CPP. In the current studies, a sedative effect of i.p. or intra-accumbal THIP was not detected. The discrepancy between these findings and those reported previously (see Chapter 2, Fig.2.6. & Fig.2.8.) may be explained by a lack of sensitivity in the method used to record locomotor activity in the CPP apparatus. In the CPP boxes locomotion is detected in just one dimension, using beam breaks. While the narrow locomotor runways used in previous experiments (see Chapter 2, Fig.2.6. & Fig.2.8.) are designed specifically to measure locomotor activity and the tracking software provides a more accurate analysis of activity.

To conclude, these data indicate that α4βδ-$GABA_A$R-mediated inhibition of NAc MSNs plays an important role in modulating cocaine-CPP and its enhancement by a cocaine challenge. Deletion of the α4 subunit selectively from D1-MSNs resulted in greater cocaine-CPP, presumably reflecting an increase in D1-MSN excitability. Furthermore, when these $GABA_A$Rs are directly activated by THIP, they suppress cocaine enhancement of the CPP effect. Thus α4βδ-$GABA_A$Rs modulation of MSN excitability plays a role in regulating dopamine's effects in the NAc. By increasing α4βδ-$GABA_A$Rs inhibition of D1-MSNs, dopamine counteracts its own excitatory actions, stabilizing MSN output. The multiple actions of dopamine on MSNs allows downstream tuning of the dopamine signal.

Chapter 5

The role of α4-containing $GABA_A$ receptors in food conditioned reinforcement and its potentiation by cocaine

5.1. Introduction

In the previous chapter it was described how neutral stimuli can acquire incentive motivational properties following Pavlovian association with an unconditioned rewarding stimulus, such as cocaine, and can subsequently elicit approach responses. Appetitive Pavlovian conditioned stimuli (CS) can also act as conditioned (secondary) reinforcers to control and maintain instrumental behaviour in the absence of the unconditioned (primary) rewarding stimulus (US), a process known as conditioned reinforcement (CRf). Understanding such appetitive processes and the neural substrates underlying them will be critical for elucidating mechanisms of learning and motivation, and their maladaption in drug addiction.

Much work has been done to resolve the neuroanatomical substrates of CRf, and the NAc, once again, appears to be a key integrating centre for excitatory inputs carrying information about stimulus-reward associations. Interestingly, there is a functional dissociation between NAc subregions in terms of the relative roles they play in mediating conditioned behaviours. Lesions of the NAc core impair Pavlovian associative learning, as well as reducing the associative control over specific instrumental responding (for a reward-paired vs non-rewarding reinforcer) following intra-accumbal amphetamine potentiation of CRf (Parkinson et al., 1999; Kravitz and Kreitzer, 2012). Conversely, lesions of the NAc shell do not affect Pavlovian or instrumental conditioning (Hall et al., 2001; Lobo et al., 2010), but are able to completely abolish the potentiating effects of intra-accumbal amphetamine on responding with CRf (Parkinson et al., 1999; Lobo et al., 2010). Thus, the NAc core is thought to be integral for instrumental learning and behavioural responses to incentive-motivational conditioned stimuli (Kelley et al., 1997; Bassareo and Di Chiara, 1999; Parkinson et al., 1999; Ito et al., 2000; Parker and Mcdonald, 2000; Parkinson et al., 2000). Whereas the NAc shell is implicated in the primary rewarding effects of unconditioned stimuli, as well as well as the potentiative effects of psychostimulants

(Chiara et al., 1993; Pontieri et al., 1995; Bassareo and Di Chiara, 1999; Cruz et al., 2008).

The NAc core receives efferent projections from the basolateral amygdala (BLA) that are reported to be critical for instrumental behaviour in response to Pavlovian stimulus-reward associations (Everitt et al., 2000; Mueller and Stewart, 2000). Excitotoxic lesions of the BLA impair responding for conditioned reinforcers, but do not affect Pavlovian conditioning (Everitt and Robbins, 1992; Gallagher and Holland, 1994; Everitt et al., 1999; Biala and Budzynska, 2006). Furthermore, BLA-lesioned rats fail to spontaneously adjust their responding to the CS after reinforcer devaluation (Hatfield et al., 1996; Parker and Mcdonald, 2000). Thus, the BLA is suggested to underlie the ability to use the CS to access the value of a specific US to guide behavioural responses (Everitt et al., 2003; Font et al., 2008).

Much less clear is the role that hippocampal projections to the NAc play in mediating CRf. Inactivation of the hippocampus by tetrodotoxin is known to block reinstatement for cocaine seeking by contextual cues (Bespalov et al., 1999; Fuchs et al., 2004), and lesions of the dorsal hippocampus block acquisition of food- and cocaine-induced CPP (Cunningham and Noble, 1992; Meyers et al., 2003; 2006; Shabani et al., 2011). These data support the notion that the hippocampus mediates conditioning for contextual or spatial stimuli, whereas the amygdala underlies conditioning to discrete CS (Selden et al., 1991; Gremel and Cunningham, 2007). It has been hypothesised that the hippocampal contextual information and amygdala discrete CS information may compete within the NAc for control over goal-directed behaviour (Cunningham and Noble, 1992; Bespalov et al., 1999; Everitt and Robbins, 2005). Indeed, selective lesions of the hippocampus not only disrupt behavioural responses under the control of contextual cues, but facilitate control by discrete cues, with the reverse being observed following amygdala lesions (Groenewegen et al., 1987; French and Totterdell, 2003; Di Ciano, 2004; Ito et al., 2006; Ambroggi et al., 2008). Interestingly, lesions of the hippocampus block the ability of intra-accumbens amphetamine to potentiate responding for CRf, suggesting that the hippocampal projection to the NAc shell may also act to mediation of dopaminergic tone (Burns et al., 1993; Ito et al., 2004; Everitt and Robbins, 2005; Mortensen et al., 2010).

The importance of NAc dopamine transmission in appetitive conditioning is confirmed by evidence that dopamine depletion of the NAc impairs the acquisition and expression of Pavlovian approach (Parkinson et al., 2002; Gerfen and Surmeier, 2011). It appears that dopamine is also implicated in mediating instrumental responses to conditioned stimuli as intra-accumbens dopamine or amphetamine produce dose-dependent increases responding with CRf (Cador et al., 1991; Gerfen and Surmeier, 2011). This responding was similarly blocked by dopamine depletion in the ventral, but not the dorsal striatum (Taylor and Robbins, 1986; Janssen et al., 2009). Interestingly, CRf responding is also dose dependently potentiated by systemic administration of dopamine receptor D2 agonists quinpirole and bromocriptine, but not D1 agonist SKF 38393 (Beninger et al., 1989; Beninger, 1992; Heiman et al., 2008). Indeed, D1 agonists SKF 82958, SKF 81297, SKF 77434 and CY 208-243 have been reported to impair responding for a conditioned reinforcer (Beninger and Rolfe, 1995; Taverna et al., 2004). However, when infused directly into the NAc, both D1 (SKF 38393) and D2 (quinpirole) agonists are able to potentiate CRf responding, and intra-accumbal D1 antagonist SCH 23390 and D2 antagonist raclopride block intra-accumbal amphetamine-induced potentiation of CRf responding (Wolterink et al., 1993; Herd et al., 2009; Vashchinkina et al., 2012). Furthermore, systemic administration of D1 antagonist SCH 23390 or D2 antagonist raclopride during Pavlovian training, increase or decrease, respectively, responding for the conditioned reinforcer on a drug-free test day (Eyny and Horvitz, 2003; Gremel and Cunningham, 2007). Although the specificity of current dopamine D1 and D2 receptor agonists/antagonists has been questioned (Gerfen and Surmeier, 2011), these data indicate that both D1 and D2 receptors may be involved in mediating CRf responding and psychostimulant potentiation of CRf responding (summarised in Table 5.1.).

Table 5.1.

	CRf responding		Psychostimulant potentiation of CRf responding	
	Systemic	**Intra-accumbal**	**Systemic**	**Intra-accumbal**
D1 agonist	No change/ decreased (Beninger et al., 1989; Beninger and Ranaldi, 1992)	Increased (Wolterink et al., 1993)	Decreased potentiation (Ranaldi et al., 1995)	?
D2 agonist	Increased (Beninger et al., 1989; Beninger and Ranaldi, 1992)	Increased (Wolterink et al., 1993)	?	?
D1 antagonist	No change/ decreased (Beninger et al., 1987; Sanger, 1987; Beninger et al., 1989)	?	?	Decreased potentiation (Wolterink et al., 1993)
D2 antagonist	Decreased (Beninger et al., 1987; 1989)	?	?	Decreased potentiation (Wolterink et al., 1993)

Table 5.1. The effect of systemic or intra-accumbal administration of dopamine D1 or D2 receptor agonists and antagonists on CRf responding, and psychostimulant-potentiated CRf responding.

As previously described, the NAc is an area predominantly expressing GABAergic neurons, however it is still unclear what role GABA and $GABA_ARs$ in the NAc may play in controlling appetitive conditioning and instrumental responding for conditioned reinforcers. Given the multitude of inputs onto NAc MSNs vying for control over goal-directed behaviour, it has been hypothesised that GABAergic inhibition may function to supress the activity of unwanted competing neurons (Taverna et al., 2004; Nicola, 2006). *In-vivo* electrophysiological studies demonstrate that NAc neuronal firing is correlated with the onset of reward-conditioned stimuli, as well as for instrumental responses for conditioned reinforcers (Nicola et al., 2004). Thus it is clear that subpopulations of NAc neurons encode the predictive value of reward-paired cues, and the instrumental behaviours required to respond to them (Nicola et al., 2004). Interestingly, response to reward-related stimuli is also associated with inhibition of a subset of accumbens neurons during appetitive behaviors (Nicola et al., 2004; Taha and Fields, 2006). It is suggested that sustained inhibition of NAc MSNs, perhaps through activation of fast-spiking GABAergic interneurons (Tepper et al., 2004), disinhibits target regions, permissively gating and maintaining appetitive behaviors (Taha and Fields, 2006). However, infusion of the $GABA_AR$ agonist muscimol directly into the NAc does not affect second-order instrumental responding for food rewards (Pulman et al., 2012). Similarly, deletion of $GABA_AR$ α2-subunits, the most widely found $GABA_AR$ subunit within the NAc (Pirker et al., 2000), does not affect Pavlovian conditioning or instrumental responding for CRf (Dixon et al., 2010). However, interestingly, α2-subunit knockout mice fail to show a potentiation of CRf responding by systemic cocaine administration (Dixon et al., 2010).

Here the effects of deletion of $GABA_ARs$ α4-subunits on Pavlovian conditioning, instrumental responding for conditioned reinforcers and potentiation of CRf by systemic cocaine are explored. Subsequently, α4βδ $GABA_ARs$ within the NAc were activated using intra-accumbal THIP during expression of CRf and cocaine-potentiated CRf in wildtype and α4-subunit knockout mice. To confirm the involvement of α4βδ $GABA_ARs$ within the NAc, CRf and its potentiation by cocaine were repeated using control and NAc-specific α4-subunit viral knockdown mice. Finally, mice in which α4-subunit were specifically ablated in either dopamine D1- or D2-expressing neurons were used to explore striatal pathway-specific effects of α4-$GABA_ARs$ (α4-$GABA_ARs$)

in Pavlovian conditioning and CRf. Intra-accumbal THIP was similarly used to specifically activate NAc α4βδ $GABA_A$Rs in these mice.

5.2. Materials and Methods

5.2.1. Animals

$GABA_AR$ α4 wildtype (WT) and knockout ($\alpha4^{-/-}$), and dopamine D1-/D2-specific α4 wildtype (WT) and knockout ($\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$) mice were on a C57Bl/6J background strain. WT littermate controls were used for each knockout line. C57Bl/6J mice (Charles River Laboratories, Kent, UK) were used for virus experiments. All mice weighed between 20-30g, were housed in groups of 2-3, or separately for surgery animals, with food and water available *ad libitum*. A 12hr light/dark cycle was used (lights on at 7:00 A.M.) with holding room temperature maintained at 21±2ºC and humidity 50±5%. All injections, infusions and behavioural testing were performed between 2:00 P.M. and 5:00 P.M. All procedures were conducted in accordance to Animals (Scientific Procedures) Act 1986, following ethical review by the University of Sussex Ethical Review Committee.

5.2.2. Stereotaxic Cannulation.

Mice anaesthetised with isoflurane were implanted stereotaxically with bilateral guide cannulae (26 ga., 10mm) aimed at NAcc (coordinates AP1.34; L+/− 1.00; DV −3.20, (Paxinos and Franklin, 2001)). Following surgery, mice were singly housed and underwent a one-week recovery/habituation period. A steel infuser (33 ga., 11 mm) connected *via* polyvinyl tubing to a (5 µl) Hamilton Gastight syringe was used to infuse 0.5µl of either saline or THIP (3 mM) bilaterally across 90 seconds and left to settle for 90 seconds before infusers were removed. Location of cannulae was confirmed histologically. Two mice were removed from data analysis due to inexact cannulae placement.

5.2.3. Stereotaxic Viral Infusion

C57BL/6J mice anesthetised with isoflurane were stereotaxically infused with Ad-NSS or Ad-shα4 (Rewal *et al.*, 2009), bilaterally into the NAc (coordinates AP1.34; L+/− 1.40; DV −4.20, (Paxinos and Franklin, 2001)). A steel infuser (33ga) connected via polyvinyl tubing to a (5µl) Hamilton Gastight syringe was used to infuse 1ul (0.5µl per side) of virus at a rate of 0.2µl/min for 5 minutes, then left to settle for an additional 5 minutes. Following surgery mice were singly housed and allowed to recover for 7 days. Both viruses contained GFP under control of the CMV promoter allowing location of

infusion to be confirmed using immunohistochemistry. Ad-NSS and Ad-shα4 adenoviruses were kindly donated by Patricia Janak (Ernest Gallo Clinic).

5.2.4. Immunohistochemistry

Mice brains were perfused via the aorta with 25ml (5 minutes of 5ml/min) of phosphate buffer solution (PBS) followed by 75ml (15 minutes of 5ml/min) of 4% paraformaldehyde (PF) (Sigma-Aldrich, St. Lois, MO, USA) in PBS. After perfusion, brains were removed and post-fixed overnight in 4% PF in PBS at 4°C, then transferred into 30% sucrose solution in PBS and left overnight again at 4°C. Coronal sections (60μm thick) were cut on a microtome and collected in PBS.

Free-floating sections were incubated in 50% alcohol, rinsed twice in PBS for 1 minute each with gentle agitation, and then incubated in blocking solution (normal donkey serum 10% in PBS, pH 7.4) for 1 hour at room temperature with gentle agitation. Slices were washed twice in PBS for 1 minute each with gentle agitation, then incubated overnight in rabbit anti-GFP polyclonal primary antibody (1:10,000, Abcam, Cambridge, UK). Slices were again washed twice in PBS for 1 minute each with gentle agitation, and then incubated for two hours in donkey anti-rabbit secondary antibody (1:3000, Jackson ImmunoResearch, West Grove, PA, USA). Following two final washes in PBS for 2 minutes each with gentle agitation, sections were mounted on Superfrost plus microscope slides (Fisher, MA, USA) and air-dried.

Images were acquired using a confocal laser scanning microscope (LSM) (Zeiss, Jena, Germany) and visualized using LSM software (Zeiss, Jena, Germany).

5.2.5. Quantitative reverse transcription PCR (qRT-PCR) RNA analysis

qRT-PCR was conducted according to the protocol previously described (see Chapter 2; 2.2.6.). Tissue punches from the NAc and dorsal striatum were collected and processed 10 days post-infusion for measuring $GABA_AR$ α4 mRNA levels in Ad-NSS control adenovirus-infused mice and Ad-shα4 adenovirus-infused α4-subunit knockdown mice. Untreated mice, which did not undergo any surgery, were also used as a control measure. GAPDH was used as an internal control (see α4 and GAPDH primer sequences described in Chapter 2; Table 2.1.).

5.2.6. Drugs

Cocaine Hydrochloride was obtained from Macfarlan Smith (Edinburgh, UK). THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) was kindly donated by Bjarke Ebert (Lundbeck, Valby, Denmark). Both drugs were dissolved in 0.9% saline, and administered i.p. at an injection volume of 10 ml/kg.

5.2.7. Apparatus

Conditioned reinforcement was measured using 8 operant chambers (Med Associates Inc, Vermont, USA), each housed within a light-resistant, sound-attenuating cubicle. Each unit consisted of two nose-poke inputs and a food magazine delivering 20mg sweetened pellets (5TUL, Cat no. 1811142; Test Diets, Indiana, USA). Head entries into the food magazine were detected using an infrared beam. A tone generator (2.9 KHz, 5 dB above background) was located above the food magazine, and two LED stimulus lights positioned on the opposite wall.

5.2.8. Pavlovian Conditioning

Following food deprivation to maintain approximately 85% of baseline body weight, mice underwent 10 consecutive daily 60 minute Pavlovian training sessions during which they were presented with the two stimuli, 16 presentations of a 10-second tone and 16 presentations of 10-second LED flashing lights. One was always associated with a food reward (CS+), and the other with no outcome (CS-). The order of stimulus presentations was randomly determined and each stimulus trial was separated by a variable, no stimulus, intertrial interval (ITI) (range of 80-120 seconds; mean (M) = 100 seconds). A single food pellet delivery occurred 5 seconds after CS+ onset. Food magazine entries during presentation of each stimulus trial (CS+ or CS-) were expressed as a percentage of total magazine entries during the session (CS+ + CS- + ITI) to give a measure of Pavlovian conditioning.

5.2.9. Conditioned Reinforcement (CRf)

On completion of Pavlovian conditioning, two nose-poke inserts were added to the operant chamber, each triggering presentation of either the CS+ or the CS-. Rates of nose-poke responses for the CS+ and CS- were measured over 60 minute sessions.

5.2.9.1. Conditioned Reinforcement in Wildtype and $GABA_AR$ α4-Subunit Knockout Mice with Cocaine Potentiation Dose-Response

Rates of nose-poke responses were recorded following i.p. cocaine (0, 3, 10, 30 mg/kg, test sessions on consecutive days in a latin square design) in WT and $\alpha4^{-/-}$ mice.

5.2.9.2. Conditioned Reinforcement in Wildtype and $GABA_AR$ α4-Subunit Knockout Mice with Cocaine Potentiation and Intra-accumbal THIP

Mice underwent stereotaxic surgery to insert guide cannulae bilaterally into the NAc, and following recovery received 10 days of Pavlovian conditioning. Cannulated WT and $\alpha4^{-/-}$ mice then underwent four test days (in a latin square design, with a day off between test days) during which they were administered intra-accumbal infusions of either saline or THIP (3μM or 3mM), followed by an i.p. injection of saline or cocaine (10mg/kg), directly prior to testing.

5.2.9.3. Conditioned Reinforcement in Scrambled Virus Control (Ad-NSS) and α4 Adenoviral Knockdown (Ad-shα4) Mice with Cocaine Potentiation and Intraperitoneal THIP

Mice underwent stereotaxic surgery to infuse Ad-shα4 adenovirus or Ad-NSS scrambled control adenovirus bilaterally into the NAc. Following recovery for 7 days, all mice underwent 10 days of Pavlovian conditioning. Control and α4-knockdown mice then underwent four test days (in a latin square design, on consecutive days) during which they were administered i.p. injections of either saline or THIP (8mg/kg) 20 minutes prior to an i.p. injection of saline or cocaine (10mg/kg) directly prior to testing. The CRf tests were conducted on days 18-21 post-infusion of the viruses, previously demonstrated to be a behaviourally relevant timescale for this virus (Rewal et al., 2009).

5.2.9.4. CRf in Wildtype and $\alpha4^{D1}$ and $\alpha4^{D2}$ Knockout Mice with Cocaine Potentiation and Intra-accumbal THIP

Following stereotaxic surgery, cannulated WT, and conditional knockout ($\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$) mice, were given 10 days of Pavlovian training and then underwent four CRf test days, during which they were administered intra-accumbal infusions of saline or THIP (3uM or 3mM) 20 min prior to an i.p. injection of saline or cocaine (10mg/kg), directly prior to testing (in a latin square design, with a day of between test days).

5.2.10. Statistical Analysis

5.2.10.1. Conditioned Reinforcement in Wildtype and $GABA_AR$ α4-Subunit Knockout Mice with Cocaine Potentiation Dose-Response

Pavlovian conditioning data were assessed in WT and $\alpha4^{-/-}$ mice using a four-way mixed-factors ANOVA, with genotype as the between-subjects factor, conditioned stimulus (CS+ or CS-) and session as the within-subjects factors, and magazine entries made during the first five seconds of conditioned stimulus presentation as the dependent variable. The dose-response of cocaine-potentiation of CRf data were analysed using a four-way mixed-factors ANOVA, with genotype as the between-subjects factor, conditioned stimulus and drug dose as the within-subjects factors, and nose-poke responses as the dependent variable.

5.2.10.2. Conditioned Reinforcement in Wildtype and $GABA_AR$ α4-Subunit Knockout Mice with Cocaine Potentiation and Intra-accumbal THIP

Pavlovian conditioning data were assessed in WT and $\alpha4^{-/-}$ mice using four-way mixed-factors ANOVAs, with genotype as the between-subjects factor, conditioned stimulus and session as the within-subjects factors, and magazine entries made during the first five seconds of conditioned stimulus presentation as the dependent variable. CRf data were analysed independently for each THIP dose using five-way mixed-factors ANOVAs, with genotype as the between-subjects factor, conditioned stimulus, infusions and injections as the within-subjects factors, and nose-poke responses as the dependent variable. *Post hoc* analyses were conducted where appropriate using paired t-tests.

5.2.10.3. RNA Analysis of Ad-shα4 Adenovirus Knockdown of $GABA_AR$ α4-Subunits

Quantitative RT-PCR data were analysed using a three-way mixed-factors ANOVA, with virus group (Ad-NSS, Ad-shα4 or untreated) as the between-subjects factor, brain region (NAc or dorsal striatum) as the within-subjects variable, and the GAPDH-controlled α4-subunit mRNA expression delta ct (see explanation in Chapter 2, 2.2.10.2.) as the dependent variable.

5.2.10.4. Conditioned Reinforcement in Scrambled Virus Control (Ad-NSS) and α4 Adenoviral Knockdown (Ad-shα4) Mice with Cocaine Potentiation and Intraperitoneal THIP

Pavlovian conditioning data were assessed in control and α4-subunit knockdown mice using a four-way mixed-factors ANOVA, with virus group as the between-subjects factor, conditioned stimulus and session as the within-subjects factors, and magazine entries made during the first five seconds of conditioned stimulus presentation as the dependent variable. CRf data were analysed using a five-way mixed-factors ANOVA, with virus group as the between-subjects factor, conditioned stimulus, drug injection and THIP injection as the within-subjects factors, and nose-poke responses as the dependent variable. *Post hoc* analyses were conducted where appropriate using paired t-tests.

5.2.10.5. Conditioned Reinforcement in Wildtype and $\alpha4^{D1}$ / $\alpha4^{D2}$ Knockout Mice with Cocaine Potentiation and Intra-accumbal THIP

Pavlovian conditioning data were assessed in WT and conditional knockout ($\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$) mice using four-way mixed-factors ANOVAs, with genotype as the between-subjects factor, conditioned stimulus and session as the within-subjects factors, and magazine entries made during the first five seconds of conditioned stimulus presentation as the dependent variable. CRf data were analysed independently for each THIP dose using five-way mixed-factors ANOVAs, with genotype as the between-subjects factor, conditioned stimulus, infusions and injections as the within-subjects factors, and nose-poke responses as the dependent variable. *Post hoc* analyses were conducted where

appropriate using paired t-tests. The conditional knockout lines with their littermate WT controls were each tested in independent experiments.

5.3. Results

5.3.1. Conditioned Reinforcement in Wildtype and $GABA_AR$ α4-Subunit Knockout Mice with Cocaine Potentiation Dose-Response

Both wildtype and α4-subunit knockout mice demonstrated a similar ability to learn the reward predictive properties of the CS+ as assessed by increased approaches to the food delivery chamber on CS+ presentation (Fig. 5.1.A; significant conditioned stimulus by session; $F_{(9,162)} = 36.42$, $p < 0.001$; non-significant session by genotype interaction; $F_{(9,162)} = 0.32$, $p = 0.96$, NS). Similarly, both genotypes accurately learned to elicit presentation of the cues via nose-poke responding, demonstrating robust conditioned responses (Fig. 5.1.B; significant main effect of conditioned stimulus, $F_{(1,18)} = 334.36$, $p < 0.001$). However, in comparison to their WT counterparts, $\alpha4^{-/-}$ mice displayed increased instrumental responding (Fig. 5.1.B; significant conditioned stimulus by genotype interaction; $F_{(1,18)} = 36.78$, $p < 0.001$). Administration of cocaine dose-dependently potentiated instrumental responding for the conditioned reinforcer equally across both genotypes, amplifying the initial cocaine-free pattern of responding (Fig. 5.1.C; significant conditioned stimulus by drug dose interaction, $F_{(3,51)} = 23.97$, $p < 0.001$, non-significant conditioned stimulus by drug dose by genotype interaction; $F_{(3,51)} = 1.99$, $p = 0.12$, NS).

5.3.2. Conditioned Reinforcement in Wildtype and $GABA_AR$ α4-Subunit Knockout Mice with Cocaine Potentiation and Intra-accumbal THIP

Following surgery, mice underwent 10 days Pavlovian training. As previously seen, WT and $\alpha4^{-/-}$ mice learnt the reward predictive properties of the CS+ to a similar degree, as assessed by increased approaches to the food delivery chamber on CS+ presentation (3μM THIP group, Fig 5.2.C; significant conditioned stimulus by session; $F_{(9,243)} = 67.69$, $p < 0.001$; non-significant session by genotype interaction; $F_{(9,243)} = 1.38$, $p = 0.19$, NS, 3mM THIP group, Fig 5.2.D; significant conditioned stimulus by session; $F_{(9,126)} = 24.27$, $p < 0.001$; non-significant session by genotype interaction; $F_{(9,126)} = 0.36$, $p = 0.95$, NS).

As previously seen, $\alpha4^{-/-}$ mice displayed increased instrumental responding in comparison to WT mice (Fig. 5.2.E; significant conditioned stimulus by genotype interaction; $F_{(1,28)} = 36.78$, $p < 0.001$, Fig. 5.2.F; significant conditioned stimulus by

genotype interaction; $F_{(1,14)} = 83.34$, $p < 0.001$). Similarly, as previous observed, a cocaine (10mg/kg) challenge potentiated the initial pattern of responding to a similar degree in WT and $\alpha4^{-/-}$ mice (Fig. 5.2.E; significant main effect of drug, $F_{(1,28)} = 50.14$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,28)} = 0.35$, $p = 0.55$, NS, Fig. 5.2.F; significant main effect of drug, $F_{(1,14)} = 81.68$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,14)} = 4.23$, $p = 0.06$, NS).

Local infusion of 3μM or 3mM THIP into the NAc via indwelling bilateral cannulae did not alter baseline CRf responding, however, 3mM but not 3μM THIP was able to decrease cocaine-potentiated responding in WT but not $\alpha4^{-/-}$ mice (3μM THIP, Fig. 5.2.E; significant conditioned stimulus by infusion by injection by genotype interaction; $F_{(1,28)} = 2.78$, $p = 0.11$, NS, 3mM THIP, Fig. 5.2.F; significant conditioned stimulus by infusion by injection by genotype interaction; $F_{(1,14)} = 20.63$, $p < 0.001$).

Figure 5.1.

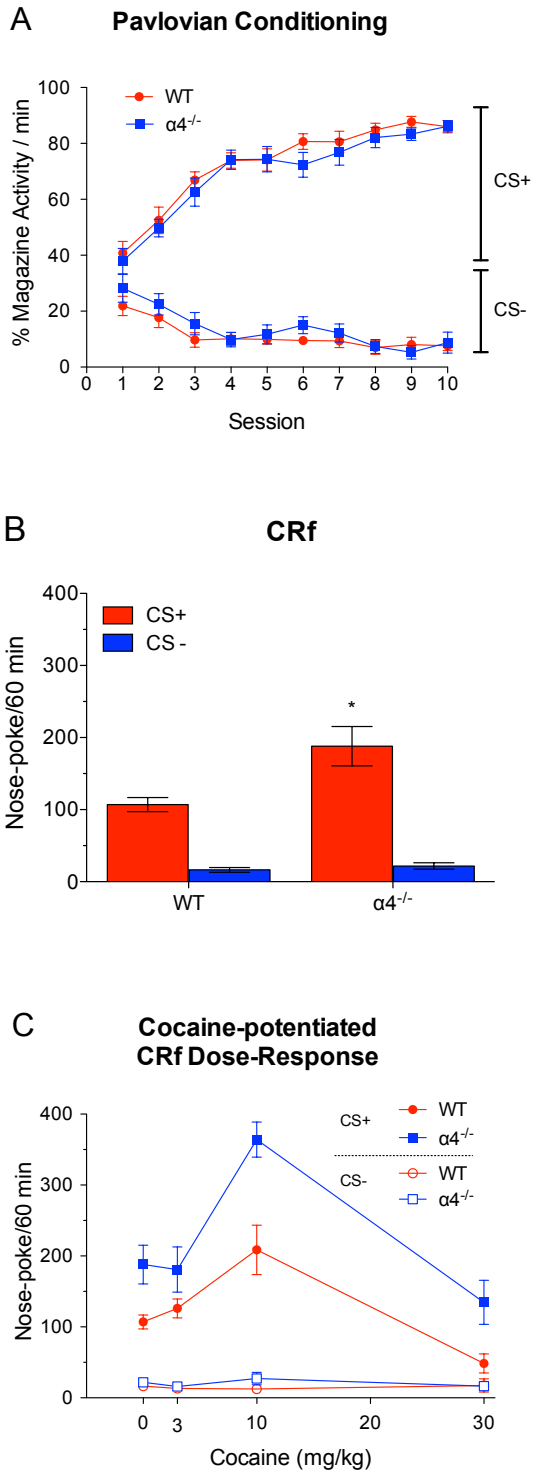
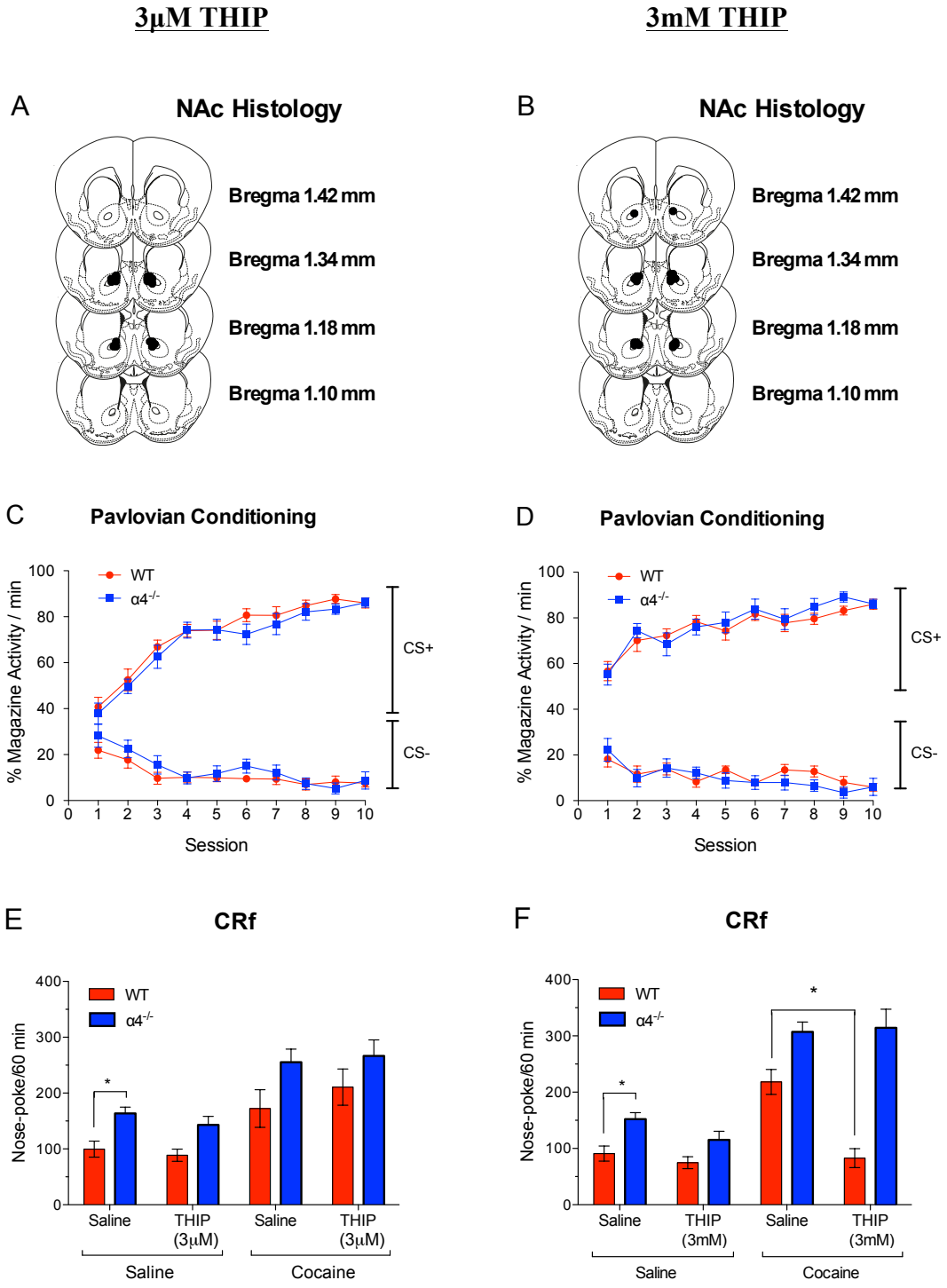


Fig. 5.1. Conditioned reinforcement in wildtype and $GABA_AR$ α4-subunit knockout mice. (**A**) Pavlovian training over 10 consecutive days of 60 minute sessions; both WT (n=10) and $\alpha4^{-/-}$ (n=10) mice learnt the association between a Pavlovian cue and a food reward to a similar degree. (**B**) Instrumental responding for a conditioned stimuli during

a 60 minute session: both genotypes preferentially responded on a nose-poke that led to CS+ presentations, compared with a CS− paired nose-poke. However, $\alpha4^{-/-}$ mice made significantly more CS+ paired lever responses than WT mice. (**C**) Cocaine facilitation of conditioned reinforcement in WT and $\alpha4^{-/-}$ mice during 60 minute sessions. Responding for the CS+ showed a dose-dependent potentiation following cocaine administration, to a similar degree across genotypes. Error bars represent SEM. *$p < 0.05$, *post hoc* paired *t* test.

Figure 5.2



A

Fig. 5.2. Conditioned reinforcement in wildtype and $GABA_AR$ α4-subunit knockout mice with intra-accumbal THIP. (**A**) Histology of intra-accumbal infusions in WT (n=12) and $\alpha4^{-/-}$ (n=11) mice in the 3µM THIP experiment. (**B**) Histology of intra-accumbal infusions in WT (n=8) and $\alpha4^{-/-}$ (n=8) mice in the 3mM THIP experiment. (**C&D**) Pavlovian training over 10 consecutive days of 60 minute sessions; as

previously seen, both WT and $\alpha4^{-/-}$ mice learnt the association between a Pavlovian cue and delivery of a food reward to a similar degree. (**E&F**) Nose-poke responses for the CS+ during 60 minute sessions following local NAc infusion of saline/THIP and an i.p injection of saline/cocaine (10mg/kg). As seen previously, responding for the CS+ was greater in $\alpha4^{-/-}$ than wildtype mice, and cocaine potentiated CS+ responding equally in both genotypes. WT but not $\alpha4^{-/-}$ mice display an attenuation of cocaine-potentiated CS+ responding following intra-NAc 3mM but not 3μM THIP infusion. Error bars represent SEM. *$p < 0.05$, *post hoc* paired *t* test.

5.3.3. RNA Analysis of Ad-shα4 Adenovirus Knockdown of $GABA_AR$ α4-Subunits

qRT-PCR analysis revealed that $GABA_A$ R α4-subunit mRNA levels were reduced within the NAc but not dorsal striatum 10 days after infusion in Ad-shα4 adenovirus α4-subunit knockdown (66 ±6.7% reduction), but not Ad-NSS adenovirus control when compared to untreated control mice (Fig. 5.3.B; significant virus group by brain region interaction, $F_{(2,15)} = 32.16$, $p < 0.001$).

5.3.4. Conditioned Reinforcement in Scrambled Virus Control (Ad-NSS) and α4 Adenoviral Knockdown (Ad-shα4) Mice with Cocaine Potentiation

As with wildtype and constitutive knockout mice, both control and α4-subunit viral knockdown mice learnt the food-predictive properties of the CS+ to a similar degree (Fig. 5.4.A; significant conditioned stimulus by session interaction; $F_{(9,162)} = 28.13$, $p < 0.001$, non significant conditions stimulus by session by genotype interaction; $F_{(9,162)} = 0.29$, $p = 0.96$, NS). Similarly, α4-subunit viral knockdown mice showed an increased instrumental responding for the conditioned reinforcer relative to controls (Fig. 5.4.B; significant conditioned stimulus by virus interaction, $F_{(1,18)} = 431.85$, $p < 0.001$). CRf responding was also equally potentiated by cocaine in both control and α4-subunit viral knockdown mice (Fig 5.4.B; significant main effect of drug injection; $F_{(1,18)} = 36.57$, $p < 0.001$, non-significant drug injection by virus interaction $F_{(1,18)} = 0.12$, $p = 0.97$, NS). However following i.p. THIP (paired with either saline or cocaine) CRf responding decreased drastically to minimal levels in both control and α4-subunit viral knockdown mice (Fig 5.4.B; significant THIP injection by drug injection interaction; $F_{(1,18)} = 9.87$, $p < 0.01$, non-significant THIP injection by drug injection by virus interaction $F_{(1,18)} = 0.13$, $p = 0.91$, NS).

Figure 5.3.

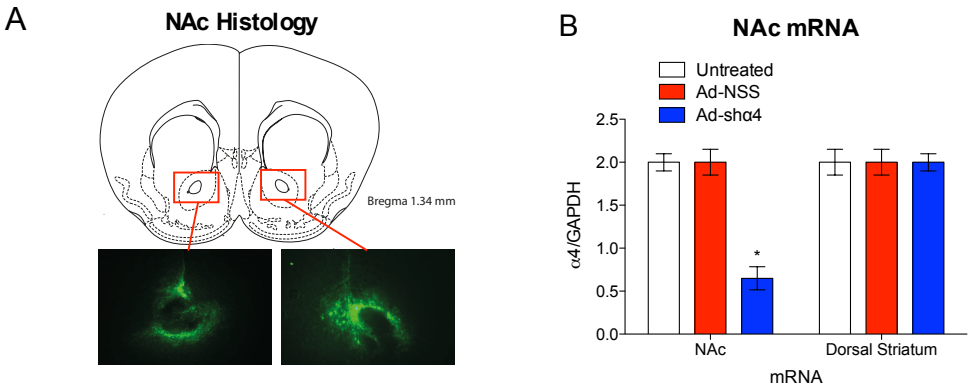


Fig. 5.3 Adenovirus knockdown of α4-subunits **(A)** Histological examination of GFP expression after virus infusion into the NAc. **(B)** Ad-shα4 but not Ad-NSS scrambled adenovirus reduced α4 mRNA expression in the NAc. Histogram depicts the mean ratio of α4 to GAPDH ±SEM (n=6 per group). Error bars represent SEM.* $p < 0.05$ *post hoc* paired *t* test.

Figure 5.4.

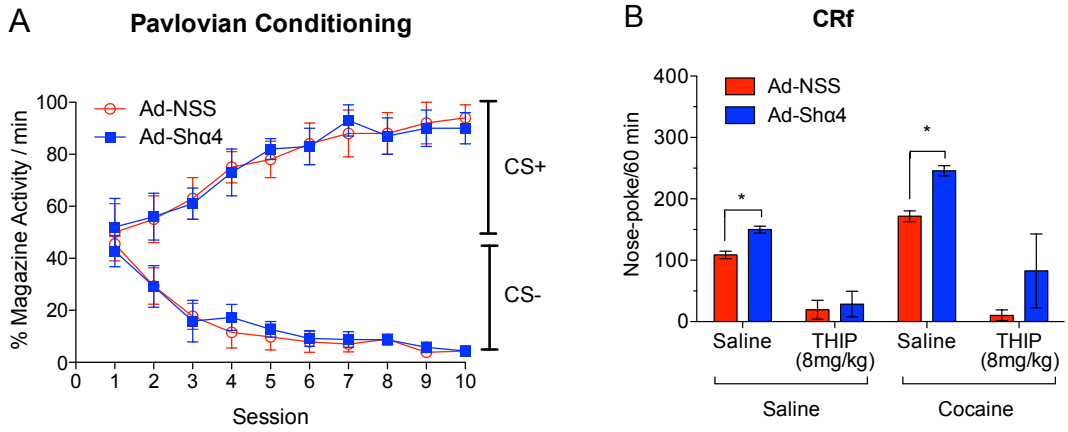


Fig. 5.4. Conditioned reinforcement in control and $GABA_AR$ α4-subunit viral knockdown mice. (**A**) Pavlovian training over 10 consecutive days of 60 minute sessions; both Ad-NSS scrambled virus control (n=10) and Ad-Shα4 α4-subunit viral knockdown (n=10) mice learnt the association between a Pavlovian cue and delivery of a food reward to a similar degree. (**B**) Ad-Shα4 α4-subunit viral knockdown mice demonstrate greater responding for the CS+ than Ad-NSS scrambled virus control mice. This effect was potentiated by cocaine to the same extent in both genotypes. Error bars represent SEM.* $p < 0.05$ *post hoc* paired *t* test.

5.3.5. Conditioned Reinforcement in Wildtype and $\alpha4^{D1}$ / $\alpha4^{D2}$ Knockout Mice with Cocaine Potentiation and Intra-accumbal THIP

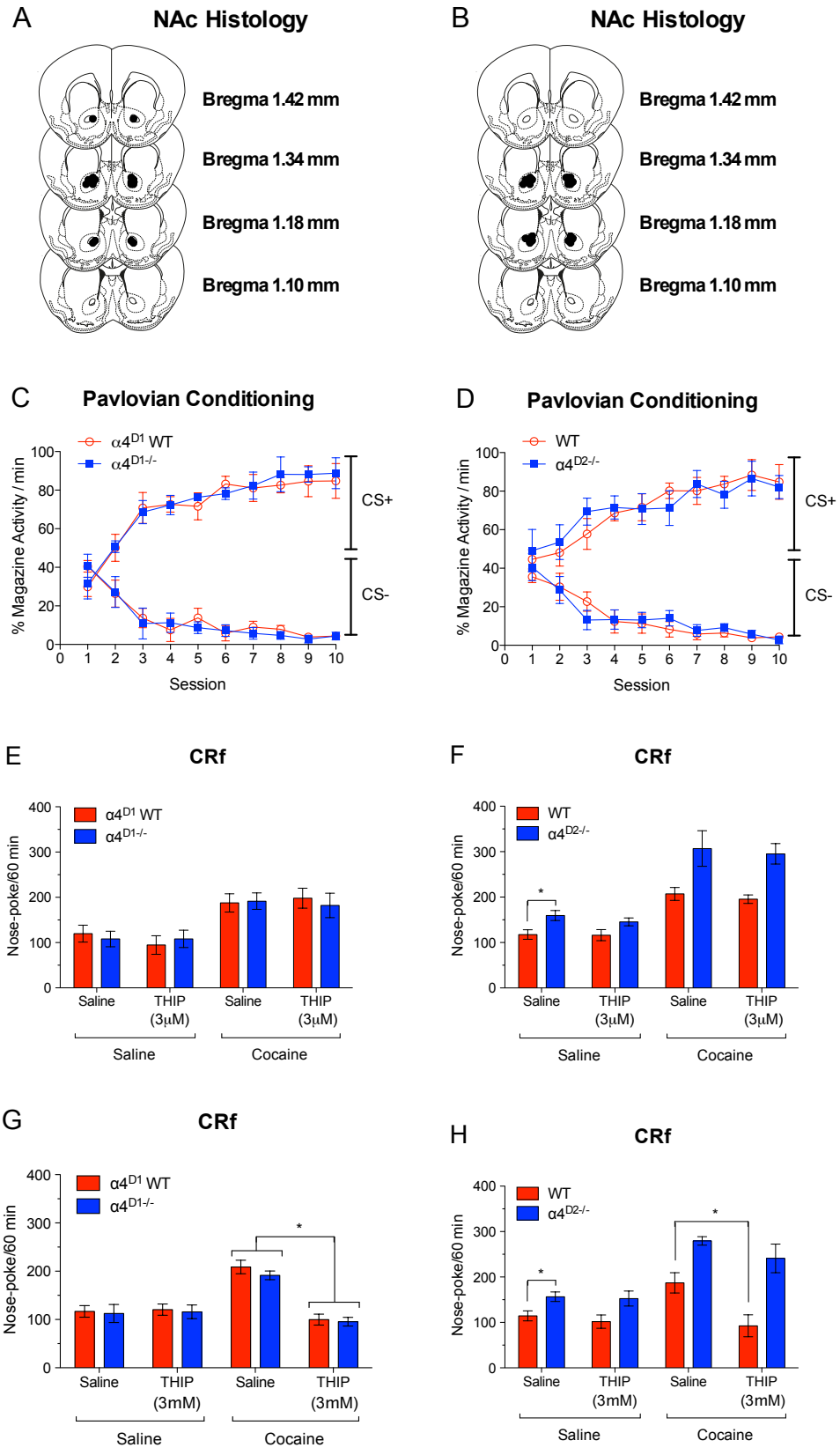
As with the previous experiments, no differences were seen between any of the genotypes in their ability to learn the reward-predictive properties of the CS+ (Fig. 5.5.C; significant session by conditioned stimulus interaction, $F_{(9,279)} = 70.69$, $p < 0.001$, non significant session by genotype interaction, $F_{(9,279)} = 1.12$, $p = 0.35$, NS; Fig.5.5.D; significant main effect of session, $F_{(9,279)} = 53.98$, $p < 0.001$, non significant session by genotype interaction, $F_{(9,279)} = 0.19$, $p = 0.99$, NS).

Similarly to global KOs, dopamine D2-specific $GABA_AR$ α4-subunit knockout mice demonstrated increased CRf responding for the CS+ when compared to wildtype controls (Fig. 5.5.F&H; significant main effect of conditioned stimulus, $F_{(1,28)} = 3903.12$, $p < 0.001$, significant conditioned stimulus by genotype interaction; $F_{(1,28)} = 229.89$, $p < 0.001$). However, this increase was absent in dopamine D1-specific $GABA_AR$ α4-subunit knockout mice (Fig. 5.5.E&G; significant main effect of conditioned stimulus, $F_{(1,28)} = 972.74$, $p < 0.001$, non-significant conditioned stimulus by genotype interaction; $F_{(1,28)} = 0.37$, $p = 0.54$, NS).

A cocaine (10mg/kg) challenge potentiated the initial pattern of responding to a similar degree in all genotypes (Fig. 5.5.E&G; significant main effect of drug, $F_{(1,28)} = 50.14$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,28)} = 0.35$, $p = 0.55$, NS, Fig. 5.5.F&H; significant main effect of drug, $F_{(1,28)} = 211.63$, $p < 0.001$, non-significant drug by genotype interaction, $F_{(1,28)} = 0.06$, $p = 0.79$, NS).

Local infusion of 3μM or 3mM THIP into the NAc did not effect CRf responding in any genotype, however, 3mM but not 3μM intra-accumbal THIP blocked potentiation of CRf responding in all WT and $\alpha4^{D1-/-}$, but not $\alpha4^{D2-/-}$ mice (Fig 5.5.E&G; significant drug by infusion by THIP dose interaction, $F_{(1,28)} = 13.98$, $p < 0.001$, non-significant drug by infusion by THIP dose by genotype interaction, $F_{(1,28)} = 2.63$, $p = 0.11$, NS, Fig.5.5.F&H; significant drug by infusion by THIP dose interaction, $F_{(1,28)} = 4.86$, $p < 0.05$, significant drug by infusion by THIP dose by genotype interaction, $F_{(1,28)} = 7.70$, $p < 0.01$).

Figure 5.5.


A
NAc Histology
Bregma 1.42 mm
Bregma 1.34 mm
Bregma 1.18 mm
Bregma 1.10 mm
B
NAc Histology
Bregma 1.42 mm
Bregma 1.34 mm
Bregma 1.18 mm
Bregma 1.10 mm
C
Pavlovian Conditioning
α4D1 WT
α4D1-/-
% Magazine Activity / min
CS+
CS-
Session
D
Pavlovian Conditioning
WT
α4D2-/-
% Magazine Activity / min
CS+
CS-
Session
E
CRf
α4D1 WT
α4D1-/-
Nose-poke/60 min
Saline
THIP (3μM)
Saline
THIP (3μM)
Saline
Cocaine
F
CRf
WT
α4D2-/-
Nose-poke/60 min
Saline
THIP (3μM)
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THIP (3μM)
Saline
Cocaine
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CRf
α4D1 WT
α4D1-/-
Nose-poke/60 min
Saline
THIP (3mM)
Saline
THIP (3mM)
Saline
Cocaine
H
CRf
WT
α4D2-/-
Nose-poke/60 min
Saline
THIP (3mM)
Saline
THIP (3mM)
Saline
Cocaine

Fig. 5.5. Conditioned reinforcement in wildtype and dopamine D1- or D2-expressing neuron specific $GABA_AR$ α4-subunit knockout mice with intra-accumbal THIP. (**A**) Histology of intra-accumbal infusions in $α4^{D1}$ WT (n=8) and $α4^{D1-/-}$ (n=8) mice. (**B**) Histology of intra-accumbal infusions in $α4^{D2}$ WT (n=8) and $α4^{D2-/-}$ (n=8) mice. (**C**&**D**) Pavlovian training over 10 consecutive days of 60 minute sessions; all genotypes learnt the association between a Pavlovian cue and a food reward to a similar degree. (**E**&**G**) Nose-poke responses for the CS+ during 60 minute sessions following local NAc infusion of saline/THIP (3μM/3mM) and an i.p injection of saline/cocaine (10mg/kg) in $α4^{D1}$ WT and $α4^{D1-/-}$ mice. In a saline/saline control test, responding for the CS+ was equivalent in both genotypes. Similarly, a challenge injection of cocaine (10mg/kg) potentiated responding to a similar degree in both genotypes. CS+ responding and cocaine-potentiated CS+ responding were not effected by an intra-accumbal infusion of THIP (3μM or 3mM) in both genotypes. Intra-accumbal 3mM but not 3μM THIP blocked the ability of cocaine to potentiate responding both genotypes. (**F**&**H**) Nose-poke responses for the CS+ during 60 minute sessions following local NAc infusion of saline/THIP (3μM/3mM) and an i.p injection of saline/cocaine (10mg/kg) in $α4^{D2}$ WT and $α4^{D2-/-}$ mice. In a saline-saline control test, $α4^{D2-/-}$ mice showed increased conditioned responding for the CS+ compared to $α4^{D2}$ WT mice. A challenge injection of cocaine- (10mg/kg) potentiated responding equally in both genotypes. CS+ responding and cocaine-potentiated CS+ responding were not effected by an intra-accumbal infusion of THIP (3μM or 3mM) in $α4^{D2}$ WT and $α4^{D2-/-}$ mice. Intra-accumbal 3mM but not 3μM THIP blocked the ability of cocaine to potentiate responding in $α4^{D2}$ WT but not $α4^{D2-/-}$ mice. Error bars represent SEM. $*p < 0.05$, *post hoc* paired *t* test.

5.4. Discussion

These data demonstrate that a global deletion of α4-$GABA_A$Rs increases instrumental responding for a conditioned reinforcer, indicating that inhibition mediated by these receptors may serve as a protective mechanism against excessive responding for reward-conditioned cues. However, activation of NAc α4βδ $GABA_A$Rs using THIP was not able to reduce baseline CRf responding at either low or high doses, indicating that the protective effect of α4-$GABA_A$R-mediated inhibition cannot be induced to a greater degree than is provided by physiological levels of GABA activation. Interestingly, a 3mM dose of THIP blocked cocaine-potentiated responding in wildtype but not α4-subunit knockout mice when infused directly into the NAc. Thus, activation of NAc α4-$GABA_A$Rs is sufficient to block cocaine potentiation of CRf. Given that the same dose of THIP was also able to block cocaine-potentiation of CPP (see Chapter 4, Fig. 4.5.F), it is possible that α4βδ $GABA_A$Rs may play a general role in modulating potentiation of behaviours by cocaine. The implications and limitations of this hypothesis will be discussed later (see Chapter 6).

The specificity of these mechanisms to the NAc was confirmed after an intra-accumbal viral knockdown of α4-subunits produced an increased CRf response comparable to that of constitutive knockout mice. The location of the viral infusions was largely confined to the NAc core, suggesting that inhibition of NAc core MSNs through α4-$GABA_A$R activity serves as a mechanism to limit levels of responding for conditioned reinforcers. This is not surprising given that previous evidence shows the NAc core rather than shell to be implicated in the expression of instrumental responses for conditioned stimuli (Kelley et al., 1997; Bassareo and Di Chiara, 1999; Parkinson et al., 1999; Ito et al., 2000; Parkinson et al., 2000). However, as the effects of viral knockdown of α4-subunits in the NAc shell are yet to be explored, their possible influence in mediating responding with CRf cannot be discounted. Administration of systemic THIP interfered with cocaine-potentiated CRf responding in both control and α4-subunit knockdown mice. It is possible that systemic THIP may have acted at α4-$GABA_A$Rs within the NAc shell (which we presume to be unaffected in both virus groups) to block the potentiation of CRf responding by cocaine. Indeed evidence indicates that the NAc shell rather than core is involved with psychostimulant potentiation of CRf responding (Parkinson et al., 1999). However, a decrease in baseline CRf responding also occurs in both control and

α4-subunit knockdown mice. This is interesting, as a decrease in cocaine-CPP is not observed following systemic THIP at the same dose (see Chapter 4, Fig. 4.4.B). It is possible that systemic THIP may inhibit the ability to perform more complex instrumental tasks such as the nose-poke responses in CRf, but not simpler motor behaviours such as Pavlovian approach in CPP. These systemic THIP effects are likely to be due to an action at δ-containing $GABA_A$Rs outside of the NAc as a decrease in baseline CRf responding does not occur following intra-accumbal administration of THIP at a dose that blocks cocaine-potentiation of CRf in wildtype mice.

Interestingly, the virus experiment indicates that α4-subunit expression within the NAc only needs to be reduced by a proportion (67%) to get a similar behavioural effect to that produced by a total deletion of α4-subunits in the constitutive knockout mice. This is further emphasized by evidence that heterozygote $\alpha4^{+/-}$ mice, with 50% expression of α4-subunits, demonstrate a similar level of CRf responding as $\alpha4^{-/-}$ mice rather than WT mice (data not shown). This suggests that α4-$GABA_A$Rs are maximally active during CRf responding, as any reduction in α4-subunits has a behavioural impact, and intra-accumbal THIP does not affect responding. Interestingly, previous evidence indicates that sustained inhibition of NAc MSNs, disinhibits target regions, permissively gating and maintaining appetitive behaviors (Taha and Fields, 2006). Tonically active extrasynaptic α4-$GABA_A$Rs may provide the mechanism by which this sustained inhibition is achieved.

Finally, the contribution of α4-$GABA_A$R–mediated inhibition of distinct striatal pathways was explored using mice in which α4-subunits had been deleted from either dopamine D1 or D2-expressing neurons. The increased CRf responding seen in both the α4- constitutive knockout and α4-viral knockdown mice was replicated in D2-specific knockout mice only, as was the THIP blockade of cocaine-potentiated responding seen in constitutive knockouts. Mice with the α4 receptor ablated from D1-expressing neurons were indistinguishable in their behaviour from wildtypes. Constitutive and dopamine D1 and D2-specific α4-KO mice, as well as α4-subunit viral knockdown mice demonstrated a similar ability to learn the reward-predictive properties of a conditioned cue as the WT mice in all experiments. Thus, α4-$GABA_A$Rs do not appear to be involved in Pavlovian associative learning processes, but rather mediate the expression of behavioural responses to conditioned stimuli.

Electrophysiological evidence indicates that activation of dopamine D2-receptors induces a reduction of α4βδ $GABA_AR$-mediated tonic currents in NAc D2-MSNs, which presumably increases the excitability of these neurons (Maguire et al, submitted). Here it is demonstrated that release of D2-expressing neurons from α4-$GABA_AR$-mediated inhibition results in increased responding for a conditioned reinforcer, suggesting that activation of D2-MSNs (possibly through dopamine agonsim) potentiates CRf responding. Indeed, this is in agreement with previous evidence that D2-receptor agonists quinpirole and bromocriptine both facilitate CRf responding (Beninger et al., 1989; Beninger and Ranaldi, 1992). The current experiments also indicate that the NAc is likely the site of action of D2-neuron-mediated potentiation of CRf responding. Mice with a viral knockdown of α4-subunits within the NAc demonstrate a similar phenotype to constitutive and D2-specific α4-subunit knockout mice, with increased responding for a conditioned reinforcer. This is supported by previous evidence that intra-accumbal administration of quinpirole increases CRf responding (Wolterink et al., 1993). The potentiation of CRf responding by psychostimulants also appears to be mediated by D2-MSNs within the NAc. In the current experiments, intra-accumbal THIP at a high dose was able to block the cocaine-potentiation of CRf responding in wildtype but not their counterpart constitutive or D2-specific α4-subunit knockout mice. This role of D2-MSNs is further supported by evidence that intra-accumbal administration of D2-antagonist raclopride blocks amphetamine-induced potentiation of CRf responding (Wolterink et al., 1993).

If an action of dopamine agonsim is to reduce tonic GABAergic currents in D2 cells, then THIP would be expected to oppose this as it would overrule decreased tonic inhibition. However, decreased tonic currents were only observed when NAc D2-MSNs were incubated in dopamine agonists, suggesting reduced inhibition is not a rapidly induced effect, and so may only come into play with sustained agonism of D2 dopamine receptors, as might be expected following cocaine administration. If under physiological conditions dopamine levels are not sufficient to switch off tonic currents then THIP has nothing to oppose, and so has no behavioural effect. Therefore, it could be predicted that in heterozygote mice THIP would produce a reduced attenuation of cocaine-potentiation of CRf responding. This would likely need to be tested with intra-accumbal THIP, as in α4-subunit viral knockdown and control mice systemic THIP reduced

baseline and cocaine-potentiated CRf responding to minimal levels, indicating it interferes with the ability to produce nose-poke instrumental responses.

The enhanced CRf responding observed following release of D2- but not D1-expressing neurons from α4-$GABA_A$R-mediated inhibition is interesting, given that D1- but not D2-specific α4-subunit knockout mice show an increased cocaine-CPP effect compared with wildtype counterparts (see Chapter 4, Fig. 4.6.A). Deletion of α4-$GABA_A$Rs from D1-expressing neurons did not increase CRf responding, and THIP-potentiated α4-$GABA_A$R-mediated inhibition of D1-expressing neurons was not able to block cocaine potentiation of CRf responding. Interestingly, previous evidence reveals that pharmacological activation of D1-receptors using full and partial agonists produces varying effects on CRf responding. Intra-accumbal infusion of the D1-receptor agonist SKF 38393 dose-dependently potentiates CRf responding (Wolterink et al., 1993). However, systemically, SKF 38393, and D1-receptor partial agonists SKF 81297, SKF 77434 and CY 208-243 do not alter CRf responding at low-to-medium doses, but all impair responding for conditioned reinforcers at high doses (Beninger and Rolfe, 1995). It is difficult to extrapolate intracranial doses to those used systemically, however these data may provide evidence for a dose-dependent inverted U-shaped potentiation of CRf responding, as is seen following systemic psychostimulant administration (Beninger and Ranaldi, 1992). A mechanism to explain this curve may be provided by electrophysiological evidence that activation of D1-receptors increases α4-$GABA_A$R-mediated tonic currents in NAc D1-MSNs, thus acting as a homeostatic control to prevent excessive neuronal excitation by dopamine at these neurons (Maguire et al, submitted). Thus when α4-$GABA_A$R-mediated inhibition is removed from D1-expressing neurons it may produce an over-activation of D1-receptors by dopamine and block any potentiating effects of activation of D1-neurons. This may explain the current experiments where D1-specific α4-subunit knockout mice demonstrate a similar level of CRf responding as their wildtype counterparts. The potentiating effects of cocaine in these mice would therefore be a product of activation of D2-receptors on D2 MSNs, masking a detrimental effect of over-activation of D1-MSNs.

Currently proposed models of striatal functioning hold that activation of MSNs within the *direct* and *indirect* pathways produce opposing effects, enhancing or inhibiting reward-seeking, respectively (Kravitz and Kreitzer, 2012; Kravitz et al., 2012).

However, it is unclear how D1- and D2-MSN control over behavioural responses to conditioned stimuli may fit into these models. D2- rather than D1-expressing MSNs appear to be important in the mediation of CRf responding, as demonstrated by an increased response following their release from α4-$GABA_A$R-mediated inhibition. The protective influence of α4-$GABA_A$Rs on D2-neurons in CRf responding and its potentiation by cocaine occurs within the NAc, therefore it could be hypothesised that α4-$GABA_A$Rs may be functioning to modulate the incentive salience of conditioned stimuli, and/or the motivation for instrumental responding. Indeed, D2-MSNs have previously been implicated in the computation of costs and benefits, as well as the mediation of motivation for effortful behaviour (Trifilieff et al., 2013). PET studies in rodents and humans indicate that D2-receptor availability in the striatum is positively correlated with optimal goal-directed behaviour and levels of positive incentive motivation (Dalley et al., 2007; Tomer et al., 2008). Similarly, overexpression of D2-receptors in the NAc of mice using a viral vector is able to increase motivation to work for a food reward without altering the representation of the value of the reward (Trifilieff et al., 2013). The increased CRf responding following disinhibition of D2-expressing neurons in the current experiments may be the result of a similar such increase in motivation to respond for a rewarding stimuli, in this case a conditioned reinforcer rather than a primary reward. As activation of α4-$GABA_A$Rs on D2-expressing neurons (by THIP) is able to block the potentiating effects of cocaine it is possible that cocaine potentiation of CRf responding is also a result of a D2-MSN-mediated increase in motivation. It is yet unclear whether this may be occurring through the same potentiating effect of increased MSN (presumably D2-MSN) excitability within the NAc core, as demonstrated by the virus experiment. Alternatively, cocaine potentiation of CRf responding may be the result of dopamine activation of D2-MSNs within the NAc shell, as previous evidence suggests (Parkinson et al., 1999).

These questions may be elucidated by further experiments employing the use of viral knockdown of α4-subunits specifically within the NAc shell. Additionally, optogenetic and chemicogenetic techniques, such as those used in CPP experiments (Hikida et al., 2010; Lobo et al., 2010), would allow direct activation or inhibition of either D1- or D2-MSNs of the *direct* and *indirect* striatal pathways, respectively. This would provide an efficacious method of testing the predictions made as a result of the current experiments regarding the role of D1- and D2-MSNs in mediating responding for CRf.

To conclude, these data suggest that α4-$GABA_AR$ inhibition of dopamine D2-expressing NAc MSNs is a critical mechanism for controlling the expression of behavioural responses to conditioned stimuli. Furthermore, these experiments provide additional evidence that α4-$GABA_ARs$ are critically involved in modulating the potentiating effects of cocaine in behaviours associated with addiction to drugs of abuse.

Chapter 6

General Discussion

The data presented within this thesis have helped to elucidate a role for α4-$GABA_A$Rs in mediating locomotor and reward-conditioned behaviours associated with addiction to drugs of abuse. α4βδ-$GABA_A$Rs have been shown to be able to modulate baseline and acute cocaine-potentiated locomotor activity, as well as the ability of repeated cocaine to produce a sensitised increase in activity. Additionally, α4-$GABA_A$Rs produce pathway-specific effects in the initiation of reward-seeking behaviours by conditioned contextual or discrete cues, and the ability of cocaine to enhance these behaviours. A discussion of the physiological mechanisms thought to underlie these findings and their wider implications will be presented here.

6.1. α4-$GABA_A$Rs in Locomotor Activity

The locomotor experiments in this thesis indicate that α4-$GABA_A$R-mediated inhibition of NAc MSNs is able to attenuate the ability of cocaine-increased dopamine-facilitation of glutamatergic NAc inputs to potentiate locomotor activity.

Deletion of the Gabra4 gene encoding α4-subunits did not affect baseline locomotor activity or the ability of cocaine to enhance locomotor activity. However, pharmacological activation of α4βδ $GABA_A$Rs using systemic THIP was able to reduce both baseline (when analysed using the first 5 minutes of activity) and cocaine-potentiated locomotor activity in WT but not $\alpha4^{-/-}$ mice. This is in agreement with previous reports of a dose-dependent reduction in baseline locomotor activity following systemic THIP (Agmo and Giordano, 1985; Herd et al., 2009; Vashchinkina et al., 2012). Similarly, a 3μM dose of intra-accumbal THIP attenuated cocaine-potentiated locomotor activity, indicating that the NAc is likely to be the site of action for effects seen with systemic THIP.

Functionally, the predominant effect of THIP in the NAc is probably a suppression of MSN excitability, due to activation of postsynaptic α4βδ receptors and the associated

decreased input resistance (Maguire et al, submitted). Thus, THIP activation of α4βδ $GABA_A$Rs on NAc MSNs will increase tonic inhibitory currents and promote a downstate in these neurons. This will presumably limit the excitability of these neurons in response to glutamatergic inputs, and reduce the ability of dopamine to facilitate excitatory inputs. As release of NAc MSNs from α4βδ $GABA_A$R-mediated tonic inhibition in $\alpha4^{-/-}$ mice did not increase locomotor activity it appears that under normal physiological conditions, there exists little tonic inhibition.

While a 3μM dose of intra-accumbal THIP is able to block cocaine-potentiation of locomotor activity, it does not alter cocaine-enhancement of cocaine-CPP or CRf responding. It is possible that during cocaine-CPP and CRf, NAc MSNs are put into an upstate by excitatory inputs in response to conditioned cues. Therefore, a greater dose of THIP would be needed to produce sufficient inhibition to block behaviour. Indeed, a 3mM dose of intra-accumbal THIP is able to block cocaine-enhancement of cocaine-CPP or CRf responding, but, interestingly, it has no affect on cocaine-potentiation of locomotor activity. When given in the absence of cocaine, this dose of 3mM intra-accumbal THIP is able to greatly increase locomotor activity in WT and, to a lesser extent, $\alpha4^{-/-}$ mice. A proportion (approximately half) of this increase appears to be mediated by receptors other than α4-$GABA_A$Rs, although possibly containing δ-subunits, as $\alpha4^{-/-}$ mice also show an increase in activity. However, an explanation for the α4-dependent proportion of the increase in WT mice is still elusive. It is possible that the increase in locomotor activity seen at this 3mM intra-accumbal dose overrides the ability of THIP to inhibit NAc MSNs and attenuate cocaine potentiation of locomotor activity.

These data indicate that α4-$GABA_A$Rs within the NAc provide an efficacious target for control of locomotor activity and its potentiation by cocaine. Interestingly, systemic or intra-accumbal THIP-induced changes in baseline- and cocaine-potentiated locomotor activity do not appear to directly affect cocaine-CPP or CRf responding.

6.2. α4-$GABA_A$Rs in Behavioural Sensitisation to Cocaine

Deletion of $GABA_A$R α4-subunits did not alter the augmentation of locomotor activity observed following repeated, intermittent cocaine, indicating that unlike α2-$GABA_A$Rs, α4-$GABA_A$Rs are not involved in the development of behavioural sensitisation to

cocaine. These data indicate that α2- and α4-$GABA_A$Rs play dissociable functional roles in the NAc. It is hypothesised that α2-$GABA_A$Rs may mediate a lateral inhibition of NAc MSNs required for behavioural sensitisation, while α4-$GABA_A$Rs likely mediate a general tonic inhibition of NAc MSNs (Dixon et al., 2010). Indeed, as with locomotor activity following acute cocaine, systemic THIP was able to reduce the sensitised increase in locomotor activity.

6.3. α4-$GABA_A$Rs in Cocaine-CPP

Investigation of the role of α4-$GABA_A$Rs in cocaine-CPP revealed that deletion of α4-subunits did not alter acquisition of cocaine-CPP. However, specific deletion from dopamine D1- but not D2-expressing neurons was able to increase the time spent in the cocaine-paired chamber, presumably due to an increase in D1-MSN excitability. This data is in agreement with previous evidence demonstrating optogenetic activation of D1-expressing NAc MSNs during cocaine-paired chamber conditioning to enhance acquisition of cocaine-CPP (Lobo et al., 2010).

A major novel finding of this thesis was that a cocaine challenge given during the test session enhanced the cocaine-CPP effect in wildtype, constitutive and dopamine D1-, but not D2-expressing neuron specific $GABA_A$R α4-subunit knockout mice. It is hypothesized that this may be explained by the interoceptive properties of cocaine increasing the salience of the CPP.

Finally, pharmacological activation of α4βδ-$GABA_A$Rs by THIP suppressed cocaine enhancement of the CPP effect in wildtype but not constitutive and dopamine D1-expressing neuron specific $GABA_A$R α4-subunit knockout mice. Thus α4βδ-$GABA_A$R modulation of MSN excitability plays a role in regulating dopamine's effects in the NAc.

6.4. α4-$GABA_A$Rs in CRf

Following global deletion of $GABA_A$R α4-subunits, Pavlovian conditioning of a discrete cue with a food reward was unaffected, but instrumental responding for this conditioned reinforcer was increased. Subsequent investigation revealed viral knockdown of α4-subunits specifically within the NAc resulted in a similar increase in CRf responding. Targeted deletion of α4-subunits from dopamine D2-, but not D1-expressing neurons also demonstrated an increased CRf responding phenotype.

CRf responding was potentiated in all genotypes by cocaine challenge during the test session. Cocaine potentiation of CRf responding was blocked by a 3mM dose of intra-accumbal THIP in wildtype and dopamine D1-expressing neuron specific, but not constitutive or D2-expressing neuron specific $GABA_AR$ α4-subunit knockout mice.

These data indicate that α4-$GABA_AR$-mediated inhibition of dopamine D2-expressing NAc MSNs is a critical mechanism for controlling the expression of behavioural responses to conditioned stimuli. In addition to the cocaine CPP experiments, these data provide evidence that α4-$GABA_ARs$ are critically involved in modulating the potentiating effects of cocaine in behaviours associated with addiction to drugs of abuse.

Table 6.1.

Behaviour	Constitutive $\alpha4^{-/-}$	$\alpha4^{D1-/-}$	$\alpha4^{D2-/-}$
Cocaine-potentiated locomotor activity	Normal	?	?
Behavioural sensitisation to cocaine	Normal	?	?
+ THIP	Not Blocked	?	?
Cocaine-CPP	Normal	Enhanced	Normal
Cocaine-enhanced Cocaine-CPP	Normal	Normal	Absent
+ THIP	Not Blocked	Not Blocked	-
CRf	Enhanced	Normal	Enhanced
Cocaine potentiated CRf	Normal	Normal	Normal
+ THIP	Not Blocked	Blocked	Not Blocked

Table 6.1. Summary of the behavioural consequences of constitutive or dopamine D1-/D2-expressing neuron specific $GABA_AR$ α4-subunit knockout mice, and pharmacological activation of α4βδ-$GABA_A$Rs by THIP.

6.5. A Dissociation Between NAc MSNs Mediating Reward-Seeking in Response to Contextual or Discrete Cues?

A main finding of this thesis is that deletion of α4-$GABA_A$Rs from D1-expressing neurons increases cocaine-CPP, while deletion from D2-expressing neurons increases CRf responding. Thus, α4-$GABA_A$Rs on NAc dopamine D1- or D2-MSNs appear to have dissociable roles in modulating various aspects of conditioned reward-seeking. Electrophysiological evidence indicates that α4-$GABA_A$Rs have a similar physiological role on both D1- and D2-MSNs, mediating a tonic inhibition that controls the ability of excitatory inputs to generate an action potential in the MSN output projections of both the indirect and direct pathways (Maguire et al, submitted). Thus, the dissociation between the neurons mediating these two behaviours may be better explained by the difference in the glutamatergic inputs onto these two neuronal groups and the information that they provide.

NAc MSNs are known to receive multiple inputs from several afferent structures, including the amygdala, PFC, and hippocampus (French and Totterdell, 2003; Stuber et al., 2011; Stamatakis and Stuber, 2012). It could be hypothesised that while glutamatergic projections from cortical and subcortical regions project to both D1- and D2-MSNs, information about contextual cues needed for cocaine-CPP may be provided by inputs onto NAc D1-MSNs, while information about discrete cues important for CRf responding arrives onto D2-MSNs. Indeed, blockade of NMDA conductance specifically in D1-MSNs by selective expression of an NR1 subunit, which contains a mutation in the pore that reduces calcium flux, decreases acquisition of cocaine-CPP (Heusner and Palmiter, 2005). The origin of these glutamatergic inputs onto D1-MSNs is currently unknown. However it is thought that projections from the hippocampus to the NAc may provide information about contextual cues during acquisition and expression of CPP (Ferbinteanu and McDonald, 2001; Britt et al., 2012). This would suggest that the enhanced cocaine-CPP seen in $\alpha4^{D1-/-}$ mice may be the result of an increased ability of glutamatergic inputs from the hippocampus to excite D1-MSNs within the NAc in the absence of α4-mediated inhibition.

At present, little is known about glutamatergic projections onto NAc D2-MSNs. The increased CRf responding following removal of α4-$GABA_AR$ tonic inhibition from D2-MSNs suggests that glutamatergic inputs onto D2-MSNs may be important for driving responding for conditioned reinforcers. Thus, activity in the indirect pathway might not necessarily be a reward-opposing, demotivating force, as some models propose (Kravitz et al., 2012), but it could simply encode a separate dimension of certain behaviors (Britt et al., 2012). Indeed, it might be that D2-MSNs are the site of input within the NAc for information about discrete cues arriving from areas including the PFC and BLA. This hypothesis is supported by evidence that the D2 receptor antagonists metoclopramide and haloperidol inhibit responding for a food-conditioned reinforcer and disrupt secondary cue-induced cocaine-seeking, respectively (Beninger et al., 1987; Gál and Gyertyán, 2006). Interestingly, deletion of mGlur5 receptors specifically from NAc D1-MSNs also attenuates CRf responding, suggesting that glutamatergic signaling at D1-MSNs may also play a role in CRf responding (Novak et al., 2010). However, in the current experiments removal of α4-$GABA_AR$-mediated tonic inhibition from D1-MSNs did not increase CRf responding. It is still unclear how these data fit together into neurobiological model of CRf.

Further support for a dissociation in the roles of direct and indirect pathways in mediating distinct reward-seeking behaviours may be provided by evidence that specific patterns of NAc dopamine release help to enhance or attenuate glutamatergic activation of either D1- or D2-MSNs. Under normal conditions, a tonic, basal level of DA release is thought to maintain the balance between limbic and cortical inputs in the NAc (Goto and Grace, 2005). Therefore, both increases and decreases in NAc dopamine release may exert a potent effect on the influence of information delivered to NAc MSNs via glutamatergic projections. D1 and D2 receptors show low and high affinities to dopamine, respectively (Creese et al., 1983). It is thought that D1 receptor activation requires phasic DA release, while D2 receptors are activated continuously by basal, tonic DA release (Grace, 1991). Within the NAc, phasic dopamine release activates D1 receptors to selectively facilitate hippocampus inputs at D1-MSNs, indicated by an increased local field potential response (Goto and Grace, 2005). On the other hand, increased or decreased tonic DA release impacts D2 receptors, producing an attenuation or facilitation, respectively, of PFC, but not hippocampal inputs at D2-MSNs (Goto and Grace, 2005). Therefore, increased NAc phasic dopamine release may enhance the influence of contextual cue information from the hippocampus onto D1-MSNs needed for cocaine-CPP. Whereas, decreased tonic NAc dopamine release may enhance the influence of discrete cue information from the PFC onto D2-MSNs important for CRf responding. However, phasic dopamine is known to be released within the NAc in response to both contextual cues in cocaine-CPP (Schiffer et al., 2009), and discrete cues associated with rewards (Schultz, 1998; Weiss et al., 2000). Thus, a mechanism must exist by which PFC inputs onto D2-MSNs that can overrule phasic dopamine-induced strengthening of hippocampal inputs onto D1-MSNs. Indeed, administration of the D2 antagonist eticlopride during phasic dopamine release is able to attenuate facilitation of hippocampus inputs (Goto and Grace, 2005). A decrease in tonic dopamine release, and the resulting facilitation of PFC inputs, would therefore attenuate hippocampus afferent drive. It is still unknown how amygdala projections onto NAc neurons may be modulated by dopamine; however given their proposed role in CRf responding (Everitt et al., 1999; 2000), it might be predicted they would also demonstrate enhanced excitement of D2-MSNs in response to decreased tonic dopamine.

The findings of this thesis reveal that activation of α4-$GABA_A$Rs specifically on D1- or D2-expressing neurons may a provide mechanism by which reward-conditioned behaviours can be controlled. It will be discussed later how targeting these receptors can be predicted to be efficacious in the treatment of addiction-related behaviours (see 6.7.1.).

6.6. Does Activation of α4-$GABA_A$Rs Attenuate Cocaine-Potentiation of Behaviours by Inhibition of MSNs Within the NAc Shell?

Given that intra-accumbal THIP is able to block cocaine-enhancement of cocaine-CPP, CRf responding and locomotor activity (albeit at different doses), it appears that α4βδ $GABA_A$Rs within the NAc play a critical role in the modulation of the behaviour-enhancing properties of psychostimulants. In rats, the ability of psychostimulant to potentiate CRf responding is reported to be mediated by NAc shell region (Parkinson et al., 1999; Ito et al., 2000). However, delineation of regions involved in psychostimulant-increased locomotor activity is more complex, with rat studies indicating the involvement of the NAc shell (Heidbreder and Feldon, 1998), core (Boye et al., 2001; Sellings and Clarke, 2003; 2006) or even both regions (Ikemoto, 2002). Here, for the first time, it is demonstrated that psychostimulants are also able to enhance cocaine-induced CPP.

The co-ordinates used for the intra-accumbal infusions in the current experiments are situated at what is described to be the dorsomedial NAc core/shell boundary (Paxinos and Franklin, 2001). Thus, it is likely that THIP would activate α4βδ $GABA_A$Rs within the both the NAc core and shell. In this case, the THIP-induced blockade of the enhancing effects of cocaine on CRf responding, CPP and locomotor activity may be due to a α4βδ $GABA_A$R-mediated tonic inhibition of MSNs within the NAc shell. Interestingly, activation of α4-$GABA_A$Rs on D1-MSNs by THIP blocks cocaine enhancement of cocaine-CPP, while removal of these receptors from D1-MSNs increases cocaine-CPP. Conversely, activation of α4-$GABA_A$Rs on D2-MSNs by THIP blocks cocaine potentiation of CRf responding, while removal of these receptors from D2-MSNs increases CRf responding. Thus, cocaine-enhancement of cocaine-CPP and CRf responding appears to be modulated by activation of α4βδ $GABA_A$Rs on the same MSN type as those on which deletion of these receptors increases cocaine-CPP and CRf

responding respectively. It is possible that the increase in cocaine-CPP and CRf responding observed in $\alpha4^{D1-/-}$ and $\alpha4^{D2-/-}$ mice, respectively, in the absence of cocaine, may also be the result of a disinhibition of NAc shell neurons, allowing a greater response to ambient levels of dopamine. However, an increase in CRf responding was also observed following a viral knockdown of α4-subunits that was largely specific to the NAc core. This indicates that, at least for CRf responding, the NAc core plays an important role in mediating behavioural responses to conditioned stimuli.

In conclusion, it is possible that there may be NAc core/shell dissociation in the mediation of the baseline effect and the enhancement by cocaine of cocaine-CPP and CRf responding. However, if this is the case, it is clear that the ability of cocaine to enhance these behaviours is complex and involves activation of the same MSN types that mediate the baseline effect. A more plausible explanation for the current data would be that the same MSN ensembles mediate the baseline effect and the enhancement by cocaine, although, at present, not enough data is available to ascertain the location within the NAc of these MSNs. Finally, it is also still unknown whether the attenuation of cocaine potentiated locomotor activity by intra-accumbal THIP is mediated by activation of α4βδ $GABA_A$Rs on D1- MSNs, D2-MSNs or the combination of both.

6.7. Implications for Drug Abuse

6.7.1. Compounds acting at α4-$GABA_A$Rs as a treatment for cocaine abuse?

The experiments of this thesis reveal that α4-$GABA_A$Rs are able to modulate behavioural responses to reward-conditioned cues, indicating that compounds acting at α4-$GABA_A$Rs may have potential therapeutic value in the treatment of some behaviours associated with drug addiction. Unfortunately, systemic or intra-accumbal administration of THIP was not able to reduce cocaine-CPP and CRf responding under drug-free (baseline) test conditions. However, THIP was able to reduce cocaine-enhancement of cocaine-CPP, CRf responding and locomotor activity, as well as behavioural sensitisation to cocaine. These data indicate that THIP is able to block the energising effects of cocaine. Although unclear what the effects may be in humans, it could be speculated that THIP may hold a potential therapeutic value in blocking the hyperlocomotor and drug-seeking potentiating effects of cocaine while the drug is still within the system. It could also be speculated that THIP may be able to block other

effects of cocaine, including the subjective euphoric effects in humans. However, when administered systemically, THIP also has sedative properties. Therefore until THIP activation of α4βδ $GABA_A$Rs can be targeted specifically to the NAc, this compound may have limited therapeutic use in treating cocaine abuse. However, an additional problem is that deletion of $GABA_A$R α4-subunits from the NAc has been revealed to reduce alcohol drinking in mice (Rewal et al., 2009; Lobo et al., 2010; Rewal et al., 2011). Therefore although targeting these subunits pharmacologically with THIP or other compounds may be beneficial for reducing the effects of cocaine, it could be speculated that they may produce other adverse effects, i.e. increased alcohol drinking.

6.7.2. Investigation of GABRA4 genes in humans

Linkage and association analysis indicates that a region of chromosome 4p (the 16-cM region), containing a cluster of genes encoding a number of $GABA_A$R subunits, including Gabra2 and Gabra4, is associated with an increased risk of drug dependence (Reich et al., 1998; Edenberg, 2002; Enoch, 2008; Enoch et al., 2013). Indeed, single nucleotide polymorphisms (SNPs) of the Gabra2 gene are associated with alcohol dependence and cocaine addiction in humans (Edenberg et al., 2004; Dixon et al., 2010). Given that this thesis reveals deletion of the Gabra4 gene in mice to alter the behavioural response to reward-conditioned cues, it could be hypothesised that SNPs or altered expression of the Gabra4 gene in humans may also be associated with drug addiction. However, although post-mortem analysis of GABAergic gene expression has revealed the Gabra2 gene to be altered in hippocampus of alcohol and cocaine addicts, Gabra4 expression was unaltered (Enoch et al., 2012; 2013). Similarly, an association study revealed that six Gabra4 SNPs were not significantly correlated with risk for alcohol dependence (Edenberg et al., 2004). Future studies may benefit from investigation of other Gabra4 SNPs and changes in Gabra4 expression within the NAc of alcohol and cocaine addicts.

6.8. Considerations

6.8.1. The use of THIP to target α4-GABA$_A$Rs

At present there are limited pharmacological tools for the specific activation of α4-GABA$_A$Rs. In this thesis the GABA$_A$R agonist THIP was used due to its preferred action at δ-subunit containing GABA$_A$Rs, which within the NAc are largely co-assembled with α4-subunits in extrasynaptic locations (Pirker et al., 2000; Belelli et al., 2005; Brickley and Mody, 2012). However, it has been reported that THIP doses over 3μM may begin to act at γ2-containing synaptic GABA$_A$Rs in addition to its action at δ-containing extrasynaptic receptors (Ebert et al., 1994; Mortensen et al., 2004; 2010). In the locomotor activity experiments presented in this thesis there is some evidence that intra-accumbal 3mM THIP may have also acted at receptors other than α4-GABA$_A$Rs. However, in the cocaine-CPP and CRf studies the behavioural effects of 3mM THIP were contingent upon the presence of α4-subunits within the NAc. Recent immunohistochemical analysis of NAc slices reveals that the expression pattern of the GABA$_A$R α4-subunit is indistinguishable from that of GABA$_A$R δ-subunits, and distinct from expression of neuroligin2 (NL2), a cell adhesion protein that is selectively expressed in inhibitory synapses (Maguire et al, submitted). Thus, there appears to be few or no synaptic α4-GABA$_A$Rs within the NAc, indicating that the α4-GABA$_A$R-mediated effects of intra-accumbal 3mM THIP observed in the cocaine-CPP and CRf experiments are highly likely to be due to an action at extrasynaptic α4βδ receptors.

The recent creation of delta-selective compound 2 (DS2), a novel positive allosteric modulator of δ-containing GABA$_A$Rs may help to specifically target extrasynaptic α4βδ receptors. An *in-vitro* concentration-response curve indicates that DS2 produces a similar peak stimulated inhibitory current as THIP in α4βδ receptors, but, unlike THIP, does not produce any response in α4βγ2 or α1βγ2 receptors even at high doses (Mortensen et al., 2010; Jensen et al., 2013). Unfortunately, when tested *in-vivo* systemic administration DS2 demonstrates a poor brain/plasma ratio, suggesting DS2 does not readily cross the blood-brain barrier (Jensen et al., 2013). Indeed, systemic doses of up to 100mg/kg fail to produce the effect in animal models of gross behavioural changes (locomotor activity, rotarod) seen with relevant doses of systemic THIP (Wafford and Ebert, 2006; Herd et al., 2009). Similarly, problems arise with intracranial administration of DS2, as unlike THIP it is not readily soluble in saline

solution. In electrophysiological studies DS2 was dissolved in OR2 buffer (composition, in mM: 90 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 5 HEPES; pH adjusted to 7.4) (Jensen et al., 2013). However, it is unknown whether this buffer may produce effects on its own when infused intracranially.

Alternatively, it may soon be possible to target α4βδ receptors specifically with the use of designer receptors. DREADD (designer receptor exclusively activated by a designer drug) receptors are receptors that have been modified such that they no longer respond to their endogenous ligand but instead can be activated by the administration of synthetic ligands. At present this technology only exists to modify G-protein coupled receptors, but similar such techniques have been used to modify ligand-gated ion channels such as $GABA_A$Rs, such that they are specifically activated by certain drugs. Zolpidem, a systemically active allosteric modulator that enhances the function of the $GABA_A$Rs, requires a phenylalanine residue (Phe77) in the γ2-subunit. By changing this residue to isoleucine, then subsequently modifying γ2-subunits on specific neurons using Cre recombinase (exchanging Ile77 for Phe77), zolpidem sensitivity can be restored to chosen cell types (Wulff et al., 2007). Similar such techniques could one day be created for δ-subunit containing $GABA_A$Rs and may be able to be used in combination with dopamine D1- or D2-cre-recombinase lines to allow these receptors to be activated specifically on D1- or D2-expressing neurons.

6.8.2. The use of mouse behavioural paradigms to model addiction-associated behaviours

It is also important to consider the limitations of the behavioural paradigms used within this thesis. Firstly, although neural and behavioural sensitisation has been widely documented in animals, it has been questioned whether such sensitisation also occurs in humans. Investigation of neuroadaptations in human cocaine addicts has produced conflicting results. While some studies report that repeated intermittent administration of amphetamine results in sensitisation of dopamine release (Boileau et al., 2006), others have found that detoxified cocaine addicts actually show a decrease in evoked dopamine release rather than a sensitised increase (Volkow et al., 1997; Martinez et al., 2007). Behaviourally, there is some evidence for progressive drug effects in humans. Repeated amphetamine increased self-report of the subjective effects of the drug (Strakowski et al., 2001; Boileau et al., 2006), and clinician-rated levels of energy and

motor activity (Strakowski et al., 1996; Strakowski and Sax, 1998). However, other studies have failed to find evidence for a sensitisation of subjective drug effects following repeated amphetamine administration (Johanson and Uhlenhuth, 1981; Kelly et al., 1991). An explanation for these conflicting reports may be that in humans, as with in animals, the expression of sensitisation is powerfully modulated by the context of the drug administration (Robinson and Berridge, 2008). Thus, a drug challenge in the test environment may not result in the expression of behavioural sensitisation as it would in the context where the drugs were previously taken. Further investigation is needed to elucidate the complexity of behavioural sensitisation in humans.

There are also a number of limitations with using CPP as a model of the drug-seeking behaviour. Firstly, it has been questioned whether pairing the drug with one context may block or impair the habituation to that environment, rendering it more novel when compared with the saline-paired chamber (Bardo and Bevins, 2000). Animals are known to prefer novel contexts over familiar contexts (Hughes, 1968; Parker, 1992). A method to overcome this problem would be to include a third chamber to the apparatus to create three distinct contexts, one that is drug-paired, one that is saline-paired, and one that is novel. Secondly, it could be questioned whether CPP is directly comparable with drug-seeking in humans as in the CPP paradigm animals passively receive drugs rather than self-administering them. Indeed, animal studies comparing CPP to drug self-administration reveal that the increase in dopaminergic activity observed with self-administration of stimulant drugs is attenuated in yoked control animals that receive the drug passively (Di Ciano et al., 1996; Hemby et al., 1997; Stefanski et al., 1999). A solution to this may be to modify the CPP design so that the animal is able to self-administer the drug or saline while confined in the drug- or saline-paired chamber.

6.8.3. Genetic limitations

A limitation of the use of mutant mouse lines is that genetic alteration of gene expression often results in compensatory changes in other gene products. Indeed, in this thesis, qRT-PCR analysis of NAc tissue samples revealed that mRNA expression levels of two $GABA_AR$ subunits were altered following global or dopamine D1-/D2-expressing neuron specific deletion of $GABA_AR$ α4-subunits. Firstly, a decrease in δ-subunits was detected. This is likely due to a lack or decrease in α4-subunits subunits for δ-subunits to associate with resulting in a downregulation. Secondly, increased

mRNA expression levels of the $GABA_AR$ α2-subunit were detected. As the Gabra4 and Gabra2 genes are located adjacently on chromosome 4 it is possible that deletion of Gabra4 may have interfered with expression of Gabra2. However, electrophysiological evidence indicates that deletion of α4-subunit has no impact on the kinetics of the phase currents mediated by synaptic receptors within the NAc (Maguire et al, submitted). Thus it appears that the increase in α2-subunits mRNA in the NAc is not translated into α2-subunit proteins in functional receptors. This could be further confirmed by investigating α2-subunit protein levels in $GABA_AR$ α4-subunit knockout mice.

6.9. Future Work

6.9.1. Further investigation of the role of α4-$GABA_ARs$ in locomotor activity and behavioural sensitisation to cocaine.

The findings from Chapters 4 and 5 indicate that α4-$GABA_ARs$ on dopamine D1- or D2-expressing neurons are able to modulate both baseline and cocaine-enhanced cocaine-CPP and CRf behaviours. However, although ability of α4-$GABA_ARs$ to modulate locomotor activity and its sensitisation by repeated, intermittent cocaine have been established, it is still unclear how α4-$GABA_ARs$ expressed on neurons within distinct striatal pathways may control these behaviours.

The investigation of the role of α4-$GABA_ARs$ in controlling locomotor activity and its potentiation by cocaine could be extended by repeating the experiments presented in Chapter 2 using D1- or D2-expressing neuron specific $GABA_ARs$ α4-subunit knockout mice. Current evidence indicates that activation of D1-expressing neurons of the direct striatal pathway facilitate locomotor activity, while activation of D2-expressing neurons of the indirect striatal pathway oppose locomotor activity (Kravitz and Kreitzer, 2012). Thus, it could be predicted that deletion of α4-$GABA_ARs$ from D1-expressing neurons may disinhibit the direct pathway and increase locomotor activity, while deletion from α4-$GABA_ARs$ from D2-expressing neurons would release the indirect pathway from inhibition and thus attenuate locomotor activity. If this is found to be correct, it might indicate that the lack of a change in baseline or cocaine-potentiated locomotor activity observed following constitutive deletion of α4-subunits is due to the effects of disinhibiting both direct and indirect pathways cancelling each other out, as was

observed with cocaine-CPP. In the cocaine-CPP and CRf experiments cocaine-enhancement of these behaviours was modulated by activation of α4βδ $GABA_A$Rs on the same MSN type as those on which deletion of these receptors increased the baseline effect. Based upon these evidence, it is hypothesised that cocaine-potentiation of locomotor activity would also be attenuated by THIP-induced activation of α4βδ $GABA_A$Rs on D1-expressing neurons, while activation of the same receptors on D2-expressing neurons would have no effect or possibly even increase cocaine-potentiated locomotor activity.

In this thesis it was revealed that deletion of α4-$GABA_A$Rs did not alter induction of behavioural sensitisation to cocaine. Nevertheless, it is possible that deletion of α4-$GABA_A$Rs from D1- or D2-expressing neurons may be able to influence this phenomenon. Indeed, transient disruption of D1-expressing *direct* striatal pathway neurons using a synthetic inhibitory G_{α_i}- coupled DREADD (designer receptor exclusively activated by a designer drug) receptor was able to impair behavioural sensitisation to amphetamine, while the reverse was reported following disruption of D2-expressing *indirect* striatal pathway neurons. (Ferguson et al., 2011). Thus, as with the hypothesis for locomotor activity, it is predicted that deletion of α4-$GABA_A$Rs from D1-expressing neurons would increase behavioural sensitisation to cocaine, while deletion of α4-$GABA_A$Rs from D2-expressing neurons would attenuate sensitisation. This could also be investigated by co-administering THIP with cocaine in during the induction of cocaine behavioural sensitisation in D1- or D2-expressing neuron specific $GABA_A$R α4-subunit knockout mice.

6.9.2. Further investigation of the role of α4-$GABA_A$Rs on D1- or D2-expressing neurons in controlling reward-conditioned behaviours.

Earlier it was hypothesised that there may be dissociation between the NAc MSN types that mediate behavioural responses to contextual cues and discrete cues. If this is indeed correct, then it would be predicted that mice in which $GABA_A$R α4-subunits are removed from D2-expressing neurons would also show a facilitation of other behaviours influenced by discrete cues, including Pavlovian-instrumental transfer (PIT). In this paradigm, discrete cues previously paired with food can markedly elevate the

rate of food-reinforced instrumental responding (Estes, 1943; Lovibond, 1983; Colwill and Rescorla, 1988). Indeed, there is some evidence to suggest that D2 receptors may play an important role in mediating PIT. Instrumental responding during the presentation of a Pavlovian cue was reduced by microinjection of the D2 receptor antagonist raclopride into the NAc shell (Lex and Hauber, 2008).

The role of α4-$GABA_A$Rs on D1- or D2-expressing neurons in controlling reward-conditioned behaviours could also be further investigated using a procedure developed by Ito et al (2006), which involves the animal making behavioural responses to both conditioned spatial and discrete cues within the same test. Under this paradigm, animals are initially trained to associate a discrete cue with delivery of a sucrose reward in a Y-maze apparatus with three topographically identical chambers. The same animals then undergo 'place/contextual conditioning' during which the conditioned discrete cue is presented in one, but not the other two chambers. The behavioural response to discrete or contextual cues could then be measured using head entries into the food magazine following CS presentation and time spent in the reward-paired chamber, respectively. Using this paradigm, Ito (2006) found lesions of the hippocampus disrupt the CPP effect but not magazine approaches following the discrete cue, with the reverse seen following BLA lesions. Based upon the findings from the cocaine-CPP and CRf experiments of this thesis, it is predicted that mice in which α4-$GABA_A$Rs are deleted from D1-expressing neurons would show an unaltered behavioural response to discrete cues but an increased CPP effect. Whereas, mice in which α4-$GABA_A$Rs are deleted from D2-expressing neurons are predicted to demonstrate an increased behavioural response to discrete cues but unaltered CPP. If these predictions are correct, these data would add credence to the hypothesis that information about contextual cues from the hippocampus is received onto NAc D1-MSNs, while information about discrete cues from the amygdala and PFC are received onto D2-MSNs.

Finally, it is important to note that in the CPP and CRf expreriments described within this thesis, the reward received by the mouse was different between these paradigms. A cocaine reward was used for conditioning in the CPP paradigm, whereas, mice received a sucrose reward in the CRf paradigm. In future, recreating the CPP experiments of this thesis using a food-CPP paradigm would allow for a more direct comparison of CPP and CRf responding. Alternatively, the Ito (2006) procedure described above, in which

sucrose is conditioned to contextual and discrete cues, would also allow for a more direct comparison of behavioural responses to contextual and discrete cues.

6.9.3. Further investigation of the role of α4-GABA$_A$Rs in the NAc core or shell

The data presented in this thesis indicate that the NAc is the site of action for α4-GABA$_A$R modulation of CRf responding and the ability of cocaine to enhance CRf responding and cocaine-CPP. However, as described above, it is still not clear whether α4-GABA$_A$Rs may have different functional roles within the NAc core and shell.

In chapter 5 (see 5.3.4.) an adenovirus was used to knockdown expression of GABA$_A$R α4-subunits. Post-infusion immunohistochemical analysis of coronal brain slices revealed that the GFP-tagged virus was largely limited to the NAc core (see chapter 5; Fig. 5.3.A.). The specificity of these infusions means that future experiments could continue to use this protocol to explore the role of NAc core α4-GABA$_A$Rs in mediating cocaine-CPP, locomotor activity and behavioural sensitisation to cocaine. Furthermore, adjustment of the infusion location could allow for specific knockdown of α4-subunits within the NAc shell, thus allowing a comparison between NAc core and shell α4-GABA$_A$Rs in the behaviours described. Indeed, there is already evidence that in rats α4-GABA$_A$Rs within specific locations of the NAc contribute to the reinforcing effects of alcohol. Viral knockdown of α4-subunits within the medial NAc shell, but not ventral or lateral shell, or core, reduced ethanol intake in a two-bottle choice test and instrumental responding for ethanol (Rewal et al., 2009; 2011).

6.9. Conclusions

In conclusion, α4-$GABA_A$Rs are potent modulators of the excitability of NAc MSNs, and therefore play an important role in controlling both locomotor and conditioned behaviours. Deletion of α4-$GABA_A$Rs specifically from D1- or D2-expressing neurons is able to increase cocaine-CPP and CRf responding, respectively, likely through a disinhibition of the separate striatal output pathways within which these neurons are contained. Furthermore, activation of α4-$GABA_A$Rs within the NAc by intra-accumbal THIP at specific doses, is able to attenuate the enhancement of locomotor activity, cocaine-CPP and CRf responding by cocaine. These data indicate α4-$GABA_A$Rs within the NAc play an important role in controlling reward-seeking behaviours and that their selective agonism provides a method by which to reduce the energising effects of cocaine.

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