Investigations into the role of the metabotropic glutamate receptor, mGluR5, in incentive learning and some behavioural and neurobiological effects of cocaine

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March 2011

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The metabotropic glutamate receptor, mGluR5, is densely expressed in brain regions involved in incentive learning processes. There is considerable evidence to suggest that following exposure to addictive drugs such as cocaine, adaptations in these brain areas may underlie the development and maintenance of behavioural responses related to addictive processes. The present thesis examines the role of mGluR5 in both incentive learning processes and some behavioural and neurobiological effects of cocaine.

First, using a novel mutant mouse line in which mGluR5 is selectively knocked down in cells that express dopamine D1 receptors (D1R), I argue that this mGluR5 population is critically important for specific incentive learning processes. By blocking mGluR5 in wild-type mice with a selective antagonist, I then propose mGluR5 as necessary for the acquisition, but not the expression of an incentive association. Next, I present data showing that mGluR5 on dopaminoceptive neurons are not necessary for the 'conditioned rewarding' properties of cocaine, measured in the conditioned place preference model, but do contribute to the psychomotor activating effects of cocaine. Finally, I present an immunohistochemistry study that examines cocaine-induced activation of the extracellular-signal related kinase (ERK) pathway. In the mGluR5 knock-down mice, activation of the ERK pathway in the striatum is disrupted following an acute injection of cocaine. Given the importance of the ERK pathway in establishing and maintaining long term memories, I propose that disruption of this pathway could contribute, in part, to some findings reported in the present thesis.

Taken together, this thesis will argue that signalling through mGluR5 on D1R expressing neurons is important for the formation of incentive associations, and may contribute to neural adaptations necessary for the development and maintenance of behavioural responses related to addictive processes.

Acknowledgments

First and foremost, to Dai, thank you for accepting me into your lab and providing endless support, encouragement and freedom to pursue my interests. Your knowledge and kindness have been inspirational. I am forever grateful.

To Hans, thank you for your support, advice and always challenging me to think twice about every experiment and its *proper control procedures*.

To Andy, thank you for opening my eyes to basic science and for supporting my move into academia. I hope we can work together again someday.

To those in the mGluR5 collaboration, in particular, Briac Halbout, Martin Novak, Jan Rodriguez Parkitna, David Engblom and Rainer Spanagel. Thank you for allowing me to work with the mGluR5$^{KD\text{-}D1}$ mice and for many great discussions. I hope there will be more to come.

To those in Safety Pharmacology, Pfizer, Sandwich, in particular, Marcus Andrews, Coralie Apfeldorfer, Clare Gardner and Hannah Brinkmann. Thank you for taking me back and for teaching me so much in 7 months. Without you, Chapter 6 would not have been possible.

A special thanks to Jesus Bertran-Gonzales for invaluable advice regarding immunoblotting and IHC, to Bruce Hope for advice with immunoblotting, to Amparo Garcia-Lopez for assistance in preparation of the IHC figures, to Sarah King and Tom MacPherson for help with the last phase of the behavioural experiment in Chapter 6, to Tamzin Ripley for assisting with the analysis of Pavlovian conditioning data and to the Ancillary Unit staff for animal husbandry.

I am grateful to the BBSRC and Pfizer for financial support.

To my labmates, Claire, Hannah, Katie, Kim, Nima, Sandra, Sophie, Simon, Tom and Yolanda. Thank you for your incredible friendship and support. You have made me laugh every day and kept my spirits up when the going got tough. I will take away many great memories. Make sure to come and visit me in Switzerland and perhaps we can hire an RV...

To my friends (and great housemates), in particular, Miguel, Amparo, Vanessa, Raj, Graham, Hamira, and Tom. Thank you for many conversations and experiences that have kept me sane outside of the lab. I look forward to many more in the future.

Last, but by no means least, to my family, Mum, Dad, Maggie and Fiona. Thank you for your constant support, encouragement and love. I could not have come this far without you.

Publications and communications arising from this thesis

O'Connor EC, Stephens DN, Crombag HS. Modelling appetitive Pavlovian-instrumental interactions in mice. Current Protocols in Neuroscience (2010), Chapter 8: Unit 8.25

Novak M*, Halbout B*, **O'Connor EC***, Rodriguez Parkitna J, Su T, Chai M, Crombag HS, Bilbao A, Spanagel R, Stephens DN, Schütz G, Engblom D. Incentive learning underlying cocaine-seeking requires mGluR5 receptors located on dopamine D1 receptor-expressing neurons. Journal of Neuroscience (2010), 30 (36): 11973-11982. **contributed equally to the work*

O'Connor EC, Mead AN, Crombag HS, Stephens DN. The mGluR5 antagonist MTEP dissociates the acquisition of predictive and incentive motivational properties of reward-paired stimuli in mice. Neuropsychopharmacology (2010), 35: 1807-1817.

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Engblom D, Novak M, Halbout B, **O'Connor EC**, Rodriguez J, Su T, Chai M, Crombag HS, Bilbao A, Spanagel R, Stephens DN, Schütz G. mGluR5 receptors on dopamine D1 receptor-expressing neurons control the incentive learning underlying cocaine relapse. FENS 7th Forum of European Neuroscience, Amsterdam, Netherlands, 2010

O'Connor EC, Novak M, Engblom D, Rodriguez Parkitna J, Schütz G, Mead AN, Crombag HS, Stephens DN. The metabotropic glutamate receptor subtype-5 located on dopamine-D1 receptor expressing neurons is necessary for conditioned reinforcement, but not Pavlovian-instrumental transfer. EBPS, 13th Biennial Meeting, Rome, Italy, 2009; published in Behavioural Pharmacology, 2009. Vol 20 (Special issue 1): S34

List of abbreviations

ACC	Anterior cingulate cortex
AMPA	α–amino-3-hydroxy-5-methylisoxazole-4-propionate
BLA	Basolateral nucleus of the amygdala
CAMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CeN	Central nucleus of the amygdala
CHPG	(R,S)-2-chloro-5-hydroxyphenylglycine
CPP	Conditioned place preference
CPu	Caudate putamen
CR	Conditioned response
CREB	cAMP response element-binding
CRf	Conditioned reinforcement
CS	Conditioned stimulus
D1R	(Dopamine) D1 receptor
D2R	(Dopamine) D2 receptor
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa
DLS	Dorsolateral striatum
DMS	Dorsomedial striatum
dsRNA	Double-stranded RNA
ES cell	Embryonic stem cell
F-IHC	Fluorescence-immunohistochemistry
FR	Fixed-ratio
GABA	γ-aminobutyric acid
GFP	Green fluorescent protein
IEG	Immediate early gene
ILC	Infralimbic cortex
IP3	Ionositol 1,4,5-trisphosphate
ITI	Inter-trial interval

KA	Kainate
KD	Knock-down
LTD	Long term depression
LTP	Long term potentiation
MAPK/ERK	Mitogen-activated protein kinase/extracellular signal-regulated kinase
mGluR1/5	Metabotropic glutamate receptor subtype 1/5
miRNA	micro-RNA
MPEP	2-methyl-6-(phenylethynyl)-pyridine
mPFC	Medial prefrontal cortex
mRNA	Messenger RNA
MSN	Medium spiny neuron
MTEP	3-[2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine
NAcc	Nucleus accumbens
NARP	Neuronal activity regulated pentraxin
NMDA	*N*-methyl-D-aspartate
NS	Not significant
O	Outcome
OFC	Orbitofrontal cortex
PIT	Pavlovian-instrumental transfer
PLC	Prelimbic cortex
PLCβ	Phospholipase Cβ
PKA/C	Protein kinase A/C
R	Response
RNA	Ribonucleic acid
RNAi	RNA interference
S	Stimulus
(S)-DHPG	(S)-3,5-dihydroxyphenylglycine
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNc/r	Substantia nigra pars compacta/ reticulata

STEP	Striatal enriched tyrosine phosphatase
STN	Subthalamic nucleus
UR	Unconditioned response
US	Unconditioned stimulus
VI	Variable interval
VLS	Ventrolateral striatum
VP	Ventral pallidum
VTA	Ventral tegmental area
WT	Wild-type

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1 General introduction

1.1 Overview

Emotions are fundamental to a normal sense of well-being and can provide motivation for many tasks encountered in daily life. Associative learning processes represent a major component of emotion and motivation. In Pavlovian incentive learning, a neutral environmental stimulus that is predictive of reward (e.g. food, water or sex) can be attributed with motivational value enabling the stimulus itself to become attractive and wanted, reinforce new learning and/or energise goal-directed action. While such learning has clear adaptive value, addictive drugs are proposed to interact and subvert the neural components of Pavlovian incentive learning. In this way, environmental stimuli that are predictive of drug experience may exert powerful control over behaviour and contribute to cardinal features of drug addiction, including relapse after an extended period of abstinence. A major goal of behavioural neuroscience is to uncover the neural mechanisms of reward processing and thereby provide new insight into the basis of clinical disorders like addiction. It is within this broad framework that the following thesis will investigate the role of the metabotropic glutamate receptor, mGluR5, in Pavlovian incentive learning and some behavioural and neurobiological effects of one addictive drug, cocaine.

1.2 Reward

Obtaining and experiencing desirable objects like food, water or even the latest iPhone can be accompanied by complex emotional feelings such as pleasure or relief. Emotions experienced during rewarding events can provide motivation for many tasks encountered in daily life, but dysregulation of emotional and reward processing is a major feature in many affective disorders including depression, schizophrenia and addiction. A major focus of behavioural neuroscience research has been to explore the neural mechanisms of reward, not least with the hope of finding new therapeutic targets for affective disorders. However, to be successful in this goal, it is first necessary to consider what reward is.

Reward is a difficult concept because it is used to refer to a wide-range of constructs including 'reinforcement', 'subjective states' and 'incentive'. Reward differs from reinforcement in a number of ways (reviewed in White, 1989). For example, the classical definition of a 'reinforcer' as an event that increases the probability of the response that precedes it, makes no mention of subjective states (Robbins and Everitt, 1996). Indeed, reward can extend beyond solely the need for explicit subjective states experienced during the pursuit of the desired object and the final interaction with it (Robbins and Everitt, 1996; Dickinson and Balleine, 2002; Berridge and Robinson, 2003; Schultz, 2006; Stephens et al., 2010). For example, as a consequence of Pavlovian incentive learning, initially neutral environmental stimuli that predict a desired goal can themselves become motivationally important (Berridge and Robinson, 1998). Thus, reward is not a unitary process, but can involve distinct emotional, incentive motivational and learning components. All of these components may be acting in concert to determine behaviour in the face of a rewarding event or a stimulus that has been associated with reward. Thus, for behavioural neuroscientists to fully appreciate the role of any neural mechanism in reward requires that reward is teased apart into its distinct components (Cardinal et al., 2002a; Berridge and Robinson, 2003; Yin et al., 2008).

A model advanced by Robinson and Berridge (Berridge and Robinson, 1998; see also Berridge and Kringelbach, 2008) provides a useful framework in which the components of reward can be deconstructed. These components consist of 1) *Liking*: defined as the affective component or hedonic impact of a reward (e.g. food), which includes core 'liking' reactions, that can be unconsciously perceived, and explicit conscious experiences of pleasure. 2) *Wanting*: defined as motivation for reward, which includes incentive processes, that are not necessarily conscious, and conscious desires for incentives or cognitive goals and 3) *Learning*: which describes the ability to form associations, representations and make predictions about future rewards based on previous experiences, which involves explicit and cognitive predictions and implicit knowledge, as well as associative conditioning, such as Pavlovian and instrumental associations (Berridge and Kringelbach, 2008).

Some experiments reported in this thesis will explore processes that fall within (and between) the incentive 'wanting' and instrumental and Pavlovian associative 'learning' domains as delineated by Berridge. For this reason, the following sections will review some basic concepts in instrumental and Pavlovian learning and some theories of motivation that are pertinent to this thesis. Aversive associative learning processes will not be formally considered here, although their role in regulating appetitive motivational processes is acknowledged (see Dickinson and Balleine, 2002 for further discussion).

1.2.1 Instrumental learning

An animal can come to influence its environment by learning that a response (R) it makes can produce an outcome (O) that is advantageous to its survival and should be repeated (positive reinforcement), and that, oppositely, some responses produce outcomes which are deleterious to survival and should be avoided (negative reinforcement and punishment) (reviewed in Bouton, 2007). The performance of reward-related actions are thought to reflect two distinct learning processes, one controlling the acquisition of goal-directed (R-O) actions and the

other the acquisition of S-R habits (Fig 1.1a; Adams and Dickinson, 1981; Dickinson et al., 1983; Dickinson and Balleine, 1994; Dickinson et al., 1995). Goal-directed learning necessitates the acquisition of a response representation and a representation of the outcome produced by that action (Dickinson and Balleine, 1994). Thus, an action is considered goal-directed when the initiation of the action is made with knowledge of the causal relationship between the action and its consequence (i.e. the contingency) and the current value of the outcome (Dickinson and Balleine, 1994). In habit learning, instrumental responses (R) are associated with stimuli (S) present during training and the reinforcer primarily serves to strengthen these S-R associations. The reinforcers do not themselves become encoded as a goal (Everitt and Robbins, 2006). Typically, as actions become well learned, they may become automatic behavioural responses triggered by the presentation of a stimulus (i.e. S-R habit), without any explicit relation to the outcome (Dickinson, 1985; Logan, 1998). It is sufficient for experiments reported later in this thesis to note that goal-directed and habitual actions can be distinguished by their relative sensitivity to devaluation of the outcome and to degradation of the action-outcome contingency (Balleine and O'Doherty, 2010).

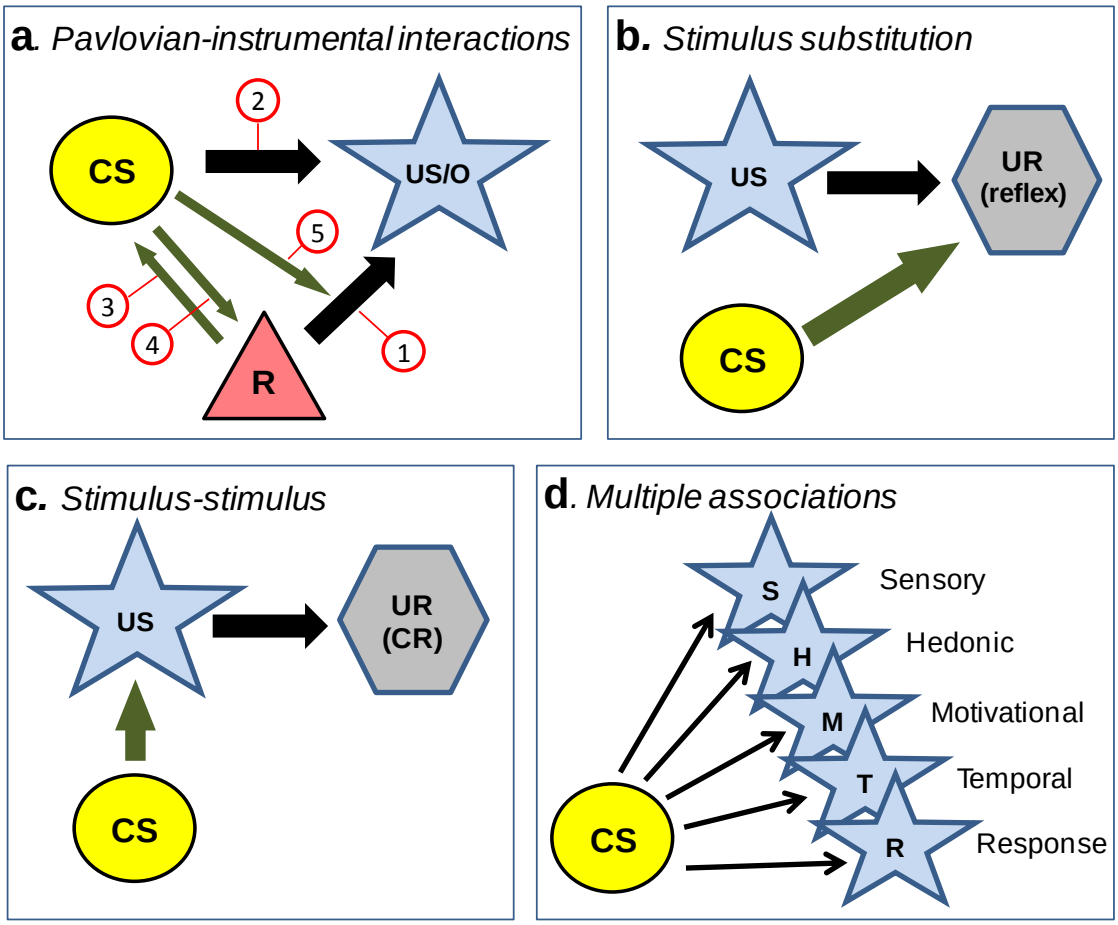


Figure 1.1 Components of associative learning (**a**) Instrumental (*1*) and Pavlovian (*2*) conditioning and some interactions (*3-5*) are illustrated. *CS*, conditioned stimulus; *US/O*, unconditioned stimulus (Pavlovian conditioning) or outcome (instrumental conditioning); *R*, instrumental response; *3*, Responses can be directly reinforced by a CS (conditioned reinforcement); *4*, A CS can trigger an instrumental response (S-R habit); *5*, A CS can motivate ongoing goal-directed actions (Pavlovian-instrumental transfer) (adapted from Bouton, 2007) (**b**) In stimulus substitution theory, a CS comes to elicit a reflex response which is identical to the unconditioned response generated to the US (e.g. salivation in response to food). (**c**) In stimulus-stimulus theory, a CS first generates a neural representation of the US, which determines the conditioned response that arises. (**d**) Multiple associations can form between the CS and features of the US, which determine how the CS can influence behaviour (redrawn from Delamater and Oakeshott, 2007).

1.2.2 Pavlovian learning

When presented with an outcome, termed the unconditioned stimulus (US), an animal may exhibit responses that allow for an appropriate reaction to that outcome. The animal may also experience behavioural or physiological changes as a result of exposure to the outcome (the unconditioned response; UR). If a neutral stimulus is associated with the US, this stimulus may take on the properties of a 'conditioned stimulus' (CS), which can subsequently evoke a conditioned response (CR) that resembles the UR, even when presented in the absence of the US. The formation of CS-US associations is central to Pavlovian conditioning (Fig. 1.1a) and the ability of a CS to elicit a CR is considered critical for behavioural adaptation to environmental events (Pavlov, 1927; Mackintosh, 1994).

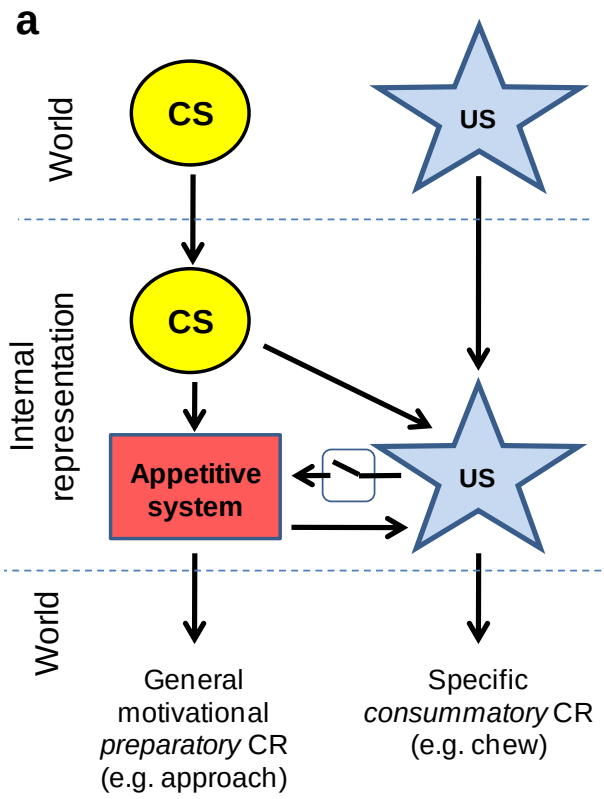
In the century that has followed Pavlov's seminal work on classical conditioning (Pavlov, 1927), many have questioned what is learned during the Pavlovian conditioning procedure, how such information is stored and represented in the brain and how Pavlovian associations interact with actions (e.g. Rozeboom, 1958; Rescorla, 1988; Cardinal et al., 2002a). Pavlov himself argued that a conditioned reflex would develop due to an acquired association between a representation of the CS and one of the US (Pavlov, 1927). However, this 'stimulus-substitution' view (Fig. 1.1b) could not readily explain how CSs were observed to modulate behaviours in more complex ways (e.g. transfer effects observed by Estes, 1948), and made no reference to motivational components of associative learning. It was not until Konorski (1967) and other contemporary learning theorists that followed (e.g. Wagner and Brandon, 1989; Holland, 1990; Dickinson and Balleine, 1994; Berridge and Robinson, 1998; Delamater and Oakeshott, 2007), that Pavlovian conditioning was recognised as having the potential to allow for multiple independent associations to form between the representation of the CS and features of the US (i.e. stimulus-stimulus associations; Fig. 1.1c-d).

1.2.3 The 'Konorskian' model

According to Konorski (1967), Pavlovian conditioning could be considered in two forms: *preparatory* and *consummatory* conditioning (reviewed in Dickinson and Balleine, 2002). Preparatory conditioning describes the acquisition of responses that are characteristic of the motivational class to which the US belongs, as determined by its autonomic (e.g. heart rate) and/or behavioural actions (e.g. approach and withdrawal). Consummatory conditioning refers to the acquisition of an association between the CS representation and a representation of the US that encodes its specific sensory-perceptual properties (e.g. salivation in response to a food US). By forming these distinct CS-US representations, a CS could elicit different CRs that, in part, also depended upon the nature of the CS itself (Konorski, 1967).

How then does motivation feature in Pavlovian learning as described by Konorski? Konorski (1967) proposed that preparatory and consummatory CRs result from CSs activating a common motivational system by direct (preparatory CS → motivational system) and indirect (consummatory CS → US representation → motivational system) pathways. Critically, feedback excitation from the motivational system to the US representation was proposed to give rise to a *Pavlovian 'desire'* (a term used by Dickinson and Balleine, 2002; but perhaps analogous to incentive 'wanting' as defined by Berridge, 1996) that motivates behaviour. In an extension to the Konorski model, Dickinson and Dearing (1979) suggested that primary motivational states could gate the capacity of an excited US representation to activate the common appetitive motivational system. In this way, motivational state could influence motivational feedback onto the US representation and, in turn, the motivational impact of the CS (reviewed in Dickinson and Balleine, 2002). In summary, this the so-called 'Konorskian' model (Fig. 1.2a) and a later version presented by Balleine (2005; Fig. 1.2b), proposed that both US-specific *consummatory* and general motivational *preparatory* associations can influence appetitive activation, which subsequently motivates goal-directed (instrumental) actions, but in distinct ways; either by generation of arousal or affect. As will be

evidenced later in this introduction, these preparatory and consummatory processes map well onto parallel learning processes that occur within, and probably between, sub-compartments of brain regions such as the amygdala (Balleine and Killcross, 2006) and ventral striatum (Shiflett and Balleine, 2010).



Panel B not shown due to copyright restrictions

For panel, see Balleine, 2005, Fig. 3.

Figure 1.2 A 'Konorskian' model of appetitive motivational influences in Pavlovian learning (**a**) Preparatory CSs directly activate an appetitive motivational system and give rise to general motivational CRs. This pathway also allows for preparatory CSs to excite performance of consummatory CRs. Consummatory CSs first activate a representation of the US, which can acquire further 'Pavlovian desire' by reciprocal connections with the appetitive system. However, this pathway is gated (blue box) by primary motivational state (e.g. hunger or thirst) (adapted from Konorski, 1967; Dickinson and Balleine, 2002). (**b**) (for panel,). A later model described by Balleine clearly incorporates the principles of the Konorskian model, with specific sensory associations (Se; i.e. consummatory) giving rise to affect, while general motivation associations (M; i.e. preparatory) giving rise to arousal, which can both influence goal-directed (instrumental) actions. Affect and arousal both activate a distributed appetitive (Ap) system and can be dissociated between different brain regions

1.2.4 The 'Incentive Salience' model

Incentive motivation concepts that were first developed around the 1970s and 1980s, most notably by Bolles (1972), Bindra (1974) and Toates (1986), closely followed principles of Pavlovian learning and provided an alternative account for how Pavlovian CSs could themselves serve as incentives to motivate behaviour (reviewed in Berridge, 2004). Bolles (1972) proposed that individuals were motivated by incentive expectancies, which he termed S-S* associations. In Pavlovian parlance, Bolles meant that the US (the S*) carried motivational value before learning had occurred, while the would-be CS (the S) did not. Thus, learning was required upon experience of S* to enable the acquisition of a predictive expectancy by the S. Bindra (1974) subsequently added to the expectancy account of Bolles that, as a consequence of classical conditioning, the CS could elicit the same incentive motivational state that had been generated by the reward itself (Bindra, 1974). Thus, the CS could be 'liked' and 'wanted', even when the US was not present, because it carried hedonic and affective qualities of the US. Finally, Toates (1986) proposed that primary drive states could also influence behaviour by enhancing the hedonic impact and incentive value of both the reward and the reward associated CS. For example, under conditions of thirst, the hedonic impact and incentive value of both a CS paired with water and of water itself would be enhanced.

In the Bolles-Bindra-Toates accounts of incentive motivation, conditioned incentives had value because they were both 'liked' (i.e. they produced pleasure) and 'wanted' (i.e. they had incentive value) as a consequence of Pavlovian learning (reviewed in Berridge, 2004). Robinson and Berridge (1993; 1998), in their incentive salience model, further advanced incentive motivation theory to propose that 'liking' and 'wanting' processes are, in fact, neurobiologically separate and experimentally dissociable. Thus, it is the attribution of incentive salience 'wanting' to a neutral perceptual or representational event (e.g. a cue) that determines the value of the incentive, controls instrumental behaviour directed toward obtaining it and enables neural representations of CSs to become highly salient, attractive and

desired (Berridge, 1996; Berridge and Robinson, 1998; Robinson and Berridge, 2001).

1.2.5 Summary

I have introduced the idea that Pavlovian learning is not a unitary process but can result in multiple independent associations between internal representations of the CS and features of the US. The content of what is learned and even the nature of the reward-paired environmental stimulus can determine how the CS subsequently influences behaviour, which can occur in many ways beyond generating simple reflexive responses as was first envisioned by Pavlov (1927). Two prominent theories of motivation in appetitive Pavlovian conditioning have also been introduced. In the 'Konorskian' model, CSs activate a general appetitive system that motivates goal-directed (instrumental) actions (Dickinson and Balleine, 2002). In this model, and subsequent extensions of it (see also Dickinson and Balleine, 1994; Cardinal et al., 2002a; Balleine, 2005; Everitt and Robbins, 2005; Yin et al., 2008) the strength of implicit stimulus-stimulus associations are emphasised and so too are effects of the S-R habits. In the incentive salience model of incentive motivation, CSs acquire incentive motivational value and emphasis is placed on the idea that CSs are 'wanted' in their own right, and that incentive salience 'wanting' can be dissociated from hedonic 'liking' aspects of reward (Berridge and Robinson, 1998). From this point onward, I will use the phrase 'Pavlovian incentive learning' in referring to learning that enables environmental stimuli associated with reward experience to influence behaviours. What is particularly interesting is that both theoretical standpoints reviewed here have made use of similar behavioural paradigms to assess Pavlovian incentive learning; which are sign-tracking (or approach towards the Pavlovian CS), conditioned reinforcement (CRf; response-contingent CS presentations) and Pavlovian-instrumental transfer (PIT; response-independent presentations of CSs). These three tests and some parameters that influence performance in them will be introduced in the following sections.

1.3 Behavioural models of Pavlovian incentive learning

1.3.1 Sign-tracking

When a localisable stimulus that is predictive of reward is presented, animals may come to approach and interact with it. I will use the term sign-tracking (Hearst and Jenkins, 1974) when referencing this phenomenon, while recognising that it has also been termed autoshaping (Brown and Jenkins, 1968), signal-centred behaviour (Jenkins et al., 1978) and conditioned (or Pavlovian) approach and contact behaviour (Peterson et al., 1972).

Sign-tracking was first reported by Brown and Jenkins (1968), who noted that a hungry pigeon would peck at a light that was predictive of food delivery, even though pecks never affected grain delivery. Notably, pecking of the stimulus persisted even if this resulted in the omission of food delivery (Williams and Williams, 1969), indicating that sign-tracking is most likely driven by Pavlovian learning, rather than implicit instrumental associations that could form as a result of the co-occurrence of reward delivery with CS directed responding. Sign-tracking has been observed in mice (Mead and Stephens, 2003b), rats (Peterson et al., 1972), monkeys (Sidman and Fletcher, 1968) and humans (Wilcove and Miller, 1974).

A number of factors influence the sign-tracking CR. The topography of the response (e.g. peck, sniff or lick) typically resembles that of the UR directed at the US indicating that sign-tracking is partly a reward-specific mechanism. For example, pigeons would make brief and forceful pecks at a stimulus predictive of food, but make relatively weak pecks accompanied by occasional licking or swallowing at a stimulus predictive of water (Wolin, 1968; Jenkins and Moore, 1973). Where the US was intravenously delivered cocaine, rats approached and investigated the lever CS, but rarely contacted it, perhaps because no actions were required to consume the drug US (Uslaner et al., 2006). Similarly, the occurrence of sign-tracking responses may depend upon the nature of the CS (e.g. visual or

auditory) and whether the CS is a natural releaser for appetitive behaviour systems in that species (Cleland and Davey, 1983). In rats, manual or oral manipulability may be a property of appetitive CSs that is important for maintaining sign-tracking responses (Holland, 1980; Cleland and Davey, 1983) and successful studies in rats have used localizable stimuli including retractable or illuminated leavers (Stiers and Silberberg, 1974; Boakes, 1977), static stimulus lamps (Holland, 1980) and even restrained conspecifics (Timberlake and Grant, 1975). More recently, approach and contact responses have been recorded in rats using computer graphic CSs presented on touch-screen panels (Bussey et al., 1994). Taking advantage of the natural tendency for mice to explore with nose-pokes, sign-tracking has been recorded in mice by placing a discrete visual CS within a hole and measuring nose-pokes into the CS-containing hole (Mead and Stephens, 2003b).

1.3.2 Conditioned reinforcement

In addition to eliciting approach responses, the attribution of incentive salience to appetitive Pavlovian CSs allows them to act as a goal for instrumental behaviour (Mackintosh, 1974; Robbins, 1978). Conditioned reinforcers can influence behaviour in a number of powerful ways, such as maintaining actions over delays between delivery of the primary reinforcer, as modelled by second-order schedules of reinforcement (e.g. Kelleher, 1966; Mead and Stephens, 2003b; Wilson and Bowman, 2004), and supporting the acquisition of a novel instrumental action (Mackintosh, 1974).

Experiments reported in Chapter 3 and chapter 4 of this thesis will focus on the acquisition of a novel instrumental response with CRf (Mackintosh, 1974). Extensive reviews on second-order schedules of reinforcement can be sought elsewhere (Everitt and Robbins, 2000; Schindler et al., 2002; Di Ciano and Everitt, 2005). The acquisition of a novel instrumental response with the CRf method is particularly advantageous as the reinforcing properties of the CS can be examined in isolation from any prior association between the operant response and a primary

reinforcer (Di Ciano and Everitt, 2004a). In this procedure, an animal is first trained a Pavlovian (CS-US) association, in which a neutral stimulus (e.g. a light or sound) is paired with the delivery of a reinforcer (e.g. liquid or solid food). Following Pavlovian conditioning, a test of CRf is undertaken in which two novel levers are inserted into the conditioning chamber. Responses on one lever result in presentation of the previously food-paired stimulus (CS+), while responding on an alternate (control) lever is without consequence. The number of responses elicited on the CS+ lever, in comparison to the control lever, provides a measure of the acquired reinforcing quality of the CS+. While most commonly used with rats (e.g. Robbins, 1978), the acquisition of a novel instrumental response with CRf procedure has successfully been adopted for use in mice (Mead and Stephens, 2003b; O'Connor et al., 2010).

1.3.3 Pavlovian-instrumental transfer

A third feature of appetitive Pavlovian CSs is that they can enhance goal-directed instrumental behaviour, termed Pavlovian-instrumental transfer (Estes, 1948; Lovibond, 1983), which may be considered ethologically as an example of a foraging behaviour (Galarce et al., 2007), and which others have referred to as conditioned motivation (Milton et al., 2008a) or cue-triggered wanting (Wyvell and Berridge, 2001). Although originally developed in rats, the PIT procedure has been adopted for use in mice (Mead and Stephens, 2003b; Yin et al., 2006a; Crombag et al., 2008a; O'Connor et al., 2010), monkeys (Henton and Brady, 1970) and humans (Hogarth et al., 2007; Talmi et al., 2008). In assessing this phenomenon, animals are first trained a Pavlovian association between presentation of a CS and the delivery of a primary reward (e.g., sucrose). Next, animals are trained to respond on a lever for the same primary reward experienced during conditioning, but in the absence of the CS. Finally, a test is undertaken in which the ability of the CS to enhance lever pressing in extinction is assessed. By performing the test under extinction conditions, neither primary nor secondary reinforcement occur, therefore enabling purely the response activating properties of CSs to be examined (Wyvell and Berridge, 2001).

In the experimental preparation described above, the training of a single CS-US (e.g. light → food) and R-O (e.g. left lever → food) association likely promotes a more general form of learning such that the CS exerts a more general enhancement of instrumental performance (termed general PIT). However, Pavlovian CSs can also exert a selective influence on instrumental performance (termed outcome-specific or selective PIT; Kruse et al., 1983). In the selective PIT procedure, an animal is first trained two separate CS-US associations (e.g. tone → food pellet, light → sucrose liquid). For the instrumental training component, animals acquire a different operant response for each of the outcomes earned during the Pavlovian conditioning phase (e.g. left lever → food pellet, right lever → sucrose). The effects of CS presentations on lever responding are then examined under extinction conditions. In selective PIT, the CS is observed to selectively enhance performance of the instrumental response associated with the same outcome. While most studies have examined either general or selective PIT within different experimental preparations (see Holmes et al., 2010 for an extensive review), Corbit and Balleine (2005) have described an elegant procedure in which general and outcome selective forms of PIT can be dissociated in the same experiment.

The study of general or specific PIT necessitates that the animal first learns a Pavlovian CS-US association, but there exist a number of crucial differences in conditioning procedures used for PIT than those for Pavlovian approach and CRf. First, a long-duration CS (e.g. 2 min) is typically used for PIT, in contrast to a relatively short-duration CS (e.g. 10 sec) used in the study of Pavlovian approach and CRf. The duration of the CS can influence the temporal dynamics of the PIT effect (Holland and Gallagher, 2003) and more robust PIT is observed when using a longer-duration CS (Crombag et al., 2008a). It is proposed that longer-duration CSs are more likely to establish preparatory or drive conditioned responses, which can exert modulatory influence over behaviours. In contrast, short-duration CSs are more likely to establish specific consummatory conditioned responses

(Konorski, 1967; Wagner and Brandon, 1989; Holland and Gallagher, 2003). Second, in conditioning for PIT, reinforcer deliveries are made on a semi-random basis during CS presentations. This is important, since CSs not only encode sensory or motivational representations of the US, but can also signal its temporal occurrence (Delamater and Oakeshott, 2007). The semi-random US presentation ensures that any influence of Pavlovian appetitive CRs on instrumental responding are not limited to discrete portions of the CS interval (Holland and Gallagher, 2003; Holland, 2004). Finally, animals are typically provided with relatively fewer presentations of the unpaired CS in the conditioning sessions for PIT. In effect, the unpaired CS serves as a neutral CS, rather than an explicitly non-reinforced CS. The purpose of this conditioning parameter is to limit the contribution of experience-dependent suppression of responding in the transfer test that may occur when using a stimulus that has been extensively non-reinforced (Holland, 2004). A truly novel stimulus is not used as a control stimulus, as this may result in unconditioned suppression of responding during the transfer test (Rescorla, 1967; Holland, 2004).

The instrumental training parameters are another important factor in the PIT effect. A variable interval (VI) or random ratio (RR) schedule is commonly used in PIT training. Extensive training under such schedules encourages instrumental responding that is supported by stimulus-response (S-R) rather than response-outcome (R-O) representations (Dickinson and Nicholas, 1983a, b; Dickinson et al., 1983; Dickinson et al., 1995). Thus, in general PIT, where transfer is most dependent upon the general motivational influence of the Pavlovian CS, the influence of the CS over instrumental responding is considered greatest when responding is least sustained by specific R-O representations (Holland, 2004).

1.3.4 Summary

Pavlovian incentive learning and some tests that can be used to explore the incentive motivational properties of CSs have been introduced from a theoretical and operational perspective. Understanding of the neural components of reward

processing has advanced significantly over the last twenty years, propelled by a greater understanding of neuroanatomy and by application of techniques that can be used to isolate discrete brain regions (e.g. lesioning), brain circuits (e.g. disconnection procedures) and neurotransmitters and receptors within those regions (e.g. behavioural pharmacology and genetics). The following sections will review studies that have investigated the neural components of the aforementioned behavioural models of Pavlovian incentive learning. In addition, studies that have examined the neural components of the acquisition and performance of an appetitive instrumental response will be reviewed, given the important role of instrumental processes in both CRf and, in particular, PIT. For each behavioural model, consideration will be given to what these studies tell us about the role of certain brain components in reward processing.

1.4 Neural components of reward

1.4.1 Sign-tracking

A series of lesioning experiments have identified interactions between the nucleus accumbens (NAcc) and its limbic cortical afferents in the acquisition of a sign-tracking CR. Thus, sign-tracking is disrupted by selective excitotoxic lesions of the anterior cingulate cortex (ACC) (Bussey et al., 1997b), orbitofrontal cortex (OFC) (Chudasama and Robbins, 2003), NAcc core (Parkinson et al., 1999; Parkinson et al., 2000b; Cardinal et al., 2002b) and pre- but not post-training lesions of the central nucleus of the amygdala (CeN) (Parkinson et al., 2000a; Cardinal et al., 2002b). A necessary role for ACC→NAcc core projections in sign-tracking has been confirmed using a double disconnection procedure, involving a unilateral lesion of the NAcc core and a contralateral unilateral lesion of the ACC (Parkinson et al., 2000b). In addition, lesions of the subthalamic nucleus (STN), both prior to or following training, facilitate sign-tracking, possibly by increasing incentive salience attribution to the CS and/or US (Uslaner et al., 2008). In contrast, sign-tracking was unaffected by lesions of the posterior cingulate cortex, prelimbic cortex (PLC) and infralimbic cortex (ILC) (Bussey et al., 1997b; Chudasama and Robbins, 2003), NAc shell (Parkinson et al., 2000a; Parkinson et al., 2000b), basolateral amygdala (BLA) and dorsal or ventral subiculum (Parkinson et al., 2000a).

Both dopamine and glutamate transmission are important for the acquisition and performance of sign-tracking responses. In the NAcc, dopamine depletions (Dalley et al., 2002; Parkinson et al., 2002) or antagonism of dopamine receptors (Di Ciano et al., 2001) impaired the acquisition and performance of sign-tracking. Using fast-scan cycling voltammetry, dopamine signalling in the NAcc core has been confirmed as an integral part of CS-US incentive learning necessary for the development of a sign-tracking response (Flagel et al., 2010). With respect to glutamate, infusion of an AMPA/KA receptor antagonist into the NAcc core impaired the expression, but not the acquisition, of sign-tracking (Di Ciano et al., 2001). In contrast, an NMDA receptor antagonist infused into the NAcc core

impaired the acquisition, but not the expression, of sign-tracking (Di Ciano et al., 2001). Notably, the particular pattern of disruption following AMPA/KA receptor antagonism (that is, increased approaches to the CS-) was also observed following ACC lesions (Bussey et al., 1997a) but not NAcc lesions, where disrupted Pavlovian approach arose from a reduction in responding to the CS+ (Parkinson et al., 1999). ACC-dependent processes may therefore interact with the NAcc in controlling the performance of sign-tracking responses through AMPA/KA receptor dependent signalling mechanisms (Di Ciano et al., 2001). Consistent with a necessary role of AMPA receptors in the performance of sign-tracking, mice lacking the GluR1 (*gria1* knock-outs), and GluR2 subunit (*gria2* knock-out) showed reduced sign-tracking responses (Mead and Stephens, 2003b, a). Finally, early post-training intra-NAcc infusions of a dopamine D1 receptor (D1R) and NMDA receptor antagonist impaired sign-tracking, while similar infusions of a dopamine D2 (D2R) receptor antagonist were without effect (Dalley et al., 2005).

So what do these lesioning, pharmacology and genetic studies tell us about the role of specific neural components in sign-tracking? The ACC plays a critical role in the formation of stimulus-reward associations, but perhaps not approach itself (Bussey et al., 1997a; Cardinal et al., 2002b), and provides a major source of projections to both the NAcc core and the CeN (Groenewegen et al., 1987; Groenewegen et al., 1999). The ACC→NAcc core pathway is proposed to subserve associative learning processes (whether attentional, impulsive or memory-based) and provide direction to the behavioural response, in terms of helping the organism to discriminate between relevant and irrelevant cues (Bussey et al., 1997a; Bussey et al., 1997b; Everitt et al., 1999; Parkinson et al., 2000b; Di Ciano et al., 2001). The ACC→CeN projection may allow for cortical control over autonomic, endocrine and behavioural reflexive components of emotional responses through projections from the CeN to the hypothalamus and brainstem (Swanson and Petrovich, 1998).

The CeN also receives information about appetitive stimuli from other inputs (including the BLA and thalamus) and projects to dopaminergic neurons of the VTA and STN (Fudge and Haber, 2000) and basal forebrain cholinergic neurons of the isodendritic core (Robledo et al., 1996; Han et al., 1997; Everitt et al., 1999). Thus, while there are no direct connections between the CeN and NAcc core, dopaminergic signalling in the CeN can markedly affect extracellular dopamine in the NAcc (Louilot et al., 1985; Simon et al., 1988). According to Everitt and colleagues, through these cholinergic and dopaminergic connections, the CeN may 1) influence attentional processing necessary for the learning about CS-US associations, 2) provide information about appetitive stimuli to the NAcc that promotes behavioural activation and 3) supervise the strengthening of associations between environmental stimuli, encoded by cortical limbic glutamatergic inputs, and motivational process in the NAcc core (Everitt and Robbins, 1992; Everitt et al., 1999; Everitt et al., 2001; Cardinal et al., 2002b). A fourth possibility from an incentive salience perspective (Berridge and Robinson, 1998), and perhaps analogous to the second point above, would propose that the ability of the CeN to influence dopamine signalling in the NAcc core may contribute directly to incentive salience attribution.

Glutamatergic and dopaminergic signalling are clearly critical within the cortical limbic striatal circuitry that converges on the NAcc core (Di Ciano et al., 2001; Flagel et al., 2010). Dopamine is proposed to play a major role in the attribution of incentive value to the reward paired CS (Yin et al., 2008; Flagel et al., 2010), distinct from its proposed role in reward prediction (Schultz et al., 1997). Both NAcc dopamine D1R and NMDA receptors appear necessary for the early consolidation of appetitive learning (Dalley et al., 2005), consistent with a role of these receptors in LTP and other forms of synaptic plasticity hypothesized to underlie associative learning (Pennartz et al., 1993; Kombian and Malenka, 1994; Kelley et al., 2003; Kelley, 2004). In contrast, AMPA receptors may be crucial for the performance of the sign-tracking CR, consistent with their role in the maintaining synaptic strength at excitatory synapses (Malenka, 2003; Kessels and Malinow, 2009).

1.4.2 Conditioned reinforcement

In comparison to the neural circuitry mediating Pavlovian approach, lesion studies have identified a distinct (but convergent) neural circuitry mediating control over the acquisition of a new instrumental response by conditioned reinforcers. Thus, the OFC (Pears et al., 2003), BLA (Cador et al., 1989; Burns et al., 1993; Whitelaw et al., 1996; but see Alderson et al., 2000), ventral subiculum (Burns et al., 1993), STN (Baunez et al., 2002) and pedunculopontine nucleus (Inglis et al., 2000) contribute to CRf. In contrast, lesions of the ACC (Everitt et al., 2001), PLC (Burns et al., 1993) and medial PFC (Pears et al., 2003) and CeN (Robledo et al., 1996) are without effect. Notably, responding for CRf can be powerfully amplified by psychomotor stimulants (Robbins, 1978; Robbins et al., 1983; Taylor and Robbins, 1984). This phenomenon critically depends upon dopamine (Taylor and Robbins, 1986; Wolterink et al., 1993), glutamate (Burns et al., 1994) and possibly GABAergic transmission (Dixon et al., 2010) in the NAcc, and integrity of the ventral subiculum (Burns et al., 1993), NAcc shell (Parkinson et al., 1999) and CeN (Robledo et al., 1996). Lesions of the NAcc core, while not affecting the acquisition of CRf, result in a loss of selectivity over stimulant-induced potentiation of CRf (Parkinson et al., 1999).

A role for dopamine and glutamate in the acquisition and performance of responding for CRf has been further explored with pharmacological and genetic approaches. Dopamine and glutamate signalling within the amygdala appears important for both the acquisition and performance of CRf (Hitchcott et al., 1997; Hitchcott and Phillips, 1997, 1998; Phillips et al., 2010). Prior to the CRf test, intra-BLA, but not CeN, infusions of a D3R antagonist 7-OH-DPAT disrupted the acquisition of a new response with CRf (Hitchcott and Phillips, 1998). Pre-test, intra-BLA infusion of an AMPA antagonist CNQX reduced responding for a drug associated CRf, but also increased responding on the control CS reinforced lever (Hitchcott and Phillips, 1997). In the amygdala, NMDA receptors may play a critical role in memory reconsolidation that leads to responding for CRf (Milton et al.,

2008b). In the ventral-subiculum, infusions of an AMPA/KA receptor antagonist CNQX selectively reduced responding for drug associated CRf (Hitchcott and Phillips, 1997). In the NAcc core, but not shell, responding reinforced by a cocaine-paired CS was dependent upon AMPA/KA receptors (Di Ciano and Everitt, 2001). In contrast, blockade of NMDA receptors in either the NAcc core or shell were without effect (Di Ciano and Everitt, 2001). Confirming a role for AMPA receptors, CRf was impaired in GluR1 knock-out mice (Mead and Stephens, 2003b) but not in GluR2 knock-out mice (Mead and Stephens, 2003a). Moreover, point mutation of the GluR1-ser831 phosphorylation site in mice resulted in impaired responding for CRf, while mutation of the GluR1-ser845 phosphorylation site was without effect (Crombag et al., 2008c).

Clearly then, the BLA, OFC and NAcc core are important for CRf, but what are the roles of these structures and signalling that occurs between them? First, dopamine transmission serves as a “gain amplification” signal, influencing the impact of the CS, but does not mediate responding for CRf *per se* (reviewed in Everitt et al., 2001). This latter role is likely provided by cortical limbic glutamatergic inputs carrying information about conditioned reinforcers and converging onto the ventral striatum, which provides the “limbic-motor interface” (Mogenson et al., 1980). In this respect, the BLA appears to play a major role. The BLA projects to the OFC, NAcc core and NAcc shell (Wright et al., 1996) and is considered to form a fronto-temporal system underlying more complex goal-directed behaviour (Everitt and Robbins, 1992; Schoenbaum et al., 1998; Everitt et al., 1999). Thus, the BLA is not necessary for Pavlovian conditioning *per se*, but may provide an affective representation of environmental stimuli to support more complex forms of goal-directed behaviour (including CRf; Cador et al., 1989; Burns et al., 1993; Whitelaw et al., 1996; Setlow et al., 2002), but not the simple elicitation of a stimulus CR (Parkinson et al., 2000a). The ventral subiculum, with an ability to influence dopamine transmission in the NAcc (Brudzynski and Gibson, 1997; Floresco et al., 1998), may provide a contextual background necessary for the potentiation of CRf (Everitt et al., 2001). Finally, a functional dissociation within the ventral striatum is

apparent, with the NAcc shell being involved in “vigour” of CRf, while “choice” or “direction” of the response involves the convergence of cortical limbic inputs at the NAcc core (Parkinson et al., 1999).

1.4.3 Pavlovian-instrumental transfer

The neural substrates mediating general PIT overlap with those described for Pavlovian approach. Thus, general PIT is disrupted by lesions of the CeN, but not the BLA (Hall et al., 2001; Holland and Gallagher, 2003) and lesions of the NAcc core, but not the NAcc shell (Hall et al., 2001) also disrupt general forms of PIT. Contrary to Pavlovian approach, there is no effect of ACC lesions on general PIT (Cardinal et al., 2003). A recent electrophysiology study suggests subsets of neurons in the PLC and OFC are involved in the integration of Pavlovian and instrumental information, which is fundamental to the general PIT effect (Homayoun and Moghaddam, 2009).

The circuitry recruited for outcome specific PIT can be dissociated from that of general PIT. Thus, lesions of the BLA (Blundell et al., 2001; Corbit and Balleine, 2005), but not the CeN (Holland and Gallagher, 2003; Corbit and Balleine, 2005), lesions of the NAcc shell, but not the core (Corbit et al., 2001) and post-training lesions of the OFC (Ostlund and Balleine, 2007), but not the PLC (Corbit and Balleine, 2003) disrupt specific PIT. Within the dorsal striatum, the dorsolateral striatum (DLS) appears vital for a CS to excite instrumental responding, while the dorsomedial striatum (DMS) is involved in the integration of CS-US associations with specific R-O associations to produce selective responding (Corbit and Janak, 2007; but see Pielock et al., 2011). Using a double disconnection procedure, BLA-NAc shell disconnection impairs the ability of outcome-specific CSs to bias instrumental actions during PIT, whereas BLA-NAcc core disconnection is without effect on selective PIT (Shiflett and Balleine, 2010).

Like Pavlovian approach and CRf, a clear contribution of dopamine transmission for PIT has been recognised. Systemic administration of the dopamine antagonists

pimozide or cis-fluphenthixol were found to disrupt general PIT (Dickinson et al., 2000). These findings may be attributable to blunted mesolimbic dopamine transmission, since PIT is also disrupted by antagonism of dopamine D1R and D2R in the NAcc core and shell (Lex and Hauber, 2008) and inactivation of the VTA (Murschall and Hauber, 2006; Corbit et al., 2007). Similar to the potentiation of CRf by psychomotor stimulants, intra-NAcc shell infusions of d-amphetamine potentiate general PIT (Wyvell and Berridge, 2000, 2001). The facilitation of PIT also occurs following intra-NAcc shell microinjections of corticotrophin releasing factor (Pecina et al., 2006). Selective PIT was intact in mice with knock-down of the dopamine transporter (Yin et al., 2006a), suggesting selective transfer may be independent of tonic dopamine levels (Yin et al., 2006a) and/or dopamine may be restricted to general excitatory effects of Pavlovian cues, with more specific motivational effects provided by other signals (Corbit and Balleine, 2005; El-Amamy and Holland, 2007).

Although glutamate transmission is likely involved in PIT, given the cortical limbic striatal circuitry recruited for PIT, there is some conflicting evidence on the particular molecular components involved. Systemic blockade of AMPA/KA and NMDA receptors were without effect on general PIT (Murschall and Hauber, 2005), while blockade of mGluR5 reduced general PIT (George et al., 2009). However, general PIT was impaired in GluR2 knock-out mice (Mead and Stephens, 2003a), but not GluR1 knock-out mice (Mead and Stephens, 2003b). Mutation of both GluR1-ser845 and GluR1-ser831 phosphorylation sites in mice resulted in a disruption of general PIT, but mutation of either site alone was without effect (Crombag et al., 2008b). General PIT was intact in mice lacking NARP (Johnson et al., 2007b), a secreted neuronal product that clusters AMPA receptors and regulates excitatory synaptogenesis (O'Brien et al., 1999). In a test of selective PIT, CS enhanced responding was impaired in GluR1 knock-out mice (Johnson et al., 2007a).

Collectively, studies on the neural components of PIT indicate that general and selective forms recruit different neural circuits that ultimately enhance activity in the NAcc core and shell, respectively. Moreover, these studies support a necessary role of the BLA in providing more outcome-specific CS-US representations, while the CeN provides more general motivational control over goal-directed behaviour (Corbit and Balleine, 2005). The CeN may well influence general PIT through its ability to influence mesoaccumbens dopamine signalling, as discussed previously. The role of the OFC in PIT is consistent with the proposed role of this structure in using the acquired S-O associations to guide appropriate goal-directed behaviour (Pickens et al., 2005; Ostlund and Balleine, 2007; Schoenbaum et al., 2009).

1.4.4 Instrumental learning

The neural substrates mediating the acquisition and transition between goal-directed and habitual responding have been discussed elsewhere (Belin et al., 2009; Balleine and O'Doherty, 2010). Notably, the acquisition, but not the expression of goal-directed behaviour requires the prelimbic PFC (Corbit and Balleine, 2003; Killcross and Coutureau, 2003; Ostlund and Balleine, 2005) while the DMS (a striatal target of the prelimbic PFC) contributes to both the learning and expression of goal-directed behaviour (Yin et al., 2005). In contrast, the DLS appears critically important for the transition to habit based responding (Yin et al., 2004, 2006b).

There is ample evidence to implicate dopamine and glutamate signalling within the cortical limbic striatal circuitry the acquisition and/or performance of a simple (R-O) appetitive instrumental response. Instrumental learning is disrupted by D1R antagonism in the NAcc core (Hernandez et al., 2005), the BLA and CeN (Andrzejewski et al., 2005) and the ventral, but not dorsal subiculum (Andrzejewski et al., 2006). With regard to glutamate, instrumental learning is disrupted by pre-trial antagonism of NMDA receptors in the mPFC (Baldwin et al., 2000), BLA and CeN (Andrzejewski et al., 2004; McKee et al., 2010), posterior lateral striatum (Andrzejewski et al., 2004) and the medial striatum (McKee et al., 2010). In

contrast, NMDA antagonism in the OFC (McKee et al., 2010), dorsal or ventral subiculum (Baldwin et al., 2000) and the anterior dorsal striatum (Andrzejewski et al., 2004) were without effect on the acquisition of an instrumental response. Co-infusion of a D1R and NMDA antagonist in the NAcc core strongly impaired the acquisition of instrumental responding, but were without effect when infused separately, highlighting the importance of dopamine-glutamate interactions in the learning of an instrumental response (Smith-Roe and Kelley, 2000). The contribution of NMDA and D1R receptors in the NAcc core to the acquisition, but not the performance of an instrumental response has been confirmed (Kelley et al., 1997; Baldwin et al., 2000; Hernandez et al., 2005). Finally, post-training interference with protein synthesis in the NAcc core impairs the acquisition of an instrumental response, further implicating synaptic alterations within this region as important for the consolidation of instrumental memories (Baldwin et al., 2002; Hernandez et al., 2002).

1.4.5 Summary

I have presented a review of studies that have investigated neural components of sign-tracking, CRf and PIT and of appetitive instrumental responding. The neural substrates underlying each behavioural model of Pavlovian incentive learning are distinct, but also show some commonality (Table. 1.1). Taken together, the studies reviewed in this section point to a critical importance for Pavlovian incentive learning of dopamine signalling in the striatum converging with excitatory glutamatergic inputs arising from limbic cortical regions such as the amygdala, prefrontal cortex and hippocampus (Fig. 1.3). Within each of these major nodes are further subdivisions (e.g. the BLA and CeN of the amygdala and the NAcc shell and core of the ventral striatum) that play functionally distinct roles in reward processing (Fig. 1.3). Lesions of specific subdivisions within these nodes can leave the function of other subdivisions intact, indicating that many neural components of reward processing learning convey parallel and independent information. This general theme is in accordance with neuroanatomical and theoretical models that support the existence of parallel and interactive neural networks in reward

processing (Haber et al., 2000; Cardinal et al., 2002a; Haber, 2003; Balleine and Killcross, 2006; DeLong and Wichmann, 2007; Belin et al., 2009; Kringelbach and Berridge, 2009; Balleine and O'Doherty, 2010). The striatum, broadly divisible into ventral and dorsal parts, is clearly an important interface between Pavlovian and instrumental processes. Is it now useful to introduce some details on the micro-circuitry of the ventral striatum, to further appreciate its role as the ‘limbic-motor interface’ (Mogenson et al., 1980).

	Ventral Striatum		Amygdala		Subiculum		Cortex				Other	
Behaviour	Core	Shell	BLA	CeN	Dorsal	Ventral	PLC	ACC	OFC	ILC	STN	VTA
Sign-tracking				Pre								
CRf	F	F		F		F						
General PIT												
Specific PIT												

Table 1.1 A summary of some brain regions involved in Pavlovian incentive learning processes. Lesion studies have identified an overlapping neural circuitry involved in different tests of Pavlovian incentive learning. Note that the picture presented here is rather simplified, since pharmacology manipulations of dopaminergic and glutamatergic signalling in each region are not incorporated. *Green boxes*, lesions disrupt behaviour; *Grey*, lesions are without effect; *Pre*, pre-training, but not post-training, lesions disrupt behaviour; *F*, Area necessary for psychostimulant facilitation of CRf. For complete details and references, see text.

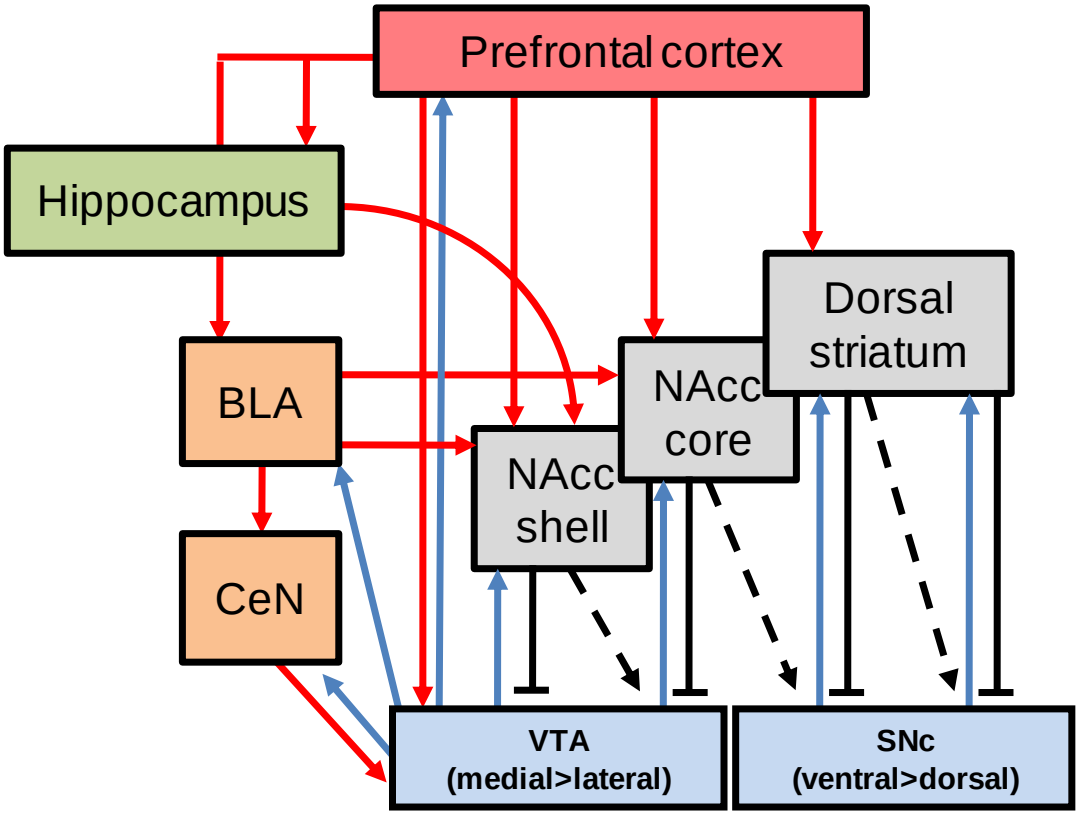


Figure 1.3 Neural circuitry of Pavlovian incentive learning. Glutamatergic (**red**) projections from the amygdala, hippocampus and prefrontal cortex arrive onto the striatum and are integrated with dopamine signals (**blue**) from the ventral midbrain. Reciprocal dopamine - GABA (**black**) connections between the midbrain and striatum result in a series of spiralling loops that allows more ventral parts of the striatum to influence more dorsal regions (DMS and DLS). The roles of each region in Pavlovian incentive learning can be briefly ascribed as follows: *Hippocampus*, contextual information; *BLA*, sensory specific information (producing affect); *CeN*, general motivation (producing arousal); *VTA*, incentive salience attribution and/or reward-prediction error learning; *Prefrontal cortex*, executive control and contingency / outcome representation; *Dorsal striatum*, control of instrumental actions; be they goal directed (DMS) or habit based (DLS); *NAcc shell,* consummatory CRs / hedonic URs; *NAcc core,* preparatory CRs / sign-tracking. Outputs of the striatum are illustrated in more detail in Figure 1.4 (adapted from; Everitt and Robbins, 2005; Yin et al., 2008).

1.5 The ventral striatum

The striatum can be broadly divided into ventral and dorsal parts (reviewed in Meredith et al., 2008; Humphries and Prescott, 2010; Sesack and Grace, 2010), although other reviewers have suggested that divisions of the striatum are best considered across a ventromedial to dorsolateral gradient (Voorn et al., 2004). The dorsal regions of the striatum (i.e. DMS and DLS) receive information from motor and somatosensory cortical inputs and have clear importance for controlling goal-directed and habit-based instrumental actions (Balleine, 2005; Faure et al., 2005; Yin et al., 2006b; Yin et al., 2008; Balleine and O'Doherty, 2010). Here I will focus on the ventral part (i.e. the accumbens), which can be further divided into the NAcc shell and NAcc core regions (Zaborszky et al., 1985; Meredith et al., 1992; Brog et al., 1993; Sesack and Grace, 2010) and which serves as a 'hub' of the limbic network, playing a central role in Pavlovian incentive learning processes.

1.5.1 Afferents

The ventral striatum receives most of its excitatory glutamatergic input from allocortical regions including the hippocampus and amygdala and neocortical areas including the prefrontal cortex (Heimer et al., 1991; Heimer and Van Hoesen, 2006; Meredith et al., 2008). Dopamine input to the ventral striatum comes from the VTA and the SN (Beckstead et al., 1979; Brog et al., 1993). There are few strong inhibitory afferents to the NAcc, although there is evidence for reciprocal GABA projections from the VP, other parts of the basal forebrain and the VTA (reviewed in Sesack and Grace, 2010). The NAcc shell also receives a projection from hypocretin neurons in the lateral hypothalamus (Peyron et al., 1998), which may have inhibitory actions on NAcc neurons (Martin et al., 2002). Serotonin and non-serotonin afferents from the dorsal raphe nucleus, and small norepinephrine projections from the locus coeruleus (LC) and the nucleus of the solitary tract, also input to the NAcc, the latter mainly to the NAcc shell (reviewed in Sesack and Grace, 2010).

Both glutamatergic and dopaminergic inputs appear differentially distributed between the NAcc core and shell compartments. For example, the ventral subiculum of the hippocampus projects primarily to the NAcc shell, while the more dorsal parts of the subiculum innervate the core (Meredith et al., 2008). Similarly, inputs from the amygdala strongly innervate the NAcc shell, but innervate only small compartments (the patches or striosomes) of the core (Ragsdale and Graybiel, 1988; Wright et al., 1996). Glutamatergic projections to the shell from the cortical regions arise from infralimbic, central and lateral orbital cortices, while projection to the NAcc core arise from more dorsal regions of the prefrontal cortex, such as the ventral and dorsal prelimbic and anterior cingulate cortices (reviewed in Yin et al., 2008). With respect to dopamine, the medial shell receives the most dense dopaminergic innervation (Voorn et al., 1986), with inputs to the NAcc shell arising almost exclusively from the VTA (Brog et al., 1993). In contrast, the NAcc core receives dopaminergic projections from both the VTA and SN (Brog et al., 1993). More specifically, dopamine neurons from the SNc project to 'patch' compartments in the ventral striatum (Gerfen et al., 1987), while dopaminergic neurons from the VTA terminate ventromedially in the NAcc shell and in medial parts of the core (Brog et al., 1993). How these different glutamatergic and dopaminergic inputs are fully integrated throughout the ventral and dorsal striatum is still under investigation, but the possibility exists that inputs from different regions can synapse onto the same principal neurons of the striatum, as has been demonstrated for inputs from the ventral subiculum and BLA and inputs from the PFC and BLA (French and Totterdell, 2002, 2003). Such connectivity may be critical for allowing hippocampal and BLA afferents to gate prefrontal influences on accumbens neurons (O'Donnell and Grace, 1995; O'Donnell et al., 1999; Grace, 2000)

1.5.2 Efferents

The major projections of the ventral striatum are GABAergic and connect with the VP, SN, VTA, hypothalamus and brainstem (reviewed in Sesack and Grace, 2010). The NAcc core projects mainly to the dorsolateral portion of the VP, the

entopenduncular nucleus and the SNr (Sesack and Grace, 2010). The NAcc shell innervates regions including the ventromedial part of the VP, SNc and VTA. Inhibitory projections from the NAcc shell to the VTA influence DA cells that, in turn, project to the NAc core, which give rise to a medial to lateral series of 'spiralling' projections which allow ventral regions of the striatum to influence transmission in more dorsal, motor-related, striatal regions (Heimer et al., 1991; Haber et al., 2000; Haber, 2003; Ikemoto, 2007).

1.5.3 Medium spiny neurons

The principle neurons in the striatum were first described as medium in size and densely spiny (Ramón y Cajal, 1911). Medium spiny neurons (MSNs) use GABA as their primary neurotransmitter, express neuropeptides, and comprise 90-95% of the total neuronal population of the striatum (Kita and Kitai, 1988; Meredith et al., 2008). However, MSNs are far from homogenous in morphology, the organisation of their inputs, the peptides and receptors they express and regions that they project to (Gerfen et al., 1990; Meredith et al., 2008; Bertran-Gonzalez et al., 2011). Morphologically, the cell bodies of MSNs are smaller in the ventral striatum than in the dorsal striatum, but the spine densities are similar (Meredith et al., 1992). However, in the medial shell, MSNs are smaller still and have fewer dendritic arbors and spines compared to MSNs in other striatal regions. Thus, it is estimated that MSNs in the medial shell have 80% less surface for synaptic contact than MSNs in the dorsal striatum (Meredith et al., 1992). With regard to organisation of inputs, MSNs receive glutamate input at synapses on the "head" of the spine (Kemp and Powell, 1971), while dopamine terminals generally contact with the "neck" of the spine (Bolam, 1984; Freund et al., 1984). This so-called 'triad of elements' is believed to enable dopamine signals to influence the efficacy of the glutamatergic signal (reviewed in Dani and Zhou, 2004). But, in the NAcc core, only about half of dopaminergic inputs contact with spine necks and in the medial shell, approximately one third of dopaminergic terminals make contact with spines (Zahm, 1992). In more medial and caudal regions of the ventral striatum, more dopaminergic contacts are found on dendrites of MSNs (Zahm, 1992).

1.5.4 Direct and indirect pathways

What receptors and peptides do MSNs express and where do they project to? These topics have been a matter of some debate and there may be ventral / dorsal striatal divisions that are not yet fully understood (for review see Voorn et al., 2004; Sesack and Grace, 2010; Bertran-Gonzalez et al., 2011). Current evidence suggests that MSNs can be segregated into approximately equal numbers based on the target of their main axon (Beckstead and Cruz, 1986). MSNs that form the 'direct' striatonigral pathway project mainly to the SNr and express the D1R as well as the neuropeptides dynorphin and substance P (Hong et al., 1977; Mroz et al., 1977). MSNs that form the 'indirect' striatopallidal pathway connect to the SNr through successive synaptic relays in the VP and STN, and express primarily D2Rs and enkephalin (Finley et al., 1981; Gerfen and Young, 1988; Gerfen et al., 1990). Uncertainty remains as to the identity and strength of connections on postsynaptic neurons to which MSNs project, but techniques such as optogenetics are beginning to prove useful in this area of research (e.g. Chuhma et al., 2011).

Finally, it is important to recognize that MSNs make contact not only with neurons outside of the striatum but also within it (Chang and Kitai, 1986; Chuhma et al., 2011). There exist MSN–MSN connections, which may allow for lateral inhibition within the NAcc, of which unidirectional (D1R-D1R or D2R-D2R) collaterals are most common and D2R→D1R collaterals are more abundant than D1R→D2R (Taverna et al., 2005; Taverna et al., 2008). MSNs may also connect with striatal inter-neurons, which together comprise < 5% of all striatal neurons, that include cholinergic tonically active neurons (TANs), fast-spiking interneurons (FSIs) and low-threshold spiking GABAergic inter-neurons (Kawaguchi et al., 1995).

1.5.5 Summary

Some of the connective intricacies of the striatum have been highlighted. The key points from this section are that, first, the striatum receives extensive dopaminergic inputs from the ventral midbrain and glutamatergic inputs from cortical and limbic

regions (Fig. 1.3). Second, how these inputs are integrated is not completely understood, but dopaminergic synapses are often well placed at the neck of spines on MSNs to modulate information encoded by glutamatergic inputs connecting at the spine head. Third, MSN projections from the striatum are broadly divisible into the direct pathways which connect with the ventral midbrain and express predominantly dopamine D1Rs and indirect pathways, which first connect with the ventral pallidum and express predominantly dopamine D2Rs (Fig. 1.4). Fourth, a series of ascending spiral loops between the striatum and midbrain allow the ventral striatum to influence activity in more dorsal striatal regions (Fig. 1.3). Finally, local connections amongst MSNs and striatal inter-neurons are likely critical for regulating overall striatal activity.

A challenge remains to understand how various components of the striatum interact at a network level in Pavlovian incentive learning processes (discussed further in Berridge, 2004; Voorn et al., 2004; Goto and Grace, 2008; Yin et al., 2008; Belin et al., 2009). The ventral striatum can be considered as a 'hub' for associative Pavlovian learning, while the dorsal striatum is a 'hub' for integrating sensorimotor actions. That neurons from the VTA and SNc project differently between ventral and dorsal parts of the striatum has led some to propose that the mesoaccumbens pathway may have a role more restricted in acquiring the value of stimuli during Pavlovian learning, while the nigrostriatal pathway may be more critical for learning the value of actions (Yin et al., 2008). However, it is important to remember that both striatal regions likely operate in parallel in 'real world' situations in which actions may lead to the presentation of conditioned incentives and where conditioned incentives may be encountered that, in turn, promote actions. Serial, dopamine-dependent connectivity linking the ventral striatum to more dorsal regions is proposed to offer a neural substrate for how incentive motivation can link to cognitive processes, with glutamate afferents onto the ventral striatum acting to push control in this circuitry toward more dorsal sites (Belin et al., 2009).

The neural circuitry of Pavlovian incentive learning is clearly important for adaptive behaviours, but its role in clinical disorders, and in particular drug addiction, is increasingly appreciated. In the next section, I will introduce some of the work which supports a role of Pavlovian incentive learning and its neural components in addiction-related behaviours.

Figure not shown due to copyright restrictions
For figure, see Seack and Grace, 2010, Fig. 2.

Figure 1.4 Outputs of the ventral striatum. Hypothetical direct and indirect output pathways whereby the NAcc core and shell may disinhibit or inhibit, respectively, adaptive motor pathways. Note that in this figure exist direct projections (i.e. D1R expressing) to the ventral mid brain, and indirect projections (i.e. D2R expressing) to the midbrain via the pallidum and subthalamic nucleus. Only major projections are shown. Red indicates inhibitory structures and pathways, whereas green indicates excitatory connections. *BF Hypoth*: basal forebrain and hypothalamus, *MD Thal*: mediodorsal thalamic nucleus, *NAc*: nucleus accumbens, *PFC*: prefrontal cortex, *SNr*: substantia nigra zona reticulata, *STN*: subthalamic nucleus, *VP dl/vm*: ventral pallidum, dorsolateral/ventromedial, *VTA*: ventral tegmental area (Sesack and Grace, 2010)

1.6 Drug addiction and Pavlovian incentive learning

"A number of detoxified cocaine users in our clinic population report experiencing intense arousal and cocaine craving when they encounter 'reminders' of their previous cocaine use: the sight of cocaine-using friends or locations, the use of alcohol, the sight of white bread, crumbs on the carpet, even the sight of talcum powder while changing a child's diaper... In our view, these cocaine 'reminders' are essentially classically conditioned stimuli which have acquired their 'reminder power' through repeated pairings with cocaine's pharmacologic effects over the natural course of a patient's drug use." (taken from Childress et al., 1988)

Drug addiction is defined as a chronic relapsing disorder characterised by loss of control over drug intake and compulsive drug taking despite adverse consequences (Everitt and Robbins, 2005; Koob and Le Moal, 2006). A cardinal feature of drug addiction is the high likelihood of relapse, even after long periods of abstinence from drug taking (DeJong, 1994). Early views of drug addiction emphasised motivation to take drugs because of their initial hedonic effects and to continue take drugs to avoid negative effects associated with drug withdrawal (Koob and Le Moal, 1997; Koob and Le Moal, 2006). However, as exemplified in the clinical observations above made by Childress and colleagues (1988) of detoxified drug users who were probably no longer experiencing withdrawal, a role for drug-associated cues is now appreciated in both the maintenance of drug seeking and taking and in precipitating relapse (Stewart et al., 1984; Robinson and Berridge, 1993; Everitt et al., 2001; Hyman and Malenka, 2001).

Addictive drugs, such as cocaine, and drug-paired CSs closely interact with and can persistently alter the neural systems involved in Pavlovian incentive learning (reviewed in Berke and Hyman, 2000; Hyman and Malenka, 2001; Kelley, 2004; Hyman et al., 2006; Kauer and Malenka, 2007; Belin et al., 2009). For example, cocaine-associated stimuli have been found to activate dorsolateral striatum and limbic cortical areas in humans (Childress et al., 1999; Garavan et al., 2000;

Volkow et al., 2006) and non-contingent presentations of drug CSs are able to increase dopamine release in the NAcc core, but not the shell of rats (Ito et al., 2000). Moreover, repeated d-amphetamine exposure can facilitate appetitive Pavlovian learning (Harmer and Phillips, 1998, 1999) and, within the striatum, exposure to addictive drugs can dramatically alter dopamine and glutamate signalling (Di Chiara and Imperato, 1988; White et al., 1995; White and Kalivas, 1998; Wolf, 1998; Ungless et al., 2001; Kalivas, 2004; Wolf and Ferrario, 2010), can cause persistent alterations in neuronal morphology (Robinson and Kolb, 2004; Crombag et al., 2005) and alter how information is processed and how actions are controlled at a network level (Schoenbaum and Setlow, 2005; Takahashi et al., 2007; Belin and Everitt, 2008). As a result of these (and many more) neurobiological changes caused by drug experience, drug-associated cues are proposed to become particularly effective in triggering drug-taking habits (Everitt and Robbins, 2005; Belin et al., 2009), or may become pathologically 'wanted' due to a sensitisation of incentive salience attribution (Robinson and Berridge, 1993).

In the following sections I will introduce the incentive-sensitisation model (Robinson and Berridge, 1993), given the experiments on behavioural sensitisation reported later in this thesis (Chapter 5). It is also valuable to introduce some ideas on how such CSs may operate in controlling addiction-related behaviours.

1.6.1 Incentive-sensitisation

Incentive motivation theory (see section 1.2.4) proposes that environmental stimuli associated with rewards can acquire incentive salience as a consequence of classical conditioning processes. Within the context of drug addiction, incentive-sensitisation theory provides an account of how addictive drugs, through their ability to activate and cause persistent neural adaptations in 'reward-related' brain areas, result in drugs and associated stimuli exerting increasingly powerful control over drug seeking and taking behaviour in some individuals (Robinson and Berridge, 1993, 2000, 2001, 2003, 2008). The four major points of the incentive

sensitisation, outlined by Robinson and Berridge (2001) are that 1) potentially addictive drugs cause long-lasting alterations in the brain 2) brain systems altered include those normally involved in incentive motivation and reward 3) these neural adaptations result in reward systems being sensitised to drug effects and drug associated stimuli and 4) these systems do not mediate pleasure or euphoric drug effects, but do mediate the incentive salience 'wanting' component of reward. Importantly, the incentive-sensitisation model proposes that drugs and drug associated stimuli may be wanted more and more even if the affective/hedonic qualities of drugs are experienced less and less. Thus, 'liking' components of reward, from the incentive-sensitisation perspective, are neither necessary nor sufficient for drug addiction to develop (Robinson and Berridge, 2001).

Measuring the psychomotor activating effects of addictive drugs has been a primary method of assessing sensitisation, based on the assumption that neural components underlying behavioural sensitisation overlap with those involved in reward processing (Robinson and Becker, 1986; Wise and Bozarth, 1987; Robinson and Berridge, 1993, 2000). It is worthwhile pointing out some parameters of behavioural sensitisation and consider what some of these may tell us about how this phenomenon reflects addiction-related processes (reviewed in Robinson and Berridge, 2001). Behavioural sensitisation is dose-dependent (Kalivas et al., 1988), typically requires repeated, intermittent drug experience (Robinson and Becker, 1986) but can also follow repeated exposure to food (Le Merrer and Stephens, 2006). Sensitisation in remarkably persistent (Robinson and Becker, 1986), can occur with drug self-administration (Phillips and Di Ciano, 1996) and shows individual differences in susceptibility (Robinson and Berridge, 2001; Flagel et al., 2008). Individual variation seen in the development of sensitisation have been found to correlate with individual variation seen in incentive salience attribution and is proposed to reflect differences in vulnerability or resistance to drug addiction and other compulsive behavioural disorders (Flagel et al., 2008; Flagel et al., 2009; Robinson and Flagel, 2009). Sensitisation is also strongly influenced by associative learning that involves circumstances surrounding drug

experience (Robinson et al., 1998). Thus, while sensitisation of neural systems can occur non-associatively, the expression of behavioural psychomotor sensitisation critically depends upon stimuli (such as the context) associated with drug experience (Pert et al., 1990; Anagnostaras and Robinson, 1996). This feature may reflect the role of drug-associated contexts in precipitating relapse (Robinson and Berridge, 2001). In addition, whether the environment is novel or familiar (i.e. a home environment) can also influence the development of sensitisation (Badiani et al., 1995; Crombag et al., 1996; Browman et al., 1998b, a; Crombag et al., 1999).

1.6.2 Sign-tracking

How might conditioned incentives influence addiction-related behaviour? Drug-paired CSs can elicit approach toward them (Uslaner et al., 2006) and, in real-world situations, individuals may be brought into close proximity with sources where drugs can be found, which may in turn influence the propensity to relapse (Stewart et al., 1984; Tiffany, 1990; Robinson and Berridge, 1993; Tomie, 1996; Di Chiara, 1998; Everitt and Robbins, 2005). Although sign-tracking effects have long been thought important for addiction-related behaviours, it has only been with recent methodological developments that sign-tracking toward drug-paired CSs has been demonstrated in animals (Krank, 2003; Kearns and Weiss, 2004; Uslaner et al., 2006; Cunningham and Patel, 2007).

1.6.3 Conditioned reinforcement

In many aspects of addiction-related behaviours, conditioned reinforcers may play a critical role. First, conditioned reinforcers may maintain drug-seeking between periods when drugs are not available, as modelled using the second-order schedule of reinforcement (Kelleher, 1966; Arroyo et al., 1998; Schindler et al., 2002; Mead and Stephens, 2003b; Wilson and Bowman, 2004; Di Ciano and Everitt, 2005). Indeed, drug-seeking for psychostimulants is particularly sensitive to omission of conditioned reinforcement during second-order schedules (Goldberg et al., 1981). Second, following extinction of an instrumental drug-seeking response, conditioned reinforcers are observed to reinstate instrumental responding on the

previously drug-paired lever (de Wit and Stewart, 1981; Kruzich et al., 2001; Highfield et al., 2002; Shalev et al., 2002; Shaham et al., 2003). This 'extinction-reinstatement model of drug seeking' may reflect the observation that humans frequently relapse after prolonged abstinence from drug taking. Third, conditioned reinforcers are able to support the acquisition of a new instrumental response (Di Ciano and Everitt, 2004a), which may model the learning of new behavioural strategies for obtaining drugs by drug addicted individuals (Everitt and Robbins, 2005). Whether these three differing operational roles of conditioned reinforcers are also neurobiologically separable remains to be determined (Stephens et al., 2010). Finally, as has been discussed previously, psychostimulants can markedly enhance responding for CRf maintained by CSs associated with natural rewards (see section 1.4.2). Everitt and Robbins (2005) propose that this feature of psychostimulants may be a critical component that contributes to their reinforcing effects; by analogy, this could be one reason why certain drugs are repeatedly taken for their ability to enhance enjoyment of music and other social experiences.

1.6.4 Pavlovian-instrumental transfer

PIT like processes may be considered as important in invigorating efforts to seek out, to consume or to 'want' drugs (Berridge and Robinson, 2003; Everitt and Robbins, 2005) and it is noteworthy that PIT effects have been shown for smokers working to gain cigarettes and money (Hogarth et al., 2007). To date, PIT like processes have not been observed with drug-associated CSs in animals. In fact, unexpected drug-CS presentations are found to suppress rather than enhance drug-seeking (Di Ciano and Everitt, 2003), which may be due to distracting effects of the highly salient 'wanted' cues (Robinson and Berridge, 1993; Belin and Everitt, 2008).

1.6.5 Summary

The present section has introduced the idea that environmental cues associated with addictive drugs, through associative learning processes, are proposed to subsequently influence drug taking, seeking and relapse in ways that reflect normal

Pavlovian incentive learning processes (i.e. sign-tracking, CRf and PIT). There has been some debate as to whether the effects of drug-associated cues arise because of 'aberrant learning' (Tiffany, 1990; Berke and Hyman, 2000; Everitt et al., 2001); in terms of strengthened stimulus-stimulus associations or dominant effects of S-R habits (discussed in Robinson and Berridge, 2003). Everitt and Robbins (2005) propose that habit does not refer to a procedural skill, but rather a persistent and repetitive initiation of an activity (e.g. drug-seeking). The incentive-sensitisation model argues that S-S associations are not pathologically strong, that learning itself is normal and learning alone cannot generate compulsive behaviour (Robinson and Berridge, 2001, 2003). Rather, Robinson and Berridge (1993, 2000, 2001, 2003, 2008) assert that the motivational impact of drug-associated CSs themselves are elevated due to sensitisation of systems (particularly mesolimbic and mesocorticolimbic dopamine systems) that control incentive salience 'wanting' attribution. However, food can also support behavioural sensitisation that shows many commonalities with drug sensitisation (Le Merrer and Stephens, 2006), suggesting that behavioural sensitisation alone cannot account for a dominance of drug-seeking behaviour supported by drug associated cues (Stephens, 2006). Nevertheless, the role of drug associated CSs and Pavlovian learning processes are likely of critical importance for many features of addiction, including the maintenance of drug-seeking and taking and of relapse after periods of abstinence.

I have now discussed at length Pavlovian incentive learning, its neural components and its relevance to drug addiction. Throughout this introduction an important and often necessary role of dopamine and glutamate signalling has been identified. In the final part of this introduction, the importance of glutamate signalling and the metabotropic glutamate receptor, mGluR5, will be further considered.

1.7 A focus on glutamate

Mesolimbic and mesostriatal dopamine has gained prominence as *the* neurotransmitter of reward-related learning (Schultz, 1997; Schultz et al., 1997; Berridge and Robinson, 1998; Wise, 2004; Flagel et al., 2010). However, as has been highlighted throughout this introduction, glutamatergic afferents to the striatum and glutamate signalling within this and other reward-related structures, including the amygdala, are clearly important for Pavlovian incentive processes (e.g. Hitchcott and Phillips, 1997; Baldwin et al., 2000; Di Ciano et al., 2001; Di Ciano and Everitt, 2001; Mead and Stephens, 2003b, a; Andrzejewski et al., 2004; Dalley et al., 2005; Crombag et al., 2008b; Crombag et al., 2008c; Milton et al., 2008b; McKee et al., 2010). In addition, dopamine outflow from the ventral midbrain is heavily influenced by glutamatergic inputs from the prefrontal cortex, ventral hippocampus and amygdala (reviewed in Sesack and Grace, 2010).

Many of the studies reviewed thus far point to dissociable roles of specific glutamate receptors in the ventral striatum, with NMDA receptors generally seen as necessary for the acquisition of learning (e.g. Kelley et al., 1997; Dalley et al., 2005), and AMPA receptors playing a more prominent role in the performance of acquired associations (e.g. Di Ciano et al., 2001). This theme has led a number of reviewers to propose that sculpting of synapses on striatal MSNs during reward-related learning may be critical for determining how these neurons subsequently respond to salient events (e.g. reward-associated CSs) and that glutamatergic mechanisms, acting in partnership with dopamine, may be critical for instigating and maintaining these experience-dependent neuroplastic changes (Kelley et al., 2003; Svenningsson et al., 2004; Dalley et al., 2005; Valjent et al., 2005; Meredith et al., 2008; Wolf and Ferrario, 2010). Moreover, glutamatergic signalling, and alterations of it following exposure to addictive drugs, may be a key component underlying much addiction-related behaviour (Kalivas and Duffy, 1998; Kalivas, 2000; Vanderschuren and Kalivas, 2000; Kalivas, 2004; Wolf et al., 2004; Conrad

et al., 2008; Engblom et al., 2008; Kalivas and O'Brien, 2008; Kalivas, 2009; Mameli et al., 2009).

In the following sections, I will briefly introduce some receptors of the glutamatergic neurotransmitter system and consider some features that underwrite their central role in synaptic plasticity, considered as the cellular correlate of learning and memory (Bliss and Lomo, 1973; Morris et al., 1986). Finally, I will introduce metabotropic glutamate receptors with a focus on group I mGluRs. Some electrophysiology and behavioural evidence will be reviewed that points to this receptor family as playing a central role in striatal synaptic plasticity and which indicates that mGluR5, in particular, could play an important role in Pavlovian incentive learning processes and the behavioural effects of addictive drugs, including cocaine.

1.7.1 Ionotropic NMDA and AMPA receptors

NMDA receptors, named after the selective agonist *N*-methyl-D-aspartate, are multimeric complexes formed of subunits belonging to three related families (NR1-3), with NR1 and NR2 required for subunit function (reviewed in Monaghan et al., 2005). An important feature of NMDA receptors is that they are both ligand gated and voltage gated, with voltage dependency due to blockade of NMDA receptor channels by Mg^{2+} ions at negative membrane potentials (Mayer et al., 1984; Nowak et al., 1984). NMDA receptors are highly permeable to Ca^{2+}, Na^{+} and K^{+} ions. Calcium permeability through NMDA receptors allows for the activation of intracellular signalling cascades; a feature that advocates NMDA receptors as critical for triggering experience-dependent plasticity (Monaghan et al., 2005). Indeed, NMDA receptor activation is critical for many forms of LTP (a long lasting enhancement of the synaptic response following high frequency stimulation of an afferent input) that have been observed in brain tissue (Collingridge and Bliss, 1995; Nicoll and Malenka, 1999), and also for the opposing phenomenon of LTD (a long lasting reduction in synaptic strength following low-frequency stimulation of an

afferent). Whether NMDA activation results in LTP or LTD may also depend, in part, upon the NMDA receptor subunit composition (Hrabetova et al., 2000).

AMPA receptors, named after the selective agonist α–amino-3-hydroxy-5-methylisoxazole-4-propionate, consist of four subunits (GluR1-4) and the composition of these subunits can dramatically alter the pharmacological and functional properties of the receptor. Notably, while agonist binding reveals a pore in AMPA receptors that can flux NA^{+} ions and allow for depolarisation of the cell, GluR2 lacking AMPA receptors can also flux Ca^{2+}, which leads to more robust changes in cellular activation (reviewed in Monaghan et al., 2005; Derkach et al., 2007; Shepherd and Huganir, 2007). AMPA receptors can also be phosphorylated at multiple sites on the intracellular C-terminal domain of their subunits, which allows for rapid and dynamic changes in the electrophysiological, morphological (e.g. trafficking and clustering) and biochemical (e.g. synthesis and subunit composition) properties of these receptors (reviewed in Wang et al., 2005; Santos et al., 2009). The correct trafficking of AMPA receptors is critical for the expression and maintenance of synaptic plasticity, with LTP generally requiring incorporation of new AMPA receptors at synapses, and LTD requiring removal of synaptic AMPA receptors (the mechanisms underlying AMPA trafficking have been extensively reviewed elsewhere; Song and Huganir, 2002; Bredt and Nicoll, 2003; Collingridge et al., 2004).

1.7.2 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluR) are categorised into three groups, based on similarities in agonist pharmacology, primary sequence and associated G-protein effector coupling; Group I (mGluR1 and mGluR5), Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7 and mGluR8) (Alexander et al., 2008). The mGluRs share a common structure of a large bi-lobed extracellular N-terminal domain, seven putative trans-membrane spanning domains separated by short intra- and extracellular loops, and a variable length cytoplasmic carboxyl-terminal domain (Fig. 1.5; Abe et al., 1992; Conn and Pin, 1997; Cartmell and

Schoepp, 2000; Kew and Kemp, 2005). Activation of mGluRs can trigger a cascade of intracellular events that indirectly alter neuronal excitability and can also produce relatively slow and delayed synaptic currents (reviewed in Ossowska, 2005).

Figure not shown due to copyright restrictions
For figure, see Conn and Pin, 1997, Fig. 4.

Figure 1.5 Schematic representation of an mGluR. The mGluRs share a common structure of a large bi-lobed extracellular N-terminal domain that contains the glutamate (Glu) biding site, seven putative trans-membrane spanning domains separated by short intra- and extracellular loops that together mediate G protein activation, and a variable length cytoplasmic carboxyl-terminal domain which regulates receptor activity and targeting by interactions with proteins including calmodulin, Homer and PICK1. Positive and negative allosteric modulators bind to the heptahelical transmembrane domain (adapted from Conn and Pin, 1997).

1.7.3 Group I mGluRs

1.7.3.1 Function

Group I mGluRs positively couple via pertussis toxin-insensitive G protein ($G_{q/11}$) to phospholipase Cβ (PLCβ), modulating cellular activity through secondary messenger cascades associated with increased phosphoinositide hydrolysis and calcium mobilization (Abe et al., 1992; Conn and Pin, 1997; Kew and Kemp, 2005). Activation of mGluR5a induces oscillations of intracellular calcium (Nash et al., 2001), whereas mGluR1a activation produces a single peak of intracellular calcium followed by a plateau (reviewed in Ossowska, 2005). Calcium mobilisation following group I mGluR activation can occur in a number of ways, including 1) production of ionositol 1,4,5-trisphosphate (IP3) due to PLCβ activation, with resultant release of Ca^{2+} from internal stores via IP3 receptors located on the endoplasmic reticulum (Fagni et al., 2000), 2) release of Ca^{2+} from internal stores via ryanodine-sensitive receptors (Fagni et al., 2000) and 3) Ca^{2+} influx through voltage dependent L-type calcium channels in the plasma membrane (Fagni et al., 2000; Ossowska, 2005). Calcium signalling by group I mGluRs is likely to involve Homer proteins, which can physically bridge these receptors to internal calcium stores (Tu et al., 1998). In a partly calcium independent manner, Group I mGluRs can positively link to the extracellular signal-regulated kinase (ERK) cascade (Peavy and Conn, 1998; Thandi et al., 2002), which has numerous functions in experience-dependent plasticity, for example, by regulating gene expression, cell proliferation and AMPA receptor trafficking (Adams and Sweatt, 2002; Boudreau et al., 2007; Patterson et al., 2010).

1.7.3.2 Localisation

In the CNS, distribution of group I mGluRs is widespread. Regarding mGluR5 (Fig. 1.6), Immunohistochemistry of mGluR5 protein and *in situ* hybridization of mGluR5 mRNA has identified intense expression predominantly in the telencephalic regions; including the cerebral cortex, hippocampus, subiculum, the olfactory bulb, dorsal and ventral striatum and the lateral septal nucleus (Abe et al., 1992; Shigemoto et al., 1993; Testa et al., 1994; Romano et al., 1995). Electron

microscopic immunocytochemistry has indicated that mGluR5 is primarily localised postsynaptically in the striatum (Shigemoto et al., 1993) and on post-synaptic dendritic spines and shafts in the cortex and hippocampus (Romano et al., 1995; Shigemoto et al., 1997). Typically, group I mGluRs are found in a perisynaptic zone that surrounds the ionotropic receptors (Luján et al., 1996). To a lesser extent, Group I mGluRs may be found pre-synaptically on glutamatergic (Herrero et al., 1992; Thomas et al., 2000), cholinergic (Marti et al., 2001; Feligioni et al., 2003) and noradrenergic (Parodi et al., 2006) nerve endings (but see Cartmell and Schoepp, 2000 for review).

The distribution of mGluR1 and mGluR5 largely overlap, although the relative expression of either receptor varies markedly amongst brain regions. Thus, the density of mGluR5 is considerably higher than mGluR1 in the basal ganglia and the CA1, CA3 and dentate gyrus of the hippocampus (Spooren et al., 2001). Expression of mGluR1 in the Purkinje cell layer of the cerebellum is high, but this area is almost devoid of mGluR5 (reviewed in Ossowska, 2005).

Figure not shown due to copyright restrictions
For figure see Shigemoto et al 1993, Fig 2

Figure 1.6 Localisation of mGluR5 receptors in the adult rat brain. (**a**) Negative image of a parasagittal section showing mGluR5 immunoreactivity. Abbreviations: *Sp*, Spinal trigeminal nucleus; *Cb*, Cerebellum; *IC*, Inferior colliculus; *Hi*, Hippocampus; *Th*, thalamus; *St*, Striatum; *Cx*, Cerebral cortex; *Ac*, accumbens nucleus; *AO*, Anterior olfactory nucleus; *AOB*, Accessory olfactory bulb, *MOB*: main olfactory bulb. Bar = 4 mm (taken from Shigemoto et al., 1993)

1.7.3.3 Pharmacology

Synthetic ligands for the mGluRs can be divided into competitive ligands which interact with the bi-lobed orthosteric agonist binding site and a relatively newer class of non-competitive ligands that interact with the transmembrane heptahelical domain, and which function as positive and negative allosteric modulators (reviewed in Kew and Kemp, 2005). Regarding competitive ligands, the most potent group I mGluR agonist, that displays reasonable selectivity over AMPA receptors and group II and III mGluRs, is (S)-3,5-dihydroxyphenylglycine [(S)-DHPG] (Schoepp et al., 1999), which exhibits low micromolar potency at mGluR1 and 5 (Kew and Kemp, 2005). The compound (R,S)-2-chloro-5-hydroxyphenylglycine (CHPG) is also a low-potency agonist at rat recombinant mGluR5 and is selective over mGluR1 (Doherty et al., 1997). Selective mGluR1 competitive antagonists have also been developed, which include (R,S)-1-aminoindan-1-5-dicarboxylic acid (AIDA) (Pellicciari et al., 1995).

Non-competitive mGluR5 antagonists have proven particularly valuable in understanding the physiological role of these receptors thanks to their useful *in vivo* pharmacokinetic profiles. Notably, 2-methyl-6-(phenylethynyl)-pyridine (MPEP) is a potent ligand of mGluR5 (IC50 = 37nM at human mGluR5a) and exhibits inverse agonism (Gasparini et al., 1999), while 3-[2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) exhibits improved selectively and CNS bioavailability over MPEP (Cosford et al., 2003). It is worthwhile noting that a number of positive allosteric modulators of group I mGluRs have also been identified, which may be particularly useful in reducing the potential for receptor desensitisation and tolerance effects associated with other ligands (reviewed in Kew and Kemp, 2005).

1.7.3.4 Role in plasticity

Group I mGluRs are well positioned at post-synaptic excitatory synapses to regulate neuronal excitability (reviewed in Ossowska, 2005) and to facilitate or induce both LTP and LTD of synaptic strength, although the cellular mechanisms through which these effects are achieved may vary amongst brain regions

(reviewed in Anwyl, 1999; Bellone et al., 2008; Anwyl, 2009; Luscher and Huber, 2010). Commonly, activation of group I mGluRs is associated with a persistent weakening of glutamatergic synapses (i.e. mGluR-LTD), which typically involves a pre-synaptic expression mechanism mediated by retrograde endocannabinoid signalling (Gerdeman and Lovinger, 2001; Sung et al., 2001; Robbe et al., 2002). However, mGluR-LTD can also occur independently of endocannabinoid signalling (Rouach and Nicoll, 2003) and can be expressed post-synaptically (Huber et al., 2000; Snyder et al., 2001; Nosyreva and Huber, 2005).

In the dorsal and ventral striatum, group I mGluRs are densely expressed on both D1- and D2-MSNs (Tallaksen-Greene et al., 1998) and can influence synaptic plasticity in both striatal regions and on both MSN populations (Surmeier et al., 2009; Luscher and Huber, 2010). In the dorsal striatum, mGluR-LTD is prevented on D1-MSNs of the direct pathway in the presence of dopamine, while mGluR-LTD on D2-MSNs of the indirect pathway is promoted (Kreitzer and Malenka, 2007; Shen et al., 2008). Both LTD mechanisms involve post-synaptic mGluRs, involve endocannabinoid signalling and are expressed pre-synaptically (reviewed in Luscher and Huber, 2010).

Within the ventral striatum, CB1 receptors are also present on glutamatergic corticostriatal afferents and activation of post-synaptic mGluR5 is both necessary and sufficient to produce retrograde endocannabinoid signalling, resulting in LTD through stimulation of these pre-synaptic CB1 receptors (termed 'eCB-LTD'; Robbe et al., 2002; Uchigashima et al., 2007). Notably, eCB-LTD in the NAcc is abolished following a single administration of cocaine (Fourgeaud et al., 2004). It is not known how dopamine regulates mGluR-dependent eCB-LTD in the ventral striatum and the relative contributions of mGluR1 and mGluR5 to mGluR-LTD in the ventral and dorsal striatum are not fully understood. Some evidence suggests that mGluR1 is the primary receptor mediating mGluR-LTD in the striatum (Gubellini et al., 2001), while both mGluR5 and mGluR1 may be necessary for corticostriatal LTP (Gubellini et al., 2003). In either case, mGluR-LTD would seem necessary to

balance activity between the direct and indirect pathways, which is normally dominated by an inhibition (i.e. LTD) of the indirect pathway (Surmeier et al., 2007). It is also noteworthy that mGluR5 participates in retrograde cannabinoid signalling in the BLA (Zhu and Lovinger, 2005) and, at excitatory synapses in the mouse prefrontal cortex, retrograde endocannabinoid signalling plays an important role in mediating LTD, with some evidence pointing to involvement of mGluR5 (Lafourcade et al., 2007).

In addition to altering pre-synaptic glutamate release through endocannabinoid signalling, post-synaptic group I mGluRs can closely interact with other post-synaptic receptors to influence neuronal plasticity. For example, both mGluR5 and dopamine D1Rs, but not D2Rs, may be involved in LTP that occurs in the NAcc core following high-frequency stimulation of glutamatergic inputs (Schotanus and Chergui, 2008), while mGluR5 is found to potentiate NMDA responses in striatal MSNs following stimulation of corticostriatal afferents (Pisani et al., 2001). In some brain regions (such as the hippocampus and VTA), mGluR-LTD can arise through alterations in post-synaptic AMPA receptor trafficking (Huber et al., 2000; Mameli et al., 2007; Luscher and Huber, 2010), which involves rapid local synthesis of proteins that include activity-regulated cytoskeletal associated protein (Arc; Park et al., 2008), microtubule associated protein 1b (MAP1b; Davidkova and Carroll, 2007) and striatal enriched tyrosine phosphatase (STEP; Moult et al., 2006; Zhang et al., 2008). At least in the VTA, mGluR-LTD requires the rapid synthesis of GluR2, which forms GluR2-containing AMPA receptors that are built within minutes and replace synaptic GluR2-lacking (calcium permeable) AMPA receptors (Mameli et al., 2007). Notably, mGluR-LTD is only observed in the VTA after synapses have been potentiated by cocaine exposure, suggesting that group I mGluRs may play a protective role by ‘depotentiating’ synapses (Bellone and Luscher, 2005, 2006; Mameli et al., 2007). Finally, interactions with the ERK-MAPK and PI3K-mTOR intracellular signalling pathways provide a mechanism through which group I mGluRs can alter translational regulation and induce plasticity (reviewed in Luscher and Huber, 2010).

1.7.3.5 Role in behaviour

Much of what is known about the role of group I mGluRs in behaviour has come from studies using the selective mGluR5 antagonists MPEP and MTEP. Both compounds have been shown to display anxiolytic and antidepressant activity (Ballard et al., 2005; Li et al., 2006; Belozertseva et al., 2007; Stachowicz et al., 2007; George et al., 2009), to reverse L-dopa induced dyskinesia in rodent models of Parkinson's disease (Dekundy et al., 2006; Levandis et al., 2008), to modulate nociceptive transmission (Neugebauer, 2002; Zhu et al., 2005; Osikowicz et al., 2008) and to modulate the reinforcing effects of drugs of abuse (Table 1.2), as discussed further below (and reviewed elsewhere; Carroll, 2008; Markou, 2009; Olive, 2009). Recent evidence also suggests that positive allosteric modulators of mGluR5 may provide a novel approach for the development of antipsychotic and precognitive agents (Liu et al., 2008). Thus, it appears that mGluR5 contributes to a variety of behavioural and emotional processing functions, which is in accordance with the predominant expression of mGluR5 within the limbic system.

In a now seminal study, mGluR5 knock-out mice were found not to self-administer cocaine and were insensitive to cocaine's locomotor stimulating effects (Chiamulera et al., 2001). Following this report, numerous studies have used mGluR5 antagonists to further understand the role of this receptor in animal models of addiction-related behaviours and behavioural effects of addictive drugs. Thus, by example of cocaine, mGluR5 antagonism has been shown to disrupt the acute psychomotor activating effects of cocaine (McGeehan et al., 2004), to block the acquisition and/or expression of cocaine sensitisation (Dravolina et al., 2006; Veeneman et al., 2010), to disrupt the acquisition of cocaine CPP (McGeehan and Olive, 2003) (but see Herzig and Schmidt, 2004), and to attenuate reinstatement of drug seeking by cocaine-associated cues and by a cocaine prime (Backstrom and Hyytia, 2006; Kumaresan et al., 2009). Indeed, mGluR5 antagonists have been shown to disrupt the reinforcing effects of addictive substances from a variety of pharmacological classes, as assessed using the drug self-administration paradigm

(Table 1.2). Interestingly, mGluR5 antagonism had no effect on the reinstatement of food seeking by a food-paired cue (Bespalov et al., 2005; Gass et al., 2008; Kumaresan et al., 2009), although reinstatement of seeking induced by a discriminative cue paired with sweetened, condensed milk was attenuated by MTEP (Martin-Fardon et al., 2009).

Taken together, these studies suggest that mGluR5 may be intimately involved in appetitive learning processes for both 'natural' and/or 'drug' rewards. However, it remains uncertain as to whether mGluR5 plays an exclusive role in primary and/or secondary reinforcement and, in fact, little work has been done to tease apart the role of mGluR5 in specific Pavlovian incentive learning processes (although see George et al., 2009). Furthermore, attempts to clarify the exact contribution and neuroanatomical location of mGluR5 in regulating responses to addictive drugs or reward-paired CSs have been complicated by the fact that pharmacological tools, such as MPEP, may induce anhedonia following intracerebral administration (Backstrom and Hyytia, 2007), or may possess intrinsic reinforcing properties (van der Kam et al., 2009b) and also because of the widespread expression of mGluR5 in the brain (Shigemoto et al., 1993).

Drug	Reference	Species	Sub species / Strain	Sex	Schedule	mGluR5 antagonist	Doses (mg/kg)	Admin Route	dose time (min)	Outcome (MED; mg/kg)
Cocaine	(Platt et al., 2008)	NHP	Squirrel Monkey	M	FR10	MPEP	0.1-1.8	i.m.		↓ (0.3)
	(Lee et al., 2005)	NHP	Squirrel Monkey	M/F	FI(FR)	MPEP	0.1-1.0	i.m.	5	↓ (0.3)
	(Tessari et al., 2004)	Rat	Wistar	M	FR2	MPEP	1.0-10	i.v.	30	↓ (10)
	(Paterson et al., 2005)	Rat	Wistar	M	PR	MPEP	1.0-9.0	i.p.	30	↓ (9)
	(Kenny et al., 2005)	Rat	Wistar	M	FR1	MPEP	1.0-9.0	i.p.	30	↓ (3)
Methamphetamine	(Osborne et al., 2008)	Rat	SD	M	FR1	MTEP	0-3	i.p.	30	↓ SA (1)
	(Gass et al., 2008)	Rat	SD	M	FR1/PR	MTEP	0-3			↓
Ethanol	(Cowen et al., 2005)	Rat	Fawn Hooded	M	FR3	MTEP	2	i.p.	20	↓ (2)
	(Cowen et al., 2005)	Rat	Alc Prefering (iP)	M	FR3	MTEP	0.5-2	i.p.	20	↓ (1)
	(Besheer et al., 2008)	Rat	Alc Prefering (P)	M	PR	MPEP	1.0-10	i.p.		↓ (3)
	(Schroeder et al., 2005)	Rat	Alc Preferring (P)	M		MPEP	1.0-10			↓ (3)
	(Cowen et al., 2007)	Mouse	C57BL/6	M	FR1	MTEP	5-40	i.p.	20	↓ (20)
	(Lominac et al., 2006)	Mouse	C57BL/6J	M	FR4	MPEP	1.0-30	i.p.	30	↓ (10)
	(Hodge et al., 2006)	Mouse	C57BL/6J	M	FR1	MPEP	1.0-10	i.p.	0	↓ (3)
Heroin	(van der Kam et al., 2007)	Rat	Long Evans		FR10	MPEP	1.25-20	i.p.	30	↓ (20)
Ketamine	(van der Kam et al., 2007)	Rat	Long Evans		FR3	MPEP	1.25-20	i.p.	30	↓ (5)
Nicotine	(Tessari et al., 2004)	Rat	Wistar	M	FR2	MPEP	1.0-10	i.v.	30	↓ (10)
	(Palmatier et al., 2008)	Rat	Sprague Dawley	M	FR1(VS/lever)	MPEP/MTEP	0-12/0-4	i.p.		↓ (6 & 2)
	(Liechti et al., 2007)	Rat	Wistar	M	FR5	MPEP	1.0-9.0	i.p.	30	↓ (6)
	(Paterson et al., 2005)	Rat	Wistar	M	PR	MPEP	1.0-9.0	i.p.	30	↓ (9)
	(Paterson, et al., 2003) (Markou, et al., 2004)	Rat	Wistar	M	FR5	MPEP	1.0-9.0	i.p.	30	↓ (3)

Table 1.2 Review of mGluR5 antagonist effects on drug self-administration. Abbreviations: NHP, Non-human primate; M, male; F, female; FR, fixed-ratio; PR, progressive ratio; i.m. intra muscular; i.v. intra venous; i.p. intra peritoneal; MED: minimum effective dose; ↓ indicates decrease in self-administration

1.7.4 Summary

Reward processing is multifaceted, but associative learning represents one major component that allows an organism to recognise and appropriately respond to environmental stimuli that are predictive of reward experience. Reward-associated stimuli can influence behaviour in many ways that are considered to reflect the formation of distinct CS-US associations. Investigations into the neural substrates of Pavlovian incentive learning have identified a forebrain circuitry involving cortical limbic and striatal regions, with glutamatergic and dopaminergic signalling recognised as important in both the formation of distinct reward-related memories and in determining responding to reward-associated stimuli. The striatum, with its ventral and dorsal parts, is a key integration point for dopaminergic signalling arising from the ventral midbrain with glutamatergic inputs from cortical and limbic regions. Outputs from the striatum to motivational and motor systems can be broadly distinguished into two neuronal populations that form the direct striatonigral (D1-MSNs) and indirect striatopallidal (D2-MSNs) pathways. A challenge remains to understand how reward experience might sculpt activity in these functionally distinct pathways, which could determine how these pathways, and presumably the organism in turn, respond to reward-associated stimuli. Moreover, understanding how addictive drugs interact with the neural mechanisms of Pavlovian incentive learning may provide insight to how drug associated stimuli come to influence drug-seeking, taking and relapse in addiction.

Within this context, mGluR5 is particularly interesting. From histology and electrophysiology studies, group I mGluRs appear ideally positioned to contribute to experience-dependent neuroplastic changes in reward-related brain areas, either through modulation of pre-synaptic neurotransmitter release, post-synaptic intracellular signalling cascades and/or post-synaptic activity of D1Rs, NMDA and AMPA receptors. With the advent of useful *in vivo* tools, behavioural studies have identified mGluR5 as important for appetitive learning for ‘natural’ and/or ‘drug’ rewards. However a number of interesting questions remain unanswered. First, it is not clear which component(s) of reward processing mGluR5 is involved in, such as

liking and/or incentive salience attribution or Pavlovian and/or instrumental learning. Second, with regard to Pavlovian incentive learning, it is not clear whether mGluR5 contributes to general motivational and/or outcome specific learning and whether mGluR5 could be involved in learning necessary to support sign-tracking, CRf and/or PIT. Third, it is not clear whether mGluR5 could play a role in the acquisition of reward-related associations and/or the expression of control over behaviour by reward-associated stimuli. Fourth, it is not clear where in the brain mGluR5 might contribute to reward processing, although the striatum would seem like one obvious candidate given the particularly high expression of mGluR5 in this region. Fifth, if striatal mGluR5 contributes to reward-related learning, it would be of interest to understand the relative contributions of mGluR5 located on MSNs of the D1R direct striatonigral pathway or the D2R indirect striatopallidal pathway. Finally, it would be of further value to understand the effect of addictive drugs on signalling mediated by mGluR5 which, combined with a better understanding of the role of mGluR5 in reward processing, may provide some insight to how drug-associated stimuli come to influence addiction-related behaviours.

As outlined in the following sections, experiments reported in this thesis will aim to address some of these issues using behavioural pharmacology techniques combined with immunohistochemistry and immunoblotting in novel mouse line in which mGluR5 has been selectively knocked-down on cells that express dopamine D1Rs (termed mGluR5$^{KD\text{-}D1}$ mice; Novak et al. 2010).

1.8 Aims and structure of this thesis

1.8.1 Chapter 2

Chapter 2 investigates the basic phenotype of mGluR5^{KD-D1} mice. Particular attention is paid to taste, vision and auditory function and motor activity. Intact function of these sensory and motor systems will be required to undertake more complex behavioural tasks in mGluR5^{KD-D1} mice. In addition, there is much evidence to support a role of mGluR5 in anxiety-related behaviours (e.g. Spooren et al., 2000b), thus potential alterations in anxiety-related behaviours in mGluR5^{KD-D1} mice will be assessed using three tests of unconditioned anxiety; open-field, light-dark box and elevated plus-maze. The experiments reported in this chapter therefore aim to discover any basic deficits in mGluR5^{KD-D1} mice that should be considered when undertaking and interpreting data from other experiments reported in this thesis.

1.8.2 Chapter 3

Chapter 3 explores Pavlovian incentive learning in mGluR5^{KD-D1} mice. Given the dense expression of mGluR5 in 'reward-related' brain areas (Shigemoto et al., 1993; Sesack and Grace, 2010) and its established role in synaptic plasticity (Bellone et al., 2008), considered as the cellular correlate of learning and memory (Kelley, 2004), it is reasonable to propose that mGluR5 may have a critical role in appetitive Pavlovian learning. To investigate this possibility, cohorts of mGluR5^{KD-D1} mice will be trained a Pavlovian association between a food reward and a simple stimulus (such as a tone or light). The ability of mGluR5^{KD-D1} mice to learn about the predictive properties of the food-paired CS will be assessed in tests of discriminated approach. The ability of the CS to acquire incentive motivational value, such that it can reinforce, attract and motivate behaviour will be assessed in tests of CRf, sign-tracking (Pavlovian approach) and PIT, respectively. To further probe neural function in mGluR5^{KD-D1} mice, the ability of cocaine to facilitate CRf will be examined.

1.8.3 Chapter 4

The aim of Chapter 4 is to investigate whether the CRf deficit in mGluR5^{KD-D1} mice reported in Chapter 3 reflected a failure of these mice to learn an incentive association between an environmental stimulus and a food reward (i.e. an acquisition deficit) and/or a failure of the conditioned incentive to subsequently exert control over behaviour (i.e. an expression deficit). Since mGluR5 is constitutively knocked-down in mGluR5^{KD-D1} mice, the experiments in Chapter 4 will take a pharmacology approach to investigate this question. Thus, wild-type mice will be injected with the mGluR5 antagonist, MTEP, to block mGluR5 function during the learning of a Pavlovian association and/or a test of CRf. In this way, the role of mGluR5 in the acquisition and/or expression of predictive and incentive properties of a reward-paired CS will be assessed.

1.8.4 Chapter 5

Findings from chapter 3 and 4 both implicate mGluR5 in the acquisition of incentive associations that subsequently enable a CS paired with a “natural” reward (food) to control behaviour. Environmental stimuli associated with addictive drugs, such as cocaine, are proposed to exert powerful control over behaviours because of the effects of such drugs on the neural circuitry that mediates incentive learning and memory processes (Robinson and Berridge, 1993; Nestler, 2001; Kelley, 2004; Hyman et al., 2006). If incentive Pavlovian learning is disrupted in mGluR5^{KD-D1} mice, it is reasonable to propose that some behavioural effects of cocaine, which reflect cocaine-induced neuronal adaptations within reward-related brain areas, may also be disrupted in mGluR5^{KD-D1}mice. Thus, Chapter 5 will use mGluR5^{KD-D1} mice to explore the contribution of mGluR5 on dopaminoceptive neurons to cocaine conditioned reward, measured with the CPP task, and to behavioural sensitisation.

1.8.5 Chapter 6

Experiments in Chapter 5 reveal that the acute psychomotor activating effects of cocaine are significantly attenuated in mGluR5^{KD-D1} mice when compared to their

wild-type counterparts. If some acute behavioural effects of cocaine, which involve glutamate and dopamine signalling in the striatum, are diminished, it may be expected that some neurobiological effects of cocaine are also disrupted in the striatum of mGluR5^{KD-D1} mice. The experiments of Chapter 6 explore this possibility using immunohistochemistry and immunoblotting techniques to measure cocaine-induced activation of the ERK1/2 pathway in the striatum of mGluR5^{KD-D1} mice. This pathway appears critical for influencing transcriptional regulation proposed to underlie the long term effects of addictive drugs (Girault et al., 2007). In addition, both cocaine and group I mGluRs are well known to regulate trafficking of AMPA receptors, a feature also considered important for neuroplastic changes underlying addiction-related behaviours (Snyder et al., 2001; Bellone and Luscher, 2005; Zhang et al., 2008; Wolf and Ferrario, 2010). The second series of experiments reported in Chapter 6 aim to explore, with immunoblotting, the extent to which loss of mGluR5 on dopaminoceptive neurons disrupts rapid changes in AMPA receptor trafficking and function in the striatum that occur following acute cocaine exposure. Finally, it must be remembered that while the experiments of this chapter tell us something about the role of mGluR5 on dopaminoceptive neurons in neuroplastic changes that may contribute to behavioural effects of cocaine, they may also serve to highlight deficits in neuroplasticity following loss of mGluR5 on these neurons that could contribute to incentive learning deficits in mGluR5^{KD-D1} mice reported in Chapter 3 of this thesis.

2 Basic phenotyping of mGluR5^{KD-D1} mice

2.1 Introduction

Advances in molecular genetics have led to a revolution in our ability to explore the function of individual genes in physiology and behaviour. From the introduction of the inbred mouse stain in the 1900s to the development of transgenic (Jaenisch, 1976) and genetically mutated mice (Thomas and Capecchi, 1987), powerful tools now exist to examine genetic components of more complex behavioural traits that characterise many human disorders. The mapping of animal genomes (Lander et al., 2001; Waterston et al., 2002) has facilitated the ability to insert or remove genes or modify their expression levels in a cell-type and time-point specific manner. However, with these advancements comes an important caveat. Genes do not work in isolation and mutation of a single gene or transgene insertion can have many effects beyond those anticipated by the investigator. The observable characteristics (that is, the phenotype) of a novel mouse line must therefore be well understood before more advanced behavioural analyses are undertaken.

The aim of this chapter will be to characterise the basic phenotype of a novel mouse line in which mGluR5 has been selectively knocked-down on cells that express dopamine D1 receptors by use of RNA interference technology. Before introducing these experiments in further detail (section 2.1.3 of this chapter), it is first necessary to appreciate some general concepts in generating transgenic mice and the use and limitations of RNA interference technology, which is a relatively recent addition to the neuroscientist toolbox. In addition, the mGluR5^{KD-D1} mouse and its biological characterisation (as reported in Novak et al., 2010) will be introduced.

2.1.1 Transgenic mice in behavioural neuroscience

Tremendous progression within molecular biology, genetics and behavioural neuroscience has uncovered great complexity in the genetic control of human

behaviour. One gene can influence multiple phenotypic traits (termed, pleiotropy; Hodgkin, 1998), indicating that genes and their associated proteins do not work in isolation but are part of a complex network of interactions. For example, transcriptional silencing of the *FMR1* gene, arising from an unstable trinucleotide (CGG) repeat expansion mutation (Verkerk et al., 1991), can result in physical perturbations, attentional deficits, stereotypic movement, anxiety, autism, susceptibility to epilepsy and language and learning impairments that characterise Fragile X syndrome (Bagni and Greenough, 2005; Mercaldo et al., 2009). Meanwhile, psychiatric disorders such as drug addiction, depression or schizophrenia can reflect the interaction of multiple genes and signals that control their expression (termed, epigenetics; Nestler, 2000; Tsankova et al., 2007). Gene-behaviour and gene-gene interactions are also influenced by environmental factors, which add another layer of complexity in determining behavioural output under normal and aberrant states (Feder et al., 2009; Bale et al., 2010). If we are to successfully parse the genetic components of human behaviours, a model organism is required in which individual genes can be readily manipulated, environment factors carefully controlled and individual behaviours that contribute to complex behavioural traits examined.

A variety of experimental species have been adopted by behavioural geneticists. Genomic alterations of species traditionally used to examine developmental processes, including the nematode *Caenorhabditis elegans,* fruit fly *Drosophila melanogaster,* and zebrafish *Danio rerio* have recently been used to study the genetic basis of associative learning processes (Glanzman, 2005; Ardiel and Rankin, 2010; Sison and Gerlai, 2010) and of more complex behavioural disorders such as Parkinson's disease (Schmidt et al., 2007; Flinn et al., 2008; Botella et al., 2009). While these species are cost effective and their genes can be rapidly manipulated, the translation of findings to mammalian systems can prove difficult (Yin et al., 2007). In contrast, rodents share ~99% of their genes with humans (Waterston et al., 2002) and the rat is perhaps one of the best characterized laboratory species with regard to its physiology, neurobiology, pharmacological

responses and behavioural repertoire. Until recently, the development of genetically modified rats was precluded by the failure to generate germline competent rat embryonic stem (ES) cells (Buehr et al., 2008; Li et al., 2008; Voigt and Serikawa, 2009). In contrast, mouse pluripotent ES cells were successfully isolated in 1981 (Evans and Kaufman, 1981), which allowed for modifications of the mouse genome to be transmitted through the mouse germline (Capecchi, 1989). Around the same time, the first transgenic mouse was generated by the stable integration of foreign DNA into the genome using pronuclear microinjection (Brinster et al., 1981; Gordon and Ruddle, 1981; Wagner et al., 1981). When combined with behavioural tasks, many of which were initially developed for use with rats, the genetically modified mouse offers a powerful model system for understanding the genetic components of complex behavioural traits.

2.1.1.1 Generation of transgenic mice and some limitations

The application of mutant mice for the exploration of gene function is dependent, in part, upon methodologies available to manipulate the genome (Branda and Dymecki, 2004). The techniques used to generate mutant mice have become increasingly sophisticated since their original description in the early 1980s and today, literally thousands of modified strains are available to the researcher (Yoshiki et al., 2009; Ringwald and Eppig, 2010). It is beyond the scope of this thesis to address the many molecular techniques that enable generation of mice with deletions, insertions, inversions or exchanges of chromosomal DNA with high fidelity and regional and temporal specificity of expression. Indeed, a number of reviews thoroughly discuss generation of mice by homologous recombination of ES cells, including site-specific recombination (for example, Cre/*lox*P and Flp-*FRT*), knock-out, knock-in and conditional gene inactivation techniques (Picciotto and Wickman, 1998; Brusa, 1999; Misra and Duncan, 2002; Branda and Dymecki, 2004; Castrop, 2010). In addition to these ‘designer’ mutant technologies, the contribution of phenotype-driven strategies in mice for identifying the genetic basis of behavioural traits, such as mapping of spontaneous or induced single gene mutations in inbred strains and QTL analysis, also deserves mention (reviewed in

Lander and Botstein, 1989; Paigen, 2003b, a; Peters et al., 2007). A more detailed consideration of the features and potential limitations of transgenic mice generated by pronuclear microinjection using RNA interference (RNAi) is warranted however, as this approach was used to generate the mGluR5^{KD-D1} strain (Novak et al., 2010).

The generation of transgenic mice by pronuclear microinjection first requires the design of a transgene expression cassette, which is then cloned into a vector, purified, microinjected into the pronuclei of fertilized eggs and reimplanted into the oviducts of a pseudo-pregnant foster mother. Newborn mice that carry the transgene are referred to as founders of the transgenic line and, all being well, the transgene is passed onto progeny in Mendelian fashion (Brusa, 1999; Auerbach, 2004). Clearly, the entire process is not quite as straightforward as the two sentence description just provided. Many factors contribute to the success of generating the novel transgenic mouse line, which include the design of the transgene cassette and the genetic material that is incorporated into the genome.

The transgene expression cassette typically contains two major components; the transcription unit of a gene of interest and regulatory elements that control its expression, which are cloned into a vector such as a bacterial or yeast artificial chromosome (BAC or YAC) (Auerbach, 2004). A reporter gene can also be incorporated into the cassette, allowing for expression of the transgene to be readily identified. Reporter gene examples include *lacZ*, which is detected by assaying for β-galactosidase, and Green Fluorescent Protein (GFP; or its enhanced version, EGFP), which can be visualized without need for a substrate (Chalfie et al., 1994).

The regulatory elements (promoter/enhancer) of the transgene cassette can provide regional specificity of transgene expression. For example, use of a small fragment of the gene promoter for tyrosine hydroxylase confers selectivity of transgene expression to dopaminergic neurons (Liu et al., 1997), while larger promoter fragments can drive gene expression in all catecholaminergic cells (Min

et al., 1994). Careful selection of neural-specific promoters can therefore offer greater precision for investigating the function of a specific gene and an associated protein within a brain region that cannot be so easily isolated with lesioning approaches or in which pharmacology is neither available or regionally or cell-type selective. Promoters can also confer temporal specificity of transgene expression. For example, a transgene with a promoter of the NR2B subunit would be expressed in neurons of the forebrain and cerebellar granule cells from embryonal to early stages of postnatal development, but would then be selectively repressed in the cerebellum after the second week (Monyer et al., 1994). Further control over transgene expression is afforded by inducible promoters, such as tetracycline-responsive promoters (Gossen and Bujard, 1992; Mayford et al., 1996), which enable the switching on and/or off of a promoter with an external stimulus. Tissue specific transgene removal and activation can also be achieved using the Cre recombinase system (Schwenk et al., 1995; Auerbach, 2004).

There are some important limitations associated with the use of pronuclear microinjection for generation of transgenic mice. A significant issue is that the efficiency of production of founder mice that appropriately express the transgene is low. First, microinjection can result in genetic mosaicism if the transgene is integrated during or after a round of replication in the fertilized egg (Palmiter et al., 1984). Second, random insertion of the transgene into the genome may result in transgene expression being influenced not only by the number of transgene copies, but also by genomic sequences that flank the integration site(s) (termed, positional effects; Palmiter et al., 1982; Overbeek et al., 1986). For example, transgene expression may be dramatically reduced or abolished if the transgene is inserted into a transcriptionally inactive region of the genome. Alternatively, flanking sequences may contain regulatory elements of neighboring genes that act on the transgene promoter as an enhancer, resulting in ectopic expression (Auerbach, 2004). Moreover, if a transgene is inserted into a transcriptionally active region, it may interrupt the normal expression of an endogenous gene and produce effects on phenotype ranging from inconsequential to lethal but, most importantly,

which are independent of the particular function of the transgene. Third, as with all approaches that seek to modify the mouse genome, compensation effects from other genes can result in rescued function of the mutated gene and may mask any phenotypic deficit due to a mutation. Finally, irrespective of any positional or compensation effects, the mutant mice phenotype will always be a result of the mutated gene and interactions with background genes. Since behavioural traits vary amongst mouse strains, the genetic background of the strain used to construct the modified mouse must always be considered when assessing behavioural traits (Gerlai, 1996; Crawley et al., 1997; Crawley, 2007).

2.1.1.2 RNA interference technology and some limitations

The inclusion of a gene of interest in the transgene cassette has traditionally restricted the pronuclear microinjection approach to 'gain of function' studies (with respect to time, place and/or level of gene expression). For example, in middle to advanced stages of Alzheimer's disease, galanin fibers and terminals are found to hyper-innervate cholinergic neurons of the nucleus basalis of Meynert (Chan-Palay, 1988). The potential consequences of galanin hyper-function for learning and memory processes have been explored, in part, by the use of transgenic mice that over-express galanin throughout the CNS using a platelet-derived growth factor promoter (Diez et al., 2000; Kuteeva et al., 2005), or where galanin over-expression is confined to adrenergic neurons by use of a dopamine β-hydroxylase promoter (Steiner et al., 2001). In many scenarios, however, a more revealing experiment is provided by the 'loss of function' study, which has traditionally been achieved by genetic disruption using ES cell technology. The identification of RNAi and the ability to deliver RNAi technology into mammalian cells using a transgene cassette has changed this situation and provided a new way to study gene function in transgenic mice (Hannon, 2002; Bartel, 2004; Prawitt et al., 2004; Gao and Zhang, 2007; Kunath, 2008).

The first demonstration of RNAi came from studies in *C. elegans* where introducing double-stranded RNA (dsRNA) homologous to a specific gene resulted in the post-

translational silencing of that gene (Fraser et al., 2000). The cellular mechanism through which silencing is achieved is particularly interesting. Initially, the dsRNA is recognized by a nuclease enzyme, named Dicer, and processed into small double-stranded molecules, termed small interfering RNA (siRNA). Next, siRNA is unwound by an RNA-induced silencing complex (RISC), which then finds homologous target mRNAs complementary to the siRNA sequence and cleaves these mRNAs (Martinez et al., 2002; Tijsterman and Plasterk, 2004). This response to dsRNA is considered a likely self-defense mechanism against viral invasion (Gao and Zhang, 2007). In mammalian cells, a similar response to dsRNA is observed, except that the end result is silencing of all protein encoding RNAs and, eventually, cell death via apoptosis (Meurs et al., 1990). However, it was later discovered that short (21 nt) RNA duplexes could induce RNAi in cultured mammalian cells without eliciting global gene silencing (Elbashir et al., 2001). Another major breakthrough for RNAi was the discovery that animal cells naturally express short (~22 nt) RNAs, termed micro-RNA (miRNA; Lagos-Quintana et al., 2001; Lee and Ambros, 2001), that arise from the processing of long primary miRNA precursors (pri-miRNA) (Bartel, 2004). These micro-RNAs play an important role in regulating gene expression through blocking translation, or inducing degradation of target mRNA (Bartel, 2004). Exploitation of these collective findings allowed molecular biologists to stably induce RNAi in mammalian cells, first using expression vectors that expressed the RNA duplex as small hairpin RNA (shRNA), which is likely processed to siRNA within the cell (Brummelkamp et al., 2002), followed by the use of artificial miRNA-expressing vectors (Silva et al., 2005). The stable expression of RNAi in transgenic mice (Peng et al., 2006) is now becoming more commonplace, providing an alternate approach to targeted gene disruption in delineating gene function. Moreover, in comparison to conditional gene deletion, RNAi can be used with only one mouse line, and offers the potential to be used, in modified forms, in other organisms where targeted mutagenesis is not feasible.

There are, of course, some important limitations of the RNAi technology for generation of transgenic mice. First, knock-down of expression is never complete and residual expression of an RNAi-targeted gene is expected (Gao and Zhang, 2007). However, this limitation may be seen as advantageous where null mutations are associated with lethality. Second, RNAi is useful for modulating the level of gene expression, but cannot be used for other genetic manipulations (e.g. point mutations). Third, not all cell types can perform RNAi, and the efficiency of cells that can varies widely amongst cell types (Peng et al., 2006). Fourth, the genetic material used to produce RNAi may have off-target effects, such as degradation of non-target mRNA (Castrop, 2010). Finally, whether a transgenic RNAi effect is permanent remains to be determined, since it is possible that an shRNA transgene could be shut down during development or turned off through epigenetic modifications (Gao and Zhang, 2007).

2.1.1.3 Characterising a new transgenic mouse

Because of the potential complications that may arise in the generation of transgenic mice and use of RNAi, phenotyping screens provide an important and necessary step in understand traits related to transgene function or unexpected abnormalities (Crawley, 2007). Phenotyping screens also complement the rigorous assessment by molecular biologists of transgene expression and the potential for compensatory or off-target effects in founder lines. Findings of gross phenotypic differences between genotypes can be used to optimise animal husbandry and/or the design of subsequent behavioural tests. Indeed, only when the basic phenotype has been characterised can conclusions from advanced behavioural tests be made with assurance that an exciting finding is not simply due to interference from unexpected behavioural traits.

The majority of phenotyping screens have adapted protocols from those originally described by Irwin (1968), which incorporated some 50 categories of observations. More recently, the SHIRPA consortium described an observation battery for mutant mice that consists of three levels of testing, starting with preliminary observations

(for example, muscle function) and progressing to more detailed assessments of behaviour and physiology (for example, feeding, anxiety, histology and magnetic resonance imaging) (Rogers et al., 1997). Whatever combination of assessments are chosen, the first point of phenotyping is to ensure that the mutant line is generally healthy, since ill mice will likely behave aberrantly in the simplest behavioural test. This initial inspection often includes assessments of sensory capabilities and basic neurological reflexes, such as the righting reflex. Any gross physical deficits will similarly impair performance in many behavioural tests. Once these compulsory observations have been completed, subsequent tests can be selected to determine adequate function for performance in specific behavioural tasks or to test specific hypotheses regarding gene function (Crawley, 2007). For example, undertaking the Morris water-maze task would require that mice could adequately swim and observations of motor activity would be necessary. Similarly, phenotyping screens for strains with mutations known to affect neuronal excitability might focus on observations of convulsions, locomotor activity and handling reactivity. Any deficits found within a particular domain can then be investigated further.

2.1.2 Introducing the mGluR5$^{KD\text{-}D1}$ mouse

To explore the role of mGluR5 in mediating appetitive behaviours and neurobiological responses to cocaine, we use a novel mouse line in which mGluR5 has been selectively knocked-down in cells that express the dopamine D1R. The generation and characterization of these mGluR5$^{KD\text{-}D1}$ mice has recently been described (Novak et al., 2010). Cell-type specific knock-down of mGluR5 is achieved using a BAC-based construct in which a conventional RNA-polymerase II promoter (the D1R-promoter) drives the expression of artificial miRNAs that target mGluR5 RNA. The coding sequence for green fluorescent protein (GFP) is introduced in tandem with the miRNAs, enabling expression of the construct to be easily tracked (Fig. 2.1a-b).

Extensive characterization of these mice has confirmed the selectivity of transgene expression to cells that express the dopamine D1R (Novak et al., 2010). Thus, immunostaining of GFP in brains from mGluR5^{KD-D1} mice identified an expression pattern that fits with that described for D1Rs, including strong expression in the dorsal striatum and nucleus accumbens (Fig. 2.1c) (Novak et al., 2010). A more detailed examination of the striatum confirmed that the transgene (GFP) was expressed in ~53% of the striatal neurons (NeuN; Fig. 2.1d). Furthermore, expression of the transgene was confined to MSNs (identified by immunostaining against DARPP-32) (Fig. 2.1e) but the transgene was not expressed in D2-MSNs (identified by immunostaining against preproenkephalin; ppEnk) (Fig. 2.1e), showing that expression is restricted to D1-MSNs. Confirmation that transgene expression reduced the abundance of mGluR5 transcript was provided by *in situ* hybridization. In the striatum, mGluR5-positive cells were reduced, while the staining-intensity of cells still expressing mGluR5 was not reduced (Fig. 2.1f), indicating strong mGluR5 knock-down selectively in the targeted cells. The abundance of mGluR5 transcript was reduced to ~40% in the homogenised striatum (Fig. 2.1g) with the corresponding protein reduced to ~50% compared to levels in wild-type mice (Fig. 2.1h). Since the expression of the construct is restricted to D1-MSNs (Fig. 2.1e), knock-down efficiency was estimated to be ~90% in the targeted cells. There was no significant reduction of mGluR5 mRNA in the cerebral cortex or in the hippocampus of mGluR5^{KD-D1} mice, which may suggest that the D1R-promotor is less strong in these regions or that D1 and mGluR5 are not expressed in the same neuronal populations (Novak et al., 2010).

In addition to enabling selective knock-down of mGluR5 in cells that express dopamine receptors, the RNAi approach used for generation of mGluR5^{KD-D1} mice is particularly impressive. First, previous use of RNAi have identified that high levels of short RNAs may result in perturbed cellular homeostasis due to over-saturating exportin 5 and thus blocking the processing of endogenous short RNAs (Grimm et al., 2006). This is not the case for the mGluR5^{KD-D1} mice, where maturation of short RNAs is normal (Novak et al., 2010). Most likely, previously

reported problems were caused by the use of tools resulting in very high levels of short RNAs, such as strong RNA polymerase III promoters or the use of shRNAs instead of artificial miRNAs (Boudreau et al., 2009). Second, while use of miRNAs may knock-down mRNAs other than mGluR5, this does not appear problematic in mGluR5^{KD-D1} mice where abundance of mRNAs with partial complementarity to the miRNAs was not affected (Novak et al., 2010). Third, no evidence has been found for a reduction in the knock-down efficiency of the mutation across generations (up to 12 generations over 3 years of breeding; personal communication with Dr. J. Rodriguez). Finally, the successful use of artificial miRNAs driven by cell-type specific promoters had previously only been reported for interference with other genes in nurse cells (Rao et al., 2006). Together with a very recent report (Garbett et al., 2010), the mGluR5^{KD-D1} line shows this approach can also be used successfully in the brain.

Figure not shown due to copyright restrictions
For figure, see Novak et al., 2010, Fig. 1.

Figure 2.1 Neurobiological characterisation of mGluR5^{KD-D1} mice. (**a**) Design of the transgene expressing GFP as a marker and two interfering RNAs (iRNAs). This construct was inserted after the translational start of the gene encoding the dopamine D1 receptor in a bacterial artificial chromosome. (**b**) Sequences of iRNAs. Interfering sequence is depicted in bold. Red arrows indicate targeted regions of mGluR5 mRNA. (**c**) Expression of the transgene in mGluR5^{KD-D1} mice (KD) as detected by immunohistochemistry for GFP in a sagittal brain section. Higher magnification showing difference between staining of cell bodies in the caudate putamen (CPu) and its projections to ventral midbrain nuclei (VMN). (**d**) The transgene (GFP; green) is expressed in ~53% of the striatal neurons (NeuN; red; → indicates examples of GFP-positive neurons and ► indicates examples of GFP-negative neurons). (**e**) The expression of the construct is selective for D1-MSNs. Thus, expression is limited to MSNs (DARPP-32; blue) and absent from D2-MSNs (labeled by red immunofluorescent labelling of pre-pro enkephalin; ppEnk). Examples of GFP-expressing (→) and non-GFP-expressing (►) MSNs. (**f**) Expression of mGluR5 in the striatum as shown with in situ hybridization. (**g**), Knock-down assessment by quantitative PCR (n = 5-8, p = 0.0027) and (**h**), western-blotting with representative blot example shown (n = 4, p = 0.0112). Data is presented as mean + S.E.M., p-value of t-test (*p < 0.05, **p < 0.01). Scale bars 20 µm. Cx, cortex; CPu, caudate putamen (dorsal striatum); VMN, ventral midbrain nuclei; Acb, nucleus accumbens (taken from Novak et al., 2010).

2.1.3 Phenotyping of mGluR5^{KD-D1} mice

In regard to the initial phenotyping of mGluR5^{KD-D1} mice that will be undertaken in the present chapter, a conventional screening approach as discussed above will be adopted. Initial observations will examine basic health and neurological reflexes to ensure no gross abnormalities that would otherwise preclude undertaking of more advanced behavioural tests. Body weights of adult mice will be also assessed, which provides a reliable indicator of factors affecting growth (Crawley, 2007). Tests of sensory function will assess visual and auditory function, since conditioning studies necessitate that mice can adequately see and hear the conditioned stimuli. Similarly, mutant mice must be able to detect a palatable food, since the majority of tests described in this thesis will use food-based reinforcement paradigms. The sucrose two-bottle choice paradigm will be used primarily to assess taste perception.

Following these initial assessments, close attention will be paid to motor activity. In addition to providing information regarding physiological function, a change in locomotor activity could also result from general ill health in mutant mice. Assessment of motor function is also necessary to permit the use of many behavioural tasks reported in this thesis. For example, if motor function is impaired, a mouse may be unable to coordinate actions necessary to explore an operant chamber, to lever press or to collect a small food pellet from a receptacle. Efforts to evaluate the psychomotor stimulating effects of cocaine or performance in a conditioned-place preference chamber (see Chapter 5) may also be confounded by any genotype differences in baseline locomotor activity. Locomotor assessments are particularly important with respect to mGluR5^{KD-D1} mice, given the widespread expression of mGluR5 within the basal ganglia motor circuitry, including the STN (Awad et al., 2000) and SNr (Hubert et al., 2001; Smith et al., 2001), and the potential for co-expression of the D1R in these nuclei (Smith and Villalba, 2008).

The final series of tests will look at anxiety-related behaviours. Soon after one of the first non-competitive mGluR5 antagonists (MPEP) was developed, a role for

mGluR5 in anxiety-related behaviours was established in rodents (Gasparini et al., 1999; Spooren et al., 2000b). The anxiolytic effects of MPEP have been demonstrated using several unconditioned (for example, social interaction, stress-induced hyperthermia, elevated plus-maze and marble burying) and conditioned tests (for example, Geller-Seifter, Vogel conflict and fear potentiated startle) (Spooren et al., 2000b; Tatarczynska et al., 2001; Brodkin et al., 2002b; Brodkin et al., 2002a; Pietraszek et al., 2005; Paterson et al., 2010). Critically, anxiolytic effects of MPEP occurred at doses that did not alter locomotor activity (Tatarczynska et al., 2001), did not show psychotomimentic effects (Spooren et al., 2000b) and did not impair working memory or spatial learning (Ballard et al., 2005). Similarly, the more potent mGluR5 antagonist, MTEP also demonstrated efficacy in several anxiety-related models in rodents (Cosford et al., 2003; Klodzinska et al., 2004; Pietraszek et al., 2005; Varty et al., 2005), and lacked side effects seen with benzodiazepines, such as sedation or ethanol interactions (Busse et al., 2004). Surprisingly few studies have examined the anxiety phenotype of mice in which mGluR5 has been genetically modified, and those few reports offer conflicting findings. In mGluR5 knockout mice, attenuated anxiety-related behaviours have been reported using stress-induced hyperthermia (Brodkin et al., 2002b) and open-field tests (Olsen et al., 2010), but not in the elevated plus-maze or a light-dark box (Olsen et al., 2010). Thus, it is of value to examine anxiety-related behaviours in mGluR5$^{KD\text{-}D1}$ mice not only to add to our knowledge of the role of mGluR5 in anxiety *per se*, but also given the potential symmetry between anxiety phenotypes and performance in appetitive learning and memory tasks that are reported in subsequent chapters of this thesis (Pecina et al., 2006; George et al., 2009).

2.2 Materials and methods

2.2.1 Animals

mGluR5^{KD-D1} mice were generated as previously described (Novak et al., 2010). In brief, shRNAs were designed and first tested in cell culture for knock-down efficiency of mGluR5 mRNA. Synthetic oligos were inserted into an artificial micro-RNA context and the construct recombined into a bacterial artificial chromosome (BAC) that harbored the mouse D1R gene (as described in Parkitna et al., 2009). Following purification of the BAC and removal of vector sequences, the transgene was injected into the pronuclei of fertilized oocytes from C57BL/6n mice. Experimental animals were generated by backcrossing of mGluR5^{KD-D1} transgenic mice to a C57BL/6n line (Novak et al., 2010). mGluR5^{KD-D1} and wild-type littermate mice used in the present experiments were gifted from Dr. J. Rodriguez Parkitna (DKFZ, Heidelberg, Germany) or obtained from a breeding colony subsequently established at the University of Sussex.

Mice (n = 85/89, WT/KD; at least 8 weeks old prior to experiment start) were maintained on a 12:12 h light-dark cycle (lights on at 07:00 h) under controlled temperature (21 ± 2 °C) and humidity conditions (50 ± 5%). Animals were housed in groups of two or three in polycarbonate cages, except for the sucrose consumption experiment where mice were singly housed. Water was available *ad libitum* in the holding room. Unless otherwise stated, mice were placed onto a restricted feeding regime designed to maintain body weights at ~85% of free-feeding weight at least 7 days prior to the experiment start. In this way, any phenotypic differences between genotypes could be used more readily to understand any differences in performance during subsequent behavioural tests where food restriction was employed. Experiments took place during the light-phase. All procedures were performed in accordance with the United Kingdom 1986 Animals (Scientific Procedures) Act, following institutional ethical review.

It is worthwhile to note here that wild-type littermates provide the most appropriate control for a mutant phenotype, since phenotype is influenced by many intrauterine and postnatal environmental factors such as parental care, temperature, noise, lighting, humidity, circadian cycle, cage cleaning, cage type, colony size and age at weaning (Crawley, 2007). Because the RNAi mutation exerts a dominant effect (Castrop, 2010), heterozygous mGluR5^{KD-D1} mice were used in all experiments such that disruption of any gene function as a result of random transgene insertion could potentially be compensated by the presence of an intact gene copy on the other allele. Heterozygous mice and wild-type littermate controls were obtained from the breeding of heterozygous and wild-type parents.

2.2.2 Genotyping

2.2.2.1 DNA extraction

Ear punches were taken and incubated for 2 hours at 55^{o}C followed by 15 min at 95^{o}C in 20 µl of a 1 mg/mL proteinase K solution (Roche Diagnostics, Burgess Hill, West Sussex, UK) in a 20 mM Tris-HCl (Sigma-Aldrich, Dorset, UK) and 10 mM EDTA (Sigma-Aldrich) buffer. The digested sample was then diluted with 80 µl of purified water.

2.2.2.2 PCR

mGluR5^{KD-D1} mice were identified using primers (ACGTAAACGGCCACAAGTTC, AAGTCGTGCTGCTTCATGTG) that amplified a 180bp sequence of the GFP-encoding region of the inserted transgene. Control primers (CCATTTGCTGGAGTGACTCTG, TAAATCTGGCAAGCGAGACG), derived from the Dicer gene, amplified a 370bp sequence and were included to confirm efficiency of the PCR reaction (Fig. 2.2). Each PCR reaction contained 0.5 µl of sample DNA, 0.5 µl of each primer and 22.5 µl of a PCR mix containing *Taq* polymerase, dNTPs and an agarose loading dye (Mega Mix-Blue; Microzone Ltd., Haywards Heath, West Sussex, UK). Using a thermal cycling PCR machine (G-Storm; GRI Ltd., UK), the PCR sample was incubated at 95^{o}C for 5 min, followed

by 35 cycles of: 95°C for 30 sec, 60°C for 1 min and 72°C for 1 min. Samples were then held at 72°C for 10 min.

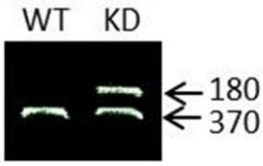


Figure 2.2 Example western blot for genotyping of mice. KD mice were identified by a band (180 bp) confirming presence of the transgene that results in mGluR5 knock-down selectively in dopamine D1R-expressing cells. The presence of a control band (370 bp) confirmed the efficiency of the PCR reaction.

2.2.2.3 DNA Detection

DNA gel electrophoresis was performed using a gel made from 2% agarose (High Res standard agarose; AGTC Bioproducts Ltd., Hessle, UK) in 1x TAE buffer (242 g/L Tris-base; 57.1 mL/L acetic acid; 500 mL/L of 50 M EDTA; all Sigma-Aldrich; in dH_2O) supplemented with 0.004% ethidium bromide (50 mg/mL stock; Sigma-Aldrich). Gels were loaded with ~12 µl of each post-PCR sample and run in 1x TAE buffer for 30 min at 140 volts prior to imaging under UV light.

2.2.3 Gross appearance, auditory and visual function and neurological reflexes

Procedure: Mice (n = 12/12, WT/KD; fed *ad libitum*) were first exposed to a battery of assessments (adapted from Crawley, 2007) designed to assess gross appearance, simple sensory responses (auditory and visual) and neurological reflexes. Assessments were made in a quiet and low lit room designated for behavioural studies. The test order of mice (wild-type or knock-down) was randomised and the experimenter was unaware of the mouse genotype. Gross appearance was first assessed by observation of mice within their home cages and

included the following; 1) *Whiskers present:* presence of whiskers, 2) *Bald patches:* missing fur, 3) *Exopthalmia:* bulging of eyes, and 4) *Piloerection:* erection of fur. A basic assessment of auditory function was then provided by making a loud clapping noise within close proximity to the mouse. Auditory function was confirmed by a Preyer startle reflex (movement of the body or ear twitch response). Assessment of visual function was provided by the visual placing test (Heyser, 2003). The mouse was held by its tail ~15 cm from a table surface and lowered toward the surface. Visual function was confirmed if the mouse extended its forepaws to make contact with the approaching surface.

After assessments of gross appearance, auditory and visual function, the following reflexive assessments were undertaken in the same mice; 1) *Air puff*: a puff of air was directed toward the back of the mouse using an empty spray bottle. A response was recorded as normal if mice turned in reaction to the air puff, 2) *Touch escape*: the experimenter attempted to pick up the mouse. A normal response was observed if the mouse made attempts to escape, 3) *Eye blink*: the corner of the eye was touched with a clean cotton swab. A normal response was defined as an eye blink in response to the cotton swab, 4) *Ear twitch*: the ear was touched with a clean cotton swab. A normal response was defined as an ear twitch in response to the cotton swab, 5) *Whisker touch*: whiskers were touched with a cotton swab. A normal response was defined as a brief cessation of whisker movement in response to the cotton swab, 6) *Wire suspension test*: Mice were placed on a wire-grid, which was then inverted for 60 seconds. A normal response was recorded if mice remained attached to the wire-grid for the duration of the test, 7) *Righting reflex*: mice were placed onto their back. A normal response was recorded if mice resumed a righting posture unaided, and finally 8) *Splay reflex*: mice were placed into an empty polycarbonate cage which was then moved from side to side and up and down. A normal response was recorded if mice elicited a postural reflex to maintain an upright, balanced position.

2.2.4 Body weight

Procedure: Mice (n = 12/12, WT/KD; as used in assessments above) were placed onto a restricted feeding regime designed to maintain body weights at approximately 85% of free-feeding weight. This was achieved by the provision of ~2 standard lab chow pellets (B&K Feeds, Hull, UK) per mouse per day. To determine whether weights differed between genotypes under basal or restricted feeding conditions, mouse body weight (g) was recorded every 48 hours for 6 days prior to and 6 days following the start of food restriction. Additional recordings were made at 32-36 days after the start of food restriction. Under restricted feeding conditions, body weights were recorded immediately prior to daily feeding.

2.2.5 Sucrose consumption

Apparatus: A two-bottle choice paradigm was used to assess sucrose consumption. The experiment was conducted in the home-cage using two bottles inserted into the front of the cage. Chow, which was normally placed into a recess at the front of the cage, was instead placed on the floor of the cage.

Procedure: Mice (n = 12/12, WT/KD) were individually housed to enable accurate recordings of consumption to be obtained from each mouse. To habituate mice to the drinking bottles, both bottles were filled with water for the first three days. For the choice phase of the experiment, one bottle was filled with water and the second with varying concentrations of sucrose (1%, 3%, 10% or 20% w/v; Tate & Lyle, Nottingham, UK). Presentation order of sucrose may be important for determining consumption in some strains but not in the majority of others (Lewis et al., 2005). Here, sucrose concentrations were presented in increasing order so that preference for a higher concentration of sucrose did not develop before being presented with a lower sucrose concentration. When comparing preference amongst different solutions (for example, sucrose, saccharin and water), testing for at least 4 days at each concentration of solution has been considered most sensitive for detecting any differences between strains (Tordoff and Bachmanov, 2002). However, shorter durations of exposure are considered sufficient for

detecting strain differences in intake when using multiple concentrations of the same solution (Glendinning et al., 2002; Lewis et al., 2005). In the present test, consumption of sucrose and water was measured every 24 hours over 3 days at each concentration. Chow was placed on the floor of the cage at the same time when bottles were weighed and/ or replenished. Although chow intake was not recorded in the current study, it is recognised that chow intake during the test can vary as a function of sucrose concentration in an effort to maintain caloric homeostasis in some strains, but not others (Lewis et al., 2005). The left and right positions of the bottles were reversed daily to control for any side preference. In addition to measuring daily sucrose and water intake (mL/Kg), a sucrose preference score was calculated by dividing sucrose intake by total fluid intake (i.e. sucrose plus water intake).

2.2.6 Activity in a novel environment

2.2.6.1 Locomotor activity

Apparatus: Locomotor activity was first assessed using nine circular runways. Each runway was constructed from a black open-ended plastic tube (24.5 cm dia. x 25.5 cm), inside which was fixed a similar smaller tube (10.5 cm dia. x 25.5 cm) thereby creating a 6.5 cm wide circular runway (Fig. 2.3a). Runways were set on a translucent Perspex platform (122 x 89 x 1 cm) mounted 127 cm above the floor. Illumination of runways was achieved by 4 fluorescent tubes (T4, 30 watt) positioned behind a second Perspex sheet suspended 17 cm above the runways. A camera (Fire-i; UniBrain, San Ramon, California, USA), which interfaced with a PC, was situated on the floor beneath the runways and captured the location of mice (Fig. 2.3b). Images were recorded and the distance travelled (m) by mice calculated using a PC running MATLAB (version R2007a; MathWorks, Cambridge, UK).

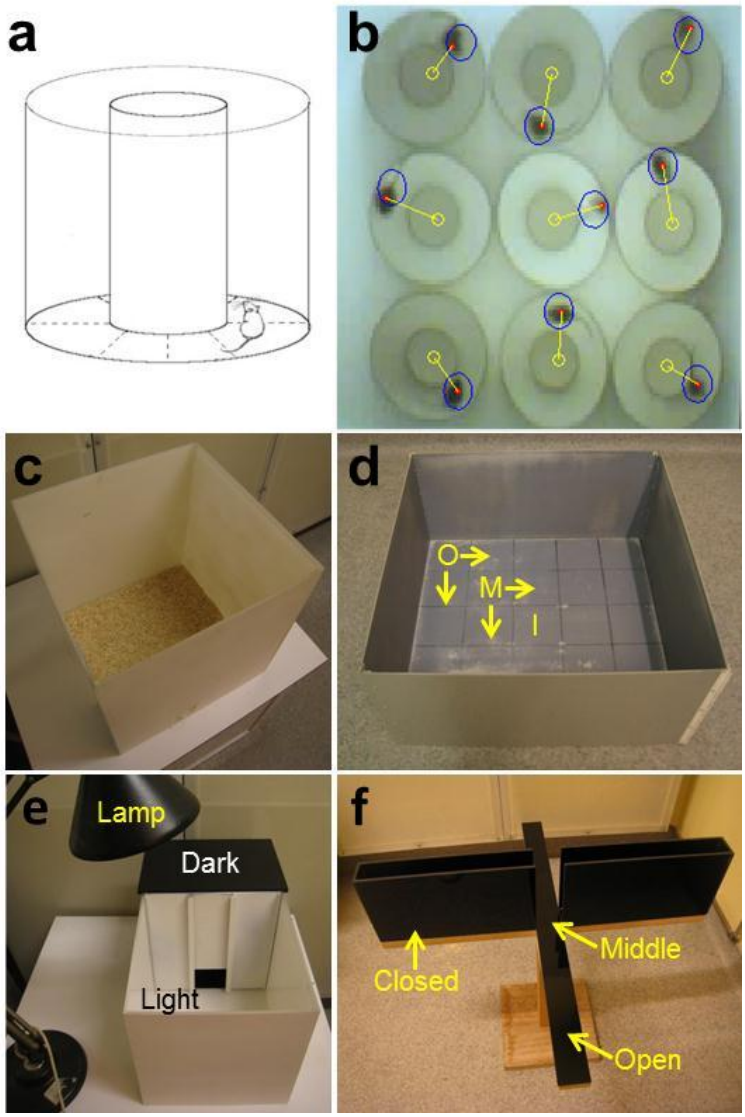


Figure 2.3 Apparatus used for behavioural phenotyping of mice. (**a**) Schematic of a circular runway used for locomotor assessment. (**b**) Video tracking of mice in circular runways. (**c**) Open-field arena used for the assessment of spontaneous activity in a novel environment. (**d**-**f**) For assessing anxiety-related behaviours: (**d**) Open-field arena indicating the outer (O), middle (M) and inner (I) areas. (**e**) Light-dark box indicating the light area illuminated by an overhead lamp and the dark area accessed by an opening in the dividing partition. (**f**) Elevated plus-maze indicating a closed and open arm and the middle area. A camera (not shown) was positioned to record mouse activity in each apparatus. *Panel **a** provided by Simon Nilsson*

Procedure: Mice (n = 28/31, WT/KD) were habituated to the circular runways across five once-daily 1 hour sessions. In this way, activity in a novel environment (session 1) and habituation between daily sessions (sessions 1-5) could be compared between genotypes. Multiple groups of mice were run each day, although each group contained mice from both genotypes. The runways and Perspex platform were cleaned between each group. Mice were assigned to the same runway for all sessions. Note that mice reported in this experiment were subsequently used for cocaine locomotor studies (reported in Chapters 5 and 6).

2.2.6.2 Spontaneous nose-poking

Apparatus: Exploratory behaviour in a novel environment, indexed by nose-poking, was assessed using eight standard mouse conditioning chambers (15.9 x 14 x 12.7 cm; Med Associates, Vermont, USA; see section 3.2.3 for further description). Each chamber was fitted with a recessed food magazine (ENV-303M, Med Associates) situated at the centre of one side wall. Two nose-poke ports (ENV-313M, Med Associates) were inserted into the wall opposite to the magazine. Infra-red beams detected head entries into each of the three apertures. The recording of beam breaks and their time of occurrence was performed using Med-PC IV (Med Associates).

Procedure: Mice (n = 24/24, WT/KD) were placed into the conditioning chambers and nose-poking activity recorded during a single 30 minute session. Multiple groups of mice were run each day, although each group contained mice from both genotypes.

2.2.6.3 Spontaneous activity in an open-field

Apparatus: A more detailed analysis of spontaneous activity was undertaken in a novel open-field arena (39 x 39 x 42 cm), constructed from white acrylic and layered with sawdust (Fig. 2.4c). A video camera (Sony B&B CCD-HAD), connected to a videocassette recorder (Sony SLV-SE70), was mounted directly above the arena.

***Procedure**:* Mice (n = 12/12, WT/KD; used also for the nose-poking assessment) were allowed to explore the area for 30 minutes. The test order of mice (wild-type or knock-down) was alternated, the arena cleaned and the sawdust replaced between each recording session. Video recordings were subsequently scored by an observer unaware of the mouse genotype. Specifically, locomotor activity was assessed by measuring the duration of the following activity levels: 1) *Static*: No ambulatory movement and no exploratory movement (e.g. sleeping), 2) *Static/Exploratory*: No ambulatory movement but exploratory movement (e.g. static and sniffing or rearing), 3) *Forward movement*: ambulatory movement around the arena, and 4) *Excited movement*: rapid movement, such as wild circular running or jumping. The duration of more specific exploratory behaviours was also measured: 1) *Wall rearing:* rearing (both forelimbs removed from the floor and extension of the body) with support from the wall, 2) *Centre rearing*: rearing without support from the wall, 3) *Grooming:* body care movements using mouth or paws, and 4) *Digging*: excavation of the sawdust with paws or head.

2.2.7 Anxiety-related behaviours

2.2.7.1 Open-field arena

Apparatus: The open-field arena (50 x 50 x 25.5 cm; Fig 2.3d) was constructed from grey Perspex. The arena floor was marked into 10 x 10 cm squares that defined the ‘outer’ (16 squares), ‘middle’ (8 squares) and ‘inner’ (1 square) areas. A video camera (Sony B&W CCD-HAD), connected to a videocassette recorder (Sony SLV-SE70), was mounted directly above the arena. The surface of the apparatus was under an illumination of 115–157 lx, provided by a 40W bulb located 80cm above the apparatus.

Procedure: Mice (n = 12/12, WT/KD; those used for assessment of gross appearance, auditory and visual function and neurological reflexes) were placed into the inner area of the open-field at the start of the test session, and allowed to freely explore the arena for 5 minutes. The test order of mice (wild-type or knock-

down) was alternated and the arena cleaned between each test. Video recordings were subsequently scored for the amount of time mice spent in each area. A measure of gross locomotor activity was provided by counting crosses over lines that marked each 10 x 10 cm square.

2.2.7.2 Light-dark box

Apparatus: The light-dark box (Fig. 2.3e) consisted of two partitioned areas. The 'light' area (27 x 27 x 30 cm) had an open top and was painted white. A lamp (60 watt) positioned 30 cm above the light area provided illumination in the range of 380-470 lx at the floor of the light area. The 'dark' area was smaller in size (27 x 18 x 30 cm), painted black and was fully enclosed. Mice could move between areas through an opening (7.5 x 7.5 cm) in the partitioning wall. Access to the opening was controlled by a removable panel. A video camera (Sony B&W CCD-HAD), connected to a videocassette recorder (Sony SLV-SE70), was mounted directly above the light-dark box.

Procedure: Mice (n = 9/10, WT/KD; fed *ad libitum*) were placed into the light area at the start of the test session. The panel was then removed, allowing mice free access between the light and dark areas. Mice were allowed to explore the light-dark box for 5 minutes. The test order of mice (wild-type or knock-down) was alternated and the apparatus cleaned between each test. Video recordings were subsequently scored for the amount of time mice spent in each area. The latency to the first escape from the light area and the latency to re-enter the light area were also measured. A measure of gross locomotor activity was provided by counting transitions between the light and dark areas.

2.2.7.3 Elevated Plus-Maze

Apparatus: The elevated plus-maze (raised 45 cm above the floor; Fig. 2.3f) was constructed form black acrylic and consisted of four arms (30 x 5 cm) connecting at right angles to a middle platform (5 x 5 cm). Two opposing arms were enclosed by a 15 cm high wall (the 'closed' arms), while the other arms were not enclosed (the

'open' arms). The open arms of the maze were under illumination of 40-48 lx. A video camera (Sony B&W CCD-HAD), connected to a videocassette recorder (Sony SLV-SE70), was mounted directly above the plus-maze.

Procedure: Mice (n=8/10, WT/KD; also used in the light-dark box experiment) were placed into the middle area of the maze at the start of the test session and allowed to explore the plus-maze for 5 minutes. The test order of mice (wild-type or knock-down) was alternated and the plus-maze cleaned between each test. Video recordings were subsequently scored for the amount of time mice spent in each area. A measure of gross locomotor activity was provided by counting transitions between each area of the plus-maze.

2.2.8 Statistical Analysis

Assessments of gross appearance, auditory and visual function and neurological reflexes were qualitative and not subject to formal statistical analysis. All other data were initially assessed for normality (Shapiro-Wilk test; assumption violated when $p \leq 0.05$) and homogeneity of variance (Levene's test, assumption violated when $p \leq 0.05$), to permit use of parametric tests. For most tests, data were first analysed by mixed-factor analysis of variance (ANOVA), with genotype (WT, KD) as a between-subjects factor. Details of the within-subjects factors used for the analysis of data by ANOVA are provided in the results section. Where significant ($p \leq 0.05$) main effects or interaction terms were found, further analysis was performed using ANOVA. Individual between genotypes comparisons were performed using an independent samples two-tailed *t*-test. Findings were considered indicative of a trend where $p \leq 0.1$. For within-subjects ANOVA, the Greenhouse-Geisser correction was used where the assumption of sphericity was violated (Mauchly's test, $p \leq 0.05$). Statistical analysis was performed with SPSS Statistics v.17 (IBM, Somers, New York, USA). All figures show group mean (± SEM).

2.3 Results

2.3.1 Gross appearance, auditory and visual function and neurological reflexes

Mutant mice were indistinguishable from their wild-type counterparts when gross appearance, auditory and visual function and neurological reflexes were assessed. Specifically, all mice assessed had whiskers present and did not present with bald patches, expothalmia or piloerection. All mice responded to a loud noise (e.g. jumped or twitched) and directed forelimbs in anticipation of an approaching surface, suggesting intact auditory and visual function, respectively. A variety of other neurological assessments failed to find any difference in responses between WT and KD mice (see Table 2.1 for summary).

2.3.2 Body weight

Body weights in adult mice (Fig. 2.4) did not differ between genotypes measured prior to the implementation of a restricted feeding regime (from days -6 to -2 prior to food restriction; main effect of Genotype, not significant (NS); Genotype x Day interaction, NS). After the restricted feeding regime had commenced, body weights declined at a similar rate in both genotypes (days 2 to 6 from restriction start; main effect of Genotype, NS; Day, $F(2,44) = 38.637$, $p < 0.001$; Genotype x Day interaction, NS). Weights did not differ between genotypes after an extended period of food restriction (days 32-36 from restriction start; main effect of Genotype, NS; Genotype x Day interaction, NS).

Genotype	WT	KD
Gross Appearance		
Whiskers present (%)	100	100
Bald Patches (%)	0	0
Exophthalmia (%)	0	0
Piloerection (%)	0	0
Sensory Responses		
Auditory reflex (%)	100	100
Visual response (%)	100	100
Reflexive Responses		
Air puff (%)	100	100
Touch escape (%)	100	100
Eye blink (%)	100	100
Ear twitch (%)	100	100
Whisker touch (%)	100	100
Wire suspension (%)	100	100
Righting reflex (%)	100	100
Splay Reflex (%)	100	100

Table 2.1 Basic assessments of gross appearance, sensory responses and neurological reflexes. KD mice were indistinguishable from WT mice in their general appearance, responses to auditory and visual stimuli and neurological reflexes assessed using a variety of simple tests. The procedure for each test and normal responses are defined in the methodology (n = 12/12, WT/KD).

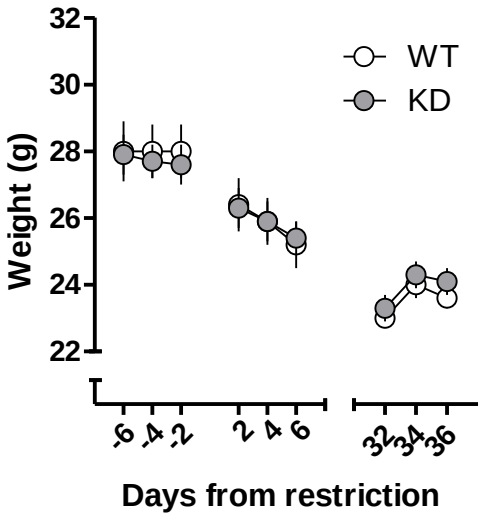


Figure 2.4 Body weights measured prior to and during food restriction. Body weights did not differ between adult WT and KD mice either prior to (days -6 to -2) or following (days 2-6 and 32-26) the implementation of a restricted feeding regime (n = 12/12, WT/KD).

2.3.3 Sucrose consumption

Prior to the two-bottle sucrose choice test, baseline water intake (ml/kg/24 h; averaged from intake recorded over the final two days of habituation to the water bottles) was lower in KD mice, although this effect did not reach significance (t = 1.723, df = 22, p = 0.099; Fig. 2.5a).

During the two-bottle choice phase of the experiment, both genotypes drank more from the bottle containing sucrose than the water bottle at all sucrose concentrations. In both genotypes, sucrose intake (ml/kg/24 h; averaged from the final two days of testing at each sucrose concentration) described an 'inverted-u' profile with consumption progressively increasing from 1-10 % sucrose and decreasing at 20% sucrose (Fig. 2.5a). These observations were confirmed by an initial analysis of intake across all sucrose concentrations (1-20%) using a mixed-factor ANOVA, with Bottle (sucrose, water) as a within-subjects factor. Intake differed according to the bottle type (main effect of Bottle, $F(1,22) = 81.97$, $p < 0.001$), but not between genotypes (main effect of Genotype, NS; Genotype x Bottle interaction, NS). A subsequent analysis of sucrose intake alone by mixed-factor ANOVA, with Concentration (1-20%) as a within-subjects factor, confirmed that sucrose intake varied with sucrose concentration (main effect of Concentration, $F(3,66) = 23.67$, $p < 0.001$), although this intake profile did not differ between genotypes (main effect of Genotype, NS; Concentration x Genotype interaction, NS).

A plot of absolute sucrose consumption (i.e., g/Kg/24 h; Fig. 2.5b) revealed that the amount of sucrose consumed increased between 1-10% sucrose and approached a plateau between 10-20% sucrose (main effect of Concentration, $F(3,66) = 93.64$, $p < 0.001$), likely reflecting satiety. This consumption profile did not differ between genotypes (main effect of Genotype, NS; Concentration x Genotype interaction, NS).

Finally, calculation of sucrose preference (that is, sucrose intake divided by total fluid intake) revealed that preference increased in line with the sucrose concentration in both genotypes, although overall sucrose preference was lower in mutant mice (Fig. 2.5c). These observations were confirmed by analysis of preference scores by mixed-factor ANOVA (main effect of Concentration, $F(3,66) = 9.04$, $p = 0.001$; Genotype, $F(1,22) = 5.17$, $p < 0.05$; and Concentration x Genotype interaction, NS).

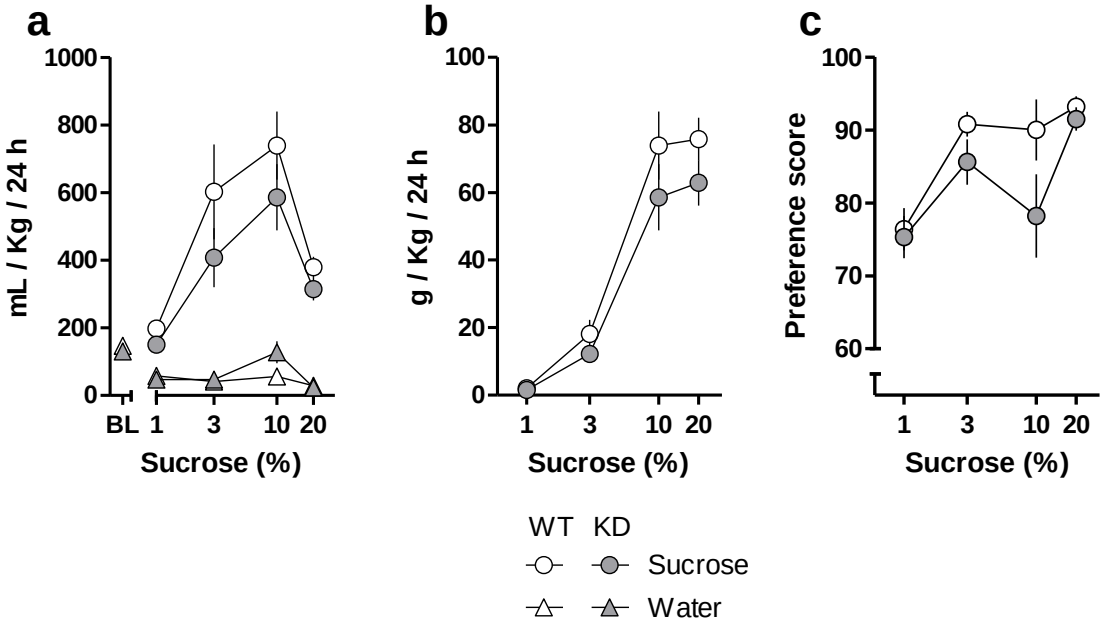


Figure 2.5 Measures of sucrose and water intake during a two-bottle choice paradigm. (**a**) Mice from both genotypes drank more from the sucrose bottle than the water bottle at all sucrose concentrations. Baseline intake of water from prior to sucrose testing is shown (BL). Each point represents the group mean intake of sucrose or water, averaged from measurements taken over two consecutive days. (**b**) The quantity of sucrose consumed is shown (**c**) Preference for sucrose (sucrose intake / total fluid intake) at each concentration is shown. Sucrose intake and consumption did not differ between genotypes, although the overall sucrose preference score was lower in mutant mice (n = 12/12, WT/KD).

2.3.4 Activity in a novel environment

2.3.4.1 Locomotor activity

In both genotypes upon exposure to the novel circular runway environment, activity (indexed by the distance travelled; m) was greatest during the first 10 minute period of the 1 hour session and declined over each subsequent 10 minute period (Fig. 2.6a; main effect of Period, $F(5,285) = 69.79$, $p < 0.001$; Period x Genotype interaction, NS). Notably, activity in KD mice during this first session was significantly reduced in comparison to their wild-type counterparts (main effect of Genotype, $F(1,57) = 6.75$, $p < 0.05$). Both genotypes habituated to the apparatus with repeated exposure to the runway over five once-daily 1 hour sessions (Fig. 2.6b). Habituation was confirmed by a significant reduction in activity over the five sessions in each genotype (WT: main effect of Session, $F(4,108) = 8.36$, $p < 0.001$; KD: main effect of Session, $F(4,120) = 3.54$, $p < 0.01$). During the fifth session (Fig. 2.6c), activity declined across each 10 minute period of the session in both genotypes (main effect of Period, $F(5,285) = 19.37$, $p < 0.001$; Period x Genotype interaction, NS), but there was no difference in overall activity between genotypes (main effect of Genotype, NS).

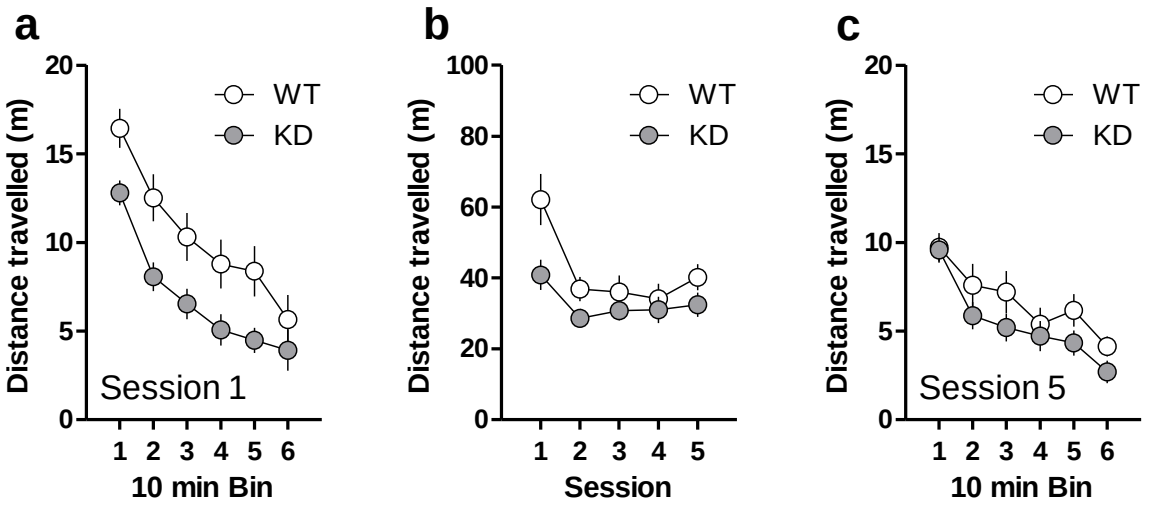


Figure 2.6 Locomotor activity in a circular runway. Mice were exposed to the locomotor apparatus over five once-daily 1 hour sessions. (**a**) During the first session (i.e. when presented as a novel environment), locomotor activity in both genotypes was greatest at the start of the 1 hour session, but declined over each subsequent 10 minute period. Activity in KD mice was significantly reduced in comparison to WT mice. (**b**) Mean total activity from each daily 1 hour session is shown. Both genotypes habituated to the circular runway, although activity in KD mice was reduced during the first session. ANOVA findings reflected this observation (main effect of Session, $F(4,228) = 12.19$, $p < 0.001$; Genotype, $F(1,57) = 4.99$, $p < 0.05$; Session x Genotype interaction, $F(4,228) = 2.35$, $p = 0.08$) (**c**) In the fifth locomotor session, activity did not differ between genotypes during any of the 10 minute periods (n = 28/31, WT/KD).

2.3.4.2 Spontaneous nose-poking

In the spontaneous nose-poking test, no difference was found between genotypes in the total number of nose-pokes made during the 30 minute session (Fig. 2.7a; $t = 0.92$, df = 46, $p > 0.05$). A plot of the time-course of nose-poke responses across each 10 minute period of the session (Fig. 2.7b) did not reveal any difference between genotypes in the response profile (Period x Genotype interaction, NS; main effect of Genotype, NS). There was no difference between genotypes in the latency to the first nose-poke response ($t = -0.31$, df = 46, $p > 0.05$; Fig. 2.7c).

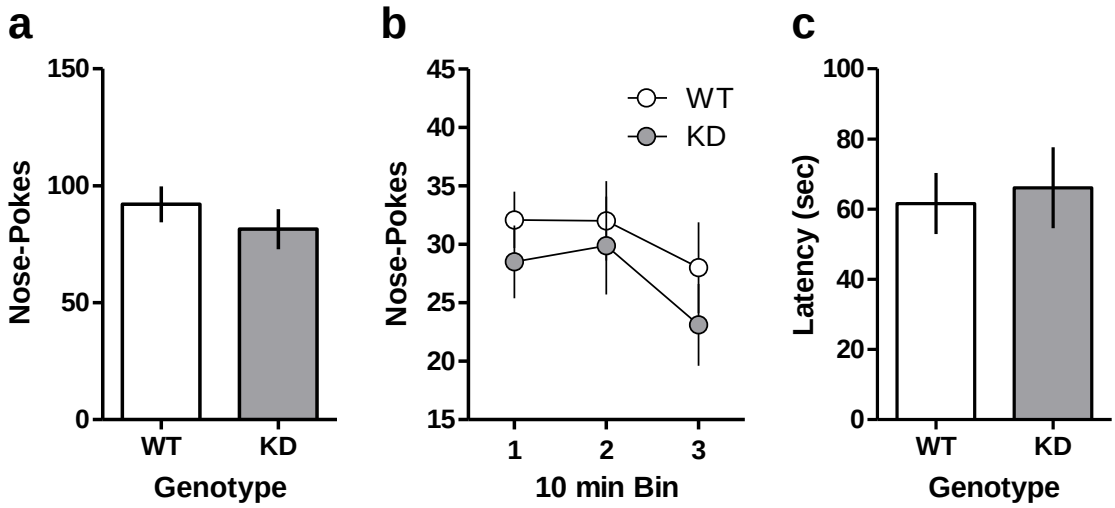


Figure 2.7 Spontaneous nose-poking in a novel environment. (**a**) There was no difference between genotypes in the mean total number of nose-pokes made during the 30 minute session. (**b**) Nose-poke activity remained stable over each 10 minute period of the session (main effect of Period, NS) and this response profile did not differ between genotypes. (**c**) The mean latency to the first nose-poke of the session did not differ between genotypes (n = 24/24, WT/KD).

2.3.4.3 Spontaneous activity in an open-field

The final assessment of activity was provided by recording behaviours of mice during a single 30 minute session in a novel open-field environment. The mean total duration of four levels of activity (static, static/exploratory, forward movement, excitatory movement) were first compared between genotypes (Fig. 2.8a). In both genotypes, the majority of session time was occupied by static/exploratory movement, followed by forward movement. Consistent with findings from the circular runway experiment, forward movement duration was significantly reduced in KD mice in comparison to WT mice (t = 2.08, df = 22, p < 0.05). Conversely, static/exploratory duration was increased in KD mice, although a between genotype comparison did not reach statistical significance (t = -1.78, df = 22, p = 0.089).

Time course plots of each activity level (Fig. 2.8c) were also consistent with findings from the circular runways. In both genotypes, forward movement duration decreased across each 10 minute period of the session (Fig. 2.8c, panel iii) while static/exploratory duration increased (Fig. 2.8c, panel ii). Static and excitatory movement duration did not differ over the course of the session, nor between genotypes (Fig. 2.8c, panels i and iv, respectively). These observations were confirmed by mixed-factor ANOVA, with each 10 minute bin represented as the within-subjects factor of Period (see Table 2.2 for ANOVA results).

From video recordings of the same 30 minute session, an analysis of more specific activities (wall rearing, centre rearing, grooming and digging) was also undertaken. In both genotypes, centre rearing occupied most session time, followed by wall rearing and digging (Fig. 2.8b). Between genotype comparisons of total activity duration identified a significant increase in centre rearing duration in KD mice (t = -2.14, df = 22, p < 0.05). Time-course plots of these more specific activities indicated that, in both genotypes, centre rearing duration increased while digging duration decreased over each 10 minute period of the session (Fig. 2.8d). Wall rearing and grooming duration not differ over the course of the session, nor

between genotypes. These findings were confirmed by mixed-factor ANOVA, which also identified a significant overall increase in centre rearing duration in KD mice (see Table 2.2 for ANOVA results).

Spontaneous Activity	Period F(2,44)	Period x Genotype F(2,44)	Genotype F(1,22)
Static/No Movement	7.24	2.86§	1.73
Static/Exploratory	44.20***	0.64	3.16§
Forward Movement	57.83***	0.44	4.34*
Excitatory Movement	3.32*	0.009	1.39
Wall Rearing	0.39	0.15	1.78
Center Rearing	9.75***	0.20	4.57*
Grooming	0.80	0.27	1.08
Digging	9.13***	0.19	1.39

Table 2.2 ANOVAs for time-course profiles of spontaneous activity in an open-field. Factors: Period (3 x 10 minute Bin of the 30 minute session); Genotype (WT, KD). ***$p < 0.001$, *$p < 0.05$, §$p < 0.1$

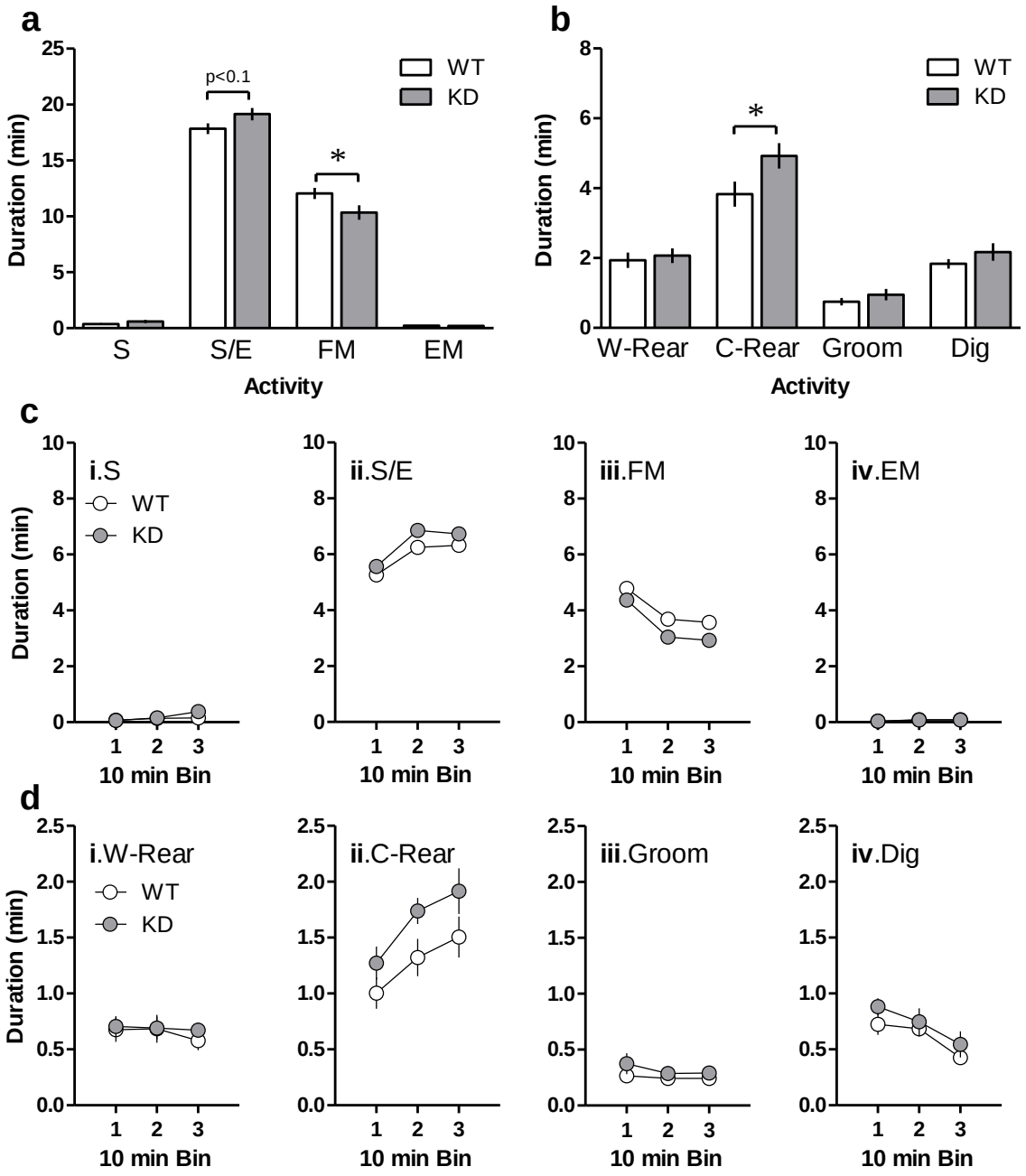


Figure 2.8 Spontaneous activity in an open-field. (**a**) The mean total duration of four levels of activity classified as static (S), static/exploratory (S/E), forward movement (FM) and excited movement (EM) are shown. In comparison to WT mice, forward movement duration was significantly reduced in KD mice, while static/exploratory movement tended to be increased. (**b**) Figure shows the mean total duration of four specific activities classified as rearing at the wall (W-Rear), rearing away from the wall (C-Rear), grooming (Groom) or digging (Dig). Rearing away from the wall duration was significantly increased in KD mice. (**c**) The time

course for each level of activity is shown. (**d**) The time course for each specific activity is shown. *$p < 0.05$, *t*-test comparison between genotypes (n = 12/12, WT/KD).

2.3.5 Anxiety-related behaviours

2.3.5.1 Open-field arena

During the 5 minute assessment of anxiety-related behaviours in an open-field arena, both genotypes spent more time in the outer area than the middle or inner areas of the arena (Fig. 2.9a). These findings were confirmed by mixed-factor ANOVA, with Area (outer, middle, inner) included as a within-subjects factor (main effect of Area, $F(2,44) = 2386.22$, $p < 0.001$; Genotype, NS). However, mutant mice spent a greater amount of time in the middle area than their wild-type littermates (Genotype x Area interaction, $F(2,44) = 4.17$, $p < 0.05$; between genotype comparison of time in middle area, $t = -2.16$, df = 22, $p < 0.05$). Although KD mice also spent less time in the outer area than WT mice, this comparison was not quite statistically significant ($t = 2.03$, df = 22, $p = 0.055$). Locomotor activity in the test session, assessed by crosses over lines that identified areas of the open-field, did not differ between genotypes ($t = 0.79$, df = 22, $p > 0.05$; Fig. 2.9b).

2.3.5.2 Light-dark box

During the 5 minute test in the light-dark box, both genotypes spent more time in the dark area than the light area. The time in each area did not differ between genotypes (Fig. 2.10a). These findings were confirmed by mixed-factor ANOVA, with Area (light, dark) included as a within-subjects factor (main effect of Area, $F(1,17) = 34.27$, $p < 0.001$; Genotype, NS; Genotype x Area interaction, NS). In both genotypes, the latency to the first escape from the light area was shorter than the latency to the first re-entry into the light area (Fig. 2.10b; main effect of Latency, $F(1,17) = 18.82$, $p < 0.001$). Latency events did not differ between genotypes (main effect of Genotype, NS; Latency x Genotype interaction, NS). Locomotor activity in the test, assessed by the total number of transitions between light and dark areas, tended to be reduced in KD mice, although this effect did not reach statistical significance ($t = 1.94$, df = 17, $p = 0.07$; Fig. 2.10c).

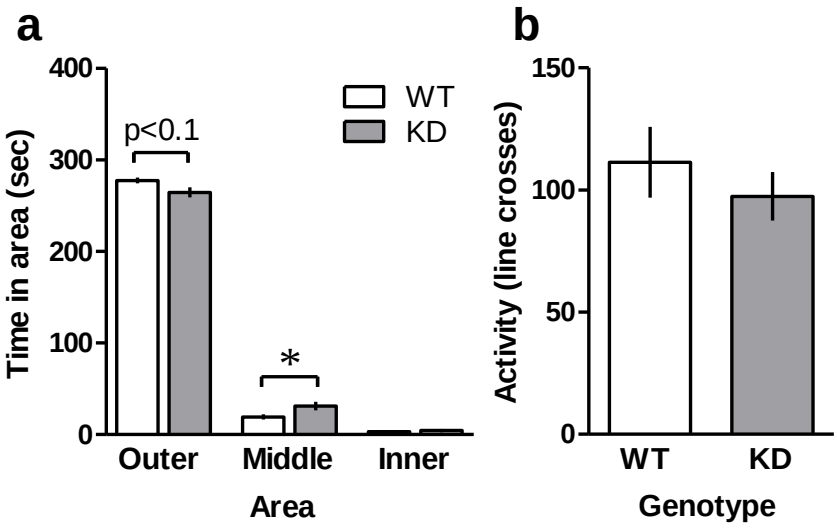


Figure 2.9 Anxiety-related behaviours in an open-field arena. (**a**) In the 5 minute session, both genotypes spent most time in the outer area of the open-field than the middle or inner areas. Mutant animals spent significantly more time in the middle area of the open field than WT littermate controls (**b**) Locomotor activity, assessed by crosses over lines that identified the areas of the open field, did not significantly differ between genotypes. *p < 0.05, t-test comparison between genotypes (n = 12/12, WT/KD).

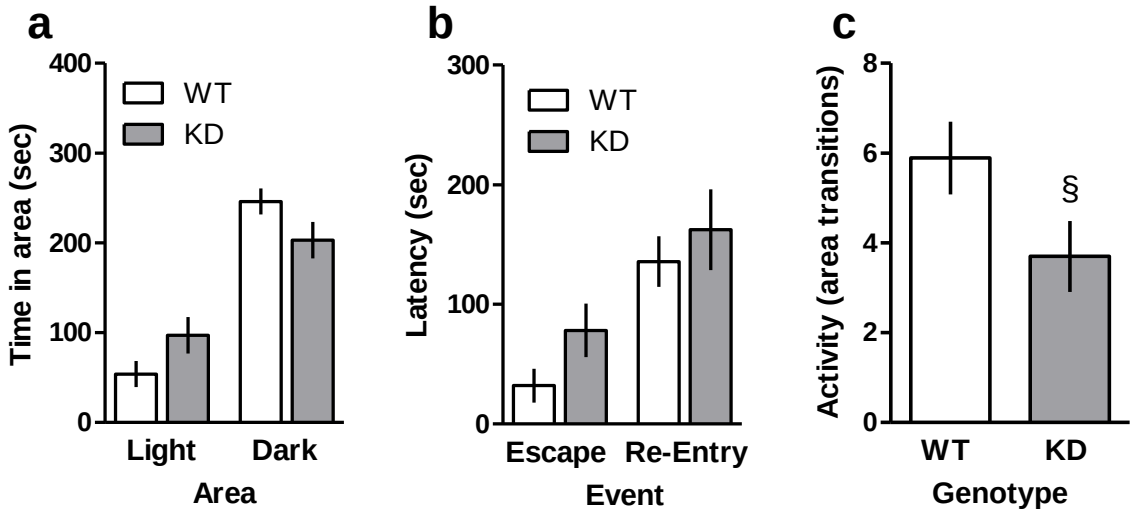


Figure 2.10 Anxiety-related behaviours in a light-dark box. (**a**) During the 5 minute test, both genotypes spent more time in the dark area than the light area. This effect did not reliably differ between genotypes. (**b**) There was no difference between genotypes in the latency to the first transition from the light area to the dark area (Escape), or in the latency to the first return to the light area from the dark area (Re-Entry). (**c**) Locomotor activity, indexed by the total number of transitions between the light and dark areas, tended to be reduced in KD mice. $^{\S}p < 0.1$ between-genotype *t*-test comparison (n = 9/10, WT/KD).

2.3.5.3 Elevated plus-maze

During the 5 minute test, mice from both genotypes spent most time in the closed arms of the elevated plus-maze than the open arms or the adjoining middle area. The time in each area did not differ between genotypes (Fig 2.11a). These findings were confirmed by mixed-factor ANOVA, with Area (open, closed, middle) included as a within-subjects factor (main effect of Area, $F(2,32) = 122.95$, $p < 0.001$; Genotype, NS; Genotype x Area interaction, NS). Locomotor activity in the 5 minute test, assessed by transitions between each of the three areas, did not differ between genotypes ($t = 0.660$, df = 16, $p > 0.05$; Fig. 2.11b). So called ethological 'risk assessment behaviours', such as stretch-attend postures and head-dips over the edges of the open sides were recorded, but the occurrence of these behaviours were insufficient to permit meaningful analysis.

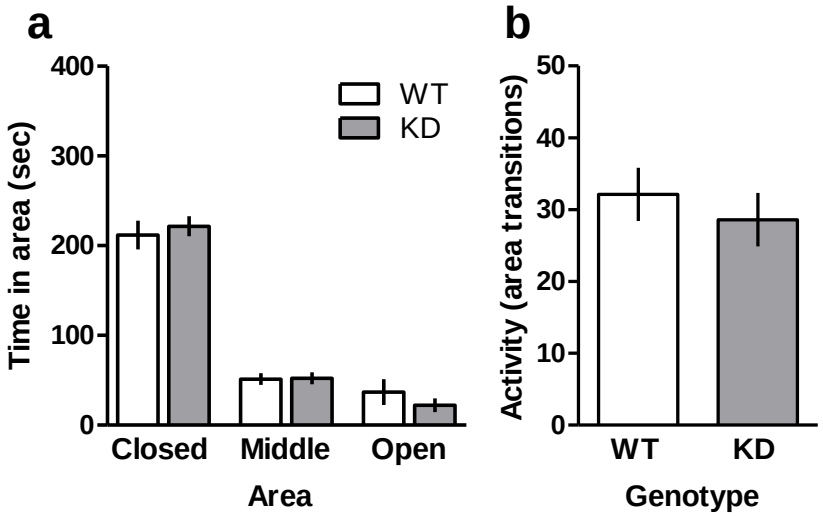


Figure 2.11 Anxiety-related behaviours in an elevated plus-maze. (**a**) During the 5 minute test, mice from both genotypes spent more time in the closed arms of the plus-maze than in the open arms or middle area that connected the arms. This activity profile did not differ between genotypes. (**b**) Locomotor activity, assessed by the total number of transitions between each of the three areas, did not differ between genotypes (n = 8/10, WT/KD).

2.4 Discussion

The present experiments examined the phenotypic traits of a novel mouse line in which cell-type specific RNA interference was used to selectively knock-down mGluR5 in cells that express the dopamine D1R. These mGluR5^{KD-D1} mice were indistinguishable from their wild-type littermates in gross appearance, simple tests of auditory and visual function and a variety of assessments of reflexive responses. Body weights in adult mice did not differ between genotypes under *ad libitum* or restricted feeding conditions. Mutant animals showed a preference for sucrose and were able to adjust their intake according to the sucrose concentration. However, locomotor activity experiments revealed that activity in a novel environment was reduced in mutant mice. Tests of anxiety-related behaviours suggested that mutant mice may have a reduced anxiety phenotype, but this effect was not reliably observed among the different tests employed.

The first series of assessments included an evaluation of general health and neurological reflexes. While these tests may appear crude, they can provide valuable information regarding gene function or unexpected effects that could severely constrain the interpretation of findings from more complex behavioural experiments. Poor body condition could indicate illness, inadequate diet, premature weaning, aberrant grooming or fighting amongst littermates. Impaired neurological reflex responses could have pointed to serious motor dysfunction including motor neuron degeneration. For example, the hanging wire test reflects motor strength and can be used to detect neuromuscular abnormalities, since balance and grip strength is required for the mouse to remain suspended from the inverted wire cage (Crawley, 2007). These tests were particularly relevant for mGluR5^{KD-D1} mice because mGluR5 is widely expressed in at many levels of the and peripheral neural circuitry involved in motor control central (Tallaksen-Greene et al., 1998; Alvarez et al., 2000; Awad et al., 2000; Hubert et al., 2001). The possibility for co-expression of the D1R in these areas could have resulted in disruption of motor control due to corresponding knock-down of mGluR5. For example, mGluR5 is

expressed within the ventral horn of the spinal cord, albeit at lower levels than the dorsal horn (Alvarez et al., 2000; Tomiyama et al., 2001; Ma et al., 2006). There is also some evidence for D1R-like expression in the ventral horn (Dubois et al., 1986; Zhu et al., 2007) and behavioural data pointing to a role of spinal D1R in the control over rhythmic movement generation (Lapointe et al., 2009). That we found no difference between mGluR5$^{KD\text{-}D1}$ and control mice in these basic assessments of health and neurological reflexes provides the first indication that loss of mGluR5 in mutant mice does not result in gross abnormalities that could restrict the use of these mice in more complex behavioural paradigms.

The use of visual and auditory stimuli for conditioning experiments reported in subsequent chapters of this thesis would necessitate that mGluR5$^{KD\text{-}D1}$ mice could adequately see and hear. Vision was assessed using the visual placing test (Heyser, 2003). While blind mice perform poorly in this test (Pinto and Enroth-Cugell, 2000), normal performance recorded in mGluR5$^{KD\text{-}D1}$ mice does not necessarily infer normal visual function. It is possible that performance in this task could be maintained despite some degree of impaired vision. However, it is also worth noting that mGluR5$^{KD\text{-}D1}$ mice typically moved directly from the light area to the dark area in the light-dark box test, indicating sufficient vision to identify the escape route from the light area. Published electrophysiology data, considered to provide the most sensitive and accurate measure of visual activity (Pinto and Enroth-Cugell, 2000), also fails to support a major role of mGluR5 in visual function (Cirone et al., 2002). Meanwhile, auditory function was provided by assessing the Preyer startle reflex. Akin to the visual placing test, a normal Preyer reflex cannot be taken as indicative of entirely normal auditory function (Astbury and Read, 1982; Horner and Barkway, 1986). However, there is a paucity of literature examining the role of mGluR5 in auditory function and expression of mGluR5 protein is low in the dorsal cochlear nucleus (Petralia et al., 1996), a major brain centre for the integration of auditory information. Collectively, the lack of evidence for impaired visual or auditory function in mGluR5$^{KD\text{-}D1}$ mice provides support for the use of visual and auditory stimuli for conditioning experiments.

The sucrose two-bottle choice test was used to primarily as an index of taste perception. However, sucrose consumption is a complex behaviour influenced by separate and dissociable orosensory and post-ingestive mechanisms (Sclafani, 1995) and can also reflect hedonic states (Papp et al., 1991; Stephens et al., 2010). With the experimental design used here, it is not possible to identify which factor was primarily responsible for driving sucrose consumption in mutant and control mice. However, the use of food restriction in the present test likely results in sucrose consumption being largely driven by the requirement for energy homeostasis (Hayward et al., 2002). Notably, sucrose consumption did not differ between genotypes and was high in both WT (4.8-28.6 mL @ 10% sucrose) and KD (3.9-25.5 mL @ 10% sucrose) mice, consistent with what has previously been reported in other C57BL/6 strains (Lewis et al., 2005). Although we were unable to determine a minimum threshold for detection of sucrose (subsequent studies would ideally test lower concentrations of sucrose), mGluR5$^{KD\text{-}D1}$ mice displayed a clear preference for sucrose at all concentrations tested. Thus, despite the complex nature of the task, the study at least demonstrates that taste functions such as simple detection and discrimination are not impaired in mutant mice under conditions of restricted feeding that would be used throughout behavioural tests in subsequent chapters of this thesis.

Other studies of sucrose consumption have reported that mice initially consume little sucrose upon first exposure, but gradually develop a sucrose preference over subsequent days (Amico et al., 2005). This neophobic response can be a useful indicator of memory and taste perception, since it reflects the ability to store representations of an experienced taste so that it can be retrieved on subsequent encounters and infers the ability to identify and respond differently to novel versus familiar tastes (Vogt and Rudy, 1984). The present study found no evidence for neophobia in either wild-type or mutant mice, as sucrose consumption in both genotypes did not significantly increase across the days of exposure to each sucrose concentration (data not shown). Absence of a neophobic response in the

present study may reflect rapid acclimatisation to sucrose due to the relatively low sucrose concentration first experienced (approximately 10 fold lower than typically used to examine neophobia) and/or heightened motivation due to food restriction overriding a neophobic response. Alternatively, the interval between measurements of sucrose intake (once every 24 hours) may have lacked sensitivity for detecting a neophobic response that may have occurred during initial exposure to the sucrose bottle. Indeed, 24 hour measurements take no account of meal size, rate of consumption, meal frequency or circadian rhythms.

An important finding in the two-bottle test was that sucrose preference was reduced in mGluR5$^{KD\text{-}D1}$ mice, which was a function of increased water intake at the 10% sucrose concentration. The reason for altered intake of water is not entirely clear, but could reflect altered metabolic function in mutant mice. Metabotropic glutamate receptors are involved in hormone secretion in the endocrine pancreas (Brice et al., 2002). Indeed, mGluR5 is expressed at the cell surface of clonal beta-cells and in purified insulin-containing granules and is required for an optimal insulin response to glucose (Storto et al., 2006). Given that pancreatic cells also possess characteristics of dopamine producing cells (Mezey et al., 1996) and dopamine-D1 receptors may be expressed by beta-cells (Rubi et al., 2005), it is plausible that mGluR5 is knocked-down in these cells, resulting in disrupted pancreatic signalling. Decreased intake of sucrose and increased water intake by animals with experimentally induced diabetes in a two-bottle choice test has been reported previously (Maller and Hamilton, 1968; Hiji, 1969). This pattern of intake is considered a strategy by which diabetic animals dilute the osmotic load of concentrated sugar solutions (Tepper and Friedman, 1991). Although we have not examined pancreatic expression of mGluR5 or assessed pancreatic function directly in mGluR5$^{KD\text{-}D1}$ mice, a ‘diabetic mouse’ hypothesis seems unlikely. First, water intake measured during the habituation phase of the two-bottle choice experiment tended to be lower in mutant mice, which is inconsistent with a polydipsia phenotype typically observed in diabetic animals (Tepper and Friedman, 1991). Second, mutant mice attained free-feeding body weights comparable to

their wild-type littermates and were able to tolerate a restricted feeding regime, which is also inconsistent with diabetic phenotypes of hyperphagia and weight loss (Tepper and Friedman, 1991).

Three tests were used specifically to examine locomotor behaviour in mutant mice. In a circular runway, a clear reduction in activity was found in mutant mice during the first test session (that is, when the test environment was novel). A more detailed assessment of activity in a novel open-field arena indicated this genotype difference likely arose from a reduction in ambulatory movement and, conversely, a tendency for an increase in the duration of static/exploratory behaviour in KD mice. One possibility is that decreased ambulatory movement in mutant mice was due to competing aberrant behaviours, such as stereotypy (that is, repetitive, invariant, and perseverative motor patterns that do not appear directed toward a purposeful action). Although stereotypy was not formally assessed in the current studies, mutant mice did not show elevated levels of grooming or digging. Similarly, static/exploratory or excitatory movement duration, which would have captured stereotyped movements (for example, wild-running, circling, excessive grooming, stereotyped sniffing, head bobbing), did not significantly differ between genotypes. After a period of habituation, baseline activity in the circular runways did not differ between genotypes, consistent with findings of no genotype difference in activity when monitored in the home-cage (personal communication with Dr. B. Halbout). Taken together, these data suggest that the locomotor responsiveness to novelty is attenuated in mutant mice, rather than general locomotor activity *per se*.

The spontaneous nose-poking task was also used as a measure of exploratory behaviour in a novel environment, taking inspiration from hole-board exploration paradigms that exploit of the tendency of mice to poke their noses into holes in a wall or floor (Boissier et al., 1964). In contrast to findings from the circular runway and the open-field, no difference was found between genotypes in exploratory behaviour that was indexed by nose-poking. This lack of difference may indicate that the behavioural measure (nose-poking) was not sufficiently sensitive to detect

locomotor differences between genotypes. Alternatively, the nose-poke response may have been simply unaffected in mutant mice, while motor behaviours reduced in the circular runway and open-field (for example, ambulatory activity) were more heavily influenced by mGluR5 loss on D1 expressing cells. Finally, if differences in motor activity between genotypes were correlated to the anxiogenic potential of the test environment, this would be consistent with a lack of difference between genotypes in the nose-poking test, which was conducted in the relatively small, dark and enclosed space of the conditioning chamber.

Locomotor activity in a novel environment, although commonly associated with dopaminergic signalling, requires the function of many central and peripheral systems (Picciotto and Wickman, 1998). It is well beyond the scope of this thesis to determine precisely where disruption has occurred in this particular network in mGluR5$^{KD\text{-}D1}$ mice, although this point will be given further consideration in the discussion in Chapter 5 and also the General Discussion of this thesis. For now, it is sufficient to recognise that this particular phenotypic trait must be considered when discussing data generated from subsequent behavioural tests, given the potential for non-specific effects associated with reduced locomotor activity under certain conditions.

Three tests were used to examine anxiety-related behaviours in mutant mice. The first test used one of the oldest and simplest (at least in design) measures of rodent emotional behaviour, the open-field (Hall, 1934, 1936). A number of behaviours recorded in a novel and inescapable open-field are proposed as indices of heightened emotionality and/or anxiety, including decreased ambulation, increased defecation, thigmotaxis (the proportion of time spent close to the wall) and decreased rearing (Archer, 1973; Walsh and Cummins, 1976; Crawley et al., 1997). In the present test, mutant mice spent significantly more time in the middle part of the arena than wild-type mice. Although rearing was not assessed in this open-field test, increased rearing was observed in mutant mice in the smaller open-field arena used to assess spontaneous activity. Together, these data

support a reduced anxiety-like phenotype in mGluR5^{KD-D1} mice. However, this conclusion was not robustly supported when anxiety-related behaviours were examined in two other tests of unconditioned behaviour, namely the light-dark box (Crawley and Goodwin, 1980; Crawley et al., 1981; Bourin and Hascoet, 2003) and the elevated plus-maze (Pellow et al., 1985; Pellow and File, 1986; Stephens et al., 1986; Lister, 1987). Both tests similarly exploit the naturalistic conflict between the tendency of mice to explore a novel environment and the aversive properties of an open space (Crawley, 1985; Belzung and Griebel, 2001). In the light-dark box, mutant mice did spend more time in the (anxiogenic) light area than wild-type controls, but this effect did not reach significance. In the elevated plus-maze, no difference in exploratory behaviour was observed between genotypes. The contrasting findings amongst the three tests are not easily reconciled, but could reflect different genetic components underlying performance in each of the paradigms (Crawley and Goodwin, 1980; Crawley et al., 1981; Mathis et al., 1994; Dawson and Tricklebank, 1995; Mathis et al., 1995).

An important consideration for interpreting findings from the tests of anxiety-related behaviours is that all are heavily dependent upon locomotor activity. In the light-dark box, there was a tendency in mutant mice for a reduction in the number of transitions between areas (a measure considered by some to be a more sensitive measure of anxiety-related behaviour than time in each area (Crawley, 1985)), although no genotype difference in activity was detected in the open-field or the elevated plus-maze. The failure to find a significant difference in activity in these tests could have been due to the sensitivity of the measures used and/or the relatively short duration of the tests. For example, the number of area transitions in the elevated plus-maze is reported to be a relatively insensitive measure of drug-induced changes in locomotor activity (Dawson et al., 1995). Since mice experienced all tests under novel environment conditions, the potential for differences in locomotor activity between genotypes contributing to an apparent reduced anxiety phenotype cannot be completely discounted.

If mGluR5 on D1R expressing cells contributes to anxiety related behaviours, it is likely that the overall contribution is small. Reports on the role of mGluR5 in anxiety propose multiple brain areas that may be involved in the anxiolytic-like effects of mGluR5 antagonists. First, the anxiolytic-like effects of mGluR5 antagonists may reflect their ability to reduce extracellular noradrenaline in the frontal cortex, as the noradrenergic locus coeruleus is an important mediator of stress responses and may contribute to affective disorders (Page et al., 2005). Second, the mGluR5 antagonist MTEP is reported to produce a dose-dependent increase in serotonin in the frontal cortex, and the anxiolytic-like effects of MTEP may necessitate activation of the serotonin system (Stachowicz et al., 2007). Third, MTEP reduced anxiety-related measures in the elevated plus-maze when injected directly into the lateral-septal nucleus (Molina-Hernandez et al., 2006), an area that likely regulates mood and motivation through connections with the mesocorticolimbic dopamine system (Sheehan et al., 2004). Finally, the anxiolytic effects of MPEP in non-conditioned tests of anxiety may by mediated by block of mGluR5 in the BLA and/or CeN, resulting in reduced glutamate transmission within the BLA and reduced glutamate output from the CeN (Perez de la Mora et al., 2006). Clearly, to determine whether function in any or all of these neural systems are impaired in mGluR5^{KD-D1} mice would require many years of investigation. For now, it is sufficient that the potential for a reduced anxiety-phenotype in mutant mice will be considered alongside any other findings reported in this thesis.

Finally, it must be acknowledged that all of the assessments reported here were undertaken in adult male mice during the light phase and phenotype/genotype interactions may have also arisen as a function of age, gender or time of day. For example, Huntington's disease transgenic mice show reduced impaired motor activity that progressively worsens with age (Mangiarini et al., 1996). Just one example of gender differences is provided by the observation that female mice of some strains typically consume greater volumes of sweet solutions than male mice (Stockton and Whitney, 1974; Ramirez and Fuller, 1976). Although all of these factors would be of interest to explore in mGluR5^{KD-D1} mice, it must also be noted

that subsequent experiments of this thesis would be conducted during the light-phase in adult male mice. Thus, the experimental parameters employed in the current chapter allowed for basic phenotype differences to be used more readily in understanding any further genotype differences that could emerge from experiments reported in the following chapters of this thesis.

3 Appetitive learning in mGluR5^{KD-D1} mice

3.1 Introduction

As a consequence of associative learning, an environmental stimulus paired with reward experience (a conditioned stimulus; CS) can not only acquire predictive properties that serve to signal the availability and/or location of the reward (discriminated approach or goal-tracking; Boakes, 1977), but may also acquire incentive properties that enable CSs to attract (auto-shaping or sign-tracking; Brown and Jenkins, 1968), directly reinforce (conditioned reinforcement; Mackintosh, 1974) or energise (Pavlovian-instrumental transfer; Estes, 1948) appetitive behaviours (see also Flagel et al., 2009; Robinson and Flagel, 2009). While the predictive and incentive functions of CSs have clear adaptive value, the neural systems that mediate the learning of incentive properties (the acquisition) and the CSs' subsequent effects on behaviour (the expression) are proposed to be subverted by drugs of abuse (Everitt et al., 2001; Kelley, 2004; Hyman et al., 2006). Thus, contemporary theories of drug addiction ascribe particular importance to the role of drug-paired CSs in maintaining drug taking and triggering relapse (Stewart et al., 1984; Robinson and Berridge, 1993; Everitt et al., 2001). The powerful influence of CSs over the consumption of natural rewards (e.g. cue-potentiated feeding; Zambie, 1973; Weingarten, 1983) has similarly led to the proposition that food-paired CSs may contribute to the development and maintenance of certain eating disorders and obesity (Holland and Petrovich, 2005; Le Merrer and Stephens, 2006; Volkow et al., 2008).

The neural circuitry underlying incentive learning and control over appetitive behaviours by CSs involves, in part, convergence within the striatum of dopaminergic projections from the ventral tegmental area (VTA) and substantia nigra (SN), with glutamatergic inputs originating in the prefrontal cortex (PFC), hippocampus and amygdala (Schultz et al., 1997; Robbins and Everitt, 2002; Cardinal and Everitt, 2004; Goto and Grace, 2008). Glutamate signalling through

ionotropic AMPA and NMDA receptors in the ventral striatum appear particularly important for mediating the control over certain appetitive behaviours by CSs (for example, sign-tracking responses; Di Ciano et al., 2001). However, much less is known about the role of metabotropic glutamate receptors in these incentive learning processes.

The group I metabotropic glutamate receptor, mGluR5, is found throughout the CNS, but is most densely expressed in the striatum, cortex and hippocampus (Romano et al., 1995). Typically located postsynaptically on dendritic spines and concentrated at perisynaptic sites (Shigemoto et al., 1993; Luján et al., 1996), mGluR5 plays a central role in different forms of synaptic plasticity, including long term potentiation (LTP; see Anwyl, 2009 for review) and long term depression (LTD; see Bellone et al., 2008 for review), that are thought to be involved in a variety of learning and memory processes (Kelley, 2004; Malenka and Bear, 2004; Hyman et al., 2006). Mechanisms by which group I mGluRs influence synaptic plasticity include control over presynaptic transmitter release via retrograde endocannabinoid signalling (Robbe et al., 2002) and changes in postsynaptic sensitivity to excitatory input through alterations in AMPA receptor expression (Snyder et al., 2001; Bellone and Luscher, 2005; Mameli et al., 2007; Jo et al., 2008; Zhang et al., 2008; Kelly et al., 2009). Thus, mGluR5 appears ideally positioned to mediate incentive learning processes necessary for the acquisition of predictive and/or incentive properties by reward-paired CSs which enable them to influence appetitive behaviours.

In the striatum, mGluR5 is expressed on both striatonigral and striatopallidal projecting MSNs (Tallaksen-Greene et al., 1998) that are characterised, in part, by predominant expression of dopamine D1R or D2R receptors, respectively (Gerfen et al., 1990; Bertran-Gonzalez et al., 2011). MSNs provide the sole striatal output to motivational and motor systems (Goto and Grace, 2008), but the role of mGluR5 on these functionally distinct neurons for incentive learning processes is not clear. Although systemic administration of an mGluR5 antagonist can disrupt behaviours

maintained by a reward-paired CS (e.g. Backstrom et al., 2004), pharmacological approaches inevitably lack the selectivity required for understanding the contribution of mGluR5 on distinct neuronal populations within the same brain locus, such as D1- and D2-MSNs within the striatum. In addition, the only published report to examine the role of striatal mGluR5 in behaviours maintained by a drug-paired CS was unsuccessful (Backstrom and Hyytia, 2007). In this study, the mGluR5 antagonist MPEP was reported to decrease cocaine seeking relative to baseline, but only because similar effects were seen following vehicle injections in the same within-subjects design. The authors suggested this may reflect conditioned anhedonic effects associated with MPEP (Backstrom and Hyytia, 2007). The mGluR5$^{KD\text{-}D1}$ mouse model therefore offers a valuable tool to examine the contribution of mGluR5 on cells that express the dopamine D1R (including striatal D1-MSNs) to incentive learning processes.

In this regard, the following experiments will see mGluR5$^{KD\text{-}D1}$ and wild-type mice exposed to tests that aim to examine the role of mGluR5 on D1R expressing neurons in predictive and incentive elements of appetitive learning. Mice will first be trained a purely Pavlovian association between the presentation of a discrete stimulus (e.g. a light) and the delivery of a food pellet. Learning about the predictive properties of the food-paired CS will be indexed by discriminated approach responses during training and, more formally, by tests of discriminated approach conducted in the absence of food delivery (i.e. extinction conditions). To assess incentive learning in mGluR5$^{KD\text{-}D1}$ and wild-type mice, three fundamental (but neurobiologically distinct; Cardinal et al., 2002a) properties of an incentive CS will be examined; the ability of a CS to 1) elicit approach toward it (sign-tracking), 2) reinforce the learning of a new response (CRf) and 3) energize ongoing instrumental actions (PIT). Two pharmacology tests will be used to further probe the role of mGluR5 in incentive learning. First, mGluR5$^{KD\text{-}D1}$ and wild-type mice will receive injections of cocaine prior to CRf tests. This experiment will inform about the contribution of mGluR5 on D1R expressing cells to the neural circuitry underlying psychostimulant potentiation of CRf. Second, wild-type mice will receive

injections of an mGluR5 antagonist, MTEP, prior to tests of CRf. In this experiment, the role of mGluR5 in the expression of control over behaviour by a conditioned incentive can be examined. Finally, two satiety-induced devaluation tests will be reported. The first devaluation test will assess whether mice can update the value of the CS elicited representation of the US. The second test will assess whether instrumental responding, after the PIT test, is goal-directed or habit based.

3.2 Materials and Methods

3.2.1 Animals

Mice (n = 40/45, WT/KD; male and at least 8 weeks old prior to experiment start) were maintained on a 12:12 h light-dark cycle (lights on at 0700 hours) under controlled temperature (21 ± 2 °C) and humidity conditions (50 ± 5%). Animals were housed in groups of two or three per cage. Water was available *ad libitum* in the holding room. At least 7 days prior to the experiment start, mice were placed onto a restricted feeding regime designed to maintain body weights at ~85% of free-feeding weight. Experiments took place during the light-phase. All procedures were performed in accordance with the United Kingdom 1986 Animals (Scientific Procedures) Act, following institutional ethical review.

3.2.2 Drugs

Cocaine hydrochloride (Macfarlan Smith, Edinburgh, Scotland, UK) was dissolved in 0.9% saline. The non-competitive mGluR5 antagonist, 3-((2-methyl-1,3-thiazol-4-yl)ethynyl)pyridine (MTEP; Sequoia Research Products, Pangbourne, UK), was dissolved in 10% v/v Tween 80 : 90% water. Injections were administered at a volume of 10 ml/kg i.p.

3.2.3 Apparatus

Behavioural training and testing were performed in eight standard mouse conditioning chambers (15.9 x 14 x 12.7 cm; Med Associates, Vermont, USA). Each chamber was housed within a sound attenuating and light-resistant cubicle, fitted with an exhaust fan that served both to ventilate the unit and mask any external noise. The front access panel, ceiling and rear wall of the conditioning chambers were constructed from clear Plexiglas and the side walls consisted of removable aluminium panels. Each chamber was fitted with a pellet dispenser system that delivered 20 mg food pellets (5TUL, Cat no. 1811142; Test Diets, Indiana, USA) into a recessed food magazine situated at the centre of one side wall (Fig. 3.1a). A retractable response lever was located on either side of the food

magazine and an LED stimulus light was positioned approximately 8 cm above each lever (Fig. 3.1a-b). A tone generator (2.9 KHz, 5 dB above background) was situated between the stimulus lights (Fig. 3.1a). On the wall opposite to the food magazine was located the house light and, for the Pavlovian approach tests, two nose-poke holes that each contained an LED stimulus light (Fig. 3.1c) Infra-red beams detected head entries into the food magazine and the nose-poke holes. Conditioning chambers were controlled and responses recorded using Med-PC IV (Med Associates).

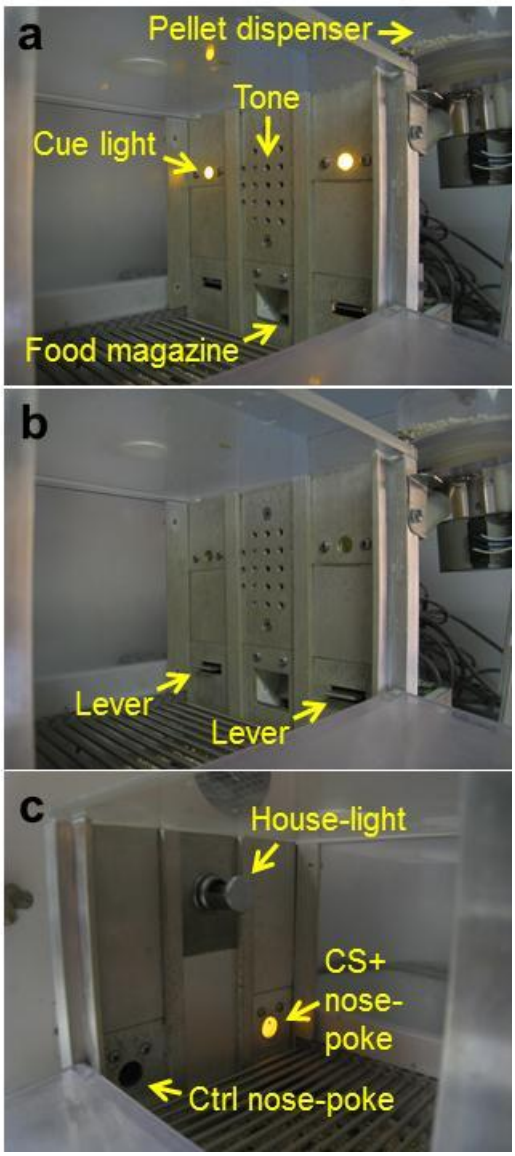


Figure 3.1 Apparatus for conditioning studies (**a**) The front wall of the conditioning chamber is shown, with the Plexiglas door that provides access to the chamber open. A waste tray filled with sawdust is located below the grid floor. (**b**) The two levers are extended into the chamber. (**c**) The rear wall of the conditioning chamber is shown. For the Pavlovian approach test, two nose-pokes are inserted into the chamber. In one nose-poke a stimulus light is presented (CS+ nose-poke), while no stimulus is presented in the control (Ctrl) nose-poke.

3.2.4 Procedural note

Four different groups of mice were used for the following experiments. An outline of the experiments conducted within each group (1-4) is provided (Fig. 3.2). Where multiple tests were conducted in the same group, Pavlovian reconditioning sessions were provided between tests to maintain baseline conditioning performance.

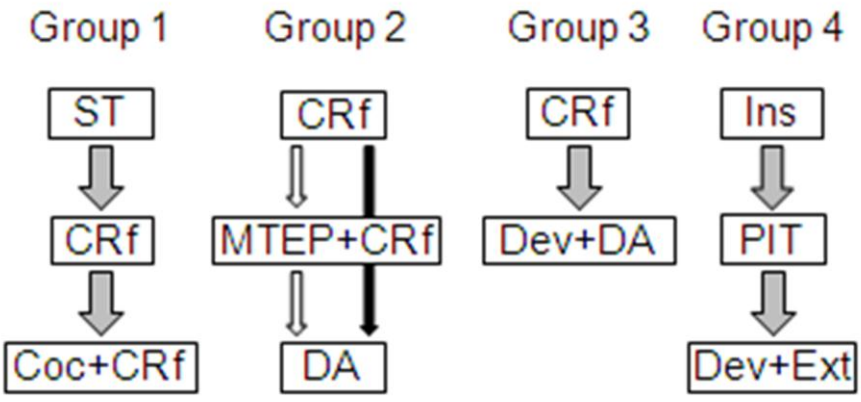


Figure 3.2 Experimental outline for conditioning studies. Four groups of WT and KD mice were used for the present experiments. All groups first received a magazine training session followed by Pavlovian conditioning. Further reconditioning sessions, represented by the block arrows, were provided between tests. **Group 1** was used to examine sign-tracking (ST), conditioned reinforcement (CRf) and cocaine facilitation of CRf (Coc+CRf). **Group 2** was used to examine CRf. Wild-type mice (open arrows) from this group were then used to examine the effect of MTEP on CRf (MTEP+CRf). WT and KD (black arrows) mice were then both assessed for discriminated approach (DA). **Group 3** was used for conditioned reinforcement (CRf) and devaluation of DA (Dev+DA). **Group 4** was used to examine Pavlovian-instrumental transfer (PIT) and devaluation of instrumental responding under extinction (Dev+Ext).

3.2.5 Magazine training

To familiarize all mice with the food used for conditioning studies, a small amount of the food was given to mice in their home cage. Mice also received a single, 30 min, magazine training session in which food pellets were delivered once every 60

sec, on average (range of 25 to 95 sec; M = 60 sec). No levers, nose-pokes or stimuli (e.g. tone or cue lights) were present during the magazine training session.

3.2.6 Group 1

Procedure overview: Mice (n = 12/12, WT/KD) were first trained a Pavlovian association between a discrete visual stimulus and the delivery of a food pellet. Following Pavlovian conditioning sessions, the ability of the food-paired stimulus to elicit approach toward it (sign-tracking) and to reinforce a novel instrumental response (CRf) was examined. Finally, I examined whether cocaine would facilitate responding for CRf in wild-type and mutant mice.

3.2.6.1 Pavlovian conditioning

Mice received eleven, once-daily, 30 min Pavlovian conditioning sessions in which 16 presentations of a 10 sec stimulus paired with food delivery (CS+; cue lights flashing at 1 Hz) occurred. A single food pellet was delivered 5 sec after CS+ onset. Each CS+ presentation was separated by a variable, no-stimulus, inter-trial interval (ITI; range of 80 to 120 sec; M = 100 sec). A number of measures were used to inform about learning in Pavlovian conditioning sessions, 1) *Magazine entries / min*: the rate of magazine entries during CS+ presentations and the ITI, 2) *% Magazine entries*: the distribution of magazine entries between stimulus and ITI periods, calculated as ((#CS+ entries / CS+ time) / ((#CS+ entries / CS+ time) + (#ITI entries / ITI time))) x 100), 3) *Discriminated approach responses*: the number of magazine entries made during the first 5 sec interval that occurred after CS+ onset but before food delivery, and 4) *Retrieval latency*: the mean latency to enter the food magazine after CS+ onset. Note that when a mouse did not enter the magazine during a CS+ trial, a time of 10 sec was designated as the retrieval latency for that trial.

3.2.6.2 Sign-tracking

For the 45 min sign-tracking test, conducted 24 h after the final conditioning session, two nose-poke holes were inserted into the conditioning chamber (Fig.

3.1c). Access to the food magazine was blocked in order to minimize the potential for competition between discriminated approach and sign-tracking responses. In one nose-poke hole, 15 x 1 min presentations of a flashing cue light (that is, the CS+) occurred. Each CS+ presentation was separated by a 2 min, no-stimulus, ITI. No stimulus presentations occurred in the second (control) nose-poke hole and no food was delivered during the test. The assignment of nose-poke holes (left or right) as CS+ and control was counterbalanced within each genotype. Entries into each nose-poke were recorded during CS+ presentations, thus providing a measure of Pavlovian approach responses toward the CS+. Entries into the nose-poke ports during the ITI were recorded to provide an indication of overall activity during the test.

3.2.6.3 Conditioned reinforcement

The 45 min CRf test commenced with insertion of both response levers into the operant chamber. A single response on one lever (termed, the CS+ lever) resulted in a 1.5 sec presentation of the flashing cue lights (that is, the CS+), whereas a single response on the alternate lever (the control lever) had no scheduled consequence. For half of the animals, the left lever was assigned as the CS+ lever and the right lever was the control lever. The remaining animals received the reverse lever-outcome pairings. The number and time of occurrence of responses on both levers were recorded. No food was delivered during the test.

3.2.6.4 The effect of cocaine on responding for conditioned reinforcement

Mice were injected with cocaine (0, 3, 10 or 20 mg/kg i.p.) immediately prior to CRf tests (undertaken as described 3.2.6.3.). Each mouse received all doses of cocaine and vehicle in a Latin-square design. One or two reconditioning sessions (as described 3.2.6.1) were provided between each CRf test.

3.2.7 Group 2

Procedure overview: Since findings from the CRf test in group 1 could have been influenced by experience of the sign-tracking test first, a second group of mice (n =

12/12, WT/KD) underwent identical Pavlovian training that was immediately followed by a CRf test. Next, the role of mGluR5 in mediating responding for CRf was further explored by injecting WT mice from this group with the mGluR5 antagonist, MTEP, prior to additional CRf tests. Finally, the ability of the food-paired stimulus to serve as a predictor of food delivery (discriminated approach) was examined in both WT and KD mice. A number of discriminated approach tests were performed to understand whether mice were using the stimulus light or perhaps another cue (such as the sound of the food pellet delivery) as a signal of oncoming food delivery.

3.2.7.1 Pavlovian conditioning

Pavlovian conditioning was undertaken as described (3.2.6.1)

3.2.7.2 Conditioned reinforcement

Conditioned reinforcement was undertaken as described (3.2.6.3)

3.2.7.3 The effect of MTEP on responding for conditioned reinforcement

Wild-type mice were injected with MTEP (0, 3, 10 or 20 mg/kg i.p.) 15 minutes prior to CRf tests (undertaken as described 3.2.6.3). Each mouse received all doses of MTEP in a Latin-square design. One or two reconditioning sessions (as described 3.2.6.1) were provided between each CRf test. Mutant mice did not undergo this experiment, since they did not demonstrate any CRf in the first test. One WT mouse that failed to elicit any responses during the first CRf test was excluded from this test, leaving a group size of n = 11.

3.2.7.4 Discriminated approach

Mice (n = 12/12, WT/KD) were given five discriminated approach tests, each of which was 15 minutes in duration. Each test contained 8 x 10 sec trials, with each trial separated by a fixed 100 sec ITI. The type of trial in each test was either 1) *CS+, US:* 10 sec presentation of the CS+, with delivery of a single food pellet 5 sec after CS+ onset (i.e. normal Pavlovian conditioning parameters), 2) *CS+, Noise:* 10

sec presentation of the CS+, with the sound of the food pellet delivery 5 sec after CS+ onset. The sound of the food pellet delivery was recreated by activating the food magazine and allowing the food pellet to fall into a metal receptacle located outside of the conditioning chamber. 3) CS+: 10 sec presentation of the CS+ only, 4) Noise: the sound of the food pellet delivery occurring 5 sec after the end of each 100 sec ITI, or 5) No: no stimuli were presented during the 10 sec trial period. Note that only one type of trial was present in a test. The order of the five discriminated approach tests was presented in a Latin-square design. One or two reconditioning sessions (as described in 3.2.6.1) were provided between each test. Magazine entry rates were recorded during each 10 second trial and the intervening ITIs.

3.2.8 Group 3

Procedure overview: Because recent evidence suggested that responding for sensory reinforcement may contribute significantly to responding for CRf where only one conditioned stimulus is used (Winterbauer and Balleine, 2007), a third group of mice (n = 9/12, WT/KD) was trained a Pavlovian association with one stimulus paired with food delivery and a second stimulus unpaired with food. A test of CRf followed in which responding on one lever led to presentation of the food paired stimulus and responding on a second lever resulted in presentation of the unpaired stimulus. A test of discriminated approach was also undertaken in these mice. However, this test contained a devaluation component that would inform about ability of the mice to use the CS for gaining access to an internal representation of the associated outcome (Holland, 2004).

3.2.8.1 Pavlovian conditioning

Mice received eleven, once-daily, Pavlovian conditioning sessions in which 16 presentations of a stimulus paired with food delivery (CS+) and 16 presentations of a stimulus paired with no outcome (CS-) occurred. The order of stimulus presentations was entirely random and each stimulus was separated by a variable no-stimulus ITI (range of 80-120 sec; M = 100 sec). For half of the mice, a constant tone (80 dB, 2,900 Hz; 10 sec) served as the CS+ and flashing cue lights (1 Hz; 10

sec) served as the CS-. The remaining animals received the reverse pairings. A single food pellet was delivered 5 sec after CS+ onset. The measures used to inform about learning in Pavlovian conditioning sessions were as described (3.2.6.1), with the modification that *magazine entries / min* and the *% Magazine entries* calculation now took into account magazine entries during both CS+ and CS- periods.

3.2.8.2 Conditioned reinforcement

The 45 min test commenced with insertion of both response levers into the operant chamber. A single response on one lever resulted in a 1.5 sec presentation of the CS+, whereas a single response on the alternate lever resulted in a 1.5 sec presentation of the CS-. For half of the mice, the left lever was designated the CS+ lever and the right lever the CS- lever. This contingency was reversed for remaining mice. No food was delivered during the test. The number of lever responses and their time of occurrence was recorded.

3.2.8.3 The effect of satiety on discriminated approach responses

The discriminated approach test contained 8 presentations of the 10 sec CS+ and 8 presentations of the 10 sec CS-. The order of stimulus presentations was entirely random and each stimulus was separated by a fixed 100 sec ITI. No food was delivered during the test. Magazine entry rates were recorded during each CS+ and CS- trial. All mice were exposed to two discriminated approach tests, with two reconditioning sessions (as described in 3.2.8.1) provided between each test. For 1 hour prior to the first test, half of the mice within each genotype received *ad libitum* access to standard lab chow in their home cage (termed, the ‘valued’ conditioned), while the other half received *ad libitum* access to the food pellets used for conditioning studies (termed, the ‘devalued’ condition), also within their home cages. For the second discriminated approach test, mice were exposed to the opposite feeding condition. To confirm whether food consumption had occurred, body weights of mice were recorded before and after the 1 hour feeding period.

3.2.9 Group 4

Procedure overview: Mice (n = 7/9, WT/KD) were first trained a Pavlovian association between a stimulus paired with food delivery and a second stimulus unpaired with food. Following lever training for food reinforcement, the ability of the food-paired stimulus to motivate on-going instrumental responding was examined in the Pavlovian-instrumental transfer test. Lever responding under extinction was then examined in these mice, which included a devaluation component to inform whether instrumental responses were goal-directed or habit based.

3.2.9.1 Pavlovian conditioning

Mice received twelve, once-daily, 30 min Pavlovian conditioning sessions in which four presentations of a 2 min stimulus paired with food delivery (CS+; an intermittent tone or flashing house light) occurred. Each stimulus event was separated by a variable, no-stimulus, ITI (range of 225 to 375 sec; M = 300 sec). Mice then received a further six 45 min conditioning sessions, in which two presentations of a 2 min stimulus paired with no outcome (CS-; the alternative stimulus) occurred, along with four reinforced presentations of the CS+. The order of stimulus presentations was randomly determined and each stimulus was separated by a variable, no-stimulus, ITI (range of 205 to 395 sec; M = 300 sec). Four food pellets were delivered during each CS+ presentation. Pellet delivery was equally likely to occur in each 10 sec time bin throughout the CS+, although a minimum time of 10 sec separated each pellet delivery. Magazine entry rates (*magazine entries / min*) were recorded and the distribution of magazine entries (*% magazine entries*) calculated for the two stimulus periods (CS+, CS-).

3.2.9.2 Instrumental training

Following Pavlovian conditioning sessions, mice were trained to lever press for food during once-daily sessions. Each food self-administration session commenced with the insertion of two levers. Responses on one lever (the active lever) resulted in food pellet delivery, while responses on the alternative lever (the inactive lever) had no scheduled consequence. Instrumental training sessions

terminated after 30 food pellets had been obtained, or 30 min had elapsed. Initially, mice were trained to respond under a fixed-ratio 1 (FR1) schedule of reinforcement followed by progressively leaner variable-interval (VI) schedules, until responding in both genotypes was stable (that is, less than 20% variation in active lever response rates between training sessions) under a VI60 sec schedule of reinforcement.

3.2.9.3 Pavlovian-instrumental transfer

The PIT test commenced with the insertion of both levers and for the first 5 min, no stimuli were presented. This period was followed by 4 presentations of the 2 min CS+ and 4 presentations of the 2 min CS-, occurring in an alternating order. Whether the CS+ or CS- occurred first was randomly determined. Each stimulus presentation was preceded by a 2 min, no stimulus ITI. No food was delivered during the test. An elevation score was calculated to assess changes in active lever response rate during CS+ and CS- presentations (elevation score = lever responses during CS+ or CS- presentations minus lever responses during the no-stimulus ITI period prior to CS+ or CS- presentations, respectively).

3.2.9.4 The effect of satiety on extinction responding

The 10 min extinction test commenced with insertion of both levers into the chamber. Responses on levers were recorded but were without consequence. All mice were exposed to two tests, with one instrumental training session (as described 3.2.9.2) provided between tests. For 1 hour prior to the first test, half of the mice within each genotype received *ad libitum* access to the food pellets used for conditioning studies in their home cage (termed, the ‘devalued’ condition), while the other half received no food (termed, the ‘valued’ conditioned). For the second extinction test, mice were exposed to the opposite condition.

3.2.10 Statistical analysis

All test data were initially assessed for normality (Shapiro-Wilk test; assumption violated when $p \leq 0.05$) and homogeneity of variance (Levene’s test, assumption

violated when $p \leq 0.05$). To permit analysis by parametric tests, appropriate transformations were undertaken to transform skewed distributions closer to a normal distribution and to reduce heterogeneity of variance (Cardinal and Aitken, 2006). Specifically, for lever responding during CRf tests (groups 1 and 2), magazine entries during the discriminated approach test (group 3) and lever responding during the devaluation test (group 4), data were square root transformed ($Y'=\sqrt{Y}$). Most data (see exceptions below) were first analysed by mixed-factor analysis of variance (ANOVA), with genotype (WT, KD) as a between-subjects factor. Details of the within-subjects factors used each ANOVA are provided in the results section. Where significant ($p \leq 0.05$) main effects or interaction terms were found, further analysis was performed using ANOVA and *post-hoc* comparisons by *t*-test. Findings were considered indicative of a trend where $p \leq 0.1$ and Bonferroni corrections were applied for multiple comparisons. For within-subjects ANOVA, the Greenhouse-Geisser correction was used where the assumption of sphericity was violated (Mauchly's test, $p \leq 0.05$).

For nose-pokes during the sign-tracking test (group 1) and magazine entries during the MTEP on CRf test (group 2), data were not amenable to transformation and were analysed by non-parametric tests. Specifically, the comparison of CS+ and control nose-pokes within each genotype was made by Wilcoxon Signed-Rank test, while comparisons of nose-pokes between genotypes was made by Mann-Whitney U test. The time course of magazine entries during the MTEP on CRf test were analysed by Friedman test. All figures show group mean (± SEM).

3.3 Results

3.3.1 Group 1

3.3.1.1 Pavlovian conditioning

Both wild-type and mutant mice acquired Pavlovian conditioning; that is, they came to enter the food magazine during presentation of the stimulus paired with food delivery (the CS+), but largely ignored the food magazine during the no-stimulus (ITI) period when food delivery never occurred. Four measures of magazine activity were used to compare the acquisition of Pavlovian conditioning between genotypes (Fig. 3.3a-d). Analyses of these data was undertaken by mixed-factor ANOVA that included Session (1-11) and Period (CS+, ITI) as within-subjects factors.

Magazine entries / min: In both genotypes, the absolute rate of magazine entries across conditioning sessions increased during CS+ presentations, but decreased during the ITI period (Fig. 3.3a). An initial analysis of entries in both periods confirmed that magazine entry rates over the conditioning sessions significantly differed depending upon the Period identity (main effect of Period, $F(1,22) = 70.05$, $p < 0.001$; Period x Session interaction, $F(10,220) = 37.34$, $p < 0.001$), but not between genotypes (Period x Session x Genotype interaction, not significant (NS)). A subsequent analysis of magazine entries in both genotypes, first for the CS+ period only, confirmed that entry rates in the CS+ period increased over conditioning sessions (main effect of Session, $F(10,220) = 26.42$, $p < 0.001$), but this effect did not differ between genotypes (Session x Genotype interaction, NS). Overall CS+ magazine entry rates tended to be lower in mutant mice, but this effect did not reach significance (main effect of Genotype, $F(1,22) = 3.51$, $p = 0.074$). Magazine entry rates during the ITI period decreased over conditioning sessions (main effect of Session, $F(10,220) = 8.94$, $p < 0.001$) in both genotypes (Session x Genotype interaction, NS). Overall magazine entry rates during the ITI were reduced in mutant mice (main effect of Genotype, $F(1,22) = 5.532$, $p < 0.05$).

% Magazine entries: The proportion of total entries made into the food magazine during CS+ presentations significantly increased over conditioning sessions (main effect of Session, $F(10,220) = 34.55$, $p < 0.001$) in both genotypes (Session x Genotype interaction, NS), such that magazine entries occurred almost exclusively during the CS+ period (Fig. 3.3b). There was no overall difference in % magazine entries between genotypes (main effect of Genotype, NS).

Discriminated approach responses: By recording magazine entries in the 5 sec interval following CS+ onset but prior to food delivery, it was possible to determine whether mice were learning to use the CS+ as a predictor of food delivery. In both genotypes, the number of entries into the magazine during this 5 sec interval significantly increased over conditioning sessions (main effect of Session, $F(10,220) = 9.14$, $p < 0.001$), in both genotypes (Session x Genotype interaction, NS). This overall response profile did not differ between genotypes (main effect of Genotype, NS; Fig. 3.3c).

Retrieval latency: The average time at which mice from both genotypes first entered the food magazine following onset of the CS+ decreased across conditioning sessions (main effect of Session, $F(10,220) = 37.51$, $p < 0.001$; Session x Genotype interaction, NS) and approached the time of food delivery (that is, 5 sec after CS+ onset; Fig. 3.3d). Although overall retrieval latencies tended to be reduced in mutant mice, in comparison to wild-types, this effect did not reach statistical significance (main effect of Genotype, $F(1,22) = 3.97$, $p = 0.059$).

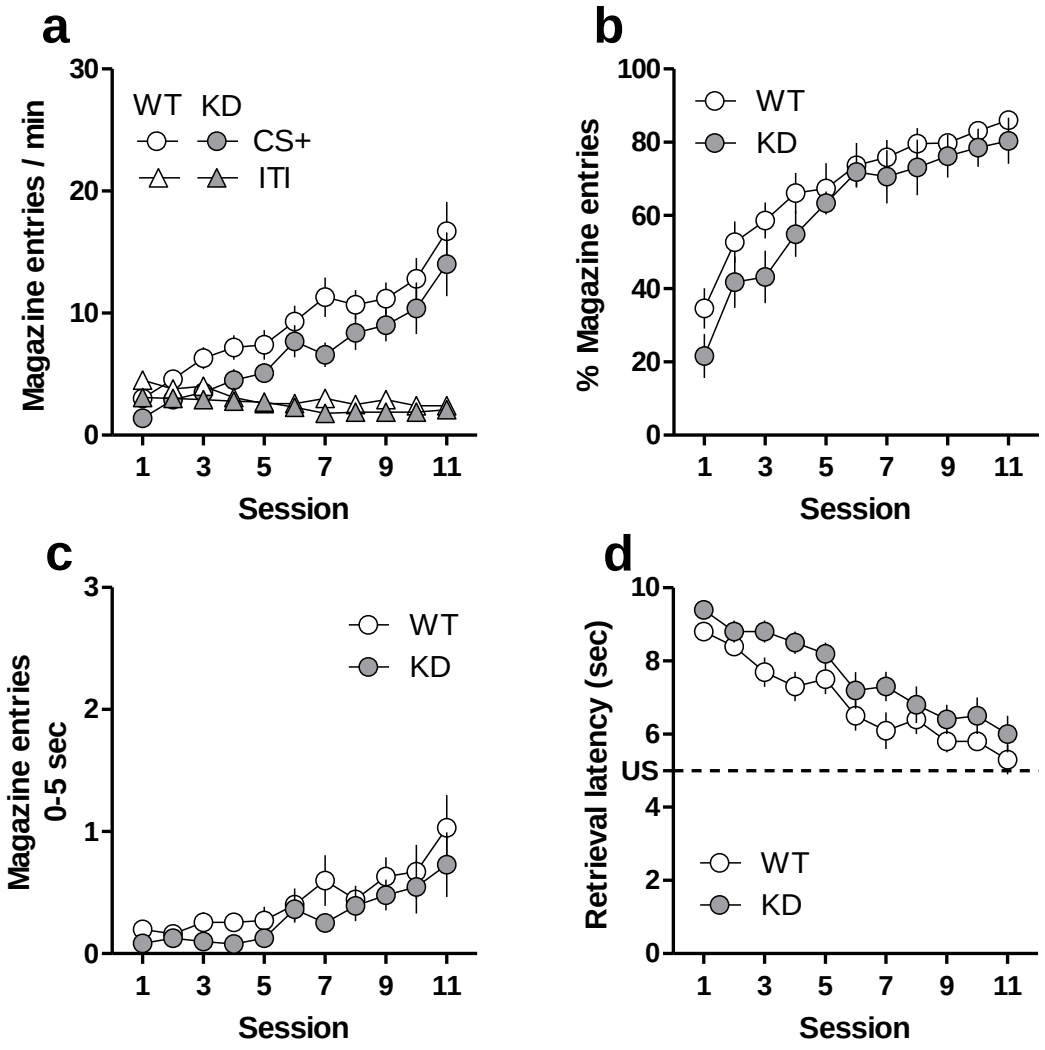


Figure 3.3 Group 1: Pavlovian conditioning (**a**) In both genotypes, the number of entries into the food magazine increased across conditioning sessions during presentation of a stimulus paired with food (CS+), while entries during the no-stimulus period (ITI) decreased. (**b**) The number of magazine entries made during presentation of the CS+, relative to the total number of magazine entries in the session (% Magazine entries), increased over conditioning sessions in both genotypes. (**c**) The number of magazine entries made during the first five seconds of CS+ onset (i.e. before food delivery), increased over conditioning sessions in both genotypes. (**d**) The average time of the first entry into the food magazine following CS+ onset (retrieval latency) decreased over conditioning sessions and approached the time of food (US) delivery (n = 12/12, WT/KD)

3.3.1.2 Sign-tracking

When the CS+ was presented in a nose-poke (the CS+ nose-poke), both genotypes in group 1 preferentially approached the location of the CS+ in comparison to a second (control) nose-poke in which no stimulus was presented (Fig. 3.4a; comparison of CS+ and control nose-pokes within each genotype; WT, $Z = -2.67$, $p < 0.01$; KD, $Z = -2.21$, $p < 0.05$). However, mutant mice made significantly fewer CS+ approaches than wild-type mice ($U = 37$, $Z= -2.06$, $p = 0.045$). Mutant mice tended to make fewer approaches into the control nose poke than wild-type mice, although this effect did not reach statistical significance ($U = 40$, $Z = -2.131$, $p = 0.068$).

During the period between CS+ presentations (ITI), nose-poking rates in the CS+ and control holes did not differ within each genotype (Fig. 3.4b; WT, $Z = -0.46$, $p = 0.65$; KD, $Z = -0.21$, $p = 0.83$), although overall rates of nose-poking were reduced in mutant mice (comparison between genotypes for the CS+ nose-poke, $U = 43.5$, $Z = -1.68$, $p = 0.1$; control nose-poke, $U = 34$, $Z = -2.22$, $p < 0.05$).

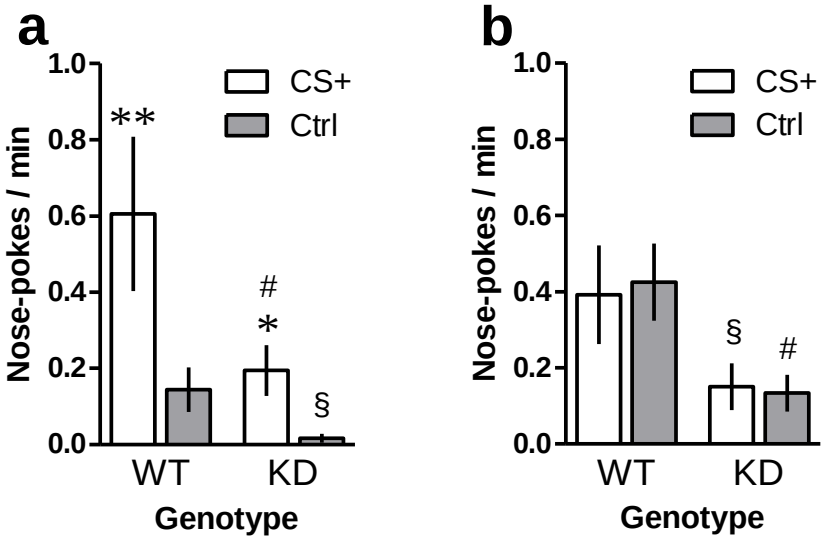


Figure 3.4 Group 1: Sign-tracking test (**a**) When the CS+ was presented in a nose-poke hole, both genotypes preferentially approached the CS+ nose-poke (CS+) than an alternate (control; Ctrl) nose-poke in which no stimulus presentations occurred. However, KD mice made significantly fewer CS+ nose-pokes than WT mice. Nose-pokes in the Ctrl hole tended to be reduced in mutant mice. (**b**) During the period between stimulus presentations (ITI), nose poking rates in the CS+ and Ctrl holes did not differ in either genotype, although overall rates over nose-poking tended to be reduced in mutant mice in comparison to wild-types. $^{**}p < 0.01$, $^{*}p < 0.05$, within-genotype comparison of CS+ and Ctrl nose-poke responses. $^{\#}p < 0.05$, $^{\S}p < 0.1$, between-genotype comparison of CS+ or Ctrl nose-poke responses (n = 12/12, WT/KD)

3.3.1.3 Conditioned reinforcement

Mice from both genotypes in group 1 demonstrated CRf; that is, responding was preferential for a CS+ reinforced lever over an alternate control lever on which responses were met with no consequence (Fig. 3.5a). Analysis of lever responding by mixed-factor ANOVA, with Lever (CS+, Control) as a within-subjects factor, confirmed that responding varied depending on the lever identity (main effect of Lever, $F(1,22)= 5.27$, $p < 0.05$) in both genotypes (Genotype x Lever interaction, NS). This analysis also indicated no overall difference in lever responding between genotypes (main effect of Genotype, NS). Although both genotypes responded more on the CS+ paired lever than the control lever, *post-hoc* comparisons of CS+ and control lever responding indicated that this conditioned reinforcement effect was not particularly robust in either genotype (WT, $t = 1.82$, df = 11, $p = 0.095$; KD, $t = 1.465$, df = 11 , $p = 0.171$).

3.3.1.4 The effect of cocaine on responding for conditioned reinforcement

Data from one mutant mouse were excluded from analysis (CS+ lever responses following 10 mg/kg cocaine exceeded the group mean responses + 2.5 x S.D.), leaving group sizes of n = 12/11 for WT/KD mice, respectively. Cocaine, at a dose of 10 mg/kg, potentiated responding for CRf in wild-type mice, but not in mutant mice (Fig. 3.5b). An initial analysis of lever responding in both genotypes by mixed-factor ANOVA, with Lever (CS+, Control) and Dose (0-20 mg/kg) included as within-subjects factors, confirmed that lever responding varied according to the lever identity (main effect of Lever, $F(1,21) = 17.18$, $p < 0.001$). This analysis also identified that lever responding varied amongst cocaine doses and between genotypes (main effect of Dose; $F(3,63) = 5.91$, $p < 0.01$; Lever x Genotype interaction, $F(1,21) = 3.46$, $p = 0.077$; Lever x Dose x Genotype interaction, $F(3,63) = 6.34$, $p < 0.01$). Further analyses were undertaken to explore these effects.

The next analyses examined lever responding in each genotype separately, across all cocaine doses. Wild-type mice showed an overall preference for the CS+ lever (main effect of Lever, $F(1,11) = 11.72$, $p < 0.01$), and lever responding varied with

cocaine dose (Lever x Dose interaction, $F(3,33) = 6.95$, $p < 0.05$). Mutant mice also showed an overall preference for the CS+ lever (main effect of Lever, $F(1,10) = 7.58$, $p < 0.05$), but lever responding did not vary with dose (Lever x Dose interaction, NS).

The third level of analyses examined CS+ and control lever responding separately in each genotype across all cocaine doses. In wild-type mice, responding varied with cocaine dose for the CS+ lever (main effect of Dose, $F(3,33) = 7.05$, $p = 0.01$) and the control lever (main effect of Dose, $F(3,33) = 8.89$, $p < 0.01$). In mutant mice, responding varied with cocaine dose for both the CS+ lever (main effect of Dose, $F(3,30) = 3.68$, $p < 0.05$) and the control lever (main effect of Dose, $F(3,30) = 5.35$, $p < 0.05$). Subsequent comparisons confirmed that 10 mg/kg cocaine facilitated CS+ lever responding in wild-type mice (10 mg/kg vs. vehicle, $t = -2.70$, df = 11, $p = 0.021$). Moreover, the effect of cocaine in wild-type mice was specific to the CS+ lever, since control lever responding was significantly decreased following 10 mg/kg cocaine (10 mg/kg vs. vehicle, $t = 2.77$, df = 11, $p = 0.020$). The facilitation effect did not occur at a higher cocaine dose. In fact, CS+ lever responding was significantly decreased in wild-type mice at 20 mg/kg cocaine (20 mg/kg vs. vehicle, $t = 2.54$, df = 11, $p < 0.028$). In mutant mice, CS+ lever responding tended to be decreased following 10 mg/kg cocaine (10 mg/kg vs. vehicle, $t = 2.17$, df = 10, $p = 0.056$), while CS+ lever responding was significantly decreased following 20 mg/kg cocaine ($t = 2.88$, df = 10, $p = 0.016$).

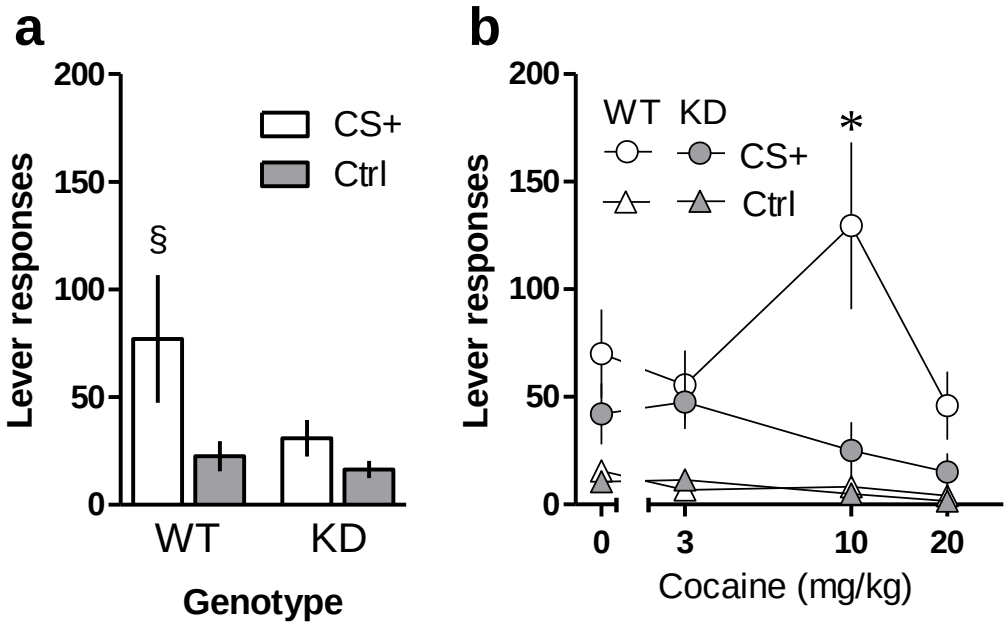


Figure 3.5 Group 1: Conditioned reinforcement and cocaine potentiation (**a**) Both genotypes preferentially responded on a novel lever that led to presentation of the food-paired stimulus (CS+) than an alternate control lever on which responding had no consequence (Ctrl). This effect was more robust in WT than KD mice. $^{\S}p < 0.1$, within-genotype comparison of CS+ and Ctrl lever responding (n=12/12, WT/KD) (**b**) Cocaine (10 mg/kg i.p.) facilitated responding for CRf in WT mice, but not KD mice. $^{*}p < 0.05$, within-genotype comparison of CS+ lever responding following 10 mg/kg cocaine and vehicle (n = 12/11, WT/KD)

3.3.2 Group 2

3.3.2.1 Pavlovian conditioning

Group 2 received identical Pavlovian conditioning to Group 1 and, in agreement, wild-type and mutant mice from this group also acquired Pavlovian conditioning (Fig 3.6a-d). For the sake of brevity, analyses of Pavlovian conditioning data are summarized in table (Table 3.1). Across conditioning sessions, findings for each of the four measures of Pavlovian conditioning were near identical to that observed in Group 1. The only notable differences were that, in Group 2, overall rates of magazine entries (magazine entries / min) during the CS+ and ITI periods and overall retrieval latencies were not significantly reduced in mutant mice, in comparison to wild-types. However, the overall proportion of magazine entries made during CS+ presentations (% Magazine entries) was lower in mutant mice, in comparison to wild-types (Table 3.1).

	Session F(10,220)	**Session x Genotype F(10,220)**	**Genotype F(1,22)**
Magazine entries/ min			
CS+ period	10.79***	0.28	0.32
ITI period	30.23***	1.65	1.24
% Magazine entries	73.97***	0.68	8.75**
Discriminated approach	3.33*	1.51	0.70
Retrieval latency	31.06***	0.92	0.17

Table 3.1 Group 2: ANOVAs for Pavlovian conditioning. Factors: Session (1-11); Genotype (WT, KD). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

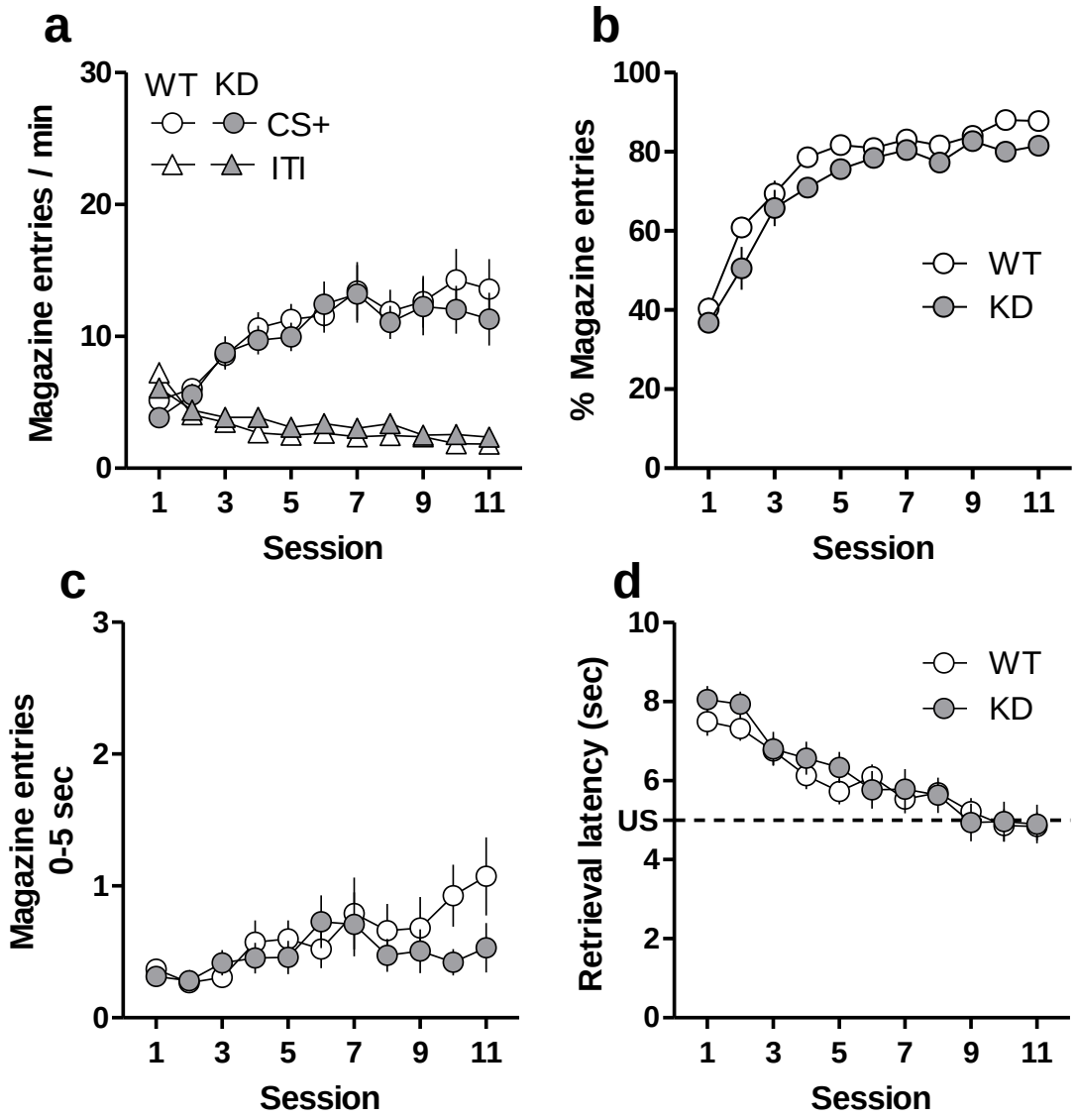


Figure 3.6 Group 2: Pavlovian conditioning. Figures show (**a**) magazine entry rates, across conditioning sessions, during presentation of the stimulus paired with food (CS+) and the no-stimulus period (ITI), (**b**) The proportion of total magazine entries made during CS+ presentations, (**c**) The number of magazine entries made in the 5 sec interval following CS+ onset but before food delivery, (**d**) The average time of the first entry into the food magazine following CS+ onset (retrieval latency) (n = 12/12, WT/KD)

3.3.2.2 Conditioned reinforcement

In group 2, responding for CRf was observed in wild-type mice, but not mutant mice (Fig. 3.7a). Analysis of lever responding in both genotypes identified that responding varied depending on the lever identity (main effect of Lever, $F(1,22)= 10.33$, $p < 0.01$). However, lever responding also varied between genotypes (Lever x Genotype interaction, $F(1,22) = 4.61$, $p < 0.05$) and overall responding was reduced in mutant mice (main effect of Genotype, $F(1,22) = 12.51$, $p < 0.01$). *Post-hoc* comparisons of CS+ and control lever responding confirmed robust CRf in wild-type mice ($t = 3.38$, $df = 11$, $p < 0.01$), but not mutant mice ($t = 0.873$, $t = 11$, $p = 0.40$). In comparison to WT mice, *post-hoc* comparisons confirmed that CS+ lever and control lever responding was significantly reduced in mutant mice (CS+ lever, $t = 3.11$, $df = 22$, $p < 0.01$; Control lever, $t = 3.28$, $df = 22$, $p < 0.01$).

A time-course plot of lever responding and magazine entries during the 45 minute CRf test (Fig. 3.7b-c) revealed that CS+ lever responding was steady across the duration of the test in both genotypes (main effect of Period, NS; Period x Genotype interaction, NS). CS+ lever responding was reduced in mutant mice over the duration of the session (main effect of Genotype, $F(1,22) = 7.26$, $p < 0.05$). Similarly, control lever responding (Fig. 3.7c) was steady in both genotypes (main effect of Period, NS; Period x Genotype interaction, NS) and was reduced overall in mutant mice (main effect of Genotype, $F(1,22) = 10.085$, $p < 0.01$). In contrast, entries into the food magazine (Fig. 3.7d) declined in both genotypes over the test (main effect of Period, $F(2,44) = 18.19$, $p < 0.001$; Period x Genotype interaction, NS) and did not differ overall between genotypes (main effect of Genotype, NS).

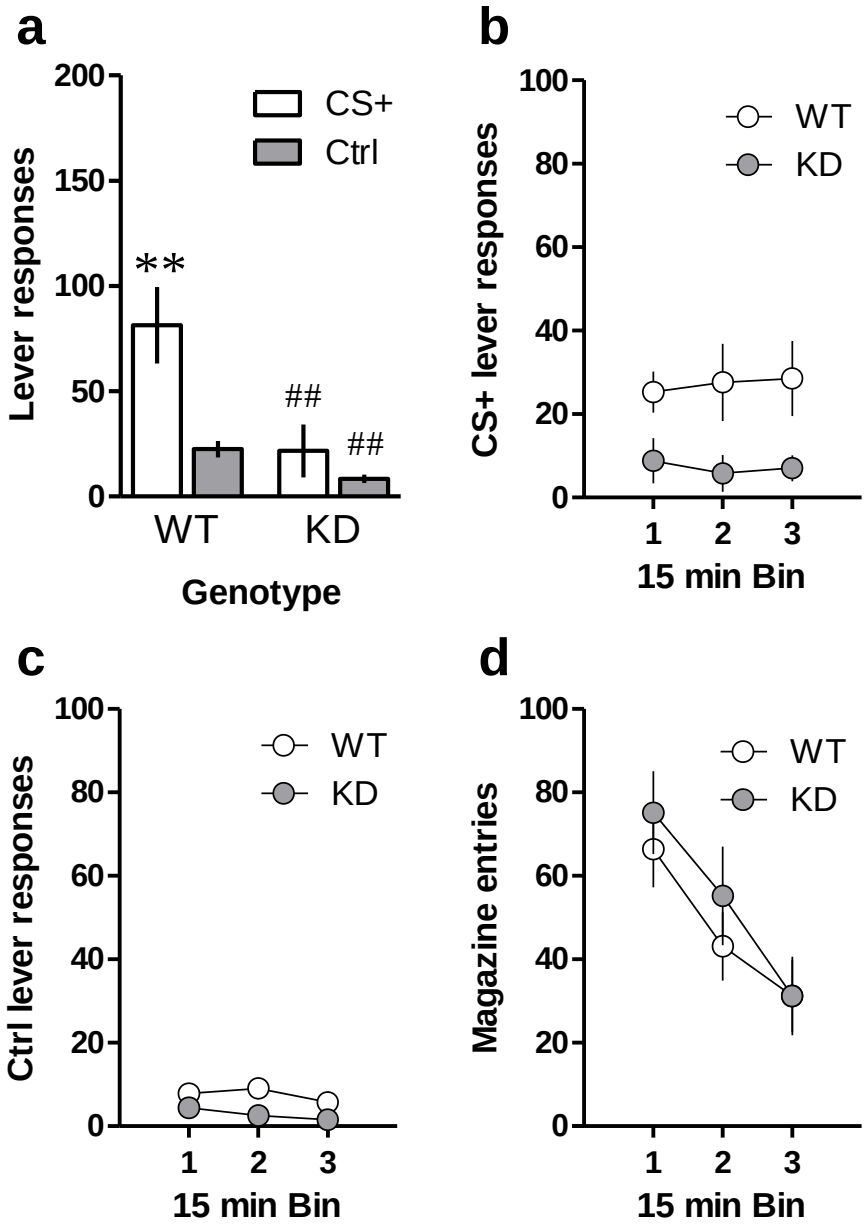


Figure 3.7 Group 2: Conditioned reinforcement. (**a**) Responding for CRf was present in wild-type, but not mutant mice. $^{**}p < 0.01$, within-genotype comparison of CS+ and Ctrl lever responding. $^{\#\#}p < 0.01$, between-genotype comparison of CS+ or Ctrl lever responding. (**b**) A time-course plot shows that CS+ paired lever responding was reduced in mutant mice over the duration of the 45 minute test. (**c**) Responding on the control (Ctrl) lever was also reduced in mutant mice over the test duration (**d**) Entries into the food magazine decreased over the course of the session, but did not differ between genotypes (n = 12/12, WT/KD).

3.3.2.3 The effect of MTEP on conditioned reinforcement

In wild-type mice, CRf was present over repeated tests that were preceded by injections of 0-20 mg/kg MTEP (main effect of Lever, $F(1,10) = 104.24$, $p < 0.001$). However, MTEP did not affect overall lever responding (main effect of Dose, NS; Dose x Lever interaction, NS). Analysis of responding on each lever separately across all MTEP doses confirmed that MTEP did not affect responding on the CS+ lever (main effect of Dose, NS), nor reliably on the inactive lever (main effect of Dose, $F(3,30) = 2.76$, $p = 0.11$). Magazine entries were unaffected by MTEP treatment (Friedman test, df = 3, $p = 0.60$).

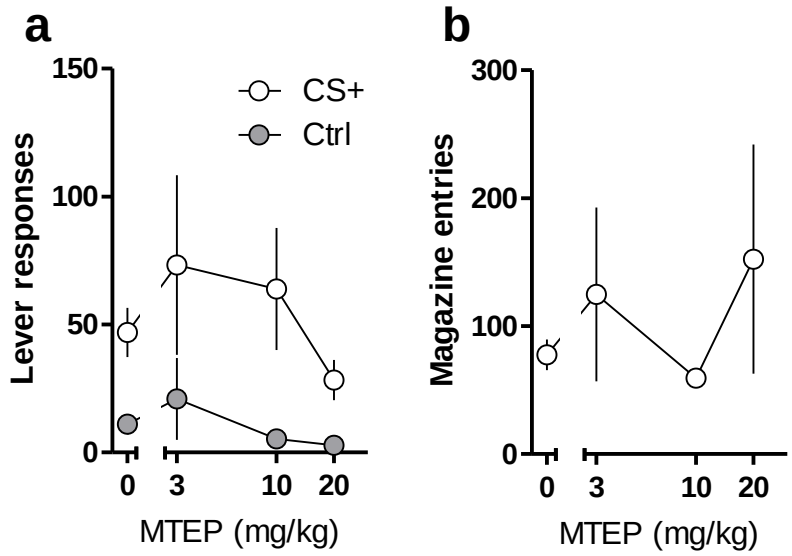


Figure 3.8 Group 2: MTEP on conditioned reinforcement in wild-type mice. (**a**) Injections of MTEP (1-20 mg/kg i.p.) 20 minutes prior to the CRf test did not disrupt responding for CRf in WT mice. (**b**) Magazine entries during the CRf test were similarly unaffected following injections of MTEP (n = 11).

3.3.2.4 Discriminated approach

Multiple tests of discriminated approach were undertaken in group 2 to determine whether mice were using the light stimulus and/or perhaps another cue (such as the noise of food pellet delivery) to guide magazine entries. Analyses of these data were undertaken for each genotype separately due to different training and test histories. Discriminated approach during a particular trial type (e.g. 'CS/US' or 'noise' test) was identified by comparing magazine entry rates during trials to entry rates in the interval between trials (ITI).

In wild-type mice (Fig. 3.9a), magazine entry rates during the two periods (trial, ITI) varied among the five different test conditions (main effect of Period, $F(1,11) = 39.52$, $p < 0.001$; Test, $F(4,44) = 15.76$, $p < 0.001$; Test x Period interaction, $F(4,44) = 15.19$, $p < 0.001$). *Post hoc* comparisons of magazine entry rates between trial and ITI periods, in each test, identified that rates were significantly increased (that is, discriminated approach was present) during trial periods for the CS+, US test ($t = 10.93$, df = 11, $p < 0.001$), the CS+, noise test ($t = 4.28$, df = 11, $p = 0.001$) and the CS+ test ($t = 3.17$, df = 11, $p < 0.01$).

In mutant mice, findings from the discriminated approach tests were near identical to those of wild-type controls. Magazine entry rates (Fig. 3.9b) during the trial period or the no-stimulus period between trials (ITI) also varied among the five test conditions (main effect of Period, $F(1,11) = 54.31$, $p < 0.001$; Test, $F(4,44) = 13.63$, $p < 0.001$; Test x Period interaction, $F(4,44) = 18.23$, $p < 0.001$). *Post-hoc* comparisons of magazine rates between trial and ITI periods, in each test, identified that rates were significantly increased during the trial period for the CS+, US test ($t = 7.80$, df = 11, $p < 0.001$), CS+, noise test ($t = 5.72$, df = 11, $p = 0.001$), the CS+ test ($t = 4.44$, df = 11, $p = 0.001$) and the noise test ($t = 3.77$, df = 11, $p < 0.01$).

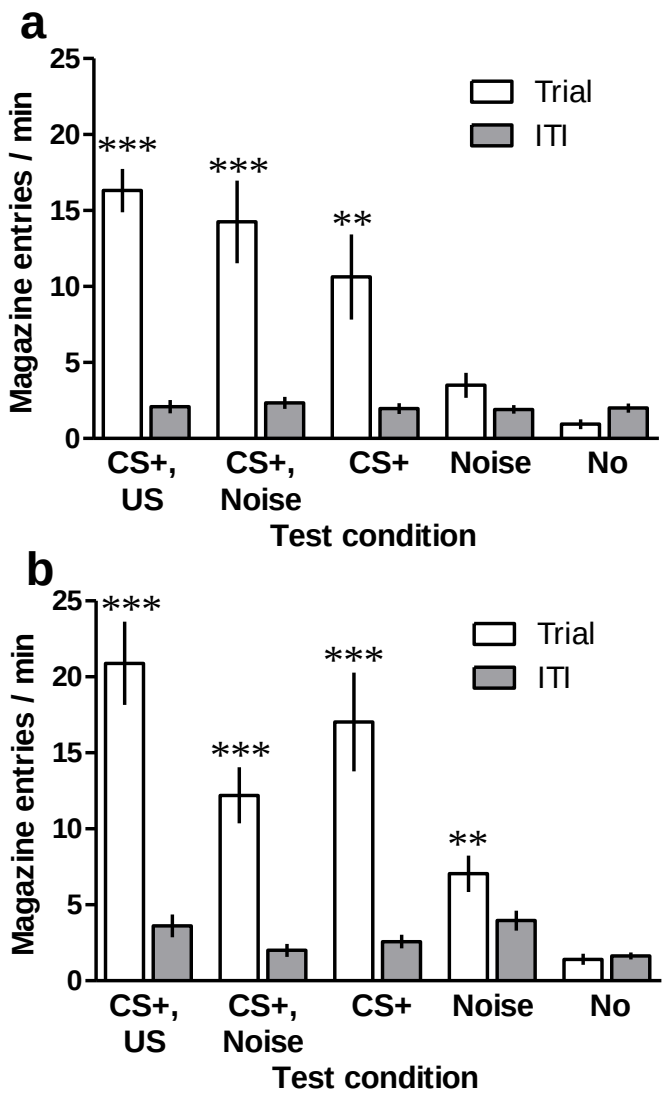


Figure 3.9 Group 2: Discriminated approach (**a**) WT and (**b**) KD mice received 5 different tests of discriminated approach. Each test consisted of 8 x 10 sec trials, each separated by a 100 sec ITI. The type of trial in each test was either 1) *CS+, US*: CS+ presentation and food delivery, 2) *CS+, Noise*: CS+ presentation and noise of food delivery only, 3) *CS+*: CS+ presentation only, 4) *Noise*: noise of food delivery only 5) *No*: no stimuli were presented in each 10 sec trial period. Due to the different training and test histories, data from WT and KD mice are presented on two different panels. Discriminated approach in each test was identified by a significant difference in magazine entry rates between trial periods and the ITI. ***$p < 0.001$, **$p < 0.01$, within-genotype comparison of trial and ITI magazine entry rates in each test (n = 12/12, WT/KD).

3.3.3 Group 3

3.3.3.1 Pavlovian conditioning

When mice were trained a Pavlovian association between a stimulus paired with food (CS+) and a second stimulus unpaired with food (CS-), mice from both genotypes came to enter the food magazine almost exclusively during presentation of the CS+ and largely ignored the magazine when the CS- was presented. As for Group 1 and 2, four different measures of conditioning performance are reported (Fig. 3.10a-d). Again, for brevity, analyses of these measures are summarised in table format (Table 3.2). Notably, in both genotypes, magazine entry rates increased during presentation of the CS+, and decreased during CS- presentations (Fig. 3.10a). Overall rates of responding were reduced during CS- presentations in mutant mice (Fig. 3.10a). The proportion of total magazine entries made during CS+ presentations increased across sessions in both genotypes, although % CS+ magazine activity tended to be reduced overall in wild-type mice (Fig. 3.10b). Discriminated approach responses increased over conditioning sessions in both genotypes, and to a similar extent (Fig. 3.10c). In both genotypes, the mean retrieval latency decreased over conditioning sessions, approaching the time of food delivery (Fig. 3.10d). The decrease in retrieval latency over conditioning sessions was less pronounced in wild-type mice than mutants, although this effect did not reach statistical significance (Table 3.2).

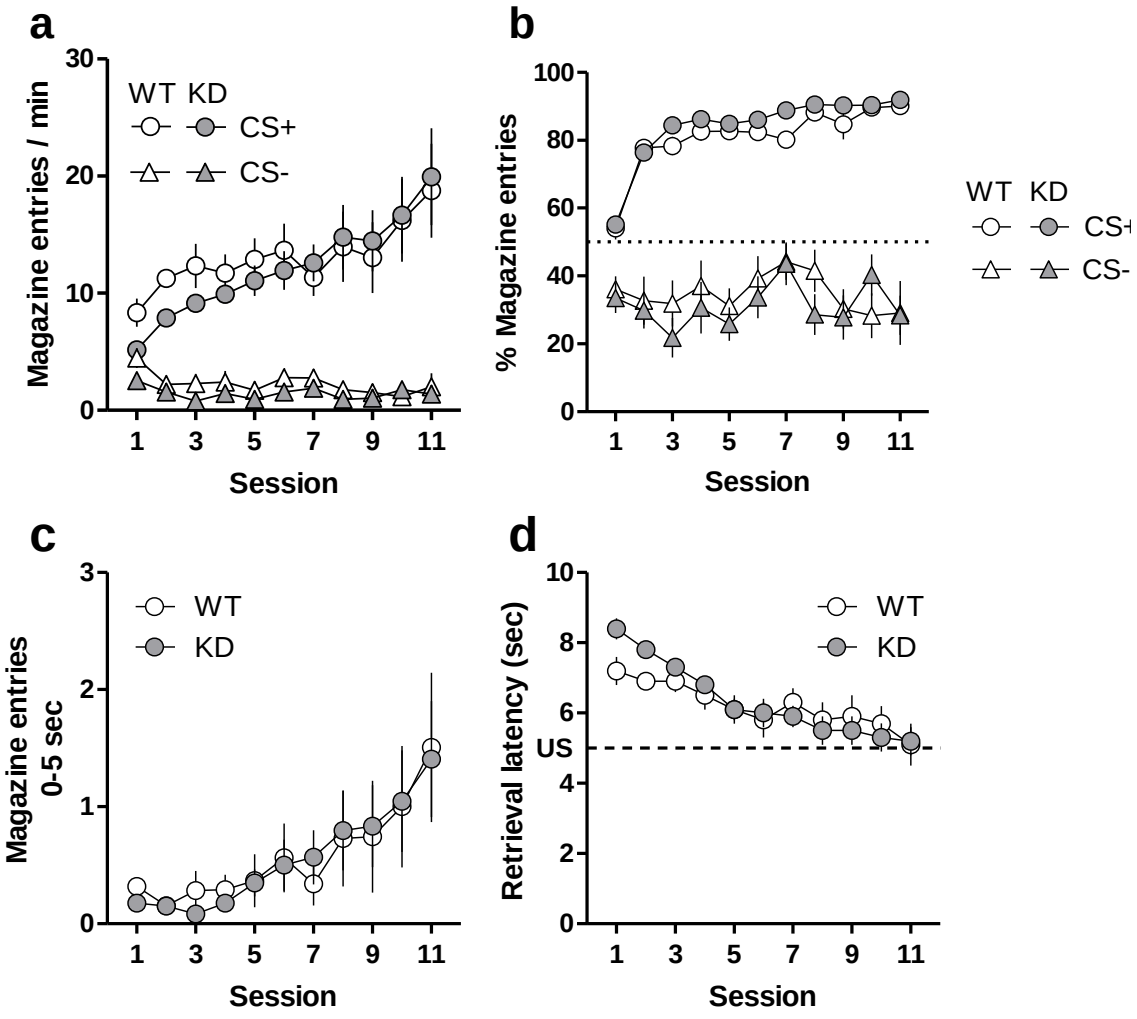


Figure 3.10 Group 3: Pavlovian conditioning. Figures show (**a**) magazine entry rates, across conditioning sessions, during presentation of the stimulus paired with food (CS+) and stimulus unpaired with food (CS-), (**b**) the proportion of total magazine entries made during CS+ and CS- presentations, (**c**) the number of magazine entries made in the 5 sec interval following CS+ onset but before food delivery and (**d**) the average time of the first entry into the food magazine following CS+ onset (retrieval latency) (n = 9/12, WT/KD).

	Session F(10,190)	Session x Genotype F(10,190)	Genotype F(1,19)
Magazine entries / min			
CS+ period	8.74**	0.72	0.14
CS- period	3.12*	0.76	5.18*
% Magazine entries			
CS+	29.66***	0.93	$3.51^{\S}$
CS-	5.57***	0.58	1.43
Discriminated approach	7.57**	0.17	0.004
Retrieval latency	19.26***	$2.10^{\S}$	0.06

Table 3.2 Group 3: ANOVAs for Pavlovian conditioning, Group 3. Factors: Session (1-11); Genotype (WT, KD). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, $^{\S}p < 0.10$

3.3.3.2 Conditioned reinforcement

In group 3, the CRf test was conducted such that responses on one lever were reinforced by the CS+ and responses on the alternate lever led to CS- presentations. Responding for CRf was observed in both genotypes, but was reduced in mutant mice (Fig. 3.11a). Analysis of lever responding in both genotypes identified that responding varied depending on the lever identity (main effect of Lever, $F(1,19) = 24.38$, $p < 0.001$). However, lever responding also varied between genotypes (Lever x Genotype interaction, $F(1,19) = 5.57$, $p < 0.05$) and overall responding was reduced in mutant mice (main effect of Genotype, $F(1,19) = 7.23$, $p < 0.05$). *Post hoc* analyses showed that more responses were made on the CS+ lever than the CS- lever in both wild-type ($t= 3.52$, $df = 8$, $p < 0.01$) and mutant ($t = 3.285$, $df = 11$, $p < 0.01$) mice. However, mutant mice made significantly fewer CS+ lever responses than KD mice ($t= 2.36$, $df = 19$, $p < 0.05$), while responding on the CS- lever did not differ between genotypes ($t = 1.47$, $df = 19$, $p = 0.158$).

A time-course plot of lever responding during the 45 minute CRf test revealed that CS+ lever responding (Fig. 3.11b) tended to increase over the duration of the test (main effect of Period, $F(2,38) = 2.96$, $p = 0.083$), in both genotypes (Period, x Genotype interaction, NS). CS+ lever responding was reduced in mutant mice over the duration of the session (main effect of Genotype, $F(1,19) = 6.92$, $p < 0.05$). CS- lever responding (Fig. 3.11c) was steady in both genotypes over the test duration (main effect of Period, NS; Period x Genotype interaction, NS) and did not differ overall between genotypes (main effect of Genotype, NS). An analysis of a time-course plot of food magazine entries (Fig. 3.11d) indicated that the profile of magazine entries over the session varied between genotypes (Period x Genotype interaction, $F(2,38) = 3.31$, $p < 0.05$), *Post hoc* comparisons indicated that mutant mice tended to make fewer head entry responses in the final 15 minute period of the session than WT controls, although this effect did not reach statistical significance ($t = 1.73$, $df = 19$, $p = 0.10$).

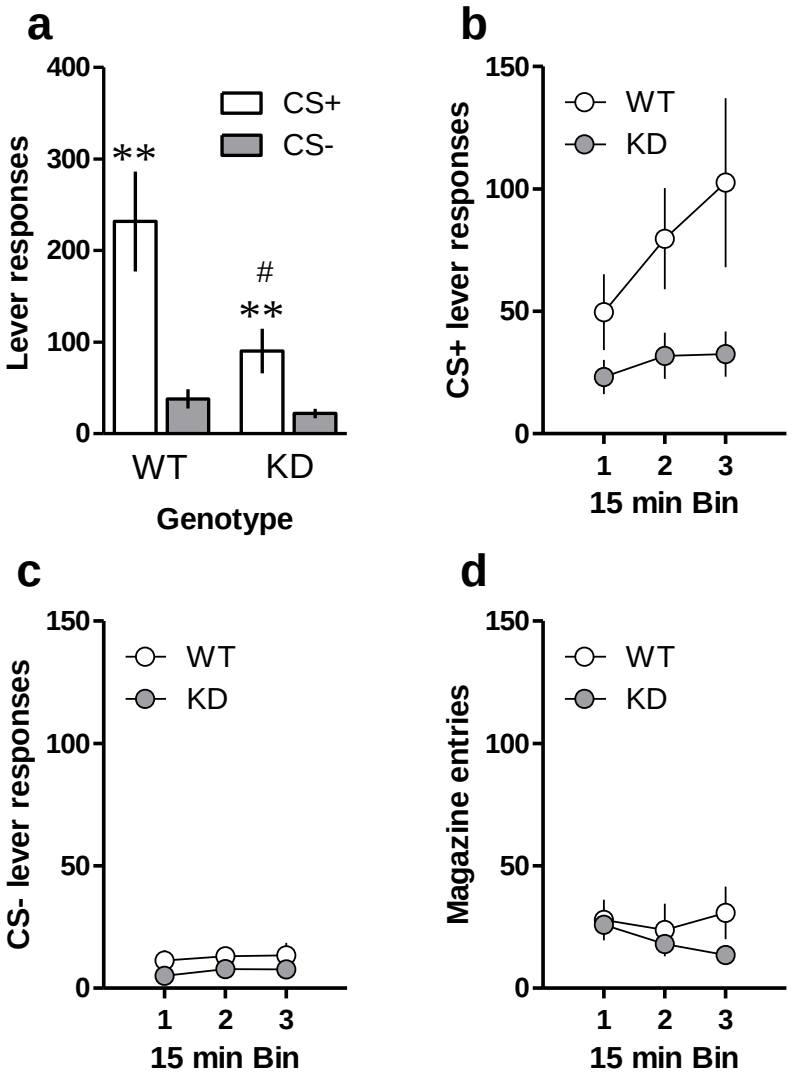


Figure 3.11 Group 3: Conditioned reinforcement (**a**) Both genotypes responding for CRf, however CS+ reinforced lever responding was significantly reduced in KD mice in comparison to WT mice. There was no between-genotype difference in lever responding reinforced by the stimulus unpaired with food (CS-). $^{**}p < 0.01$, within-genotype comparison of CS+ and Ctrl lever responding. $^{\#}p < 0.05$, between-genotype comparison of CS+ lever responding. (**b**) A time-course analysis shows that CS+ paired lever responding was reduced in mutant mice over the duration of the 45 minute test. (**c**) CS- paired lever responding did not differ between genotypes over the test duration (**d**) Magazine entries tended to be reduced in mutant mice in the final 15 minute period of the test (n = 9/12, WT/KD).

3.3.3.3 The effect of satiety on discriminated approach responses

In both genotypes from group 3, mice in the 'devalued' condition made fewer magazine entries during presentation of the CS+ in a test of discriminated approach than mice in the 'valued' condition (Fig 3.12). Analysis of these data was first performed by mixed-factor ANOVA with Stimulus (CS+, CS-) and Test (Valued, Devalued) as within-subjects factors. Discriminated approach was confirmed by higher rates of magazine entries during the CS+ than the CS- across all conditions (main effect of Stimulus, $F(1,19) = 35.52$, $p < 0.001$) in both genotypes (Stimulus x Genotype interaction, NS). Magazine entries varied with the test identity (main effect of Test, $F(1,19) = 23.34$, $p < 0.001$). An analysis of CS+ magazine entries only, in both genotypes across the two tests, confirmed that entries were decreased in the devalued condition (main effect of Test, $F(1,19) = 32.59$, $p < 0.001$), but this effect did not differ between genotypes (main effect of Genotype, NS ; Genotype x Test interaction, NS). *Post hoc* comparisons confirmed that CS+ magazine entries were significantly reduced in the devalued condition in both WT ($t = 3.68$, $df = 8$, $p < 0.01$) and KD mice ($t = 4.40$, $df = 11$, $p = 0.001$), in comparison to the valued condition. In contrast, CS- magazine entries were unaffected by the test condition (main effect of Test, NS), and did not differ between genotypes (main effect of Genotype, NS; Genotype x Test interaction, NS).

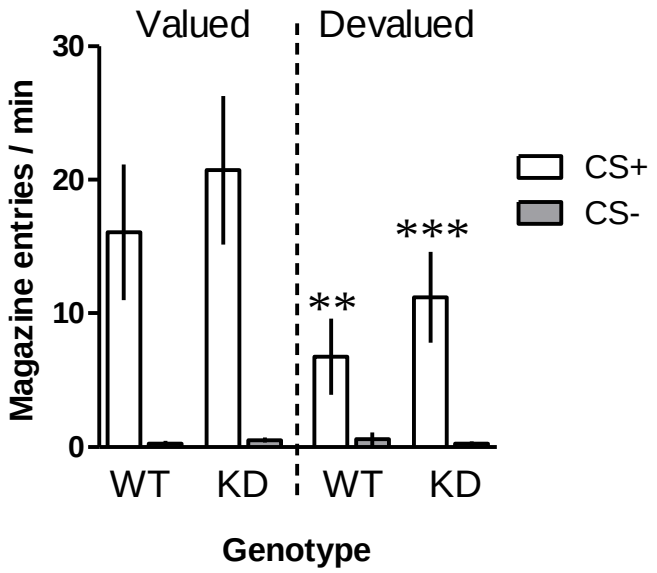


Figure 3.12 Group 3: Devaluation of discriminated approach. In both genotypes, magazine entries during presentation of the food-paired stimulus (CS+) were significantly reduced when mice were allowed *ad libitum* access to the food used for conditioning (Devalued), in comparison to mice given *ad libitum* access to a different food (Valued). Magazine entries during presentation of the unpaired stimulus (CS-) were unaffected by the test condition. ***$p < 0.001$, **$p < 0.01$, within-genotype comparison of CS+ magazine entries between the two feeding conditions (n = 9/12, WT/KD)

3.3.4 Group 4

3.3.4.1 Pavlovian conditioning

In preparation for the PIT test, when mice were trained a Pavlovian association between a stimulus paired with food delivery (CS+) and a second stimulus unpaired with food (CS-), both genotypes entered the food magazine during CS+ presentations and largely avoided the magazine during CS- presentations (Fig. 3.13a). In the first portion of conditioning, when only the CS+ was presented, magazine entries increased over the twelve conditioning sessions (main effect of Session, $F(11,154) = 4.0$, $p < 0.01$), in both genotypes (Session x Genotype interaction, NS; main effect of Genotype, NS). When the CS- was introduced, magazine entry rates were higher during the CS+ period over the six conditioning sessions (main effect of Stimulus, $F(1,14) = 113.15$, $p < 0.001$), in both genotypes (Session x Genotype interaction, NS, Stimulus x Session x Genotype interaction, NS). An analysis of CS+ magazine entries over these six conditioning sessions indicated that entry rates did not significantly vary over the sessions (main effect of Session, NS), and did not differ between genotypes (Genotype x Session interaction, NS; main effect of genotype, NS). Magazine entry rates during the CS- period neither varied across the six conditioning sessions (main effect of Session, NS), nor between genotypes (Genotype x Session interaction, NS; main effect of Genotype, NS).

The proportion of total magazine entries made during CS+ presentations increased over the first twelve conditioning sessions (main effect of Session, $F(11,154) = 9.16$, $p < 0.001$) in both genotypes (Session x Genotype interaction, NS; main effect of Genotype, NS; Fig. 3.13b). When the CS- was introduced for a further six conditioning sessions, a greater proportion of magazine entries occurred during CS+ presentations than CS- presentations (main effect of Stimulus, $F(1,14) = 192.73$, $p < 0.001$), in both genotypes (Stimulus x Genotype interaction, NS; main effect of genotype, NS). An analysis of magazine entry distribution during each stimulus period, separated, for both genotypes confirmed that the distribution of entries did not significantly differ between genotypes during the CS+ period

(Stimulus x Genotype interaction, NS; main effect of Genotype, NS), or the CS-period (Stimulus x Genotype interaction, NS; main effect of Genotype, NS).

3.3.4.2 Instrumental training

Both genotypes acquired instrumental responding reinforced by food delivery (Fig. 3.13c). Analysis of lever responding was performed by mixed-factor ANOVA, with Lever (active, inactive) and Session (1-4) included as within-subjects factors. Analysis was restricted to the first four and last four sessions of instrumental responding to compare the initial acquisition of instrumental responding and performance of the well learned instrumental response between-genotypes, respectively. Over the first four sessions of responding, under an FR1 schedule, responding was preferential for the lever that resulted in food delivery (active lever) over the alternate lever on which responding had no consequence (inactive lever; main effect of Lever, $F(1,14) = 54.84$, $p < 0.001$). This response profile did not differ between genotypes (Lever x Genotype interaction, NS; main effect of Genotype, NS). Similarly, over the final four instrumental training sessions, under a VI60s schedule, responding was preferential for the active lever (main effect of Lever; $F(1,14) = 40.35$, $p < 0.001$), and lever response rates did not differ between genotypes (Lever x Genotype interaction, NS; main effect of Genotype, NS).

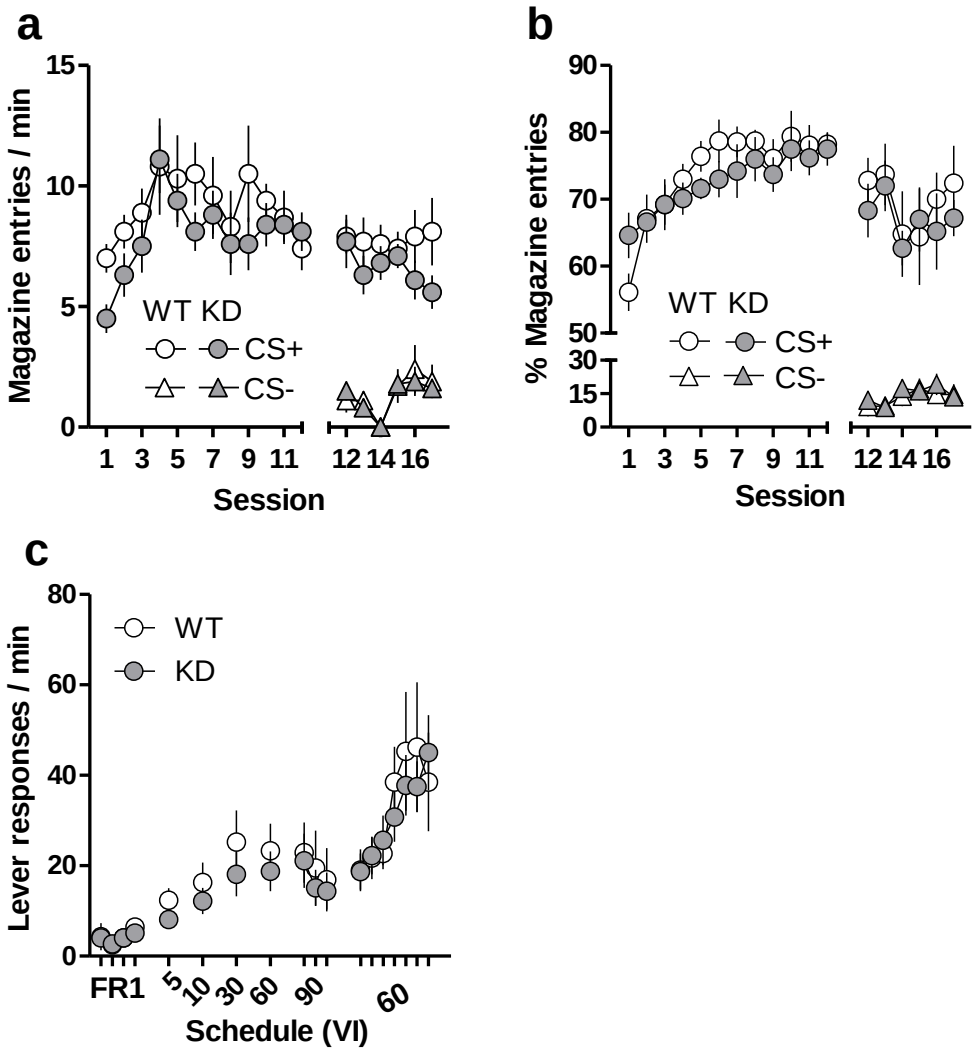


Figure 3.13 Group 4: Pavlovian conditioning and instrumental training. (**a**) Mice from both genotypes made more magazine entries during the presentation of a 2 min stimulus paired with food (CS+), than during presentations of a 2 min stimulus unpaired with food (CS-). (**b**) In both genotypes, the majority of magazine entries occurred when the CS+ presented, while only a small proportion of entries occurred during CS- presentations. (**c**) Responses on a lever that resulted in food delivery (the active lever) increased over training, initially under a fixed-ratio 1 (FR1) schedule then under variable interval (VI) schedules of reinforcement. A second lever was present during instrumental training on which responding had no consequence (the inactive lever). Inactive lever response rates are not presented as these were low (< 2 responses/ min) in both genotypes over the duration of training (n = 7/9, WT/KD).

3.3.4.3 Pavlovian-instrumental transfer

Responses on a lever that previously led to the delivery of food significantly increased during presentations of the previously food-paired stimulus (CS+), in comparison to a decrease in responding during CS- presentations (Fig. 3.14a; main effect of Stimulus, $F(1,14) = 20.93$, $p < 0.001$). There was no difference in PIT between genotypes (Stimulus x Genotype, NS; main effect of Genotype, NS). *Post-hoc* comparisons of CS+ and CS- elevation scores confirmed that lever responding was significantly increased during CS+ presentations, in comparison to responding during CS- presentations, in both WT ($t = 3.18$, df = 6, $p = 0.019$) and KD mice ($t = 3.46$, df = 8, $p = 0.009$).

A plot of elevation scores over the four trials in WT mice (Fig. 3.14b, panel i) revealed that lever responding was increased during CS+ presentations, in comparison to responding during CS- presentations (main effect of Stimulus, $F(1,6) = 10.015$, $p < 0.05$) over all trials (main effect of Trial, NS; Stimulus x Trial interaction, NS). An identical profile of elevation scores over trials was observed for mutant mice (Fig. 3.14b, panel ii; main effect of Stimulus, $F(1,8) = 9.05$, $p < 0.05$; main effect of Trial, NS; Stimulus x Trial interaction, NS).

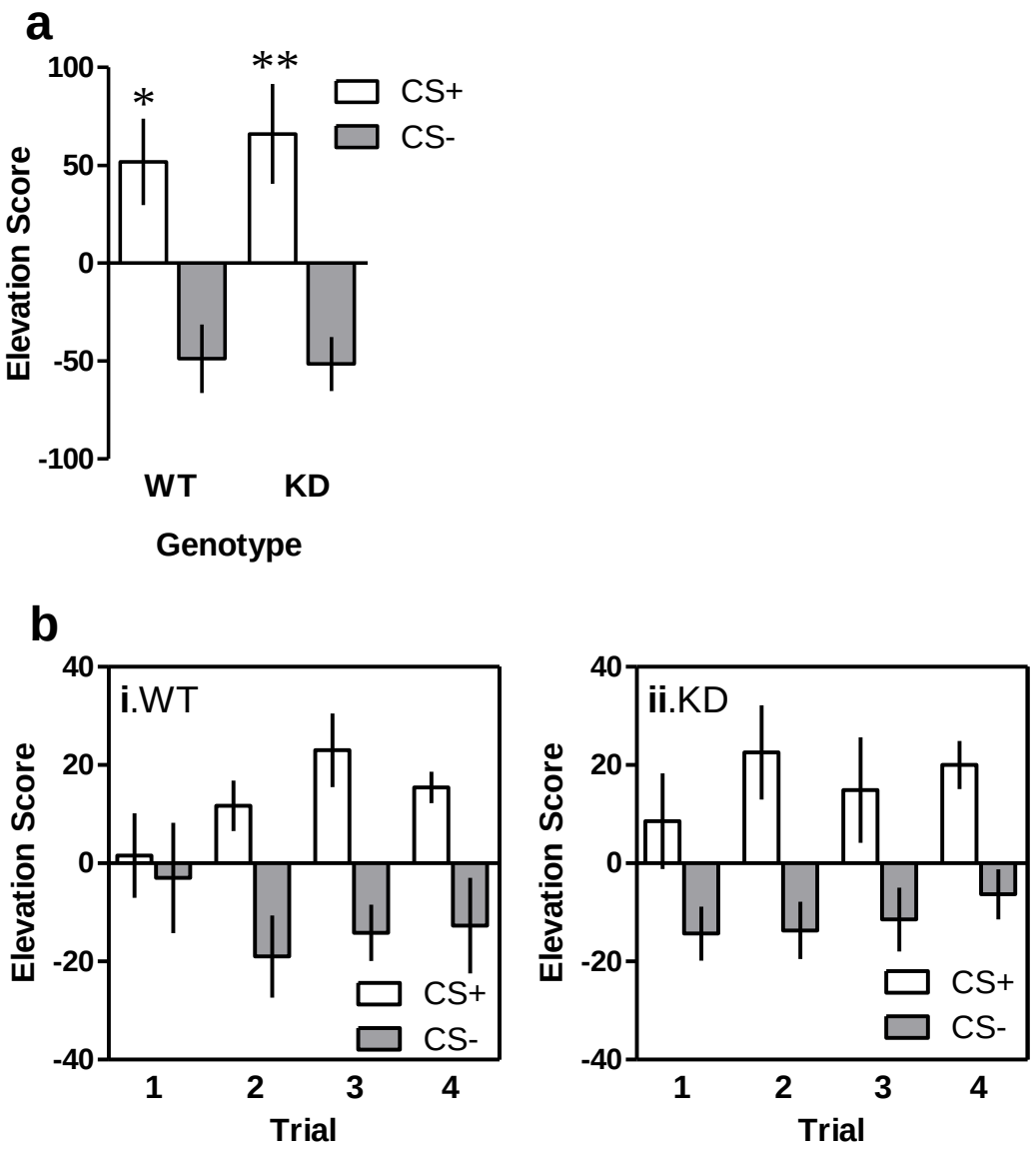


Figure 3.14 Group 4: Pavlovian-instrumental transfer test (**a**) In both genotypes, presentation of the previously food-paired stimulus (CS+) elevated responding on the lever previously associated with food, in comparison to a decrease in responding observed during presentations of the stimulus unpaired with food (CS-). The elevation score is calculated as responses during CS minus responses pre CS. (**b**) The elevation scores for each stimulus presentation trial in the PIT tests for WT (panel **i**) and KD (panel **ii**) mice are shown. $^{**}p < 0.01$, $^{*}p < 0.05$, within-genotype comparison of CS+ and CS- elevation scores (n = 7/9, WT/KD).

3.3.4.4 The effect of satiety on extinction responding

In both genotypes from group 4, mice in the devalued condition made fewer responses of the active lever in a test of extinction than mice in the valued condition (Fig. 3.15a). Analysis of these data was first performed by mixed-factor ANOVA with Lever (active, inactive) and Test (valued, devalued) as within-subjects factors. Preferential active lever responding across all tests was confirmed (main effect of Lever, $F(1,14) = 106.51$, $p < 0.001$), in both genotypes (Lever x Genotype interaction, NS). Lever responding varied with the test condition (Lever x Test interaction, $F(1,14) = 38.36$, $p < 0.001$), so further analyses were performed, first for active lever responding in both genotypes and tests. This analysis confirmed that active lever responding was significantly reduced in the devalued condition (main effect of Test, $F(1,14) = 46.67$, $p < 0.001$), but this effect did not differ between genotypes (main effect of Genotype, NS; Genotype x Test interaction, NS). *Post hoc* comparisons confirmed that active lever responding was reduced in the devalued condition, in comparison to the valued condition, in both WT ($t = 3.12$, df = 6, $p < 0.01$) and KD mice ($t = 6.014$, df = 8, $p < 0.001$). In contrast, inactive lever responses were unaffected by the test condition (main effect of Test, NS) and did not reliably differ between genotypes (main effect of Genotype, NS; Genotype x Test interaction, $F(1,14) = 3.19$, $p = 0.10$).

Finally, an analysis of magazine entry rates during these extinction tests (Fig 3.15b), indicated the entry rates were also reduced under the devalued test condition (main effect of Test, $F(1,14)=31.46$, $p < 0.001$), in both genotypes (main effect of Genotype, NS; Genotype x Lever interaction, NS). *Post-hoc* comparison of magazine entry rates between tests in confirmed that entry rates were significantly reduced in KD mice ($t = 10.97$, df = 8, $p < 0.001$) and tended to be reduced in WT mice ($t = 2.07$, df = 6, $p = 0.084$).

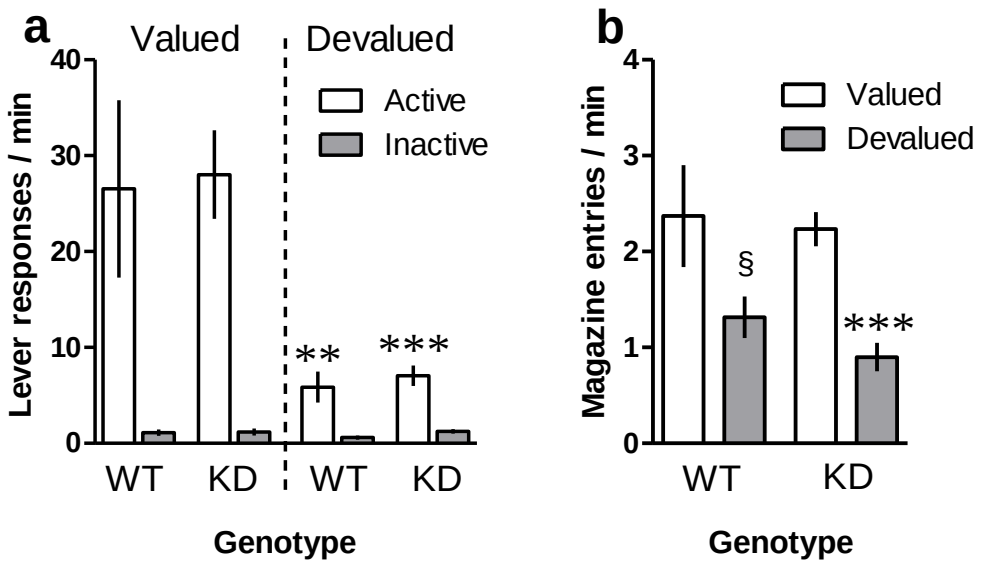


Figure 3.15 Group 4: Devaluation on instrumental responding. (**a**) In both genotypes, responses on the active lever were significantly reduced when mice were allowed *ad libitum* access to the food used for instrumental conditioning prior to test of extinction responding (Devalued), in comparison to mice that were not given access to food prior to the test (Valued). (**b**) In both genotypes, magazine entries were reduced in the devalued test condition. ***$p < 0.001$, **$p < 0.01$, $^{\S}p < 0.1$, within-genotype comparison of active lever responding, or magazine entries, between test conditions (n = 7/9, WT/KD).

3.4 Discussion

The present chapter examined distinct features of appetitive learning in mGluR5^{KD-D1} and wild-type mice. Both genotypes readily learned about the predictive properties of the food-paired CS. Thus, presentation of the CS elicited entries into the food hopper, even when food was not delivered (discriminated approach). Both genotypes were also able to adjust responding to the CS, based on the current value of the CS triggered US representation (devaluation on discriminated approach). However, assessments of incentive learning revealed specific deficits in mGluR5^{KD-D1} mice. First, the ability of the CS to serve as a 'motivational magnet' (Berridge and Robinson, 2003) and elicit approach toward it (sign-tracking) was impaired. Second, the acquisition of a novel response reinforced by the reward-paired CS (CRf) was also impaired in mGluR5^{KD-D1} mice. Further tests of CRf revealed that cocaine potentiated CRf responding in wild-type, but not mGluR5^{KD-D1} mice. In addition, blockade of mGluR5 in wild-type mice with a selective antagonist (MTEP) failed to disrupt responding for CRf. In contrast to deficits in sign-tracking and CRf, incentive learning necessary for a reward-paired CS to motivate ongoing goal-directed actions (generalised PIT) was intact in mutant mice. Finally, although this thesis primarily concerns Pavlovian appetitive learning, it is worthwhile to note that mGluR5^{KD-D1} mice readily acquired an instrumental response reinforced by a food reward and instrumental responding remained sensitive to variation in the outcome value in both genotypes (devaluation on instrumental responding). Taken together, these data indicate mGluR5 on D1R expressing cells as necessary for specific Pavlovian incentive learning processes.

Different conditioning procedures were used in the present report, including the pairing of a short duration (10 sec) stimulus with the delivery of a single food pellet (Groups 1, 2 and 3; CRf experiments), and the pairing of a longer duration (2 min) stimulus with the delivery of multiple food reinforcers (Group 4; PIT experiment). Since PIT was normal in mGluR5^{KD-D1} mice, but CRf was disrupted, could it be that mGluR5^{KD-D1} mice simply failed to acquire any CS-US association (incentive or

predictive) because a short duration CS was used, or that a longer duration CS was more permissive for incentive learning in mGluR5^{KD-D1} mice? To address this question empirically would require that both genotypes were tested for CRf and PIT after receiving conditioning with the longer duration and short duration CS, respectively. Although this experiment would be interesting, this overall proposal seems unsatisfactory. First, mGluR5^{KD-D1} mice readily acquired a predictive CS-US association under both conditioning parameters, as indexed by the different measures of discriminated approach recorded during conditioning. Discriminated approach performance in mGluR5^{KD-D1} mice typically approached or reached asymptotic levels at the end of conditioning and was largely indistinguishable from wild-type mice. In some groups, overall rates of magazine entries were lower in mGluR5^{KD-D1} mice (for example, Group 1, magazine entries/min and %magazine entries; Fig. 3.3a-b). However, a subsequent between-genotype comparison of the slopes of these measures, which perhaps provides a better measure of the *rate* of learning, indicated no difference in the rate of change of each measure across conditioning sessions (data not shown). Second, the use of different Pavlovian conditioning procedures for CRf and PIT studies was in recognition of data indicating that these procedures were most suitable for supporting subsequent CRf or PIT behaviour in mice (Crombag et al., 2008a). Longer-duration CSs are more likely to establish 'drive' conditioned responses that exert more modulatory influence over behaviours (this effect being fundamental to PIT) (Konorski, 1967; Wagner and Brandon, 1989; Holland and Gallagher, 2003). In contrast, short-duration CSs are more likely to establish specific 'consummatory' conditioned responses (Konorski, 1967; Wagner and Brandon, 1989; Holland and Gallagher, 2003). Thus, the conditioning parameters used in the present report should have been optimal for promoting learning necessary for supporting CRf and sign-tracking in mGluR5^{KD-D1} mice.

In the sign-tracking test, mGluR5^{KD-D1} mice made fewer approaches toward the food-paired CS than wild-type mice, but overall rates of nose-poking in the period between CS presentation trails (the ITI) were also reduced in mGluR5^{KD-D1} mice. A

finding of impaired sign-tracking in mGluR5^{KD-D1} mice may have simply been an artefact of reduced exploratory activity. Arguing against this possibility is that the latency to the first nose-poke response did not differ between genotypes (data not shown) and, in a separate test of spontaneous activity (Chapter 2, section 2.2.6.2), no difference in spontaneous nose-poking was found between genotypes. One possibility is that presentation of the CS+ may have been more effective in stimulating conditioned activity (or general behavioural activation) in wild-type mice, leading to an increase in overall rates of nose-poking. The presentation of food-paired CSs can trigger a variety of conditioned responses (Holland, 1977) but, in the current preparation, we were unable to record activity levels (beyond nose-poking) in the operant chamber during Pavlovian conditioning and the test.

It must also be considered whether impaired responding for CRf in mGluR5^{KD-D1} mice was due to a general reduction in activity and/or impairment in ability to acquire a lever response. These possibilities can be ruled out on a number of accounts. First, magazine entries elicited during the CRf tests did not reliably differ between genotypes, providing some evidence that overall activity levels in the CRf tests were comparable between mGluR5^{KD-D1} and wild-type mice. Second, there was no difference between genotypes in the latency to the first lever press (data not shown), providing evidence that mGluR5^{KD-D1} mice did explore the response levers. Third, mGluR5^{KD-D1} mice readily acquired an instrumental response that was reinforced by food and could respond at high rates, indistinguishable from their wild-type counterparts. Similarly, mGluR5^{KD-D1} mice also learned to lever press for cocaine in identical fashion to wild-type mice (Novak et al., 2010). Taken together, these data support a dissociable role for mGluR5 on D1R expressing cells in the primary versus secondary (that is, conditioned) reinforcing effects of both natural and drug reinforcers.

Although CS reinforced responding in mGluR5^{KD-D1} was generally reduced in comparison to wild-type mice in both the sign-tracking and CRf tests, mGluR5^{KD-D1} mice could nevertheless discriminate between CS+ and control nose-pokes (sign-

tracking test) or CS+ and CS- paired levers (CRf test). This profile of responding mirrors that reported in an autoshaping procedure following lesions of the NAcc core or its dopamine depletion, which are characterized by decreased approach toward the CS+ (Parkinson et al., 1999) and/or a more global reduction in approach responses (Parkinson et al., 2002). In contrast, lesions of the anterior cingulate cortex resulted in a general disruption of autoshaping by increasing approaches made toward the CS- (Bussey et al., 1997a; but see; Cardinal et al., 2002b). The findings reported here suggest that mGluR5 on D1R expressing neurons may be particularly important for determining the 'vigour' of the CS controlled response output, while having less influence over the 'direction' of the behavioural response and without disrupting 'drive' conditioned responses that are fundamental to PIT.

In the first two tests of CRf (Group 1 and 2), mice were presented with one lever reinforced by the food-paired CS (a light) and a second lever on which responses had no consequence. Under these conditions, mGluR5$^{KD\text{-}D1}$ mice did not demonstrate reliable CRf (Group 1 and 2) or responding for CRf was reduced in comparison to wild-type mice (Group 2). Criticism of this test design has been raised, suggesting that responding for 'sensory reinforcement' (that is, responding for stimuli without acquired reinforcing properties) may underlie a large component of CS reinforced responding (Winterbauer and Balleine, 2007). This issue is particularly pertinent for the current thesis, given a recent report indicating that the acquisition of an instrumental response reinforced solely by a novel visual stimulus is impaired in mGluR5 knock-out mice (termed, operant sensation seeking; Olsen et al., 2010). While a 'sensory reinforcement' or 'operant sensation seeking' account cannot be ruled out with regards to findings from the first two CRf tests, responding for CRf was also impaired in mutant mice in the third CRf test that employed proper control procedures (Rescorla, 1967). Thus, where responses on one lever were reinforced by a food-paired stimulus (CS+) and responses on the second lever by a stimulus unpaired with food (CS-), mutant mice demonstrated

reduced responding for CRf in comparison to wild-types, supporting our initial assertion of a specific incentive learning deficit in mGluR5^{KD-D1} mice.

When cocaine was injected prior to tests of CRf, responding for CRf was potentiated in wild-type mice. In contrast, cocaine did not facilitate CS+ reinforced responding in mutant mice. One possible account for the absence of cocaine facilitation of CRf in mutant mice is that preference for the CS+ reinforced lever was simply reduced in mGluR5^{KD-D1} mice during this test. Thus, perhaps there was no real CRf response to facilitate in these mice. However, psychostimulants can markedly potentiate the incentive effects of a reward-paired CS, even when the baseline effects of the CS alone are small (e.g. amphetamine on PIT; Wyvell and Berridge, 2001) or have been reduced due to lesioning (e.g. BLA lesions on CRf; Burns et al., 1993). The present data suggest that loss of mGluR5 on D1 expressing neurons may play a critical role in the 'gain-amplification' process that depends heavily upon dopamine and glutamate innervation in the ventral striatum (Taylor and Robbins, 1986; Wolterink et al., 1993; Burns et al., 1994), and integrity of the ventral subiculum (Burns et al., 1993), NAcc shell (Parkinson et al., 1999) and CeN (Robledo et al., 1996).

If mGluR5 on D1R expressing cells are necessary for CRf, one might have expected blockade with a selective mGluR5 antagonist (MTEP) in wild-type mice to similarly impair CS reinforced responding. This was not the case, as MTEP failed to significantly alter responding for CRf in wild-type mice. One possibility is that the doses of MTEP used were too low to cause a sufficient block of mGluR5. However, 3 mg/kg i.p. MTEP was reported to achieve >75% receptor occupancy for at least 15 min post-dosing in mice (Anderson et al., 2003) and doses of 30 mg/kg i.p. MTEP or higher reduced locomotor activity in mice (Cowen et al., 2007). In the current chapter, MTEP was injected prior to the test, thereby examining the role of mGluR5 in the expression of control over responding by the CS. Alternatively, mGluR5 may be critically involved in the acquisition of incentive value by a CS. Some support for this proposal is provided from studies of conditioned fear. Thus,

MTEP impaired the acquisition (and the expression) of hippocampus-dependent contextual fear conditioning, but impaired the expression (but not the acquisition) of hippocampus-independent auditory fear conditioning (Gravius et al., 2006).

Not all appetitive behaviours were impaired in mGluR5^{KD-D1} mice. Mice were able to use the CS as a predictive signal of food delivery, as indicated by the discriminated approach tests. A satiety-induced devaluation test indicated that mGluR5^{KD-D1} mice were able to adjust responding to the CS, based on the current value of the CS triggered US representation. In the 'devalued' condition, mice were given access to the same food used for conditioning (sucrose pellets), while in the 'valued' condition, mice were given access to a different food (chow). At first sight, the reduction in discriminated approach responses in the devalued *vs*. the valued condition might suggest that the CS triggered a specific representation of the outcome (i.e. sucrose pellets). However, mice from both genotypes ate significantly more sucrose pellets than chow in the 1 hour period before the devaluation test (data not shown). Thus, the data from the current experimental preparation can be used only to support a notion that mice had encoded a CS representation of the US, but not the nature (general or specific) of that representation.

The finding that a reward-paired CS was able to motivate ongoing goal-directed actions in mGluR5^{KD-D1} mice highlights an important dissociation in the role of mGluR5 on D1R expressing neurons for incentive learning processes. Since the underlying neural circuitry of the behavioural models used is relatively well characterised (see General Introduction for review), this dissociation can be used to propose neural 'node(s)' in which mGluR5 loss on D1R expressing neurons could contribute to the present findings. For now, I wish to speculate about the role of mGluR5 on D1R expressing neurons (i.e. D1-MSNs) within the ventral striatum, given the contribution of this structure to responding for CRf (Parkinson et al., 1999; Ito et al., 2004), the development of sign-tracking responses (Parkinson et al., 2000b; Di Ciano et al., 2001) and PIT (Hall et al., 2001). Notably, the NAcc is not required for CRf *per se* but can influence the vigour (NAcc shell) and direction

(NAcc core) of CS-reinforced behaviour (Cardinal et al., 2002a). In addition, lesions of the NAcc core, but not the shell abolish PIT (Hall et al., 2001). The mGluR5^{KD-D1} mouse would therefore appear similar to NAcc shell lesioned animals, and one might speculate that glutamatergic signalling from cortical limbic inputs onto D1-MSNs in the NAcc shell may be disrupted in mGluR5^{KD-D1} mice. However, lesions of the NAcc core, but not the NAcc shell abolish autoshaping in rats (Parkinson et al., 2000b). Thus, if mGluR5^{KD-D1} mice were completely analogous to NAcc shell lesioned animals (and moreover NAcc shell lesioned *rats*) we ought to have seen normal sign-tracking in mutant mice, which was not the case. The effects of lesioning will be far more disruptive for node function than a cell-type specific manipulation and the findings reported with mGluR5^{KD-D1} mice may underline further functional dissociations within the ventral striatum, not only between the core and shell regions, but also within the distinct MSN populations in each region. Finally, it is worthwhile noting that post-training intra-NAcc infusions of an NMDA and D1R antagonist, but not a D2R antagonist, impaired the consolidation of appetitive Pavlovian learning necessary for supporting autoshaping (Dalley et al., 2005). While infusions in this study were primarily targeted at the NAcc core region, a small number of injector tips were located in the ventrolateral shell region (Dalley et al., 2005), suggesting that dopamine and glutamate signalling in this region may contribute, in part, to incentive learning necessary for sign-tracking. Whether in the NAcc core or shell, the findings reported here with mGluR5^{KD-D1} mice suggest that glutamate and dopamine signalling in the ventral striatum, particularly though cells that express the dopamine D1R, are necessary for specific incentive learning processes.

In summary, the experiments reported here identify a necessary role of mGluR5 on D1R expressing neurons for incentive learning processes that endow a reward-paired CS with the ability to both reinforce and attract motivated behaviours. A critical question outstanding from the current chapter is whether mGluR5 in necessary for the acquisition of conditioned incentive value by reward-paired CSs

or the expression of control over responding by a conditioned incentive. This issue will be addressed in the next chapter.

4 The role of mGluR5 in the acquisition and expression of incentive learning

4.1 Introduction

In the previous chapter, mGluR5 on D1R expressing neurons was revealed as necessary for incentive learning that enables a reward-paired CS to both attract and directly reinforce behaviour. In contrast, mGluR5 on D1R expressing neurons was not necessary for learning about the predictive properties of the reward-paired CS. These experiments employed a mutant mouse line in which mGluR5 was constitutively knocked-down on D1R expressing cells. As such, it was not possible to determine whether mGluR5 was necessary for the acquisition of the incentive association, or for the expression of control over behaviour by the reward-paired CS. Using the selective mGluR5 antagonist MTEP, in wild-type mice, the following experiments will explore the role of mGluR5 in the acquisition and/or expression of predictive (discriminated approach or goal-tracking; Boakes, 1977) and incentive (conditioned reinforcement; Mackintosh, 1994) properties of a reward-paired CS.

There are numerous examples where neural nodes or molecular substrates play dissociable roles in the acquisition and expression of an appetitive Pavlovian association. For example, the BLA is required for the attribution of cues with incentive properties of outcomes, but may not (under some conditions) be required for maintaining these CS-US representations in memory or updating them with new information (Setlow et al., 2002; Blundell et al., 2003; Pickens et al., 2003; Johnson et al., 2009). With respect to the sign-tracking CR, lesion studies have indicated that the CeN plays a specific role in the acquisition of this CR, but not in the performance of it (Parkinson et al., 2000a; Cardinal et al., 2002b). Lesion studies have also indicated that some neural nodes are involved in both the acquisition and performance of a CR, but pharmacological approaches can reveal further acquisition/expression dissociations within these nodes. For example, the NAcc

core and its dopaminergic innervation contribute both to the acquisition and performance of a sign-tracking CR (Parkinson et al., 2000a; Cardinal et al., 2002b; Parkinson et al., 2002). However, dopamine and NMDA receptors within the NAcc core contribute selectively to the acquisition (but not the expression) of a sign-tracking CR, while AMPA/KA receptors in the NAcc core are necessary for the expression (but not the acquisition) of this response (Di Ciano et al., 2001). The role of NAcc core dopamine (presumably acting at D1Rs rather than D2Rs; Dalley et al., 2005) and NMDA receptors in the consolidation of appetitive learning is in agreement with the role of these receptors in LTP and other forms of synaptic plasticity hypothesised to underlie associative learning (Pennartz et al., 1993; Kombian and Malenka, 1994; Kelley et al., 2003; Kelley, 2004). In contrast, AMPA receptors are particularly important for maintaining synaptic strength at excitatory synapses (Malenka, 2003; Kessels and Malinow, 2009), which is in broad agreement with their role in the performance of CS controlled appetitive behaviours.

With regards to mGluR5, there is much evidence to suggest that it contributes to the *expression* of control over behaviours by reward paired-CSs. For example, cue-induced reinstatement of cocaine seeking, which is largely analogous to responding for CRf, is significantly attenuated by the mGluR5 antagonist, MPEP (Backstrom and Hyytia, 2006). In the Pavlovian-instrumental transfer procedure, MPEP attenuates the ability of a food-paired CS to motivate ongoing instrumental responding (George et al., 2009). In the conditioned place preference model, mGluR5 antagonism blocks the expression of place preference established with drugs, including morphine (Herzig and Schmidt, 2004) and amphetamine (Herzig et al., 2005). Few studies have directly examined the possibility that mGluR5 may be involved in the *acquisition* of an incentive CS-US association. There is some evidence that mGluR5 could play a role here, since MPEP given during conditioning with cocaine or morphine, attenuated the subsequent establishment of place preference (Popik and Wrobel, 2002; McGeehan and Olive, 2003). However,

whether the expression of CPP requires incentive learning processes is questionable (Stephens et al., 2010).

More robust support for a role of mGluR5 in the acquisition of conditioned associations can be found in studies of conditioned fear. Using the fear potentiated startle paradigm in rats, MPEP dose dependently blocked the acquisition of fear and, at a higher dose, also blocked the expression of fear (Schulz et al., 2001). When infused into the lateral-amygdala (LA), MPEP blocked the acquisition, but not the expression, of auditory and contextual fear conditioning in rats (Rodrigues et al., 2002). The role of mGluR5 in conditioned fear appeared restricted to the acquisition phase, since post-training intra-LA infusions had no effect on the consolidation of fear conditioning (Rodrigues et al., 2002). Moreover, bath application of MPEP disrupted LTP at thalamic input synapses to the LA, indicating that mGluR5 has a central role in establishing plasticity in the amygdala that may be necessary for aspects of emotional learning (Fendt and Schmid, 2002; Rodrigues et al., 2002).

As previously discussed (see section 1.4), incentive learning involves a fairly well circumscribed forebrain circuitry involving cortical limbic and striatal regions (Everitt et al., 1999). Within many components of this circuitry, mGluR5 is implicated in the associative strengthening of neural connections during learning (reviewed in Bellone et al., 2008; Anwyl, 2009). For instance, blockade of mGluR5 disrupts hippocampal LTP (Bashir et al., 1993), and mice lacking mGluR5 show a reduction in LTP in NMDA-receptor dependent pathways, including the CA1 region and dentate gyrus of the hippocampus (Lu et al., 1997). In dopaminergic cells of the VTA, the AMPA/NMDA EPSC ratio is reduced in mice lacking mGluR5 (Bird et al., 2010). In the striatum, mGluR5 regulates excitability of MSNs (D'Ascenzo et al., 2009), and is necessary for the induction of synaptic plasticity in the NAcc core that occurs following stimulation of glutamatergic cortical inputs (Schotanus and Chergui, 2008), but may not be so critical for the maintenance of corticostriatal plasticity following its induction (Sung et al., 2001; Gubellini et al., 2003). Taken

together, there is ample evidence to suggest that mGluR5 could contribute to the formation of appetitive CS-US associations.

In this chapter, the role of mGluR5 in the acquisition and/or expression of Pavlovian incentive learning is explored in mice using the mGluR5 antagonist, MTEP. By administering MTEP to mice during the learning of this stimulus-reward association (Pavlovian conditioning), we were able to examine the contribution of mGluR5 to the acquisition of predictive properties by the food-paired CS that serve to signal the availability of reward at its location (goal-tracking test), and incentive properties necessary to reinforce an entirely novel instrumental response (CRf test). To determine whether mGluR5 was necessary for the expression of control over behaviours by the CS, we administered MTEP during the tests of goal-tracking and CRf to mice that had received vehicle during Pavlovian conditioning sessions. Critically, tests of goal-tracking and CRf were performed under extinction conditions, therefore allowing the predictive and incentive motivational features of the CS to be examined without interference from presentation of the primary reward.

4.2 Materials and methods

4.2.1 Animals

Mice (n = 62; male C57BL6 x Sv129; derived in house; minimum 8 weeks old) were housed in groups of two or three and allowed to habituate to the holding room for one week prior to beginning the experiment. Animals were maintained on a 12:12 h light-dark cycle (lights on at 0700 hours) under controlled temperature (21 ± 2 °C) and humidity conditions (50 ± 5%). Body weights were maintained at approximately 85% of free-feeding weight by the provision of a limited amount of standard lab chow (B&K Feeds, Hull, UK) approximately 2 hours after daily experiment completion. Experiments took place during the light-phase between 0900 and 1500 hours. All procedures were performed in accordance with the United Kingdom 1986 Animals (Scientific Procedures) Act, following institutional ethical review.

4.2.2 Drugs

All injections were administered at a volume of 10 ml/kg i.p. The non-competitive mGluR5 antagonist, 3-((2-methyl-1,3-thiazol-4-yl)ethynyl)pyridine (MTEP; Sequoia Research Products, Pangbourne, UK), was dissolved in 10% v/v Tween 80 : 90% water.

4.2.3 Apparatus

Behavioural training and testing were performed in eight standard mouse operant chambers (as described in section 3.2.3)

4.2.4 Procedure

A summary of the experimental design is shown in Figure 4.1. Mice were allocated to one of three Pavlovian conditioning (PC) treatment groups that received injections of either vehicle (PC: Veh group; n = 22), 3 mg/kg (PC: 3; n = 19) or 10 mg/kg (PC: 10; n = 21) i.p. MTEP prior to each Pavlovian conditioning session (Phase 1). Following conditioning, each conditioning treatment group (PC: Veh, 3

and 10) was exposed to two tests of CRf (CRf; Phase 2). Mice from each conditioning treatment group were injected with vehicle during one CRf test and MTEP during the other CRf test, the order of CRf test treatment (i.e. Veh or MTEP) being counterbalanced. Specifically, group PC: Veh received 10 mg/kg MTEP during one CRf test, while groups PC: 3 and PC: 10 received 3 and 10 mg/kg MTEP during one CRf test, respectively. Each conditioning treatment group was then exposed to two tests of goal-tracking (GT; Phase 3). As described for the CRf tests, each conditioning treatment group was injected with MTEP during one of the GT tests and vehicle during the other test; the order of GT test treatments being counterbalanced. Two further Pavlovian conditioning sessions were conducted between each CRf test and each GT test. Mice received injections of vehicle (PC: Veh group) or 3 or 10 mg/kg MTEP (PC: 3 and 10 groups, respectively) prior to each reconditioning session to ensure that learning conditions were identical to those experienced during the initial conditioning phase. All drug injections were made 20 min prior to the start of the experimental sessions. The doses of MTEP used have previously been shown to not affect locomotor activity in mice (Cowen et al., 2007), and 3 mg/kg i.p. MTEP was reported to achieve >75% receptor occupancy for at least 15 min post-dosing in mice (Anderson et al., 2003).

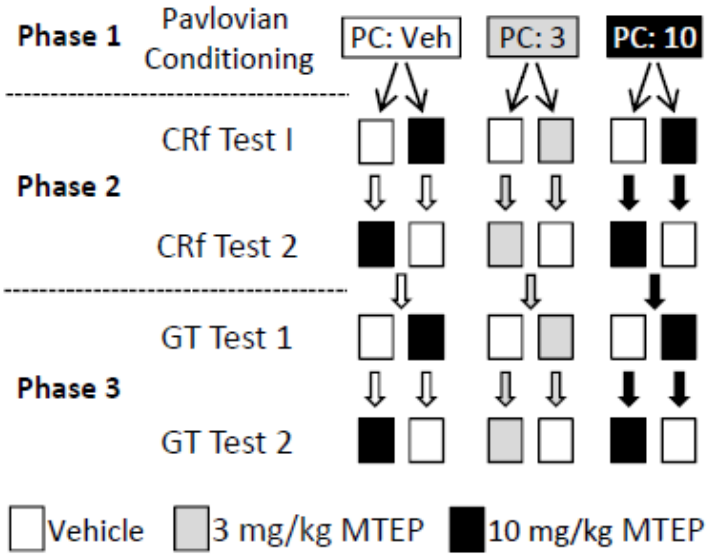


Figure 4.1 Experimental design summary. Mice were allocated to one of three groups that received injections of vehicle (PC: Veh), 3 mg/kg (PC: 3) or 10 mg/kg (PC: 10) MTEP prior to eleven, once daily, Pavlovian conditioning sessions (Phase 1). Two tests of conditioned reinforcement (CRf; Phase 2) and goal-tracking (GT; Phase 3) were subsequently undertaken in each group. Injections of vehicle or MTEP were given before each test, the order of treatments being counterbalanced. Two Pavlovian conditioning sessions were conducted between each test (block arrows). See methods section for further details**.**

4.2.4.1 Magazine training

To familiarize mice with the food reinforcer used in Pavlovian conditioning sessions, a small amount of the food was provided to mice in their home cage. The following day, mice received a single 30 min magazine training session in which food pellets were delivered once every 60 sec, on average (range of 25-95 sec). No drug injections were made prior to the magazine training session and no stimuli or response levers were presented.

4.2.4.2 Phase 1: Pavlovian conditioning

Commencing 24 h after the magazine training session, mice received eleven, once daily, Pavlovian conditioning sessions. Each 60 min session consisted of 16 trials in which presentation of a stimulus was paired with food delivery (CS+) and 16 trials in which presentation of an alternative stimulus was not paired with food (CS-). The order of stimulus presentations was randomly determined and each stimulus trial was separated by a variable, no-stimulus, inter-trial interval (ITI; range of 80-120 sec; M = 100 sec). For half of the mice a constant 10 sec tone served as the CS+ and the 10 sec flashing (1 Hz) of both cue lights served as the CS-. This contingency was reversed for the remaining mice. A single food pellet was delivered 5 sec after CS+ onset. The total number of entries made into the food magazine during each stimulus trial (CS+ or CS-) was recorded and expressed as a percentage of total magazine entries made during the session (% magazine entries). Food magazine entries that occurred in the first five seconds following CS+ onset (i.e. prior to food delivery) were recorded to provide a preliminary assessment of the acquisition of goal-tracking responses. The latency to enter the food magazine following onset of the CS+ (retrieval latency) was also measured.

4.2.4.3 Phase 2: Conditioned reinforcement

The 60 min CRf test commenced with insertion of both response levers into the operant chamber. A single response on one lever resulted in a 1.5 sec presentation of the CS+, whereas a single response on the alternate lever resulted in a 1.5 sec presentation of the CS-. For half of the mice, the left lever was

designated the CS+ lever and the right lever the CS- lever. This contingency was reversed for remaining mice. No food was delivered during the test. The ability of the CS+ to serve as a conditioned reinforcer is demonstrated by a greater number of responses on the CS+ lever than on the CS- lever.

4.2.4.4 Phase 3: Goal-tracking

The GT test was 30 min in duration and consisted of 8 trials of the CS+, and 8 trials of the CS-. The order of stimulus presentations was randomly determined and each stimulus trial was separated by a 100 sec fixed, ITI, during which no stimuli were presented. No food was delivered during the test. The total number of entries made into the food magazine during each stimulus trial was recorded. Four mice died before completion of the GT tests, reducing the size of groups PC: 3 and PC: 10 to n = 17 and n = 19, respectively.

4.2.5 Statistical analysis

Data were initially analysed by mixed-factor analysis of variance (ANOVA), where the three conditioning treatment groups (PC: Veh, 3 or 10) were represented by the between-subjects factor of PC treatment. The drug treatment (Veh or MTEP) administered to each of the three conditioning treatment groups during subsequent CRf and GT test sessions was included in analyses as a within-subjects factor of CRf treatment or GT treatment, respectively. Where a significant ($p \leq 0.05$) main effect or interaction term was found, further analysis was performed using ANOVA and *post-hoc* comparisons by two-tailed *t*-tests. To permit analysis by parametric tests, appropriate transformations were undertaken to transform skewed distributions closer to a normal distribution and to reduce heterogeneity of variance (Cardinal and Aitken, 2006). Specifically, for analysis of % magazine entries (Phase 1), data were arcsine transformed ($Y' = \arcsin\sqrt{(Y)}$). For analysis of magazine entries made during the first five seconds of CS+ presentations in conditioning sessions (Phase 1), lever responses and magazine entries in the test of CRf (Phase 2) and magazine entries in the test of goal-tracking (Phase 3), data were square root transformed ($Y' = \sqrt{Y}$). For within-subjects ANOVA, the

Greenhouse-Geisser correction was used where the assumption of sphericity was violated. All figures show group mean (± SEM).

4.3 Results

4.3.1 Phase 1: Pavlovian conditioning

Pavlovian conditioning performance did not differ among groups of mice that received vehicle (PC: Veh group), 3 mg/kg (PC: 3) or 10mg/kg (PC: 10) MTEP prior to each conditioning session. Across conditioning sessions, mice from all three conditioning treatment groups (PC: Veh, 3 or 10) directed a greater proportion of total session entries into the food magazine (% magazine entries; Fig. 4.2a) during presentations of the food-paired stimulus (CS+) than during presentations of the unpaired stimulus (CS-). This finding was confirmed by a mixed-factor ANOVA, which included Stimulus (CS+ or CS-) and Session (1-11) as within-subjects factors. A significant difference in responding to the two stimuli across conditioning sessions was identified (main effect of Stimulus, $F(1,59) = 1432.62$, $p < 0.001$; Stimulus x Session interaction, $F(10,590) = 83.26$, $p < 0.001$). However, there was no difference between the three conditioning treatment groups in % magazine entries directed toward the stimuli (Stimulus x Session x Conditioning treatment interaction, not significant (NS)).

The number of magazine entries made during the first five seconds of CS+ presentations (i.e. before delivery of the food reward; Fig. 4.2b) increased across conditioning sessions (main effect of Session, $F(10,590) = 22.01$, $p < 0.001$), but did not differ among the conditioning treatment groups (Session x Conditioning treatment interaction, NS). In contrast, the total number of magazine entries made during CS- presentations decreased across conditioning sessions (main effect of Session, $F(10,590) = 43.91$, $p < 0.001$), but also did not differ among the conditioning treatment groups (Session x Conditioning treatment interaction, N.S; data not shown).

Mice came to enter the food magazine at 4-5 sec after CS+ onset (retrieval latency; Fig.4.2c), corresponding with the time of food delivery. The mean retrieval latency to enter the food magazine following activation of the CS+ significantly decreased

across conditioning sessions (main effect of Session, $F(10,590) = 43.23$, $p < 0.001$), and there was no difference in retrieval latencies among the three conditioning treatment groups (Conditioning treatment x Session interaction, NS).

Stability of conditioning performance (indicated by asymptotic responding) prior to the first test of CRf was observed from the eighth conditioning session. % magazine entries (Fig. 4.2a) did not differ across sessions 8-11 (main effect of Session, NS), and there was no difference between conditioning treatment groups (Stimulus x Session x Conditioning treatment interaction, NS). Similarly, magazine entries made in first five seconds of CS+ presentations (Fig. 4.2b) and mean retrieval latencies (Fig. 4.2c) did not differ across sessions 8-11 (main effect of Session, NS), nor between conditioning treatment groups (Session x Conditioning treatment interaction, NS). No further change in conditioning performance was observed during any of the subsequent Pavlovian reconditioning sessions that occurred between the CRf and GT tests.

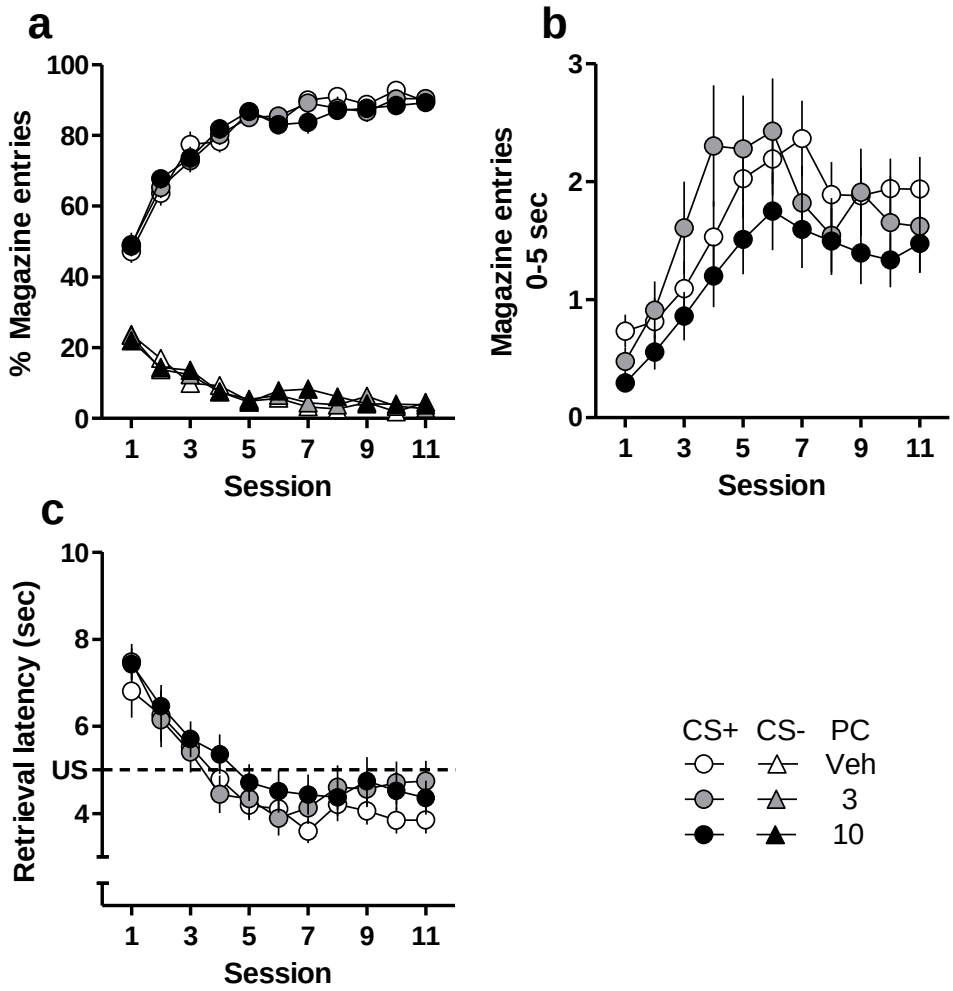


Figure 4.2 Measures of food magazine entry activity during eleven Pavlovian conditioning sessions (Phase 1) in which mice received presentations of a stimulus paired with food delivery (CS+) and a second, unpaired stimulus (CS-). Mice were injected with either vehicle or 3 or 10 mg/kg i.p. MTEP (PC: Veh, 3, or 10) 20 min prior to each conditioning session. (**a**) Magazine entries during presentation of the CS+ and CS-, expressed as a percentage of total session entries (% magazine entries), did not differ between conditioning treatment groups and stabilized from session 8 onward. (**b**) Magazine entries made during the first five seconds of the CS+ presentation (i.e. prior to food delivery) increased across conditioning sessions and were unaffected by treatment with MTEP. (**c**) The mean retrieval latency to enter the food magazine following CS+ activation stabilized at 4-5 sec, which corresponded with the time of food (US) delivery. Retrieval latencies did not differ among the three groups.

4.3.2 Phase 2: Conditioned reinforcement

Conditioned reinforcement was influenced by the MTEP treatment given prior to Pavlovian conditioning sessions, but not by the MTEP treatment given during the CRf tests (Fig. 4.3a). An initial mixed-factor ANOVA, which included Lever (CS+ or CS- paired) as a within-subjects factor, confirmed that lever responding significantly differed as a result of the treatment received during conditioning sessions (Lever x Conditioning treatment interaction, $F(2,59) = 3.80$, $p < 0.05$). However, lever responding did not reliably differ as a result of the MTEP treatment received during the CRf test (Lever x CRf treatment interaction, NS).

Within-subjects ANOVA comparisons of CS+ and CS- lever responding, which included both CRf treatment conditions (Veh or MTEP), were undertaken to determine whether each conditioning treatment group demonstrated CRf (i.e. more responding on the CS+ lever than the CS- lever). Conditioned reinforcement was demonstrated in the PC: Veh group (main effect of Lever, $F(1,21) = 26.53$, $p < 0.001$) and in the PC: 3 group (main effect of Lever, $F(1,18) = 8.55$, $p < 0.01$). However, the PC: 10 group failed to show any difference in CS+ and CS- lever responding (main effect of Lever, NS).

The impairment in responding for CRf in the PC: 10 group was due to a specific reduction in responding for the food-paired stimulus (CS+), rather than a general reduction in the ability of these mice to perform an instrumental response. A mixed-factor ANOVA, performed for each stimulus-paired lever, demonstrated that CS+ lever responding was significantly influenced by the treatment received during conditioning (main effect of Conditioning treatment, $F(2,59) = 3.59$, $p < 0.05$). By contrast, CS- lever responding was unaffected by the treatment received during conditioning (main effect of Conditioning treatment, NS). *Post-hoc* comparisons indicated that CS+ lever responding was significantly reduced in the PC: 10 group, in comparison to the PC: Veh group during CRf tests that were preceded by injection of vehicle ($t = 2.68$, df = 41, $p < 0.05$) and by 10 mg/kg MTEP ($t = 2.70$, df = 41, $p < 0.05$). Consistent with a dose-related effect of MTEP, there were no

differences in CS+ lever responding between the PC: Veh and PC: 3 groups or the PC: 3 and PC: 10 groups in either the CRf test (*t*-test comparisons, NS).

Although CRf was not impaired by pre-test administration of 10 mg/kg MTEP in the PC: Veh group, a possibility existed that the temporal profile of lever responding may have been altered by acute 10 mg/kg MTEP treatment. Further analysis was therefore performed to determine whether administration of 10 mg/kg MTEP in the test of CRf had any effect on the temporal profile of lever responding (Fig. 4.3b). Conditioned reinforcement (i.e. greater responding on the CS+ lever) was evident in each 15 min time period of the 60 min CRf test in the PC: Veh group (main effect of Lever, $F(1,21) = 25.81$, $p < 0.001$), but not in the PC: 10 group (main effect of Lever, NS). In the PC: Veh group, 10 mg/kg MTEP during the test of CRf did not alter the temporal profile of either CS+ lever responding (Period x CRf treatment interaction, NS), or CS- lever responding (Period x CRf treatment interaction, NS).

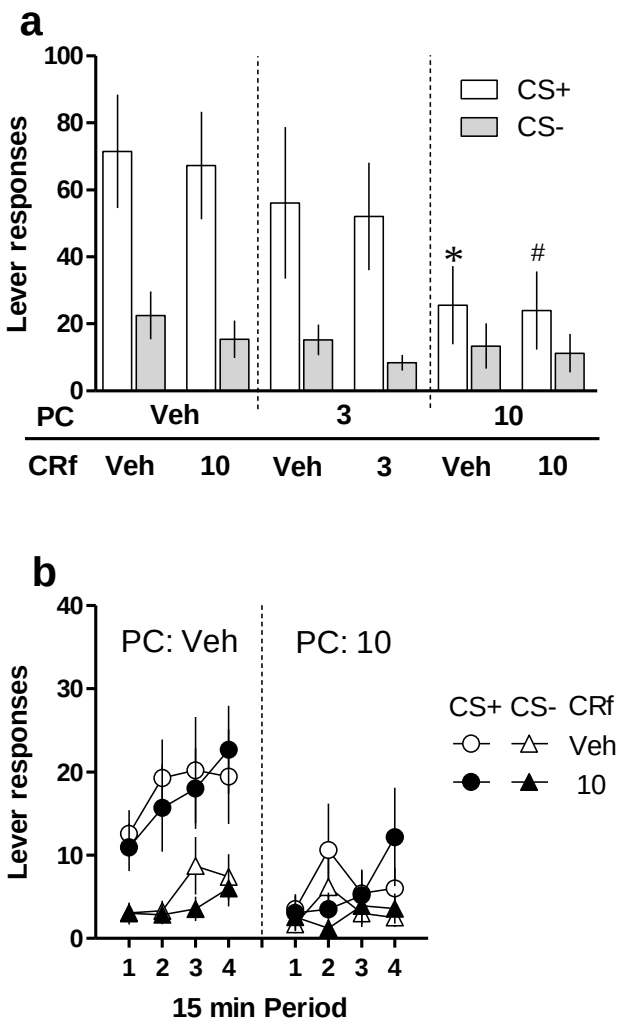


Figure 4.3 Lever responding in tests of conditioned reinforcement (**a**) Responding for CRf was observed in mice that received vehicle or 3 mg/kg MTEP during Pavlovian conditioning (PC: Veh and 3, respectively). CRf was significantly impaired in mice that received 10 mg/kg MTEP during conditioning (PC: 10). In contrast, 10 mg/kg MTEP during the CRf test did not impair CRf in mice that received vehicle during conditioning (PC-CRf: Veh-10). *$p < 0.05$ *Post-hoc*, *t*-test comparison between Veh-Veh and 10-Veh CS+ lever responses; $^{\#}p < 0.05$ *Post-hoc*, *t*-test comparison between Veh-10 and 10-10 CS+ lever responses. (**b**) 10 mg/kg MTEP did not alter the temporal profile of lever responding in mice that received vehicle during conditioning (PC: Veh). Mice that received 10 mg/kg MTEP during conditioning (PC: 10) failed to show any significant difference in CS+ and CS- lever responding in any 15 min period of each CRf test.

Magazine entry activity during CRf tests was also examined (Table 4.1), as this could provide further indication of whether MTEP administration had any gross effects on activity. A mixed-factor ANOVA of mean total magazine entries, indicated that entries were significantly increased during CRf tests in which MTEP was administered (main effect of CRf Treatment, $F(1,59) = 11.19$, $p < 0.01$), but that the effect of MTEP on magazine entries did not differ among conditioning treatment groups (CRf treatment x Conditioning treatment interaction, NS). Analysis of the time course of magazine entries during the CRf tests indicated that entries decreased over the course of the test session (main effect of Period, $F(3,177) = 7.0$, $p < 0.01$), but the effects of MTEP given during the CRf test did not reach statistical significance (CRf treatment x Period interaction, NS).

Group	Magazine Entries	15 min Period			
(PC-CRf)	Total	1	2	3	4
Veh-Veh	62.5 (8.5)	23.0 (3.4)	11.9 (2.2)	12.5 (3.5)	15.2 (3.2)
Veh-10	77.7 (11.0)	23.0 (3.8)	16.9 (3.3)	18.5 (3.7)	19.2 (3.0)
3-Veh	61.5 (13.4)	13.3 (2.6)	14.7 (3.0)	13.6 (4.0)	19.9 (7.4)
3-3	71.8 (13.6)	16.9 (2.6)	17.3 (4.5)	20.2 (4.5)	17.4 (4.3)
10-Veh	51.8 (7.7)	17.2 (3.4)	14.5 (3.0)	9.5 (2.0)	10.6 (2.9)
10-10	81.5 (13.1)	28.7 (3.5)	19.0 (3.9)	19.8 (3.6)	14.0 (4.0)

Table 4.1 Head entries into the food magazine during tests of Conditioned Reinforcement (CRf). Total magazine entries were significantly increased when MTEP was administered during the CRf test; however this effect did not differ between Pavlovian conditioning (PC) treatment groups. Magazine head entries decreased across each of the 15 min periods (1-4) of the 60 minute CRf test. Table shows group mean (±SEM).

4.3.3 Phase 3: Goal-tracking

Presentation of the food-paired stimulus (CS+), in the absence of food delivery, elicited approach responses into the food magazine (i.e. towards the goal). Mice made fewer head entry responses into the magazine during presentation of the unpaired stimulus (CS-), indicating that the CS+ was able to serve as a predictor of food availability (Fig. 4.4a). There was no effect of MTEP given during the Pavlovian conditioning phase, or MTEP given during the GT test, on goal-tracking responses. These findings were confirmed by a mixed-factor ANOVA, which included Stimulus (CS+, CS-) as a within-subjects factor. Mean total magazine entry responses significantly differed depending on the identity of the stimulus (main effect of Stimulus, $F(1,55) = 200.51$, $p < 0.001$), but there was no effect of either the treatment received during conditioning (Stimulus x Conditioning treatment interaction, NS) or during the GT test (Stimulus x GT treatment interaction, NS) on goal-tracking responses.

Analysis of magazine entries made during each stimulus trial was performed to determine whether acute 10mg/kg MTEP treatment altered the profile of goal-tracking responses in the PC: Veh group and whether response profiles differed between the PC: Veh and PC: 10 groups (Fig. 4.4b). For both PC: Veh and PC: 10 groups, the number of magazine entries made during each CS+ trial decreased across the course of the GT test and few responses were made across all CS- trials. Analysis of magazine entries during CS+ trials was performed using a mixed-factor ANOVA, which included Trial (1-8) as a within-subjects factor. This analysis confirmed that magazine entries made during each CS+ trial significantly decreased with successive trials (main effect of Trial, $F(7,273) = 17.215$, $p < 0.001$), but that this profile of responding was unaffected by the treatment received during conditioning (Trial x Conditioning treatment interaction, NS), or the treatment received during the GT test (Trial x GT treatment interaction, NS).

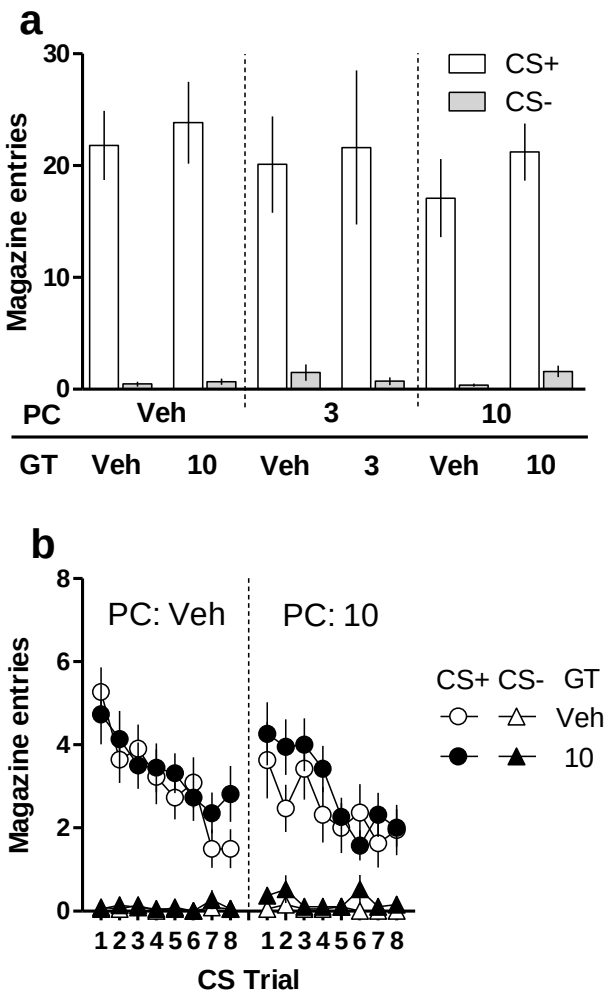


Figure 4.4 Food Magazine entries in tests of goal tracking (GT), which examines the ability of a CS to elicit approach responses to the place of food delivery. No food was delivered during each GT test. (**a**) Mice that received vehicle, 3 or 10 mg/kg i.p. MTEP during conditioning sessions (PC: Veh, 3 and 10, respectively) made more entries into the food magazine during presentation of the food-paired stimulus (CS+) than during presentation of the unpaired stimulus (CS-). There was no difference in magazine activity between the conditioning treatment groups, and magazine activity was not altered by the MTEP treatment received during the GT test. (**b**) Magazine entries made in each CS+ stimulus trial decreased across successive trials. The number of magazine entries made during each CS+ stimulus trial was unaffected by the treatment (vehicle or 10 mg/kg MTEP) received during the GT test in PC: Veh and PC: 10 groups.

4.4 Discussion

The present study explored the effects of the selective mGluR5 antagonist, MTEP, on the acquisition of a Pavlovian association that enables a food-paired stimulus to acquire predictive properties that signal reward availability (goal-tracking) and incentive properties necessary to reinforce a novel instrumental response (conditioned-reinforcement). We report that MTEP did not affect performance during Pavlovian conditioning sessions, indicating that the overall motivation to obtain food and the ability of mice to discriminate between the food-paired stimulus and the stimulus not paired with food was unaffected by blockade of mGluR5. In addition, mGluR5 function was not required for the acquisition of predictive properties necessary for the control over goal-tracking responses by the food-paired stimulus. However, mGluR5 function was critical for the associative learning processes necessary for the acquisition of properties by the CS that allow the CS to serve as a conditioned reinforcer, i.e. providing the CS with incentive value. Once incentive learning had taken place, mGluR5 function was not required for the expression of this CS-reinforced behaviour, which has been proposed to depend upon CS elicited representations of general affect (Parkinson et al., 2005; Burke et al., 2007). These findings add important new information regarding the function of mGluR5 in the control over appetitive behaviours by reward-paired stimuli.

A potential explanation for the findings reported here is that impaired CRf in mice that had received MTEP during conditioning sessions (PC: 10 group) was due to a state-dependent learning process (Stephens et al., 2000). That is, MTEP may have induced an interoceptive state during conditioning sessions and the subsequent retrieval of the CS memory during the CRf test may have been disrupted due to the presence of a different interoceptive state, namely the absence of MTEP. However, this account is unlikely since CRf responding was also impaired in the PC: 10 group when 10 mg/kg MTEP was given during the CRf test to induce the same state that existed during conditioning sessions.

That we found contrasting effects of MTEP on responding for CRf and goal-tracking responses may have been due to mice having experienced relatively more stimulus-food (CS-US) pairings prior to the GT tests than the CRf tests. Thus, goal-tracking responses may have been less susceptible to the effects of MTEP due to strengthened CS-US associations. At variance with this possibility is the observation that mice came to use the CS+ as a predictor of food delivery even during Pavlovian conditioning sessions that preceded the first CRf test (Fig. 4.2b-c). Critically, the acquisition of these goal-tracking responses were unaffected by administration of MTEP, thereby supporting our proposition that mGluR5 plays a dissociable role in the acquisition of predictive and incentive motivational properties by CSs.

Our findings that CRf was not impaired by administration of 10 mg/kg MTEP, during the test only, in mice that had received vehicle prior to conditioning sessions (PC: Veh group) is in apparent contrast to behavioural studies of cue-induced reinstatement that have reported a role of mGluR5 in the expression of control over responding maintained by both natural- and drug-paired CSs (Tessari et al., 2004; Bespalov et al., 2005; Backstrom and Hyytia, 2006; Schroeder et al., 2008; Gass et al., 2009; Kumaresan et al., 2009; Martin-Fardon et al., 2009). Since it is possible that higher doses of MTEP would have reduced the expression of CRf in our study, our findings do not exclude a role of mGluR5 in the control over appetitive behaviours by reward-paired stimuli. Alternatively, subtle methodological differences may have contributed to this apparent contrast in findings. Firstly, in our study, the CS reinforced an instrumental response that had not previously been associated with primary reinforcement. Secondly, mice were trained a purely Pavlovian (stimulus-outcome) association, while an instrumental (response-outcome) component is embedded in the acquisition of associations between environmental stimuli and reward in studies of self-administration and cue-induced reinstatement. Finally, we examined instrumental responding supported by a CS immediately following the conditioning phase, while extinction learning or periods of withdrawal are commonly employed in studies of cue-induced reinstatement and

which may contribute to neural changes mediating the subsequent expression of control over appetitive behaviours by CSs (Grimm et al., 2003; Lu et al., 2005; Conrad et al., 2008; Ghasemzadeh et al., 2009b).

Our finding that mGluR5 antagonism was effective in reducing a CS-reinforced behaviour when administered during the acquisition of a Pavlovian association shares some similarity with studies examining the role of mGluR5 in conditioned place preference (CPP) learning. Administration of the mGluR5 antagonist 6-methyl-2-(phenylethynyl)pyridine (MPEP), during conditioning (i.e. the acquisition phase), reduced the development of cocaine CPP in mice while having no effect on the development of amphetamine, ethanol, morphine or nicotine CPP (McGeehan and Olive, 2003). Another study reported that higher doses of MPEP attenuated both the acquisition and expression of morphine CPP in mice (Popik and Wrobel, 2002). In rats, the expression of cocaine CPP was unaffected by a dose of MPEP that reduced the expression of morphine CPP (Herzig and Schmidt, 2004). Thus, mGluR5 can contribute to the acquisition of associations that enable reward-paired, contextual stimuli to mediate CPP and can also influence the expression of CPP, a finding that may depend on the extent of mGluR5 blockade and/or the primary reward experienced during conditioning. However, the expression of CPP may be due to either predictive or incentive motivational associations formed between the contextual cues and the paired outcome (Stephens et al., 2010). While acknowledging that substantial differences exist between contextual vs. discrete cue conditioning, our findings may provide further insight into the psychological mechanisms underlying these earlier CPP reports by identifying a specific role of mGluR5 in the acquisition of incentive associations between an environmental stimulus and reward, while the ability of a reward-paired stimulus to acquire predictive properties is unaffected by mGluR5 antagonism.

What is surprising about the present findings is the degree of overlap between the response profile of mGluR5$^{KD\text{-}D1}$ mice in the CRf test, and that seen in wild-type mice that had received 10 mg/kg MTEP injections prior to conditioning sessions. In

both cases, a reduction in CS+ reinforced responding was observed, however discrimination between the CS+ and CS- lever was maintained. Thus, mGluR5 seems to have a particular contribution to the vigour of the response that is under the control of the reward-paired CS, rather than the direction of the behavioural output. The similarity of these results is also particularly surprising since MTEP was administered systemically in the present study, and therefore lacks any cell-type selectivity afforded by the mGluR5^{KD-D1} mouse model. These findings suggest at least two possibilities. First, that knock-down of mGluR5 is not actually cell-type specific in the mutant mouse model or, second, that only mGluR5 located on D1R expressing cells contribute to the acquisition of incentive learning necessary for CRf.

The first suggestion mentioned above seems unlikely, given the extensive characterisation of the mGluR5^{KD-D1} model (Novak et al., 2010). The second possibility would be remarkable, but not entirely inconceivable when considering some other reports that have explored the neural mechanisms of appetitive Pavlovian learning. For example, systemic administration of an NMDA antagonist in mice blocked the acquisition and expression of nicotine (Papp et al., 2002) and cocaine CPP (Maldonado et al., 2007). Similarly, mice with inactivation of NMDA receptors specifically in dopamine neurons (NR1-KO) or mutation of NMDA receptors specifically on dopamine D1R expressing neurons (DR1-NR1^{m}) also show impaired nicotine (Wang et al., 2010) and cocaine CPP (Heusner and Palmiter, 2005), respectively. These reports confirm that systemic drug treatments can produce the same behavioural phenotype as cell-type specific mutations, but whether these manipulations are working through the same processes (e.g. incentive or predictive learning or something entirely different) to disrupt CPP is not clear. Indeed, there is a dearth of literature that allows for comparison of systemic drug treatments and cell-type specific mutations in more rigorously defined appetitive learning preparations such as CRf, sign-tracking and PIT. This likely reflects the compromising side-effect profiles and/or receptor subtype selectivity of many drugs that target receptors implicated in incentive learning processes (e.g.

NMDA, AMPA, and dopamine D1Rs), rather than a scarcity of the genetic mouse models.

Until an inducible cell-type specific knock-out model is generated that allows for temporal control of mGluR5 expression on dopamine-D1R neurons, it will be impossible to formally determine the contribution of mGluR5 on D1R expressing neurons for the acquisition and/or expression of specific incentive learning processes. However, the findings reported in this chapter, together with a failure of 20 mg/kg i.p. MTEP to disrupt the expression of responding for CRf in wild-type mice (reported in Chapter 3, section 3.3.2.3), strongly favours a role of mGluR5 on D1R expressing neurons in neural adaptations that are necessary for the acquisition, rather than the expression, of an incentive CS-US association. A pertinent question that arises from the studies reported thus far is, what role might mGluR5 on D1R expressing neurons play in behavioural effects of addictive drugs, such as cocaine, which are thought to reflect the ability of such drugs to engage with the neural systems that mediate the learning of incentive associations (Stewart et al., 1984; Robinson and Berridge, 1993; Everitt et al., 2001). This issue will be addressed in the next chapter

5 Some behavioural effects of cocaine in mGluR5^{KD-D1} mice

5.1 Introduction

The previous chapters have established that glutamate signalling through mGluR5, on cells that also receive dopaminergic input, is necessary for the formation of incentive associations between environmental stimuli and natural rewards. In the striatum, the interaction of glutamate and dopamine is critical for long-term plasticity underlying a variety of adaptive learning and memory processes, but which may also subserve the behavioural effects of addictive drugs (Nestler, 2001; Kelley, 2004; Malenka and Bear, 2004; Hyman et al., 2006). The following experiments will explore the contribution of mGluR5 on dopaminoceptive neurons to cocaine conditioned reward and behavioural sensitisation; two behavioural effects of cocaine that reflect drug induced neuronal adaptations within cortical limbic striatal networks (Wolf, 1998; Vanderschuren and Kalivas, 2000; Girault et al., 2007).

Acute exposure to psychostimulants, such as cocaine, can increase extracellular dopamine and glutamate in defined brain regions, such as the ventral striatum (Reith et al., 1986; Di Chiara and Imperato, 1988; Reid et al., 1997), resulting in augmented locomotor activity (Kelly et al., 1975; Clarke et al., 1988; Delfs et al., 1990) and the induction of intracellular signalling cascades (Graybiel et al., 1990; Valjent et al., 2005). With repeated intermittent psychostimulant exposure, long lasting neuronal adaptations can occur, including changes in synaptic strength (Thomas et al., 2001b; Borgland et al., 2004) and dendritic morphology (Robinson and Kolb, 2004). Such neuronal changes are mirrored by a persistent, sensitised locomotor response to cocaine in animals (Segal et al., 1980; Robinson and Becker, 1986) and are proposed to contribute to behavioural changes underpinning

addiction in humans (Robinson and Berridge, 1993; Nestler, 2001; Kauer and Malenka, 2007; Thomas et al., 2008).

There is ample evidence that glutamatergic signalling, particularly at ionotropic NMDA and AMPA receptors, is involved in the acute psychomotor activating effects of psychostimulants (Witkin, 1993) and induction and expression of neuroplastic changes that follow repeated intermittent psychostimulant exposure (Wolf, 1998; Vanderschuren and Kalivas, 2000; Kalivas, 2009; Schmidt and Pierce, 2010). For example, glutamatergic signalling at NMDA is necessary for the induction of cocaine sensitisation (Karler et al., 1989; Kalivas and Alesdatter, 1993; Stewart and Druhan, 1993; Wolf and Jeziorski, 1993; Haracz et al., 1995; Khan and Shoaib, 1996), while AMPA receptors contribute to both the induction and expression of sensitisation (Karler et al., 1990; Karler et al., 1991). Repeated cocaine exposure also leads to changes in the expression of these ionotropic glutamate receptors in both the VTA (Fitzgerald et al., 1996) and ventral striatum (Boudreau and Wolf, 2005). In addition, after repeated cocaine exposure, basal extracellular glutamate is depressed in the NAcc (Baker et al., 2003) and VTA (Kozell and Meshul, 2003), but the amount of glutamate released in the NAcc is significantly increased following an injection of cocaine (Pierce et al., 1996; McFarland et al., 2003). With respect to the metabotropic receptor, mGluR5, both its mRNA and protein are augmented in the NAcc of cocaine-sensitised animals (Ghasemzadeh et al., 1999; Ghasemzadeh et al., 2009a) and mGluR5 appears necessary for the expression of behavioural sensitisation to morphine (Kotlinska and Bochenski, 2007), cocaine (Kotlinska and Bochenski, 2009) (but see, Dravolina et al., 2006) and nicotine (Tessari et al., 2004). Only recently has a necessary role for mGluR5 in the induction of behavioural sensitisation to cocaine (but not morphine) been reported using the mGluR5 antagonist, MTEP, in rats (Veeneman et al., 2010). In consideration of these pharmacology studies, the mGluR5$^{KD\text{-}D1}$ mouse provides an ideal tool to further probe the contribution and neuroanatomical location of mGluR5 in cocaine sensitisation.

In addition to behavioural sensitisation, animals can learn to associate the effects of cocaine with the environment in which cocaine was administered and will demonstrate preference for that place (Spyraki et al., 1982b). Conditioned place preference (CPP) relies on the formation of a Pavlovian association between a biologically relevant stimulus with neutral contextual cues and has been established with a variety of “natural” reinforcers, including food (Spyraki et al., 1982a) and conspecifics interaction (Calcagnetti and Schechter, 1992), but also many addictive drugs (Tzschentke, 2007). Although the precise psychological basis of CPP has not yet been established (Stephens et al., 2010), its neurobiological substrates clearly overlap with those required for other appetitive Pavlovian conditioning tasks. Thus, the BLA and NAcc core and their serial connectivity are necessary for discrete-cue based CPP (Everitt et al., 1991; Fuchs et al., 2002; Ito et al., 2006), while the HPC and NAcc shell and serial connectivity between these two structures are necessary for idiothetic spatial-cue based CPP (Ito et al., 2008). The acquisition of CPP has also been shown to involve the ventral pallidum and medial dorsal thalamus (McAlonan et al., 1993) and specifically the dorsal, but not ventral, hippocampus (Meyers et al., 2003).

There is also ample evidence pointing to glutamate transmission at the ionotropic NMDA and AMPA receptors as important for the acquisition and expression of cocaine-CPP (Cervo and Samanin, 1995; Kaddis et al., 1995; Kim et al., 1996; Mead and Stephens, 1999; Harris and Aston-Jones, 2003; Dong et al., 2004; Maldonado et al., 2007). However, the role of mGluR5 in CPP supported by natural or drug reinforcers is not at all clear. Using mGluR5 knock-out mice, mGluR5 has been proposed as necessary for cocaine reinforcement in one study (Chiamulera et al., 2001), but not required for cocaine reward in another (Olsen et al., 2010). Using the mGluR5 antagonist, MPEP, both a “necessary role” and “no role” of mGluR5 have been proposed for CPP established with morphine (Popik and Wrobel, 2002; McGeehan and Olive, 2003), ethanol (McGeehan and Olive, 2003; Lominac et al., 2006), nicotine (McGeehan and Olive, 2003; Yararbas et al., 2010), amphetamine (McGeehan and Olive, 2003; Herzig et al., 2005) and cocaine

(McGeehan and Olive, 2003; Herzig and Schmidt, 2004). These conflicting findings could be due to any number of procedural differences but, where MPEP has been effective in drug-CPP, it is not known whether the effects of MPEP on drug-CPP are due to disruption of drug reinforcement (Paterson et al., 2003; Kenny et al., 2005), discriminative drug effects (Lee et al., 2005; Zakharova et al., 2005; Besheer et al., 2006) or more general spatial learning impairments (Balschun and Wetzel, 2002; Naie and Manahan-Vaughan, 2004). In addition, there is now substantial evidence that MPEP is itself reinforcing (van der Kam et al., 2009b, a; Rutten et al., 2010) and the effects, or lack thereof, of MPEP on drug-CPP could be due to a potentiation, rather than an attenuation, of any rewarding drug effects (Rutten et al., 2010). Given this confound, the examination of food- and cocaine-CPP in mGluR5^{KD-D1} may provide some additional clarity on the role of mGluR5 in conditioned reward. Moreover, mGluR5^{KD-D1} mice may provide valuable insight to the psychological basis of CPP. Incentive learning necessary for CRf is clearly impaired in these mice, but CRf has been suggested as one mechanism whereby contextual stimuli may support instrumental locomotor responses necessary for approaching and contacting the paired compartment during the test of place preference (Everitt et al., 1991). If CRf was necessary for CPP, one could predict that CPP would be impaired in mGluR5^{KD-D1} mice.

The following experiments will first examine the effects of acute and repeated intermittent cocaine exposure on locomotor activity in mGluR5^{KD-D1} mice. The development of behavioural sensitisation may result from an additive effect of unconditioned locomotor activity to the drug and conditioned locomotor activity induced by the formation of Pavlovian associations to the drug-paired context (Stewart, 1983; Pert et al., 1990; Crombag et al., 1996; Le Merrer and Stephens, 2006). Thus, a test will be undertaken to explore the ability of the environment in which cocaine is experienced to augment activity in the absence of cocaine (termed, conditioned activity; Stewart, 1983). The second series of experiments reported in this chapter will see mGluR5^{KD-D1} mice exposed to tests of food- and

cocaine-CPP to examine the contribution of mGluR5 on dopaminoceptive neurons to learning about conditioned food- and drug-reward, respectively.

5.2 Materials and methods

5.2.1 Animals

Mice (n = 27/28, WT/KD; male and at least 8 weeks old prior to experiment start) were maintained on a 12:12 h light-dark cycle (lights on at 0700 hours) under controlled temperature (21 ± 2 °C) and humidity conditions (50 ± 5%). Animals were housed in groups of two or three in polycarbonate cages. Water was available *ad libitum* in the holding room. At least 7 days prior to the experiment start, mice were placed onto a restricted feeding regime designed to maintain body weights at ~85% of free-feeding weight. Experiments took place during the light-phase. All procedures were performed in accordance with the United Kingdom 1986 Animals (Scientific Procedures) Act, following institutional ethical review.

5.2.2 Drugs

Cocaine hydrochloride (Macfarlan Smith, Edinburgh, Scotland, UK) was dissolved in 0.9% saline. Injections were administered at a volume of 10 ml/kg i.p.

5.2.3 Apparatus

Conditioned place preference: Behavioural training and testing were performed in eight identical, three-compartment conditioned place preference chambers (Fig. 5.1a). Each chamber consisted of two ‘outer’ compartments (ea. 20 x 20 x 20 cm), adjoined by a smaller ‘middle’ compartment (20 x 5 x 20 cm). Access amongst the three compartments was controlled by a removable panel (5 x 20 cm) in the partitioning walls of the outer compartments and middle compartment. Each outer compartment was differentiated by tactile and visual cues. One outer compartment (context A) consisted of black and white walls (each wall split along the diagonal with the top half white and the bottom half black) and a smooth Perspex floor. The second compartment (context B) consisted of white walls and a perforated steel floor. The activity and location of mice was recorded using five infra-red beams distributed amongst the three compartments (Fig. 5.1b), which interfaced with a PC running data collection and analysis software (written by A.N. Mead).

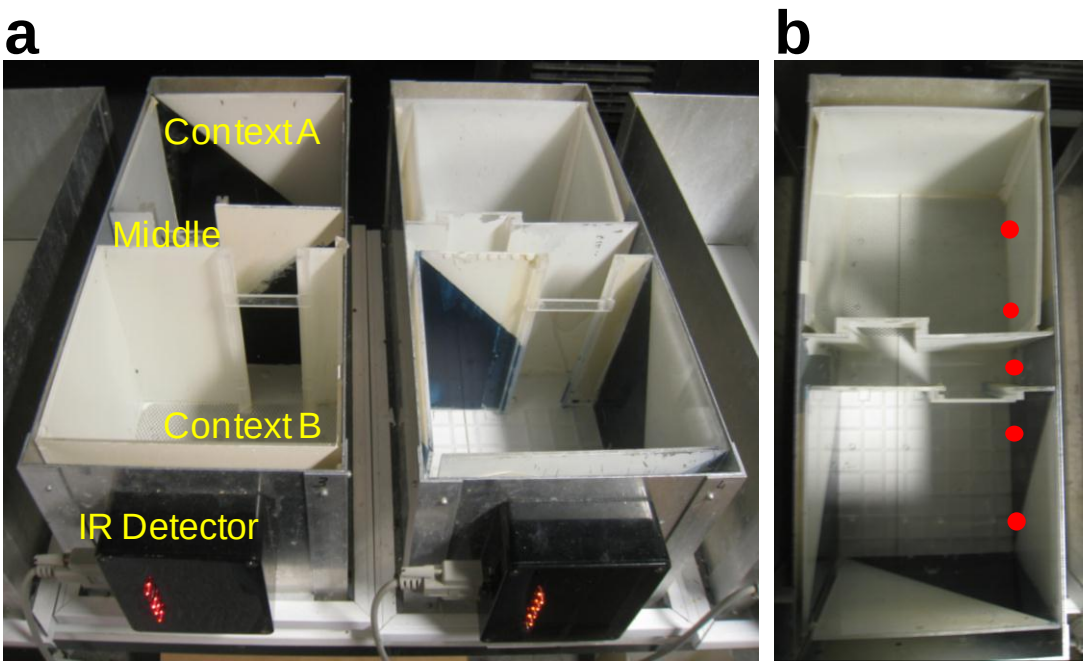


Figure 5.1 Conditioned place preference apparatus. (**a**) Two CPP chambers are shown. Each apparatus consists of two outer compartments (context A and B) differentiated by tactile (floor material) and visual (wall pattern) cues. Access between the outer compartments is made through a smaller middle compartment. (**b**) Overview of one CPP chamber. The red dots indicate the position of infra-red beams used to detect the location of mice and which also provide a measure of activity.

Locomotor activity: Locomotor activity experiments were performed using nine circular runways, as described previously (see Chapter 2, section 2.2.6.1).

5.2.4 Experiment 1: Locomotor activity

Procedural note: Initially, 9 mice per genotype were assigned to this study. One wild-type mouse showed high levels of activity during the sham injection test (> 2 x S.D. from group mean). This mouse was excluded from testing, reducing group sizes to n = 8 and n = 9 for knock-down and wild-type mice, respectively. The remaining mice were used for all the following locomotor activity experiments reported here. Mice were assigned to the same circular runway for all sessions. Multiple groups of mice were run in the locomotor apparatus each day, although each group contained mice from both genotypes. The circular runways and Perspex platform were cleaned between each group.

5.2.4.1 Habituation to a novel environment and sham injection

Mice were habituated to the circular runways across five, once-daily, 1 h sessions. On the sixth day, following 30 min of activity recording, mice were removed from the circular runways and were injected with vehicle (10 ml/kg saline i.p.), in order to acclimatize them to the injection procedure. Activity was recorded for a further 60 min post-vehicle injection.

5.2.4.2 Cocaine-locomotor dose-response (pre-sensitisation)

Mice were habituated to the circular runways for 30 min, removed and then injected with cocaine (1, 3 or 10 mg/kg i.p.) or vehicle. Activity was recorded for a further 60 min post-injection. Each mouse received each dose, separated by 48 h intervals, in a Latin-square design.

Because activity was not markedly increased by 10 mg/kg cocaine in mutant mice, a second cocaine-locomotor test was performed using a higher cocaine dose. As before, mice were habituated to the circular runways for 30 min, removed and then injected with cocaine (20 mg/kg i.p.) or vehicle. Activity was recorded for a further

60 min post-injection. These two additional tests were separated by 48 h and the order of injections (cocaine or vehicle) was counterbalanced.

5.2.4.3 Cocaine-locomotor sensitisation

Mice received repeated, intermittent injections of 10 mg/kg cocaine at 3-4 day intervals over 12 sessions. During each session, mice were habituated to the circular runways for 30 min, removed and then injected with cocaine. Activity was recorded for a further 60 min post-cocaine injection.

5.2.4.4 Conditioned activity

Three days after the final sensitisation session, mice were habituated to the circular runways for 30 min, removed and then injected with vehicle. Activity was recorded for a further 60 min post-vehicle injection. A comparison of activity following the post-sensitisation vehicle injection was made with activity following the pre-sensitisation (sham) vehicle injection (5.2.4.1).

5.2.4.5 Cocaine-locomotor dose-response (post-sensitisation)

Mice were habituated to the circular runways for 30 min, removed and then injected with cocaine (3, 10 or 20 mg/kg i.p.) or vehicle. Activity was recorded for a further 60 min post-injection. Each mouse received each dose, separated by 48 h intervals, in a Latin-square design.

5.2.4.6 Cocaine-locomotor dose-response (2 months post-sensitisation)

Commencing two months after the final post-sensitisation dose-response test, a second test was performed (as described 5.2.4.5.) to examine the persistence of cocaine-locomotor sensitisation.

5.2.4.7 Statistical Analysis

All locomotor activity data were first analysed by mixed-factor analysis of variance (ANOVA), with genotype (WT, KD) as a between-subjects factor. Details of the within-subjects factors used for each ANOVA are provided in the results section.

Where significant ($p \leq 0.05$) main effects or interaction terms were found, further analysis was performed using ANOVA and individual between- or within-genotype comparisons by *t*-test. Findings were considered indicative of a trend where $p \leq 0.1$ and Bonferroni corrections were applied for multiple comparisons. For within-subjects ANOVA, the Greenhouse-Geisser correction was used where the assumption of sphericity was violated (Mauchly's test, $p \leq 0.05$).

5.2.5 Experiment 2: Conditioned place preference (CPP)

Procedural note: An equipment malfunction meant that the location of two mutant mice from the food CPP study and one wild-type mouse from the cocaine CPP study were not recorded during the pre-conditioning session. These mice were excluded from further testing, leaving group sizes of n = 10 and n = 9 for WT and KD mice, respectively in the food CPP study, and n = 7 and n = 8 for WT and KD mice, respectively in the cocaine CPP study.

5.2.5.1 Food CPP

On day 1 (pre-conditioning), mice were placed into the apparatus and allowed to explore all three compartments for 20 min. Mice were then assigned one outer compartment (A or B) as food-paired and the other compartment as food-unpaired. The assignment of compartments as food-paired or food-unpaired was counterbalanced. For the next 8 days (conditioning), mice received access to 20 mg food pellets (5TUL, Cat no. 1811142; Test Diets, Indiana, USA) presented in a small dish in the food-paired compartment, or access to an empty dish in the unpaired compartment. Each 30 min, once-daily, conditioning session was alternated between food-paired and food-unpaired compartments, but the order of exposure to each compartment was counterbalanced. Mice were confined to the outer compartment during each conditioning session. On day 10 (post-conditioning), mice were placed into the apparatus and allowed to explore all three compartments for 20 min to determine post-conditioning preference. No food was presented during the post-conditioning test.

5.2.5.2 Cocaine CPP

Because data from the food CPP study suggested that baseline compartment preference tended to differ between the genotypes, mice for the cocaine CPP study were first allowed to explore the entire the apparatus during two, once-daily, 20 min sessions in an effort to reduce any baseline compartment preference. The following day, a 20 min pre-conditioning test of preference was conducted (as described above). Mice were then assigned one outer compartment (A or B) as cocaine-paired and the other compartment as the vehicle-paired. The assignment of compartments as cocaine-paired or vehicle-paired was counterbalanced. For the next 8 days (conditioning), mice were injected with 10 mg/kg cocaine or vehicle (10 ml/kg saline) immediately prior to placement in one compartment (cocaine- or vehicle-paired, respectively). Each 30 min, once-daily, conditioning session was alternated between cocaine-paired and vehicle-paired compartments, but the order of exposure to each compartment was counterbalanced. During each conditioning session, mice were confined to the outer compartment. Twenty four hours after the final conditioning session, mice were placed into the apparatus and allowed to explore all three compartments for 20 min to determine post-conditioning preference. No injections were given on the post-conditioning test.

5.2.5.3 Statistical Analysis

Comparison of pre-conditioning or post-conditioning preference between genotypes (that is, time spent in each outer compartment) was performed by mixed-factor ANOVA, with genotype (WT, KD) as a between-subjects factor and compartment (two levels: A, B or food-paired, food-unpaired or cocaine-paired, vehicle-paired) as a within-subjects factor. Compartment preference was also indexed by a preference score calculation (post-conditioning time minus pre-conditioning time) to inform about the change in place preference following conditioning. Preference scores were also compared between genotypes by mixed-factor ANOVA, with compartment (two levels: food-paired, food-unpaired or cocaine-paired, vehicle-paired) as a within-subjects factor. Where significant ($p \leq 0.05$) main effects or interaction terms were found, comparisons of the time spent

in either compartment were made by *t*-test to determine any place preference in each genotype.

In both the food- and cocaine-CPP studies, a between-genotype comparison of activity in the two outer chambers during pre- and post-conditioning preference tests (indexed by beam breaks in the compartments) was performed by mixed-factor ANOVA, including conditioning (Pre, Post) and compartment (food-paired, unpaired or cocaine-paired, vehicle-paired) as a within-subjects factor. For the cocaine CPP study, a comparison of activity between genotypes during conditioning sessions, following injections of cocaine or vehicle, was performed by mixed-factor ANOVA, including conditioning session (1-4) and compartment (cocaine-paired, vehicle-paired) as within-subjects factors. Where significant ($p \leq 0.05$) main effects or interaction terms were found, further analysis was performed by ANOVA. All figures show mean ± SEM.

5.3 Results

5.3.1 Experiment 1: Locomotor activity

5.3.1.1 Habituation to a novel environment

The findings for habituation in mGluR5$^{KD\text{-}D1}$ and wild-type mice have been reported previously (see Chapter 2, section 2.3.4.1). For the cohort of mice used in the present studies, upon exposure to the novel circular runway environment, activity (indexed by the distance travelled; m) was greatest during the first 10 min period of the 1 h session and declined over each subsequent 10 min period (Fig. 5.2a; main effect of Period, $F(5,75) = 7.66$, $p = 0.001$; Period x Genotype interaction, NS). Notably, activity in KD mice during this first session tended to be reduced in comparison to their wild-type counterparts (main effect of Genotype, $F(1,15) = 3.04$, $p = 0.10$). Both genotypes habituated to the apparatus with repeated exposure to the runway over five, once-daily, 1 h sessions (Fig. 5.2b). Habituation was confirmed by a significant reduction in mean total activity over the five sessions (main effect of Session, $F(4,60) = 8.57$, $p < 0.01$), in both genotypes (main effect of Genotype, NS; Genotype x Session interaction, NS). During the fifth session (Fig. 5.2c), activity declined across each 10 min period of the session in both genotypes (main effect of Period, $F(5,75) = 5.03$, $p < 0.01$; Period x Genotype interaction, NS), but there was no overall difference in activity between genotypes (main effect of Genotype, NS).

On the sixth day, when mice were given a sham-vehicle injection (Fig. 5.2d), activity did not differ between genotypes over the 30 min habituation period (main effect of Genotype, NS; Genotype x Period interaction, NS) or the 60 min post-injection period (main effect of Genotype, NS; Genotype x Period interaction, NS).

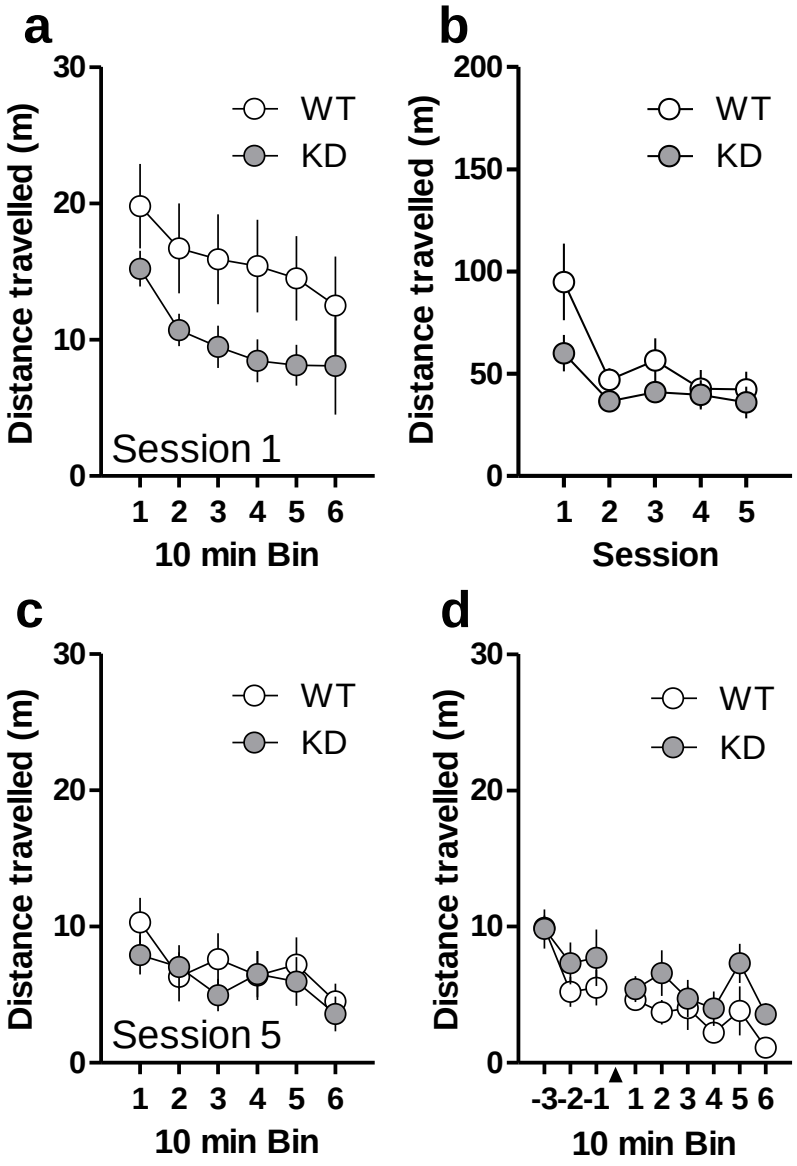


Figure 5.2 Cocaine-locomotor studies: Habituation and sham-vehicle injection. (**a**) Locomotor activity was reduced in KD mice during the first session. (**b**) Both genotypes habituated to the circular runways over five, once-daily, 1 h sessions. (**c**) Activity did not differ between genotypes over each 10 min period of the fifth session. (**d**) Mice were given a sham-injection (10 ml/kg saline; ▲) after a period of 30 min in the locomotor apparatus and activity recorded for 1 h post sham-injection. Activity did not differ between genotypes either pre- or post-sham injection. (n = 8/9, WT/KD; except for the 30 min habituation period in the sham dose test, where a data collection error resulted in group sizes of n = 6 and n = 7 for wild-type and mutant mice, respectively).

5.3.1.2 Cocaine-locomotor dose-response (pre-sensitisation)

In the first test, the effect of 0-10 mg/kg cocaine on locomotor activity was examined (Fig. 5.3a). Overall activity varied with cocaine dose (main effect of Dose, $F(3,45) = 2.46$, $p < 0.05$), but the effect of cocaine on activity also varied between genotypes (Dose x Genotype interaction, $F(3,45) = 3.90$, $p < 0.05$; main effect of Genotype, NS). Analysis of activity, for each genotype separately, indicated that cocaine increased activity in wild-type mice (main effect of Dose, $F(3,21) = 10.24$, $p < 0.001$), but not mutant mice (main effect of Dose, NS). *Post-hoc* comparisons of activity between each cocaine dose and vehicle confirmed that 10 mg/kg cocaine significantly increased mean total activity in wild-type mice ($t = 8.46$, df = 7, $p < 0.017$).

In the second test, the effect of 0-20 mg/kg cocaine on locomotor activity was examined (Fig. 5.3b). Overall activity varied with dose (main effect of Dose, $F(1,15) = 51.67$, $p < 0.001$) but the effect of cocaine on activity also tended to vary between genotypes (Dose x Genotype interaction, $F(1,15) = 4.51$, $p = 0.051$; main effect of Genotype, $F(1,15) = 3.015$, $p = 0.10$). Individual comparisons of activity between cocaine and vehicle confirmed that 20 mg/kg cocaine increased activity in wild-type mice ($t = -5.47$, df = 7, $p < 0.01$) and mutant mice ($t = -4.48$, df = 8, $p < 0.01$). *Post hoc* between-genotype comparisons of activity at each dose confirmed that the psychomotor activating effects of 20 mg/kg cocaine tended to be attenuated in mutant mice ($t = 1.96$, df = 15, $p = 0.069$), but activity did not differ between genotypes following vehicle ($t = 0.102$, df = 15, $p = 0.92$).

The effects of cocaine on locomotor activity were most pronounced immediately following injection, as revealed by activity time-course plots (Fig. 5.3d). For brevity, analyses of activity time-course data from both dose-response tests are summarised in table format (Table 5.1). Notably, the activity profile differed between genotypes following 20 mg/kg cocaine (Table 5.1: Test 2 and Fig. 5.3d). Subsequent analysis confirmed that activity varied over the post-injection period

following 20 mg/kg cocaine in wild-type mice (main effect of Period, $F(5,35) = 15.58$, $p < 0.001$) but not in mutant mice (main effect of Period, NS).

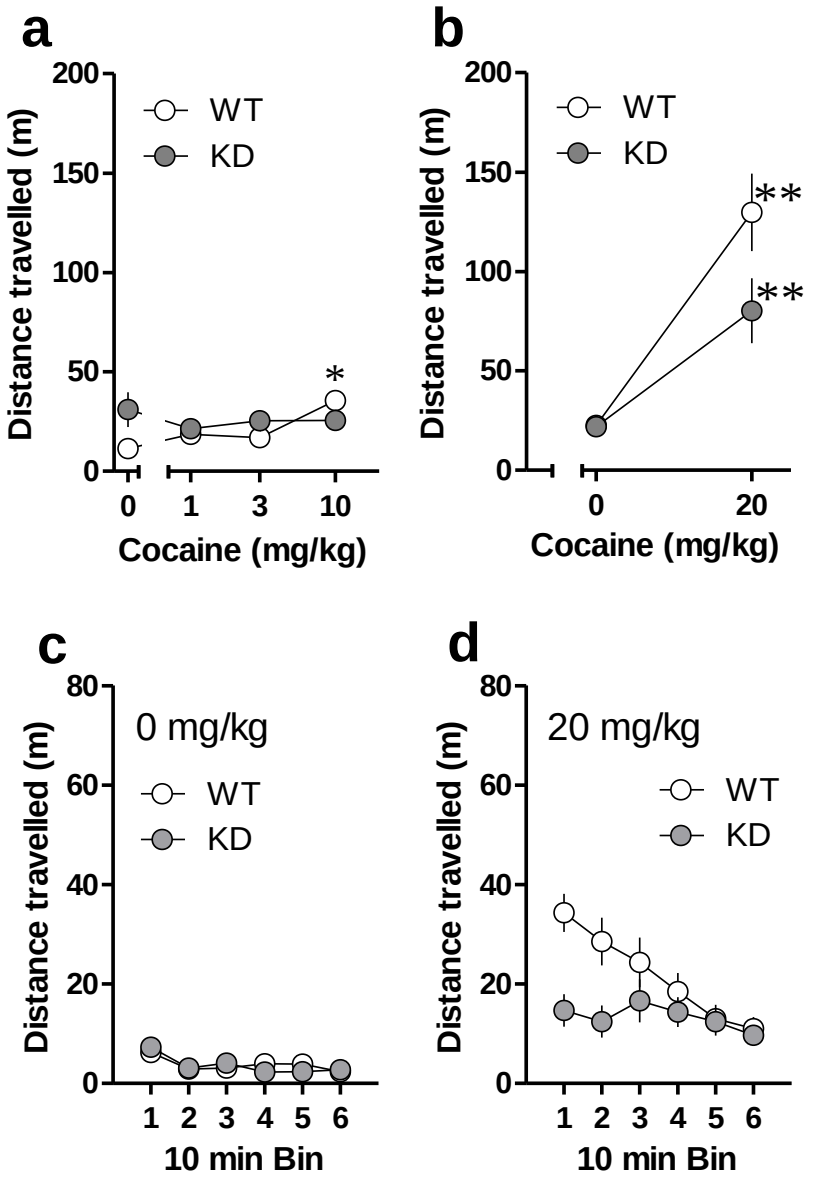


Figure 5.3 Cocaine-locomotor dose-response test (pre-sensitisation). (**a**) 10 mg/kg cocaine increased mean total locomotor activity during a 1 h session in wild-type mice, but not mutant mice. *p < 0.017, Bonferroni t-test comparison, between vehicle and 10 mg/kg cocaine (WT mice only) (**b**) 20 mg/kg cocaine increased mean total activity in both genotypes. The psychomotor stimulating effects of 20 mg/kg cocaine were attenuated in KD mice. **p < 0.01, Individual t-test comparisons between vehicle and 20 mg/kg cocaine. (**c-d**) Activity time-course plots from the second dose-response test. Note the distinct activity profiles between mutant and wild-type mice, following 20 mg/kg cocaine (n = 8/9, WT/KD).

Time course analysis	Period F(5,75)	Period x Genotype F(5,75)	Genotype F(1,15)
Test 1			
Vehicle	2.19	0.82	4.09§
1 mg/kg cocaine	1.74	0.45	0.22
3 mg/kg cocaine	2.64*	1.75	2.18
10 mg/kg cocaine	0.91	0.68	2.21
Test 2			
Vehicle	7.00***	1.12	0.01
20 mg/kg cocaine	14.17***	7.82***	3.83§

Table 5.1 ANOVAs for cocaine-locomotor dose-response time-course profiles (pre-sensitisation). Factors: Period (1-6); Genotype (WT, KD). ***$p < 0.001$, *$p < 0.05$, §$p < 0.10$

5.3.1.3 Cocaine-locomotor sensitisation

In both genotypes, mean total activity increased by a similar extent over 12 sessions of repeated, intermittent cocaine (Fig 5.4a; main effect of Session, $F(11,165) = 6.12$, $p < 0.001$; Genotype, NS; Session x Genotype interaction, NS). Restricting the analysis to the first 10 min period post-cocaine injection (i.e. where the locomotor activating effects of cocaine were greatest) also confirmed that activity increased in both genotypes to a similar extent over the 12 sessions (Fig. 5.4b; main effect of Session, $F(11,165) = 10.31$, $p < 0.001$; Genotype, NS; Session x Genotype interaction, NS).

In order to compare the rate of sensitisation between the two genotypes, linear regression analysis was performed for mean total activity data and slope coefficients compared between genotypes by *t*-test. Sensitisation is indicated by a positive slope (slope coefficient > 0), and the greater the slope co-efficient, the greater the rate of sensitisation (Crombag et al., 1999; Mead et al., 2004). Sensitisation was present in wild-type mice (mean slope co-efficient = 7.40 ± 2.38) and mutant mice (mean slope co-efficient = 3.30 ± 1.45) and the slope co-efficients did not significantly differ between genotypes ($t = 1.51$, df = 15, $p = 0.15$).

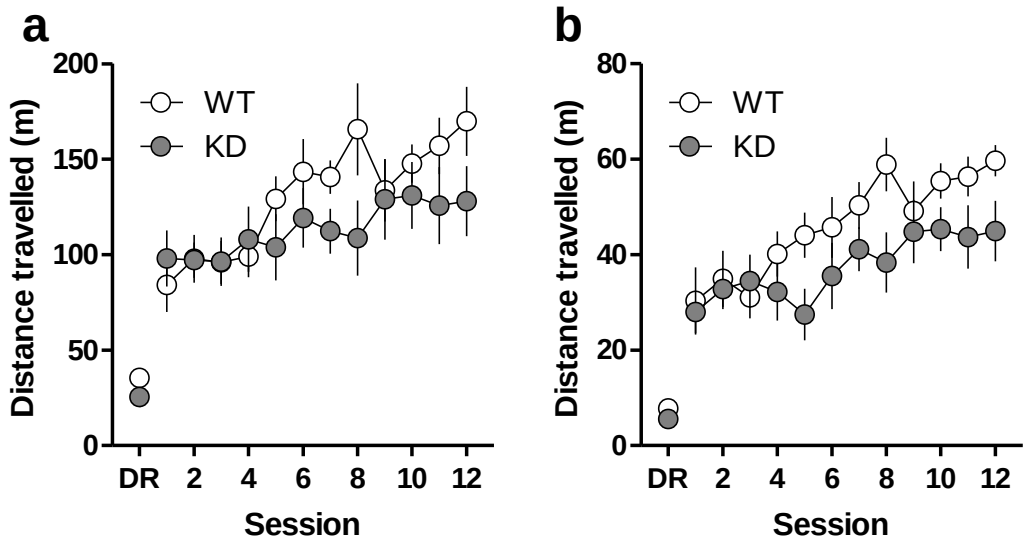


Figure 5.4 Cocaine locomotor sensitisation (**a**) Repeated, intermittent 10 mg/kg cocaine augmented mean total locomotor activity, during 60 min sessions, in both WT and KD mice. (**b**) Activity during the first 10 min period following repeated, intermittent 10 mg/kg cocaine is shown. The acute locomotor response to 10 mg/kg cocaine, obtained during the dose-response test, is shown in both figures (DR) (n = 8/9, WT/KD).

5.3.1.4 Conditioned activity

The comparison of activity following the post-sensitisation vehicle injection with activity following the pre-sensitisation vehicle injection was restricted to the first 30 min post-injection because of unstable activity in the pre-sensitisation test at 30-60 min post-injection (Fig. 5.5a). Mean total activity in this period (Fig. 5.5b) was significantly increased following sensitisation (main effect of Sensitisation, $F(1,15) = 9.64$, $p < 0.01$), and did not differ between genotypes (main effect of Genotype, NS; Genotype x Session interaction, NS).

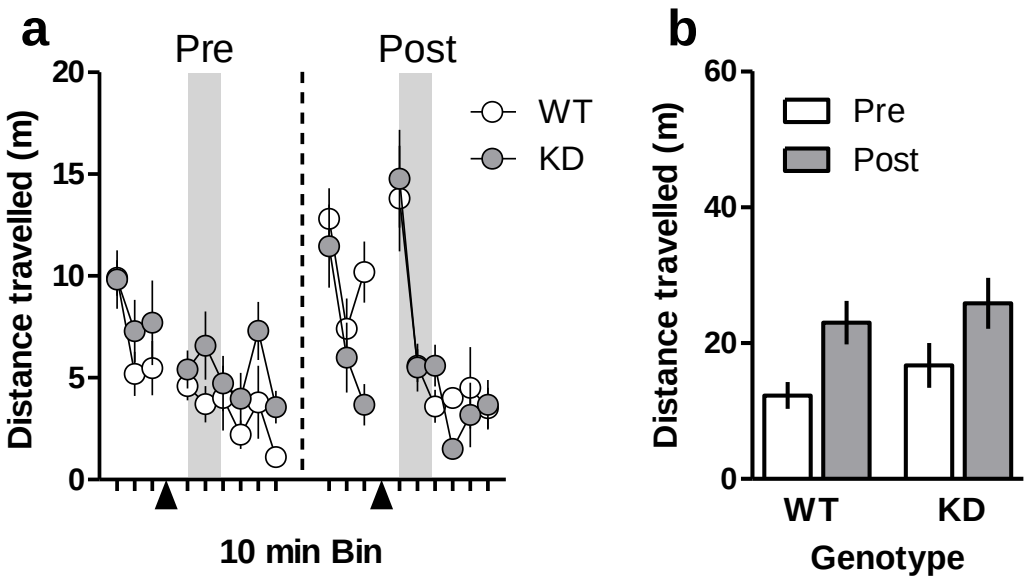


Figure 5.5 Conditioned activity in the runways in which cocaine was experienced. (**a**) The complete activity time-course is shown for a session before any cocaine was experienced (Pre) and during a session after cocaine sensitisation (post). In both sessions, mice were injected with saline (▲) after a 30 min habituation period and activity recorded for a further 60 min post-injection. The comparison of activity pre- and post-sensitisation was restricted to mean total activity from the first 30 min post-injection period (shaded area). (**b**) In both genotypes, mean total activity during the first 30 min period following a post-sensitisation saline injection (Post) was increased in comparison to activity during the same period following a pre-sensitisation saline injection (Pre) (n = 8/9, WT/KD).

5.3.1.5 Cocaine-locomotor dose-response (post-sensitisation)

Mean total locomotor activity was increased by cocaine (Fig. 5.6a; main effect of Dose, $F(3,45) = 107.25$, $p < 0.001$) and by a similar extent in both genotypes (main effect of Genotype, NS; Genotype x Dose interaction, NS). *Post-hoc* comparisons of activity between each cocaine dose with vehicle confirmed that cocaine significantly increased activity at the 10 mg/kg (WT, $t = -5.81$, df = 7, $p < 0.017$; KD, $t = -6.46$, df = 8, $p < 0.017$) and 20 mg/kg (WT, $t = -8.68$, df = 8, $p < 0.017$; KD: $t = -8.23$, df = 8, $p < 0.017$) doses. Activity time-course profiles following cocaine and vehicle injections were plotted (Fig 5.6b) and, for brevity, analyses of these data are summarised in table format (Table 5.2: Time-course analysis). Most notably, the effect of acute 20 mg/kg cocaine on locomotor activation was attenuated in mutant mice, in comparison to wild-types.

The effect of sensitisation on the acute locomotor response to cocaine was examined by comparing activity following post-sensitisation cocaine injections, with activity following pre-sensitisation cocaine injections (Fig. 5.6c). This comparison was restricted to the activity from the first 10 min post-injection period (i.e. where the locomotor stimulating effects of cocaine were greatest) and also included post-vehicle injection activity, to provide an additional measure of conditioned activity. Analyses of these data are summarised in table format (Table 5.2: Pre vs. Post analysis). Notably, following sensitisation, activity was significantly increased in both genotypes by all cocaine doses (including vehicle). However, at 20 mg/kg cocaine, the locomotor stimulating effect of cocaine was attenuated in mutant mice, relative to their wild-type counterparts. Subsequent between-genotype comparisons confirmed that psychomotor activation following 20mg/kg cocaine was attenuated in mutant mice pre-sensitisation ($t = 3.93$, df = 15, $p < 0.01$) and tended to be attenuated post-sensitisation ($t = 1.88$, df = 15, $p = 0.08$).

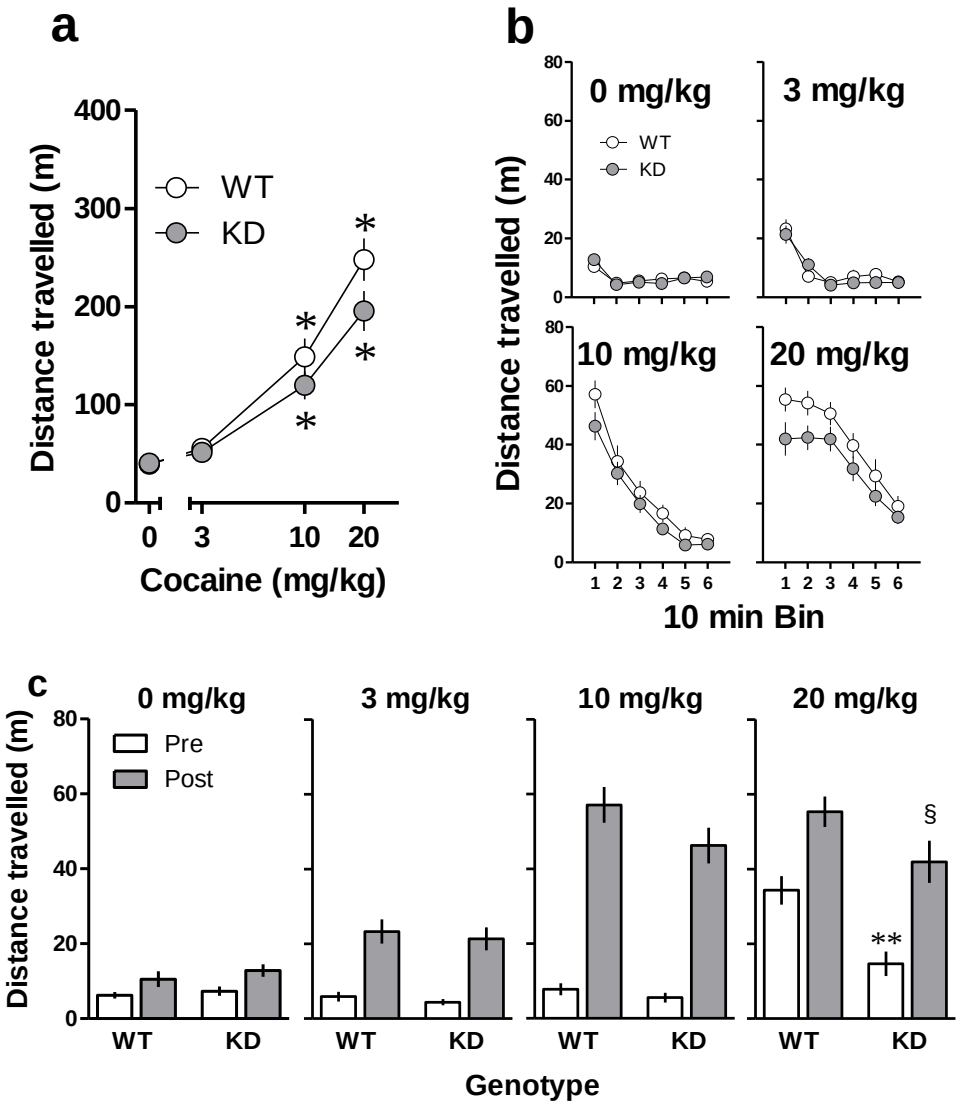


Figure 5.6 Cocaine-locomotor dose-response tests (post-sensitisation). (**a**) In both genotypes, cocaine significantly increased mean total activity at the 10 and 20 mg/kg doses. *$p < 0.017$, within-genotype, Bonferroni *t*-test comparison between each cocaine dose and vehicle (**b**) Time-course plots of activity at each dose. There was a trend for an overall reduction in activity in mutant mice at the 20 mg/kg cocaine dose. (**c**) In the first 10 min period post-injection, activity was significantly increased in both genotypes at all doses after cocaine sensitisation (Post), in comparison to the same period before sensitisation (Pre). At the 20 mg/kg cocaine dose, activity was reduced in mutant mice both pre- and post-sensitisation. **$p < 0.01$, $^{§}p < 0.10$, between-genotype *t*-test comparisons of activity pre- or post- sensitisation.

Time-course analysis	Period F(5,75)	Period x Genotype F(5,75)	Genotype F(1,15)
Vehicle	9.69***	0.73	0.02
3 mg/kg cocaine	39.71***	1.35	0.15
10 mg/kg cocaine	116.91***	0.99	1.55
20 mg/kg cocaine	53.65***	0.94	3.08§
Pre vs. Post analysis	**Sensitisation F(1,15)**	**Sensitisation x Genotype F(1,15)**	**Genotype F(1,15)**
Vehicle	16.98***	0.26	0.87
3 mg/kg cocaine	45.43***	0.008	0.66
10 mg/kg cocaine	216.82***	1.98	2.72
20 mg/kg cocaine	43.76***	0.72	11.1**

Table 5.2 ANOVAs for cocaine-locomotor dose-response tests (post-sensitisation). Factors: Period (1-6); Sensitisation (Pre, Post); Genotype (WT, KD). ***$p < 0.001$, **$p < 0.01$, §$p < 0.10$

5.3.1.6 Cocaine-locomotor dose-response (2 months post-sensitisation)

Mean total locomotor activity was increased by cocaine (Fig. 5.7a; main effect of Dose, $F(3,45) = 73.77$, $p < 0.001$), although the effect of cocaine on activity tended to vary between genotypes (Dose x Genotype interaction, $F(3,45) = 3.17$, $p = 0.077$). In addition, overall activity was significantly lower in mutant mice than wild-types (main effect of Genotype, $F(1,15) = 5.97$, $p < 0.05$). In both genotypes, *post-hoc* comparisons of activity between each cocaine dose with vehicle confirmed that cocaine significantly increased activity at the 10 mg/kg (WT, $t = -8.22$, df = 7, $p < 0.017$; KD, $t = -6.30$, df = 8, $p < 0.017$) and 20 mg/kg (WT, $t = -7.44$, df = 8, $p < 0.017$; KD: $t = -6.22$, df = 8, $p < 0.017$) doses. Individual between-genotype comparisons at each dose confirmed that, in comparison to wild-type mice, psychomotor activation was significantly attenuated in mutant mice following vehicle ($t = 2.93$, df = 15, $p < 0.05$) and 20 mg/kg cocaine ($t = 2.25$, df = 15, $p < 0.05$), and tended to be attenuated following 10 mg/kg cocaine ($t = 1.97$, df = 15, $p = 0.067$). Activity time-course profiles following cocaine and vehicle injections were plotted (Fig 5.7b) and, for brevity, analyses of these data are summarised in table format (Table 5.3: Time-course analysis). Most notably, the locomotor activating effects of 20 mg/kg cocaine were significantly attenuated in mutant mice and tended to be attenuated at the 10 mg/kg cocaine dose (Table 5.3: Time-course analysis and Fig 5.7b) in comparison to wild-type mice.

The persistence of sensitisation was examined by comparing activity following 2 month post-sensitisation cocaine injections, with activity following pre-sensitisation cocaine injections (Fig 5.7c). This comparison was restricted to the activity from the first 10 min post-injection period and also included post-vehicle injection activity, to provide an additional measure of conditioned activity. Analyses of these data are summarised in table format (Table 5.3: Pre vs. 2 months Post analysis). Notably, 2 months following repeated cocaine, activity was significantly increased in both genotypes at all doses, although this effect was not quite significant in the vehicle condition. In comparison to wild-type mice, the locomotor activating effects of cocaine were significantly attenuated in mutant mice at the 10mg/kg and 20 mg/kg

cocaine doses. Following 10 mg/kg cocaine (Fig. 5.7c) activity in mutant mice was attenuated, in comparison to wild-type mice, at 2 months post-sensitisation (t = 2.21, df = 15, $p < 0.05$). Following 20 mg/kg cocaine (Fig. 5.7c), activity in mutant mice was attenuated, in comparison to wild-type mice, both pre-sensitisation (t = 3.93, df = 15, $p < 0.01$) and 2 months post-sensitisation (t = 2.83, df = 15, $p <$ 0.05).

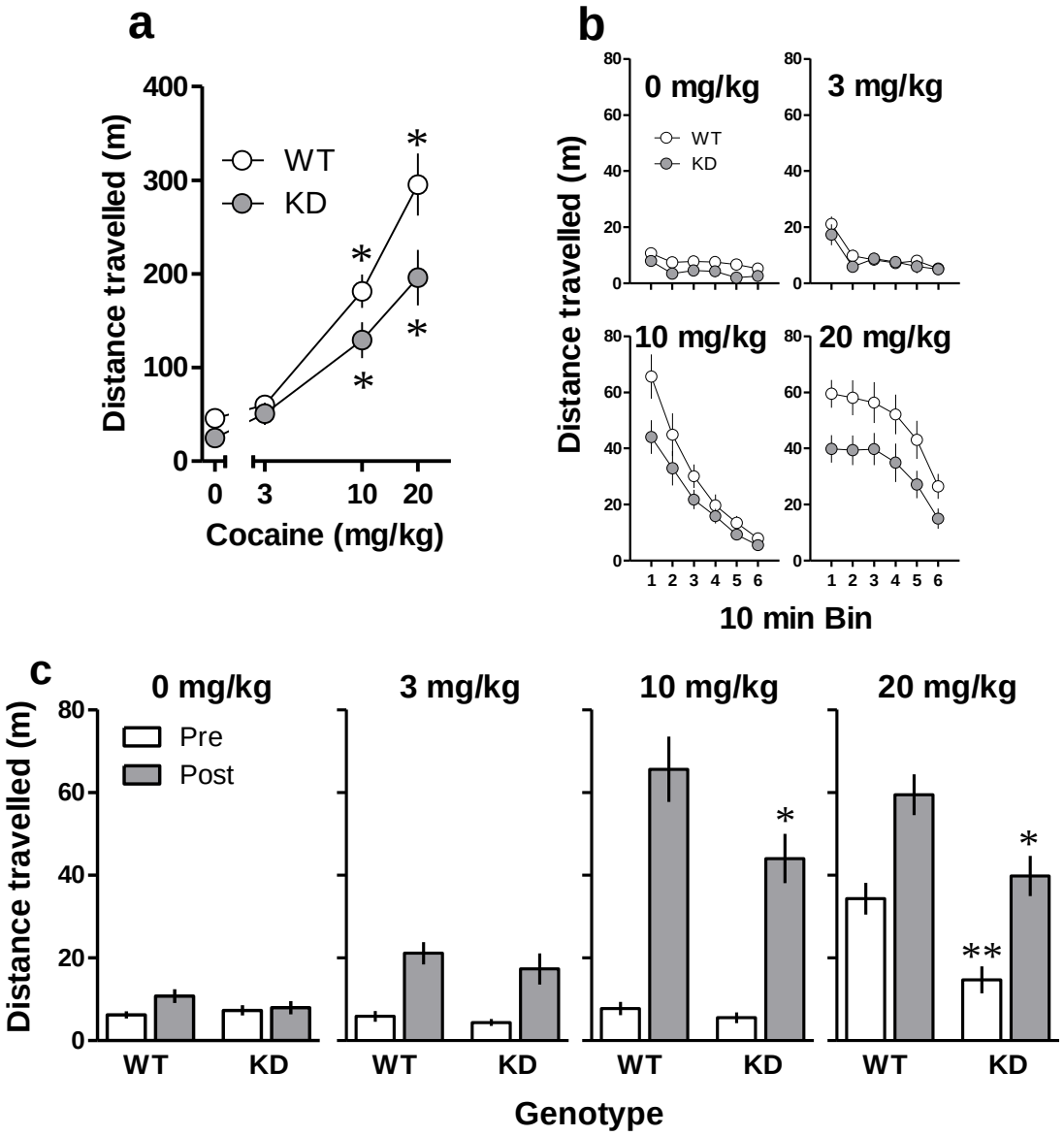


Figure 5.7 Cocaine-locomotor dose-response tests (2 months post-sensitisation). (**a**) Cocaine increased mean total activity in both genotypes, although overall activity was reduced in mutant mice. **p* < 0.017, within-genotype, Bonferroni *t*-test comparison between each cocaine dose and vehicle (**b**) Time-course plots of activity at each dose. Overall activity was reduced in mutant mice at the vehicle, 10 and 20 mg/kg cocaine doses. (**c**) In the first 10 min period post-injection, activity was increased at all doses after cocaine sensitisation (Post), in comparison to the same period before sensitisation (Pre). At the 10 and 20 mg/kg cocaine doses, activity was reduced pre and/or 2 months post-sensitisation in mutant mice, in comparison to wild-types. ***p* < 0.1, **p* < 0.05, between-genotype *t*-test comparisons of activity pre- or 2 months post-sensitisation

Time-course analysis	Period F(5,75)	Period x Genotype F(5,75)	Genotype F(1,15)
Vehicle	6.55***	0.26	8.59**
3 mg/kg cocaine	23.38***	0.92	0.42
10 mg/kg cocaine	48.56***	1.91	3.90§
20 mg/kg cocaine	37.43***	0.59	5.04*
Pre vs. 2 months Post analysis	**Sensitisation F(1,15)**	**Sensitisation x Genotype F(1,15)**	**Genotype F(1,15)**
Vehicle	3.38§	1.87	0.39
3 mg/kg cocaine	31.17***	0.20	1.15
10 mg/kg cocaine	91.48***	3.70§	5.77*
20 mg/kg cocaine	40.97***	0.00	18.19***

Table 5.3 ANOVAs for cocaine-locomotor dose-response tests (2 months post-sensitisation). Factors: Period (1-6); Sensitisation (Pre, Post); Genotype (WT, KD). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, §$p < 0.10$.

5.3.2 Experiment 2: Conditioned place preference

5.3.2.1 Food CPP

In the 20 min pre-conditioning session, base-line preference for the outer compartments (A and B) differed between the two genotypes (Fig. 5.8a; Compartment x Genotype interaction; $F(1,17) = 5.66$, $p < 0.05$). Mutant mice spent significantly more time in compartment A than wild-type mice ($t = -2.59$, df = 17, $p < 0.05$) and, conversely, tended to spend less time in compartment B than their wild-type counterparts ($t = 1.81$, df = 17, $p = 0.088$). However, once these compartments had been assigned as food-paired or food-unpaired, there was no overall difference in pre-conditioning preference between genotypes (Fig. 5.8b; main effect of Compartment, NS; Genotype, NS; Genotype x Compartment interaction, NS).

In the 20 min post-conditioning session, mice from both genotypes spent more time in the food-paired than the food-unpaired compartment (Fig. 5.8c; main effect of Compartment, $F(1,17) = 8.13$, $p < 0.05$; Genotype, NS; Genotype x Compartment interaction, NS). Subsequent within-genotype comparisons confirmed preference for the food-paired compartment, although the effect did not reach statistical significance in wild-type ($t = 2.15$, df = 9, $p = 0.06$) or mutant ($t = 1.89$, df = 8, $p = 0.095$) mice. Calculating a preference score indicated that, following conditioning, the amount of time spent in the food-paired compartment increased while the amount of time spent in the food-unpaired compartment decreased in both genotypes (Fig. 5.8d; main effect of Compartment; $F(1,17) = 15.25$, $p < 0.01$; Genotype, NS; Genotype x Compartment interaction, NS). Subsequent within-genotype comparisons confirmed that, following conditioning, preference for the food-paired compartment increased relative to a decrease in preference for the food-unpaired compartment, in both wild-type ($t = 3.10$, df = 9, $p < 0.05$) and mutant ($t = 2.45$, df = 8, $p < 0.05$) mice.

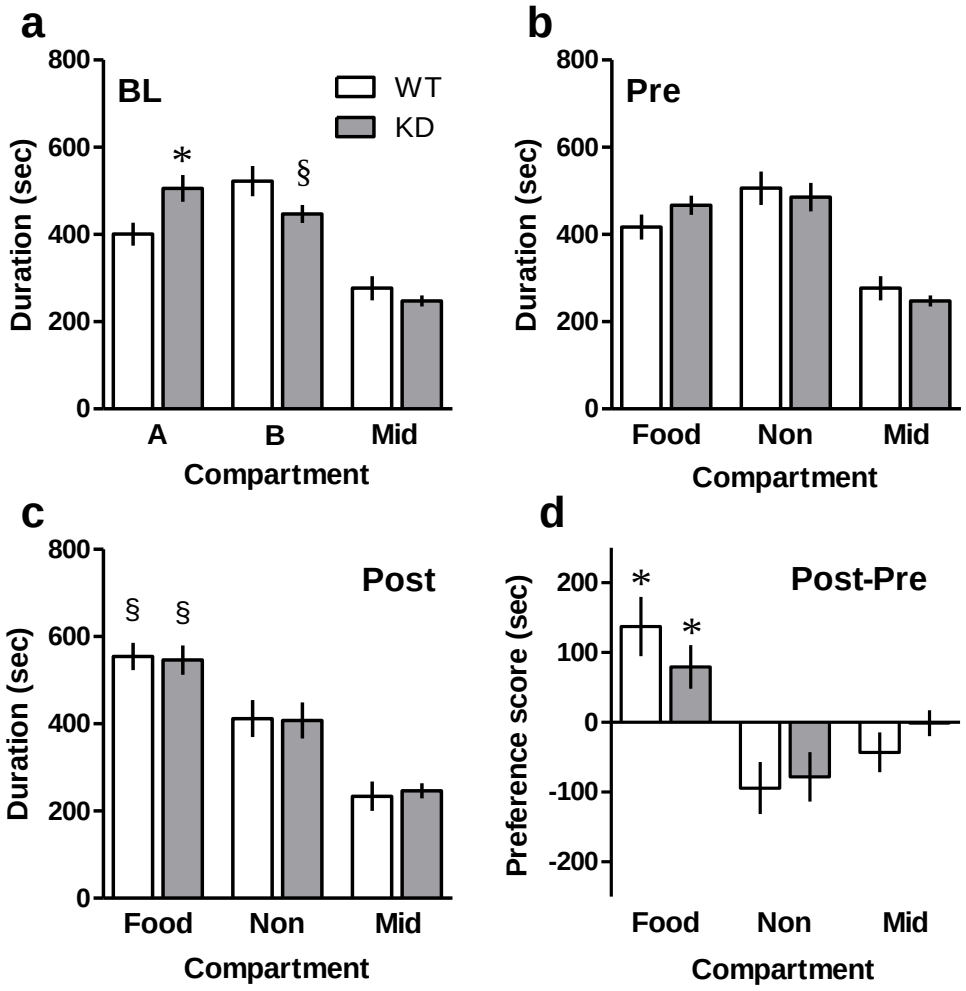


Figure 5.8 Conditioned place preference for food. (**a**) During a 20 min test of baseline preference (BL), both genotypes spent most time in the outer compartments (A and B) than the adjoining middle compartment (Mid). *$p < 0.05$, $^{\S}p < 0.1$, between-genotype *t*-test comparison of time spent in compartment A or B (**b**) Prior to conditioning (Pre), there was no between genotype difference in the time spent in each compartment assigned as food-paired (Food) or food-unpaired (Non) (**c**) Post-conditioning (Post), both genotypes spent more time in the food-paired compartment, than the food-unpaired. $^{\S}p < 0.10$, within-genotype *t*-test comparisons of time spent in the food-paired and unpaired compartments. (**d**) The preference score (post-conditioning time minus pre-conditioning time) shows that time in the food-paired compartment increased, while time in the food-unpaired context decreased, following conditioning. *$p < 0.05$, within-genotype *t*-test comparisons of food-paired and unpaired preference scores (n = 10/9, WT/KD).

In both genotypes, post-conditioning activity in the food-paired compartment was increased in comparison to post-conditioning activity in the food-unpaired compartment (Fig. 5.10a). An initial analysis of activity during pre- and post-conditioning preference tests, in both genotypes, revealed only that activity in the compartments varied with conditioning (Conditioning x Compartment interaction, $F(1,17) = 18.29$, $p = 0.001$), and further ANOVAs were performed to explore this effect. First, analysis of pre-conditioning activity in the two compartments in both genotypes confirmed that activity in the compartments did not differ depending upon the compartment identity (main effect of Compartment, NS), nor between genotypes (main effect of Genotype, NS; Genotype x Compartment interaction, NS). A similar analysis of post-conditioning activity confirmed that activity was greater in the food-paired compartment (main effect of Compartment, $F(1,17) = 6.93$), $p < 0.05$) and to a similar extent in both genotypes (main effect of Genotype, NS; Genotype x Compartment interaction, NS).

5.3.2.2 Cocaine CPP

In the 20 min pre-conditioning session, there was no overall base-line preference for the two outer compartments (A and B) in either genotype (Fig. 5.9a; main effect of Compartment, NS; Genotype, NS; Genotype x Compartment interaction, NS). Once the outer compartments had been assigned as cocaine- or vehicle-paired, there was a tendency for an overall pre-conditioning preference for the cocaine-paired compartment (Fig. 5.9b; main effect of Compartment, $F(1,13) = 3.85$, $p = 0.072$; Genotype, NS; Genotype x Compartment interaction, NS). However, subsequent within-genotype comparisons did not identify a significant pre-conditioning preference for the cocaine-paired compartment in either wild-type ($t = 1.81$, df = 6, $p = 0.12$) or mutant ($t = 0.71$, df = 7, $p = 0.50$) mice.

In the 20 min post-conditioning session, mice from both genotypes spent more time in the cocaine-paired compartment than the vehicle-paired compartment (Fig. 5.9c; main effect of Compartment, $F(1,13) = 21.60$, $p < 0.001$; Genotype, NS; Genotype x Compartment interaction, NS). Subsequent within-genotype comparisons

confirmed significant preference for the cocaine-paired compartment in both wild-type (t = 3.20, df = 6, p < 0.05) and mutant (t = 3.37, df = 7, p < 0.05) mice. Calculating a preference score indicated that, following conditioning, the amount of time spent in the cocaine-paired compartment increased while the amount of time spent in the vehicle-paired compartment decreased in both genotypes (Fig. 5.9d; main effect of Compartment; F(1,13) = 10.71, p < 0.01; Genotype, NS; Genotype x Compartment interaction, NS). Subsequent within-genotype comparisons confirmed that, following conditioning, preference for the cocaine-paired compartment increased relative to a decrease in preference for the vehicle-paired compartment, in mutant mice (t = 3.26, df = 7, p < 0.05) but not wild-type mice (t = 1.51, df = 6, p = 0.18).

In both genotypes, post-conditioning activity in the cocaine-paired compartment was increased in comparison to post-conditioning activity in the vehicle-paired compartment (Fig. 5.10b). An initial analysis of activity during pre- and post-conditioning preference tests, in both genotypes, revealed that activity in the compartments varied with conditioning (Conditioning x Compartment interaction, F(1,13) = 10.75, p < 0.01), and further ANOVA were performed to explore this effect. First, analysis of pre-conditioning activity in the two outer compartments in both genotypes confirmed that activity in the compartments did not differ depending upon the compartment identity (main effect of Compartment, NS), nor between genotypes (main effect of Genotype, NS; Genotype x Compartment interaction, NS). A similar analysis of post-conditioning activity confirmed that activity was greater in the cocaine-paired compartment (main effect of Compartment, F(1,13) = 14.57, p < 0.01) and to a similar extent in both genotypes (main effect of Genotype, NS; Genotype x Compartment interaction, NS).

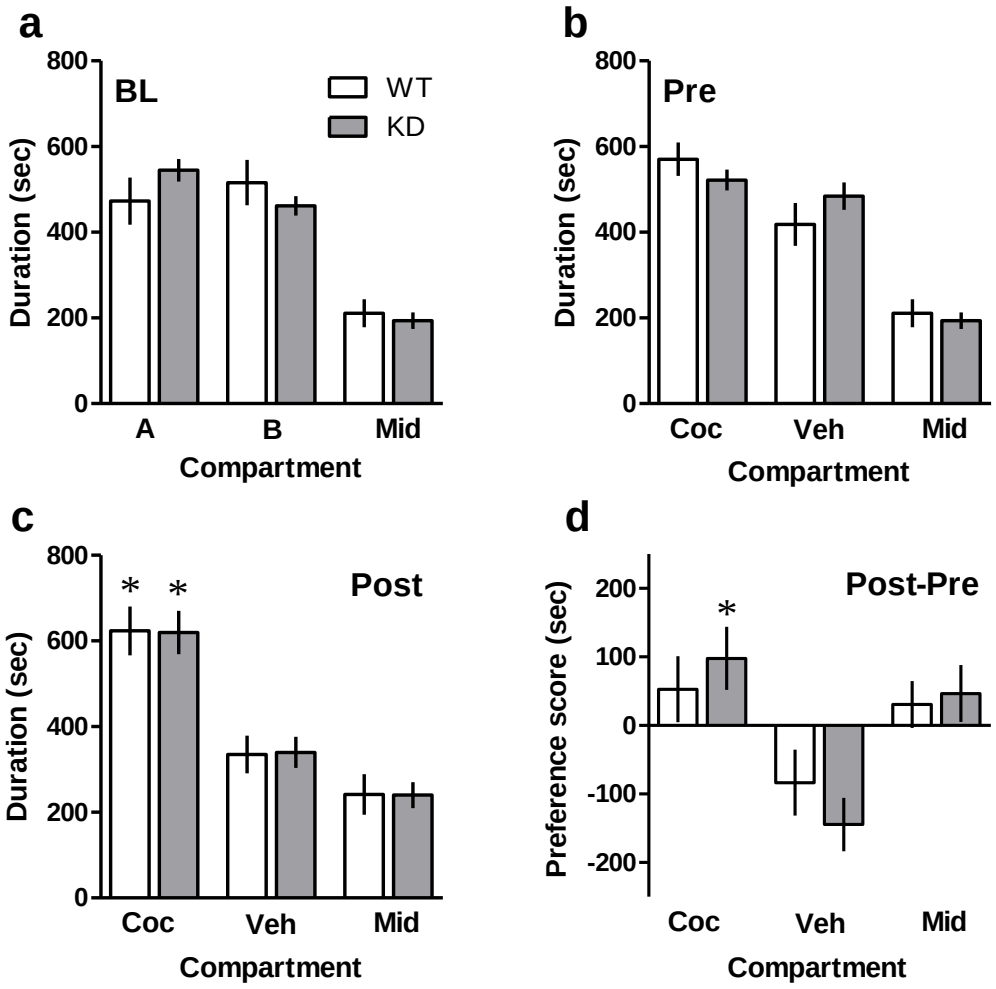


Figure 5.9 Conditioned place preference for cocaine. (**a**) During a 20 min test of baseline (BL) preference, both genotypes spent most time in the outer compartments (A and B) than the adjoining middle compartment (Mid) (**b**) Prior to conditioning (Pre), there was no significant genotype difference in the time spent in compartments assigned as cocaine-paired (Coc) or vehicle-paired (Veh) (**c**) Post-conditioning (Post), both genotypes spent more time in the cocaine-paired than the vehicle-paired compartment. *$p < 0.05$, within-genotype *t*-test comparisons of time spent in the cocaine-paired and vehicle-paired compartments (**d**) The preference score (post-conditioning time minus pre-conditioning time) shows that time in the cocaine-paired compartment increased, while time in the vehicle-paired compartment decreased, following conditioning. *$p < 0.05$, within-genotype *t*-test comparison of cocaine-paired and vehicle-paired preference scores (n = 7/8, WT/KD).

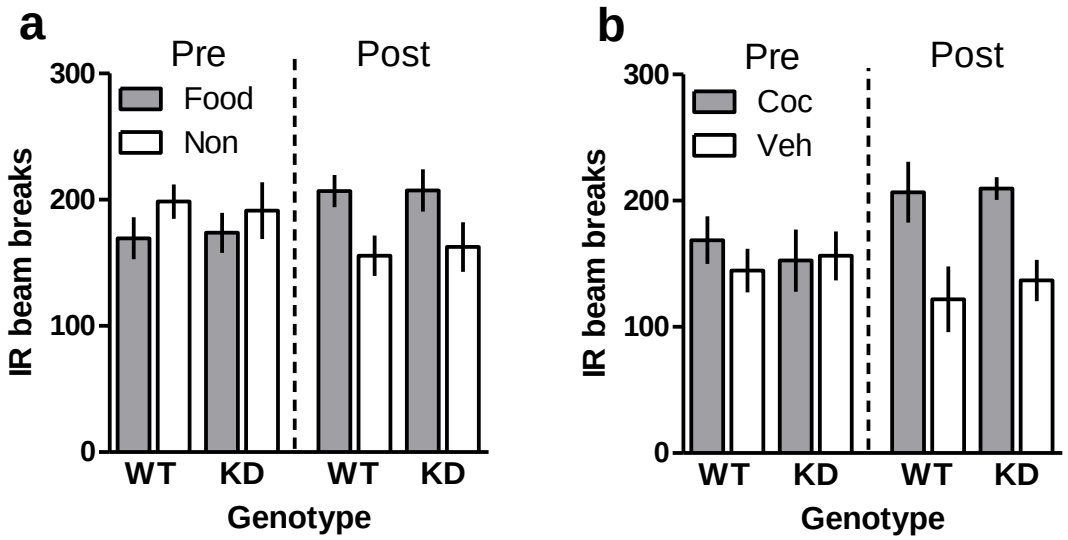


Figure 5.10 Activity during pre- and post-preference tests for the food and cocaine CPP studies. (**a**) For food CPP, in the post-conditioning test of preference (Post), activity in both genotypes was greater in the food-paired compartment (Food) than the food-unpaired compartment (Non), in both genotypes. Activity in the pre-conditioning test of preference (Pre) did not significantly differ between genotypes or compartments (n=10/9, WT/KD). (**b**) For cocaine-CPP, activity in the post-conditioning test of preference (Post) was greater in the cocaine-paired compartment (Coc) than the vehicle-paired compartment (Veh), in both genotypes. Activity in the pre-conditioning test of preference (Pre) did not significantly differ between genotypes or compartments (n = 7/8, WT/KD).

Activity in the cocaine and vehicle-paired compartments was also recorded over the eight 30 min conditioning sessions (Fig. 5.11). Overall activity was higher in the cocaine-paired compartment in both wild-type mice (main effect of Compartment, $F(1,6) = 13.19$, $p < 0.05$) and mutant mice (main effect of Compartment, $F(1,7) = 69.23$, $p < 0.001$). In addition, the profile of activity in the two outer compartments differed over conditioning sessions in mutant mice (Compartment x Session interaction, $F(3,21) = 7.12$, $p < 0.05$), but not wild-type mice (Compartment x Session interaction, NS). In mutant mice, activity in the cocaine-paired compartment increased over conditioning sessions (main effect of Session, $F(3,21) = 6.83$, $p < 0.05$), while activity in the vehicle-paired compartment did not change (main effect of Session, NS).

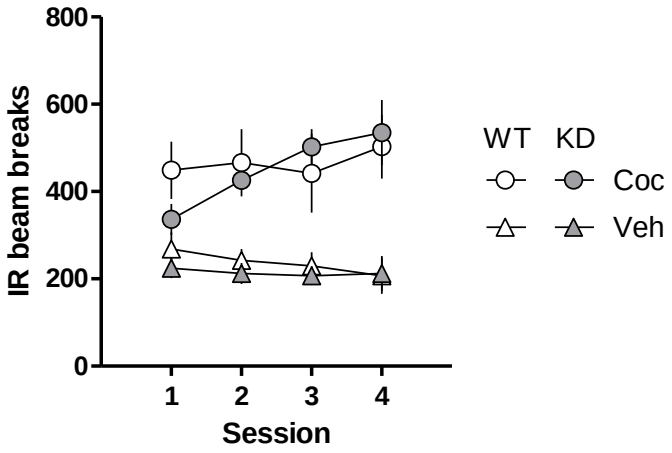


Figure 5.11 Activity during conditioning sessions in the cocaine CPP study. In both genotypes, overall activity (indexed by interruptions of two infra-red beams located in each compartment) was higher in the cocaine-paired (Coc) than the vehicle-paired compartment (Veh). In mutant mice, activity in the cocaine-paired compartment increased over conditioning sessions while activity in the vehicle-paired compartment did not change. In wild-type mice, activity in both compartments did not significantly change over the course of conditioning (n = 7/8, WT/KD)

5.4 Discussion

The present data indicate that knock-down of mGluR5, on cells that receive dopaminergic input via D1Rs, results in a marked attenuation of the acute psychomotor activating effects of cocaine. However, repeated intermittent cocaine does induce behavioural sensitisation in mGluR5$^{KD\text{-}D1}$ mice that persists for at least two months after the final cocaine injection. In the CPP studies, mGluR5$^{KD\text{-}D1}$ mice showed preference for an environment paired with food availability and non-contingent cocaine, indicating that mGluR5 on dopaminoceptive neurons are not required for learning about natural or drug rewards that supports place preference.

As reported previously (Chapter 2), mGluR5$^{KD\text{-}D1}$ mice showed reduced activity in a novel environment, relative to wild-type mice, which is in agreement with studies showing that the mGluR5 antagonist MPEP reduces spontaneous locomotor activity in rats (Spooren et al., 2000a; Herzig and Schmidt, 2004), but does contrast with spontaneous hyperlocomotion reported in mGluR5 knock-out mice (Bird et al., 2010; Olsen et al., 2010). Notably, the group I mGluR agonist (S)-DHPG elevated motor activity after micro-injection into either the VTA or NAcc (Swanson and Kalivas, 2000) and, in the NAcc, the group I mGluR antagonist S-4-CPG prevented increases in locomotor activity produced by the D1-like receptor agonist SKF 38393, while having no effect when injected alone (David and Abraini, 2001). In contrast, S-4-CPG had no effect on locomotor responses produced by the selective D2-like receptor agonist LY17155 (David and Abraini, 2001). Taken together, our data with mGluR5$^{KD\text{-}D1}$ mice provides further evidence that spontaneous activity in a novel environment may require the integrity of dopamine and glutamate signalling on neurons that project monosynaptically to the substantia nigra pars reticulata (SNr) and which constitute the ‘direct’ striatonigral pathway (Alexander and Crutcher, 1990).

A critical finding in the present series of experiments was that the acute locomotor activating effects of cocaine were attenuated in mGluR5$^{KD\text{-}D1}$ mice, both before and

following the induction of sensitisation. The psychomotor effects of stimulants are often strongly dependent on the novelty of the environment in which the stimulant drug was administered (Badiani et al., 1995), and perhaps the decreased locomotor response of mGluR5^{KD-D1} mice to acute cocaine was due, in part, to the reduced response to the novelty of the test environment. Although mutant mice were thoroughly habituated to the apparatus before the cocaine-locomotor dose-response test was conducted, differences in baseline locomotor activity were still occasionally apparent between mGluR5^{KD-D1} and wild-type mice (for example, Fig 5.7a, but see Fig. 5.3b & 5.6a), suggesting that an interaction between the drug and environment could contributing to differences in the locomotor activating effects of cocaine between genotypes. It would certainly be of interest to examine the effect of cocaine in mGluR5^{KD-D1} mice while situated in their home cages. Against this general idea is the finding that mGluR5 knockout mice are hyper-responsive in a novel environment, yet show a blunted locomotor response to acute cocaine (Chiamulera et al., 2001; Olsen et al., 2010), indicating that mGluR5 may be integral for the acute psychomotor activating effects of cocaine.

The locomotor activating effects of psychostimulants, such as cocaine, are typically attributed to their enhancement of dopamine neurotransmission in mesolimbic brain regions (Swerdlow et al., 1986; Delfs et al., 1990; Neisewander et al., 1995; Giros et al., 1996). There is much evidence to suggest that striatal mGluRs, including mGluR5, interact with dopamine to regulate the locomotor response to stimulant drugs (Meeker et al., 1998; Verma and Moghaddam, 1998). For example, although systemic MPEP is without effect on basal extracellular striatal dopamine (Golembiowska et al., 2003; Tronci and Balfour, 2011), it can block increases in extracellular striatal dopamine produced by nicotine (Tronci and Balfour, 2011). It is also noteworthy that extracellular striatal dopamine is increased by intra-striatal infusions of MPEP (Golembiowska et al., 2003), but not by a group I mGluR agonist (Hu et al., 1999; although see Canales et al., 2003). Taken together, these data might suggest that loss of mGluR5 on striatal D1-MSNs may result in altered dopaminergic transmission in response to psychostimulants which, in turn, results

in a reduction of locomotor activity. Although glutamate spillover from corticostriatal synapses can depress DA transmission, this effect is most likely attributable to pre-synaptic group I mGluR receptors (Zhang and Sulzer, 2003). How loss of (post-synaptic) mGluR5 on D1R expressing neurons may translate to disrupted dopaminergic signalling in response to psychostimulants at a synaptic level is not clear but, at a network level, could involve altered input of GABAergic MSNs onto the ventral midbrain.

In addition to elevating extracellular striatal dopamine, cocaine also results in elevated glutamate in the ventral striatum (Smith et al., 1995; Reid and Berger, 1996; Reid et al., 1997; Zhang et al., 2001) and block of glutamate receptors in the NAcc disrupts the acute locomotor activating effects of psychostimulants (Pulvirenti et al., 1989, 1991). Although mGluR5 is typically located post-synaptically on MSNs in the striatum (Tallaksen-Greene et al., 1998), it can influence pre-synaptic glutamate release (Thomas et al., 2001a) through retrograde endocannabinoid signalling (Robbe et al., 2001). Thus, in addition to the potential for disrupted dopaminergic transmission in mGluR5^{KD-D1} mice, loss of mGluR5 in MSNs may have also translated to disrupted glutamatergic transmission in response to cocaine. Further studies (for example, microdialysis or voltammetry) are required to directly test whether the (pre-synaptic) dopaminergic and/or glutamatergic response to cocaine is attenuated in mGluR5^{KD-D1} mice, but it is interesting to note that mGluR5 knockout mice show elevations in cocaine induced extracellular striatal dopamine that are comparable to wild-type mice (Chiamulera et al., 2001), which perhaps favours a glutamatergic dysregulation account.

Finally, a third, but not entirely incongruent hypothesis that could explain the attenuated psychomotor response to cocaine in mGluR5^{KD-D1} mice comes from recognising that the locomotor activating effects of psychostimulants typically require a substantial D1-like receptor, but not D2-like receptor component (Moratalla et al., 1996a; Fritts et al., 1997; Badiani et al., 1999; O'Neill and Shaw, 1999; Kim et al., 2001). As mentioned above, intra-NAcc antagonism of group I

mGluRs alters motor responses arising from stimulation of NAcc D1-like receptors, but not D2-like receptors (David and Abraini, 2001), but also suppresses the acute locomotor activating effects of intra-NAcc amphetamine (David and Abraini, 2003). When our present data with mGluR5^{KD-D1} mice are placed in the context of these reports, there is compelling evidence to propose that the acute locomotor activating effects of cocaine are critically dependent upon the (post-synaptic) interaction of mGluR5 with D1-like, but not D2-like receptors on striatonigral MSNs.

With repeated intermittent cocaine infusions the locomotor response (measured by distance travelled) to cocaine was augmented in both genotypes. Thus, mGluR5^{KD-D1} mice did show behavioural sensitisation to cocaine. This finding is surprising on a number of accounts. First, a role of mGluR5 in the induction and expression of cocaine sensitisation has been demonstrated using an mGluR5 antagonist (Kotlinska and Bochenski, 2009; Veeneman et al., 2010) (but see, Dravolina et al., 2006). Second, the role of glutamate transmission through ionotropic NMDA and AMPA receptors in the induction and expression of psychostimulant sensitisation is well established (e.g. Karler et al., 1989; Karler et al., 1990; Karler et al., 1991; Kalivas and Alesdatter, 1993; Stewart and Druhan, 1993) and mutation of NMDA on D1R expressing neurons (i.e. non-dopaminergic neurons) prevents the induction of cocaine sensitisation (Heusner and Palmiter, 2005), while knock-out of NMDA on dopaminergic neurons is without effect (Engblom et al., 2008). Because mGluR5 can powerfully modulate NMDA-mediated responses (Pisani et al., 2001) and can also influence AMPA receptor expression (Zhang et al., 2008), these reports would predict that mGluR5, particularly on D1R expressing neurons, should play a role in behavioural sensitisation. Finally, the propensity to to attribute incentive salience to environmental stimuli associated with reward experience has been associated with susceptibility to behavioural sensitisation (Flagel et al., 2008). Thus, given clear incentive learning deficits in mGluR5^{KD-D1} mice (Chapter 3), impaired behavioural sensitisation might have been expected.

Could it be that the experimental design in the present study was not sufficient for detecting differences in sensitisation between genotypes? Locomotor activity is often considered as a relatively insensitive indicator of behavioural sensitisation (Ferrario et al., 2005; Flagel and Robinson, 2007; Flagel et al., 2008) and it would be valuable to examine more detailed motor behaviours in mice during the induction of sensitisation, such as stereotypy, rearing or head bobbing (Tolliver and Carney, 1994). In addition, it has been proposed that the relative degree of sensitisation (that is, the change in activity over sessions) may be a critical measure of neurobehavioural plasticity, rather than whether or not a drug produces sensitisation *per se* (Ferrario et al., 2005; Flagel and Robinson, 2007; Flagel et al., 2008). The degree of sensitisation was lower in mGluR5$^{KD\text{-}D1}$ mice (see comparison of slopes over sessions 1-11), but did not significantly differ from wild-type mice. However, it was clear that a robust sensitised response was seen in both genotypes following the first 10mg/kg cocaine injection of the sensitisation test, indicating that exposure to the cocaine-locomotor dose-response test had produced some neural adaptations necessary for supporting sensitisation. Thus, the comparison of slopes in the present study was unable to take into account the initial induction period of sensitisation. Thus, while the present data suggest no differences in behavioural sensitisation between genotypes, additional studies are warranted to assure this finding.

Sensitisation involves both unconditioned drug effects on motor activity and conditioned responses to the context in which the animals receive the drug (Pert et al., 1990; Crombag et al., 1996; Stephens, 2006). Thus, the environment in which the drug was experienced can act as a CS, causing behavioural activation even in the absence of the drug (Stewart, 1983). In the present report, conditioned activity was observed in both genotypes, and to a similar extent, when mice were injected with saline and placed into the runway in which cocaine had previously been experienced. A potential confound of the conditioned activity test is that conditioned activity was determined by comparing activity in the chambers after cocaine had been experienced with activity in the same animals, but before

cocaine was first experienced (i.e. activity in the sham vehicle injection session). It could be conditioned activity was simply a reflection of a sensitised stress response as a result of repeated injections in the runway, rather than due to acquired associations between the runway and the effects of cocaine. Although this possibility cannot be ruled out, it seems unlikely given that repeated injections failed to give rise to hyperlocomotion during the four saline conditioning trials in the CPP test.

The second series of experiments examined food- and cocaine-CPP. In both CPP tests, mutant mice displayed preference for the environment previously associated with the reward. Thus, mGluR5 on D1R expressing neurons is not necessary for learning that supports performance in the place preference test. But, what learning is required to support place preference? The ability of the paired context to generate approach behaviour in a CPP test of preference could reflect any number of distinct psychological processes (Robbins and Everitt, 2002). Contextual stimuli may acquire the ability to elicit simple Pavlovian approach (i.e. sign-tracking) responses (Tomie, 1996; Krank, 2003; Mead et al., 2005; Cunningham and Patel, 2007), or may act as conditioned reinforcers that strengthen the instrumental locomotor response of approaching and contacting the paired compartment during the CPP choice test (Everitt et al., 1991). Alternatively, the reward associated stimuli may acquire incentive motivational properties that energize the sign-tracking response or the instrumentally reinforced approach response. Indeed, despite CPP being described as a measure of conditioned reward, it is plausible that incentive learning is not required for CPP and the reward-paired compartment simply acts as a predictor of reward availability. Any or all of these processes may be contributing to the development and expression of place preference, and deficits in any one learning strategy may be compensated for by another. Nevertheless, that food- and cocaine-CPP are normal in mGluR5$^{KD\text{-}D1}$ mice suggests that incentive learning necessary for CRf and sign-tracking is not necessary for performance in the CPP choice test. This proposal garners some support from studies identifying distinct

neuronal nodes involved in the reconsolidation of CPP and CRf related memories (Theberge et al., 2010).

A potential concern over the CPP paradigm is that, since animals typically prefer a novel context over a familiar one (Hughes, 1968), place preference may be influenced by novelty exploration during the choice test. This may occur in one of two ways. First, in the three-compartment CPP procedure used in the present report, the middle choice compartment remains relatively novel as animals are only exposed to this compartment during tests of preference. Exploration of the central compartment during a test of preference may further enhance food- or drug-CPP, by reducing time spent in the relatively less novel unpaired compartment, whilst having less of an effect on time spent in the reward-paired compartment (Bardo et al., 1995). In the current CPP studies, preference was determined by comparing time spent in the two outer compartments, and it is plausible that exploration of the central compartment differentially contributed to place preference seen in both genotypes. However, in both CPP tests, a comparison of time spent in the middle compartment before and after conditioning failed to find any significant difference between genotypes (data not shown), indicating that enhanced exploration of the middle compartment did not contribute to CPP in the present studies. Second, an argument based on state-dependency, would propose that the drug-paired compartment is more novel on the test day because the compartment is experienced in a drug-free state. Although this account cannot be ruled out in the present study, since we did not look at preference when animals were in a drugged state, other studies have shown that animals prefer the drug-paired compartment regardless of whether they are tested with or without drug (Mucha and Iversen, 1984), which provides some evidence to discount this novelty effect.

In the cocaine CPP study, preference for the cocaine-paired compartment was not particularly robust in wild-type mice when assessed using a preference score (that is, post- minus pre- conditioning preference). This study used a non-biased CPP design, in which no preference for either outer compartment existed prior to, or

following the assignment of compartments as cocaine-paired or vehicle-paired. However, a small (albeit non-significant) bias for the cocaine-paired compartment was present in wild-type mice during the pre-conditioning test. A pre-conditioning bias may influence the development of CPP in two ways. First, a “motivational interaction hypothesis” would suggest that a drug interacts with some unconditioned motivational state reflected in the initial compartment bias (Cunningham et al., 2003). Thus, a drug might support CPP by reducing ‘fear’ associated with a non-preferred environment or, conversely, potentiate the motivational value of a preferred environment (Schenk et al., 1985; Cunningham et al., 2003). Second, pairing a drug with an initially non-preferred compartment may simply provide a greater window to measure CPP and, conversely, pairing the drug with an initially preferred compartment may obscure any conditioning properties of the drug due to a ceiling effect (Cunningham et al., 2003). This second idea seems more probable in explaining the weak cocaine CPP in wild-type mice, when assessed by calculation of a preference score, since CPP was clearly robust when comparing only post-conditioning preference between the vehicle- and cocaine-paired compartments.

One advantage of the CPP apparatus used here is that locomotor activity during preference tests and conditioning trials can be measured. Confirming findings from the cocaine behavioural sensitisation study, mutant mice displayed a sensitised locomotor response to cocaine over conditioning trials. Cocaine did augment locomotor activity in wild-type mice, but did not produce locomotor sensitisation over conditioning trials. It is unlikely that the lack of robust sensitisation in wild-type mice explains the weak cocaine-CPP in these animals, since locomotor sensitisation and CPP can be considered as dissociable phenomena (Shimosato and Ohkuma, 2000). During the food-CPP study, activity tended to be reduced in the food-paired compartment relative to the food-unpaired compartment during conditioning trials in both genotypes (data not shown), which probably reflects the occurrence of food consumption competing with exploratory behaviour during these conditioning trials. It is also important to note that, in both genotypes, post-

conditioning activity in the cocaine-paired and food-paired compartments was increased in comparison to the non-reward-paired compartments. These data confirm and extend the findings of the cocaine conditioned activity study above by demonstrating that both a food and cocaine-paired environment can stimulate conditioned activity in mGluR5^{KD-D1} mice in a manner indistinguishable from wild-type mice.

Although a role for glutamate signalling through ionotropic NMDA and AMPA receptors in the acquisition and/or expression of cocaine-CPP has been established (e.g. Cervo and Samanin, 1995), previous studies exploring the role of mGluR5 in conditioned drug reward using MPEP have produced conflicting results. Thus MPEP has been found to reduce the acquisition of cocaine CPP in rats in one study (McGeehan and Olive, 2003), but not in another (Herzig and Schmidt, 2004). These findings may be explained, in part, by the intrinsic reinforcing properties of MPEP producing drug-substitution like effects (van der Kam et al., 2009b, a; Rutten et al., 2010). Our data with mGluR5^{KD-D1} mice indicate that mGluR5 on D1R expressing neurons are not necessary for learning that supports cocaine-CPP and this result is in agreement with a recent study using mGluR5 KO mice in which cocaine CPP was normal (Olsen et al., 2010). It has been proposed that loss of mGluR5 may potentiate the rewarding properties of (Rutten et al., 2010) or increase behavioural sensitivity to (Bird et al., 2008) some addictive drugs. Our cocaine-CPP study does not exclude the possibility that cocaine reward is potentiated in mGluR5^{KD-D1} mice (i.e. a leftward-shift of the minimal effective dose required to induce CPP). However, it is worthwhile noting that in a self-administration preparation the fixed-ratio cocaine dose-response profile in mGluR5^{KD-D1} mice is indistinguishable from wild-type mice (Novak et al., 2010), suggesting that mGluR5^{KD-D1} mice do not show enhanced sensitivity to the primary reinforcing effects of cocaine.

Finally, our findings do not exclude the possibility that mGluR5 on D1R expressing neurons may be involved in other behavioural effects of cocaine. For example, this

population of mGluR5 could contribute to extinction learning in the CPP paradigm (but not extinction of instrumental responding; Novak et al., 2010), or the reinstatement of CPP. Some evidence for this first hypothesis comes from appreciating the importance of glutamate in extinction learning. Thus, enhancement of glutamate transmission with an NMDA partial agonist (D-cycloserine) can facilitate the extinction of conditioned fear and drug-associated memories (Botreau et al., 2006; Davis et al., 2006). In mice lacking GluR1 or NR1 selectively in dopamine neurons, both cocaine CPP and sensitisation was normal, but extinction of cocaine CPP and reinstatement of CPP by cocaine were abolished in GluR1^{DATCre} and NR1^{DATCre} mice, respectively (Engblom et al., 2008). Similarly, GluR1 knock-out mice show extinction deficits, despite normal cocaine self-administration (Mead and Stephens, 2003b) and viral over-expression of GluR1 and GluR2 in the NAcc facilitates extinction learning (Sutton et al., 2003). Because mGluR5 is closely linked to NMDA and AMPA (see discussion above), it would seem plausible that activation of mGluR5 could facilitate the extinction of cocaine-CPP memories. In addition, knock-out of neuronal activity regulated pentraxin (NARP), a neuronal IEG that encodes a secreted protein which may influence AMPA trafficking or clustering (O'Brien et al., 1999), also interferes with inhibitory learning necessary for extinction, but does not disrupt the initial acquisition of a conditioned association (Crombag et al., 2009). This observation is interesting with respect to this thesis, since neuronal pentraxins play a necessary role in LTD induced by group I mGluR stimulation (Cho et al., 2008). More direct evidence for a role of mGluR5 in extinction learning comes from a recent study by Gass and Olive (2009), who demonstrated that a positive allosteric modulator of mGluR5, CDPPB, facilitated extinction of cocaine-CPP. This effect was reversed by MTEP and MK801, suggesting a contribution of both mGluR5 and NMDA receptors. The authors noted that the neuroanatomical regions that mediate these effects are not clear, but likely include the infra-limbic prefrontal cortex (Zavala et al., 2003; Hsu and Packard, 2008), BLA (Fuchs et al., 2002) or hippocampus (Meyers et al., 2003), given the role of these structures in extinction learning, stimulus-reward learning and drug-context associations in CPP, respectively. It

would be of great interest to examine extinction learning in mGluR5^{KD-D1} mice to further probe the potential neuroanatomical location of mGluR5 involved in this effect.

In summary, the present study identifies an important role of mGluR5 on neurons that receive dopamine input in the acute psychomotor activating effects of cocaine. In contrast, this population of mGluR5 is not required for neuroplastic changes that support learning about cocaine conditioned reward, measured by CPP, and are not necessary for cocaine sensitisation. However, this latter finding should be interpreted with caution given the potential for differences in the degree of sensitisation to be revealed using more sensitive measures of motor activity. Whether the reduced locomotor effects of cocaine can be ascribed to altered pre-synaptic glutamate and/or dopamine transmission in the striatum and/or disrupted output of neurons that constitute the direct nigrostriatal pathway remains to be determined. However, psychostimulants, such as cocaine are known to produce robust neurochemical changes in the striatum (Reith et al., 1986; Di Chiara and Imperato, 1988; Reid et al., 1997), which in turn lead to the induction of post-synaptic signalling cascades (Graybiel et al., 1990; Young et al., 1991; Valjent et al., 2005). If mGluR5^{KD-D1} mice have impaired regulation of pre- or post-synaptic signalling in the striatum, then this may be revealed by examining activation of intra-cellular signalling cascades in this brain region in response to acute cocaine challenge. This possibility will be explored in the next and final experimental chapter.

6 Some neurobiological effects of cocaine in mGluR5^{KD-D1} mice

6.1 Introduction

The most notable finding of the previous chapter was that the acute psychomotor activating effects of cocaine were significantly attenuated in mice lacking mGluR5 on cells that receive dopamine input via D1Rs. If some behavioural effects of cocaine, which involve glutamate and dopamine signalling in the striatum, are diminished, it may be expected that some neurobiological effects of cocaine in the striatum are also disrupted in mutant mice. The following experiments will explore the ability of acute cocaine to produce rapid changes in synaptic function in the striatum and to activate an intracellular signalling cascade in striatal MSNs, which may be required for cocaine to produce long lasting changes in synaptic structure and function.

As stated in the preceding chapter, cocaine, like many addictive drugs, can increase levels of extracellular dopamine and glutamate in the accumbens (Di Chiara and Imperato, 1988; Reid et al., 1997; McKee and Meshul, 2005). Elevated dopamine and glutamate can augment locomotor activity (Kelly et al., 1975; Clarke et al., 1988; Delfs et al., 1990) and trigger intracellular signalling cascades in MSNs and alter the expression of immediate early genes (IEGs) (Young et al., 1991; Hope et al., 1992; Moratalla et al., 1993; Berke et al., 1998; Valjent et al., 2000). Some of these IEGs encode transcription factors (for example, c-fos, JunB, *zif*268) that may be associated with long lasting changes in synaptic structure and function (Bai and Kusiak, 1997; Thomas et al., 2003; Kim et al., 2009; Ren et al., 2010). Since MSNs provide the sole striatal output to motivational and motor systems (Goto and Grace, 2008), cocaine-induced adaptations in these neurons are likely to be of considerable importance for the development and maintenance of behavioural responses related to addictive processes (Berke and Hyman, 2000).

The mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway is a major effector of signal transduction from the neuronal cell surface to the nucleus (Grewal et al., 1999; Valjent et al., 2001; Adams and Sweatt, 2002) and may be a critical component enabling drug-induced adaptations in striatal MSNs (Girault et al., 2007). Inactive ERK1 (44KDa) and ERK2 (42KDa) are found mainly in somatic and dendritic compartments of neurons (Ortiz et al., 1995). Activated ERKs can phosphorylate a wide range of substrates within the cytoplasm, including membrane associated proteins such as EGF receptors, phospholipase A2 and cytoskeletal proteins, including microtubule associated proteins and neurofilaments (Grewal et al., 1999). In this way, ERKs can contribute to rapid changes in synaptic efficacy that underlie the induction of plasticity and short term memory formation. Activated ERKs can also translocate to the nucleus (Chen et al., 1992) and phosphorylate major transcriptional regulators, including ELK-1 and CREB (cyclic AMP response element binding protein), which bind to promoters SRE (serum response elements) and/or CRE (cAMP-response elements) of IEGs (Sgambato et al., 1998; Vanhoutte et al., 1999; Davis et al., 2000; Choe and McGinty, 2001; Valjent et al., 2001; Choe et al., 2002; Mattson et al., 2005; Radwanska et al., 2005; Valjent et al., 2005). This ERK1/2-mediated burst of gene transcription likely drives long term changes in synaptic function necessary for establishing and maintaining long term memories (Adams and Sweatt, 2002; Mazzucchelli et al., 2002; Selcher et al., 2002; Sweatt, 2004).

In striatal MSNs, one well characterised mechanism of ERK1/2 control involves convergent dopamine and glutamate signalling at D1Rs and NMDARs, respectively, and co-operative intracellular signalling pathways (Valjent et al., 2000; Valjent et al., 2005; Pascoli et al., 2011). One pathway involves a D1R-PKA mediated inhibition of PP-1, requiring phosphorylation of DARPP-32 at Thr-34 and resulting in de-phosphorylation of STEP46, thus preventing de-phosphorylation of ERKs (Valjent et al., 2005). This first pathway likely potentiates activity in a second pathway of ERK1/2 control that requires NMDAR-Ca^{2+}-Ras mediated activation of

MEK, resulting in activation of ERKs by phosphorylation at Thr^{202} and Tyr^{204} residues (Valjent et al., 2005; Pascoli et al., 2011). The requirement for both dopamine and glutamate signalling for ERK1/2 activation points to this pathway as a key element in plasticity of striatal neurons triggered by activity of glutamatergic neurons encoding behavioural choice and stimulus properties (Kalivas and Volkow, 2005) together with dopaminergic signals providing reward error prediction signals (Schultz et al., 1997) and/or influencing attention (Mackintosh, 1975; Roesch et al., 2010). Given that psychostimulants strongly activate ERK1/2 the striatum (Choe et al., 2002; Rajadhyaksha et al., 2004; Valjent et al., 2004; Zhang et al., 2004) with resultant induction of IEGs (Valjent et al., 2000; Choe et al., 2002; Salzmann et al., 2003; Zhang et al., 2004; Brami-Cherrier et al., 2005), the ERK1/2 pathway would also appear critical for influencing transcriptional regulation proposed to underlie the long term effects of addictive drugs (Hope et al., 1994; Moratalla et al., 1996b; Nestler, 2001; Zhang et al., 2004; Lu et al., 2006).

More recently, evidence has been provided for a role of mGluR5 in activating ERK1/2. Thus, in addition to glutamate signalling through ionotropic NMDARs and AMPARs activating ERKs (Sgambato et al., 1998; Mao et al., 2004), stimulation of group I mGluRs leads to ERK1/2 activation in the spinal cord (Karim et al., 2001), glial cell cultures (Peavy and Conn, 1998), cultured striatal neurons (Voulalas et al., 2005) and the striatum (Choe and Wang, 2001). Moreover, stimulation of mGluR5 appears sufficient for ERK1/2 activation in cultured striatal neurons with resultant phosphorylation of Elk-1, CREB and IEG expression (Mao et al., 2005). In this system, activation of at least two distinct intracellular signalling pathways is required. The first pathway involves Ca^{2+}-dependent kinase activation of ERK1/2, triggered by the 'conventional' mGluR5 derived PLCβ1/IP_3/ Ca^{2+} cascade (Mao et al., 2005). A second dominant pathway involves the Homer family of scaffold proteins, homer1b/c, which directly couple with mGluR5 to enable ERK1/2 activation through a currently undefined mechanism (Mao et al., 2005). Other studies provide evidence for mGluR5-dependent ERK activation in mediating responses to addictive drugs. Thus, antagonism of mGluR5 *in vivo* blocks

amphetamine-induced ERK1/2 activation and IEG (P-CREB, P-ELK-1 and Fos) induction in the dorsal striatum (Choe et al., 2002) and mGluR5 antagonism blocks cue-induced reinstatement of alcohol seeking and associated ERK1/2 activation in the NAcc shell and BLA (Schroeder et al., 2008).

In summary, ERKs likely play an important role in both short and long term changes in drug-induced synaptic plasticity. Striatal ERK1/2 activation requires glutamate and dopamine signalling through NMDA and D1Rs, respectively, but mGluR5 stimulation may also be sufficient. Since psychostimulants are known to trigger ERK1/2 activation almost exclusively in those MSNs that express predominantly D1Rs (that is, MSNs of the direct pathway) (Bertran-Gonzalez et al., 2008), the mGluR5$^{KD\text{-}D1}$ model provides an excellent tool to assess the contribution of mGluR5 on this population of MSNs to cocaine-induced striatal ERK activation. The following experiments will use immunohistochemistry and immunoblotting to examine activation of ERK1/2 in areas of the dorsal and ventral striatum following a single injection of cocaine in wild-type and mGluR5$^{KD\text{-}D1}$ mice.

In addition to ERK1/2 activation, drug-induced alterations of AMPA receptors at glutamatergic corticostriatal synapses are considered important for neuroplastic changes underlying addiction-related behaviours, including control over drug-seeking by drug-associated stimuli (Di Ciano and Everitt, 2001; Shepherd and Huganir, 2007; Conrad et al., 2008; Wolf and Ferrario, 2010). Stimulation of group I mGluRs, including mGluR5, can produce changes in synaptic plasticity through trafficking of AMPA receptors (Snyder et al., 2001; Bellone and Luscher, 2005; Mameli et al., 2007; Jo et al., 2008; Waung et al., 2008; Zhang et al., 2008; Kelly et al., 2009) and recent evidence suggests that, at least in the hippocampus, stimulation of group 1 mGluRs can drive internalisation of AMPA receptors, in part, requiring activation of ERK1/2 and the subsequent rapid translation of STEP61 (Zhang et al., 2008). Phosphorylation of AMPA receptors, which can result to altered receptor conductance, subunit composition and/or trafficking (Wang et al., 2005), provides an additional mechanism through which addictive drugs, such as

cocaine, can influence neuronal plasticity and addiction-related behaviours (Snyder et al., 2000; Famous et al., 2008). In the striatum, activation of mGluR5 appears necessary for phosphorylation of GluR1-Ser845 (Ahn and Choe, 2009), a PKA and cGMP-dependent protein kinase II phosphorylation site (Roche et al., 1996; Serulle et al., 2007), GluR1-Ser831 (Ahn and Choe, 2009), a PKC and CAMKII phosphorylation site (Roche et al., 1996; Mammen et al., 1997) and GluR2-Ser880 (Ahn and Choe, 2010), also a PKC site (Chung et al., 2000). Taken together, these studies suggest that glutamate signalling through striatal mGluR5 may play an important role in cocaine-induced alterations of AMPA receptor-mediated neuronal plasticity by influencing AMPA receptor trafficking and/or phosphorylation state. To examine this possibility, the following immunoblotting experiments will also examine STEP protein expression and alterations in AMPA receptor phosphorylation state in areas of the dorsal and ventral striatum following acute cocaine challenge in mGluR5$^{KD\text{-}D1}$ and wild-type mice.

6.2 Materials and methods

6.2.1 Animals

Mice (n = 20/21, WT/KD; male and at least 8 weeks old prior to experiment start) were bred in house and housed in groups of two or three. Animals were maintained on a 12:12 h light-dark cycle (lights on at 0700 hours) under controlled temperature (21 ± 2°C) and humidity (50 ± 5%) conditions. Body weights were maintained at approximately 85% of free-feeding weight by the provision of a limited amount of standard lab chow (B&K Feeds, Hull, UK) approximately 2 h after daily experiment completion. Food restriction was used in these animals to match the conditions of other studies reported in this thesis, such that effects of cocaine could be paralleled between studies without confounds arising from the use of different feeding conditions (Deroche et al., 1993). Experiments took place during the light-phase between 1200 and 1900 hours. All procedures were performed in accordance with the United Kingdom 1986 Animals (Scientific Procedures) Act, following institutional ethical review.

6.2.2 Drugs

Cocaine hydrochloride ($C_{17}H_{21}NO_4 \cdot HCl$; MacFarlan Smith, Edinburgh, UK) was dissolved in 0.9% saline. All injections were administered at a volume of 10 ml/kg i.p.

6.2.3 Locomotor apparatus and behavioural procedure

Locomotor activity was recorded using nine circular runways, as previously described (see Chapter 2, section 2.2.6.1). Mice were first habituated to the locomotor apparatus during five, once-daily, 1 h sessions. On the sixth day, following 30 min of activity recording, mice received a vehicle sham injection (10 ml/kg saline i.p.) designed to acclimatize them to the injection procedure. Activity was monitored for 10 min post-sham injection. On the seventh day, locomotor activity was again recorded for 30 min. Mice were then injected with either cocaine (20 mg/kg i.p.) or vehicle and activity recorded for a further 10 min post-injection.

Mice were then immediately removed from the locomotor apparatus and used for either immunohistochemistry or immunoblotting, as described below. Five blocks of locomotor sessions were run each day, with each block containing mice from both genotypes. The assignment of treatment condition (cocaine or saline) and study destination (immunohistochemistry or immunoblotting) was counterbalanced within each genotype and each block.

6.2.4 Immunohistochemistry

6.2.4.1 Tissue preparation

Saline (n = 5/6, WT/KD) and cocaine (n = 5/6, WT/KD) treated mice were injected with pentobarbital (0.2 ml of 200 mg/ml stock; Euthatal; Merial Animal Health, Harlow, Essex) and perfused transcardially with 4% (w/v) paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 7.5). PFA was delivered rapidly (20 ml/min) with a peristaltic pump for 5 min (as reported in Bertran-Gonzalez et al., 2008). Brains were post-fixed overnight in the same solution at 4°C and then blocked, sectioned and placed in cassettes (Tissue-Tek Uni-Cassettes; Sakura, Torrance, California, USA) to process for paraffin fixation (Tissue-Tek VIP 5; Sakura). In brief, brain sections were dehydrated in increasing concentrations of ethanol (70% for 1 h, 80% for 1 h and 5 x 100% each for 1 h), cleaned in xylene (2 x 1 h and 1 x 2 h) and incubated in molten paraffin (4 x 1 h; Richard-Allan Scientific Histoplast IM; Thermo Scientific, Waltham, Massachusetts, USA), before being embedded in paraffin blocks (Tissue-Tek TEC 5; Sakura). From these blocks, 4 µm sections of medial striatum (corresponding to 1.18 mm from bregma; Franklin and Paxinos, 2008) were cut with a microtome (RM2255; Leica, Milton Keynes, Bucks, UK), placed on charged slides (SuperFrost Plus; Menzel-Gläser, Brunswick, Germany), air-dried overnight at 37°C and stored at room temperature prior to staining.

6.2.4.2 Staining

Immunohistochemistry was performed using a Discovery XT Autostainer (Ventana Medical Systems, Tucson, Arizona, USA). Sections underwent de-paraffinization,

heat-induced epitope retrieval (CC1 solution, mild protocol; Ventana Medical Systems) and staining using a heat protocol. Sections were incubated for 1 h with primary antibodies raised against DARPP-32 (1:100, mouse monoclonal, 611520, lot 58902; BD Biosciences, Franklin Lakes, New Jersey, USA) and phospho-Thr202-Tyr204-ERK1/2 (P-ERK1/2; 1:200, 9101, lot 26; Cell Signaling Technology, Danvers, Massachusetts, USA). For detection of P-ERK1/2 and DARPP-32, slides were incubated for 30 min with goat anti-rabbit Cy3-conjugated (1:200, A10520; lot 771575, Invitrogen) and goat anti-mouse Cy5-conjuated (1:200, A10524, Lot 731500; Invitrogen) secondary antibodies, respectively. All antibodies were diluted in a Tris-based diluent (PSS diluent, 760-212, Ventana Medical Systems). On completion of staining, slides were removed from the Autostainer, washed in warm soapy water and cover slipped using ProLong® Gold anti-fade reagent (P36930; Invitrogen). Sections for P-ERK1/2 detection were cover slipped using the same reagent supplemented with 4',6-diamidino-2-phenylindole (DAPI; P36931; Invitrogen), a DNA intercalating fluorescent molecule.

6.2.4.3 Controls

As a negative control, the same immunohistochemical protocol was performed except that primary antibodies were omitted and replaced by fractions of rabbit IgG (for P-ERK1/2; V0415; Vector laboratories, Burlingame, California, USA) or mouse IgG2a (for DARPP-32; X0943; Dako, Ely, Cambridgeshire, UK), used at identical concentrations to the respective primary antibodies. In addition, primary antibodies were preadsorbed for at least 1 h with varying dilutions of the control peptide for DARPP-32 (1081, lot 1; Cell Signaling Technology) or P-ERK1/2 (1150, lot 4; Cell Signaling Technology).

6.2.4.4 Imaging and analysis of fluorescence

Images (375 x 375 µm; see panel b in Figs. 6.3-6.6 for illustration of sample area) from the dorsomedial (DM) and ventrolateral (VL) striatum, NAcc core and NAcc shell of the medial striatum were obtained bilaterally using sequential laser scanning confocal microscopy (SP5; Leica; using 40x dry objective, APO, 0.85

N.A.). For quantification of P-ERK1/2 +ve neurons, the Cy3 immunofluorescent nuclei were counted bilaterally from two sections from each mouse. Cell counts were performed by an observer unaware of the treatment condition (cocaine or saline) or the genotype (WT or KD) of mice.

6.2.5 Immunoblotting

6.2.5.1 Sample preparation

Saline (n = 5/5, WT/KD) and cocaine (n = 5/5, WT/KD) treated mice were decapitated and their brains rapidly (30-60 sec) extracted and snap-frozen in chilled isopentane (-50°C). Using a cryostat (CM3050S; Leica) frozen brains were cut in the coronal plane until the rostral striatum was exposed (Fig. 6.1b; equivalent to 1.54 mm from bregma; Franklin and Paxinos, 2008). A ~1 mm section that encompassed rostral, medial and caudal parts of the striatum was manually cut (Fig. 6.1a), from which bilateral punches (1.5 mm dia. dermal biopsy punch; Kai Medical, Honolulu, Hawaii) of the dorsal and ventral striatum were taken (for example see Fig. 6.1c) and stored at -80°C.

Frozen samples were briefly sonicated in ~100 µl of ice cold RIPA lysis buffer (1x of 10x stock: 0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA: 20-188; Millipore, Temecula, California, USA) supplemented with phosphatase inhibitors (1x phosSTOP; Roche Diagnostics, Burgess Hill, West Sussex, UK) and protease inhibitors (1x c*O*mplete mini, EDTA free; Roche Diagnostics). Lysate protein concentrations were determined by Bradford assay. Specifically, 5 µl of each sample was added to 250 µl of Bradford Reagent (B6916; Sigma, Saint Louis, Missouri, USA) and incubated at room temperature for a minimum of 5 min. Each lysate was assayed in duplicate. Protein concentrations were determined by comparing mean assay absorbance values measured at 590 nm (PowerWave HT Microplate Spectrophotometer; Biotek, Winooski, Vermont, USA) against a standard curve generated using known concentrations of Bovine Serum Albumin (BSA, A/1278/46; Fisher Scientific, Loughborough, Leicestershire, UK) dissolved in the same lysis buffer.

Equal amounts of lysates were prepared for gel electrophoresis by the addition of protein loading buffer (1x of 4x stock: 125 mM Tris-HCl, pH 6.8, 50% glycerol, 4% SDS, 0.2% (w/v) Orange G: 928-40004; Li-Cor Biosciences, Lincon, Nebraska, USA) and reducing agent (1x of 10x stock: 500 mM dithiothreitol: NP0004; Invitrogen, Paisley, UK), made to volume (15 µl per well) with deionized water (Milli-Q, Millipore). Loading samples were heated at 70°C for 10 min prior to charging gels.

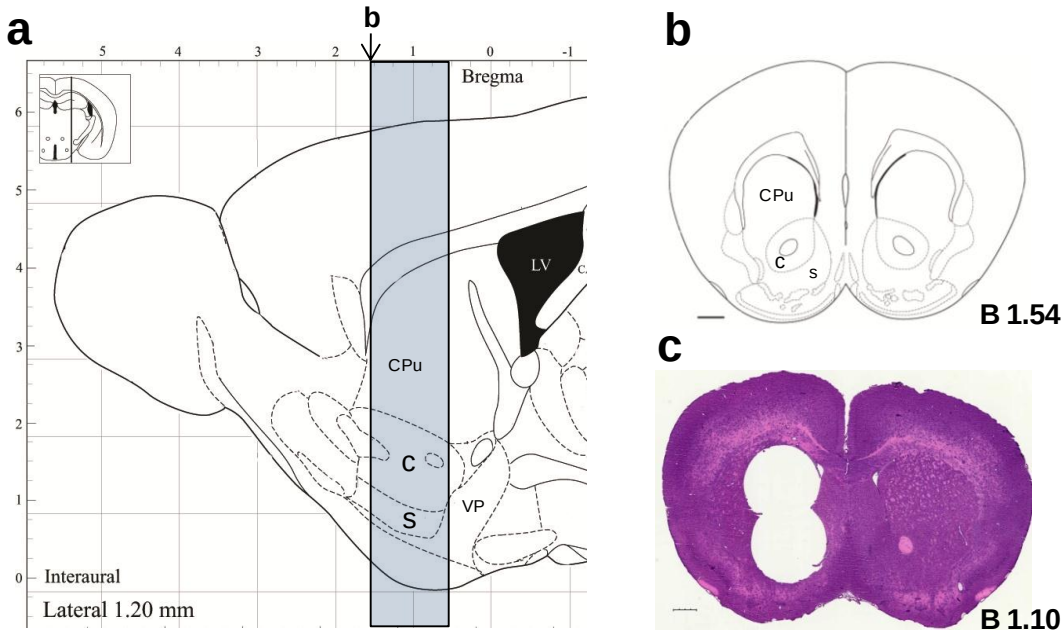


Figure 6.1 Punches for immunoblotting. Frozen brains were cut until the rostral striatum was visualised (panel **b**; corresponding to 1.54 mm from Bregma; Franklin and Paxinos, 2008). A 1 mm slice was then manually cut that encompassed rostral, medial and caudal striatum (shaded area of panel **a**). From this slice, 1.5 mm dia. bilateral punches of dorsal and ventral striatum were taken for immunoblotting. (**c**) Brain section (15 µm thick, equivalent to 1.10 mm from Bregma), stained with haematoxylin and eosin, illustrating dorsal and ventral punches taken from a single hemisphere. *CPu*: Caudate Putamen, *C*: NAcc core, *S*: NAcc shell, *VP*: Ventral Pallidum. Scale bar, 500 µm

6.2.5.2 Electrophoresis

Protein gel electrophoresis was undertaken with the NuPage® pre-cast gel system (all reagents and equipment from Invitrogen unless otherwise stated) using Novex 4-12% Bis-Tris gels (NP0322) and MOPS SDS running buffer (1x of 20x stock, NP0001-02) with added Antioxidant (NP0005). Molecular weight markers (Two-Color Protein Molecular Weight Marker, 928-40001; Li-Cor Biosciences; or SeeBlue Plus2 Pre-stained Standard, LC5925) were run alongside samples to confirm antibody specificity. Gels were run for 50 min at 200 v (PowerEase500) and proteins transferred to a 0.2 µm nitrocellulose membrane (IB3010-01) using iBlot (IB1001UK).

6.2.5.3 Western blotting

Blots were blocked in Li-Cor blocking buffer (927-40000; Li-Cor Biosciences) for 1 h at room temperature and incubated overnight at 4°C with primary antibodies raised against STEP (1:500, mouse monoclonal, 4396, lot 1; Cell Signaling Technology), ERK1/2 (1:1000, rabbit polyclonal, 9102, lot 20; Cell Signaling Technology), phospho-Thr202-Tyr204-ERK1/2 (P-ERK1/2; 1:500, rabbit polyclonal, 9101, lot 26; Cell Signaling Technology), GluR1 (1:500, rabbit monoclonal recombinant, 05-855R, lot NG1683901; Millipore), phospo-Ser831-GluR1 (P-GluR1-s831; 1:200, rabbit polyclonal, ab5847, lots NG1731388, JC1676088; Millipore), phospho-Ser845-GluR1 (P-GluR1-s845; 1:200, rabbit monoclonal, 2491-1, lots YH011804C, YF052719C; Epitomics, Burlingame, California, USA), GluR2 (1:500, goat polyclonal, N-19, sc-7661, lot B1908; Santa Cruz Biotechnology, Santa Cruz, California, USA) and phospho-Ser880-GluR2 (P-GluR2-s880; 1:200, rabbit polyclonal, 07-294; Millipore). All antibodies were diluted in fresh Li-Cor blocking buffer supplemented with 0.1% Tween 20.

After four 5 min washes in DPBS/0.1 % Tween 20, blots were incubated for 1 h at room temperature with appropriate donkey anti-mouse (680, 926-32222, lot B81023-03; 800, 926-32212, lot B70820-02), anti-rabbit (680, 926-32223, lot C00118-0; 800, 926-32212, lot B90713-02) and/or anti-goat (680, 926-32224, lot

C00426-06) secondary antibodies labelled with IRDyes 680 or 800 (Li-Cor Biosciences). Blots were washed and then imaged with an Odyssey infrared fluorescence scanner (Li-Cor Biosciences).

To control for sample loading variation, membranes were stripped (10 min incubation with 1x of a 5x NewBlot Nitro stripping buffer stock; 928-40030; Li-Cor Biosciences) and incubated for 1 h at room temperature with an antibody raised against β-actin (1:5000, mouse monoclonal, clone AC-15, A1978, lot 056K4796; Sigma; or 1:1000, rabbit monoclonal, clone 13e5, 4970, lot 3; Cell Signaling Technology). After washing, blots were incubated for 1 h at room temperature with donkey anti-mouse or anti-rabbit secondary antibodies labeled with IRDye 800 (Li-Cor Biosciences). Following a final wash step, blots were again imaged for fluorescence detection.

Band intensity was determined using Odyssey v2.1 software. Quantification was performed by subtracting background readings from the relative intensity of each sample band and normalizing band intensity with that of actin. The band intensity for each sample in a gel was divided by the mean band intensity for the control sample condition in the same gel (i.e. WT mice treated with saline) to provide a '% of Control' value (100% of Control value would indicate no change in band intensity between the treatment group and the control condition).

6.2.6 Statistical Analysis

All test data were initially assessed for normality (Shapiro-Wilk test; assumption violated when $p \leq 0.05$) and homogeneity of variance (Levene's test, assumption violated when $p \leq 0.05$). To permit analysis by parametric tests, appropriate transformations were undertaken to transform skewed distributions closer to a normal distribution and to reduce heterogeneity of variance (Cardinal and Aitken, 2006). Specifically, for P-ERK1/2 +ve cell counts in the immunohistochemistry study, all data were square root transformed ($Y' = \sqrt{Y}$).

For the locomotor habituation phase and sham-injection day, data were analysed by mixed-factor analysis of variance (ANOVA), with genotype (WT, KD) as a between-subjects factor and session (1-5) as a within-subjects factor. Where significant ($p \leq 0.05$) main effects or interaction terms were found, further analysis was performed using ANOVA and *post-hoc* comparisons by *t*-test, with Bonferroni corrections applied for multiple comparisons. For within-subjects ANOVA, the Greenhouse-Geisser correction was used where the assumption of sphericity was violated (Mauchly's test, $p \leq 0.05$).

For the cocaine/vehicle injection day, immunohistochemistry and immunoblotting studies, the effect of treatment (cocaine or vehicle) and genotype (WT or KD) on the respective dependent variables (distance travelled, P-ERK1/2 +ve cell counts or % of control) was analysed by univariate ANOVA. Where a significant ($p \leq 0.05$) main effect or interaction term was found, between-group comparisons were performed by independent samples *t*-test. Findings were considered indicative of a trend where $p \leq 0.1$ and all figures show group means (± SEM). Statistical analysis was performed with SPSS Statistics v.17 (IBM, Somers, New York, USA).

6.3 Results

6.3.1 Locomotor activity

As has been reported previously (Chapter 2, section 2.3.4.1), activity of KD mice was reduced in a novel environment (Fig. 6.2a, session 1, *post-hoc* Bonferroni t = 2.76, df = 40, $p < 0.01$). Following a period of habituation there was no difference in locomotor activity between WT and KD mice (Fig. 6.2a, session 5, *post-hoc* Bonferroni *t*-test comparison, NS). Locomotor activity in the first 10 min period following the vehicle sham-injection (Fig. 6.2b) did not differ between genotypes (*t*-test comparison, NS). When injected with cocaine, locomotor activity significantly increased in both WT and KD mice (Fig. 6.2c; main effect of Treatment, $F(1,37) = 35.56$, $p < 0.001$). However, the locomotor response to cocaine was significantly attenuated in KD mice (Treatment x Genotype interaction; $F(1,37) = 11.79$, $p = 0.001$; *t*-test comparison of WT cocaine vs. KD cocaine, $t = 3.70$, df = 19, $p < 0.01$). Locomotor activity following injection with vehicle did not differ between genotypes (*t*-test comparison, NS). The locomotor response to either treatment did not differ depending on the fate of mice (immunoblotting or immunohistochemistry; data not shown).

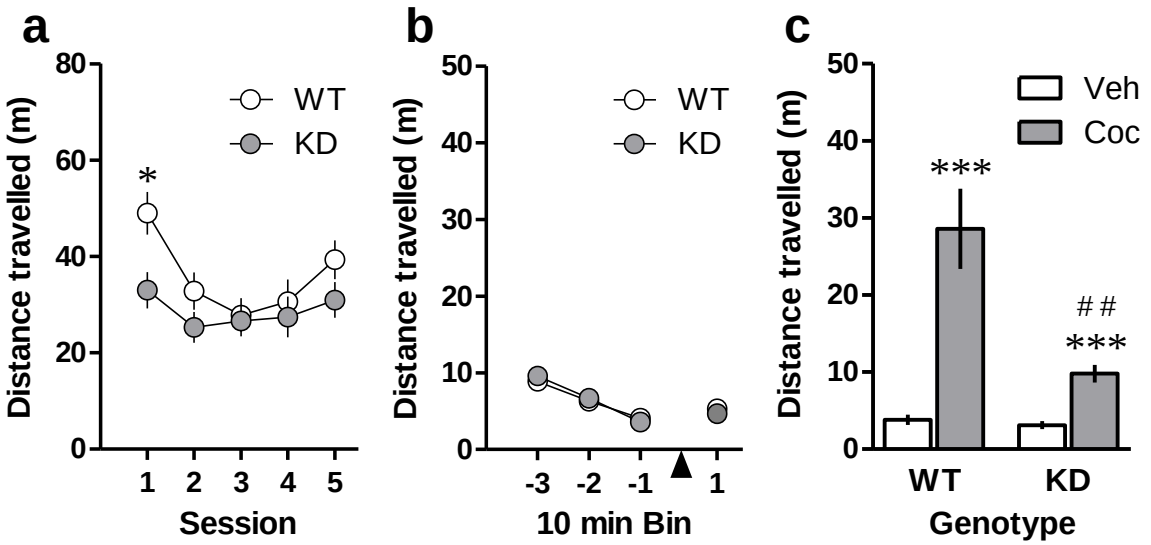


Figure 6.2 Locomotor activity (**a**) Activity was reduced in mutant mice when exposed to the novel locomotor apparatus (session 1), but activity did not differ between genotypes following a period of habituation (session 5). Each data point represents mean total activity (distance travelled) from once-daily 1 h sessions. *p < 0.01, between-genotype Bonferroni *t*-test comparison (**b**) During the sixth session, a vehicle sham injection (▲) was given after a 30 min habituation period. Locomotor activity did not differ between genotypes either prior to or following the injection. (**c**) Activity significantly increased in both genotypes during the 10 min period following 20 mg/kg cocaine (Coc), but was attenuated in KD mice. Activity following a vehicle injection (Veh) did not differ between genotypes. ***p < 0.001, within-genotype *t*-test comparison of vehicle and cocaine activity, $^{\#\#}p$ < 0.01, between-genotype *t*-test comparison of cocaine activity (n = 20/22, WT/KD; except for 30 min habituation period before the sham injection where n = 17/21 due to data recording error).

6.3.2 Immunohistochemistry

6.3.2.1 P-ERK1/2

Striatal ERK1/2 activation following cocaine and saline injections in wild-type and mutant mice was assessed by counting P-ERK1/2 +ve cells in the dorsal and ventral compartments of the medial striatum (a summary of the analyses is provided in Table 6.1). In the dorsomedial part of the striatum (Fig. 6.3a-c), cocaine did not increase P-ERK1/2 +ve cell counts (main effect of Treatment, NS), but overall P-ERK1/2 +ve cell counts were reduced in mutant mice (main effect of Genotype: $F(1,18) = 5.59$, $p < 0.05$). In the ventrolateral part of the medial striatum (Fig. 6.4a-c), cocaine did not reliably increase P-ERK1/2 immunoreactivity (main effect of Treatment, NS) and there was no overall difference between genotypes in P-ERK1/2 +ve cell counts (main effect of Genotype, NS). In the NAcc core (Fig. 6.5a-c), cocaine did increase P-ERK1/2 +ve cell counts, although this effect was not quite significant (main effect of treatment: $F(1,18) = 3.50$, $p = 0.078$). The effect of cocaine on P-ERK1/2 immunoreactivity in the NAcc core did not differ between genotypes (main effect of Genotype, NS; Genotype x Treatment interaction, NS). In the NAcc shell (Fig. 6.6.a-c), cocaine produced a robust increase in P-ERK1/2 immunoreactivity (main effect of Treatment, $F(1,18) = 11.01$, $p < 0.01$) and overall P-ERK1/2 immunoreactivity tended to be reduced in mutant mice (main effect of Genotype, $F(1,18) = 3.85$, $p = 0.065$). However, the effect of cocaine on P-ERK1/2 +ve cell counts did not reliably differ between genotypes (Treatment x Genotype interaction, NS). Subsequent within-genotype comparisons indicated that cocaine significantly increased P-ERK1/2 +ve cell counts in WT mice ($t = -2.48$, df = 8, $p < 0.05$), although this effect was not so robust in mutant mice ($t = -2.11$, df = 10, $p = 0.061$).

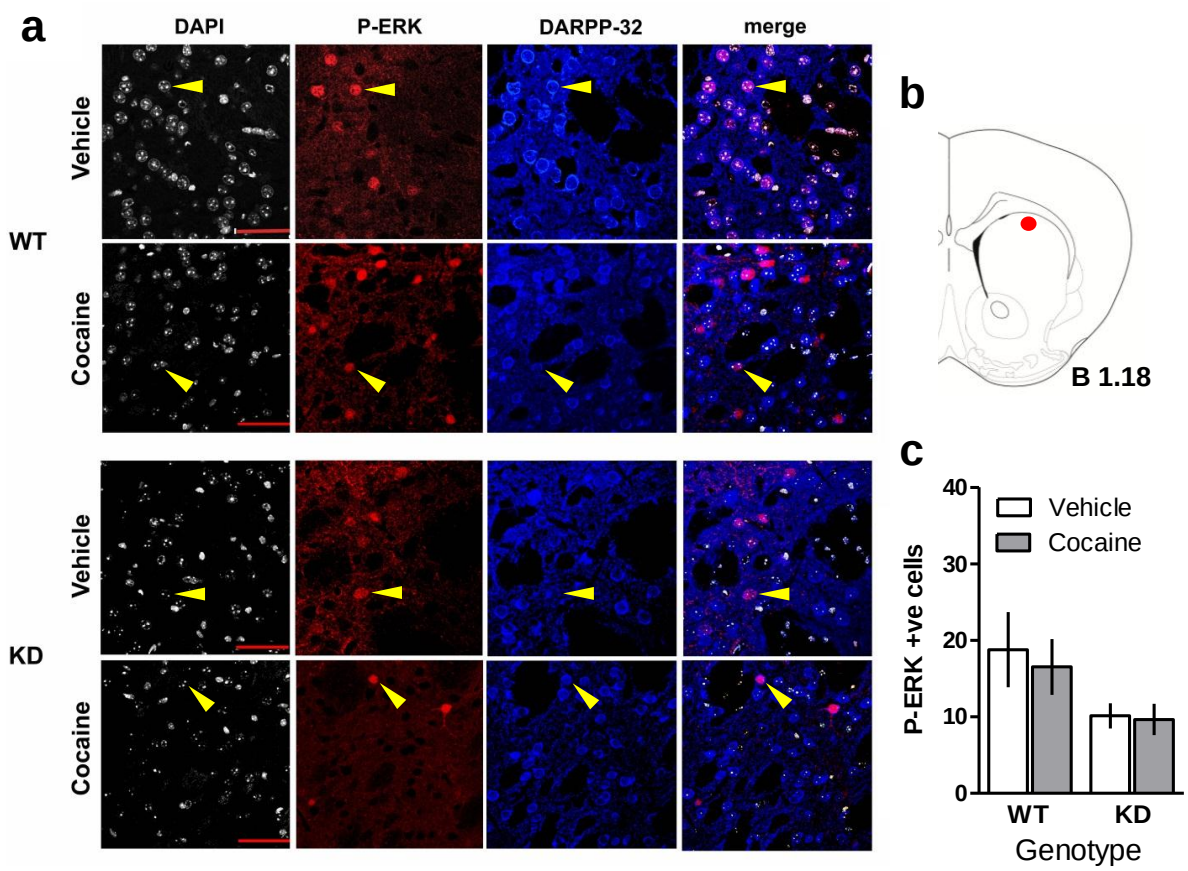


Figure 6.3 Immunohistochemistry of P-ERK1/2 in the dorsomedial striatum. (**a**) P-ERK1/2 immunoreactivity (red) was detected together with DAPI (white; a marker of DNA) and DARPP-32 (blue; a marker of MSNs) in the dorsomedial part of the striatum (red dot in panel **b;** equivalent to 1.18 mm from Bregma; Franklin and Paxinos, 2008) of wild-type and mGluR5$^{KD\text{-}D1}$ (KD) mice in a triple-fluorescence analysis. Arrows indicate the position of P-ERK1/2 +ve neurons. Merged images show that P-ERK1/2 +ve neurons are also MSNs. Scale bars, 50 μm. (**c**) Cocaine did not increase P-ERK1/2 immunoreactivity in either genotype, although P-ERK immunoreactivity was reduced overall in mutant mice in this striatal region.

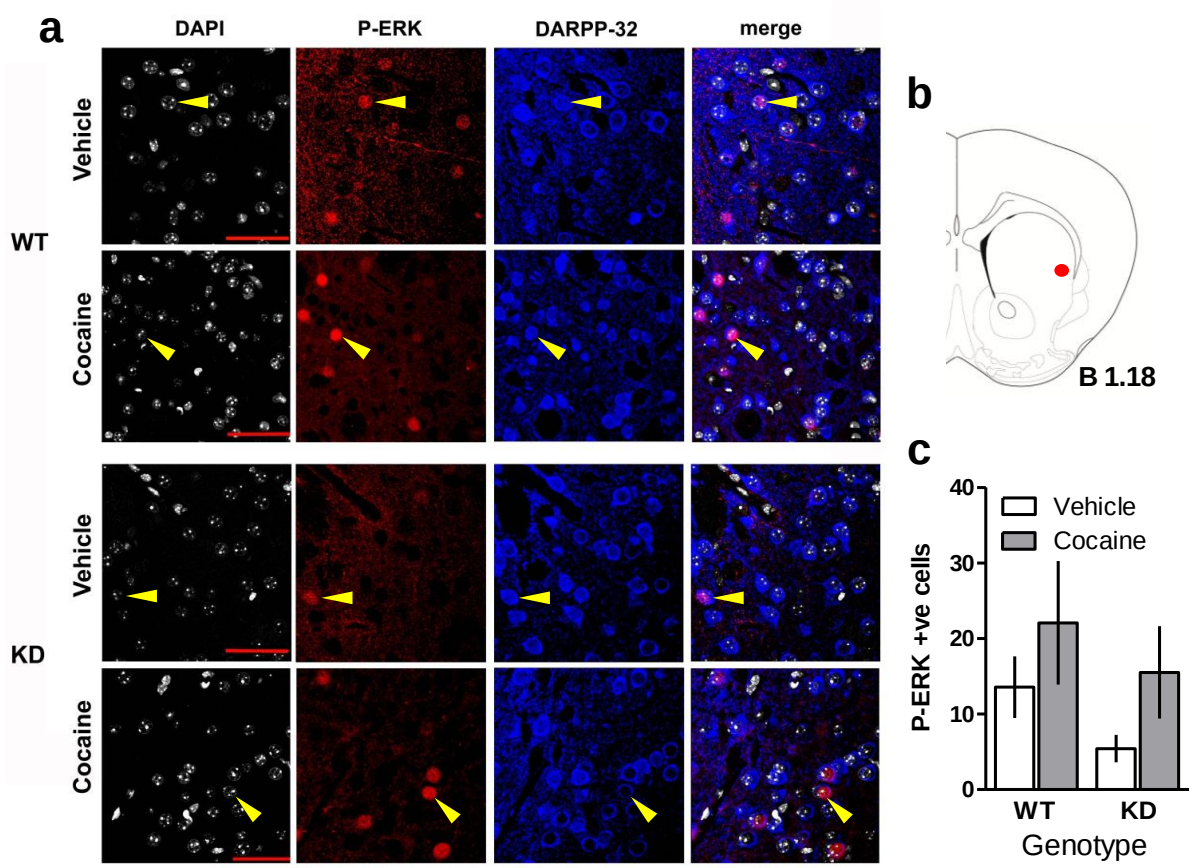


Figure 6.4 Immunohistochemistry of P-ERK1/2 in the ventrolateral striatum. (**a**) P-ERK1/2 immunoreactivity (red) was detected together with DAPI (white) and DARPP-32 (blue) in the ventrolateral part of the striatum (red dot in panel **b;** equivalent to 1.18 mm from Bregma; Franklin and Paxinos, 2008) of wild-type and mGluR5$^{KD\text{-}D1}$ (KD) mice. Arrows indicate the position of P-ERK1/2 +ve neurons. Scale bars, 50 µm. (**c**) Cocaine did not reliably increase P-ERK1/2 +ve cell counts in either genotype in this striatal region.

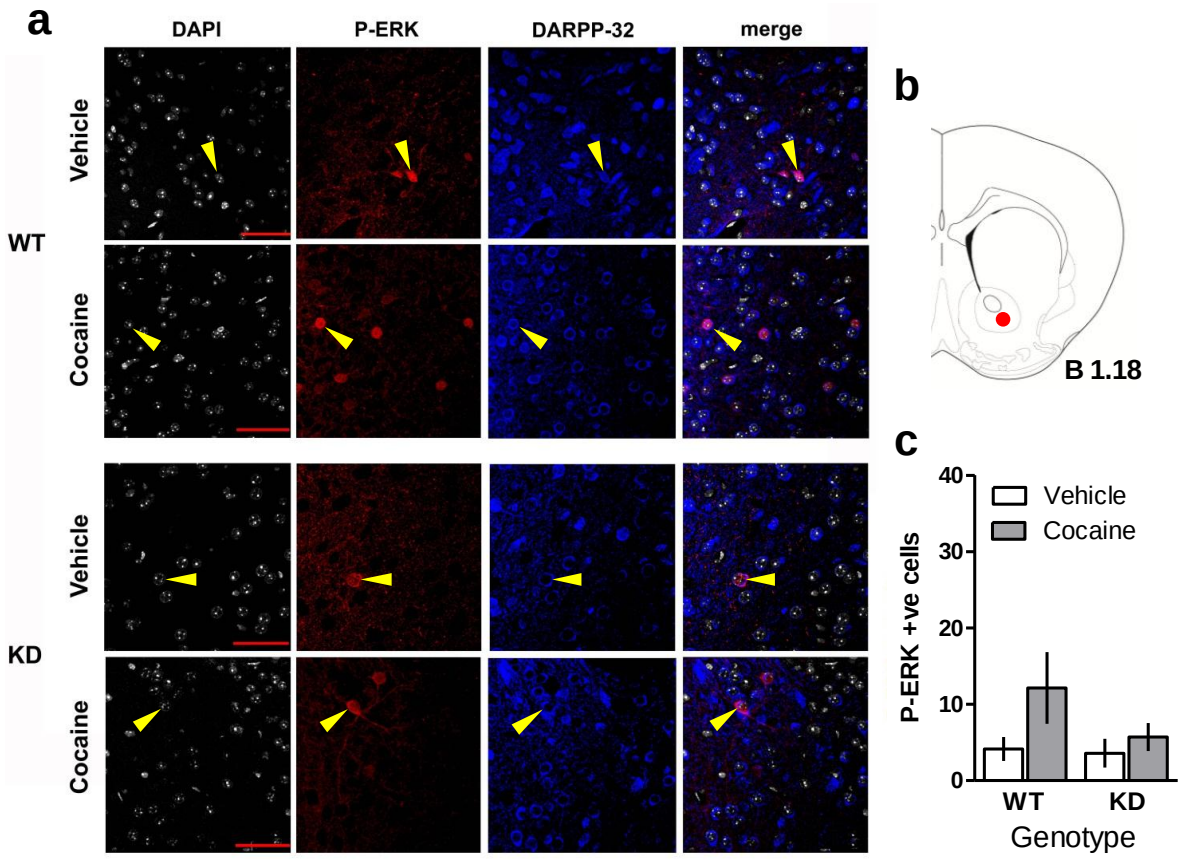


Figure 6.5 Immunohistochemistry of P-ERK1/2 in the NAcc core. (**a**) P-ERK1/2 immunoreactivity (red) was detected together with DAPI (white) and DARPP-32 (blue) in the NAcc core of the medial striatum (red dot in panel **b;** equivalent to 1.18 mm from Bregma). Arrows indicate the position of P-ERK1/2 +ve neurons. Scale bars, 50 µm. (**c**) Cocaine increased P-ERK1/2 immunoreactivity in the NAcc core, although this effect was not quite statistically significant (main effect of Treatment, $p = 0.065$).

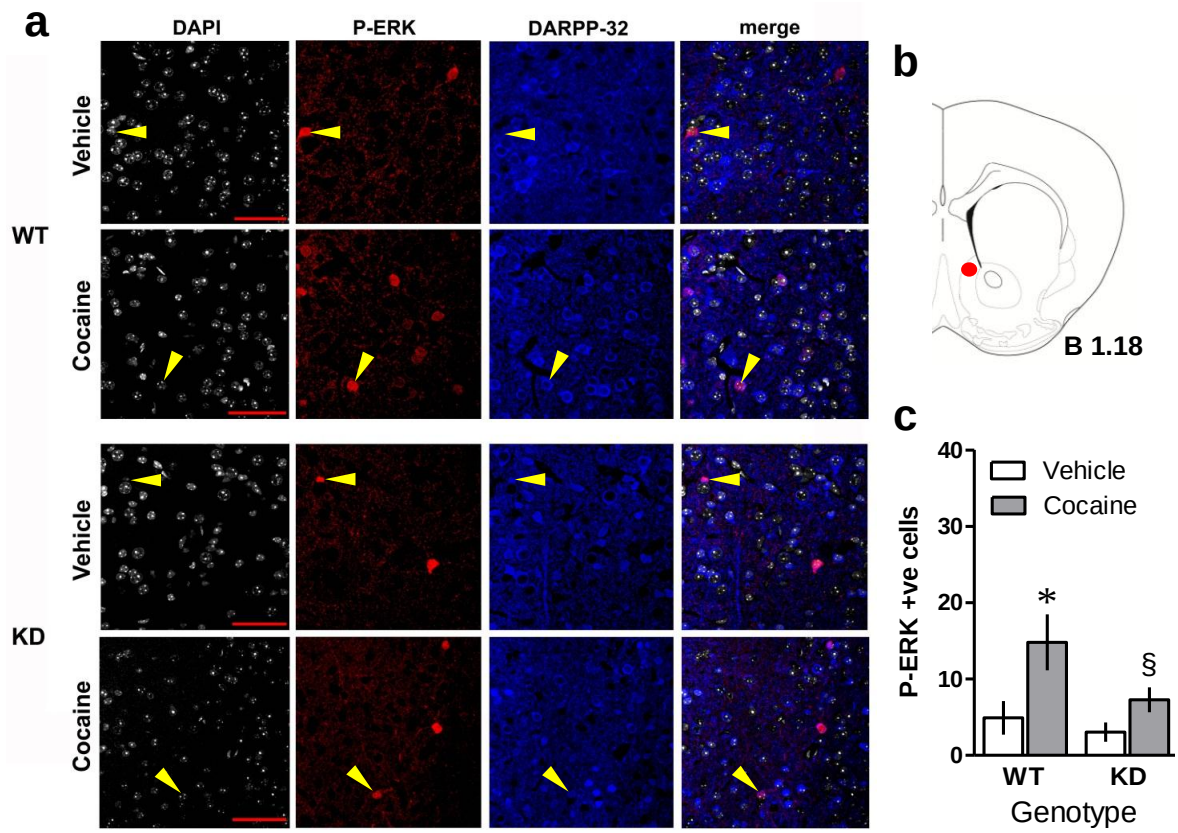


Figure 6.6 Immunohistochemistry of P-ERK1/2 in the NAcc shell. (**a**) P-ERK1/2 immunoreactivity (red) was detected together with DAPI (white) and DARPP-32 (blue) in the NAcc shell of the medial striatum (red dot in panel **b;** equivalent to 1.18 mm from Bregma). Arrows indicate the position of P-ERK1/2 +ve neurons. Scale bars, 50 μm. (**c**) Cocaine increased P-ERK1/2 immunoreactivity in both genotypes, although this effect was not so robust in mutant mice $^{*}p < 0.05$, $^{\S}p < 0.1$ within-genotype t-test comparison between vehicle and cocaine.

F-IHC: P-ERK1/2	Treatment F(1,18)	Genotype F(1,18)	Treatment x Genotype F(1,18)
Dorsomedial striatum	0.180	5.59*	0.036
Ventrolateral striatum	2.48	2.33	0.20
NAcc core	$3.50^{\S}$	1.58	0.50
NAcc shell	11.01**	$3.85^{\S}$	0.58

Table 6.1 ANOVAs for fluorescence immunohistochemistry (F-IHC) of P-ERK1/2 cell counts. Factors: Treatment, (Cocaine, vehicle); Genotype (WT, KD), $^{**}p < 0.01$, $^{*}p < 0.05$, $^{\S}p < 0.1$

6.3.2.2 Controls

No cell-type specific fluorescent signal was detected when IgG fractions were replaced for respective primary antibodies (images not shown). In the preadsorption assays, the control peptide for DARPP-32 blocked DARPP-32 immunoreactivity in a concentration dependent manner (Fig. 6.7). Although the control peptide for P-ERK1/2 blocked P-ERK1/2 immunoreactivity, no concentration dependent block of P-ERK1/2 was demonstrated (i.e. the peptide was still effective at the lowest dilution tested; 0.3 µg of peptide per 1 µg of P-ERK1/2 antibody; images not shown).

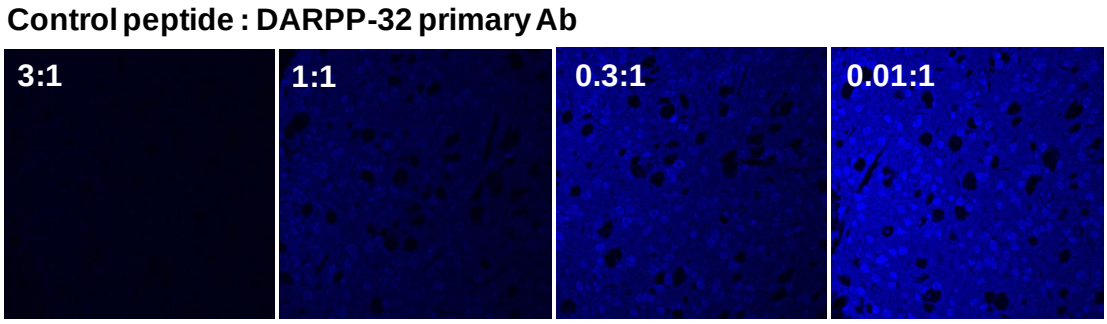


Figure 6.7 DARPP-32 preadsorption control. DARPP-32 immunoreactivity was blocked by its control peptide in a concentration dependent manner. Each 375 x 375 µm panel indicates the ratio of control peptide preadsorbed to DARPP-32 primary antibody. Note that all images are of the same brain region in the same wild-type mouse. Images were captured with identical confocal settings and brightness adjusted for display purpose to the exact same extent.

6.3.3 Immunoblotting

Tissue from one animal was lost during punch collection, reducing group sizes to n = 5/5 (WT/KD) for the saline injected animals and n = 4/5 (WT/KD) for the cocaine treated mice. In addition, two samples intended for detection of total ERK1/2 in the dorsal striatum did not run correctly in the gel, reducing the group sizes for this particular condition to n = 5/4 (WT/KD) for the saline condition and n = 4/4 (WT/KD) for the cocaine condition.

Bands for GluR2, GluR1, P-GluR1-s845 and P-GluR1-s831 were observed at ~100 KDa (Fig. 6.8). The antibody used for detection of P-GluR2-s880 was not of sufficient quality to permit reliable identification of the target phosphorylated protein (not shown), and thus no data for GluR2 or P-GluR2-s880 will be reported in this thesis. Consistent with reported identification of STEP isoforms (Lombroso et al., 1993; Sharma et al., 1995), two major bands at 61 and 46 KDa were observed for STEP, with additional minor bands at 37, 33, and 20 KDa (Fig. 6.8). For total ERK1/2 and P-ERK1/2, two bands were observed at 44 and 42 KDa corresponding with ERK1 and ERK2, respectively (Fig. 6.8). Both actin antibodies detected a single band, observed at 42 KDa (A1978; Sigma shown in Fig. 6.8). It is worthwhile to note that, under the same immunoblotting conditions described above, little success was had with other antibodies raised against GluR1 (sc-13152, lot H1109; Santa Cruz), phospho-Ser845-GluR1 (AB5849, lot JC1650292; Millipore), phospo-Ser831-GluR1 (04-823, lot DAM1557561; Millipore), and GluR3 (mab5416, lot JC1629918; Millipore). In addition, antibodies for ERK1/2 (06-182, lot DAM1644549; Millipore) and phospho-Tyr204-ERK1/2 (sc-7383, Lot H0409; Santa Cruz) did produce bands at the predicted size, but were not used in the present report.

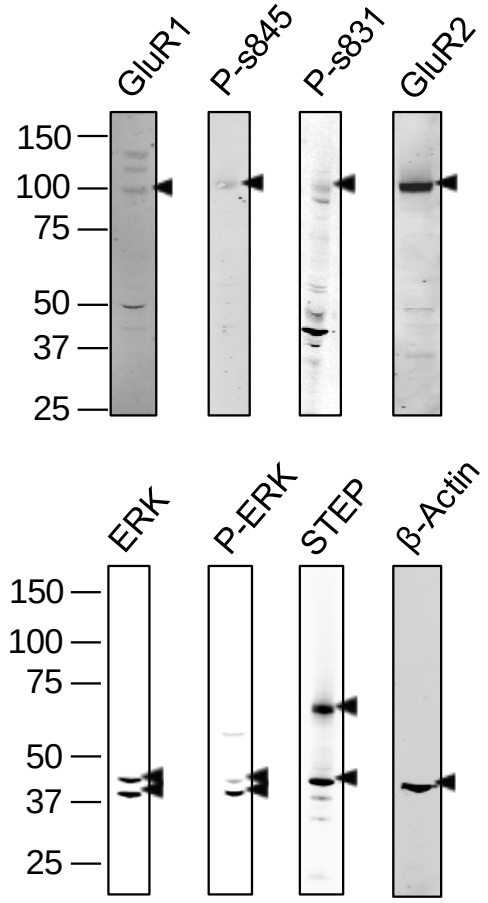


Figure 6.8 Example western blots. Full blots are shown for antibodies raised against GluR1, P-GluR1-s845 (P-s845), P-GluR1-s831 (P-s831), GluR2, ERK1/2 (ERK), P-ERK1/2 (P-ERK), STEP and β-Actin. Arrow heads indicate bands selected for analysis, based on predicted protein weight.

6.3.3.1 Total ERK1/2 and P-ERK1/2

No differences in either total ERK1/2 or P-ERK1/2 were found in the dorsal striatum of WT and KD mice treated with cocaine or vehicle (Fig. 6.9a-b, DStr; for each ERK and P-ERK isoform: main effect of Genotype, NS; Treatment, NS; Genotype x Treatment interaction, NS). Similarly, in the ventral striatum, total ERK1/2 levels did not differ between genotypes or as a function of treatment (Fig 6.9a-b, VStr; for each ERK: main effect of Genotype, NS; Treatment, NS; Genotype x Treatment interaction, NS). Although P-ERK1/2 levels in the ventral striatum were elevated overall in KD mice (P-ERK1: main effect of Genotype, $F(1,15) = 4.93$, $p < 0.05$; P-ERK2: main effect of Genotype, $F(1,15) = 5.16$, $p < 0.05$), P-ERK1/2 levels were unaffected by cocaine (for each P-ERK isofrom: main effect of Treatment, NS; Genotype x Treatment interaction, NS). A summary of the analyses is provided in Table 6.2.

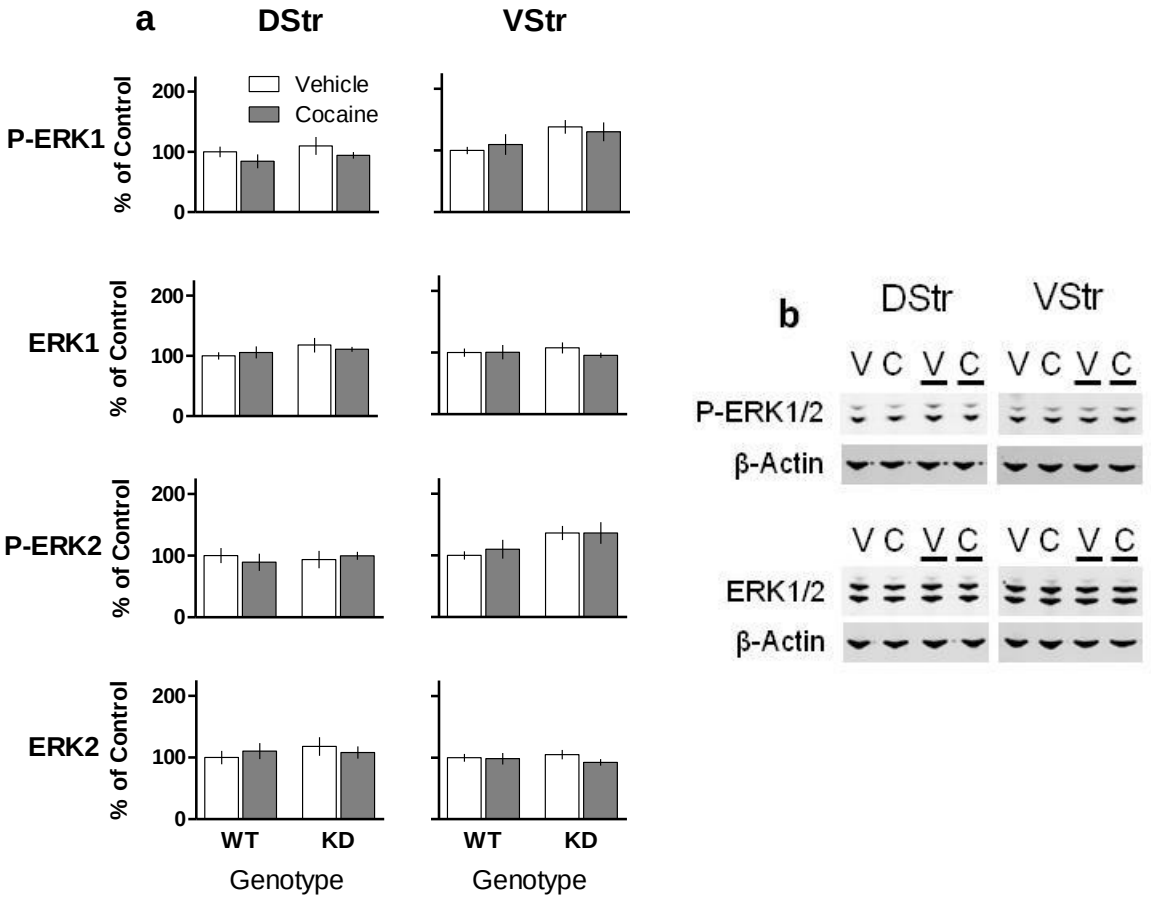


Figure 6.9 Immunoblotting of total ERK1/2 and P-ERK1/2 (**a**) 20 mg/kg cocaine did not alter total ERK1/2 protein or its active form P-ERK1/2 in either the dorsal (DStr) or ventral (VStr) striatum of wild-type or mGluR5$^{KD\text{-}D1}$ mice. Overall levels of P-ERK1 and P-ERK2 showed a small but significant increase in mGluR5$^{KD\text{-}D1}$ mice in comparison to wild-types (n = 4-5 per group). (**b**) Example blots are shown from wild-type or mGluR5$^{KD\text{-}D1}$ (bold line) mice injected with vehicle (V) or 20 mg/kg cocaine (C).

6.3.3.2 STEP

Total STEP46 protein did not differ in mice given cocaine or saline, in either the dorsal or ventral striatum (Fig. 6.10a-b; in each area: main effect of Genotype, NS; Treatment, NS; Genotype x Treatment interaction, NS). Although STEP61 protein was not altered in the ventral striatum of mice in either treatment condition (Fig. 6.10a-b; main effect of Genotype, NS; Treatment, NS; Genotype x Treatment interaction, NS), expression of this isoform in the dorsal striatum showed a small but significant variation depending on the genotype and treatment condition (Treatment x Genotype interaction, $F(1,15) = 6.81$, $p < 0.05$; main effect of Genotype, $F(1,15) = 3.11$, $p = 0.098$). Subsequent comparisons revealed a trend for increased STEP61 expression in WT mice given cocaine, in comparison to WT mice injected with saline ($t = -1.92$, df = 8, $p = 0.09$). In contrast, STEP61 expression was significantly reduced in KD mice given cocaine, in comparison to WT mice injected with cocaine ($t = -3.38$, df = 8, $p = 0.01$). A summary of the analyses is provided in Table 6.2.

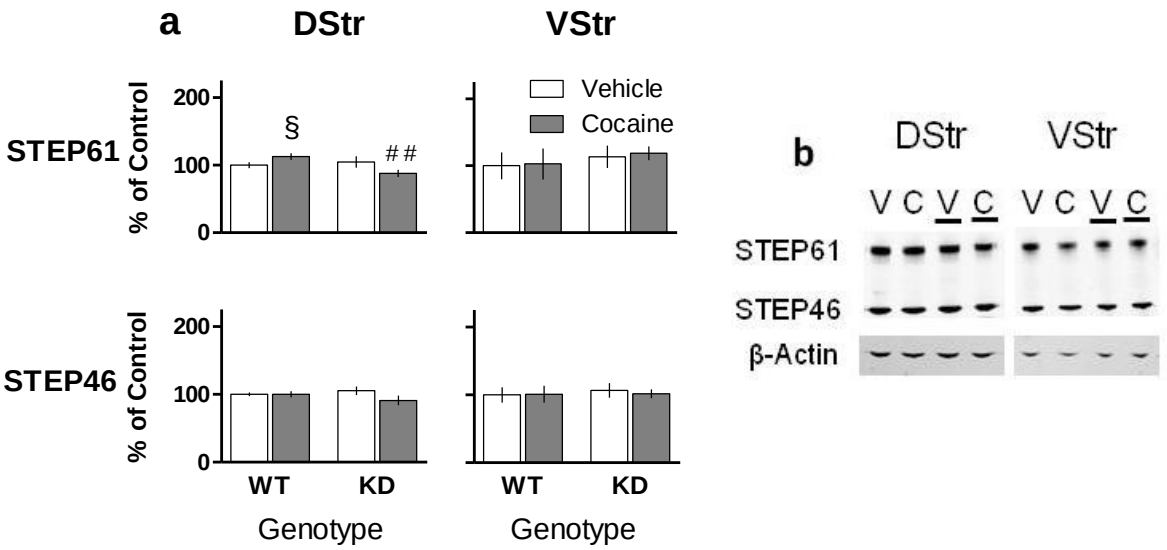


Figure 6.10 Immunoblotting of STEP. (**a**) Following 20 mg/kg cocaine, STEP61 expression tended to increase in the dorsal striatum (DStr) of WT mice, while STEP61 expression was significantly decreased in KD mice injected with cocaine compared to WT mice. There were no between group differences in expression of either STEP isoform in the ventral striatum (VStr). $^{\S}p < 0.1$ within-genotype *t*-test comparison of cocaine and vehicle, $^{\#\#}p < 0.01$ between-genotype *t*-test comparison of cocaine (n = 4-5 per group) (**b**) Example blots are shown from wild-type or mutant (bold line) mice injected with vehicle (V) or 20 mg/kg cocaine (C).

6.3.3.3 P-GluR1-s845 and P-GluR1-s831

In the dorsal striatum, P-GluR1-s845 levels were significantly increased following 20 mg/kg cocaine (Fig. 6.11a-b, DStr; main effect of Treatment, $F(1,15) = 13.19$, $p < 0.01$) but this effect differed between genotypes (Treatment x Genotype interaction, $F(1,15) = 7.77$, $p < 0.05$). Subsequent comparisons revealed that cocaine significantly increased P-GluR1-s845 levels in WT mice in comparison to WT mice injected with vehicle ($t = -4.2$, df = 8, $p < 0.01$), but no similar change was found in KD mice (*t*-test comparison, NS). Moreover, P-GluR1-s845 levels significantly differed between WT and KD mice injected with cocaine ($t = 2.83$, df = 8, $p < 0.05$). In the ventral striatum, cocaine again increased P-GluR1-s845 levels (Fig. 6.11a-b, VStr; main effect of Treatment, $F(1,15) = 5.39$, $p < 0.05$), although this effect did not differ between genotypes (Treatment x Genotype interaction, NS). In marked contrast, P-GluR1-s831 levels did not vary in either the ventral or dorsal striatum region as a function of drug treatment, nor between genotypes (in both regions: main effect of Treatment, Genotype and Treatment x Genotype interaction, NS). A summary of the analyses is provided in Table 6.2.

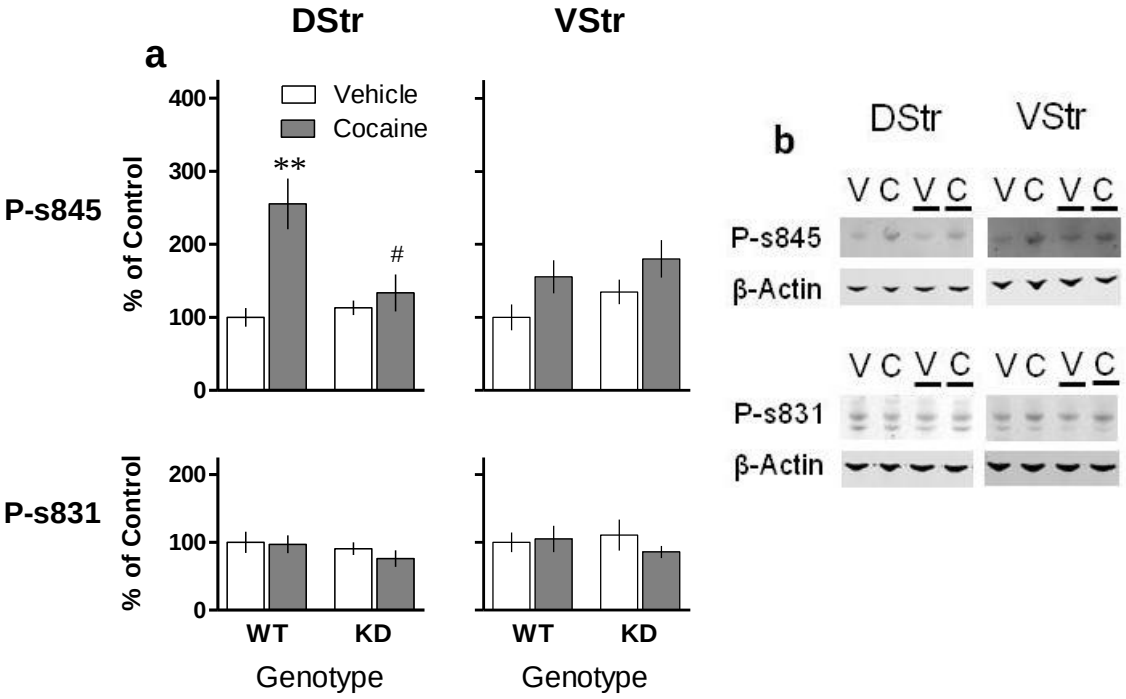


Figure 6.11 Immunoblotting of P-GluR1-s845 and P-GluR1-s831 (**a**) 20 mg/kg cocaine significantly increased levels of P-GluR1-s845 in the dorsal striatum (DStr) in wild-type mice, but not mutant mice. In the ventral striatum (VStr), P-GluR1-s845 was increased by cocaine, but this effect did not differ between genotypes. Levels of P-GluR1-s831 did not differ following cocaine in either brain region. $^{**}p < 0.01$ within-genotype *t*-test comparison of vehicle and cocaine, $^{\#}p < 0.05$ between-genotype t-test comparison of cocaine (n = 4-5 per group). (**b**) Example blots are shown from wild-type or mutant (bold line) mice injected with vehicle (V) or 20 mg/kg cocaine (C).

Immunoblotting	Treatment F(1,15)	Genotype F(1,15)	Treatment x Genotype F(1,15)
Dorsal Striatum			
P-ERK1	2.32	0.96	0.00
P-ERK2	0.037	0.028	0.51
Total ERK1[#]	0.003	1.92	0.58
Total ERK2[#]	0.001	0.40	0.68
STEP61	0.142	3.11[§]	6.81*
STEP46	1.72	0.12	1.79
P-GluR1-s845	13.19**	5.04*	7.77*
P-GluR1-s831	0.43	1.31	0.20
Ventral Striatum			
P-ERK1	0.004	4.93*	0.43
P-ERK2	0.13	5.16*	0.13
Total ERK1	0.47	0.026	0.57
Total ERK2	0.92	0.003	0.58
STEP61	0.046	0.63	0.005
STEP46	0.035	0.12	0.079
P-GluR1-s845	5.39*	1.88	0.56
P-GluR1-s831	0.36	0.063	0.81

Table 6.2 ANOVAs for immunoblotting studies. Factors: Treatment (Cocaine, vehicle); Genotype (WT, KD), $^{**}p < 0.01$, $^{*}p < 0.05$. [#]For total ERK1/2 in the dorsal striatum, F(1,14).

6.4 Discussion

In the present chapter, immunohistochemistry and immunoblotting techniques were used to explore some neurobiological effects of acute non-contingent cocaine in wild-type and mGluR5$^{KD\text{-}D1}$ mice. The immunohistochemistry study revealed that a single cocaine injection was sufficient to produce a robust activation of ERK1/2 in the NAcc shell, with some activation also seen in the NAcc core. Moreover, at least in the NAcc shell, the effect of cocaine on ERK1/2 activation tended to be attenuated in mGluR5$^{KD\text{-}D1}$ mice. In the dorsal striatum, cocaine did not reliably increase ERK1/2 activation in either the dorsomedial or ventrolateral regions; although overall levels of ERK1/2 activation in the dorsal striatum were reduced in mGluR5$^{KD\text{-}D1}$ mice when compared to their wild-type counterparts. In contrast to the immunohistochemistry study, immunoblotting did not identify increased ERK1/2 activation in either the dorsal or ventral striatum in response to a cocaine injection. However, immunoblotting did reveal that translation of STEP61 and levels of P-GluR1-s845 were increased following a cocaine injection in the dorsal striatum of wild-type mice, but not mutant mice. In the ventral striatum, cocaine increased levels of P-GluR1-s845 in both genotypes to a similar extent. By contrast, cocaine had no effect on levels of P-GluR1-s831 in the ventral or dorsal striatum of either genotype. Taken together, these data suggest that regulation of basal levels of ERK1/2 activation in the dorsal striatum and activity of this pathway in response to acute cocaine challenge in the ventral striatum may involve glutamate signalling through mGluR5 located on D1-MSNs. In addition, mGluR5 on D1-MSNs may play an important role in regulating AMPA receptor trafficking and/or phosphorylation state of AMPA receptors in the striatum following acute cocaine experience.

A general limitation of the immunohistochemistry approach is that only cells which show robust activation of ERK1/2 are counted and it is not possible to distinguish between the degree of ERK1/2 activation amongst neurons. It is possible that less robust ERK1/2 activation occurred in other neurons of wild-type and mGluR5$^{KD\text{-}D1}$ mice, which may still have important physiological consequences (Valjent et al.,

2004). Oppositely, it is not known whether ERK1/2 activation observed in either genotype in the present study was sufficient to activate associated transcriptional regulators, such as ELK-1 and CREB (Konradi et al., 1994; Yang et al., 2004). In future studies, examining the induction of such regulators and associated immediate early genes (for example, c-fos) would be highly valuable to further understand the role of mGluR5 on D1-MSNs in gating neuronal adaptations that occur following exposure to psychostimulants.

In the immunohistochemistry study, cocaine had no significant effect on ERK1/2 activation in the dorsal striatum, which contrasts with other reports (most notably those of Valjent and colleagues; Valjent et al., 2005; Bertran-Gonzalez et al., 2008). The lack of more robust and widespread ERK1/2 activation could reflect a number of factors. First, it may be that the time of sampling after the cocaine injection in the present study (mice were perfused immediately after the 10 min locomotor session) was not optimal. The time course of striatal ERK1/2 activation in response to cocaine has been characterised and varies between the dorsal and ventral striatum (Bertran-Gonzalez et al., 2008). Thus, activation of ERK1/2 was greatest in all striatal regions measured (dorsal striatum, NAcc core and shell) 2 min after cocaine injection, declined rapidly from 15 min post-cocaine, but was prolonged in the dorsal striatum compared to the ventral striatum (Bertran-Gonzalez et al., 2008). Although we did observe robust ERK1/2 activation in the ventral striatum, but not the dorsal striatum, it would still be valuable to examine additional time points post-cocaine injection in future studies. Second, ERK1/2 activation was assessed only in the medial part of the striatum and it is possible that more robust effects of cocaine would have been recorded in more rostral striatal regions, since, at least in the ventral striatum, a rostro-caudal gradient for cocaine induced ERK1/2 activation has been described (Bertran-Gonzalez et al., 2008). Third, mice experienced only one vehicle sham-injection prior to the test day and it may be that stress arising from the injection procedure contributed to ERK1/2 activation, which masked the effects of cocaine (particularly in the dorsal striatum where basal levels of ERK1/2 activation were greater than that seen in the

ventral striatum). Stress has been noted to activate ERK1/2 in many brain regions, including the striatum (Shen et al., 2004) and it is notable that in a study of Bertran-Gonzalez and colleagues (2008), mice received vehicle injections in each of the three, 2 hour, once-daily habituation sessions that occurred before the actual experiment in which the effect of cocaine on striatal P-ERK1/2 was assessed.

A final procedural difference that may contribute to the differences in ERK1/2 activation observed in our current report with those reported by Valjent and colleagues was the use of food restriction in our current protocol, while mice in studies reported by Valjent and colleagues were provided with *ad libitum* access to food (Valjent et al., 2005; Bertran-Gonzalez et al., 2008). Numerous studies have identified wide ranging influences of food restriction on behavioural responses to cocaine and other addictive substances. For example, in rats, food restriction increased sensitivity to the locomotor-stimulating effects of cocaine (Cabeza de Vaca and Carr, 1998; Stamp et al., 2008), amphetamine (Campbell and Fibiger, 1971; Deroche et al., 1993; Stuber et al., 2002) and morphine (Deroche et al., 1993) and increased the expression of CPP mediated by cocaine (Bell et al., 1997) and amphetamine (Stuber et al., 2002). At the neurobiological level, food restriction in drug naïve rats produced a small but significant enhancement of expression of 35-37 KDa isoforms of Delta-FosB in the NAcc, but not the CPu (Stamp et al., 2008). However, dopamine release in the NAcc following a single amphetamine injection was comparable between *ad libitum* fed and food deprived rats (Stuber et al., 2002), suggesting that alterations in behavioural responses to psychostimulants mediated by food deprivation are unlikely to be determined by the level of extracellular dopamine in the striatum. More convincingly, a clear role for endogenous corticosterone secretion in modulating responses to addictive drugs by food restriction has been demonstrated (Deroche et al., 1993; Stamp et al., 2008). Thus, one possibility is that hyper secretion of corticosterone as a result of food restriction in the present studies may have masked some of the effects of cocaine on ERK1/2 activation. This idea would require further study, but evidence for a role of corticosterone in regulating ERK1/2 activation in limbic regions and the

dorsal striatum has been provided (Gourley et al., 2008) and food restriction results in enhanced sensitivity to ERK1/2 activation under basal conditions in rats (Pan et al., 2006).

There is much evidence that activation of ERK1/2 in response to psychostimulants occurs almost exclusively in MSNs of the direct striatonigral pathway although, under certain behavioural (e.g. home vs. novel environment) or genetic (e.g. ERK1 knock-out mice) manipulations, some activation of ERK1/2 in response to psychostimulants in the indirect striatopallidal pathway has been observed (Valjent et al., 2000; Mazzucchelli et al., 2002; Ferguson and Robinson, 2004; Valjent et al., 2005; Ferguson et al., 2006; Bertran-Gonzalez et al., 2008). In the present study, ERK1/2 activation did occur almost exclusively in MSNs (identified with DARPP-32), and it would have been valuable to formally distinguish between ERK1/2 activation in MSNs of the striatonigral or striatopallidal pathway. Striatonigral neurons are typically characterised by enriched expression of substance P and dynorphin, while striatopallidal neurons contain enkephalin (Chesselet and Graybiel, 1983; Beckstead and Kersey, 1985). In pilot studies (not reported here), antibodies raised against prepro-dynorphin (guinea pig polyclonal, GP10110; Neuromics Inc, Minneapolis, Minnesota, USA) and substance-P (guinea pig polyclonal, ab10353, lot 866742; Abcam) showed little cell-type specificity, despite some reports to the contrary (Martella et al., 2009). To date, it would appear that the most reliable segregator of MSNs by immunohistochemistry is provided by antibodies raised against enkephalin (e.g. Novak et al., 2010). Unfortunately, anti-enkephalin antibodies (produced in rabbit) were not compatible with the combination of antibodies used in the present report to identify P-ERK1/2 (produced in rabbit) and DARPP-32 (produced in mouse). Although it is tempting to propose that loss of mGluR5 on D1-MSNs was concomitant with an attenuation of ERK1/2 activation specifically in D1-MSNs in response to cocaine, it will be of critical importance for any future studies to determine whether cocaine induced ERK1/2 activation in wild-type and mGluR5$^{KD\text{-}D1}$ mice occurred in the same population of MSNs.

While the immunohistochemistry study identified increased ERK1/2 activation in the ventral striatum following cocaine, this finding was not replicated in the immunoblotting studies. These findings are perhaps disconcerting, but not entirely unexpected; others have reported increased ERK1/2 activation in the ventral striatum following cocaine when measured with immunohistochemistry but not immunoblotting (Marin et al., 2009). The total number of P-ERK1/2 +ve cells in the immunohistochemistry study only ever represented a small minority of total cells in each region of interest. In the immunoblotting experiments, homogenate samples incorporated rostral, medial and caudal striatum and could not distinguish between sub-regions in the ventral (that is, NAcc core and shell) or dorsal striatum (e.g. dorsomedial and ventrolateral striatum). Moreover, punches of the ventral striatum likely incorporated dorsal parts of the striatum and some part of the ventral pallidum. Thus, it is likely that small changes in ERK1/2 activation were obscured in the immunoblotting studies by unaltered or even decreased ERK1/2 phosphorylation levels in the majority of neurons within the homogenate sample. Even so, most reports do identify robust increases in P-ERK1/2 with immunoblotting following cocaine, suggesting that there is some room for optimising the experimental protocol used in the present report with respect to the time of sampling, habituation procedures and the use of food restriction (as discussed above).

If future studies confirm disrupted ERK1/2 signalling in the striatum of mGluR5$^{KD\text{-}D1}$ mice, then what might be the implications for neuronal function and behavioural responses to cocaine? First, ERK1/2 is closely involved in synaptic plasticity (reviewed in Thomas and Huganir, 2004; Thomas et al., 2008). For example, in mice lacking the ERK1 isoform, striatal LTP induced by cortical stimulation is dramatically enhanced (Mazzucchelli et al., 2002). The ERK1/2 pathway has also been directed linked to alterations in AMPA receptor surface expression (Boudreau et al., 2007; Zhang et al., 2008) and regulation of AMPA by ERK1/2 may be critical for the induction of LTP at excitatory synapses (Patterson et al., 2010). Thus, it

seems reasonable to propose that plasticity at excitatory corticostriatal synapses may be disrupted in mGluR5^{KD-D1} mice, in part, because of impaired activation of ERK1/2. Second, given the proposed role of corticostriatal plasticity in certain addiction-related behaviours, including drug seeking under the control of drug-associated cues (Kalivas, 2009; Wolf and Ferrario, 2010), it would also be reasonable to propose that such behaviours could also be impaired in mGluR5^{KD-D1} mice. Indeed, recent data has provided direct support for this proposal, since mGluR5^{KD-D1} mice show deficits in cue-induced reinstatement of cocaine seeking (Novak et al., 2010). Whether corticostriatal plasticity is impaired in mGluR5^{KD-D1} and whether this is linked to disruption in ERK1/2 signalling requires further empirical study, but there is considerable evidence to implicate ERK1/2 as a central signalling pathway involved in the learning and expression of control over behaviours by environmental stimuli associated with drug reinforcement (Lu et al., 2005; Valjent et al., 2006).

The immunoblotting studies reported here also examined the role of mGluR5 on D1R expressing neurons in regulating STEP, a family of proteins expressed in dopaminoceptive neurons of the CNS and enriched in the basal ganglia and related structures (Lombroso et al., 1991; Lombroso et al., 1993). STEP family members are produced by alternative splicing with variants including STEP61, a membrane associated protein, and STEP46, a cytosolic protein (Sharma et al., 1995; Bult et al., 1996; Bult et al., 1997). The role of STEP proteins in synaptic plasticity has been reviewed by others (Braithwaite et al., 2006a), but notably, at least in the hippocampus, stimulation of group I mGluRs causes rapid translation of STEP61 protein, which is necessary for the endocytosis of AMPA receptors (Zhang et al., 2008). Moreover, data would suggest that STEP61 translation in the hippocampus requires mGluR5-, not mGluR1-, mediated ERK1/2 activation (Zhang et al., 2008). This report is particularly interesting with respect to findings of the current chapter, because an increase in total STEP61 protein was found in the dorsal striatum of wild-type mice injected with cocaine, but not mGluR5^{KD-D1} mice. If STEP61 translation promotes AMPAR receptor internalisation in the striatum, as

has been reported in the hippocampus (Zhang et al., 2008), it would be exciting to propose that AMPA receptor trafficking in MSNs in response to acute cocaine challenge necessitates translation of STEP61 following stimulation of mGluR5 on D1-MSNs. However, the increase in STEP61 protein levels following cocaine in wild-type mice was small and further studies would be required to explore whether AMPA receptor trafficking was disrupted in mGluR5^{KD-D1} mice because of alterations in STEP translation.

At least two further functions of STEP in regulating neuronal activity warrant further investigation in mGluR5^{KD-D1} mice. First, STEP associates with the NMDA complex in postsynaptic terminals, constitutively inhibits NMDA channel function (Oyama et al., 1995; Pelkey et al., 2002) and can regulate LTP, in part, through modulation of NMDA trafficking (Snyder et al., 2005; Braithwaite et al., 2006b). Thus, it would be of interest to explore the extent to which NMDA channel function is altered, if at all, in mGluR5^{KD-D1} mice. Second, through direct protein-protein interactions, active (dephosphorylated) STEP can inhibit the duration of ERK1/2 activation by dephosphorylating the tyrosine residues in the ERK1/2 activation loop (Pulido et al., 1998; Paul et al., 2000; Paul et al., 2003). STEP activation is under the opposing control from dopamine and glutamate signalling in the striatum. Thus, in MSNs, dopamine signalling through D1 receptors and activation of cAMP-dependent PKA inactivates STEP by phosphorylation and thus prevents STEP from dephosphorylating ERK (Pulido et al., 1998; Paul et al., 2000). The maintenance of inactive (phosphorylated) STEP by dopamine likely involves a PKA mediated phosphorylation of DARPP-32 at Thr34 and subsequent inhibition of PP-1, which would normally serve to activate STEP (Svenningsson et al., 2004; Valjent et al., 2005). Oppositely, glutamate mediated activation of NMDA receptors leads to activation of STEP, by dephosphorylation, which in turn limits the duration of ERK1/2 activation (Paul et al., 2003). Since the present immunohistochemistry studies suggest that activation of ERK1/2 in response to cocaine is attenuated in mutant mice, it would be of interest to further explore the contribution of STEP to this finding; the hypothesis being that reduced ERK1/2 activation could be due to

increased levels of active (dephosphorylated) STEP in mGluR5^{KD-D1} mice, possibly arising from loss of PKA-mediated inactivation of STEP. On measuring the phosphorylation state of STEP, it is noteworthy that others have reported STEP phosphorylation to be observed by an increase in the apparent molecular weight of the 46KDa STEP isoform (Valjent et al., 2005; Sun et al., 2007). No such shift in STEP46 was found in the present immunoblotting studies, suggesting that optimisation of the immunoblotting protocol (with respect to the primary antibody, gel concentration and electrophoresis conditions) will be required to fully understand any changes in STEP activity in the striatum of mGluR5^{KD-D1} mice.

Phosphorylation of the GluR1 subunit of AMPA at Ser845 was increased in the dorsal striatum of wild-type mice following cocaine but, remarkably, no such change was found in mGluR5^{KD-D1} mice. Phosphorylation of GluR1-s845 by cocaine necessitates activation of dopamine D1R/cAMP-dependent PKA (Snyder et al., 2000; Valjent et al., 2005) and GluR1-s845 phosphorylation is often used as a surrogate measure for PKA activity. That levels of P-GluR1-s845 were not increased by cocaine in mGluR5^{KD-D1} mice, at least in the dorsal striatum, provides critical support for the proposal that ERK1/2 signalling, and indeed regulation of DARPP-32 and STEP activity, could well be disrupted in mGluR5^{KD-D1} mice due to loss of PKA activity. The GluR1-Ser845 site is also functionally important, since its phosphorylation results in enhanced current of GluR1 containing AMPA receptors and thus promotes increased neuronal activity (Banke et al., 2000). In the hippocampus, phosphorylation of GluR1-s845 is modulated during LTP and LTD and may affect membrane insertion of GluR1-containing AMPA receptors (Lissin et al., 1999; Lee et al., 2003). Moreover, mice with point-mutations of GluR1-s845 show impaired memory deficits in spatial learning tasks (Lee et al., 2003). By analogy, it is tempting to propose that impaired corticostriatal plasticity and associated learning impairments in mGluR5^{KD-D1} may be due, in part, to disrupted PKA activation and regulation of GluR1-s845 phosphorylation.

An important limitation of the immunoblotting studies is that levels of phosphorylated protein (e.g. P-GluR1-s845) were not corrected for levels of total protein (i.e. total GluR1). It is therefore possible that changes in P-GluR1-s845 simply reflected a change in total GluR1 protein. Arguing against this possibility is that P-GluR1-s831 levels did not change following cocaine, which would have been expected had total GluR1 protein been altered. It is noteworthy that others have also reported no change in P-GluR1-s831 protein, which necessitates activation of PKC and CAMKII, in the striatum of cocaine treated animals (Roche et al., 1996; Mammen et al., 1997; Snyder et al., 2000). Nevertheless, cocaine is well known to modulate cell surface expression of AMPA receptor subunits in many brain regions (reviewed in Wolf and Ferrario, 2010) (but see Lu et al., 2002) and it would be imperative that any future studies applied this control measure. However, even if phosphorylated proteins had been corrected against total protein, it must be recognized that AMPA receptor subunits can rapidly cycle between intracellular pools and the cell surface (Shepherd and Huganir, 2007) and even total protein levels may provide a poor control measure. Thus, under ideal conditions, phosphorylated and total protein levels in these distinct cellular compartments would be analysed separately by the use of cross-linking (e.g. Boudreau and Wolf, 2005) or subcellular fractionation (e.g. Ghasemzadeh et al., 2009a) techniques.

In summary, the present data suggest that neuroplastic changes triggered by acute non-contingent cocaine experience, including ERK activation and AMPA receptor trafficking and phosphorylation state, may be disrupted in mice lacking mGluR5 on D1-MSNs. Whether disruption of these neuroplastic events reflects the necessity for glutamate signalling through mGluR5 on D1-MSNs *per se*, interactions between mGluR5 and NMDARs or D1Rs, or generally disrupted dopaminergic signalling in mGluR5$^{KD\text{-}D1}$ mice remains to be determined. This question will be touched upon again in the general discussion, but it is interesting to note that mGluR5 KO mice show a normal increase in extracellular dopamine in the accumbens following acute cocaine challenge (Chiamulera et al., 2001). Many further studies are required to formally confirm or disprove the present findings and suggestions for

how to optimise such studies have been provided. The early neurobiological effects of cocaine assessed in the present chapter can have important long term consequences for neuronal function, including altered strength of corticostriatal synapses (Thomas et al., 2001b; Borgland et al., 2004) and changes in dendritic morphology (Robinson and Kolb, 2004; Crombag et al., 2005; Ren et al., 2010). Thus, given the present findings, it will also be valuable to assess electrophysiological and morphological characteristics of striatal MSNs in mGluR5$^{KD\text{-}D1}$ mice both following acute and repeated cocaine exposure and following a period of cocaine withdrawal, ideally from response-contingent (i.e. self-administration) cocaine experience.

7 General discussion

7.1 Review of general aims

The ability to learn about and subsequently use environmental stimuli associated with reward experience to guide our behaviour has clear adaptive value. Addictive drugs are proposed to closely interact with neural mechanisms of learning and memory, enabling environmental stimuli associated with drug experience to promote drug-seeking, drug-taking and to trigger relapse (Stewart et al., 1984; Robinson and Berridge, 1993; Everitt et al., 2001; Kelley, 2004; Hyman et al., 2006). Uncovering the neural mechanisms of reward-related learning may therefore provide valuable insight into the basis of clinical disorders like drug addiction. The group I metabotropic glutamate receptor, mGluR5, is particularly interesting in this respect. It is densely located within brain regions that contribute to reward-related learning (Shigemoto et al., 1993; Romano et al., 1995; Tallaksen-Greene et al., 1998) and electrophysiology and biochemistry studies point to mGluR5 as a key regulator of synaptic plasticity (e.g. Mao et al., 2005; Bellone et al., 2008; Anwyl, 2009), considered as the cellular correlate of learning and memory. Using a novel mouse line in which mGluR5 is selectively knocked-down on cells that express the dopamine D1R (Novak et al., 2010), the present thesis aimed to further understand the role of mGluR5 located on dopaminoceptive neurons in Pavlovian incentive learning processes for a natural reward and in some behavioural and neurobiological effects of one addictive drug, cocaine.

7.2 Summary of key findings

A summary of the key findings of this thesis is provided in Table 7.1. Before undertaking more complex behavioural assessments, it first was necessary to understand the basic phenotype of mGluR5^{KD-D1} mice (Chapter 2). On casual observation, mGluR5^{KD-D1} mice were indistinguishable from their wild-type counterparts, but the experiments of Chapter 2 identified two interesting traits in this novel mouse line. First, mGluR5^{KD-D1} mice displayed a reduced anxiety-like phenotype in tests of unconditioned anxiety-related behaviours, although this effect was not particularly robust across the three different tests employed. Second, in comparison to their wild-type counterparts, mutant mice showed a clear reduction in exploratory locomotor activity when placed into a novel inescapable environment. However, after a period of habituation, basal locomotor activity did not differ between genotypes.

Studies on Pavlovian incentive learning in mGluR5^{KD-D1} mice (Chapter 3) identified mGluR5 on dopaminoceptive neurons as critically important for incentive learning that enables an environmental stimulus associated with reward to became attractive and elicit approach toward it (sign-tracking) and to support the learning of a new instrumental response (CRf). It is also noteworthy that a psychostimulant failed to facilitate responding for CRf in mGluR5^{KD-D1} mice; a phenomenon that is critically dependent upon dopamine and glutamate signalling within the ventral striatum (Taylor and Robbins, 1986; Wolterink et al., 1993; Burns et al., 1994; Parkinson et al., 1999). In contrast to these select deficits in Pavlovian incentive learning processes, mGluR5 on dopaminoceptive neurons appeared to play no role in learning about the predictive relationship between the reward-paired stimulus and the reward itself (discriminated approach), the acquisition of a simple food-reinforced instrumental response, or incentive learning that allows the food-paired CS to motivate ongoing, goal-directed actions (PIT). Studies using wild-type mice injected with the selective mGluR5 antagonist, MTEP, (Chapter 4) indicated that mGluR5 was particularly important for the acquisition of an incentive Pavlovian

association necessary to support CRf, rather than the expression of the acquired incentive association.

In Chapter 5, I examined the possibility that some behavioural effects of cocaine, which involve neural components implicated in Pavlovian incentive learning processes, might also be disrupted in mGluR5^{KD-D1} mice. Mutant mice demonstrated normal performance in a test of learning about cocaine and food conditioned reward (i.e. CPP). The psychological processes involved in CPP are not fully understood (see discussion of Chapter 5), but data from this Chapter suggested that learning and/or performance in this task does not require Pavlovian incentive learning processes that are necessary for sign-tracking and conditioned-reinforcement responses. In a series of locomotor studies reported in Chapter 5, the acute psychomotor activating effects of cocaine were found to be attenuated in mGluR5^{KD-D1} mice, in comparison to wild-type animals. Notably, mGluR5^{KD-D1} mice did develop a sensitised locomotor response to cocaine, which persisted for at least 2 months after its induction. However, it is important to note that the rate of sensitisation was slightly reduced in mutant mice (although not significant) and the possibility remains that more robust differences in sensitisation between genotypes may have been uncovered had sensitisation been performed in animals without prior cocaine experience and/or by the inclusion of other measures of activity, including the development of stereotypies.

Finally, in Chapter 6, a series of experiments were undertaken to explore some neurobiological effects of acute cocaine exposure in mGluR5^{KD-D1} mice. The purpose of these experiments was twofold. First, to understand the role of mGluR5 on dopaminoceptive neurons in neuroplastic changes that may contribute to long term behavioural effects of cocaine and second, to highlight any deficits in neuroplasticity that could have contributed to incentive learning deficits observed in mGluR5^{KD-D1} mice. Notwithstanding the technical limitations raised in the discussion of Chapter 6, it appeared that activation of the ERK1/2 intracellular signalling cascade was disrupted in the ventral striatum of mGluR5^{KD-D1} mice

following cocaine (immunohistochemistry studies), and some evidence pointed to disruption of AMPA receptor trafficking and/or regulation of its phosphorylation by PKA at the GluR1-s845 site, at least in the dorsal striatum (immunoblotting studies).

Test	KD	WT
Chapter 2		
Basic observations (body weight, reflexive responses)	✓	
Sucrose consumption	✓	
Locomotor activity in a novel environment	×	
Time in middle zone of open-field	×	
Chapter 3		
Discriminated approach	✓	
Sign-tracking	×	
Conditioned reinforcement	×	
Cocaine facilitation of CRf	×	
Instrumental responding for food	✓	
Pavlovian instrumental-transfer	✓	
Chapter 4		
MTEP on the acquisition of incentive learning		×
MTEP on the expression of incentive learning		✓
Chapter 5		
Acute cocaine locomotor response	×	
Cocaine sensitisation	✓ / **?**	
Conditioned activity	✓	
Is cocaine sensitisation persistent?	✓	
Food CPP	✓	
Cocaine CPP	✓	
Chapter 6 (In response to acute cocaine...)		
ERK1/2 activation in the ventral striatum	×	
STEP61 translation in the dorsal striatum	×	
P-GluR1-s845 in the dorsal striatum	×	

Table 7.1 Summary of key findings from each chapter. Normal performance (✓) and altered performance (×) in mGluR5$^{KD\text{-}D1}$ mice (KD) relative to wild-type (WT) littermates is indicated. Studies in Chapter 4 were conducted only in wild-type mice, and comparisons here are made with wild-type mice treated with vehicle. For the cocaine sensitisation study, a question mark is given; see comment in main text above.

7.3 Is mGluR5 on D1R expressing neurons involved in specific sensory or general preparatory conditioning processes?

In the introduction of this thesis I introduced two accounts of motivation; a 'Konorskian' model (Konorski, 1967; Dickinson and Balleine, 2002; Balleine, 2005) and an 'incentive salience' model (Robinson and Berridge, 1993; Berridge, 1996; Berridge and Robinson, 1998; Berridge, 2004), both of which described how environmental stimuli associated with reward experience could come to influence behaviours through Pavlovian learning processes. According to the incentive salience model, mGluR5$^{KD\text{-}D1}$ mice show impaired incentive salience attribution necessary for the acquisition of sign-tracking and CRf CRs, but a valuable question to consider is whether impaired learning in mGluR5$^{KD\text{-}D1}$ mice relates more to specific consummatory or general motivational conditioning processes, as described in the Konorskian model.

In answering this question, it is first necessary to consider whether the three tests of Pavlovian incentive learning represent specific consummatory or general motivational conditioning processes. Conditioned reinforcement may be mediated by activation of both general motivational or specific outcome representations, which are proposed to operate in concert under normal conditions (Burke et al., 2007, 2008). Sign-tracking responses often acquire a topography that resembles that of the UR directed at the US (Wolin, 1968; Jenkins and Moore, 1973), indicating that sign-tracking is partly a reward-specific mechanism. The conditioning parameters used for PIT in the present thesis likely favoured the formation of a more general motivational CS-US association due, in part, to the longer duration CS (Konorski, 1967; Dickinson and Balleine, 1994; Holland and Gallagher, 2003; Crombag et al., 2008a), but also because only one CS-US outcome was trained. Taken together, it would appear that mGluR5$^{KD\text{-}D1}$ mice fail to acquire more specific consummatory associations (Fig. 7.1). As such, it may be predicted that other Pavlovian learning processes that involve sensory specific

learning, such as outcome-selective PIT, would also be disrupted in mGluR5$^{KD\text{-}D1}$ mice.

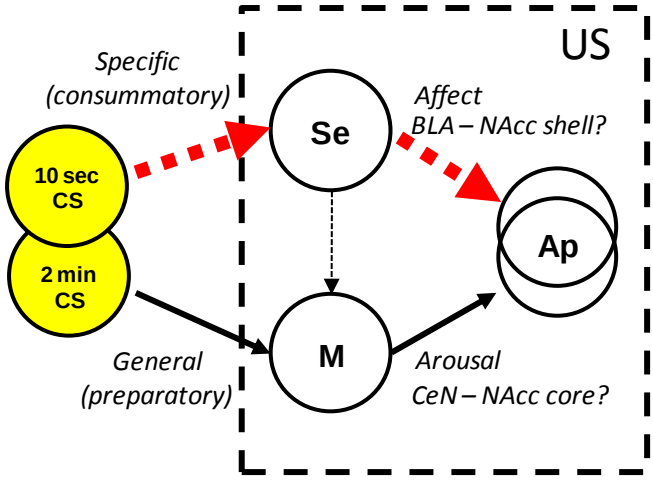


Figure 7.1. mGluR5^{KD-D1} mice show impaired specific consummatory conditioning. Both sign-tracking and CRf processes employ a short duration CS (10 sec CS) and may require the formation of specific consummatory associations to activate the appetitive system (Ap) through increased affect (red arrows indicate impairment in mGluR5^{KD-D1}). In contrast, Pavlovian-instrumental transfer was normal in mGluR5^{KD-D1} mice, which employs a long duration CS (2 min CS) and depends upon the formation of more general preparatory CS-US associations that give rise to arousal. A connection between sensory specific (Se) and motivational (M) features is thought not functional for the role of reward-related cues on activating instrumental performance (adapted from Balleine, 2005).

7.4 Does Pavlovian incentive learning involve mGluR5-mediated regulation of plasticity in D1-MNSs?

Deficits in the acquisition of sign-tracking and CRf in mGluR5^{KD-D1} mice and a failure of cocaine to facilitate CRf in mutant animals closely echo results from studies in rats that received selective excitotoxic lesions of the ventral striatum, or in which glutamatergic signalling in this region was blocked (Burns et al., 1994; Parkinson et al., 1999; Parkinson et al., 2000b; Di Ciano et al., 2001; Di Ciano and Everitt, 2001; Cardinal et al., 2002b; Ito et al., 2004; Dalley et al., 2005; Backstrom and Hyytia, 2007). Such studies (reviewed more extensively in the general introduction), have led a number of reviewers to propose that neuroplastic changes on MSNs in the ventral striatum during reward-related learning may be key for determining how these neurons subsequently respond to salient events (e.g. reward-associated CSs), and that glutamatergic mechanisms, acting in partnership with dopamine, may be critical for initiating and maintaining these neuroplastic changes (Kelley et al., 2003; Svenningsson et al., 2004; Dalley et al., 2005; Valjent et al., 2005; Meredith et al., 2008; Wolf and Ferrario, 2010). Findings in the present thesis suggest that mGluR5 on D1-MSNs in the ventral striatum may play a critical role in controlling neuroplastic changes that are necessary for supporting the acquisition of specific Pavlovian incentive learning processes. A key question is through which cellular mechanisms does mGluR5 exert such control?

In the general introduction (Chapter 1), studies were highlighted that identified an important role of group I mGluRs in mediating LTD at excitatory synapses in the dorsal and ventral striatum, which likely involves a retrograde-endocannabinoid signalling mechanism (Robbe et al., 2002; Kreitzer and Malenka, 2007; Uchigashima et al., 2007; Shen et al., 2008). It is possible that this ‘post-synaptic induction/pre-synaptic expression’ mechanism of mGluR-LTD could be disrupted in mGluR5^{KD-D1} mice and could account for observed incentive learning deficits. There is also evidence that mGluR5 can contribute to corticostriatal LTP (Gubellini et al., 2003; Schotanus and Chergui, 2008) and ‘post-synaptic induction/post-

synaptic expression' mechanisms of neuroplasticity could also account for incentive learning deficits in mGluR5^{KD-D1} mice. Two such mechanisms, although not mutually exclusive, can be proposed; one involving mGluR5-mediated regulation of AMPA receptor activity and a second involving regulation of the ERK1/2 signalling cascade. These intracellular interactions are illustrated in Figure 7.2.

Stimulation of group I mGluRs, including mGluR5, can produce changes in the post-synaptic expression of AMPA receptors (Snyder et al., 2001; Bellone and Luscher, 2005; Mameli et al., 2007; Jo et al., 2008; Waung et al., 2008; Zhang et al., 2008; Kelly et al., 2009) and, in the striatum, activation of mGluR5 is required for phosphorylation of striatal GluR1-Ser831 and -Ser845 (Ahn and Choe, 2009), and GluR2-Ser880 residues (Ahn and Choe, 2010). Such changes in AMPA receptor trafficking and/or activity may be critical for experience-dependent alterations in synaptic plasticity and which subsequently determine the sensitivity to control over appetitive behaviours by reward-paired CSs (Di Ciano et al., 2001; Mead and Stephens, 2003b, a; Conrad et al., 2008; Crombag et al., 2008b; Crombag et al., 2008c). Whether mGluR5 is important for regulating trafficking and/or phosphorylation of AMPA receptors during Pavlovian incentive learning processes requires further empirical study, but some support for the proposal that this processes is disrupted in mGluR5^{KD-D1} mice is offered by the immunoblotting experiments reported in Chapter 6 (see Table 7.1 for summary).

Group I mGluRs can positively link to the extracellular signal-regulated kinase (ERK) cascade (Peavy and Conn, 1998; Thandi et al., 2002), which has numerous functions in experience-dependent plasticity (Adams and Sweatt, 2002; Boudreau et al., 2007; Patterson et al., 2010; Shiflett and Balleine, 2011). Activation of ERK1/2 in the striatum requires co-stimulation of D1R and NMDARs (Valjent et al., 2005) and it is noteworthy that both D1R and NMDA receptors in the accumbens were identified as critical for the early consolidation of appetitive Pavlovian memories (Dalley et al., 2005). These reports are particularly relevant in the

context of this thesis, given the close interactions between striatal mGluR5 and D1Rs (involving an enhanced cAMP response; Paolillo et al., 1998; Voulalas et al., 2005; Schotanus and Chergui, 2008), and striatal mGluR5 and NMDA receptors (Pisani et al., 2001; Mao and Wang, 2002; Yang et al., 2004; Choe et al., 2006). Regarding the mGluR5-NMDA interaction, it is likely that this involves a PKC dependent mechanism (Kelso et al., 1992; Fitzjohn et al., 1996; Doherty et al., 1997; Pisani et al., 1997; Awad et al., 2000; Skeberdis et al., 2001) in which PKC phosphorylates the ion channel associated with NMDA receptors that increases NMDA sensitivity and activity leading to enhanced Ca^{2+} influx (Chen and Huang, 1992; Lan et al., 2001; Skeberdis et al., 2001). Some reports have also proposed that stimulation of mGluR5 alone is sufficient for ERK1/2 activation in striatal neurons (Choe et al., 2002; Mao et al., 2005). Thus, whether through co-operative interactions between mGluR5 and D1Rs and/or NMDARs, or as a direct consequence of mGuR5 stimulation, activation of ERK1/2 involving mGluR5 on D1-MSNs in the ventral striatum may be a key component necessary for the formation of Pavlovian incentive associations. Some support for the proposal that activation of ERK1/2 is disrupted in $mGluR5^{KD\text{-}D1}$ mice is offered from immunohistochemistry studies of Chapter 6 (see Table 7.1 for summary).

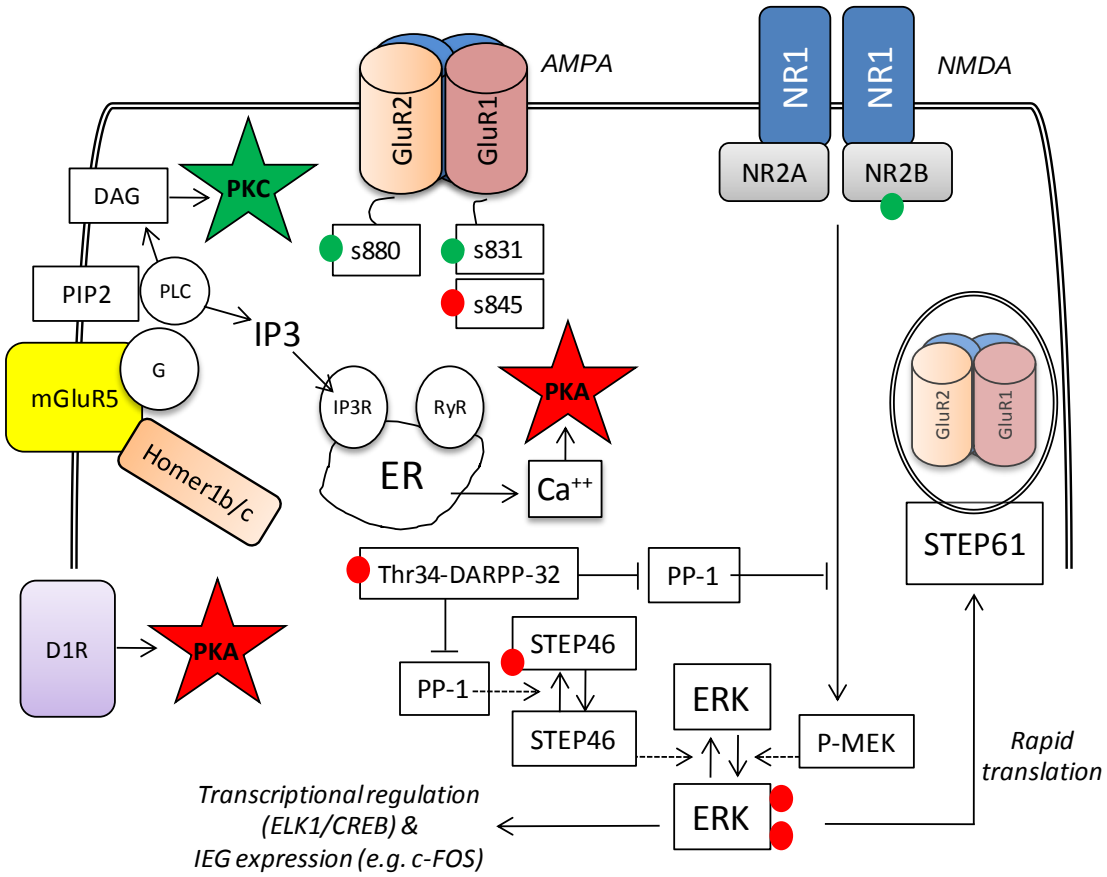


Figure 7.2 Post-synaptic induction/post-synaptic expression mechanisms of mGluR5 signalling in striatal neurons. In a simplified and largely hypothetical post-synaptic D1-MSN synapse, stimulation of perisynaptic mGluR5 may result in activation of PKC and PKA, the latter involving endogenous release of calcium. Activity of a number of substrates is dependent upon phosphorylation regulated by PKA (red dots; note STEP46 and ERK are not direct substrates of PKA, but lie downstream of PKA phosphorylation of DARPP-32 at Thr34) and PKC (green dots). Phosphorylation of AMPA receptor subunits can lead to alterations in activity and/or trafficking. Activation of ERK by phosphorylation may result in the rapid synthesis of STEP61 protein, at least in the hippocampus, which promotes internalisation of AMPA receptors. Coupling between mGluR5 and the ERK cascade is also achieved by the scaffolding protein Homer1b/c. (image based on findings reported by; Snyder et al., 2001; Mao et al., 2005; Ossowska, 2005; Valjent et al., 2005; Zhang et al., 2008; Ahn and Choe, 2009, 2010).

7.5 Does Pavlovian incentive learning require a balance of output between direct and indirect pathways?

In the classical model of basal ganglia function, the D1R-expressing 'direct' pathway and D2R-expressing 'indirect' pathway act in opposition to balance behavioural output (Albin et al., 1989; Gerfen, 1992). Thus, activation of the direct pathway increases locomotor activity and activation of the indirect pathway exerts a tonic inhibitory tone. A disruption in the balance of activity in these two pathways is a central feature of motor disturbances in Parkinson's disease and in Parkinsonian models, an absence of mGluR-LTD on the D2R indirect pathway has been reported, which appears to shift the balance of plasticity in this pathway toward LTP (Kreitzer and Malenka, 2007; Shen et al., 2008). The resulting enhancement of activity of the indirect pathway is proposed to give rise to excessive inhibition of movement (Kreitzer and Malenka, 2007; Shen et al., 2008; Luscher and Huber, 2010). Dysregulation in the balance of activity between D1- and D2-MSNs has also been proposed to contribute to maladaptive learning processes in addictions (Shen et al., 2008). Thus, one account to explain findings in the present thesis is that loss of mGluR5 on D1R-expressing neurons of the direct pathway results in a dysregulation in the balance of output from the striatum, which clearly would stem from a failure to appropriately integrate dopamine and glutamate signalling as discussed in section 7.4.

Some recent evidence provides support for this proposal. Bateup and colleagues (2010) generated mice in which DARPP-32, a central signalling protein, was selectively knocked out in D1R or D2R expressing neurons. In mice lacking DARPP-32 in D1R striatonigral neurons, spontaneous locomotor activity was reduced in comparison to controls and so too were the acute psychomotor activating effects of cocaine (Bateup et al., 2010). In mice lacking DARPP-32 in D2R striatopallidal neurons, spontaneous locomotor activity and the psychomotor activating effects of cocaine were both increased (Bateup et al., 2010). Durieux and colleagues (2009) reported that selective ablation of D2R MSNs in the entire

striatum resulted in hyperlocomotion, while ablation of D2R MSNs solely in the ventral striatum increased amphetamine CPP. Similarly, Lobo and colleagues (Lobo et al., 2010) reported that optogenetic stimulation of D1+ve neurons in the ventral striatum enhanced cocaine CPP, while stimulation of D2+ve neurons inhibited this measure of cocaine conditioned reward. Taken together, it seems plausible that disrupted activity in neurons of the D1R direct pathway of mGluR5^{KD-D1} mice could account for a reduction in both spontaneous locomotor activity and the psychomotor activating effects of cocaine. Moreover, it is tempting to propose that the appropriate integration of dopaminergic and glutamatergic signals in the direct pathway, involving mGluR5, and the resultant balance of activity between direct and indirect MSNs, may be a critical for appropriately responding to reward-associated CSs (Fig. 7.2). Notably, this model would predict a reduction of activity in the D1R direct pathway of mGluR5^{KD-D1} mice (either dominant LTD and/or a loss of LTP), but further studies would clearly be required to explore this hypothesis.

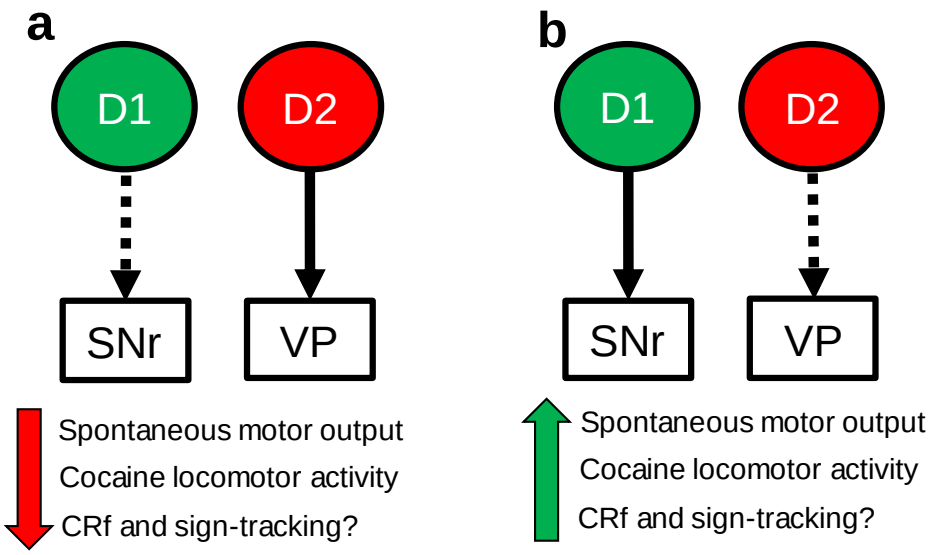


Figure 7.3 Disruption of output from the basal ganglia accounts for features of mGluR5^{KD-D1} mice. (**a**) When the function of GABAergic D1-MSNs (green) of the direct striatonigral pathway is disrupted (dotted line), spontaneous motor output diminishes and so too do the effects of cocaine on locomotor activity (Bateup et al., 2010). Such behaviours are also found in mGluR5^{KD-D1} mice, and thus loss of mGluR5 on D1-MSNs may well result in a disruption of D1-MSN function. Further, loss of mGluR5 from D1-MSNs in the ventral striatum may account for deficits observed in specific Pavlovian incentive learning processes. (**b**) When function of D2-MSNs (red) of the indirect striatopallidal pathway is disrupted, spontaneous motor output increases, and so too do the psychomotor activating effects of cocaine (Durieux et al., 2009; Bateup et al., 2010).

7.6 Implications for drug addiction

Early neuroplastic changes in response to addictive drugs, while in no way constituting the complete picture of addiction, may represent critical first steps in the remodelling of reward-related brain areas that underlie the development of more persistent addiction-related behaviours (Nestler, 2001; Valjent et al., 2001; Hyman et al., 2006; Kauer and Malenka, 2007). In this regard, mGluR5 on D1-MSNs may play a role, since activation of ERK1/2 and activity and/or trafficking of AMPA receptors appeared disrupted in striatal regions of mGluR5^{KD-D1} mice following a single cocaine challenge (Chapter 6). It is beyond the scope of this thesis to cover the literature on the role of ERK1/2 signalling and AMPA receptor function in addiction-related behaviours but, needless to say, both components are thought to play central roles in enabling environmental stimuli associated with drug experience to exert influence over addiction-related behaviours (Wolf, 1998; Thomas and Huganir, 2004; Wolf et al., 2004; Lu et al., 2006; Wolf and Ferrario, 2010).

Behavioural sensitisation is often used as a surrogate measure of the ability of repeated cocaine experience to produce long-lasting changes in brain systems that mediate incentive salience attribution (Robinson and Berridge, 1993, 2000, 2001). Since incentive salience attribution was disrupted in mGluR5^{KD-D1} mice, it was predicted that behavioural sensitisation would also have been disrupted in mutant animals, in accordance with similar correlations reported elsewhere (Flagel et al., 2008). However, this was not the case; mutant mice developed a persistent, sensitised cocaine-locomotor response (Chapter 5). It must be stressed that more sensitive measures of behavioural activity may have revealed differences in the *rate* of sensitisation between wild-type and mGluR5^{KD-D1} mice, which may be a critical component of neurobehavioural plasticity (Flagel and Robinson, 2007; Flagel et al., 2008). Thus, based on the present data, we cannot entirely exclude the possibility that mGluR5 on D1R expressing neurons play an important role in

drug-induced neuroplastic changes that are proposed to result in a sensitisation of incentive salience attribution.

That mGluR5^{KD-D1} mice were impaired in sign-tracking and CRf, suggests that such incentive learning processes may also be disrupted in mGluR5^{KD-D1} mice during learning about drug-associated CSs. Cocaine self-administration studies in mGluR5^{KD-D1} mice provide direct confirmation of this proposal (Novak et al., 2010). Mutant mice were observed to self-administer cocaine under a fixed-ratio schedule of reinforcement, but cue-induced reinstatement of drug-seeking, considered as an animal model of relapse vulnerability (Shaham et al., 2003; Sanchis-Segura and Spanagel, 2006; Stephens et al., 2010), was attenuated in mutant mice (Novak et al., 2010). This finding mirrors the failure of mGluR5^{KD-D1} mice to respond for CRf (Chapter 3) and supports a common notion that the cue-induced reinstatement model is heavily influenced by CRf related learning processes. A valuable series of future experiments would be to test mGluR5^{KD-D1} mice in second-order schedules of reinforcement and also in approach toward a drug-paired CS (e.g. Uslaner et al., 2006). These models are thought to reflect aspects of drug-seeking and taking and the ability of drug-CSs to attract drug addicts toward places where drugs can be found (reviewed in the General Introduction, section 1.6). Here, the expectation would be that responding under second-order schedules and approach toward a drug-paired CS would be disrupted in mGluR5^{KD-D1} mice.

In summary, our present findings, together with self-administration studies in mGluR5^{KD-D1} mice (Novak et al., 2010), suggest that mGluR5-mediated neuroplastic events on D1-MSNs are crucial for the formation of psychologically distinct associations between environmental stimuli and rewards that endow reward-paired stimuli with the subsequent ability to both reinforce and attract motivated behaviours. Furthermore, recent reports have revealed that mGluR5-mediated striatal plasticity is involved in, or affected by, cocaine experience (Fourgeaud et al., 2004; Moussawi et al., 2009; Hao et al., 2010). Data from mGluR5^{KD-D1} mice provide a psychobiological context for these findings by pointing

to glutamate signalling at mGluR5 on striatal D1-MSNs as a key mediator through which repeated cocaine experience (and presumably exposure to other drugs of abuse) produces a persistent increase in the susceptibility to relapse triggered by environmental stimuli associated with drug use.

7.7 Limitations

In generating mGluR5^{KD-D1} mice, cell-type specific knock-down of mGluR5 was achieved by driving the expression of artificial micro-RNAs that targeted mGluR5 mRNA with a D1R promoter (Novak et al., 2010). Thus, mGluR5 knock-down was restricted to cells that express the D1R. The percentage of striatal MSNs that exclusively express D1Rs or D2Rs or express both D1Rs and D2Rs has been the focus of much debate (for review see Bertran-Gonzalez et al., 2011), with some reports indicating little overlap (Gerfen et al., 1990), and others suggesting many if not all MSNs contain both D1Rs and D2Rs (Surmeier et al., 1992; Aizman et al., 2000). Key arguments of this thesis rest on the position that there is some degree of segregation, which is supported by the characterisation of mGluR5^{KD-D1} mice in which mGluR5 transcript and protein levels are reduced to ~40 and ~50%, respectively, of wild-type levels (Novak et al., 2010). However, if there is a high degree of overlap it cannot be concluded that some MSN populations are more important than others for Pavlovian incentive learning. Nevertheless, our data do highlight the importance of glutamate signalling through mGluR5, on cells that also receive dopamine input, for Pavlovian incentive learning processes and some behavioural and neurobiological effects of cocaine.

Much of this thesis has focused on the role of mGluR5 in the ventral striatum, in part because of the large volume of corroborating literature that identifies this region as important for Pavlovian incentive learning processes, but also because little characterisation has yet been performed to understand the extent of mGluR5 loss within other brain regions in mGluR5^{KD-D1} mice. The possibility remains that mGluR5 loss outside the ventral and dorsal striatum could have contributed to findings reported in this thesis. For example, group I mGluRs function at multiple levels within the subcortical nuclei that constitute the basal ganglia to regulate locomotor activity (DeLong and Wichmann, 2007). In the STN, activation of mGluR5 on postsynaptic neurons results in a switch from a characteristic single-spike firing mode to a burst-firing mode (Awad et al., 2000); a firing state commonly

observed in Parkinsonian rats and non-human primates (Hollerman and Grace, 1992; Bergman et al., 1994). Similarly, mGluR5 is distributed throughout the SNr (Hubert et al., 2001) and activation of mGluR5 mediates depolarization of these neurons, albeit only under conditions of dopamine blockade (Marino et al., 2002). In the mGluR5$^{KD\text{-}D1}$ mouse model, whether mGluR5 is knocked-down in these regions will depend upon whether D1R receptors are expressed in the same neuronal populations, and further characterisation is warranted. For example, while there is some evidence to suggest that the D1R promoter may be active in the STN, its pre- or post-synaptic location is debated (Fremeau et al., 1991; Mansour et al., 1992; Smith and Kieval, 2000; Smith and Villalba, 2008).

Another structure that should not be overlooked within the context of this thesis is the amygdala, which provides a key link between brain regions processing sensory information and those involved in more fundamental motivational aspects of reward-processing. In particular, the BLA and its interactions with the NAcc appear critical for turning associative information into goal-directed actions (Cador et al., 1989; e.g. Burns et al., 1993; Di Ciano and Everitt, 2004b), while the CeN may influence the impact of the motivational value of a CS via its connections with the ventral midbrain (Robledo et al., 1996). Could mGluR5 loss from D1R expressing neurons in the amygdala account for findings reported in this thesis? In the amygdala, mGluR5 has been found on dendritic shafts and spines in the lateral nucleus, post-synaptic to auditory thalamic inputs, and contributes to plasticity in this thalamo-amygdala pathway (Fendt and Schmid, 2002; Lee et al., 2002; Rodrigues et al., 2002). A retrograde endocannabinoid mechanism involving mGluR5 has been identified in isolated BLA neurons with GABAergic input neurons attached (Zhu and Lovinger, 2005) and mGluR5 also contributes to plasticity at BLA→CeN synapses (Neugebauer et al., 2003; Kolber et al., 2010). Further work is required to understand the extent of mGluR5 and D1R co-localisation in amygdala neurons and the studies mentioned here have only considered the role of mGluR5-mediated plasticity in the amygdala with respect to anxiety- (e.g. Rodrigues et al., 2002) and pain- (Kolber et al., 2010) related behaviours. Few

studies have explored the role of mGluR5 in the amygdala in appetitive learning tasks. One notable exception is from Schroeder and colleagues (2008), who reported that cue-induced reinstatement of alcohol seeking behaviour was associated with a robust increased in P-ERK1/2 in the BLA and NAcc shell, and both reinstatement and ERK1/2 activation was blocked by systemic MPEP administration. Thus, there is some evidence that mGluR5 in the amygdala may be play an important role in responding to reward-paired CSs, but further investigations are clearly required.

Only one mutant mouse line was used for experiments on the present thesis, and we cannot exclude the possibility that behavioural effects in mGluR5$^{KD\text{-}D1}$ mice were due to compensatory effects and/or interactions between the genetic manipulation and the background strain used (Crawley et al., 1997). Indeed, this idea also extends to pharmacology studies (Chapter 4) where the effect of mGluR5 antagonism may vary amongst strains and between species. For example, published reports reveal little consistency in the role of mGluR5 in locomotor activity in a novel environment. Antagonism of mGluR5, which can be considered analogous to an mGluR5 knock-out model, reduces locomotor activity in rats (Spooren et al., 2000a; Varty et al., 2005) and C57BL/6J mice (Cowen et al., 2007), but can also result in hyperactivity in DBA/2J (McGeehan et al., 2004) and C57BL/6J mice (Halberstadt et al., 2010). In genetic models, complete knock-out of mGluR5 in mice results in hyperactivity in a novel environment (Gray et al., 2009; Bird et al., 2010; Halberstadt et al., 2010; Olsen et al., 2010). Thus, it would be highly valuable to examine the effect of mGluR5 knock-down on D1R expressing cells in different strains.

Phenotyping tests (Chapter 2) were selected to determine whether deficits existed in mutant mice that could affect performance during subsequent behavioural experiments. A number of parameters were not measured in mGluR5$^{KD\text{-}D1}$ mice which could have contributed to deficits in learning reported here. For example, we did not assess parental behaviours, nor did we examine feeding in terms of meal

size, rate of consumption, meal frequency, nutrient preference, taste aversion, circadian rhythms and orofacial motor components of ingestion; considered to reflect hedonic aspects of reward experience (Berridge, 1996; Berridge and Kringelbach, 2008). Social behaviours, including exploratory social interactions, aggressive behaviours and juvenile play were not formally assessed. Emotional responses including conditioned fear, depression- and schizophrenia-related behaviours were also not assessed. Thus, while no overt differences were reported in mGluR5$^{KD\text{-}D1}$ and wild-type mice, a host of subtle factors remain unexplored that may reveal important functional roles for mGluR5 on D1R-expressing neurons.

In experiments that assessed Pavlovian incentive learning (Chapter 3-4), the main measures of activity were lever pressing and nose-poking into the food magazine or into nose-poke holes. How animals respond to cue activation can result in important differences in conditioning and the conditioned response that develops (e.g. Holland, 1980) and simple frequency measures are not sensitive to differences in the topography of the response and only account for behaviour in a tiny proportion of the entire session. It would be highly valuable to closely monitor behaviour of mGluR5$^{KD\text{-}D1}$ mice, with video capturing, to see whether further behavioural differences could be identified that could account for impaired learning in these animals. Similarly, in locomotor studies (Chapter 5-6), the main measure of activity was distance travelled, which fails to capture the variety of spontaneous behaviours that may be emitted during the course of these sessions (as assessed in Chapter 2). Again, video monitoring of locomotor activity would be valuable to more closely examine how activity, particularly in response to cocaine, differs between mGluR5$^{KD\text{-}D1}$ mice and wild-type mice.

7.8 Future research

To test the idea that mGluR5 on D1R expressing neurons are involved in more sensory specific consummatory conditioning, further tests could be undertaken in mGluR5^{KD-D1} mice that more closely examine and compare sensory specific learning with more general forms of conditioning. Notably, Corbit and Balleine (2005) have developed a PIT procedure in which both the general motivational and the specific sensory effects of Pavlovian CSs can be examined in the same rat. The testing of selective PIT would be valuable in mGluR5^{KD-D1} mice and some studies indicate that testing of outcome-selective PIT is possible in mice, although the effect sizes tend to be rather small (Yin et al., 2006a; Johnson et al., 2007a). Burke and colleagues (2007, 2008) have used reinforcer devaluation and transreinforcer blocking procedures to generate CSs that are considered to evoke representations of general affect (that are devaluation-insensitive) or outcome-specific representations (that are devaluation-sensitive), and both CS forms support CRf. To further explore whether Pavlovian learning in mGluR5^{KD-D1} mice can support the formation of outcome-specific associations, it would be valuable to attempt such procedures in mGluR5^{KD-D1} mice.

One of the main proposals of this thesis is that incentive learning deficits arose due to loss of mGluR5 on D1R expressing neurons, resulting in disrupted plasticity in D1-MSNS in the ventral striatum. A number of options exist to further investigate this possibility, beyond the obvious need for extensive electrophysiological characterisation of mutant mice. First, targeted disruption of mGluR5 solely in the ventral striatum during the learning and/or performance of the CRf task would be valuable to confirm that mGluR5 in this brain region was primarily responsible for deficits in the acquisition of incentive learning following systemic MTEP treatment (Chapter 4). The need for repeated intracerebral administration of MTEP during conditioning sessions would undoubtedly cause confounding damage to the infused brain region and thus an alternate approach could be to use virally mediated knock-down of mGluR5. Alternatively, displacement of mGluR5 from

Homer scaffolding proteins regions using a TAT decoy peptide may offer a viable alternate approach to disrupt mGluR5 mediated signalling in specific brain regions (e.g. Tronson et al., 2010). Second, if loss of a post-synaptic LTP-like expression mechanism in the ventral striatum, involving a reduction in activity of AMPA receptors, was responsible for impaired incentive learning in mGluR5^{KD-D1} mice, one prediction might be that administration of a positive allosteric modulator of AMPA receptors (i.e. an ampakine) into the accumbens could restore performance deficits in CRf and/or sign-tracking in mGluR5^{KD-D1} mice. In this respect, it is interesting that the ampakine CX546 was found to restore prepulse inhibition and latent inhibition deficits in mGluR5 knock-out mice (Lipina et al., 2007). Third, if mGluR5-mediated ERK1/2 signalling in D1-MSNs is particularly important for the formation of Pavlovian incentive associations, then manipulations that disrupt ERK1/2 signalling in the ventral striatum of wild-type mice during conditioning trials would be expected to mimic the phenotype of mGluR5^{KD-D1} mice. Support for this proposal can be taken from a study of Dalley and colleagues (2005), in which post-training infusions of a D1R and NMDA antagonist into the accumbens (i.e. blocking receptors that are critical for ERK1/2 activation) disrupted the formation of Pavlovian incentive memories. Fourth, if STEP protein is critically involved in mGluR5-mediated neuroplastic changes, then disruption of STEP signalling in the ventral striatum during conditioning may also give similar rise to deficits reported in mGluR5^{KD-D1} mice. It is noteworthy that Tashev (2009) and colleagues reported that a substrate trapping form of STEP (TAT-STEP) blocked the induction of LTP and potentiated LTD in acute striatal slices. Finally, if co-operative signalling between mGluR5 and D1Rs and/or NMDARs are important for determining appropriate cellular responses in the ventral striatum, then determining a locomotor-dose effect profile in mGluR5^{KD-D1} mice following intra-accumbens infusions of a D1R agonist (e.g. SKF 38393) or NMDA antagonist (e.g. PCP) may provide a useful starting point to understand whether the function of these receptors is disrupted following mGluR5 loss.

To test the proposal that a dysregulation in the balance of D1R direct and D2R indirect striatal output pathways may be important for incentive learning processes, optogenetic tools may prove useful (e.g. Lobo et al., 2010) where pharmacology experiments may be precluded by damage associated with repeated intracerebral infusions and further limited by a lack of regional and/or cell-type specificity. In this scenario, optogenetic stimulation of D1-MSNs during Pavlovian conditioning would be predicted to result in enhanced performance during a subsequent test of CRf and/or sign-tracking. Oppositely, selective stimulation of D2-MSNs during conditioning would be predicted to result in impaired performance during a subsequent test of the incentive motivational value of a reward paired CS. A critical question remains as to how such manipulations of striatal outputs (and indeed impairments in striatal signalling proposed in mGluR5$^{KD\text{-}D1}$ mice) may influence output from the ventral midbrain that may, in turn, be a central feature in the attribution of incentive value to reward-associated CSs (Flagel et al., 2010). In this respect, microdialysis studies following acute cocaine challenge and/or fast-scan cyclic voltammetry during conditioning sessions would be valuable to understand whether dopamine signalling is disrupted in mGluR5$^{KD\text{-}D1}$ mice.

7.9 Conclusion

The acquisition of incentive associations is necessary for many aspects of adaptive behaviours, but conditioned incentives are also proposed to contribute to compulsive drug seeking and relapse observed in drug addiction (Stewart et al., 1984; Robinson and Berridge, 2000; Everitt et al., 2001). The findings of the present thesis identify glutamate signalling through mGluR5, on cells that also receive dopamine input, as critical for the attribution of incentive motivational value to environmental stimuli associated with reward experience. Together with cocaine self-administration studies conducted in mGluR5$^{KD\text{-}D1}$ mice (Novak et al., 2010), our data suggest that mGluR5-mediated plasticity on D1-MSNs in the ventral striatum may be critical in the attribution of incentive value to drug-paired cues that enable them to support drug seeking and trigger relapse.

8 References

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