THE ROLE OF 5-HT_{2C}R MODULATION IN A REVERSAL LEARNING MODEL OF COGNITIVE FLEXIBILITY IN MICE

Maxine Borton

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DECLARATION

This thesis, whether in the same or different form, has not been previously submitted to this or any other University for a degree.

M.Borton

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University of Sussex

Maxine Borton **PhD**

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SUMMARY

Previous research employing modulation of 5-HT_{2C} receptors (5-HT_{2C}Rs) in rodents has identified a potential role in mediating cognitive flexibility. The work presented in this thesis explores the effects of systemic administration of the selective 5-HT_{2C}R antagonist SB242084 on a range of Pavlovian and operant learning paradigms used to model cognitive flexibility and reward-based learning in mice.

Based on a key design difference in previous research reporting discrepant outcomes, trial initiation requirements were manipulated. However the effect of SB242084 administration relative to vehicle-treatment was consistent with prior reports of impaired reversal performance following reductions in 5-HT_{2C}R activity, regardless of whether trials were automatically or self-initiated. In contrast, performance on a probabilistic reversal learning task was enhanced by drug-treatment, raising the possibility that task difficulty mediates the effect of this manipulation on performance. A drug-related enhancement in the ability to overcome learned non-reward at the previously incorrect location was additionally demonstrated under probabilistic reversal conditions, with no effect on perseverance at the previously correct location. However, performance of drug-treated animals in two closely related tasks demonstrated impaired extinction learning but intact development of latent inhibition to a pre-exposed stimulus. The effect of SB242084 on incentive motivation was additionally explored, but did not impact upon the acquisition of a sign-tracking response to a conditioned stimulus, or a subsequent reversal; suggesting that 5-HT_{2C}Rs may be more critically involved in instrumental than Pavlovian learning.

These experiments reveal a complex picture for the involvement of 5-HT_{2C}Rs in flexible cognition, however, systemic manipulations may not be optimal for dissecting their role. Therefore, a final study explored the expression of *c-Fos* immunoreactivity in response to reversal learning. A broad network was activated by elements of the reversal task, including regions of the prefrontal cortex and amygdala, providing a basis for future studies targeting components of this circuitry.

ABBREVIATIONS:

5,7-DHT	5,7-dihydroxytryptamine
2CKO	5-HT_{2C}R knockout
1CSRTT	1-choice serial reaction time task
5CSRTT	5-choice serial reaction time task
5-HT	5-hydroxytryptamine (serotonin)
5-HT_{2C}R	Serotonin 2C receptor
5-HTT	5-hydroxytryptamine (5-HT) transporter
5-HTTLPR	Serotonin transporter (5-HTT) linked polymorphic region
Ac	Anterior cingulate
ADHD	Attention deficit hyperactivity disorder
AI	Agranular insular cortex
APD	Atypical antipsychotic drug
ATD	Acute tryptophan depletion
BLA	Basolateral amygdala
CIC	Chronic intermittent cold (stress)
COMT	catechol-o-methyltransferase
CP	Caudate putamen
CR	Conditioned response
CRF	Continuous reinforcement
CS	Conditioned stimulus
DA	Dopamine
dACC	Dorsal anterior cingulate cortex
DCS	D-cycloserine
fMRI	Functional magnetic resonance imaging
GABA	*gamma*-Aminobutyric acid
GT	Goal-tracker
ICV	Intra-cerebroventricular
ID/ED	Intra-dimensional/Extra-dimensional (set-shift)
IEG	Immediate early gene
IL	Infralimbic cortex
IR	Immuno-reactivity
ITI	Inter-trial interval

LI	Latent inhibition
LO	Lateral orbital cortex
MAO	monoamine oxidase
MD	Miodorsal thalamic nucleus
MO	Medial orbital cortex
mPFC	Medial prefrontal cortex
NA	Noradrenaline
NAc	Nucleus accumbens
NAcC	Nucleus accumbens core
NAcS	Nucleus accumbens shell
NPE	Non-preexposed
OFC	Orbital frontal cortex
PCA	Pavlovian conditioned approach
*p*CA	parachloroamphetamine
PCP	phencyclidine
PCPA	para-chlorophenylalanine
PCR	Punished correct response
PE	Preexposed
PFC	Prefrontal cortex
PREE	Partial reinforcement extinction effect
PRF	Partial reinforcement
PrL	Prelimbic cortex
PRL	Probabilistic reversal learning
RCZ	Rostral cingulate zone
RIR	Rewarded incorrect response
SNc	Substantia nigra compacta
SSRI	Selective serotonin re-uptake inhibitor
SSRTT	Stop-signal reaction time task
ST	Sign-tracker
UCR	Unconditioned response
US	Unconditioned stimulus
VO	Ventral orbital cortex
VTA	Ventral tegmental area
WCST	Wisconsin Card Sort Task

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CHAPTER 1

INTRODUCTION

1.1. Introduction

The ability to acquire associations between stimuli, actions and environmental outcomes, and to flexibly adapt ongoing behaviour in the context of changing environmental and contextual demands is essential for successful goal-directed behaviour, carrying significant survival value (Kehagia et al. 2010), and is a characteristic of vertebrate groups including mammals, birds and reptiles (Bitterman 1975; Wilkinson & Huber 2012). Understanding the biological basis of such behaviour is an important area of research not only because these processes underpin normal human behaviour, but because they are a core abnormal behavioural component of a broad range of neuropsychiatric conditions (King et al. 2008). Deficits in flexible cognition have been observed in schizophrenia (Ceaser et al. 2008); autism (Hill 2004); obsessive compulsive disorder (Head et al. 1989); unipolar (Preiss et al. 2009) and bipolar depression (Preiss et al. 2009); attention-deficit hyperactivity disorder (Chamberlain et al. 2011); addiction (Verdejo-Garcia et al. 2006); as well as neurodegenerative diseases such as Parkinson's (Freedman & Oscar-Berman 1989; Owen et al. 1991), and Alzheimer's disease (Freedman & Oscar-Berman 1989). Whilst a loss of cognitive flexibility typically represents just one of a broad number of symptoms, decline in this function is shown to positively correlate with severity of symptoms (Addington et al. 1991; Hermesh 2003), as well as long-term health outcomes (Green, 2006; Holthausen et al., 2007; Keefe et al., 2006), making it a crucial therapeutic target. However, there is currently a lack of treatments which effectively tackle these cognitive impairments (Weiss et al. 2002). The search for novel therapeutic compounds which reverse these deficits, or which serve to improve normal cognitive function ('cognitive enhancers'), is currently an active area of neuropsychological research; and drugs acting at specific serotonin (5-hydroxytryptamine, 5-HT) receptors offer a promising target for investigation (King et al. 2008).

Anatomical evidence demonstrates that the serotonergic system, deriving mainly from neurons in the dorsal and ventral raphe nuclei, has projections to virtually every brain region associated with cognition (Baker et al. 1991; Halliday et al. 1988; Halliday et al. 1990; Roth et al.

2004); and this innervation is closely associated with expression of a range of 5-HT receptors (King et al. 2008). Yet, whilst 5-HT has long been recognized to play a role in mood disorders, anxiety, and depression (Asberg et al. 1976; Coccaro et al. 1990; Deakin & Graeff 1991; Graeff et al. 1996), its role in cognition has become a popular area of investigation only more recently, with growing evidence that serotonergic manipulations can have a significant impact on measures of learning and memory ability in humans (e.g. Park et al. 1994; Riedel et al. 1999) and in animal models (e.g. Barnes et al. 1990; Meneses 2003). Converging clinical and experimental evidence has also been suggestive of a critical role for 5-HT in neuropsychiatric conditions with associated cognitive dysfunction. For example, alterations in 5-HT levels have been associated with impulsive, disinhibited behaviours symptomatic of many conditions (e.g. Beninger & Phillips 1979; Wogar et al. 1993); and receptor occupancy studies have shown that second generation antipsychotics such as clozapine, olanzapine and risperidone have much higher occupancy of 5-HT_{2A} than D_2 receptors (Arnt & Skarsfeldt 1998; Meltzer et al. 1989), and demonstrate improved efficacy in the treatment of neurocognitive deficits in schizophrenia not seen following administration of first-generation antipsychotics (Lee et al. 1999), which have primary affinity for the dopamine D_2 receptor (Meltzer et al. 1989). However, the evidence concerning the relative efficacy of first-generation (FGA) and second-generation (SGA) antipsychotics largely comes from short-term trials with high drop-out rates, and several meta-analyses have cast doubt on the size and significance of these effects (Leucht et al. 2003a, 2003b). More recent large-scale randomised controlled trials have suggested there may in fact be little difference in efficacy between these drug classes in terms of symptoms, adverse effects, and quality of life (Jones et al. 2006; Lieberman et al. 2005; Rosenheck et al. 2003). Although there was evidence of a benefit in terms of improved cognition for some SGAs (Rosenheck et al. 2003), this must be also be weighed against accompanying problems of weight gain, and the higher cost of these drugs relative to FGAs.

Flexible cognition, as with many other executive functions, is thought to depend upon the prefrontal cortex (PFC) and its interactions with the basal ganglia; an ability which continues to mature in late adolescence in close association with development of this region (Huttenlocher 1990), and showing impairment following lesions or damage to this structure (Dias et al. 1996a, 1996b, 1997). Abnormalities in metabolic activity (Rubin et al. 1995) and structure (Baaré et al. 1999) of the PFC have also been reported in patients with neuropsychiatric conditions such as major depression and schizophrenia, prompting researchers to investigate the role of this region in the overlapping deficits seen in such conditions. Behavioural assays designed to tease apart different forms of cognitive flexibility however, have elucidated far greater specificity of brain

regions involved, pointing to a segregation of PFC function, and a dissociation of neural substrates sub-serving these distinct functions.

Until recently, the most widely used task for assessing cognitive flexibility in humans was the Wisconsin Card Sort Task (WCST) (Milner 1963), a task which integrates multiple measures of executive function, often causing problems for interpreting the profile of deficits observed (Tchanturia et al. 2012). In fact, the inability to distinguish between diverse neuropathologies on the basis of the cognitive deficits observed may point to the rather crude nature of the measurements used. The more recent intra- and extra-dimensional (ID/ED) attentional set-shifting task of the Cambridge Neuropsychological Test Automated Battery (CANTAB; Roberts et al. 1988) allows selective measurement of multiple components of flexible cognition, including discriminative learning, reversal learning, forming of an attentional set, and shifting of attention within the same (intra-dimensional shift, ID) or between different (extra-dimensional shift, ED) perceptual dimensions. Through use of this more refined task, which has also been adapted for use in non-human primates (Dias et al. 1996b) and rodents (McAlonan & Brown 2003), it has been possible to identify a functional specialisation of the brain circuitry involved in different aspects of flexible cognition, demonstrating the importance of function-specific tasks for understanding the neural basis of behaviour. Furthermore, these tasks demonstrate the similarity of brain circuitry involved across species.

Through use of this task, evidence for a double dissociation between reversal learning and higher level attentional set-shifting has since been demonstrated, with orbital prefrontal activity found to underlie the ability to reverse a learned discrimination, whereas more lateral (in primates and humans) or medial (in rodents) PFC circuitry has been shown to mediate higher level flexibility such as the ability to perform extra-dimensional (ED) shifts; as evidenced by deficits in reversal learning following focal lesions of the ventral PFC (Fellows & Farah 2003) and orbitofrontal cortex (OFC) in humans (Hornak et al. 2004), rats (Bissonette et al. 2008; Chudasama & Robbins 2003; Ghods-Sharifi et al. 2008; McAlonan & Brown 2003; Schoenbaum et al. 2003), and non-human primates (Dias et al. 1996a, 1996b, 1997; Jones & Mishkin 1972), which have been shown to leave ED shift capability intact. Conversely, lesions of the dorsolateral PFC in marmosets (Dias et al. 1996a, 1996b, 1997) and humans (Hornak et al. 2004; Owen et al. 1991), and the medial PFC in rodents (Birrell & Brown 2000; Bissonette et al. 2008; Floresco et al. 2008) affect the ability to complete an ED shift whilst leaving reversal learning unimpaired. Investigations into the role 5-HT plays in cognitive flexibility also point to a regional specificity of neuromodulatory effects, with evidence that prefrontal 5-HT plays a critical role in reversal learning (Clarke et al. 2004, 2005), that is not required for successful higher level attentional

shifting, which is modulated by prefrontal DA (Crofts et al. 2001; Roberts et al. 1994; Rogers et al. 1999). Moreover, depletions of 5-HT selective to the OFC demonstrate marked impairments of reversal performance not seen following DA depletions in this region (Clarke et al. 2007). However, reversal learning has also been shown to be dependent upon the integrity of the dorsal striatum (Kirkby 1969; Ragozzino et al. 2002) where it is DA rather than 5-HT signalling which appears to mediate performance (Clarke et al. 2011; Clatworthy et al. 2009).

Whilst the precise role 5-HT circuitry plays in cognition more broadly does not have a well-developed framework (Cools et al. 2011), there is now a growing body of evidence demonstrating its importance to reversal learning. Therefore, clarification of the role serotonergic mechanisms play in this distinct aspect of cognitive flexibility may not only be of great clinical importance, in terms of identifying novel pharmacotherapies for the treatment of cognitive deficits seen across a broad range of neuropsychiatric conditions, but may also be key to characterizing the broader role of 5-HT in cognition. This chapter reviews the current state of evidence concerning the role of 5-HT in mediating flexible cognition, as measured by reversal learning tasks, as well as significant discrepancies and unanswered questions.

1.2. Measuring cognitive flexibility

The ID/ED set-shifting task is one of several measures of cognitive flexibility which has been adapted for use across species. It comprises of several test stages of increasing difficulty. Subjects are typically tested first on simple two-choice discrimination where one stimulus signals reward (CS+) and another punishment or non-reward (CS-); followed by a compound discrimination where a second, irrelevant stimulus or perceptual dimension is introduced, but the correct and incorrect stimuli remain unchanged, which encourages the formation of an attentional set. The ability to perform an intra-dimensional (ID) shift is then tested, where the learned stimuli are replaced with novel exemplars, but the relevant and irrelevant dimensions remain unchanged. This is followed by the more difficult test of extra-dimensional (ED) or 'attentional' shifting, where the previously relevant dimension becomes irrelevant and the previously irrelevant dimension relevant. Any one of these stages can also be followed by a reversal test where the reward contingencies switch.

Versions of the ID/ED task have been successfully developed for use in animals, including a rodent bowl-digging version (McAlonan & Brown 2003) as well as operant tasks (e.g. Scheggia et al. 2014), and have been used to measure cognitive flexibility alongside more simple reversal learning tasks, where an initial two choice simple discrimination is learned, followed by a switch

in outcome contingencies such that the CS+ becomes the CS-, and the CS- becomes the CS+; sometimes followed by further serial reversal shifts where these contingencies shift back and forth multiple times, in either a within-session or between-session design (e.g. Boulougouris et al. 2007; Bushnell & Stanton 1991; Roberts et al. 1990). The stimulus modalities employed in these tasks range from visual, spatial, visuospatial, and in rodents, olfactory and tactile.

Probabilistic outcomes can also be superimposed on to this task (e.g. Lawrence et al. 1999; Swainson et al. 2000). In Probabilistic Reversal Learning (PRL) tasks, spurious error and correct feedback is experienced at a specified probability for correct and incorrect responses, respectively, such that a small proportion (e.g. 20%) of correct responses are punished and incorrect responses rewarded. Thus, subjects must instead learn to discriminate the most often rewarded stimulus. This task is typically employed to increase task difficulty for use in human subjects, but has been adapted for use in rodents (Bari et al. 2010), though it is not currently clear whether they adopt similar strategies to solve the task as compared to primates and humans (see Ineichen et al. 2012). By assessing patterns of responding on subsequent trials following false-positive or false-negative feedback, these tasks have the added benefit of measuring components of reward and punishment sensitivity, which might also mediate reversal performance.

Finally, the simultaneous discrimination reversal tasks described above are accompanied by the less frequent use of successive discrimination tasks, where each discriminative stimulus is presented on discrete trials. This most often takes the form of a 'Go/No-Go' discrimination task, where subjects must learn to initiate a specific response during presentation of the CS+ ('Go' trials), but to withhold responding when the CS- is presented ('No-Go' trials). Correct responding leads to reward on 'Go' trials and avoidance of punishment on 'No-Go' trials (e.g. Schoenbaum et al. 2002), or in symmetrically reinforced tasks, reward is given for a correct response to both trial types (e.g. Harrison et al. 1999). Although this task is typically used as a measure of response inhibition, these predictive associations can also be reversed, providing a measurement of the ability to update stimulus-response associations.

1.3. Effects of 5-HT manipulations on reversal learning

The majority of evidence derived from a broad range of specific serotonergic manipulations in both humans and experimental animals demonstrates a significant bidirectional relationship between 5-HT signalling and cognitive flexibility, and suggests that reduced 5-HT signalling impairs reversal performance, whilst increasing 5-HT facilitates it. The

following sections review existing evidence for the relationship between 5-HT and reversal learning, as well as highlighting some key inconsistencies in the literature.

1.3.1. Decreased 5-HT signalling:

One of the few methods available for reducing 5-HT function in humans is through lowering brain availability of the amino acid precursor L-tryptophan, which acts to decrease 5-HT synthesis and release (Biggio et al. 1974; Gartside et al. 1992; Williams et al. 1999). In healthy human volunteers, acute tryptophan depletion (ATD) has been found to reduce the number of subjects successfully reversing the compound discrimination of a visual ID/ED task relative to non-depleted controls (Rogers et al. 1999), as well as increasing the number of errors made during the reversal stages of this task (Park et al. 1994; Rogers et al. 1999). However, this is not a consistently reported effect. Employing a probabilistic version of a visual reversal learning task, Evers et al. (2005a) and Murphy et al. (2002) report slower, but no less accurate, responding during reversal, which could nevertheless suggest a speed/accuracy trade-off consistent with an ATD impairment effect; but other authors report no difference in reversal performance following this manipulation (Evers et al. 2005b; Talbot et al. 2006). However, there is evidence of considerable variability in the neuromodulatory effects of ATD across individuals (Neumeister et al. 2004; Pergadia et al. 2004), and this manipulation has been found to exert directionally opposite effects depending on certain trait characteristics (Bjork et al. 2000) and genetic factors (Crean et al. 2002).

By comparison, there are many more methods for depleting 5-HT in experimental animals. Administration of the selective 5-HT toxin parachloroamphetamine (*p*CA) (10 mg/kg) has been shown to significantly impair performance on a 'Go/No-Go' reversal task in rats (Masaki et al. 2006b), where subjects were required to reverse a learned response of nose-poking during house-lights on to during house-lights off; with evidence of a significant negative correlation between the number of sessions required to reach reversal criterion and 5-HT concentrations in the OFC, mPFC and amygdala. In light of evidence supporting a role for 5-HT in mediating anxiety (Deakin & Graeff 1991; Graeff et al. 1996) and motor activity (Jacobs 1991; Jacobs & Fornal 1999), which might have had a general effect on performance, this same manipulation was also shown to have no effect on tests of novelty-induced anxiety and spontaneous locomotion (Masaki et al. 2006).

Similarly, reductions of 5-HT via administration of the tryptophan hydroxylase inhibitor para-chlorophenylalanine (PCPA) (4 x 200mg/kg/day), which was shown to deplete 97% of prefrontal 5-HT, significantly impaired the first reversal learning stage of a bowl-digging task in

rats (Lapiz-Bluhm et al. 2009), as did exposure to Chronic Intermittent Cold (CIC) stress, which is of particular clinical relevance given that chronic stress is a risk factor for many psychiatric disorders. This CIC-induced impairment was also associated with reduced 5-HT transmission in the OFC, and was attenuated through acute administration of the selective serotonin reuptake inhibitor (SSRI) citalopram (5mg/kg); important for demonstrating the serotonergic basis of this effect given that chronic stress causes dysregulation of other monoaminergic neurotransmitter systems (Pardon et al. 2003). However, using a touch-screen based version of the task, similar doses of PCPA (250mg/kg x 3 days) resulted in normal visual reversal learning capabilities in both mice (Brigman et al. 2010), and rats (Izquierdo et al. 2012), whilst a higher dose (500mg/kg) prevented subjects from approaching the reward-predictive stimulus in pre-training, demonstrating a more general disruptive effect on learning (Izquierdo et al. 2012). Although Brigman et al. (2010) report a marked depletion of 5-HT in the cortex and hippocampus in PCPA-treated mice, Izquierdo et al. (2012) report no difference in 5-HT tissue content in PCPA-treated rats for either dose group relative to saline treated controls, possibly suggestive of neuroplastic compensatory adaptations; an important consideration for studies employing chronic manipulations of 5-HT function.

In further support of a role for 5-HT specifically within the OFC in mediating cognitive flexibility, a series of elegant studies by Clarke et al. (2004, 2005, 2007) demonstrate that selective ablation of prefrontal and OFC-specific 5-HT through administration of the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) impairs performance of a single visual discrimination reversal in marmosets by increasing the number of early errors (made before chance levels of responding, <50%) relative to sham lesioned controls. Rygula et al. (2015) also demonstrate impaired reversal performance following selective 5,7-DHT lesions of the OFC in marmosets completing a probabilistic version of this visual reversal task. However, animals in this study also demonstrated impaired acquisition of a novel discrimination post-lesion; possibly indicative of the increased difficulty of the probabilistic discrimination. Individual differences in reversal performance in normal behaving animals can also be predicted from 5-HT and 5-HT transporter (5-HTT) levels in the OFC in rodents (Barlow et al. 2015; Stolyarova et al. 2014), and in vervet monkeys (Groman et al. 2013); identifying the OFC as a particularly critical region for studying the effects of 5-HT manipulations on reversal.

Although the amygdala is part of the circuitry believed to be support flexible cognition (Cools et al. 2002; Ghahremani et al. 2010), and there is some evidence that 5-HT levels within the basolateral amygdala (BLA) correlate with reversal performance (Masaki et al. 2006b), a causal role for 5-HT in the BLA in mediating reversal learning is yet to be clearly displayed.

Although BLA-specific 5-HT depletion was also found to impair performance in marmosets completing the probabilistic reversal task previously described (Rygula et al. 2015), it reportedly had no effect on performance in a deterministic version of this task (Ochoa et al. 2015). The nature of the impairment observed following OFC and BLA depletion in the probabilistic task was also found to differ, with BLA-specific 5,7-DHT lesions additionally heightening sensitivity to misleading feedback of both a positive and negative valence, suggesting an impaired ability to integrate reward information across time; an effect not seen following OFC-specific lesions (Rygula et al. 2015). This could suggest that the OFC and BLA have functionally dissociable roles in supporting flexible cognition, and that the BLA is only recruited under conditions of greater uncertainty. This would be consistent with theorising that the BLA is selectively involved in updating responses to changes in reward value (Baxter & Murray 2002). However, the timing of the lesions might also be responsible for this discrepancy, since animals had experience of both acquisition and reversal of a visual discrimination in the Ochoa et al. (2015) study prior to lesioning, whilst animals in the Rygula et al. (2015) design received no prior reversal experience. Given the building evidence that 5-HT exerts its effects on reversal at an early stage, and typically only in the first of a series of reversals, this could be a critical design difference.

1.3.2. Increased 5-HT signalling

Although there is less evidence concerning the effects of increased brain 5-HT on reversal performance, there are some reports of a beneficial effect. Inhibiting 5-HT re-uptake through administration of selective serotonin reuptake inhibitors (SSRIs) is one of the most commonly explored manipulations. Chronic administration of the SSRI fluoxetine (15mg/kg) was found to improve performance during the early stage of a single visual reversal in mice (Brigman et al. 2010), whilst acute administration of a single high dose (10mg/kg) of citalopram increased the number of reversals completed by rats performing a serial probabilistic task (Bari et al. 2010), possibly mediated by its ability to reduce sensitivity to misleading negative feedback (i.e. the probability of shifting responding to the incorrect choice after receiving no reward on a correct trial, 'lose-shift' behaviour). Acute administration of the potent SSRI escitalopram (0.3 and 1mg/kg) has also been shown to improve probabilistic spatial reversal learning in rats (Brown et al. 2012). However the nature of the performance enhancement did differ from previous findings, since there was a specific reduction in the number of late, rather than early reversal errors, seemingly suggesting a role for 5-HT in maintaining a new choice once selected. The high dose was also found to exert a beneficial effect on 'win-stay', rather than 'lose-shift' behaviour, increasing the probability of staying at the correct location following reinforcement. This

difference in effect might be accounted for by the significantly greater potency of escitalopram at the 5-HT transporter site relative to citalopram (Rausch et al. 2004).

In an interesting additional experiment, escitalopram was also shown to improve the ability to shift responding away from a naturally prepotent response bias when an alternative choice pattern was optimal (Brown et al. 2012), which is of clinical relevance given that individuals with autistic spectrum disorder and schizophrenia exhibit deficits in both. Furthermore, Brown et al (2012) also demonstrate that this effect cannot be explained by a more general anxiolytic effect - acting to reduce anxiety levels felt when a response pattern is no longer reinforced to allow a more rapid switch in responding - by demonstrating that SSRI treatment has no effect on performance of the elevated plus maze; a reliable measure of anxiety in rats (Pellow et al, 1985). However contradictory evidence is reported following the administration of citalopram (30mg) to healthy human volunteers however, which acts to increase reversal errors and 'lose-shift' behaviour relative to controls (Chamberlain et al. 2006).

The effects of pharmacological manipulations can be complemented by experiments exploring the effects of genetic variation in endogenous 5-HT function; though genetic constitutive loss of the 5-HT transporter (5-HTT) in mice was found to have a more generalised effect on performance than pharmacological blockade, with 5-HTT null mutant mice making fewer errors across the entire reversal test, not specifically during early or late stages of testing (Brigman et al. 2010). However, in this same study constitutive genetic loss of 5-HT in Pet-1 null mice, resulting in a substantial loss of 5-HT neurons and decrease in cortical and hippocampal 5-HT tissue content compared to wild-type controls, had no effect on reversal performance, suggesting that genetically-induced modulation of 5-HT and 5-HTT function may have different effects on cognitive flexibility.

Further evidence linking 5-HTT function to reversal performance can be derived from experiments phenotyping polymorphisms which affect the transcription and functional efficacy of the 5-HTT. Non-human primates expressing the putatively lesser functioning (S-allele) orthologue of the human 5-HTT linked polymorphic region (5-HTTLPR) show improved reversal performance (Jedema et al. 2010); as do monkeys with a different genetic variation in the 3' untranslated region of the 5-HTT which is also predicted to result in higher 5-HT levels (Vallender et al. 2009). Complicating findings however, Izquierdo et al. (2007) found the opposite pattern of results, with rhesus monkeys homozygous for the S-allele (SS) showing poorer, rather than enhanced, reversal learning relative to heterozygous (S/L) or homozygous L-allele (LL) carriers.

1.4. Reconciling inconsistencies:

Despite a growing body of evidence taken across lesion, pharmacological and genetic inactivation studies to support a key role for 5-HT in reversal learning, the nature of the effect is sometimes found to differ, with evidence for effects specific to the early or late stage reported, and effects on win-stay or lose-shift performance in probabilistic tasks evident. There are also several inconsistent reports of a lack of effect, or even directly contradictory effects of 5-HT manipulations on reversal learning performance. However, consideration of differences in methodology and task design can help reconcile some of these inconsistencies. The following section reviews a number of important considerations for studies of flexible cognition following manipulations of 5-HT transmission.

1.4.1. Differences in task design:

A key design difference in reversal learning tasks is whether subjects are tested on performance of a single reversal shift or across multiple, serial reversals. The serial reversal learning task has been hypothesised to encourage an automatized switching tendency, measuring rule learning and the acquisition of a reversal learning 'set' as well as prospective planning for anticipated reward contingencies (Murray & Gaffan 2006), and these task details theoretically correspond to different underlying neural mechanisms (Izquierdo & Jentsch 2012). Evidence suggests that 5-HT may be less involved in the processes mediating acquisition of a reversal rule, since effects of 5-HT manipulations are most typically observed early in reversal and are transient (Clarke et al. 2004; Clarke et al. 2005; Clarke et al. 2007; Lapiz-Bluhm et al. 2009; Park et al. 1994). Therefore, whilst the use of serial reversal learning tasks can offer insight into the nature of learning that occurs during testing, it is important that these tasks be sufficiently broken down for analysis. Analysing multiple reversal switches together in the serial reversal design employed by Evers et al. (2005b), performed to enable blocked fluorescence magnetic resonance imaging in future studies, is likely to have obscured any possible effects of tryptophan depletion on early reversal performance. Similarly, the use of practice reversal sessions prior to the introduction of the relevant experimental manipulation, as observed in the Evers et al. (2005b) and Ochoa et al. (2015) design, might be responsible for the lack of significant findings observed.

A further complication in the role for 5-HT in reversal learning emerges when comparing tasks of different modalities. Van der Plasse & Feenstra (2008), in a study attempting to uncover the differential contribution of 5-HT in early versus late reversal learning in male Wistar rats, failed to find an effect of tryptophan depletion at either stage of testing on a two-lever reversal

learning task, where the discriminative cue was the spatial location of the lever (left or right), which could suggest that the role of 5-HT in reversal learning is modality-specific. There are several reports which suggest that, unlike visual and olfactory learning, reversal learning of spatial information depends on the mPFC (De Bruin et al. 2000; Kolb et al. 1974; Mishkin 1964; Salazar et al. 2004). However, 5,7-DHT lesions of mPFC have been found to have no effect on either a spatial or odour based reversal learning task (van der Plasse et al. 2007). Taken alongside observations of increased DA efflux in the mPFC during early reversal learning when employing a spatial two-lever task (van der Meulen et al 2007), this could suggest a possible role for DA rather than 5-HT during reversal of a discrimination when a spatial element is included.

Central 5-HT manipulations are also known to impact upon certain forms of impulsivity (Winstanley et al. 2003, 2004a, 2004b, 2005), an effect which appears to be mediated through actions at the level of the nucleus accumbens rather than the PFC (Robinson et al. 2008), and it is possible that different tasks engage impulsivity processes to a different extent. Manipulation of 5-HT_{2C}R function has been found to have the opposite effect on reversal performance in a standard operant reversal task as compared to a visual touchscreen task (Nilsson 2012), where animals are presented with stimuli on a touchscreen and must nosepoke the area of the screen corresponding to the CS+ to earn reward. Administration of the selective 5-HT_{2C}R antagonist SB242084 improved reversal performance in the operant task relative to vehicle-treated controls, but impaired performance in the touchscreen task; an effect which was accompanied by faster stimulus response latencies. This was interpreted as evidence that the touchscreen procedure may have greater sensitivity to manipulations of accumbal DA levels and impulsivity, which would mask any effects of SB242084 on prefrontally mediated reversal learning. Given that even small manipulations of task parameters, such as the length of the inter-trial interval (ITI), are known to have a significant bearing on measures of impulsivity (e.g. Amitai & Markou 2011; Mirza & Stolerman 1998), greater attention will need to be paid to seemingly minor design differences across tests of cognitive flexibility, particularly when interpreting the effects of 5-HT manipulations which are known to interact with mesolimbic DA signalling to affect impulsivity.

1.4.2. Differences in 5-HT manipulation:

Aside from possible differences in outcome relating to extent of 5-HT depletion caused by PCPA, *p*CA or 5,7-DHT lesions, as compared to more moderate manipulations of 5-HT function through acute SSRI administration or tryptophan depletion, there are reports of different outcomes even when employing seemingly similar manipulations. Though the majority of reports suggest a beneficial effect of SSRI treatment on reversal learning performance, there are

conflicting reports of impaired performance following acute administration of these drugs (Chamberlain et al. 2006). However, several lines of research suggest that the relative dose of SSRIs can significantly alter the directional effect these substances have on 5-HT levels. *In vivo* microdialysis studies demonstrate an increase in 5-HT levels in the PFC of freely moving rats after a 10 mg/kg dose of citalopram which is not seen following a 1 mg/kg dose (Invernizzi et al., 1992), and electrophysiological evidence demonstrates that low SSRI doses temporarily inhibit the firing of 5-HT neurons by flooding somatodendritic 5-HT_{1A} autoreceptors in the raphe nuclei, resulting in inhibition of 5-HT release and synthesis (Artigas, 1993; Barton & Hutson, 1999; Hjorth & Auerbach, 1994).

Directly testing these effects, Bari et al. (2010) report that a single low dose (1mg/kg) of citalopram administered to rats 30 minutes prior to testing reduced the number of reversals completed in a serial probabilistic reversal learning task, as well as increasing 'lose-shift' behaviour, whilst a single higher dose (10mg/kg) yielded the opposite effect on both measures. Though difficult to compare doses across human and animal studies, the close similarity of effects reported following acute low-dose administration in this study to those reported by Chamberlain et al. (2006), of impaired reversal learning alongside increased negative feedback sensitivity, suggests the 30mg dose used in their study was sufficient to inhibit 5-HT release in healthy human participants.

In addition to identifying a critical role for dose on the acute effects of SSRIs in reversal performance, Bari et al. (2010) also identify a difference in the nature of effect conferred by chronic as compared to acute manipulations of 5-HT function. Both repeated (5mg/kg 30 minutes prior to testing for 7 days), and sub-chronic (10mg/kg twice a day for 10 days) citalopram treatment was found to increase the number of reversals completed in this design. However, as opposed to the changes in negative feedback sensitivity seen following acute treatment, chronic treatment was found to specifically affect reward sensitivity, observed as an increased probability of staying at the same correct response location following a reward ('win-stay'). By contrast, long-term 5-HT depletion by 5,7-DHT infusions had the opposite effect on both measures, with fewer reversals completed and a selective reduction in win-stay behaviour observed. Although acute administration of escitalopram was previously found to affect win-stay rather than lose-shift behaviour (Brown et al. 2012), this compound, as well as being more potent, shows faster onset of anti-depressant activity (Gorman et al. 2002; Sanchez et al. 2003). The difference in acute versus chronic effects could be attributable to the down-regulation of 5-HT receptor subtypes after repeated SSRI treatment, and/or to alterations in the coupling of the 5-HT system with DA (Bari et al. 2010), which is more closely implicated in reward sensitivity

(Nestler & Carlezon 2006). This points to the importance of considering homeostatic changes following more long-term manipulations of the 5-HT system.

Such considerations are also relevant to the differences observed in reversal learning following pharmacological versus genetic manipulation of 5-HT function (e.g. Brigman et al. 2010). Constitutive changes within the serotonergic system are likely to have effects far more wide-reaching than acute pharmacological manipulations, particularly in light of evidence that 5-HT regulates brain connectivity by modulating developmental cellular migration and cytoarchitecture (Daubert & Condron 2010). 5-HTT knockout (KO) mice display functional and anatomical disturbances in corticolimbic circuitry (Wellman et al. 2007), and display reduced density of 5-HT_{1A} receptors in the dorsal raphe (Li et al. 2000); an effect which is also observed in human S-allele carriers of the serotonin-transporter-linked polymorphic region (5HTTLPR) which may be associated with lower levels of 5-HTT mRNA transcription (David et al. 2005). This suggests that loss of 5-HT function during development can lead to changes in the function or sensitivity of 5-HT receptor subtypes. Evidence for interactive effects between genetic variations and the impact of 5-HT depletion during reversal learning also raises the possibility that genotypic differences in populations across studies might account for some inconsistencies. Finger et al. (2007) report that tryptophan depletion alone produced no measurable impact on performance of a reversal learning task in healthy humans, and was only evident once genotypic variation in the 5-HTTLPR was taken into account.

The site of action in many of these studies is also unclear, and there may be a different role for 5-HT transmission at cortical and subcortical levels, or across different cortical regions. Region selective neuron destruction and depletions demonstrate a role for several regions in reversal, but their effects are found to differ. As discussed above, sensitivity to misleading feedback in probabilistic tasks appears to be predominantly controlled by 5-HT function within the amygdala, whereas the nature of effect following OFC depletion was attributed to poor response inhibition (Rygula et al. 2015). And it is DA, rather than 5-HT, function which appears to mediate reversal performance at the level of the dorsal striatum (Clarke et al. 2011; Clatworthy et al. 2009) though, despite evidence of considerable interactions between 5-HT and DA systems, most evidence displays an effect of 5-HT on mesolimbic/mesostriatal DA, and effects on nigrostriatal DA are currently under debate (De Deurwaerdere & Spampinato 2001; Di Matteo & Esposito 2001). Effects on mesostriatal DA will need to be considered when manipulating 5-HT function however, since DA levels in the ventral striatum appear to mediate impulsivity (Cole & Robbins 1989; Economidou et al. 2012; Pezze et al. 2007), which can act as a significant confound in reversal learning tasks.

1.5. Consideration of 5-HT receptor subtype mechanisms

In addition to the complexities described above, it is apparent that actions of 5-HT at different receptor subtypes must be taken into consideration when interpreting the effects of global manipulations, particularly when considering the complexity of the 5-HT system. 5-HT produces its effects through a wide variety of membrane-bound receptors which are located in both the peripheral and central nervous system (Hoyer et al. 2002), where it is known to influence multiple and diverse processes including vasoconstriction, food intake, circadian rhythm, aggression, locomotion, and thermoregulation, as well as facilitating the formation of synapses in the developing and adult brain (Terry et al. 2008). Seven families of 5-HT receptors have been identified. With the exception of the 5-HT_3Rs (5-HT_{3A}, 5-HT_{3B}, and 5-HT_{3C}) which are believed to function as ligand-gated ion channels, they belong to the G protein-coupled receptor superfamily. The metabotropic 5-HT receptor subtypes consist of seven transmembrane domains and are classified into four groups based on the type of G proteins to which they are coupled. The 5-HT_1Rs (5-HT_{1A}R, 5-HT_{1B}R, 5-HT_{1D}R, 5-HT_{1E}R, and 5-HT_{1F}R) couple to Gαi/Gαo proteins, whereas the 5-HT_2Rs (5-HT_{2A}R, 5-HT_{2B}R, and 5-HT_{2C}) couple to Gαq proteins, and the 5-HT_4R, 5-HT_6R, and 5-HT_7R couple to Gαs proteins. For the 5-HT_5Rs (5-HT_{5A}R and 5-HT_{5B}R) G-protein coupling has not yet been established (Stiedl et al. 2015).

With at least 14 distinct receptors so far identified, the 5-HT system represents one of the most complex families of neurotransmitter receptors (Terry et al. 2008). Additional complexity derives from evidence of multiple splice variants (5-HT_4R and 5-HT_7R) and RNA edited isoforms (5-HT_{2C}R) which demonstrate altered affinity for 5-HT (for review see Werry et al. 2008), as well as the recent identification of constitutive activity (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_7) where receptors spontaneously activate intracellular signalling pathways in the absence of the endogenous ligand, and correspondingly display inverse agonist properties in the presence of certain antagonists (Barker et al. 1994; Egan et al. 1998; Thomas et al. 1995; Thomas et al. 1998). Given the degree of 5-HT receptor heterogeneity, as well as the complex interactions observed between 5-HT neurons and other neuronal phenotypes (e.g. Di Giovanni et al. 2008; Guiard et al. 2008), inconsistencies in tests of cognitive function following global manipulations of 5-HT function are perhaps unsurprising. A better understanding of the contributions of individual receptor subtypes will therefore be essential to furthering our understanding of the role of the 5-HT system in these cognitive processes.

Very few studies have so far examined the effects of 5-HT receptor specific manipulations on reversal performance, but it has been suggested that the effects of global or

sub-region specific manipulations of 5-HT are related to the altered activity at 5-HT_{2C}Rs (Boulougouris & Robbins 2010; Roberts 2011), and a functional role for these receptors in cognitive flexibility is starting to be identified. Following a brief overview of the neuropharmacology of the 5-HT_{2C}R, the following section reviews existing evidence concerning the effect of 5-HT_{2C}R subtype specific manipulations on performance of reversal learning tasks.

1.5.1. The 5-HT_{2C} receptor

The distribution of 5-HT_{2C}Rs is limited to the choroid plexus and the CNS, where they are prominently expressed in neurons throughout the limbic-corticostriatal circuit (Clemett et al. 2000). The distribution of 5-HT_{2C}R protein closely tracks that of the transcript in regions that receive innervation from 5-HT neurons arising from the midbrain raphe nuclei (Lopez-Gimenez et al. 2001; Mengod et al. 1990; Wright et al. 1995), suggesting that these receptors are predominantly located post-synaptically, although they may be pre-synaptically localized in some brain regions (Lopez-Gimenez et al. 2001; Mengod et al. 1990; Wright et al. 1995). Research exploring the functional role of 5-HT_{2C}Rs has been hampered by a lack of selective ligands, though RO600175 and MK 212 display moderately selective agonist activity (Millan et al. 1997; King et al. 1989), whilst SB242084 represents a high-affinity selective antagonist, with more than 100 fold selectivity for the 5-HT_{2C}R relative to 5-HT_{2A}R or 5-HT_{2B}R, and more than 200-fold selectivity for the 5-HT_{2C}R over 5-HT_1R, 5-HT_4R, 5-HT_6R, 5-HT_7R, as well as the dopamine D_2R and D_3R (Kennett et al. 1997). Although there is some suggestion that SB242084 could have inverse agonist rather than antagonist affinity at the 5-HT_{2C}R (Herrick-Davis et al. 2000), there is as yet no evidence to support this.

5-HT_{2C}Rs are believed to exert a constitutive inhibitory influence upon frontocortical dopaminergic and noradrenergic, but not serotonergic, transmission, and administration of SB242084 (Millan et al. 1998) or the mixed 5-$HT_{2B/2C}$R antagonist SB206553 (Gobert & Millan 1999; Gobert et al. 2000) results in increased DA and noradrenaline (NA) dialysate levels in the PFC without affecting 5-HT. 5-HT_{2C} receptors also play a prominent role in the control of mesocorticolimbic DA-mediated function. The mixed $5HT_2$ receptor agonists mCPP and MK212 (Di Giovanni et al. 2000) as well as the selective 5-HT_{2C}R agonist RO600175 (Di Matteo et al. 2000) are found to have an inhibitory effect on both the activity of VTA DA-containing neurons and the release of DA from the nucleus accumbens (NAc), whilst administration of SB242084 blocks the effects of RO600175 (Di Matteo et al. 2000). Systemic, intra-VTA, or intra-PFC infusions of the $5HT_{2C}$R antagonists SB206553 and SB242084 alone also potently elevate VTA

DA-neuronal firing and DA dialysate levels in the NAc (Di Giovanni et al. 1999; Di Matteo et al. 1999).

These effects are believed to be mediated by indirect actions at GABAergic interneurons, consistent with the presence of 5-HT_{2C}R transcript and protein in VTA and substantia nigra compacta (SNc) GABA neurons (Bubar & Cunningham 2007; Eberle-Wang et al. 1997), and with the ability for 5-HT_{2C}Rs to modulate GABA function within these regions (Bankson & Yamamoto 2004; Di Giovanni et al. 2001; Invernizzi et al. 2007). Although the ventral striatum does not appear to play a critical role in reversal learning (Burk & Mair 2001; Castañé et al. 2010; Stern & Passingham 1995), effects on DA signalling within this region are believed to be the cause of the elevated motor impulsivity and hyperactivity effects observed following 5-HT_{2C}R antagonist administration (Fletcher et al. 2007, 2009; Winstanley et al. 2004). By contrast, the dorsal striatum has been implicated in reversal learning (Clarke et al. 2011; Clatworthy et al. 2009; Kirkby 1969; Ragozzino et al. 2002), but there is some debate as to whether 5-HT_{2C}Rs play a relevant role in the control of nigrostriatal DA. Although elevated striatal DA-levels and SNc DA-neuron firing have been observed in the 5-HT_{2C}R KO mouse (Abdallah et al. 2009), systemic administration of SB242084 is most often without effect on nigrostriatal DA-signalling in rats, under doses ranging from 0.16 to 10mg/kg (De Deurwaerdere et al. 2004; Di Matteo et al. 1999; Gobert et al. 2000; Navailles et al. 2006).

There is also some suggestion that 5-HT_{2C}Rs might exert inhibitory control over 5-HT cell firing in raphe nuclei via a negative feedback loop (Sharp et al. 2007). GABAergic interneurons containing 5-HT_{2C}R mRNA have been identified within the dorsal raphe nucleus (Serrats et al. 2005), and evidence that 5-HT_{2C}Rs exert constitutive inhibitory control over 5-HT neuronal firing in this region is taken from findings that administration of 5-HT_{2C} agonists RO600275 and WAY 161503 inhibit 5-HT neuron firing in the dorsal raphe nucleus; an effect which can be reversed through administration of SB242084 (Quérée et al. 2009). Although pre-treatment with SB242084 alone was without effect in this study, the dorsal raphe does project to the OFC (Goncalves et al. 2009; Morecraft et al. 1992), potentially enabling 5-HT_{2C}R manipulations to effect 5-HT levels within the OFC, a brain region known to be important to reversal.

1.5.2. Effect of 5-HT_{2C} receptor subtype specific manipulations on reversal performance

Despite some question as to how 5-HT_{2C}Rs might exert control over cognitive flexibility, there is growing evidence that actions at these receptors make an important contribution to reversal learning. Administration of the selective 5-HT_{2C}R antagonist SB242084 (0.1/0.3/1.0 mg/kg) was found to improve performance of a serial two-lever visuospatial reversal task in rats

(Boulougouris et al. 2008), with all three doses leading to a reduction in the number of trials required to reach criterion, and the two highest doses additionally decreasing the number of incorrect responses and early-stage errors (Boulougouris et al. 2008). These findings have since been extended to demonstrate the neuroanatomical specificity of this effect, with evidence for dose-dependent effects of 5-HT_{2C} antagonism following targeted infusion in the OFC similar to those seen following systemic administration, but which were absent following infusions into the mPFC or Nac (Boulougouris & Robbins 2010). In line with these findings, decreased activity at the 5-HT_{2C}R has been shown to improve aspects of reversal learning in mice using a similar serial visuospatial reversal task, but where the operant response was a nose-poke rather than a lever-press (Nilsson et al. 2012). Following either pharmacological inactivation (SB242084 0.5mg/kg) or genetic ablation (2CKO mice) of the 5-HT_{2C}R, there was a reduction in the number of trials and omissions to reversal criterion, and a reduction in correct rather than incorrect responses to criterion. Although neither manipulation was found to effect performance in a subsequent spatial T/Y-maze reversal test (Nilsson et al. 2013), this inconsistency was attributed to the different neural mechanisms which likely mediate visuospatial as compared to egocentric reversal learning, which appears to depend more heavily on the integrity of the dorsal striatum (Mitchell & Hall 1988; Packard et al. 1989; Ragozzino et al. 2002). Whilst evidence of a reversal-learning enhancement effect following reduced 5-HT_{2C}R activity appears to contradict the retarding effects of global or OFC-specific 5-HT depletions, this has previously been explained through incomplete lesions causing super-sensitivity of 5-HT_{2C}Rs (Boulougouris & Robbins 2010; Roberts 2011).

What is more difficult to reconcile however, is evidence of significantly impaired reversal learning in 2CKO mice using the same serial visuospatial task as previously employed by Nilsson et al. (2012), who reported a beneficial effect of 5-HT_{2C}R ablation (Pennanen et al. 2013). Pennanen et al. (2013) reported a significant impairment across all three serial reversal tests of the task in 2CKO mice relative to wildtype controls, requiring more sessions, trials and incorrect responses to reach criterion, and making more early as well as late-stage errors. Targeted mutations which cause constitutive loss of specific components of 5-HT systems often cause adaptations in addition to the mutation, leading to behavioural effects which differ from those of acute pharmacological blockade. Specifically, the 2CKO mutant shows markedly elevated levels of dialysate DA in the dorsal striatum (Abdallah et al. 2009), yet pharmacological inactivation is without effect on DA levels in this area (Di Matteo & Esposito 2001; Gobert et al. 2000). Furthermore, elevated DA levels in the dorsal striatum have been associated with impaired reversal performance (Clatworthy et al. 2009; Swainson et al. 2000), an effect which

might explain the retarded learning reported by Pennanen et al. (2013). However, why studies which employ the same manipulation, within the same species, using seemingly similar tasks and experimental designs should report such conflicting outcomes is currently not clear.

Along with the more traditional methods of modulating serotonergic function discussed above, recent developments in optogenetic and chemogenetic technologies will likely be instrumental in improving our understanding of the role of serotonergic systems in cognition in the future, by allowing for more selective targeting of specific neural circuits. The emergence of genetic techniques to selectively and reversibly manipulate cellular activity has revolutionised the neurosciences, and these techniques are starting to be more frequently used to investigate the neural mechanisms of behaviour. Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are synthetically derived receptors which can be introduced into neural tissue through a range of gene transfer strategies and which can be transiently activated by otherwise inert exogenous ligands (Smith et al. 2016; Whissell et al. 2016). Optogenetics can also be used to reversibly manipulate the activity of cells, through the introduction of genes encoding light-sensitive transmembrane ion conductance regulators to enable excitation or inhibition of targeted cells, offering a high degree of temporal as well as spatial resolution (Aston-Jones & Deisseroth, 2013). Such techniques can be used to explore neurotransmitter specific functions by taking advantage of receptors associated with specific transmitter molecules. So far, receptors activated solely by synthetic ligands have been developed for studying the 5-HT_4 (Claeysen et al. 2003) and 5-HT_{2A} (Kristiansen et al. 2000) receptors through the introduction of mutations that abolish the ability of 5-HT to activate the receptor without affecting the efficacy of many synthetic agonists; though these are yet to be explored in relation to effects on cognitive flexibility. A light-activated G protein-coupled receptor that targets into 5-HT_{1A} receptor domains has also been created (Oh et al. 2010). The creation of a light-activated or an engineered receptor/ligand pair for the 5-HT_{2C}R would be a significant development in enabling more precise control over this receptor subtype in the future and for understanding the relevance of these receptors to cognitive function.

In summary, although the precise mechanisms through which 5-HT_{2C}Rs effect reversal learning are not currently clear, these receptors do appear to offer a promising target for improving cognitive flexibility, with several reports of improved reversal performance following reduced activity at these receptors. However, recent contradictory evidence of effects following genetic ablation of 5-HT_{2C}Rs in seemingly similar tasks requires further exploration. Just as inspection of task differences has helped to reconcile some of the inconsistencies reported in the literature on global 5-HT manipulations, it is likely that exploration of differences in

experimental design can help to resolve such discrepancies and shed further light on the delicate balance of neuronal functions seemingly under the control of 5-HT_{2C}Rs.

1.6. The need for greater dissection of reversal learning components

In addition to exploring the role of task differences in mediating the effects of 5-HT manipulations upon reversal, there is a need to clarify the specific components of reversal learning which are being affected by these manipulations. It is not currently clear whether 5-HT circuitry serves a general role in reversal learning or is involved only in specific reversal problems. Although the tasks described above dissect the components of cognitive flexibility to a greater extent than the WCST, even within a seemingly simple reversal learning task, numerous, often concurrent, processes are involved. Any number of these may be affected by a certain neuropathology or experimental manipulation.

The initial discrimination of a reversal learning task involves the acquisition of associations between cues, responses and outcomes. These associations can range from simple, habit-like associations to more cognitive associations by which cues contain information about the predicted value and/or sensory features of outcomes. Furthermore, these associations may be rewarding or aversive, adding a dimension of value. During reversal, successful performance depends upon the ability to simultaneously adapt all of these associations, which requires the need to signal accurate predictions in order to engage error and attentional mechanisms to drive learning. Reversal also introduces the concurrent need for behavioural inhibition and engagement, with subjects having to withhold a previously learned response whilst also engaging a previously withheld response. Because reversal performance is assessed in a single format, with multiple processes concurrently taking place, it can therefore be difficult to pinpoint where experimental manipulations exert their effects (McDannald et al. 2014).

Several studies have suggested that 5-HT exerts its effects early in reversal, which is typically taken as evidence for an effect on perseverative responding for the old CS+ (e.g. Boulougouris et al. 2007; Butter 1969; Chudasama & Robbins 2003; Jones & Mishkin 1972). However, early errors can equally be evidence of a reduced ability to generate responding for the new CS-. In reversal learning, the initial two-choice discrimination could be reduced to an excitatory CS-US association, eliciting approach and contact, and an inhibitory CS –'no US' association, eliciting withdrawal (Mackintosh 1983). After the subsequent contingency shift, the CS predicting the US becomes associated with 'no US', a process opposed by perseverance, and the CS initially predicating 'no US' now predicts the US, a process opposed by learned non-reward (Nilsson et al. 2012). Deficits in reversal could therefore be due to a failure to overcome

either or both associations of previous positive (perseverance) and negative (learned non-reward or learned irrelevance) valence; though there is even evidence to suggest that learned non-reward contributes more than perseverance to the difficulty of a reversal learning task (e.g. Beran et al., 2008; Goulart et al., 2005). Despite these observations, very few researchers have so far attempted to fractionate the contribution of 5-HT to these separable processes.

It is also notable that the vast majority of reversal tasks have to date employed operant procedures, and studies using a classical conditioning approach have been rare (Bushnell & Stanton 1991). Chudasama & Robbins (2003) point out that reversal learning tasks can combine both Pavlovian and instrumental components, since classical conditioning processes can be sufficient to elicit the approach and contact response to a CS+ needed to 'solve' a discrimination, without any need for the intervention of instrumental processes. This potential confound is of vital importance given that any individual variation in the degree of incentive value ascribed to Pavlovian cues during acquisition might affect the ability to subsequently reverse these learned associations, making it unclear to what extent reversal behaviour is mediated by instrumental learning principles. Although in the Izquierdo et al. (2012) study previously discussed, the authors report impaired approach to reward-paired stimuli in PCPA treated mice, the contribution of 5-HT to this effect is not clear, given that 5-HT tissue levels did not differ from saline treated controls. Pavlovian conditioned approach does appear to depend upon similar structures as are implicated in reversal learning however, with OFC lesions acting to impair the acquisition of Pavlovian conditioned approach to the CS+ in an autoshaping task as well as increasing perseverative errors in an operant reversal learning task (Chudasama & Robbins 2003). One prior study also reports that OFC lesions impaired reversal of a Pavlovian discrimination by preventing the development of normal responding to the previously unrewarded cue (Burke et al. 2009). There is currently little evidence of the extent to which 5-HT manipulations affect reversal of Pavlovian conditioned responses however, and an understanding of the role of 5-HT in reversal learning may benefit from such an approach.

1.7. Conclusions and thesis aims

Unravelling the biological underpinnings of behaviour is one of the most challenging problems of modern biology (Crusio & Gerlai 1999). Over the years, increasingly more advanced molecular genetic tools have become available, which have allowed for precise, controllable and selective investigations into the systems and circuits associated with a variety of complex brain functions. However, the significance of studies into complex brain functions will always critically

depend upon the significance of the behavioural paradigms available to assess these functions (Colacicco et al. 2002). Even within reversal learning tests, numerous, often concurrent processes are involved, any number of which may be affected by a certain experimental manipulation (McDannald et al. 2014), and further dissection of function will be required to gain a better understanding of the role of 5-HT systems in cognitive flexibility.

Although actions at 5-HT_{2C}Rs may be responsible for mediating some of the effects of changes in global 5-HT transmission, and have been identified as potentially useful targets for improving cognitive function, the effect of 5-HT_{2C}R manipulations have rarely been investigated in tasks of learned non-reward and perseverance, or even in related tasks of extinction and latent inhibition. Furthermore, reversal learning tasks additionally involve reward and punishment feedback processing, and the updating of associations between cues, responses and outcomes, and it cannot be determined through use of standard operant reversal tasks what effect 5-HT_{2C}R manipulations may have on these separable processes. Additionally, there are discrepant reports in the literature for the effect of reducing 5-HT_{2C}R function in seemingly similar reversal tasks, which closer exploration of behavioural designs may also help to reconcile.

In a bid to resolve discrepancies in the literature and to clarify the behavioural and/or cognitive processes affected by 5-HT_{2C}R mechanisms, this thesis will describe a set of experiments reporting a detailed analysis of behavioural design differences in prior tasks manipulating 5-HT_{2C}R function. The effects of 5-HT_{2C}R antagonism will be explored in several tasks designed to measure distinct components of reversal learning and immunohistochemical methods will be used to identify CNS structures that are preferentially activated during the performance of discrimination and reversal tasks.

Since the key aims of this thesis are to attempt to reconcile differences in outcome in previous reports following manipulation of 5-HT_{2C}R activity, as well as to identify the precise cognitive/behavioural mechanisms through which 5-HT_{2C}Rs may affect reversal performance, the experiments presented in this thesis employ a single dose of the 5-HT_{2C}R antagonist SB242084 throughout, since the number of experimental groups in these designs made testing of a broader dose-range unfeasible. This drug was chosen on the basis that it represents the most potent and selective 5-HT_{2C}R antagonist currently available (Kennett et al. 1997), and is the compound previously administered in studies of reversal learning that I am seeking to replicate and expand upon (e.g. Boulougouris et al. 2008; Boulougouris & Robbins, 2010; Nilsson et al. 2012). The chosen dose (0.5mg/kg) was selected on the basis that it matches that previously used by Nilsson et al. (2012) who report its efficacy in reversal tasks; and is similar to the most

effective dose used by Boulougouris et al. (2008) and Boulougouris and Robbins (2010) of 0.3mg/kg, whereas the lower dose (0.1mg/kg) used in these studies was without effect on some reversal measures, and the higher dose (1.0mg/kg) showed non-specific effects, acting to improve retention of learning prior to reversal. A carry-over effect of this dose is also reported by Nilsson (2012), who reports impaired performance during a final drug-free reversal test for animals previously administered 1mg/kg of SB242084, not seen in animals administered the 0.5mg/kg dose. These findings suggest that higher doses offer decreased pharmacological specificity and/or might additionally activate a subpopulation of 5-HT_{2C}Rs in different cellular compartments (Marek et al. 2005). As such, a 0.5mg/kg dose minimises the possibility of non-specific effects of higher doses whilst offering superior sensitivity over lower doses to detect significant reversal effects. Furthermore, doses of SB242084 at and above the 1mg/kg dose range are shown to more reliably produce effects on measures of anxiety (Martin et al. 2002) and motivation (Fletcher et al. 2010; Simpson et al. 2011); which act as significant potential confounds when attempting to explore the effects of this drug on flexible cognition, providing further justification for the selected dose.

Thesis aims:

1. **To investigate the role of task differences in mediating the effect of 5-HT_{2C}R manipulations upon reversal learning, as identified from prior studies reporting discrepant outcomes.**
2. **To dissect reversal learning tasks into their key constituent components, and identify the effect of reduced 5-HT_{2C}R activity in these tasks.**
3. **To provide preliminary identification of areas of neuronal activation during the performance of an operant spatial discrimination and reversal task.**

CHAPTER 2

THE ROLE OF TASK PARAMETERS IN MEDIATING REVERSAL LEARNING OUTCOMES FOLLOWING 5-HT$_{2C}$R ANTAGONISM

2.1. Introduction:

Although previous research has highlighted a possible role for the 5-HT$_{2C}$R in reversal learning, there is conflicting evidence regarding the nature and direction of effect following manipulations of 5-HT$_{2C}$R function. The reasons for these discrepancies require further exploration to enable a better understanding of the precise function of 5-HT in flexible cognition. The current chapter provides a more detailed examination of the existing evidence concerning the effect of 5-HT$_{2C}$R manipulations on reversal learning, with a particular focus on identifying critical differences in behavioural design which might account for discrepant outcomes, which will then be tested.

Indirect evidence of a role for 5-HT receptors in flexible cognition was originally taken from observations that typical and atypical antipsychotic drugs (APDs) demonstrate different efficacy in reversal learning tasks. Though both act as antagonists at dopamine D$_2$ receptors, atypical APDs show lower D$_2$ receptor occupancy, and additionally act as potent 5-HT$_{2A}$ receptor antagonists, whilst some also have affinity for the 5-HT$_{1A}$R and 5-HT$_{2C}$R (Meltzer 1999). Sub-chronic (2mg/kg twice daily for 7d, plus 7d washout; Abdul-Monim et al. 2006) or acute (1.0/1.5mg/kg; Abdul-Monim et al. 2003) administration of the NMDA receptor antagonist phencyclidine (PCP), often used to model the cognitive symptoms of schizophrenia, has been shown to cause a deficit in reversal learning in rats, acting to reduce the percent of correct responses made. This PCP-induced reversal deficit can be counteracted by atypical, but not typical APDs (Abdul-Monim et al. 2003, 2006), an effect which has therefore been proposed to be mediated via the additional action of atypical APDs at 5-HT$_{2A}$ receptors. This suggestion is supported by the finding that the 5-HT$_{2A}$ receptor antagonist M100,907 also ameliorates the reversal learning deficit induced by PCP (Idris et al. 2010). However, selective 5-HT$_{2C}$R inverse agonist SB243213A is also able to attenuate this PCP-induced reversal learning deficit in rats (McLean et al. 2009), suggesting that the ability for atypical APDs to rescue the PCP-induced reversal deficit is at least partially 5-HT$_{2C}$R mediated.

A specific role for 5-HT_{2C}R in reversal learning is supported by the finding of enhanced reversal performance following systemic administration of the 5-HT_{2C}R antagonist SB242084 (Boulougouris et al. 2008), which reduced the number of trials and incorrect responses to criterion during the first of three reversals in a serial spatial discrimination task, as well as decreasing the number of perseverative errors to reversal criterion (errors committed before attainment of chance-level performance). By contrast, the selective 5-HT_{2A}R antagonist M100907 had the opposite effect on all three measures, significantly impairing reversal. Improved reversal performance was also seen following intra-OFC administration of SB242084 (Boulougouris & Robbins 2010), which was again mediated by a reduction in trials and perseverative errors to criterion, and restricted to the first reversal test. Such findings are supplemented by evidence of enhanced visuospatial reversal learning following both pharmacological and genetic inactivation of the 5-HT_{2C}R, with evidence of reduced trials and response omissions to criterion during a single reversal test in 5-HT_{2C}R knockout (2CKO) mice, and over the first two reversals in a serial reversal task in SB242084-treated animals (Nilsson et al, 2012); though there was no evidence for an effect on incorrect responding in either manipulation.

Whilst pharmacological enhancement of reversal performance might therefore be extended to genetic manipulation of 5-HT_{2C}R function, performance differences were evident, since pharmacological blockade was associated with a reduction in the number of correct trials to criterion and a decrease in response latencies; effects not observed in 2CKO mice. Though this could be related to the difference in manipulation, no effect on response omissions or latencies was previously reported following SB242084 administration (Boulougouris et al. 2008; Boulougouris & Robbins 2010). Therefore, there are slight differences in outcome reported in previous studies, though the overall direction of effect of 5-HT_{2C}R manipulations was the same. However, a more recent experiment demonstrates impaired rather than improved learning in a very similar serial visuospatial reversal task in 2CKO mice (Pennanen et al. 2013). Despite displaying similar levels of performance to wildtype controls on the first of three reversal tests, these animals failed to continually improve performance over successive reversals, resulting in a greater number of trials and incorrect responses to criterion (Pennanen et al. 2013). Although genetic alteration of 5-HT_{2C}R activity is likely to have different effects on performance than pharmacological blockade, not least because there is a loss of receptor function throughout the task, evidence for a difference in outcome using this same genetic manipulation cannot be readily explained.

The reversal tasks used in all four of these previous studies were similar, in that they employed a visuospatial discrimination task where responses at one of two spatially separated (left/right) levers (Boulougouris et al. 2008; Boulougouris & Robbins 2010) or nosepoke holes (Nilsson et al. 2012; Pennanen et al. 2013) were rewarded during acquisition, which switched location during reversal. However, these studies demonstrate a number of differences across key task parameters (see Figure 2.1 and 2.2) which may be an important consideration for understanding discrepant outcomes, since even small manipulations of task parameters have been found to have profound effects on performance. Pennanen et al. (2013) employed a within-session design, requiring recall of the previously learned discrimination immediately prior to each reversal shift. These task requirements differed quite markedly from that of the between-session paradigm used by Nilsson et al. (2012), in which acquisition, retention and reversal occurred on separate test days. The within-session design could ensure contingency shifts are more salient and easier to detect, or could equally render response requirements unclear, given that both possible responses have been associated with reward within a single test session. However, the Pennanen et al. (2013) design was based on the within-session task used by Boulougouris et al. (2008, 2010), who reported a beneficial effect of 5-HT_{2C}R antagonism on reversal performance, so this is unlikely to be a critical design difference for producing discrepant outcomes.

The trial timings in these studies also differed quite considerably. In the Nilsson et al. (2012) study, animals were required to make a response within 12s of trial initiation before the trial terminated and an omission was recorded, similar to that permitted for animals in the Boulougouris et al. (2008, 2010) design (10s). By comparison, Pennanen et al. (2013) allowed a significantly longer 60s response interval (see Figure 2.2). Although often regarded as a control for motivational or motor effects, response omissions during reversal can indicate uncertainty over the correct response, and can thus provide an additional measure of learning; this is consistent with evidence that omissions decrease over successive serial reversal tests as performance improves (Boulougouris et al. 2008). The extended response interval permitted by Pennanen et al. (2013) led to a considerably lower level of recorded omissions in comparison to previous tasks, possibly obscuring group-related differences on this informative measure. However, response omissions were not found to differ in SB242084-treated animals relative to controls in the Boulougouris et al. (2008, 2010) experiments, despite drug-treated animals showing improved reversal performance; demonstrating that these two performance measures are dissociable. Furthermore, it is not immediately obvious how this timing difference could be responsible for opposing outcomes in terms of the number of trials needed to reach criterion.

Another key design difference across previous studies is the rate of stimulus presentation. Pennanen et al. (2013) set the inter-trial interval (ITI) at just 2s, compared with the 15s ITI employed by Nilsson et al. (2012). The relevance of this might seem questionable given that Boulougouris et al. (2008, 2010) report beneficial effects of $5HT_{2C}R$ antagonism when using an ITI of only 5s (see Figure 2.2), however, there are several lines of reasoning to suggest this difference might be critical. Though Pennanen et al. (2013) and Boulougouris et al. (2008, 2010) do not report reward retrieval latencies, results from the Nilsson et al. (2012) study show that it took just over 2s on average for 2CKO animals and controls simply to retrieve rewards, though for SB242084-treated animals there was a slight, but significant, reduction in retrieval latency. An ITI of 2s therefore allows little time for animals to retrieve and consume rewards, and to be oriented, attentive and motivated to commence new trials.

Differences in the rate of stimulus presentation as small as this have also been found to have a significant bearing on performance accuracy in discriminative learning tasks. In a conditional visual discrimination task where fast and slow pulses of light signified which of two levers to press for reward, reducing the inter-trial interval (ITI) by just 3 seconds significantly impaired discriminative accuracy in rats (Ward et al. 1999). Furthermore, manipulations of the rate of stimulus presentation (ITI) had a differential effect for 5-HT lesioned rats as compared with sham-operated controls, highlighting the sensitivity of performance to serotonergic manipulation following small changes in the ITI. Forebrain 5-HT lesions given either pre- or post-acquisition improved performance accuracy compared with sham-operated controls, and protected animals from the disruptive effects of reducing the ITI from 8s to 5s. However when the ITI was further reduced to just 2s, response accuracy no longer differed and both groups of animals were significantly impaired. The beneficial effect of 5-HT lesions was reportedly specific to trials with consecutive different stimuli (i.e. fast-slow or slow-fast), suggesting that this manipulation reduced the amount of proactive interference caused by the previous response, an effect which the authors speculate would increase as ITI decreases (Ward et al. 1999). This is clearly of relevance to reversal learning designs where response perseveration at the previously correct location must be overcome, and could suggest that at certain rates of stimulus presentation the benefits of 5-HT manipulations are seen, whilst perseverative responding may be particularly difficult to overcome when the ITI is reduced below a certain level. In light of this study, although the ITI used by Boulougouris et al (5s) was also considerably shorter than that used by Nilsson et al (15s), it could be sufficiently longer than that used by Pennanen et al (2s) so as to allow the beneficial effects of $5\text{-}HT_{2C}R$ antagonism to be seen. However, whilst this might explain why a beneficial effect of 5-HT manipulation is not observed under very short ITIs, it does

not clearly explain why a performance deficit should be seen relative to controls, suggesting a need to look to other design differences to explain this result.

The design difference most likely to affect experimental outcome might therefore be the requirement to self-initiate trials, versus automatically programmed trial initiation. Each of the prior experiments which report beneficial effects of 5-HT_{2C}R manipulations on reversal performance required animals to nosepoke within the food magazine to commence each new trial following the ITI (Boulougouris et al. 2008, 2010; Nilsson et al. 2012), yet trials in the Pennanen et al. (2013) study were programmed to automatically initiate after the ITI. In a purely mechanistic sense, requiring animals to initiate trials in a central food magazine gives some confidence that they are oriented in a central position prior to the commencement of new trials, and reduces the possibility of using mediating behaviours to solve the discrimination, such as waiting at the correct stimulus location prior to trial onset. Although such behaviour might be beneficial during acquisition, it could impair performance during reversal when the correct location changes position.

The difference in trial initiation requirements might also alter the degree of impulsive responding that is recruited by these tasks. As discussed in Chapter 1, reducing 5-HT_{2C}R function produces performance deficits in touchscreen-based reversal learning tasks (Nilsson 2012), as compared to the performance enhancing effects observed under standard operant conditions. This difference was hypothesised to be caused by the greater engagement of striatally-mediated impulsivity processes in the touchscreen task, possibly obscuring the beneficial effects on PFC-mediated reversal learning. Automatic trial initiation conditions might also give rise to the type of rapid responding observed in touchscreen-based tasks. Unfortunately, Pennanen et al. (2013) do not report response latencies, so further tests will be needed to determine whether automatic trial conditions give rise to faster, more impulsive responding relative to self-initiating trial conditions.

Alternatively, it is possible that automatic and self-initiated trial requirements affect the nature, rather than the degree of impulsive responding that must be overcome during reversal. Impulsivity is unlikely to reflect a unitary construct, and different aspects of impulsivity are shown to have independent underlying biological and neurochemical mechanisms (Winstanley et al. 2004). If the process of initiating a trial can be conceived of as the first element of a response sequence, self-initiated trial conditions could be said to probe the ability to inhibit or cancel an already initiated action. By contrast, automatic trial initiation tests present the conditioned stimuli to animals before a response is initiated, and response inhibition might be

said to take the form of action restraint, rather than action cancellation. There is evidence to suggest that 5-HT_{2C}R antagonism has opposing effects on these two forms of impulsivity. In the stop-signal reaction time task (SSRTT) a 'No Go' signal is randomly presented on a proportion of trials halfway through execution of a learned, rewarded behavioural sequence, and animals must inhibit ongoing behaviour in order to earn reward. Administration of SB242084 has been found to improve response control in the stop-signal reaction time task (SSRTT) in mice (Humby et al. 2013), with a dose-dependent increase in successful stopping and a significant reduction in stopping time relative to vehicle-treated controls (Humby et al. 2013). Though localised infusion studies are presently lacking, this effect could be mediated by actions in the mPFC, since lesions of this region were also found to impair stopping (Humby et al. 2013). However, this same manipulation resulted in increased premature responding in the five-choice serial reaction time task (5CSRTT) (Winstanley et al. 2004), which measures the ability to withhold responses over time to an affectively charged stimulus. The locus of action for this effect is proposed to be striatal, with evidence that targeted infusion of SB242084 into the NAc, but not the PrL or IL, increased premature responding in this task (Robinson et al. 2008). This could suggest a (potentially mPFC-mediated) enhancement of action cancellation following 5-HT_{2C}R antagonism, accompanied by a striatally-mediated impairment of inhibition during action selection. Therefore, systemic administration of SB242084 might act to reduce impulsive responding under self-initiated trial conditions, allowing the beneficial effect of the treatment on cognitive flexibility to be observed; but increase impulsive responding under automatic trial conditions, potentially masking any beneficial effect of 5-HT_{2C}R antagonism on cognitive flexibility.

Another line of evidence to suggest that trial initiation requirements might have a significant effect on performance can be taken from studies which demonstrate a link between effort and reinforcer value. Both mice (Johnson & Gallagher 2011) and pigeons (Clement et al. 2000) have been found to demonstrate a choice preference for rewards and cues which have been associated with high-effort, over those associated with low-effort training. Significantly, this was true whether the difference in effort (high/low) was required to directly earn one of two reinforcers (Johnson & Gallagher 2011); or simply to elicit presentation of one of two S+/S- discriminations, even when the response requirement to subsequently earn reinforcers was the same (Clement et al. 2000). This demonstrates that a difference in response requirement during trial initiation could have a significant bearing on the motivational value of rewards subsequently earned. Pennanen et al. (2013) themselves state that new trial initiation is linked to reward-seeking and therefore provides a measure of motivation, creating difficulties in determining whether animals in their task were motivated to perform. The authors in fact report higher

omission rates amongst 2CKO animals relative to controls across all stages of the task, not specific to reversal, suggesting there may have been a general motivational or attentional impairment in this group. Though this could be related to the effects of the genetic manipulation, this same deficit was not seen in 2CKO mice in the Nilsson et al. (2012) study, raising the possibility that 5-HT_{2C}R blockade/loss leads to reduced motivation to earn rewards relative to controls under low-effort conditions. It should be noted however that in the Clement et al. (2000) study the difference in response accuracy in the high effort (85.2%) and low-effort (79.1%) discrimination task did not reach significance; therefore reduced motivation for reward might not translate into a significant performance deficit.

	Boulougouris et al (2008, 2010)	**Nilsson et al (2012)**	**Pennanen et al (2013)**
$5HT_{2C}$R manipulation	SB242084	SB242084 & **5-HT_{2C}R KO**	**$5HT_{2C}$R KO**
Reversal performance	IMPROVED (reduced trials, incorrect, and perseverative responses to criterion)	IMPROVED (reduced trials and omissions to criterion)	**IMPAIRED** (increased trials, and incorrect responses to criterion)
Reversal design	**Within-session**	Between-session	**Within-session**
Response Interval	10s	12s	**60s**
ITI	**5s**	15s	**2s**
Trial initiation	Self-Initiated	Self-Initiated	**Automatic**

Figure 2.1. Experimental design of previous reversal learning tasks. *Pertinent differences in design/outcome highlighted in bold. Beneficial effects of experimental manipulation appear in green fill, deleterious effects in red.*

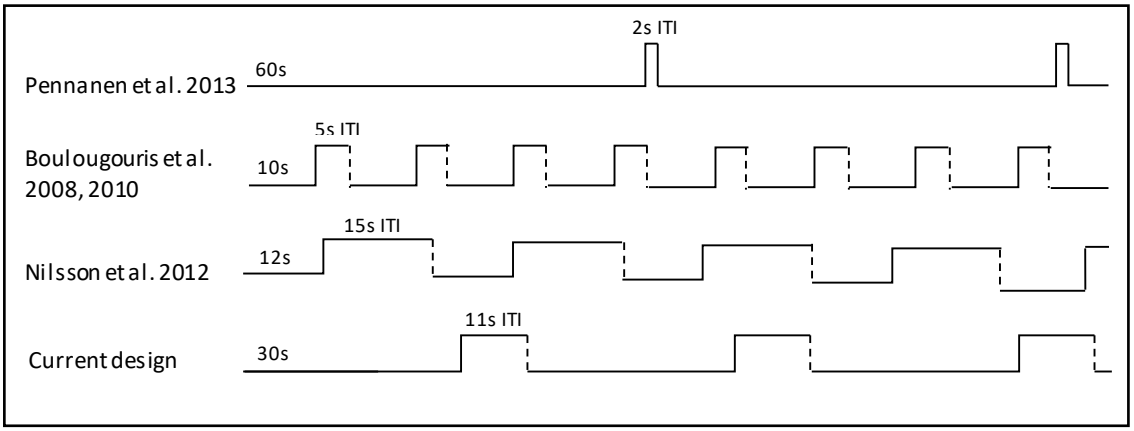

Figure 2.2. Illustration of trial timings in prior reversal tasks. *Bottom line represents response intervals (60s, 10s, 12s, 30s) indented lines represent inter-trial intervals (2s, 5s, 15s, 11s). Dashed lines demonstrate where animals are required to self-initiate the following trial by making a nosepoke response in the magazine.*

In sum, there are key differences in task design across prior studies reporting a different impact of 5-HT_{2C}R manipulations on reversal learning performance. Such design discrepancies, though seemingly small, may be critically affecting behaviour in these tasks, essentially presenting very different challenges to animals. Trial initiation requirements present the clearest design discrepancy between experiments reporting positive versus negative outcomes of 5-HT_{2C}R manipulations, and the preceding paragraphs have established a firm theoretical grounding for why differences might be expected across these tasks. With these considerations in mind, the present chapter seeks to establish whether this specific design alteration might account for divergent outcomes, by investigating the effect of automatic and self-initiated trial conditions on performance of a serial reversal learning task following pharmacological inactivation of the 5-HT_{2C} receptor, where other task parameters are set at an intermediate point derived from these prior studies (see Figure 2.2). A further control group of animals was also included, in order to rule out the contribution of differences in the rate of trial presentation for animals under automatic conditions, who receive trials on a fixed interval schedule, as compared with animals in the self-initiated condition who may take up to a further 20 seconds to initiate each new trial. This control group was yoked to a subset of animals in the self-initiating condition, and received automatic trials but with an additional delay prior to trial onset, matched to the average trial initiation latency of its partner from the previous test day. This ensured that any differences in performance between automatic and self-initiated trial conditions were not simply related to differences in rate of stimulus presentation.

2.2. EXPERIMENT 1

2.2.1. Materials and methods:

Animals

This study used 40 male C57BL/6J mice (Charles River, UK), weighing an average of 24.2 g (SEM ± 0.6g) at the start of behavioural testing. Animals were housed in pairs (except where persistent aggressive behaviour necessitated separation) in a controlled environment held at 21±2°C and 50±15 % relative humidity with a 12:12 h light-dark period (lights on 07:00 h). Behavioural testing was carried out between 09:00 and 17:30 hours during the animals' light phase. Starting 2 weeks prior to behavioural training, animals were food deprived to 90% of their *ad libitum* weight. Animals received three sham saline injections (4 ml/kg) administered at the end of Day 1 and 2 of spatial discrimination testing, and 30 min prior to Day 3 of spatial discrimination testing, for habituation to the injection procedure. All experiments described in this thesis were licensed under the UK Animals

(Scientific Procedures) Act 1986 (Project License 70/7808) following approval by the University of Sussex Local Ethical Review Committee.

Behavioural apparatus:

Training and testing was conducted in eight operant conditioning chambers (22.5 × 18 × 13 cm; Med Associates, Georgia, VT, USA), located within sound-attenuating wooden boxes fitted with a fan for the purpose of ventilation and masking external noise. Located in one side wall of each chamber was a centrally located food magazine (*W*=2.5 cm *H*=2 cm), which automatically delivered 20mg sucrose pellets (Sandown Scientific, Middlesex, UK) via an external pellet dispenser. The opposite wall of the chamber was fitted with three nosepoke holes (3.2 cm diameter), located 6.5 cm apart and 5.5 cm from the grid-floor. Each nosepoke contained a light-emitting diode (LED) located in the recess of each port, which could be illuminated to indicate it was active, and a houselight was located centrally above the nosepoke ports, 9 cm from the grid-floor. Entries made into nosepoke ports and magazine were detected by an infrared photocell beam crossing each entrance. The apparatus was controlled by Med-PC (version 4) and tasks were programmed in Medstate Notation.

Drugs:

SB242084 (Tocris, Bristol, UK) was initially dissolved in PEG400 (Sigma-Aldrich, Poole, UK) at 20% of the final required volume, which was then made with 10% (*w/v*) hydroxypropyl-beta-cyclodextrin (Fluka, Poole, UK). Stock solution was aliquoted and frozen at -80°C in 4 ml vials. Each animal was dosed 0.5 mg/kg subcutaneously (*s.c.*) at a volume of 4 ml/kg 30 min prior to each day of reversal testing. The 0.5 mg/kg dose was chosen as it replicates the dose used in the Nilsson et al. (2012) study.

Behavioural procedures:

Habituation

Before training, animals were adapted to the food pellets (20mg sucrose pellets) by receiving them in their home cage over two consecutive days prior to habituation. Animals were then exposed to operant chambers for 1h in the dark, with the magazine loaded with 20mg sucrose pellets.

Stage 1: Training to nosepoke for reward

A trial began with the offset of the houselight and onset of a single nosepoke hole LED in the centre of the three nosepoke holes, with the remaining nosepoke holes covered with metal plates. For the first trial in each session only, there was a 30s delay after the offset of the houselight before onset of the central cue light, to allow exploration. For subsequent trials a delay of 3s was used, to maximise saliency of the cue light. Responding in the central nosepoke hole led to the nosepoke LED

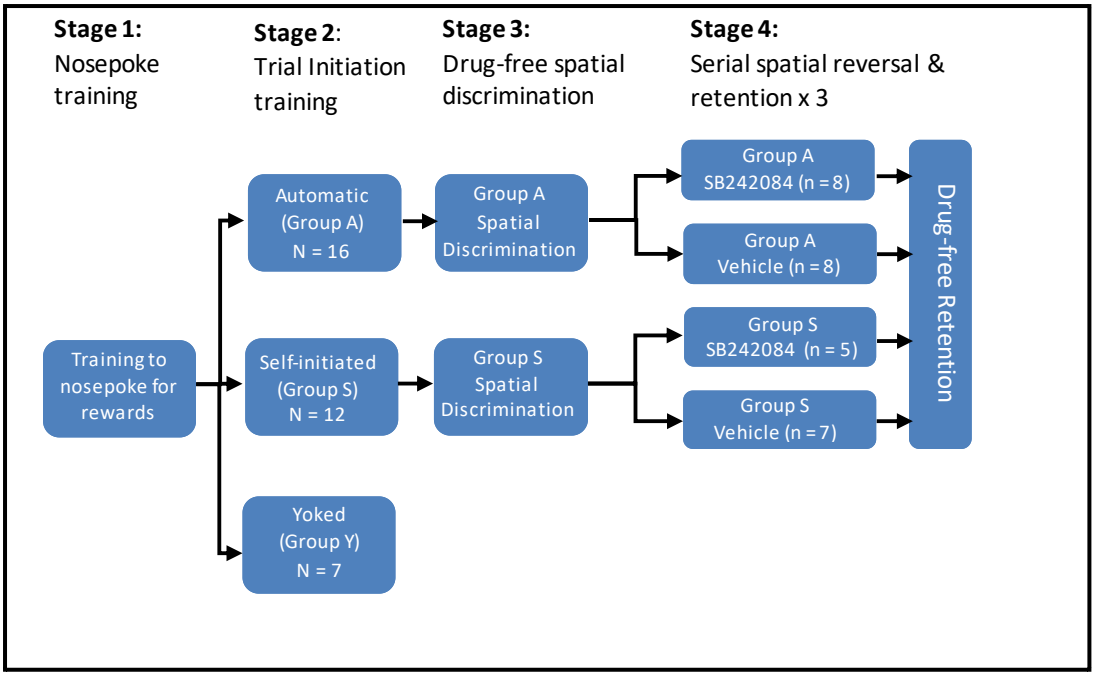

Figure 2.3 Overview of experimental design. *For Group A, trials automatically commenced immediately after the ITI. For Group S, trials would commence after the first head entry made into the magazine after the ITI. For Group Y, trials automatically commenced after the ITI, but only after a delay set to the average trial initiation latency of its yoked pair from Group S on the previous day of training.*

switching off, and delivery of one sucrose pellet. This initiated an 11 s ITI: 4s houselights off, 4s houselights on, 3s houselights off, followed by automatic initiation of the next trial. Each session lasted 60 minutes or consisted of a maximum of 60 trials, with one session per day. Animals received seven daily sessions, and were required to complete 20 trials in any one session by the final training day, or continued to receive daily testing sessions until this criterion was reached; with a complete trial classed as any trial in which the animal responded in the central nosepoke hole and retrieved the reward within the 11 s ITI prior to onset of the next trial.

Stage 2: Self-initiation/ automatic initiation/ yoked training

Once reliably nosepoking to obtain rewards, animals were divided into three groups. Two groups, counterbalanced for trials completed during the final training session, went on either to receive training on self-initiating trials (n=16), or continued to receive automatically initiated trials (n=16). To ensure that any difference between test conditions could not be ascribed to differences in trial onset delay for automatic and self-initiating animals, a third group of yoked controls (n=8) were included, pair-matched to a subset of animals from the self-initiate group for trials completed during the final training session. For this group, trials were automatically initiated but occurred following an imposed delay set to the average trial initiation latency of its' yoked partner from the previous day.

Group S, Self-Initiated training:

As training stage 1, but animals were now required to self-initiate each trial. As before, the start of a trial was signalled by the offset of the houselight. A nosepoke in the magazine now triggered the immediate onset of the central nosepoke hole LED. Responding in the lit-up nosepoke hole led to the LED turning off, delivery of the reward, and the start of the ITI. An omission was now recorded if an animal failed to self-initiate a trial by a nosepoke in the magazine within 20 s, as well as if failing to nosepoke the relevant hole within 20 s of having initiated a trial (see Figure 2.5). Animals were required to complete 49 correct responses over 70 trials (≥70% correct) in two consecutive test sessions to reach criterion, or received continued daily sessions until this criterion was met.

Group A, Automatically Initiated training:

As training stage 1, except animals were now required to respond in the central nosepoke hole within 20 s of programmed automatic trial initiation, with failure to do so being recorded as an omission (see Figure 2.5), and animals were required to respond correctly on 70% of 70 trials over two consecutive sessions to reach criterion. Following slow acquisition of this training phase within Group A in early test sessions, a trial initiation delay was introduced from session 8 onwards for this group, set to the average initiation latency of Group S on the previous test day. This served to improve performance, and was therefore imposed for Group A during all subsequent test stages. Given that this made the automatic delay largely equivalent to that used in the yoked condition, Group Y was not included in any further stages of testing.

Group Y, Yoked training:

As automatic initiation group, with a delay imposed on each new trial between offset of the houselight and onset of the central cue light, which was set to the average trial initiation latency of its' yoked pair on the previous day of testing.

Stage 3: Drug-free Spatial Discrimination:

During this stage of training, both left and right nosepoke holes were lit whilst the central nosepoke hole was covered. A nosepoke into the correct hole (with correct nosepoke fully counterbalanced across left or right between conditions), led to the nose poke lights turning off, delivery of reward, and beginning of the ITI (4 s houselights off, 4s houselights on, 3 s houselights off), whilst an incorrect response led to nosepoke lights turning off and onset of ITI (8 s houselights on, 3 s delay to next trial). Animals now had 30 s to respond; with failure to do so resulting in the onset of the ITI, and a cue response omission being recorded. For the self-initiated condition group, initiation omissions were also recorded if an animal failed to self-initiate a trial within 20 s. Animals

received 7x10 trial blocks per session, and received a minimum of three daily test sessions. Animals were required to respond correctly in 9 out of 10 trials within a single block (after which the session terminated) over two consecutive daily sessions to reach criterion. Animals failing to reach criterion were tested on subsequent days until criterion was reached.

Stage 4: Multiple serial reversals

Half the animals within each condition (counterbalanced over spatial location of correct nosepoke hole) were dosed with 0.5 mg/kg of SB242084 subcutaneously 30 min prior to each day of reversal testing. Nosepoke hole reinforcement contingencies were repeatedly reversed within both groups over a series of three reversals, where the opposite nosepoke hole to that rewarded during the previous stage became correct (see Figure 2.4). Again, animals were required to respond correctly on 9 out of 10 trials within any single block of 7x10 trials over two consecutive sessions to reach criterion. Animals in both groups were then required to demonstrate successful retention on a separate drug-free testing day by responding correctly on 9 out of 10 trials within any single block of 7x10 trials, before the reinforced location was reversed.

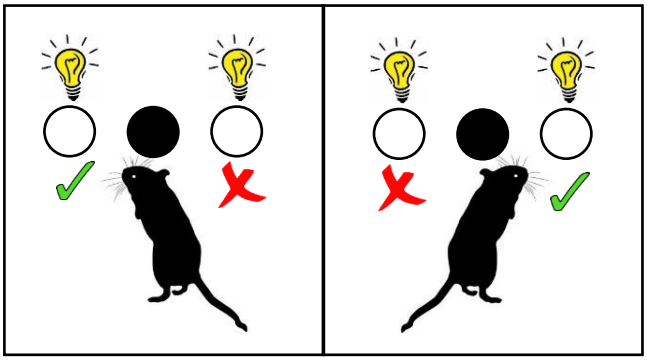

Figure 2.4*. Representation of task requirements during acquisition of the spatial discrimination (Stage 3), and subsequent reversal (Stage 4). After trial initiation (automatic/self-initiated), left and right nosepoke holes are illuminated (open circles), and animals are required to make 9/10 nosepoke responses at the correct location within a 10-trial block over two consecutive test sessions, prior to correct and incorrect response locations reversing. Central nosepoke hole is covered throughout (filled circle).*

Statistical Analyses:

Number of sessions and trials to criterion (including trials where a response omission was recorded) were measured for each animal at each stage of testing. During spatial discrimination and reversal, trials were further broken down into correct and incorrect trials to criterion. Omissions were recorded as trial initiation omissions (Group S only), response omissions and reward retrieval omissions. Latency to initiate a trial (Group S only), make a response and to retrieve a reward were additionally recorded.

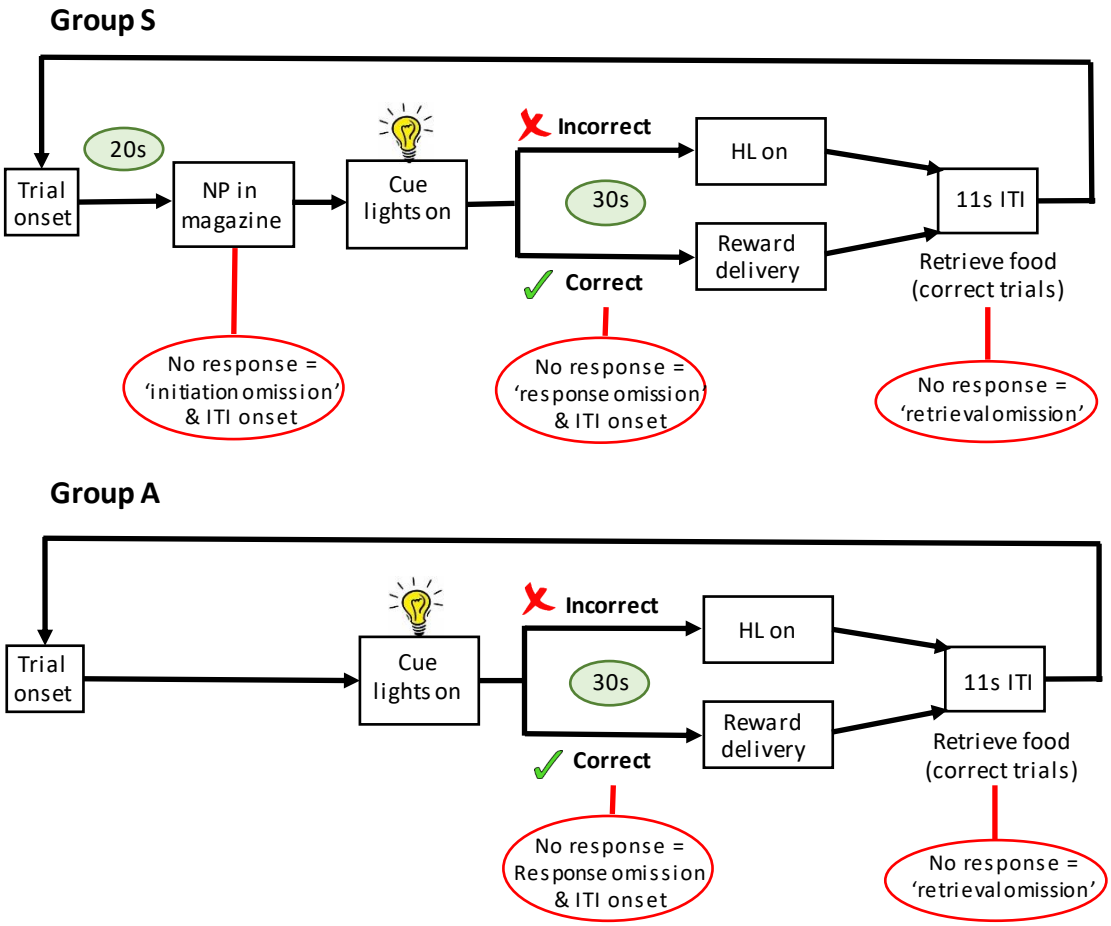

Figure 2.5. Representation of individual trial structure during acquisition and reversal testing for Group S and Group A. *Group S are required to make a nosepoke response in the magazine within 20s of trial onset in order to illuminate cue lights in the left and right nosepoke locations (see Figure 2.4), otherwise an 'initiation omission' is recorded. For Group A, cue lights are automatically illuminated either 3s after trial onset (Stage 1,) or following a delay set to the mean initiation latency of Group S on the previous test day (Stages 2-4). Both groups are required to make a response (correct/incorrect) in one of the illuminated noseoke holes within 30s, or a 'response omission' is recorded; and on correct trials, animals must make a response within the food magazine within the 11s ITI, or a 'retrieval omission' is recorded. Abbreviations: HL = houselights, NP = Nosepoke, ITI = Inter-trail interval.*

Performance of animals in each test condition (Group A/Group S/Group Y) during Stage 1 and 2 of training was compared using one-way ANOVA; to explore the effects of trial timing on performance (since Group Y only differed from Group A in length of ITI). Significant effects were further explored using pairwise comparisons with Holms-Sidak correction. Because Group Y was not included in further stages of testing, baseline performance of animals later assigned to a drug or vehicle treatment group were also analysed using 2 (Condition: Group A/Group S) × 2 (Drug: SB242084/Vehicle) independent measures ANOVA, for performance during Stage 1 & 2 of training and during acquisition of the Spatial Discrimination. Performance during the serial reversal and retention tests was analysed using 2 x 2 x 3 mixed ANOVA, with test stage (Reversal/Retention stage: 1,2,3) as an additional repeated measures factor. Data relevant to self-initiate animals only (initiation

omissions, trial initiation latency) were submitted to independent measures t-tests to examine differences between drug groups during training stage 1, 2 and spatial discrimination; and to 2 × 3 mixed ANOVA for reversal and retention data, with test stage as a repeated measures factor. Any main effect of test stage was followed up with post-hoc pairwise comparisons with Holms-Sidak correction, and where significant interactions emerged, simple effects analyses. Where assumptions of normality or homogeneity of variance were violated, data underwent log or square root transformation. All reported means ± SEM are untransformed.

2.2.2. Results:

Three animals from Group S failed to reach criterion level of responding within 20 sessions of training stage 2. One further animal (from Group S/SB242084) became unwell during reversal testing and was culled. Data from these animals were excluded from subsequent analyses. The final group sizes were: Group S/Vehicle n = 7; Group S/SB242084 n = 5; Group A/Vehicle, n = 8; Group A/SB242085 n = 8.

Training:

Effect of ITI (comparison of Group A / Group S / Group Y):

There were no baseline differences in performance across initiation conditions during Stage 1 of training, when animals learned to make a response in the central nosepoke hole to earn rewards (data not shown). When the different trial initiation requirements were introduced during Stage 2 of training however, there was a significant difference in performance across conditions on all measures except reward retrieval latency (see Table 2.1). Pairwise comparisons demonstrated an impairment in Group A relative to the other groups, whilst Group Y performed similarly to Group S on all measures (all *p*'s > .05), thus providing the justification for not running this group in the remaining stages of the experiment.

Group A required significantly more sessions and trials to reach stage 2 criterion than group S (all *p*'s < .01), as well as Group Y (though these comparisons failed to reach significance, Sessions*: p* = .081; Trials: *p* = .059). Group A also made significantly more response omissions, and were slower to make a response than either Group Y (all *p*'s < .001) or Group S (*p*'s < .05). This might suggest a motivational impairment in Group A; however, this group made fewer reward retrieval omissions (all *p*'s < .05) and were just as fast to collect rewards as other groups (see Table 2.1). This pattern of data strongly suggests that it was the short ITI, rather than the automatic initiation of trials, which was affecting training performance; since Group Y only

differed from Group A in the rate of trial presentation, and showed no such performance deficit. In fact, performance in Group A was so impaired it was necessary to add an extra delay to the ITI from session 8 of Stage 2 training, after which performance improved (see Figure 2.6). This delay was set to the average trial initiation latency of Group S on the previous test day (mean across training = 7.2 seconds), which was employed in all subsequent test stages.

	Automatic *(n = 16)*	Self-Initiate *(n = 12)*	Yoked *(n = 7)*	$F_{2,32}$
Sessions to criterion	8.0 ± 0.8	4.7 ± 0.6	5.3 ± 0.7	**6.39****
Trials to criterion	573.1 ± 56.5	311.8 ± 35.9	350.0 ± 30.6	**8.61*****
Response omissions	25.6 ± 1.1	17.0 ± 1.3	16.7 ± 1.3	**22.9*****
Response latency (s)	8.4 ± 0.2	7.6 ± 0.3	6.6 ± 0.3	**11.05*****
Retrieval omissions	3.4 ± 0.3	5.7 ± 0.6	6.0 ± 1.3	**6.37****
Retrieval latency (s)	2.4 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	1.35

Table 2.1 *Results of one-way ANOVA comparing mean (±SEM) performance across trial initiation conditions during Stage 2 training. Significant differences highlighted in bold. Trials and retrieval omissions data were log10 transformed due to unequal variances (Means and SEM displayed are untransformed).* ****p < .05, ** p < .005, *** p ≤ .001***

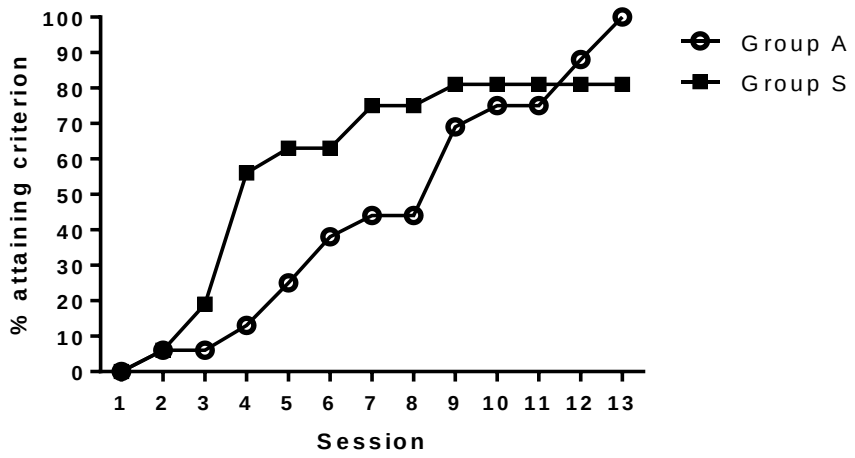

Figure 2.6 *Acquisition curves for Stage 2 training for Group A (automatic) and Group S (self-initiated). Impaired acquisition can be seen within Group A until a trial initiation delay is introduced from session 8. Note: Three animals from Group S failed to reach criterion, maximum acquisition rate for this group is 80%.*

Drug-free baseline performance (Group A/Group S):

There were no baseline differences in performance between trial initiation conditions (Group A/S) or drug treatment groups (Vehicle/SB242084) during Stage 1 training, and no significant interaction effects (data not shown).

During Stage 2 of training, the differences in performance between Group A and Group S seen when comparing performance with Group Y were replicated. Group A required significantly more sessions and trials to reach criterion, responded more slowly, and made more response omissions than Group S; but made fewer retrieval omissions (see Table 2.2). In addition, there was one marginally significant baseline difference in performance between drug groups during (drug-free) stage 2 training, with animals subsequently assigned to receive SB242084 treatment making significantly more reward retrieval omissions than controls (see Table 2.2).

There were no further significant differences in performance between animals subsequently assigned to Vehicle/SB242084 treatment, and no significant interaction effects. Within Group S, there were also no baseline differences between drug-treated animals and controls for the number of trial initiation omissions to criterion (Vehicle: 243.4 ± 74.4; SB242084: 366.8 ± 77.3; t_{10} = -1.12, p > .05) or the latency to initiate a trial (Vehicle: 7.18 ± 0.14; SB242084: 7.18 ± 0.15; t_{10} = 0.01, p > .05).

	Group A		Group S		Condition	(Drug)	Condition x (Drug)
	Vehicle (n = 8)	SB242084 (n = 8)	Vehicle (n = 7)	SB242084 (n = 5)	*F(1, 24)*	*F(1, 24)*	*F(1, 24)*
Sessions to criterion	7.1 ± 1.0	8.9 ± 1.2	4.0 ± 0.6	5.6 ± 1.1	***10.13****	*3.05*	*0.07*
Trials to criterion	507.5 ± 74.5	638.8 ± 83.1	267.9 ± 32.0	373.2 ± 68.9	***13.08*****	*3.21*	*0.04*
Response omissions	24.1 ± 1.7	27.0 ± 1.1	17.0 ± 1.1	17.0 ± 2.8	***25.99*****	*0.75*	*0.73*
Response latency (s)	8.1 ± 0.2	8.7 ± 0.3	7.8 ± 0.3	7.2 ± 0.6	***6.74****	*<0.01*	*2.91*
Retrieval omissions	2.9 ± 0.5	3.8 ± 0.3	5.1 ± 0.5	6.7 ± 1.2	***17.45*****	***4.45****	*0.53*
Retrieval latency (s)	2.3 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.6 ± 0.2	*0.75*	*1.53*	*0.25*

Table 2.2: *Results of two-way ANOVA comparing mean (±SEM) performance across trial initiation conditions and drug-treatment groups during (drug-free) Stage 2 training. Significant differences highlighted in bold. Sessions and trials to criterion data were log10 transformed to correct for unequal variances (Means and SEM displayed are untransformed).* ***p < .05, ** p < .005, *** p ≤ .001**.

Acquisition (drug-free):

During drug-free acquisition of the spatial discrimination, the pattern of responding between Group S and Group A differed from that seen during training. There was no longer a performance impairment on the number of sessions or trials (either correct or incorrect) taken to reach criterion for Group A (see Table 2.3), suggesting that the introduction of a longer ITI was successful at alleviating the deficit seen earlier during training. Additionally, animals in Group A were now faster than Group S to make a response, but slower to retrieve rewards. No other differences emerged between conditions, no baseline differences between drug groups were evident at this stage of testing, and there were no interaction effects (see Table 2.3).

	Group A		Group S		*Condition*	*Drug*	*Condition x Drug*
	Vehicle (n = 8)	SB242084 (n = 8)	Vehicle (n = 7)	SB242084 (n = 5)	*F(1, 24)*	*F(1, 24)*	*F(1, 24)*
Sessions	3.38 ± 0.3	3.0 ± 0	3.0 ± 0	3.0 ± 0	*1.47*	*1.47*	*1.47*
Trials	112.5 ± 17.1	86.3 ± 17.1	70.7 ± 18.3	78.0 ± 21.6	*1.80*	*0.26*	*0.81*
Correct	77.5 ± 40.1	63.13 ± 16.66	50.71 ± 9.64	56.8 ± 13.03	*3.08*	*0.19*	*1.17*
Incorrect	27.0 ± 27.95	19.0 ± 14.47	14.0 ± 7.37	16.2 ± 12.79	*1.28*	*0.17*	*0.53*
Response omissions	8.0 ± 14.20	4.13 ± 6.56	6.0 ± 9.0	5.0 ± 6.78	*0.02*	*0.40*	*0.14*
Response latency (s)	5.55 ± 1.51	4.59 ± 1.04	6.49 ± 1.42	5.91 ± 1.5	***4.66****	*2.16*	*0.13*
Retrieval omissions	5.88 ± 7.9	4.38 ± 7.19	5.57 ± 6.24	4.6 ± 5.18	*<0.01*	*0.22*	*0.01*
Retrieval latency (s)	2.31 ± .35	2.31 ± .34	2.08 ± .23	1.98 ± .12	***6.25****	*0.19*	*0.17*

Table 2.3: *Mean (±SEM) number of sessions, trials (correct and incorrect), response omissions and retrieval omissions made to criterion during drug-free acquisition of spatial discrimination, across trial conditions (Group S/Group A) and drug treatment groups (Vehicle/SB242084), as well as mean latency (s) to make a response and retrieve rewards.* ****p < .05****.*

Retention (drug-free):

All animals reached criterion within 1 session during each retention test (1, 2, 3). Because there was no effect of retention test stage on performance, this factor was removed from analyses, and average retention performance was analysed using 2 (Condition: Group A/S) x 2 (Drug: Vehicle/SB242084) independent measures ANOVA. There were no significant performance differences between drug-treatment groups. Though incorrect responses were very low, Group A did make significantly more incorrect responses than Group S (Group A = 5.1 ± 2.2; Group S = 2.7 ± 2.5; $F_{1,24}$ = 6.78, p < .05). There were no further significant effects of initiation condition, or any interaction effects (data not shown).

Reversal:

SB242084-treated animals showed a significant reversal impairment relative to vehicle-treated controls across both trial initiation conditions, requiring more sessions and trials (both correct and incorrect) to reach criterion (see Table 2.4 and Figure 2.7a-d). They were also significantly faster to make a response and retrieve rewards than controls (see Table 2.4 and Figure 2.7g & h). Although omissions were very low for all animals throughout reversal testing, drug-treated animals made fewer response omissions than controls (see Table 2.4 and Figure 2.7e), though this effect just failed to reach significance ($p = .053$).

Trial initiation condition also affected performance, with animals under automatic conditions making more correct trials to criterion (see Table 2.4 and Figure 2.7c & d), and responding faster (see Table 2.4 and Figure 2.5g) than those under self-initiated conditions.

There was little evidence of a difference in performance between drug-treated animals across different trial initiation conditions however; with the exception that SB242084-treated animals made more retrieval omissions than controls under automatic conditions ($F_{1, 24} = 5.60$, $p < .05$), but not under self-initiated conditions ($F_{1, 24} = 2.35$, $p > .05$) (see Table 2.4 and Figure 2.7f). Performance across serial reversal tests did not differ, and there were no further two or three way interaction effects.

Within the self-initiated condition, trial initiation omissions did not differ across drug groups ($F_{1,10} = 0.05$, $p > .05$), and there was no effect of reversal stage ($F_{2,20} = 0.56$, $p > .05$) or an interaction effect ($F_{2,20} = 0.16$, $p > .05$). Trial initiation latency also did not differ between drug-treatment groups in this condition (Vehicle: 5.6s ± 0.3; SB242084: 5.2s ± 0.4; $F_{1,10} = 0.61$, $p > .05$), and there was no effect of reversal test ($F_{2,20} = 2.99$, $p > .05$) or interaction ($F_{2,20} = 0.69$, $p > .05$).

	Drug	Condition	Drug x Condition	Reversal test	Reversal test x Drug	Reversal test x Condition	Reversal test x Drug x Condition
	$F_{1, 24}$ (F2,20)	$F_{1, 24}$	$F_{1, 24}$	$F_{2, 48}$	$F_{2, 48}$	$F_{2, 48}$	$F_{2, 48}$
Sessions to criterion	***6.95****	*0.12*	*0.64*	*3.09*	*0.71*	*2.73*	*1.37*
Trials to criterion	***6.70****	*0.21*	*0.59*	*3.05*	*0.58*	*2.89*	*1.49*
Correct to criterion	***6.91****	***4.50****	*<0.01*	*1.62*	*0.96*	*1.53*	*0.21*
Incorrect to criterion	***8.56*****	*2.56*	*2.48*	*2.28*	*0.03*	*1.98*	*2.66*
Response omissions	***4.16~***	*2.08*	*3.09*	*0.03*	*0.22*	*0.53*	*0.75*
Response latency (s)	***28.53*****	***4.72****	*1.04*	*2.88*	*0.54*	*0.24*	*0.98*
Retrieval omissions	*0.14*	***25.31*****	***7.30****	*0.75*	*0.23*	*2.47*	*0.13*
Retrieval latency (s)	***5.71****	*1.36*	*0.85*	*1.44*	*3.11*	*0.99*	*0.33*

Table 2.4. *Results of 2 (Drug: Veh/SB) x 2 (Condition: Group A/S) x 3 (Reversal Test 1, 2, 3) mixed ANOVA comparing reversal performance. Retrieval omissions and response omissions data were Log10 transformed to correct for violation of normal distribution.* ****p < .05, **p <.01, ***p<.001***.

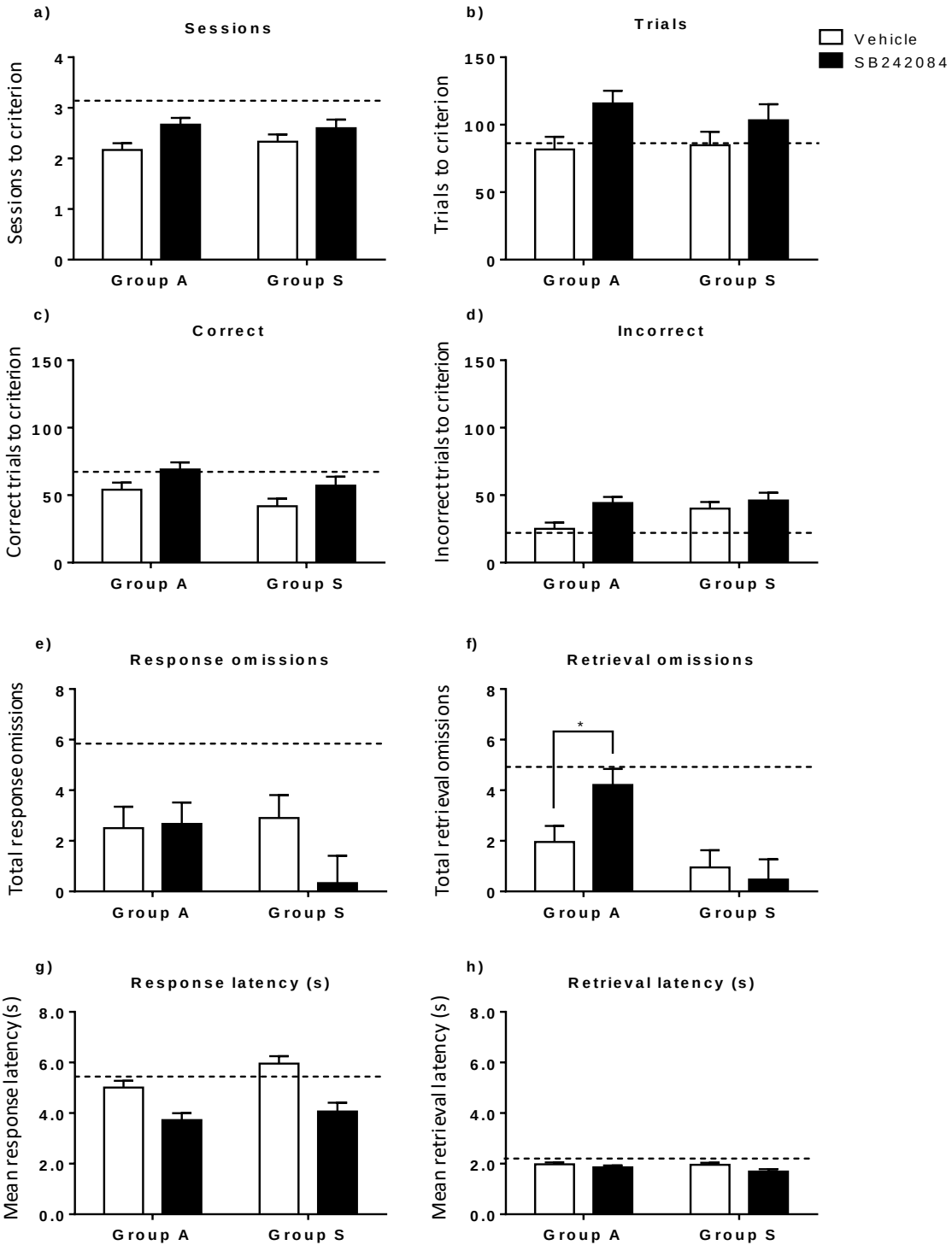

Figure 2.7. Mean performance across drug-treatment group (Veh/SB) and trial initiation condition (Group A/S) across Reversal Test 1-3. a) *Sessions to criterion, significant main effect of drug treatment ($p < .05$).* ***b)*** *Trials to criterion, significant main effect of drug treatment ($p < .05$).* ***c)*** *Correct to criterion, significant main effect of drug treatment ($p < .05$) and initiation condition ($p < .05$).* ***d)*** *Incorrect trials to criterion, significant main effect of drug treatment ($p < .01$).* ***e)*** *Response omissions to criterion, borderline significant effect of drug treatment ($p = .053$).* ***f)*** *Retrieval omissions to criterion, significant main effect of initiation condition ($p < .001$), and drug x initiation condition interaction ($p < .05$).* ***g)*** *Response latency, significant main effect of drug ($p < .001$) and initiation condition ($p < .05$).* ***h)*** *Reward retrieval latency, significant main effect of drug ($p < .05$). Dashed line represents mean acquisition performance across groups. Results quoted are derived from 2 x 2 x 3 ANOVA with reversal test as a repeated measures factor.*

2.2.3. Discussion:

Although the present study reveals significant performance differences under automatic and self-initiated trial conditions, there is little evidence that the behavioural requirement to initiation new trials significantly mediates the relationship between 5-HT_{2C}R blockade and performance on a reversal learning task. Instead, the evidence shows a significant detrimental effect of SB242084 treatment on reversal learning across both trial initiation conditions, consistent with findings reported by Pennanen et al. (2013) following genetic deletion of the 5-HT_{2C}R. Drug-treatment also led to animals in both conditions being more likely to make a response, and to respond more quickly than vehicle-treated controls, consistent with reports from Nilsson et al. (2012), which could therefore reflect motor impulsivity effects of drug treatment. The current findings do support the assertion that shorter inter-trial intervals (ITIs) can have a detrimental effect on learning however, with evidence that an ITI of 11 seconds led to a performance deficit during training relative to a control group with an average ITI of approximately 18 seconds. The relevance of these findings in relation to prior tasks and outcomes will be discussed.

A considerable difference in performance between automatic and self-initiated trial conditions emerged during training to nosepoke for food rewards, with animals in the automatic condition taking nearly twice as long to reach criterion. Because performance in the yoked control group did not differ from animals in the self-initiated condition, the cause of this deficit could be localised to the rate of stimulus presentation, rather than to the use of automatic trials. Introducing an additional delay which essentially served to lengthen the ITI, was effective at removing this deficit. The need to introduce this initiation delay during training means the effect of the shorter ITI on acquisition and reversal of the spatial discrimination could not be verified, but the training deficit does suggest that the short ITI used by Pennanen et al. (2013), though unlikely to account for differences in 5-HT_{2C}R-related effects reported in previous studies, may have made the task comparably more difficult, presumably by increasing attentional demands.

During acquisition and reversal of the spatial discrimination, when the performance deficit caused by the shorter ITI had been eliminated, animals tested under automatic conditions did show some performance differences relative to self-initiating animals, which could therefore be attributed specifically to automatic trial initiation conditions. Automatic conditions allowed for faster responses to the stimuli than self-initiating conditions during both acquisition and reversal. This was predicted on the basis that animals in the automatic condition were more likely to be nearer the stimuli between trials, since they were not required to make a head entry

into the magazine on the opposite chamber wall. This might also explain why automatic conditions allowed animals to make more correct responses during reversal, if they were able to wait near the new correct location between trials, and/or to more successfully hold the correct location in short-term memory; though the overall number of trials and sessions to criterion did not differ between groups.

The automatic condition also resulted in slower retrieval of rewards from the food magazine during acquisition testing. This could support the hypothesis that this condition result in lower motivation for rewards due to the low effort involved in acquiring them, as has previously been demonstrated following low-effort training conditions (Clement et al. 2000; Johnson & Gallagher 2011), however, there was no difference in speed of reward retrieval between groups during reversal. Though it is possible that the change in reward contingency during reversal renewed motivation in this group, faster reward-retrieval in the self-initiate group during acquisition is more likely to reflect the fact that animals in this group were initially more drawn to or familiar with the food magazine location, given its additional role in initiating trials.

Despite evidence for some difference in performance under automatic and self-initiated trial conditions, there was little evidence that these conditions played a role in mediating the effect of 5-HT_{2C}R blockade during reversal learning, which might have accounted for previous outcome discrepancies. Drug-treatment caused a significant impairment in reversal performance across both trial initiation conditions. Although observation of the data shows that the impairment was generally larger under automatic conditions, there were no significant interactions between drug treatment and initiation condition, except in relation to the number of reward retrieval omissions made. SB242084-treated animals made significantly more reward retrieval omissions under automatic conditions than animals in any other group. Omissions can provide a measure of learning in reversal tasks, since they are reported to reduce in line with improvements in performance (Boulougouris et al. 2008; Nilsson et al. 2012), which could suggest a specific learning deficit caused by 5-HT_{2C}R blockade which was only apparent under automatic trial conditions. Given that this effect was not reflected in key reversal performance measures, such as the number of sessions or trials taken to reach reversal criterion, this makes it of limited value in explaining the discrepancy of outcomes in previous studies. Instead, evidence of retarded reversal following SB242084-treatment is consistent with findings reported by Pennanen et al. (2013), suggesting that reduced 5-HT_{2C}R activity can have a negative impact on flexible cognition under certain test conditions. Drug-treatment also led to a general speeding of responding during reversal and a reduction in the number of response omissions,

consistent with the general motor impulsivity effects reported by Nilsson et al. (2012), which demonstrates that the drug was effective in the current context.

Although the overall effect of drug-treatment was consistent with the impairment effect reported by Pennanen et al. (2013) in 2CKO mice, the pattern of findings across serial reversals does differ quite considerably however. Pennanen et al. (2013) report a significant difference in performance between 2CKO mice and wildtype controls during the second and third serial reversal tests which was not apparent in the first, interpreted as a failure for 2CKO animals to continually improve responding over successive reversals as controls had (termed the 'serial reversal effect'). However, in the current task, performance in both groups did not change over successive reversals, and the drug-related performance deficit was apparent from the first reversal. This could be related to differences in within-session and between-session reversal testing, however Nilsson et al. (2013) also report this serial reversal effect in control animals using a between-session design. Given that control animals showed no such performance improvement in the current task, this could suggest the existence of floor effects.

Comparing performance during the first reversal test across control groups, animals in the current study reversed within an average of just over 80 trials, which is slightly lower than that observed within the Pennanen et al. (2013) study, where the control group required approximately 110 trials to reach reversal criterion (albeit with a lower criterion threshold of 8 consecutive correct, rather than 9/10 correct responses in any 10 trial bin). It is also considerably lower than that reported in both Boulougouris et al. (2008, 2010) experiments, of 130-170 trials to reach criterion, and in the Nilsson et al. (2012) study, where controls required more than twice as many trials to reach criterion as in the current design, at approximately 200 trials to criterion (all three employing the same criterion threshold of 9/10 correct in any 10 trial bin). Comparing acquisition to reversal performance within studies, control animals in both the Pennanen et al. (2013) and Nilsson et al. (2012) studies required more trials to reach reversal criterion than were needed to acquire the spatial discrimination (acquisition data is not provided by Boulougouris et al. 2008, 2010); whilst in the present study, animals actually required marginally fewer. The ease with which controls performed the first reversal in the current study suggests they might already have been performing at optimal levels, which might explain why a serial reversal effect was not seen.

The reason for this performance advantage is not clear, given that most other task parameters were set to an average level derived from previous studies, though it could suggest that there is considerable variance in reversal performance capability within the general

population. Evidence that the two experiments which report a retarding effect of reduced 5-HT_{2C}R activity were also those reversal tasks which were most readily solved, whilst those reporting a beneficial effect took considerably longer, could also suggest a significant interaction with task difficulty. To further examine this possibility, it may therefore be necessary to employ a more challenging reversal learning task, to examine the effect of SB242084 treatment against a higher performance baseline.

The current results provide little support for the assertion that trial initiation requirements mediate the role of $5HT_{2C}$ receptor blockade in reversal learning, and provide additional evidence that this manipulation can have a negative impact on performance. However, the relative difficulty of the reversal tasks used across studies appears to vary considerably, with the current task proving particularly easy to solve. This might help to explain previous result discrepancies, with the possibility that there is an interactive effect of 5-HT_{2C}R manipulations with reversal task difficulty. Future studies might therefore require use of a more challenging reversal task in order to provide a higher baseline for which to compare drug treatment effects. Understanding under what conditions 5-HT_{2C}R manipulations can be detrimental, as well as beneficial, to reversal learning will be critical in understanding the precise role these receptors play in cognitive flexibility, and should be a key focus of research.

CHAPTER 3

THE EFFECT OF 5-HT_{2C}R ANTAGONISM IN PROBABILISTIC REVERSAL LEARNING (PRL) TASKS

3.1. Introduction:

Although the use of simple reversal learning tasks in animals has been a useful tool for modelling human cognitive flexibility, these tasks typically employ 100 per cent accurate feedback; rewarding every correct and punishing every incorrect response made. Environmental stimuli are rarely linked with such certainty to reward or punishment in the real world. Therefore, for an organism to maintain successful goal-directed behaviour it must not only be able to adapt responses to stable changes in environmental contingencies, but must also maintain successful strategies in the face of short-term and non-deterministic fluctuations in expected outcome. Probabilistic reversal learning (PRL) tasks, widely adopted in human clinical settings (e.g. Budhani et al. 2006; Cools et al. 2001; Swainson et al. 2000), are designed to assess the ability to adapt response-outcome associations in the face of occasionally misleading information. In addition to accurate response feedback, spurious error and correct feedback is also experienced at a specified probability for correct and incorrect responses, respectively. In animal models, this translates into a further subset of correct responses being punished (or non-rewarded), and incorrect responses being rewarded (see Table 3.1).

Unlike deterministic reversal learning tasks, which can be solved using a trial-by-trial strategy of consistently staying with the same response option on the next trial when rewarded (win-stay), and shifting to the alternate response when punished (lose-shift), successful performance in probabilistic tasks necessitates the adoption of a model-based strategy - acquiring a 'response set' that is rewarded on a high proportion of occasions. This requires the ability to integrate reinforcement history over multiple trials as well as to regulate responding to local reinforcement (Bari & Robbins 2013). Such tasks not only offer far better ecological validity and translational value than deterministic reversal tasks, but additionally provide a measure of sensitivity to reward and negative feedback not offered by conventional tasks. This is achieved by examining patterns of behaviour; assessing the probability of win-stay and lose-shift behaviour in response to accurate and misleading feedback trials (see Table 3.1).

Response trial *n*	A Correct				B Incorrect			
Trial outcome	Win (Rewarded) $p = 0.8$		Lose (Punished) $p = 0.2$		Lose (Punished) $p = 0.8$		Win (Rewarded) $p = 0.2$	
Feedback accuracy	Accurate		Misleading		Accurate		Misleading	
Response trial *n*+1	Stay (A)	Shift (B)	Stay (A)	Shift (B)	Shift (A)	Stay (B)	Shift (A)	Stay (B)
Response Pattern	Correct Win-Stay	Correct Win-Shift	Correct Lose-Stay	Correct Lose-Shift	Incorrect Lose-Shift	Incorrect Lose-Stay	Incorrect Win-Shift	Incorrect Win-Stay
Feedback sensitivity	Reward Sensitivity		Negative Feedback Sensitivity		Negative Feedback sensitivity		Reward Sensitivity	

Table 3.1: *Representation of possible responses and outcomes for a two-choice probabilistic reversal learning (PRL) task set to a probability of inaccurate feedback of p = 0.2, including all possible patterns of responding on the subsequent trial, and the type of feedback sensitivity it is proposed to measure. Once animals demonstrate successful acquisition of the task, the correct (A) and incorrect (B) responses are reversed (A = incorrect, B = correct).*

Examining the immediate effects of reward and punishment on subsequent choices alongside longer-term behavioural flexibility allows for greater dissection of the underlying components thought to be involved in real-world flexible cognition; recognising affective requirements beyond pure executive function. Furthermore, given that differences in sensitivity to reward and punishment are seen in many pathological conditions, such as anxiety, depression, attention deficit hyperactivity disorder (ADHD) and oppositional defiance disorder (Matthys et al. 2004; Wilbertz et al. 2012), use of PRL tasks in animal models may be more effective in uncovering clinically relevant differences in performance.

Regions within the orbitofrontal cortex (OFC) have been shown to play a key role in supporting flexible cognition (e.g. Butter 1969; Dias et al. 1996; Iversen & Mishkin 1970; Jones & Mishkin 1972). These same regions are believed to facilitate the comparison of choice-options varying along parameters such as delay, magnitude and probability (e.g. Kable & Glimcher 2009); as well as in assigning credit to a particular stimulus (Walton et al. 2011), which becomes more difficult with increasing variability of choice-outcome histories. As such, the stochastic environment of PRL tasks may render any effect of experimental manipulations on cognitive flexibility particularly evident, making PRL tasks an especially sensitive tool. This has been clearly demonstrated with the implementation of a spatial maze reversal learning task which compared a deterministic (100% accurate) feedback condition to a probabilistic feedback condition (with a probability of misleading feedback of $p = 0.2$) in C57BL/6J and BTBR T+tj/f mice, which are used

as an animal model for the restricted interest features of Autistic Spectrum Disorders. This allowed the researchers to uncover a strain-dependent difference in cognitive flexibility not apparent under deterministic conditions, and which had therefore never previously been reported, with BTBR mice demonstrating a deficit in maintaining the correct response strategy during reversal (Amodeo et al. 2012). Similarly, Lawrence et al. (1999) report a deficit in reversal learning in Huntington's Disease patients when tested on a probabilistic, but not a deterministic task. This increased sensitivity may make PRL tasks especially useful for discriminating the effects of serotonergic manipulations on flexible cognition, where research employing deterministic reversal tasks have reported outcome discrepancies. Findings presented in Chapter 2 suggested that the deterministic reversal task used was readily solved, creating problems with potential floor effects and a high-performing control group as the baseline to compare drug performance. The use of a PRL task may provide a more challenging test, as well as helping to distinguish any potential role of 5-HT_{2C}Rs in affective processing not possible under deterministic conditions, and which could potentially contribute to reversal performance.

Many experimenters have proposed a role for the serotonergic systems in mediating aversive signalling and anxiety-induced avoidance (Deakin 1983, 2013, 2014; Deakin & Graeff 1991; Faulkner & Gray 1982; Lowry 2002; Paul & Lowry 2013; Tye et al. 1977), which is consistent with the observed role of serotonergic signalling in a range of mood and anxiety disorders (Anderson et al. 1990; Blier & Montigny 1999; Deakin 1991; Young et al. 1985), and with empirical evidence of altered sensitivity to threat and negatively-valenced stimuli following serotonergic alterations (Cools et al. 2005; Evers et al. 2006; van der Veen et al. 2007). Correspondingly, a change in negative feedback sensitivity has been reported in probabilistic feedback tasks following manipulations of serotonergic function, as reviewed in Chapter 1. For example, inhibition of central 5-HT reuptake by acute challenge with citalopram is shown to enhance the ratio of lose-shift behaviour on correct trials in a PRL task in healthy human subjects, as well as impairing reversal performance (Chamberlain et al. 2006), an effect which has been replicated using a low dose (1mg/kg) of the drug in rats (Bari et al. 2010). This low-dose effect is thought to be mediated by activity at 5-HT_{1A} auto receptors in the raphe nuclei, which act to attenuate 5-HT system activity (Sprouse & Aghajanian 1987). Reduction of central 5-HT activity through acute tryptophan depletion (ATD) has also been shown to potentiate neural activity in the dorsomedial PFC in response to negative feedback in the PRL task in humans, though without affecting behavioural measures of reversal performance (Evers et al. 2005). By contrast, increasing 5-HT function through a single high dose (10/mg/kg) of citalopram has been shown to have the opposite effect on behaviour, reducing lose-shift ratios and

increasing the number of reversals completed (Bari et al. 2010). Although chronic citalopram treatment and long-term 5-HT depletion by 5,7-DHT infusions have been found to affect reward rather than punishment sensitivity (Bari et al. 2010), this difference may be attributable to homeostatic changes following long-term manipulations of the 5-HT system, which might alter the coupling of the 5-HT system with DA. Overall, such findings support suggestions that 5-HT acts in mutual opponency to DA, signalling a negative reward-prediction error (Boureau & Dayan 2011; Cools et al. 2011; Daw et al. 2002).

No experiment has to date examined the effect of specific manipulations of 5-HT_{2C}R activity on performance in the PRL task, as far as I am aware. Although activity at 5-$HT_{2/1C}$ receptors is proposed to play a specific role in mediating the avoidance reaction to negative stimuli (Deakin & Graeff 1991), given that 5-HT_{2C}Rs also exert a direct inhibitory influence on DA neurons (Di Giovanni et al. 1999; Di Matteo et al. 1999, 2001), it is not clear precisely what effect SB242084 might have on sensitivity to reward and/or punishment within this task, given the established role of DA in appetitive motivation (Schultz et al. 1997). Further complicating the picture, there are recent reports that 5-HT depletion in the amygdala and OFC impairs PRL performance in marmosets by increasing responsivity to misleading feedback more generally, following both probabilistic reward and punishment (Rygula et al. 2015b). This impaired ability to inhibit responses to immediate, local feedback fits more closely with the proposed role of 5-HT in inhibitory response control (e.g. Harrison et al. 1997; Harrison et al. 1999; Winstanley et al. 2004a), for which signalling at the 5-HT_{2C}R is known to be important (e.g. Robinson et al. 2008; Winstanley et al. 2004b). Many of the PRL studies described above report only patterns of responding on correct trials, and may therefore have missed an effect of misleading wins on incorrect trials. Given the consistently reported effects of SB242084 in reducing omissions and response latencies during reversal, it will be important to rule out explanations centred on changes in inhibitory responding, by exploring response patterns to both correct and incorrect trials. The use of probabilistic tasks can therefore clearly contribute to a better understanding of the role of 5-HT_{2C} receptors in affective processing, cognitive flexibility and/or response inhibition, by assessing patterns of responding to different trial outcomes. The increased difficulty of these tasks could also help to elucidate outcome discrepancies in prior research, as well as offering findings more directly relevant to human behaviour. What is less clear however, is whether rodents are capable of acquiring the 'response set' necessary to maintain accurate reward prediction in PRL tasks; an assumption which underlies the human PRL test (Evers et al. 2005; Murphy et al. 2003) and is therefore critical for determining the relevance of the model.

Humans are shown to demonstrate a fairly consistent 'stay' strategy on correct trials irrespective of reward or punishment, with win-stay ratios close to 1.0 and lose-shift ratios around 0.1 (Taylor Tavares et al. 2008). This level of response pattern accuracy, though unlikely to be fully replicated in animal models, has reportedly been difficult to approximate in the few rodent studies that have attempted PRL tasks to date. Whilst the results of the Amodeo et al. (2012) study in different strains of mice show the potential utility of the spatial maze PRL task in rodents, Ineichen et al. (2012) point to data from this experiment which suggests that subjects were unable to maintain accurate reward prediction, since the ratio of win-stay behaviour on correct trials was only 0.5, i.e. chance level. Employing the same level of probabilistic feedback ($p = 0.2$), but in an operant visuospatial design, Bari et al. (2010) report evidence which suggests rats may be capable of more accurate reward-prediction in PRL tasks, accomplishing substantially higher correct win-stay ratios of around 0.8. Given this finding, Ineichen et al. (2012) chose to remove the rewarded incorrect response (RIR) element of the task in an attempt to establish an operant version of the PRL task in mice, hypothesising it would increase the cognitive demands to too high a level. They employed the same within-session reversal design as previously used (Bari et al. 2010), assessing the number of reversal switches an animal could complete within a single test session; and animals were required to reach a criterion of 8 consecutive correct responses before contingencies were reversed. They manipulated the probability of punished correct responses (*p*PCR) to 0, 0.1, 0.2 and 0.3, to assess its effect on the ability to maintain accurate reward prediction, whilst incorrect responses were consistently punished. As *p*PCR increased they observed a significant monotonic decrease in correct win-stay behaviour and number of reversals completed within a session; still, the ratio of win-stay responses closely tracked the actual changes in the likelihood of receiving reward at each stage. This was taken as evidence that the removal of rewarded incorrect response (RIR) trials had enabled more accurate reward prediction in mice, with animals displaying win-stay behaviour at levels well above the chance levels previously reported (Amodeo et al. 2012), and at levels very similar to that reported in rats when comparing the same probability of misleading feedback (Bari et al. 2010).

These data seemingly advocate the removal of RIR trials when adapting PRL tasks for use in mice. Although important to consider cross-species differences from an ethological perspective, these considerations must also be balanced against the gain in explanatory power that can be derived from assessing reactions to spurious feedback of both a positive and negative valence. Removal of RIR trials could be problematic for ruling out the contribution of more general changes in response inhibition to task performance following serotonergic

manipulations. Furthermore, the response to misleading reward on incorrect trials is likely to be a more sensitive measure of reward-sensitivity than responses to accurate reward outcomes. Given the potential value of RIR trials, it is therefore vital to ensure such a manipulation is truly necessary for use in mouse models; yet several lines of evidence suggest that the difficulties observed in mice in the previous task may have been unrelated to this particular design difference. The task requirements for successful completion of the spatial maze task employed by Amodeo et al. (2012) in mice are likely to be quite different to those involved in the operant tasks employed by both Ineichen et al. (2012) and Bari et al. (2010), and may place a higher cognitive demand on subjects which contributed to their poorer performance. The criterion for successful acquisition of this maze task and subsequent reversal was also lower, with mice needing to make 6, rather than 8, consecutive correct responses. This lowers exposure to misleading feedback, and could allow for completion of the task without necessitating a successful model to deal with uncertain outcomes, i.e. the task could be completed without win-stay ratios needing to increase above chance level. The comparable win-stay performance of rats and mice in the Bari et al. (2010) and Ineichen et al. (2012) tasks, rather than being evidence of increased response accuracy in mice following the removal of RIR trials, might instead be attributed to a similarity of performance across species on a comparable task, regardless of the inclusion or omission of RIR trials. The possibility that mice are capable of completing a PRL task which includes RIR trials therefore requires further exploration.

Ineichen et al. (2012) also reported a significant effect of *p*PCR (0, 0.1, 0.2, 0.3) on negative feedback sensitivity (NFS), or lose-shift behaviour, on correct trials in their study. However, unlike win-stay behaviour this relationship was not monotonic, with wildtype mice demonstrating a lose-shift ratio at *p*PCR = 0.1 that was significantly above chance level (0.81), whilst it did not differ from chance at *p*PCR = 0.2 or 0.3. Given the scarcity of correct punished trials at *p*PCR = 0.1 this high level of lose-shift behaviour was deemed to reflect accurate punishment expectancy, despite being far higher than the actual chances of receiving punishment at that location. The chance levels of lose-shift behaviour reported at higher levels of *p*PCR were taken as evidence that animals no longer displayed accurate punishment prediction, therefore *p*PCR = 0.1 was the level the authors recommended as optimal for the adaptation of PRL tasks in mice. However, evidence that animals were predominantly adopting a lose-shift pattern of responding at the correct location under this schedule shows they had failed to develop a model-based response strategy that allowed them to deal effectively with misleading punishment. This arguably makes it far from the ideal schedule to allow comparison to human tasks, where the ratio of lose-shift behaviour on correct trials is generally much lower.

Therefore, the reduction in lose-shift behaviour seen at higher values of *p*PCR, despite only reaching chance levels, could be interpreted as evidence of more accurate response strategies which are in fact more comparable to human performance. Although not entirely consistent with evidence of a decrease in win-stay behaviour over increasing levels of *p*PCR, this could suggest that more stochastic conditions can enhance the accuracy of response strategies, at least in response to misleading feedback, despite seemingly increasing the level of task complexity.

This interpretation of the data is supported by findings from a functional magnetic resonance imaging (fMRI) study in humans during performance of a PRL task which manipulated the probability of receiving misleading feedback. A significantly stronger response in the rostral cingulate zone (RCZ) was recorded in response to negative feedback when the *p*PCR was low (0.1), compared to when it was high (0.25) (Jocham et al. 2009). The high probability condition was also found to improve the ability to ignore irrelevant information by reducing sensitivity to local feedback on individual trials, but this consequently made reversal shifts harder to detect. The authors suggest that activity in the human RCZ varies to determine the extent to which the negative reward-prediction error is used to update action values, with more predictable environments promoting greater behavioural adaptation. This reduction in lose-shift behaviour and impairment of reversal performance over increasing levels of misleading feedback closely reflects the pattern of behaviour seen in mice in the Ineichen et al. (2012) study, with the exception that win-stay behaviour was unaffected by the manipulation in human volunteers. Thus, humans and rodents seemingly respond in similar ways to probabilistic outcomes, with higher levels of probabilistic feedback improving the accuracy of response strategies whilst simultaneously impairing the ability to detect reversal shifts.

Nevertheless, evidence that lose-shift responding in mice never reduced below chance levels in the Ineichen et al. (2012) study, even at the highest level of *p*PCR, does suggest that an accurate strategy for dealing with misleading feedback had not fully developed. However, mice in the this study did receive extensive training on a deterministic reversal learning task, both before being allowed to progress to the PRL test stage and during PRL testing, where two PRL tasks and three deterministic reversal tasks were interspersed throughout each of the eight weeks of testing. This could clearly make the acquisition of a successful strategy for dealing with misleading feedback particularly difficult. However, chance-level shifting on PCR trials was also apparent in the vehicle-treated rats of the Bari et al. (2010) study, where no deterministic reversal task was ever employed. The within-session design used in these two tasks might readily account for such findings however, since this design feature renders probabilistic outcomes

indistinguishable from within-session reversal shifts. If the correct response alternates repeatedly within an individual test session, it becomes increasingly more difficult to tell whether losses at the correct location and wins at the incorrect location are a result of probabilistic outcomes that should be ignored as 'noise', or due to yet another reversal of response-outcomes that signals the need to adapt behaviour. Probabilistic outcomes might become more easily distinguishable from the need for behavioural adaptation were animals to be tested across discrete acquisition and reversal sessions, possibly allowing for response strategies which more closely approximate human performance.

A previous study examining probabilistic reversal in rats has employed both a within-session and between-session design to examine the effect of isolation rearing on cognitive flexibility, which included misleading RIR as well as PCR trials (Amitai et al. 2014). Results indicate that during between-session reversal, where animals were required to learn a probabilistic discrimination prior to a separate reversal test stage, all animals successfully increased win-stay and reduced lose-shift behaviour at the correct location across the first three days of reversal. Conversely, they increased lose-shift and decreased win-stay behaviour at the incorrect location, providing evidence they were developing a successful model-based strategy at both locations and in response to both accurate and misleading feedback. Additionally, socially reared animals demonstrated a faster increase in correct win-stay and decrease in incorrect win-stay strategies than isolation-reared animals. However, during the within-session task, animals only managed to display a significant decrease in lose-shift behaviour at the incorrect location across the entire course of reversal testing, and no significant rearing-related differences in response strategies emerged. Although the criterion thresholds did differ slightly between the two tasks, this suggests that within-session reversal was indeed more difficult, and did not allow for easy acquisition of a model-based response strategy to deal with probabilistic outcomes. Mice might therefore be capable of more accurate responses to misleading feedback if a between- rather than within-session PRL design were used.

Probabilistic reversal learning (PRL) tasks can offer many advantages over standard deterministic reversal tasks if they can be successfully employed in rodent models, as evidenced by the discovery of a strain-related difference in performance in mice under probabilistic conditions not previously reported under deterministic conditions (Amodeo et al. 2012). However, current evidence of the ability for rodents, and in particular mice, to demonstrate an accurate model-based response strategy, which approximates that seen in humans, is currently lacking. Of the three PRL tasks that have previously been conducted in rodents to date, one employed a maze design that may have introduced extra cognitive demands and set a criterion

threshold too low to ensure accurate response strategies were necessary (Amodeo et al. 2012), whilst the other two employed a within-session design that may make probabilistic outcomes too difficult to differentiate from reversal shifts (Bari et al. 2010; Ineichen et al. 2012). Given the potential value of including both RIR and PCR trials for discriminating the effects of serotonergic manipulations on reward/punishment sensitivity from effects on response inhibition more generally, experiments presented in the current chapter seek to explore whether an accurate model-based response strategy can be achieved in mice without the need to remove RIR trials, by employing a similar operant task to that used by Ineichen et al. (2012) but using a between-session design. Given the lack of effect of SB242084 on performance under deterministic reversal conditions in the previous chapter, and the possibility that this task was too easily solved, use of a PRL task may also offer improved sensitivity for detecting the effects of this drug on cognitive flexibility, as well as being able to explore any potential effects on reward and punishment sensitivity, where the role of 5-HT_{2C}Rs is currently unclear. Before assessing reversal performance under these conditions however, it will first be necessary to establish whether accurate response strategies can be demonstrated during acquisition of an operant two-choice discrimination, since neither of the previous studies performed in mice (Amodeo et al. 2012; Ineichen et al. 2012) report evidence from this initial acquisition stage. The effect of altering the probability of misleading feedback on performance will also be assessed, in order to establish whether mice are capable of maintaining accurate responding across increasing task complexity, and to help clarify whether more stochastic environments lead to impaired (as suggested by Ineichen et al. 2012) or improved (as suggested by Jocham et al. 2009) acquisition of an accurate model-based response-strategy.

In light of this discussion, this chapter will present the results of three experiments. The first will explore whether mice are capable of successfully acquiring a two-choice visuospatial discrimination on the basis of probabilistic feedback, including both RIR and PCR trials. This experiment will additionally manipulate the probability of misleading feedback to assess its effect on the ability to maintain accurate reward and punishment prediction. The second experiment will seek to establish whether mice can successfully learn to reverse a probabilistic discrimination, as well as exploring whether 5-HT_{2C}R antagonism has any impact on various performance measures, which may help to shed light on previous outcome discrepancies and exclude possible confounding factors. The third experiment will further explore which specific aspects of reversal performance are affected by drug treatment, by isolating the effect of SB242084 treatment in tests of learned non-reward and perseverance under probabilistic conditions.

3.2. EXPERIMENT 1

ACQUISITION OF A PROBABILISTIC SPATIAL DISCRIMINATION IN MICE

In a similar design to that employed by Ineichen et al. (2012), but with the inclusion of rewarded incorrect response (RIR) trials, the current experiment tests the ability for mice to maintain performance during acquisition of a visuospatial two-choice discrimination task across increasing task complexity (increased probability of misleading feedback). Evidence that more stochastic schedules lead to a decrease in sensitivity to immediate trial outcomes (Ineichen et al. 2012; Jocham et al. 2009) means this increase may actually produce more accurate strategies for dealing with misleading feedback, but could also impair the ability to detect a reversal. In order to balance demands for an accurate model-based response strategy at acquisition with the need for an achievable reversal task when response-outcome relationships are later altered, the probability of misleading feedback was therefore modified to levels marginally lower than those tested by Ineichen et al. (2012), to p = 0.10, 0.15 and 0.20. Based on prior studies it was predicted than an increase in the probability of misleading outcomes would lead to reduced sensitivity to misleading feedback, with a decline in lose-shift ratios following a correct response, and in win-stay ratios following an incorrect response.

3.2.1. Materials and methods:

Animals and Design:

This experiment used seven C57BL/6J mice (Charles River, UK) weighing an average of 22.2g (SEM ± 0.8g) at the start of behavioural testing. Housing conditions, behavioural apparatus, and food deprivation procedures were the same as previously stated in Chapter 2. Animals underwent acquisition training on a 100% accurate reinforcement schedule of a two-choice spatial discrimination task (Stage 1: Probability of inaccurate feedback, p = 0), which was identical in design to the training and acquisition stage for Group S in Chapter 2, Experiment 1.

Briefly, after initiating a trial by a head entry into the magazine, two nosepoke holes were illuminated. Correct and incorrect locations were counterbalanced so that the target aperture was located on the right for half the animals and on the left for the other half. Responses at the correct location resulted in delivery of one sucrose pellet reward into the food magazine, whilst responses at the incorrect location resulted in immediate trial termination, illumination of the houselight and omission of reward. Mice were subsequently tested on three further stages, with increasing levels of probabilistic punished correct responses (PCR) and rewarded incorrect responses (RIR): Stage 2, p = 0.10 (10% PCR and RIR); Stage 3, p = 0.15 (15% PCR and RIR); and Stage 4, p = 0.20 (20% PCR and

RIR). Animals were tested for 60 trials a day (excluding trial initiation omissions), over five consecutive days at each stage.

Measures and Statistical Analyses:

Average performance across the final 3 days of each test stage were analysed, where behaviour was most stable and therefore most representative of the typical level of learning possible. Response accuracy was assessed as the percentage of correct responses made, excluding response omissions (percent correct = [total correct trials / total correct + incorrect trials] x 100). In order to establish if a model-based strategy had been successfully acquired, patterns of responding were assessed according to the outcome of the preceding trial. Any complete trial in which an animal made a response that was immediately followed by another complete trial (i.e. not followed by an omission) was included for analysis. There were insufficient incorrect trials to permit analysis of patterns at this location, so analysis was restricted to correct trials only, with the conditional probability of staying at the this location following a reward (win-stay) and shifting following a punishment (lose-shift) expressed as ratios (win-stay ratio = total win-stay trials/total rewarded trials; lose-shift ratio = total lose-shift trials/total punished trials). Due to the absence of punished-correct responses (PCR) during Stage 1 (p = 0), lose-shift analysis was restricted to the three probabilistic stages (p = 0.10, 0.15, 0.20). Response accuracy and response strategies were compared across schedules using repeated measures ANOVA, with post hoc pairwise comparisons to identify where significant differences lie. To limit artificially imposed ceiling or floor effects in percentage and ratio data, all data (throughout the chapter) were subject to arcsine-SQRT transformation. Where Mauchly's test for sphericity violated the equality assumption, a Greenhouse-Geisser correction was applied to adjust the degrees of freedom accordingly.

3.2.2. Results:

Results of repeated measures ANOVA show that mice continued to respond accurately despite an increase in the probability of inaccurate feedback. Correct responses were high throughout, and there was no significant effect of test stage on percent of correct trials completed ($F_{3, 18} = 1.77$, $p > .05$, see Figure 3.1). Analysis of response pattern data at the correct location shows that the increase in inaccurate feedback did not degrade responding on accurate feedback trials, with no change in win-stay ratios across test stages ($F_{1.4, 8.5} = 0.26$, $p > .05$), and subjects were consistently very likely to stay at the same location following a win (see Figure 3.2a). However, there was a significant difference in responding to misleading punishment across probabilistic test stages. As the probability of receiving inaccurate feedback increased, animals became less likely to shift from the correct location on the subsequent trial despite a loss ($F_{2, 12} = 9.53$, $p < .01$, see Figure 3.2b). Pairwise comparisons show that this decline in lose-

shift behaviour was significant between Stage 2 and 3, and Stage 2 and 4 (all *p*'s < .05), but the reduction between stage 3 and 4 was not significant. Although there were insufficient incorrect trials after Stage 1 to permit analysis of response strategies across test stages, animals demonstrated accurate responding to punishment under the deterministic conditions of stage 1, with a very high average incorrect lose-shift ratio of 0.93 (SEM = 0.05).

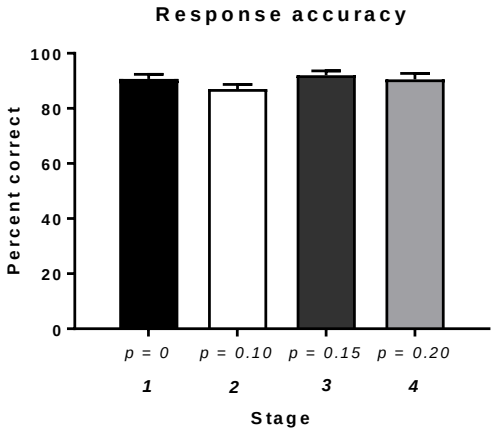

Figure 3.1: *Mean (+/-SEM) accuracy of responding on the spatial discrimination task across increasing levels of rewarded incorrect response (RIR) and punished correct response (PCR) outcomes (Stage 1: p = 0; Stage 2: p = 0.10; Stage 3: p = 0.15; Stage 4: p = 0.20). Accuracy was calculated as percentage of all trials (excluding response omissions) in which a correct response was made.*

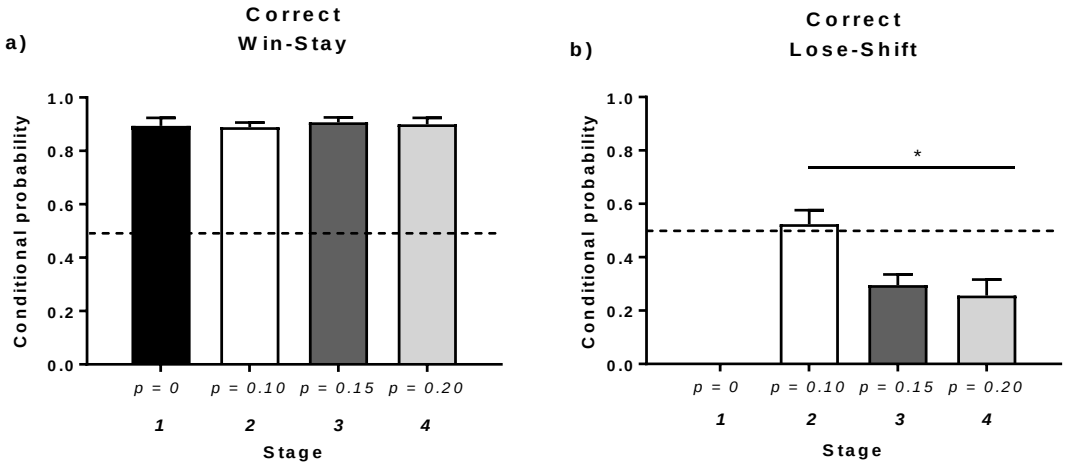

Figure 3.2: *Response strategies of animals at the correct location across increasing probabilities of inaccurate feedback (Stage 1: p = 0; Stage 2: p = 0.10; Stage 3: p = 0.15; Stage 4: p = 0.20).* ***a)*** *Win-stay behaviour: Ratio of all correct rewarded trials (wins) followed by another response in the same location* ***b)*** *Lose-Shift: Ratio of all correct punished trials (losses) followed by a shift in responding to the alternate location. The increase in inaccurate feedback probability had no effect on win-stay behaviour, which assesses responses to accurate reward; but significantly affected lose-shift behaviour (*p < .01) which assesses responses to misleading punishment. Dashed line represents chance level performance.*

3.2.3. Discussion:

Results of Experiment 1 demonstrate that the introduction of probabilistic feedback and its increase over successive test stages had no effect on performance accuracy, which was maintained at very high levels throughout. Therefore, accurate performance of an operant spatial discrimination appears to be possible in mice, even when exposed to rewarded incorrect responses (RIR), and when misleading feedback was set to a probability as high as $p = 0.2$. Effects of the increase in stochasticity on response patterns were also in line with that previously reported in both mice (Ineichen et al. 2012) and humans (Jocham et al. 2009) during reversal testing, with evidence of reduced sensitivity to misleading feedback (on correct trials) as the likelihood of experiencing it increased. This reflects the development of a successful model-based response strategy, rather than a trial-by-trial 'win-stay/lose-shift' strategy; demonstrating equivalence to human strategies under probabilistic conditions.

During the first stage of testing ($p = 0$) where only incorrect responses were ever punished, losses accurately signalled a response error and the need to switch responding, and lose-shift behaviour on incorrect trials was correspondingly high. Following the introduction of probabilistic feedback in stage 2 ($p = 0.10$), there was evidence of some initial uncertainty over how to respond to misleading losses, with chance levels of lose-shift behaviour recorded on correct trials. However, as training progressed through Stages 3 and 4, and the likelihood of receiving a punished correct response (PCR) increased, animals became less likely to shift in the face of misleading punishment. Presumably, losses no longer reliably signalled a need to change response strategy, enabling mice to ignore the 'noise' of inaccurate feedback and maintain stable and accurate performance. These findings are comparable to those reported by Ineichen et al. (2012) on lose-shift responding across increasing levels of misleading feedback during reversal, with the exception that in the present study, lose-shift behaviour dropped below the chance levels they report at the two highest schedules of testing. This is also despite the two highest schedules being marginally leaner than those employed by Ineichen et al. (2012). Although this difference is likely due to the relative ease of acquisition relative to reversal learning, and the lack of a criterion cut-off point for performance in the present design, these results demonstrate that mice are capable of acquiring accurate response strategies in probabilistic tasks, despite the inclusion of RIR trials. These findings also support the assertion by Jocham et al. (2009) that higher, rather than lower levels of inaccurate feedback are best for establishing accurate response strategies in probabilistically reinforced tasks, contrary to the recommendations of Ineichen et al. (2012).

Responding to accurate reward feedback (correct win-stay) appeared to be unaffected by the experimental manipulation however, which contrasts with prior reports of a monotonic decline in this ratio in mice as the probability of PCR increased (Ineichen et al. 2012), though this is also likely related to the relative ease of acquisition over reversal performance. Despite the low incidence of incorrect trials, which prohibited analysis of response strategies to RIR trials, this seemingly demonstrates that the inclusion of (and increase in) occasional misleading rewards had no adverse effect on the ability to predict reward at the correct location. This suggests that the exclusion of RIR trials may not be necessary to elicit accurate performance strategies in mice, at least during acquisition of a probabilistic discrimination task. However, the low number of errors also means that animals rarely experienced RIR trials; therefore, the relationship between correct responses and reward was unlikely to be degraded to a large degree, which could equally explain the maintenance of a high ratio of win-stay behaviour across test schedules. During reversal testing, when the number of incorrect responses is expected to significantly increase relative to acquisition levels, it will be important to dissect response strategies to both correct and incorrect trials where possible, to more clearly assess whether an accurate response set can be successfully acquired with the inclusion of RIR.

There is a clear confound of experience on performance in the current design, given that stage order was not counterbalanced, so the data do not rule out the possibility that schedules associated with a higher probability of misleading feedback would be more difficult to acquire without prior experience. The change in lose-shift performance may be the result of increased experience of probabilistic outcomes over time, rather than related to the change in probability of them occurring, although the concurrence of these findings with previous results under fully counterbalanced conditions (Ineichen et al. 2012; Jocham et al. 2009) suggests otherwise. Nevertheless, this increase in experience might account for the ability to maintain such high win-stay ratios at the correct location across schedules relative to previous reports (Ineichen et al. 2012).

The purpose of the current experiment was to determine whether a response set could be acquired and maintained across schedules however, which has been clearly demonstrated. This challenges the assumption that mice are unable to acquire a model-based response strategy under probabilistic schedules, which is an important assumption of human PRL tasks and therefore necessary for maximising translational value. Were similarly accurate performances not demonstrated during acquisition in subsequent tasks employing the same level of probabilistic feedback not incrementally introduced, this might suggest that longer periods of training were responsible for the current results. Nevertheless, the current results demonstrate

that it is possible to achieve a stable model-based response strategy in mice under probabilistic feedback conditions, even if longer training of probabilistic outcomes is required.

Deciding upon a suitable schedule of probabilistic feedback to carry forward into future tests is important, in order to maximise the accuracy of response strategies without making a reversal shift too difficult to detect. The difference in lose-shift performance between the final two test stages of this task ($p = 0.15$ and 0.20) was not significant, therefore either schedule could be implemented. However, given the analysis issues that arose from the low incidence of incorrect responses in this task (which could explain the tendency in prior research to ignore incorrect trials during analysis of response patterns), the more challenging schedule could be useful for creating a higher number of RIR trials. The use of higher probability schedules, beyond the 0.10 schedule Ineichen et al. (2012) recommend, may therefore be important not only for reducing sensitivity to local feedback, but in generating sufficient data for analysis in probabilistic tasks. What remains to be convincingly demonstrated however is whether mice can achieve accurate response strategies during reversal of probabilistic outcomes under more challenging schedules, given evidence that reversal shifts also become more difficult to detect (Ineichen et al. 2012; Jocham et al. 2009). Having established that mice can acquire a successful response strategy (on correct trials) during a probabilistically rewarded spatial discrimination task with a schedule of $p = 0.20$ and with the inclusion of RIR trials, Experiment 2 will test the ability of mice to successfully reverse a learned discrimination under these conditions using a between-session reversal design, whilst simultaneously assessing the role of SB242084 on performance in this task. The results of this experiment also provide a basis for the design of future experiments of this type, particularly in relation to the appropriate probability parameters for such experiments.

3.3. EXPERIMENT 2

REVERSAL OF A PROBABILISTIC SPATIAL DISCRIMINATION IN MICE

The previous experiment established that mice demonstrate accurate performance and a successful model-based strategy during a probabilistic spatial discrimination task, where the probability of inaccurate feedback was set to $p = 0.20$ and RIR trials were included. However, it will be important to replicate these findings under conditions where this level of probabilistic feedback is not incrementally introduced. Given that higher levels of inaccurate feedback are proposed to reduce sensitivity to immediate outcomes (Jocham et al. 2009), this higher probabilistic schedule could also create difficulties in being able to detect a reversal shift; and it

is not clear from previous research if mice can successfully adapt response strategies in a PRL task with such a high probability of inaccurate feedback (Amodeo et al. 2012; Ineichen et al. 2012). The current experiment tests the ability for mice to reverse responding under these same conditions.

The role of 5-HT_{2C}Rs in PRL tasks is also yet to be established, though there is some suggestion that antagonism at these receptors may reduce punishment sensitivity, due to the possible role of 5-HT_{2C}Rs in mediating responding to aversive stimuli; and/or to an increase in reward sensitivity, by removing the tonic inhibition 5-HT_{2C}Rs exert on mesocorticolimbic DA output (Di Giovanni et al. 1999; Di Matteo et al. 1999, 2001). However, there may also be an effect on response inhibition more generally, which would be reflected in altered responding to misleading feedback of both a positive and negative valence, hence the value of examining responses to both RIR and PCR trials.

The stochastic environment of PRL tasks may also offer a more sensitive tool for further elucidating the role of 5-HT_{2C}Rs in cognitive flexibility, which is currently less than clear from previous tasks using deterministic reinforcement schedules. The current experiment therefore examined the effects of SB242084 treatment during reversal of a probabilistic spatial discrimination in mice, in a task similar to that employed by Ineichen et al. (2012), but using a between-session design and including RIR trials.

3.3.1. Materials and methods:

Animals:

This study used 24 male C57BL/6J (Charles River, UK), weighing 22.3g (SEM ± 0.9g) at the start of the experiment. Housing conditions, behavioural apparatus, food deprivation procedures and production of SB242084 were the same as previously stated.

Behavioural procedures

Stages 1 & 2 of testing were identical to Group S training in Chapter 2, Experiment 1. Briefly, during Stage 1 animals were trained to reliably nosepoke into a single, central aperture to earn rewards (20mg sugar pellets). During Stage 2 animals were additionally required to make a head entry into the food magazine to initiate each new trial, until they reached a criterion of 49 complete responses within 70 trials (≥70%) over two consecutive test sessions. A complete trial was any trial in which the animal initiated a trial (within 20s), responded in the central nose poke hole (within 20s), and retrieved the reward (within the 11s ITI).

Stage 3: *Two-choice training*

Animals received one session of two-choice training consisting of 30 trials. During this stage either the left or the right nosepoke hole was illuminated on each trial, and served as the rewarded stimulus. This served to give animals experience of sampling from both spatial locations, and to teach them that both apertures can be associated with reward. Animals were required to complete 15 nosepoke responses at each illuminated spatial location. Initial location of the illuminated cue was randomly determined, but the location was constrained once either location received a total of 10 rewarded responses, until 10 responses were achieved at both locations. The program then reverted to random presentation until 15 responses were registered at any one location, followed by constrained location until 15 responses were registered at both locations. This was to control for effects of side bias, ensuring an equal number of reinforcers were associated with each spatial location. Animals were subsequently trained against any evident side bias (with a side bias defined as 8 or more consecutive responses at the same location). Animals now had 20s to initiate a trial and 30s to register a response, before an initiation omission or response omission was recorded, respectively. Responding in the lit nosepoke hole led to the LED turning off, delivery of reward, and the start of the ITI; whilst responding in the non-illuminated nosepoke hole had no programmed consequence.

Stage 4: *Forced-choice probabilistic training*

To ensure animals gained experience of the probabilistic reward outcomes of both available locations during probabilistic spatial discrimination, animals received two sessions of forced-choice probabilistic training. Animals were again presented with one illuminated nosepoke hole either to the left or right of the central aperture; however one nosepoke hole now served as the 'correct' location, and was rewarded on 80% of trials, whilst the other was 'incorrect' and rewarded on 20% of trials (with the probability of reward on each trial following a pseudo-randomized schedule). Location of the correct nosepoke hole remained stable for each animal throughout both sessions, and was assigned against any evident side bias from stage 3, or, where no side bias was evident, was counterbalanced. Animals received a total of 20 trials per session (excluding omissions), with location of the cue light remaining stable for 10 trials before switching to the opposite location. Order of presentation (i.e. correct or incorrect first) was fully counterbalanced across subjects, and switched between session 1 and session 2. Animals were required to complete 20 responses at each illuminated spatial location in total across the two sessions, therefore experiencing 4 rewarded incorrect responses (RIR) and 4 punished correct responses (PCR) overall. A rewarded trial (at both 'correct and 'incorrect' locations) led to the nosepoke light turning off, delivery of reward, and beginning of the ITI, whilst a punished trial (at both 'correct and 'incorrect' locations) led to nosepoke light turning off and immediate onset of houselights for the duration of the ITI. Animals again had

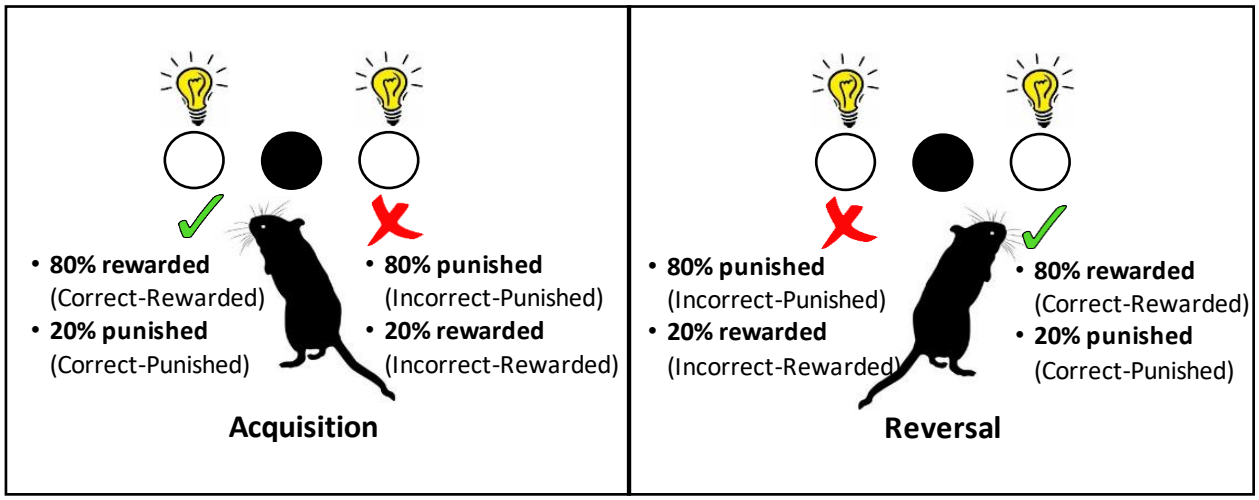

Figure 3.3. Representation of task requirements and probabilistic response outcomes during acquisition and reversal of spatial discrimination. *Following trial initiation by a response in the magazine, left and right nosepoke holes are illuminated (open circles), and animals are required to reach a criterion of 70% correct responses in any one test session, before correct and incorrect response locations reverse (in subsequent test session). Central nosepoke hole is covered throughout (filled circle).*

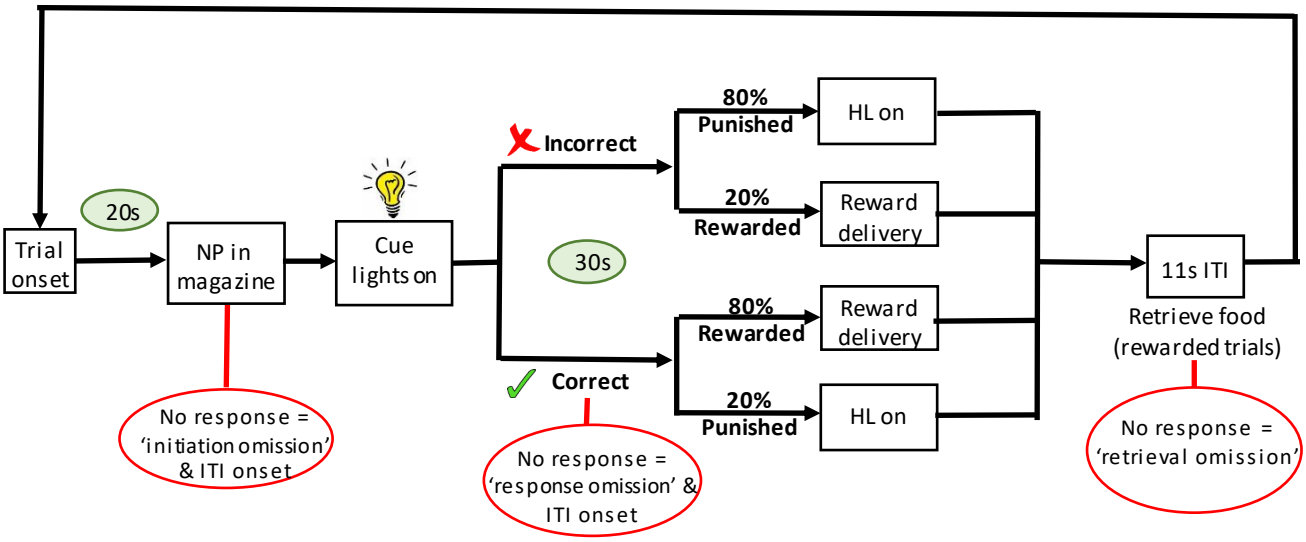

Figure 3.4. Representation of individual trial structure during acquisition and reversal of a probabilistic spatial discrimination. *Animals are required to make a nosepoke response in the magazine within 20s of trial onset in order to illuminate cue lights in the left and right nosepoke locations (see Figure 3.5), otherwise an 'initiation omission' is recorded. Animals must then register a response (correct/incorrect) in one of the illuminated nosepoke holes within 30s, or a 'response omission' is recorded. Reponses at the 'incorrect' nosepoke location are predominantly punished (80%) but occasionally rewarded (20%), whilst responses at the 'correct' nosepoke location are predominantly rewarded (80%) and occasionally punished (20%). Following a rewarded trial (correct or incorrect), animals must make a response within the food magazine within the 11s ITI, or a 'retrieval omission' is recorded. Abbreviations: HL = houselights, NP = Nosepoke, ITI = Inter-trail interval.*

20 s to initiate a trial and 30 s to respond; with failure to do either resulting in onset of the ITI, and an omission being recorded. Whilst both left and right nosepoke holes were available to the animal, so choice was not truly 'forced', responses into the non-illuminated location had no programmed outcome.

Stage 5: *Probabilistic spatial discrimination*

During this stage of training, both the left and right nosepoke holes were illuminated (see Figure 3.3). Animals were now able to choose between 'correct' and incorrect' nosepoke holes, with the location of the 'correct' aperture in the same place for each animal as in the previous stage of training. The outcome of a rewarded and punished trial was the same as during the previous stage (see Figure 3.4). Animals received 60 trials per session and were required to respond correctly on 70% of trials (42 correct responses) in any one session to reach criterion. Despite evidence from the previous experiment that mice are capable of reaching a more stringent accuracy criterion during acquisition of the spatial discrimination task, this threshold was reduced slightly from the 75% criterion used in the previous chapter due to evidence that errors increase significantly during reversal of response-outcomes under probabilistic compared with deterministic reinforcement schedules (Ineichen et al. 2012; Jocham et al. 2009).

Stage 6: *Multiple serial reversals*

Half the animals were dosed with 0.5 mg/kg of SB242084 subcutaneously 30 min prior to each day of reversal testing, with the other half receiving a vehicle injection, fully counterbalanced across left and right 'correct' locations. Reinforcement contingencies were repeatedly reversed within both groups over a series of three between-session reversals, where the opposite nosepoke hole to that rewarded during the previous stage became correct (see Figure 3.3). Again, animals were required to complete 42 correct responses within a single 60-trial session (70% correct) to reach criterion. Animals in both groups were then required to demonstrate successful retention on a separate drug-free testing day by completing 42 correct responses within a single 60-trial session, before the reinforced location was reversed.

Measures and Statistical analyses:

Number of sessions and trials (correct and incorrect) to criterion during acquisition and reversal were the primary performance measures. The total number of initiation omissions, nosepoke omissions and reward retrieval omissions to criterion were also measured, as well as the average latency to initiate a trial, make a response and retrieve rewards. For acquisition data, baseline (drug-free) performance between drug-treatment groups was compared using independent-measures t-tests. For reversal and retention data, mixed 2 x 3 ANOVA were

performed with Drug (Veh/SB) as an independent factor and Reversal Test (Reversal 1, 2 and 3) as a repeated measures factor. However, evidence for an interaction between drug-treatment and reversal test on the primary performance measures revealed the drug-related effect to be restricted to the first reversal only. Data from the first reversal were therefore analysed using independent-measures t-tests. Response patterns following each trial outcome were also analysed.

There were four possible trial outcomes (Correct-Rewarded; Correct-Punished; Incorrect-Rewarded; Incorrect-Punished), following which an animal could choose to either stay or shift (excluding omissions), see Table 3.1. The dependent variable used for analyses was 'Stay' behaviour on correct trials but 'Shift' behaviour on incorrect trials (following both wins and losses), since this reflects the accurate response strategy for each respective trial-type, and allows direct comparison of the relative accuracy of responding at each location and in response to accurate and misleading feedback. This gave four outcomes: Correct win-stay; Correct lose-stay; Incorrect win-shift; Incorrect lose-shift; with high levels of conditional probability of each reflective of accurate responding. The probability of each response type was calculated as a ratio, as previously described.

Response pattern data were subjected to arcsine-SQRT transformation and analysed using mixed 2 (Independent-measures variable: Drug: Veh/SB) x 4 (Repeated-measures variable: Trial-type: correct-rewarded; correct-punished; incorrect-rewarded; incorrect-punished) ANOVA for performance during acquisition, reversal, and retention; as well as during late-stage reversal (>50% correct) to assess performance when the impact of early perseveration was minimised. Response strategy accuracy was analysed using 2 x 4 mixed ANOVA, with Drug (Vehicle/SB242084) as a between-subjects variable and Trial-type (Correct-Win; Correct-Lose; Incorrect-Win; Incorrect-Lose) as a repeated measures variable. During acquisition testing, where there were insufficient Incorrect-Win trials, this restricted comparison to three levels of the repeated measure variable using 2 × 3 mixed ANOVA; and during late-stage reversal where there were insufficient incorrect trials of either type (win/loss), comparison was restricted to just two levels, using 2 x 2 mixed ANOVA. Significant main effects were further explored where necessary using pairwise comparisons with Holms-Sidak adjustment, and significant interaction effects were explored with simple main effects analyses. Data violating the assumption of normal distribution, and all latency data were Log10 transformed. Reported Means and SEMs are untransformed.

3.3.2. Results:

Two animals failed to learn to nosepoke for rewards during stage 1 of training, and two further animals (one from vehicle group, one from SB242084 group) failed to reverse the learned association within 18 test sessions. These animals were excluded from further testing and data from these animals were not included in analyses.

Acquisition (drug-free):

Results of independent t-tests show there were no baseline differences in performance between the SB242084 and vehicle group during the acquisition of the spatial discrimination prior to reversal on any measure of interest (see Table 3.2). Incorrect responses were low, suggesting the probabilistic task was readily acquired, however this meant there were insufficient RIR trials to permit analysis of patterns of responding to these trials. Comparison of the remaining three measures demonstrates that animals were responding accurately, as there was no significant difference in correct win-stay, correct lose-stay, or incorrect lose-shift ratios ($F_{2, 36}$ = 1.73, p > .05). Therefore, there was an equally high likelihood of shifting from the incorrect location as of staying at the correct location; and animals were just as likely to stay at the correct location whether the trial was rewarded or punished (see Figure 3.5). There was no significant difference in average response pattern ratios between drug groups ($F_{1, 18}$ = 0.35, p > .05) and no significant drug x response pattern interaction ($F_{2, 36}$ = 1.75, p > .05).

	Vehicle (n = 10)	*SB242084 (n = 10)*	t_{18}
Sessions	1.3 ± 0.2	1.3 ± 0.2	*0.00*
Trials	82.1 ± 9.2	80.6 ± 9.1	*0.12*
Correct	59.2 ± 3.3	63.5 ± 5.8	*-0.64*
Incorrect	18.8 ± 6.1	14.5 ± 4.1	*0.58*
Initiation omissions	36.1 ± 6.5	37.2 ± 4.1	*-0.14*
Response omissions	4.1 ± 1.1	2.6 ± 0.8	*1.12*
Retrieval omissions	1.6 ± 0.7	0.7 ± 0.3	*1.33*
Initiation latency (s)	6.7 ± 0.3	7.0 ± 0.2	*-0.81*
Response latency (s)	7.3 ± 0.7	5.9 ± 0.4	*1.84*
Retrieval latency (s)	2.0 ± 0.1	1.9 ± 0.1	*0.77*

Table 3.2: *Results of independent measures t-tests comparing baseline (drug-free) performance of the SB242084 and Vehicle control groups during acquisition of the probabilistic spatial discrimination task. There were no significant differences for any measure.*

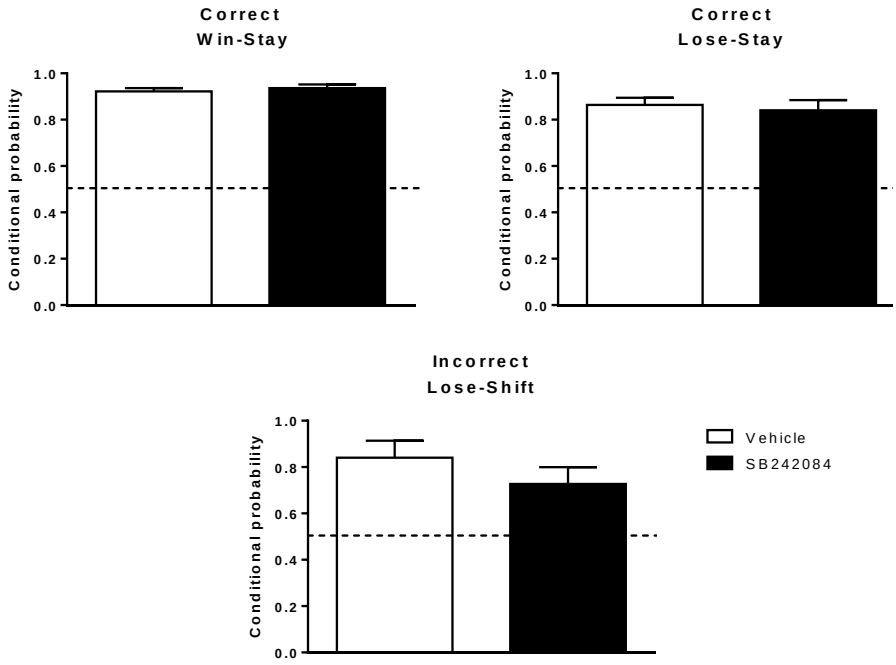

Figure 3.5: Response pattern data by trial-type during drug-free acquisition of probabilistic spatial discrimination task*. Results of 2 (Drug: Vehicle/SB242084) x 3 (Trial Type: Correct-Win; Correct-Lose; Incorrect-Lose) mixed ANOVA shows no significant difference in performance across trial-types, or between drug groups, and no interaction effect. The dependent measure was ratio of trials followed by a 'stay' response for correct trials, and ratio of trials followed by a 'shift' response for incorrect trials. Note how all animals show a high probability of staying at the correct location following both wins and losses, and of shifting from the incorrect location following losses, demonstrating accurate model-based response strategies. There were insufficient Incorrect-Rewarded trials to permit analysis of Win-Shift behaviour following an incorrect response. Dashed lines represent chance-level performance.*

Reversal:

There was a significant drug-related enhancement of reversal performance relative to controls, which was specific to the first reversal test only (see Figure 3.6). Mixed 2 (Drug: Veh/SB) x 3 (Reversal Test: 1, 2, 3) ANOVA reveal there was a significant main effect of reversal test on the number of sessions ($F_{2, 36} = 4.32$, $p < .05$) and trials ($F_{2, 36} = 3.91$, $p < .05$) required to reach criterion, but there were no significant pairwise differences between reversal tests (all p's > .05). There was no main effect of drug-treatment on performance (Sessions: $F_{1, 18} = 1.99$, $p > .05$; Trials: $F_{1, 18} = 2.24$, $p > .05$), but there was a significant interaction between reversal test and drug-treatment for trials to criterion ($F_{2, 36} = 3.30$, $p < .05$), and a borderline significant interaction for sessions to criterion ($F_{2, 36} = 3.13$, $p = .056$). Simple main effects analyses showed that SB242084-treated animals required significantly more sessions and trials to reach reversal criterion than controls during the first reversal test only ($p < .05$). Given evidence that the drug-related performance effect was restricted to this first reversal, the 'Reversal Test' factor was removed from analyses, and performance during the first reversal test was examined using independent measures t-tests.

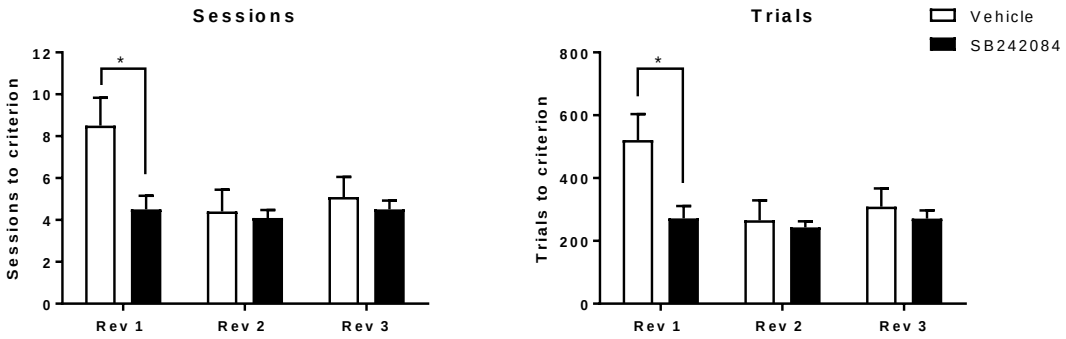

Figure 3.6: Sessions and trials to reach reversal criterion by drug-group during each reversal test (1, 2, 3). *Results of 2 x 3 ANOVA show no main effect of drug treatment for either measure (Sessions: $F_{1, 18} = 1.99$, $p > .05$; Trials: $F_{1, 18} = 2.24$, $p > .05$), a significant main effect of Reversal Test (Sessions: $F_{2, 36} = 4.32$, $p < .05$; Trials: $F_{2, 36} = 3.91$, $p < .05$), and a significant drug x reversal test interaction for trials to criterion ($F_{2, 36} = 3.30$, $p < .05$), and a borderline significant interaction effect for session to criterion ($F_{2, 36} = 3.13$, $p = .056$). Simple main effects analyses show that SB242084-treated animals required significantly fewer sessions and trials to reach criterion in reversal test 1. *p < .05.*

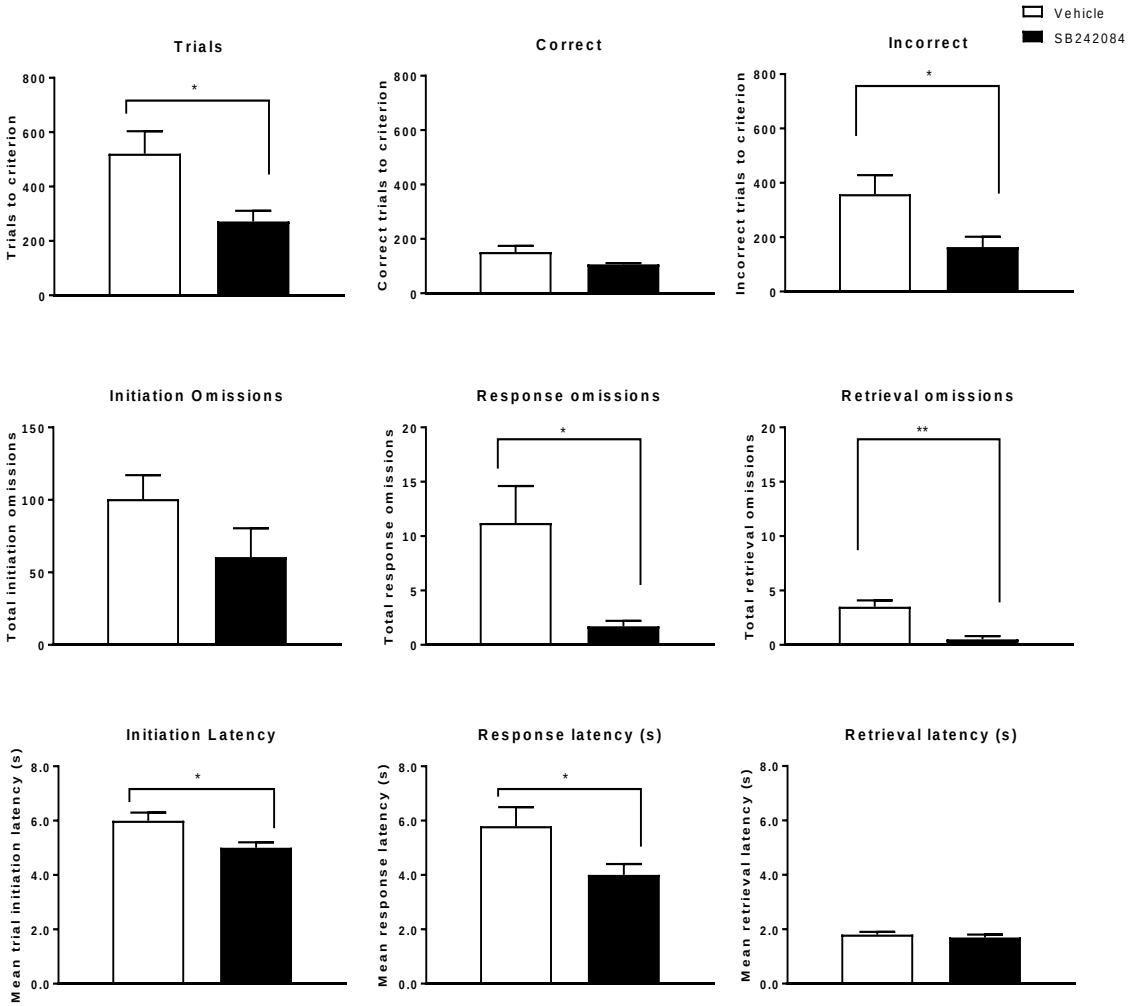

Figure 3.7: *Results of independent-measures t-tests comparing performance of the SB242084-treated animals and vehicle-treated controls during Reversal Test 1 of probabilistic spatial discrimination. *p <. 05, **p <.001.*

Analysing performance during the first reversal test confirmed a significant SB242084-related enhancement of performance, with drug-treated animals requiring significantly fewer sessions (Vehicle: 8.5 ± 1.3; SB242084: 4.5 ± 0.7; $t_{13.0} = 2.69$, $p < .05$) and trials ($t_{12.9} = 2.74$, $p < .05$, see Figure 3.5) to reach criterion than vehicle-treated controls. This effect only reached significance for incorrect trials, with drug treated animals requiring significantly fewer incorrect ($t_{13.8} = 2.43$, $p < .05$), but not correct ($t_{9.8} = 2.00$, $p > .05$) trials to reach criterion (see Figure 3.7). Whilst no drug-related difference in the number of trial initiation omissions emerged ($t_{18} = 1.55$, $p > .05$), SB242084-treated animals were found to make fewer response omissions ($t_{9.4} = 2.79$, $p < .05$) and reward retrieval omissions ($t_{18} = 4.45$, $p < .001$) than controls (see Figure 3.7). SB242084 was also found to speed responding on most latency indices, with drug-treated animals being faster to initiate trials ($t_{18} = 2.77$, $p < .05$), and to make a response ($t_{18} = 2.32$, $p < .05$) than controls (see Figure 3.7). There was no difference in reward retrieval latency between groups ($t_{18} = 1.87$, $p > .05$); however this could reflect a floor effect given the speed of retrieval (see Figure 3.5).

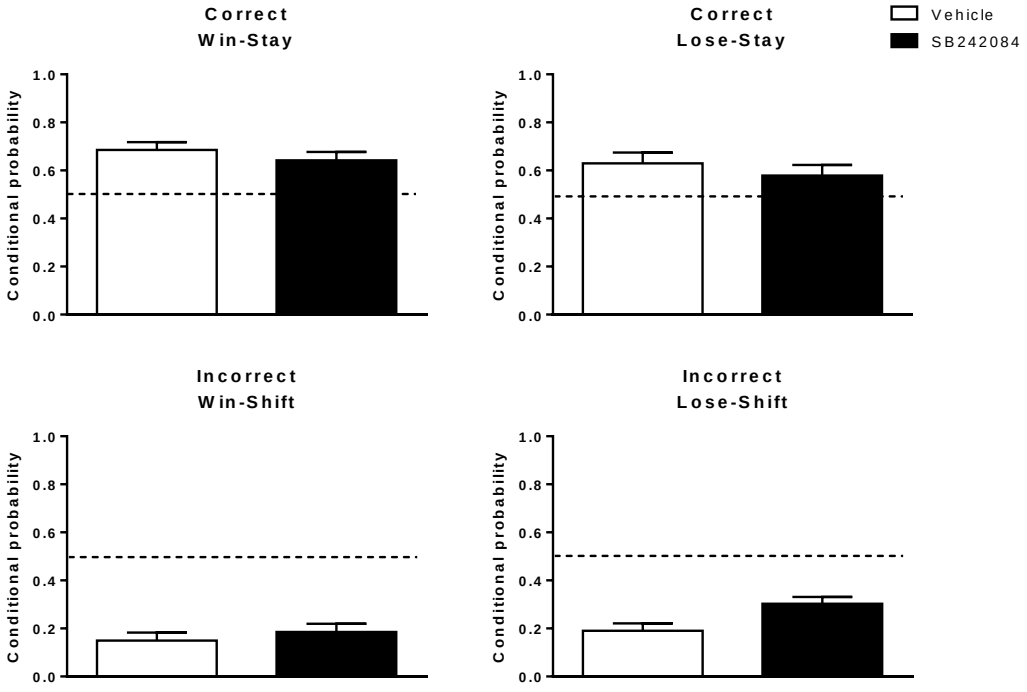

Figure 3.8: Response pattern data by trial-type during Reversal Test 1. *Results of a 2 (Drug: Vehicle/SB242084) x 4 (Trial Type: Correct-Win; Correct-Lose; Incorrect-Win, Incorrect-Lose) mixed ANOVA shows a significant difference in performance across trial-types ($F_{3, 54} = 82.95$; $p < .001$). The dependent measure was ratio of trials followed by a 'stay' response for correct trials, and ratio of trials followed by a 'shift' response for incorrect trials. Stay behaviour on correct trials was significantly higher than shift behaviour on incorrect trials ($p < .05$), demonstrating a tendency to perseverate at the incorrect location. Stay behaviour on correct trials did not differ following a win or loss ($p > .05$), but shift behaviour on incorrect trials was higher following a loss than a win ($p < .05$). There was no main effect of drug treatment, or interaction between trial-type and drug. Dashed lines represent chance level performance.*

Inspection of response-pattern data during reversal suggests that animals were responding relatively accurately at the correct location, staying at that location on the majority of subsequent trials, in response to both wins and losses. Responding at the incorrect location appeared far less accurate, with low levels of shifting behaviour, suggestive of perseverance. Additionally, responses to incorrect trials appeared more sensitive to trial outcome, with higher levels of shifting apparent following losses than wins (see Figure 3.8). These effects were confirmed by results of the mixed ANOVA, with a significant main effect of trial-type on response-pattern ($F_{3, 54}$ = 82.95; p < .001), with pairwise comparisons demonstrating no difference in win-stay and lose-stay ratios on correct trials (p > .05), but significantly higher lose-shift than win-shift ratios on incorrect trials (p < .05). Furthermore, shifting on incorrect trials was significantly lower than staying on correct trials, following both wins and losses (all p's < .05). There was no significant main effect of drug-treatment on average response pattern ratio ($F_{1, 18}$ = 0.45, p > .05), and no interaction effect ($F_{3, 54}$ = 2.22, p > .05).

Response-pattern data therefore shows that animals were predominantly staying at the incorrect location during reversal, which could suggest they had difficulty dealing with the probabilistic outcomes of the task. However, this data is skewed by responses made early in reversal, when animals show a tendency to perseverate with the previously correct response pattern. To accurately deduce whether animals were capable of acquiring an accurate response strategy by the end of the task, behaviour during the late stages of reversal (after animals had reached a criterion of 50% correct i.e. chance responding) was analysed. Due to low levels of incorrect responding at this stage, particularly among drug-treated animals, there were insufficient RIR trials to permit analysis of responses to this trial-type. Inspection of the remaining data suggests that animals had acquired a successful response strategy, and were just as likely to shift from the incorrect location following a loss as they were to stay at the correct location following either a win or a loss (see Figure 3.9). Results of mixed ANOVA confirm there was no longer a significant main effect of trial type on response pattern ratios ($F_{2, 36}$ = 3.07, p > .05), as well as no main effect of drug ($F_{1, 18}$ = 0.05, p > .05) or interaction effect ($F_{2, 36}$ = 0.31, p > .05).

In summary, SB242084 improved performance of the PRL task relative to controls, reducing the number of sessions and incorrect trials required to reach criterion during the first of three serial reversals. Drug treatment also lead to reduced omissions, and faster trial initiation and response execution. However, SB242084 had no effect on patterns of responding to misleading reward or punishment (non-reward). Overall, animals displayed accurate model-

based responding at the correct location during the first reversal test, and appear to have acquired accurate responding at the incorrect location by the late stages of testing.

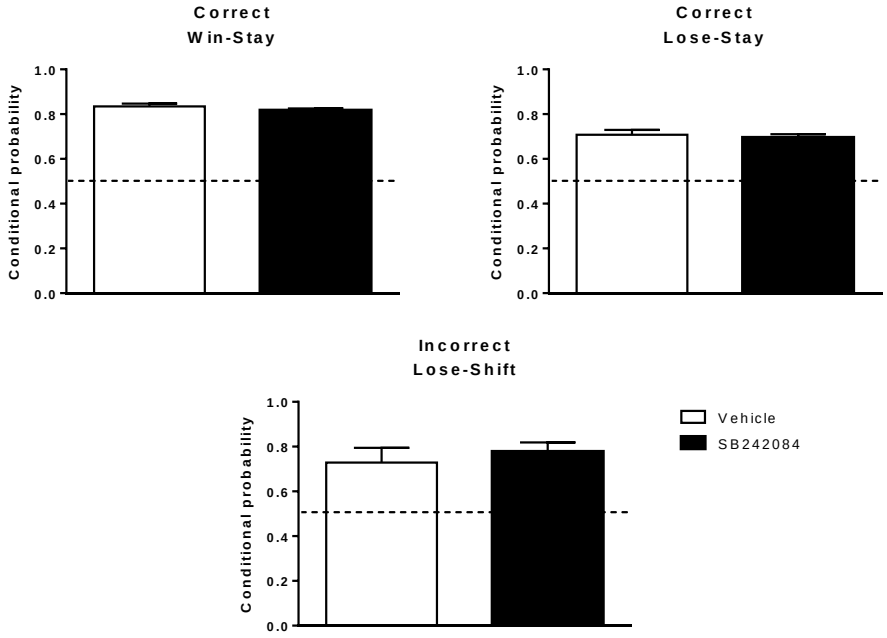

Figure 3.9: Response pattern data by trial-type during late-stage reversal (from 50% correct to criterion). *Results of a 2 (Drug: Vehicle/SB242084) x 3 (Trial Type: Correct-Rewarded; Correct-Punished; Incorrect-Punished) mixed ANOVA shows no significant difference in performance across trial-types, no effect of drug-treatment and no interaction. The dependent measure was the percent of trials followed by a 'stay' response for correct trials, and percent of trials followed by a 'shift' response for incorrect trials. Note that all animals show a high probability of staying at the correct location (whether rewarded or punished) and of shifting from the incorrect location when punished, demonstrating accurate response strategies. There was insufficient Incorrect-Rewarded data to permit analysis. Dashed lines represent chance level performance.*

Retention (drug-free):

All animals showed drug-free retention of reversal performance within one test session. Independent measures t-tests show there were no significant differences in performance between drug-groups on any measure (all *p*'s > .05, data not shown). Response patterns could only be analysed for correct trials due to insufficient incorrect responses of either type (rewarded or punished). 'Stay' behaviour was very high on correct trials, and did not differ following either reward (95.1% ± 1.2) or punishment (91.4% ± 1.8) (trial-outcome: $F_{1, 18} = 1.33$, $p > .05$). There was no difference in 'stay' behaviour between drug groups (drug: $F_{1, 18} = 0.44$, $p > .05$), and no interaction effect ($F_{1, 18} = 2.14$, $p > .05$).

3.3.3. Discussion:

The results of the current study show that mice are not only capable of acquiring accurate model-based response strategies during a probabilistic spatial discrimination task which includes rewarded incorrect responses (RIR), with a probability of misleading feedback set as high as $p = 0.2$, but can also successfully learn to reverse strategies under these conditions. This suggests that the between-session reversal design currently employed may be more appropriate for use in rodents than the within-session designs previously implemented, where poorer performance has been reported.

Employing this more complex probabilistic version of the task also revealed a significant performance-enhancing effect of 5-HT_{2C}R antagonism during reversal which was not evident using the same task under deterministic feedback conditions, where the opposite effect was reported (see Chapter 2). Taken alongside previous reports, this could indicate that task difficulty plays a key role in the relative efficacy of SB242084 treatment. Despite prior reports that serotonergic manipulations can affect punishment sensitivity, and the suggestion that 5-HT_{2C}Rs could be involved in mediating reward sensitivity due to their role in regulating mesocorticolimbic DA transmission, there was no evidence of a difference in responding to either wins or losses in drug-treated animals relative to controls, making this an unlikely mechanism through which SB242084 exerts its overall effects on performance accuracy. Neither was there any evidence of a consistent effect on responses to misleading feedback more generally, which might suggest an effect of the manipulation on response inhibition. Therefore, despite evidence of reduced omissions and response latencies following treatment with SB242084, in line with prior findings, this does not appear to translate into a reduced ability to inhibit inappropriate responding. Furthermore, there was no evidence of increased sensitivity to feedback on individual trials, reflected by a move towards a 'win-stay/lose-shift' pattern of responding, which would improve reversal by allowing faster detection of a change in response-outcome contingencies. Therefore, drug-treatment seemingly confers a benefit in degree of response accuracy in PRL tasks without affecting the nature of responding to feedback on individual trials.

All animals that completed training in this study went on to successfully acquire the probabilistic spatial discrimination under drug-free conditions, and displayed accurate model-based responding. This was demonstrated by the fact that animals stayed at the correct location on the vast majority of trials, irrespective of outcome. Although data on responses to RIR trials was lacking, there was also evidence that animals predominantly shifted from the incorrect

location when punished. This demonstrates that, prior to reversal, mice are able to cope with spurious feedback and do not simply adapt responses on a trial-by-trial 'win-stay/lose-shift' basis; which is essential to the ability to relate findings to the human literature. Win-stay performance on correct trials closely matched that reported in Experiment 1 under this same schedule of probabilistic feedback, whilst lose-stay performance showed even greater accuracy. Having removed the possible confound of experience on behaviour in Experiment 1, this provides stronger evidence that mice are capable of acquiring accurate model-based response strategies in probabilistic discrimination tasks with a probability of inaccurate feedback set at $p = 0.2$.

Though lower than acquisition levels, analysis of response pattern ratios during reversal shows that win-stay behaviour at the correct location very closely approximated that reported by Ineichen et al. (2012) under $p = 0.2$ probability of punished correct responses (PCR) (~70%). This suggests that the inclusion of rewarded incorrect responses (RIR) in the current design did not impair reward prediction during reversal as Ineichen et al. (2012) predicted. Lose-stay ratios at the correct location were also higher than the chance levels previously reported under this level of probabilistic feedback in both rats (Bari et al. 2010) and mice (Ineichen et al. 2012), and did not differ significantly from win-stay behaviour. Animals were therefore able to demonstrate an accurate response-set at the correct location, not previously demonstrated at this level of probabilistic feedback.

The reason for this discrepancy may relate to the use of a between-session rather than a within-session reversal design. The within-session design employed by Ineichen et al. (2012) and Bari et al. (2010), where the correct location switches back and forth multiple times within an individual test session, is commonly employed in human PRL tasks (e.g. Swainson et al. 2000). Whilst this added complexity is often necessary in human experiments, where the response requirements of PRL tasks are readily acquired, rodents may find within-session tasks particularly taxing. Using a between-session design, where acquisition and reversal occur during discrete test stages, reversal shifts become more clearly distinguishable from probabilistic outcomes, reducing the likelihood of inappropriate shifting. This underlines the very different nature of the two types of task. Within-session reversal examines the ability to cope with constantly fluctuating changes in reward contingencies online, over a small time period, which restricts the ability to form stable, learned representations; whilst between-session reversal taps into the ability to adapt to changes in reasonably stable learned outcomes. This is arguably more relevant to the type of flexibility typically required for real-world cognition, where there tends to be a degree of stability in response-outcome associations over time; but could pose problems

for comparison of results to the human literature, where within-session reversals are standard practice. Nevertheless, if between-session tests allow rodents to acquire an accurate response set, not always clearly demonstrable in within-session tasks, these advantages are likely to outweigh the comparative shortcomings.

In comparison to responses at the correct location, response patterns on incorrect trials could suggest very poor response accuracy during reversal however. During the first reversal test animals in both groups were very unlikely to shift from the incorrect location, both in response to losses and wins; suggesting animals never acquired a successful response set at this location. However, this data may require a different breakdown in order to be properly reflective of performance. Early perseverative behaviour at the incorrect location is likely to skew response data to reflect a difficulty in performance not evident once animals have detected the need to alter response strategy. Since incorrect responses become low once animals have acquired a successful strategy, data registered early in reversal contribute most to overall patterns of behaviour at the incorrect location. Analysis of response patterns over incremental test stages would therefore be ideal for assessing drug-related differences in performance strategy over the course of reversal. Unfortunately, due to the scarcity of correct responses early in testing (leading to very few PCR trials), and of incorrect responses late in testing (leading to few RIR trials) a full breakdown of responses was not possible in the current study. This highlights a limitation of probabilistic tasks, one which has seemingly not been resolved in existing research judging by the scarcity of incorrect response pattern analyses. Analysing data from an intermediate performance point (50% correct > criterion) was useful for assessing response patterns on incorrect-punished trials when the influence of this early data was removed however, providing evidence that animals were shifting from the incorrect location on the majority of trials (though there were still insufficient RIR trials to assess responses to misleading reward). Late-stage response pattern data in fact appeared very similar to acquisition performance, providing evidence that, when early stages of learning are excluded from analyses, mice demonstrate both accurate reward and punishment prediction in this probabilistic task.

The drug-related performance enhancement reported in the current PRL design contrasts quite markedly to the impairment effect reported in the previous chapter when employing a deterministic feedback task. This could suggest that these two tasks recruit distinct brain regions which are differentially sensitive to 5-HT_{2C}R antagonism. However, most evidence suggests that similar brain regions are involved in reversal learning and learning in stochastic environments, such as PRL tasks. Furthermore, the current findings are largely consistent with results reported by Nilsson et al. (2012) during reversal under deterministic feedback conditions,

with an almost two-fold drug-related reduction in the number of sessions and trials needed to reach criterion relative to controls. Given that vehicle-treated animals in the current study, as well as in the Nilsson et al. (2012) task, took considerably longer to solve the reversal compared to both previous studies which report a negative impact of $5HT_{2C}R$ blockade/deletion (Chapter 2; Pennanen et al. 2013), this could instead suggest that task-difficulty is the important factor mediating the effect of SB242084 treatment on reversal performance. Under conditions of low cognitive effort 5-$HT_{2C}R$ antagonism may lead to impaired performance accuracy, whereas under higher-effort conditions this same manipulation might support improved performance, possibly through interactions with motivational mechanisms; similar to the relationship between arousal and task performance (e.g. Hebb 1955; Teigen 1994; Yerkes & Dodson 1908). Though it is clear that probabilistic tasks introduce a level of complexity relative to accurate feedback tasks, it is not clear exactly which aspect of the Nilsson et al. (2012) design may have been responsible for the increased task difficulty; though the longer ITI relative to other studies may have served to increase memory demands (see Chapter 2).

Despite a similarity in the degree of drug-related reversal enhancement reported by Nilsson et al. (2012), the specific enhancement effect conferred by SB242084 in the current task was found to differ. I report that drug-treated animals required fewer incorrect, but not correct trials to reach criterion; possibly suggesting a pattern of perseverative behaviour in vehicle-treated controls that was ameliorated by drug treatment. This is in contrast to findings of a drug-associated reduction in correct but not incorrect trials to criterion under full reversal conditions in the Nilsson et al. (2012) design, interpreted as evidence that drug-treatment reduced avoidance of the previously incorrect location brought about through the accumulation of 'learned non-reward' during acquisition. Although Nilsson et al. (2012) note that correct and incorrect responses during reversal can both be produced by perseverance at the previously correct location or avoidance of the previously incorrect location; they confirmed their hypothesis by establishing that SB242084 led to a reduction in correct trials to criterion during a 'learned non-reward' test (where the influence of the previously correct response option was removed), but not in a 'perseverance' test (where the influence of the previously non-rewarded response option was removed). This could therefore suggest that the effect of SB242084-treatment in probabilistic tasks is not related to effects on learned non-reward, as demonstrated under accurate feedback conditions, but to a reduction in perseverative behaviour at the incorrect response location. However, this difference could more simply be related to the fact that all animals in the current task required more incorrect than correct trials to reach criterion, whereas in previous tasks animals required more correct than incorrect trials (Chapter 2,

Experiment 1; Nilsson et al. 2012; Pennanen et al. 2013), thus the nature of the drug-enhancement effect may only appear to differ. The probabilistic nature of the current reversal design encourages animals to persist longer with an inaccurate response strategy during reversal by making them less sensitive to immediate trial outcomes, thus increasing the number of errors made (e.g. Ineichen et al. 2012; Jocham et al. 2009). It will be necessary to test the effects of SB242084 on performance using specific tests of 'perseverance' and 'learned non-reward' under probabilistic conditions in order to establish whether its effect truly differs in type from that seen under deterministic feedback conditions.

In summary, these results demonstrate that between-session PRL tasks can be successfully employed in mouse models, at a probability of inaccurate feedback of $p = 0.2$, and without the need to remove RIR trials. Between-session reversal tasks likely tap into very different learning capacities compared to within-session tasks, but nevertheless hold strong ecological validity, as well as being able to overcome some of the performance deficits previously believed to render mice unsuitable for use in probabilistic designs. These tasks can clearly contribute important additional information to the study of cognitive flexibility, by assessing patterns of responding on accurate and misleading feedback trials and in response to wins and losses; though the current results indicate that drug-treatment had little bearing on reward or punishment sensitivity; responsivity to individual trial-by-trial outcomes; or the ability to inhibit inappropriate responses to misleading feedback more generally. The results are consistent with the drug-related benefits previously reported by Nilsson et al. (2012), with the exception that the current study reports a specific drug-related reduction in incorrect, rather than correct, trials to criterion. This disparity, possibly related to differences in design, could also have important implications for the role of SB242084 in modulating perseverance and learned non-reward, which can both contribute to reversal performance. This possibility will therefore explored by testing the effects of SB242084 on performance during a probabilistic learned non-reward and perseverance task, used to separate out the contribution of these two elements to reversal performance.

3.4. EXPERIMENT 3

EFFECT OF SB242084 IN PROBABILISTIC TESTS OF PERSEVERANCE AND LEARNED NON-REWARD

Experiment 2 found that drug-treated mice required fewer incorrect but not correct trials to reach criterion compared to controls during the first of three serial reversals; suggesting a pattern of perseverative behaviour that is ameliorated through drug treatment. This is in contrast to prior findings of a drug-associated reduction in correct but not incorrect trials to criterion under full reversal and 'learned non-reward' conditions, which was not seen under 'perseverance' conditions (Nilsson et al. 2012). However, probabilistic tasks encourage an increase in reversal errors relative to deterministic tasks, so the drug-related benefit may only appear to differ in kind. The aim of the present study was to expand upon findings from the previous full reversal test, to examine what effect SB242084 has on responding within a 'learned non-reward' and 'perseverance' test under the same probabilistic reward conditions. Since the results presented in Chapter 2 identified a significant effect of drug treatment which was confined to the first reversal test only, this experiment implemented a single reversal test.

3.4.1. Materials and methods:

Animals and behavioural procedures:

This study used 40 male C57BL/6J (Charles River, UK), weighing 22.3g (SEM ± 0.6g) at the start of the experiment. One animal assigned to the Perseverance/SB242084 group became unwell during testing and was culled.

Stages 1-5 of training were identical to Experiment 2. During acquisition of the spatial discrimination, left and right nosepoke holes were available and the central nosepoke hole was covered. Responding at one nosepoke location (counterbalanced across left/right) was 'correct' and led to reward, and responding at the other was 'incorrect' and led to omission of reward. Animals were required to reach a criterion of 70% correct responses within a single 60-trial session.

Stage 6: Learned Non-Reward / Perseverance Test:

After successful acquisition of the spatial discrimination, animals were assigned to one of two test conditions (Learned Non-Reward/Perseverance). Half the animals in each test condition were administered 0.5 mg/kg of SB242084 subcutaneously 30 min prior to each day of testing, with the other half receiving a vehicle injection. Groups were counterbalanced for left and right 'correct' locations and matched for trials to acquisition criterion. Final groups sizes

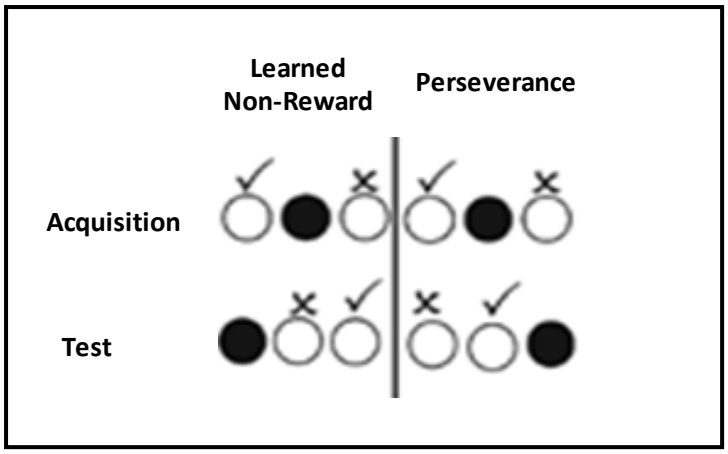

Figure 3.8: *Response options across conditions during acquisition of spatial discrimination and during testing. Open circles represent available nosepoke hole locations, closed circles represent unavailable (covered) nosepoke hole locations. Learned non-reward condition removes the previously correct response option during testing, to isolate the influence of 'learned non-reward' at the previously incorrect location. Perseverance condition removes the previously incorrect response option during testing, to isolate the influence of 'perseverance' at the previously correct location. Note: Correct and incorrect response locations were counterbalanced across left and right across conditions; diagram only shows left set-up. Diagram adapted from Nilsson et al. (2012).*

were n = 10 for groups 'Non-Reward/Vehicle'; 'Non-Reward/SB242084' and 'Perseverance/ Vehicle' and n = 9 for 'Perseverance/SB242084'. Criterion was set to the same threshold as during acquisition (70% correct within any one 60-trial session), after which they were required to demonstrate successful retention on a separate drug-free test day.

Learned Non-Reward:

The central nosepoke hole was uncovered and the previously correct nosepoke hole was covered. Responses at the previously incorrect location were rewarded, and responses at the novel, central location were punished (see Figure 3.8).

Perseverance:

The central nosepoke hole was uncovered and the previously incorrect nosepoke hole was covered. Responses at the previously correct location were now punished, and responses at the novel, central location were rewarded (see Figure 3.8).

Measures and statistical analyses:

All measures taken were the same as described in Experiment 2. The effects of test condition (Learned Non-Reward/ Perseverance) and drug (Vehicle/SB242084) on performance were analysed using 2 x 2 independent-measures ANOVA during acquisition, reversal and

retention. Response pattern data were arcsine-sqrt transformed, and latency measures were log10 transformed (Means and SEM shown are non-transformed).

3.4.2. Results:

Acquisition (drug-free):

There were no performance differences between groups during acquisition of the spatial discrimination, with 2 x 2 ANOVA revealing no significant effect of drug-treatment, condition, or interaction effects for any measure (see Table 3.3). Inspection of response-patterns suggested the development of accurate strategies, as animals stayed at the correct location and shifted from the incorrect location on the vast majority of trials. Analysis confirms there was no main effect of trial-type on performance, with subjects just as likely to stay at the correct location as they were to shift from the incorrect location, following both wins and losses. There were no baseline differences in response-patterns between groups, with no main effect of test condition or drug-treatment, and no two-way or three-way interaction effects (see Table 3.5).

	Learned Non-Reward		Perseverance		*Drug*	*Condition*	*Drug x Condition*
	Vehicle *(n = 10)*	**SB242084** *(n = 10)*	**Vehicle** *(n = 10)*	**SB242084** *(n = 9)*	$F_{1,35}$	$F_{1,35}$	$F_{1,35}$
Sessions	2.1 ± 0.3	2.1 ± 0.4	1.7 ± 0.2	2.4 ± 0.6	*0.94*	*0.01*	*0.94*
Trials	128.4 ± 19.6	128.7 ± 24.6	104.4 ± 13.6	151.4 ± 34.5	*1.00*	*< 0.01*	*0.98*
Correct	81.9 ± 10.3	76.2 ± 12.3	72.0 ± 7.1	94.1 ± 19.5	*0.04*	*0.01*	*1.20*
Incorrect	43.8 ± 10.5	49.8 ± 13.9	30.0 ± 7.3	52.6 ± 14.6	*1.50*	*0.22*	*0.50*
Initiation omissions	66.3 ± 17.5	40.6 ± 13.6	64.2 ± 17.6	56.8 ± 9.3	*1.22*	*0.22*	*0.37*
Response omissions	3.1 ± 1.1	2.7 ± 1.1	2.7 ± 0.9	4.8 ± 1.7	*0.46*	*0.46*	*0.99*
Retrieval omissions	1.5 ± 0.4	2.0 ± 0.6	2.9 ± 1.2	2.1 ± 0.7	*0.03*	*0.92*	*0.67*
Initiation latency (s)	6.4 ± 0.3	5.9 ± 0.2	6.9 ± 0.3	6.6 ± 0.4	*2.16*	*3.12*	*0.08*
Response latency (s)	6.7 ± 0.3	6.9 ± 0.4	6.6 ± 0.4	6.5 ± 0.3	*0.03*	*0.65*	*0.05*
Retrieval latency (s)	1.8 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	*0.33*	*0.18*	*0.09*

Table 3.3. *Results of 2 (Drug: Vehicle/SB242084) x 2 (Condition: Learned Non-Reward/Perseverance) ANOVA comparing performance during drug-free acquisition of the spatial discrimination, to ensure no baseline performance differences between groups.*

Reversal:

SB242084 enhanced performance specifically under 'Learned Non-Reward' conditions. There was no main effect of drug, but a significant effect of test condition, and drug x test condition interaction for the number of sessions and trials to reversal criterion (see Table 3.4 and Figure 3.9). Simple main effects analysis revealed that drug-treated animals were faster to reach criterion than vehicle-treated controls in the 'Learned Non-Reward' (*p's* < .05), but not the 'Perseverance' (*p's* > .05) condition, and control animals took just as long to reach criterion in both conditions (p's > .05). Drug-treated animals in the 'Learned Non-Reward' condition recorded both fewer correct and incorrect trials to reach criterion than controls, and analyses revealed a significant drug x condition interaction for correct trials, and a trend for an interaction for incorrect trials to criterion (*p* = .064). Despite this interaction not reaching significance, the pattern of responses across groups was very similar for both correct and incorrect trials (see Figure 3.9), and simple main effects analyses showed that drug-treated animals required significantly fewer correct and incorrect trials to reach criterion than vehicle-treated controls during the 'Learned Non-Reward' (*p's* < .05), but not the 'Perseverance' test (*p's* > .05). Control animals required the same number of correct (p's > .05) and incorrect (p's > .05) trials to reach criterion in both test conditions. Drug-treatment also significantly reduced trial-initiation omissions, trial-initiation latency and response latency across test conditions, with a significant main effect of drug treatment on these measures (See table 3.4 And Figure 3.9).

	Drug	*Condition*	*Drug x Condition*
	$F_{1,35}$	$F_{1,35}$	$F_{1,35}$
Sessions	*1.27*	***7.15****	***4.70****
Trials	*1.49*	***7.34****	***4.80****
Correct	*2.09*	***7.53****	***4.37****
Incorrect	*1.29*	***4.73****	*3.65~*
Initiation omissions	***9.59****	*0.41*	*1.46*
Response omissions	*3.18*	*0.18*	*0.17*
Retrieval omissions	*2.60*	*0.42*	*1.37*
Initiation latency (s)	***4.14****	*0.80*	*0.27*
Response latency (s)	***15.25*****	*1.48*	*3.05*
Retrieval latency (s)	*0.85*	*0.22*	*0.23*

Table 3.4. *Results of 2 (Drug: Vehicle/SB242084) x 2 (Condition: Learned Non-Reward/Perseverance) ANOVA comparing reversal performance. *p < .05, **p <.01, ***p < .001, ~p < .07.*

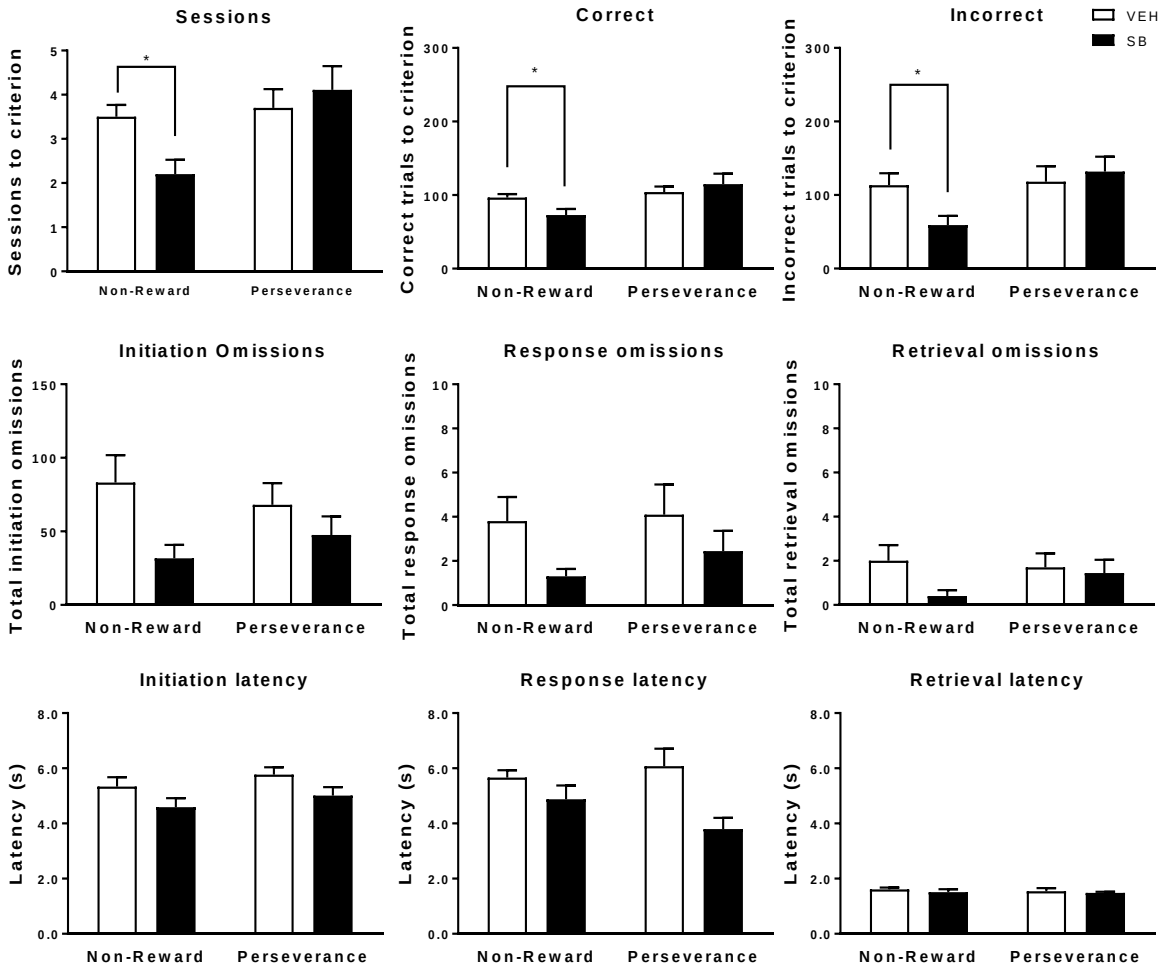

Figure 3.9. *Reversal learning results by test condition (Learned Non-Reward/Perseverance) and drug-treatment group (Vehicle/SB242084). Drug treatment reduced the number of sessions and trials (correct and incorrect) to criterion relative to controls in the Learned Non-Reward condition. Additionally, drug treatment led to a general speeding of responding (trial initiation latency and response latency) and reduced the number of trial initiation omissions across both conditions. *p < .05.*

Animals appeared to show a more accurate response strategy on correct than incorrect trials during reversal. This was partially confirmed by the analysis of response-pattern ratios, where a significant main effect of trial-type emerged (see Table 3.5); and pairwise comparisons showed that the probability of shifting on incorrect trials (following wins or losses) was significantly lower than the probability of staying on correct trials following a win (*p's* < .05), but not following a loss (*p* > .05). However, neither stay behaviour at the correct location nor shift behaviour at the incorrect location significantly differed in response to wins or losses (*p*'s > .05); suggesting animals were not sensitive to misleading feedback. There was no main effect of drug treatment on response-patterns; however, consistent with results on the main reversal measures, drug-treatment was found to enhance response-pattern accuracy specifically in the 'Learned Non-Reward' condition, with a significant drug x condition interaction (see Table 3.5). Simple main effects analyses showed that SB242084-treatment enabled a more accurate

response strategy (higher stay ratios on correct trials and shift ratios on incorrect trials) relative to vehicle-treated controls under 'Learned Non-Reward' ($p < .05$), but not 'Perseverance' ($p > .05$) conditions. This same pattern of results was seen when analysis was restricted to early reversal (0-50% correct), however drug-treatment was additionally found to convey a specific performance-enhancing effect on incorrect-rewarded trials (drug x trial-type interaction, see Table 3.5 and Figure 3.10), with greater 'shifting' relative to controls (simple main effects: $p = .005$); which could suggest reduced reward-sensitivity. Although this effect was greater in the 'Learned Non-Reward' condition (see Figure 3.10), the three-way drug x condition x trial-type interaction was not significant (see Table 3.5).

	Drug	*Condition*	*Trial-Type*	*Drug x Condition*	*Drug x Trial-type*	*Condition x Trial-type*	*Drug x Condition x Trial-type*
	$F_{1,35}$	$F_{1,35}$	$F_{2.0, 70.8}$ $F_{2.5, 9.6}$ $F_{2.1, 74.4}$ $F_{1, 35}$	$F_{1,35}$	$F_{2.0, 70.8}$ $F_{2.5, 9.6}$ $F_{2.1, 74.4}$ $F_{1, 35}$	$F_{2.0, 70.8}$ $F_{2.5, 9.6}$ $F_{2.1, 74.4}$ $F_{1, 35}$	$F_{2.0, 70.8}$ $F_{2.5, 9.6}$ $F_{2.1, 74.4}$ $F_{1, 35}$
Acquisition	*0.17*	*0.53*	*2.00*	*0.33*	*0.75*	*1.17*	*0.51*
Reversal	*0.70*	*0.96*	***8.97******	***4.16****	*2.51*	*1.50*	*1.05*
Early Reversal	*1.46*	***6.31****	***10.12*****	***7.05****	***5.42****	*1.94*	*0.97*
Retention	*0.36*	*<0.01*	*<0.01*	*1.31*	*0.36*	*0.83*	*1.11*

Table 3.5. *Results of 2 (Drug: Vehicle/SB242084) x 2 (Condition: Learned Non-Reward/Perseverance) x 4 (Trial-Type: Correct-Rewarded/ Correct-Punished/ Incorrect-Rewarded/ Incorrect-Punished) ANOVA assessing response-pattern behaviour (stay/shift) on subsequent trial, during each stage of testing. Early reversal refers to performance up to a criterion of 50% correct responses in any one session. The dependent measure was the percent of trials followed by a 'stay' response for correct trials, and percent of trials followed by a 'shift' response for incorrect trials. For retention data only, due to a lack of incorrect trials, results refer to a 2 x 2 x 2 ANOVA with only correct-rewarded and correct-punished trial-types. *p < .05, **p <.01, ***p < .001*

Retention (drug-free):

There were no significant differences in performance between groups during drug-free retention of reversal performance (data not shown). There were insufficient incorrect trials to analyse incorrect-shift response-patterns, but stay behaviour on correct rewarded and correct punished trials did not differ, and there was no effect of drug, condition, or any two- or three-way interaction effects (see Table 3.5).

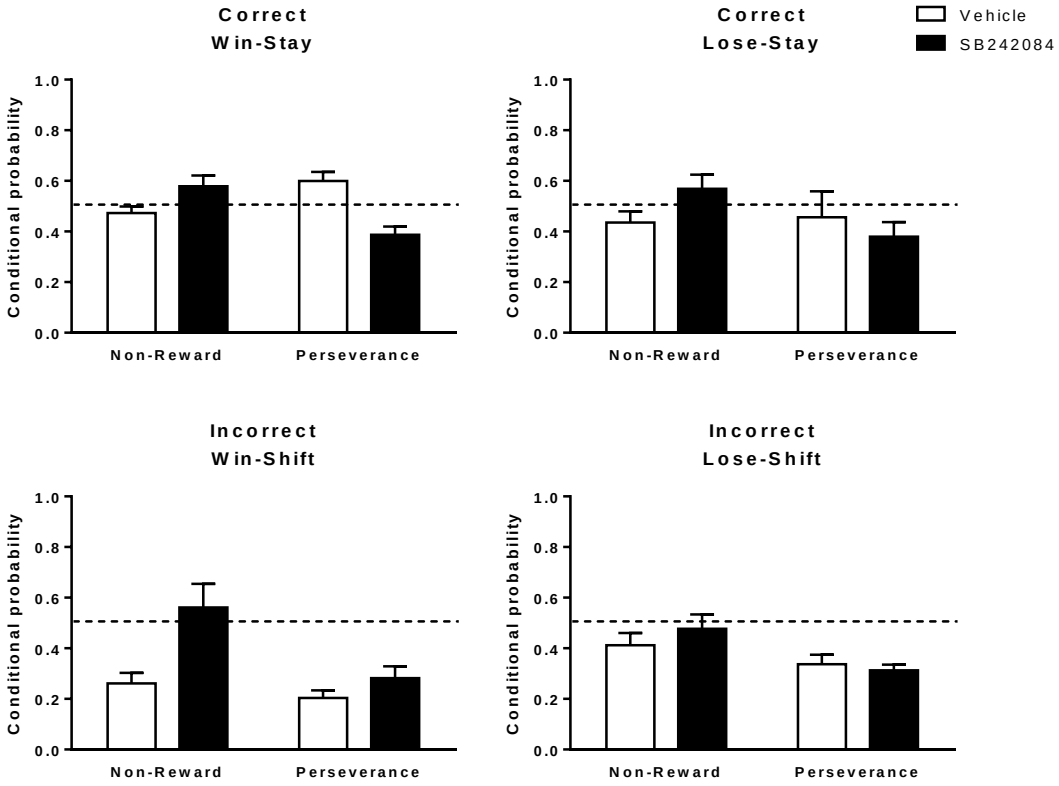

Figure 3.10. *Response-pattern data by drug and condition during early-stage reversal (0-50% correct) according to trial-type. Dashed lines represent chance level performance.*

3.4.3. Discussion:

These results support prior reports that SB242084-treatment specifically enables animals to better overcome learned non-reward at the previously correct location during reversal, without affecting perseverance at the previously incorrect location (Nilsson et al. 2012); demonstrating a consistency in the nature of drug effect across probabilistic and deterministic tasks. Analysis of response pattern data additionally identified a drug-related difference in performance not seen in the full-reversal test, with an increased ability to ignore misleading wins at the incorrect response location. A lack of effect on misleading losses suggests that this effect does not reflect enhanced ability to withhold inappropriate responses more generally, and could instead suggest reduced reward-sensitivity. However, this effect is not likely to mediate the performance-enhancing effects of drug treatment on reversal performance, as it was seen in both 'learned non-reward' and 'perseverance conditions', albeit to a differing degree. Nevertheless, this effect highlights the potential gains in explanative value PRL tasks can deliver.

Evidence for a reduction in the number of sessions and trials taken to reach reversal criterion following treatment with SB242084, which was specific to the 'Learned Non-Reward' test, is consistent with previous reports by Nilsson et al. (2012); providing evidence of 5-HT_{2C}R involvement in this particular aspect of reversal, even under probabilistic test conditions. Therefore, the drug-related reduction in the number of incorrect responses to criterion observed under the full reversal test of Experiment 2, despite being suggestive of reduced perseverance, appears instead to be driven by reduced avoidance of the previously incorrect location. Though incorrect trials to criterion are often construed as a proxy measure of perseveration, errors can just as easily be caused by an inability to overcome learned non-reward. Likewise, the number of correct responses to criterion can be driven either by the ability to overcome learned non-reward or perseveration. This further highlights the need to separate out the two different elements that contribute to reversal performance through the use of specific test conditions, rather than relying on simplistic inferences from the number of correct or incorrect trials observed.

Although this strongly suggests that the drug-related benefit observed during the PRL task is the same as that seen under deterministic conditions, enhancing the ability to overcome learned non-reward, other possible performance-related changes can be assessed through analysis of response-pattern data. Because probabilistic conditions make reversal shifts more difficult to detect by reducing sensitivity to individual feedback trials, a drug-related enhancement of performance could also be created by restoring sensitivity to immediate feedback, which would be reflected in a move towards a 'win-stay/lose-shift' pattern of responding. However, there was no evidence of this trial-by-trial response strategy during reversal for any group. Quite to the contrary, drug treatment was found to enhance model-based responding under 'Learned Non-Reward' conditions by increasing the likelihood of staying on correct trials and of shifting on incorrect trials, regardless of outcome. During early performance, there was even evidence of a drug-related reduction (rather than enhancement) of feedback sensitivity. This effect was restricted to RIR trials, with drug-treated animals better able to ignore this misleading feedback and shift away from the incorrect location. This drug-related reduction in sensitivity to misleading punishment was observed across both test conditions however, suggesting it was not responsible for the reversal advantage seen under 'learned non-reward' conditions. This shows that the nature of the drug-enhancement effect was not to allow faster detection of the need for behavioural adaptation.

Rygula et al. (2015) also report changes in sensitivity to misleading feedback in marmosets following manipulations of 5-HT during performance of the PRL task. Localised 5,7-

DHT-induced depletions of 5-HT in the amygdala led to an increase in the likelihood of obeying false information, as well as less discrimination between misleading and accurate feedback. However, this effect was accompanied by reduced suppression of responding during punished trials in a variable interval test of punishment sensitivity; which could suggest impaired ability to inhibit inappropriate responses. In light of these findings, the current results could be interpreted as evidence of better integration of long-run reinforcement histories and/or improved ability to inhibit inappropriate responding. This latter interpretation does not fit with evidence from this, and previous studies, of a drug-related reduction in omissions and response latencies however. SB242084 was shown to reduce trial initiation omissions, response omissions and reward retrieval omissions, though unlike for the full reversal test the drug effect did not reach significance for the latter two measures. This is likely due to floor effects, since both groups made very few response and retrieval omissions compared to the full reversal test. As during full reversal, there was also a drug-related reduction in latency to initiate a trial and to make a response, but the effect on reward retrieval latency was not significant, again possibly due to floor effects. The size of drug effect was also similar across both test conditions, consistent with suggestions that this is a general motor impulsivity effect of drug treatment. This is consistent with findings from Chapter 2, where the same general speeding was reported despite the reversal impairment effect of SB242084; suggesting this effect is unrelated to reversal performance.

The specificity of the current finding, of reduced sensitivity to misleading RIR but not PCR trials, also tends to rule out simple response-inhibition explanations, as well as signifying an effect on reward rather than punishment sensitivity. Although no change in responding to reward on correct trials was evident following drug-treatment, responding to misleading feedback is likely to be a more sensitive measure of reward-sensitivity. The nature of this effect may not appear to fit with existing evidence of the role of 5-HT in emotional processing, given growing evidence for a role in mediating response to losses rather than rewards, but there are several lines of research showing the possible involvement of 5-HT in coding reward value. Electrophysiological recordings of putative 5-HT neurons in the dorsal raphe nucleus display tonic activity during anticipation of reward but show no change in firing following an unexpected reward omission (Miyazaki et al. 2011). Using a four-armed bandit paradigm, where the probability of receiving reward and punishment varied independently from each other, and across locations, Seymour et al. (2012) were able to demonstrate that central 5-HT loss through dietary tryptophan depletion, selectively impaired the behavioural and neural representations of reward outcome value during a probabilistic instrumental learning task in humans. Bari et al.

(2010) have also previously reported changes in both reward sensitivity and punishment sensitivity during the PRL task, depending on the serotonergic manipulation employed. Acute manipulations affected punishment sensitivity, whilst long-lasting treatments specifically affected reward sensitivity. The authors suggest that these latter effects might result from interactions between the 5-HT system and DA, which is more strongly implicated in reward processing (Nestler & Carlezon 2006). Despite the acute manipulation used in the current design, this effect on reward-sensitivity might also be related to interactions with the dopaminergic system, given the known relationship between 5-HT_{2C}Rs and DA transmission.

It is perhaps the direction of the current effect that is most at odds with existing research however. Whilst the data relating specifically to the role of 5-HT_{2C}Rs is lacking, the current finding of reduced reward-sensitivity following administration of SB242084 is inconsistent with evidence that this drug acts to increase dopaminergic neurotransmission in the mesolimbic DA system, which would in turn be expected to increase responsivity to reward-related cues. Though, within the context of the PRL task, increased shifting in response to reward at the incorrect location would in the long-run enable animals to experience more reward (since it increases the number of correct responses). Further research may therefore be needed to establish the role of SB242084 in reward processing using tasks that can more clearly separate out changes in reward and punishment sensitivity, such as that employed by Seymour et al. (2012); though how such complex tasks might be adapted for use in animals models is a complication which must be overcome. Although this potential effect on reward-sensitivity could be of great clinical interest, given the number of conditions associated with impaired reward processing, evidence that this SB242084 effect was seen in both the 'learned non-reward' and 'perseverance' test conditions (albeit to a lesser degree), suggests this effect may be independent from its effects on cognitive flexibility demonstrated under learned non-reward conditions. This fits with previous findings in the human literature, with evidence that a polymorphism in the gene encoding the 5-HT transporter (SERT) affects lose-shift behaviour in a PRL task without affecting reversal performance (den Ouden et al. 2013). Consequently, the effects of SB242084 on reward-sensitivity, though far from clear, do not appear to contribute to its ability to affect reversal learning performance.

The current results support previous findings that SB242084 confers a significant beneficial advantage in the ability to overcome learned non-reward without affecting perseverative behaviour. These results also help to rule out several further possible explanations for the drug-related enhancement conferred. Firstly, evidence that drug-treatment led to a general enhancement of response-pattern accuracy within the Learned Non-Reward condition,

by both increasing 'shifting' at the incorrect location as well as 'staying' at the correct location, tends to rule out explanations surrounding a drug-related change in sensitivity to immediate feedback that would encourage a 'win-stay/lose-shift' pattern of responding. Secondly, during early reversal performance drug-treatment was found to specifically increase shifting in response to misleading reward, but not misleading punishment, ruling out the possibility of changes in response inhibition or more efficient detection of misleading feedback more generally. This could suggest diminished reward-sensitivity under drug-treatment, but as the effect was seen under both learned non-reward and perseverance conditions, it cannot account for the drug-related enhancement of learned non-reward. Therefore, the role of SB242084 in cognitive flexibility appears to be specifically related to the ability to overcome learned non-reward at the previously incorrect response location, in line with evidence from deterministic tasks.

3.5. GENERAL DISCUSSION:

The current chapter provides evidence that mice are capable of acquiring, maintaining and reversing learned associations on the basis of probabilistic feedback. This contests previous suggestions that mice may not be capable of accurate response strategies under conditions that include rewarded incorrect trials, and set a probability of inaccurate feedback as high as $p = 0.2$. This difference in outcome is most likely explained by one major design amendment – the use of between-session rather than within-session reversals. The use of distinct acquisition and reversal test stages, where response-outcomes are relatively stable across time, provides an environment that more readily allows for model-based response strategies to form and for probabilistic outcomes to be more clearly distinguished from reversal shifts. This modification allowed us to demonstrate that mice are not only capable of responding efficiently under more challenging PRL tasks, but that the method used to solve this task is similar to that seen in humans. Mice were not adopting a trial-by-trial 'win-stay/lose-shift' strategy, but rather implementing a response-set that was rewarded on the majority of trials, attesting to the translational value of the task.

There was also evidence for a drug-related enhancement of reversal learning performance during the full reversal test, which was not seen under the accurate feedback conditions of Chapter 2. Nevertheless, this is in line with prior findings reported by Nilsson et al. (2012) using a deterministic feedback task, so is unlikely to be related to the use of the PRL task specifically. Comparison of control group performance during each of these prior studies

suggests that task difficulty might instead interact with drug-treatment, with more challenging reversal tasks resulting in a drug-related enhancement of performance, compared to the impaired performance seen when vehicle-treated controls reversed more quickly. Although this requires further exploration, this is an intriguing possibility that is not without precedent in the literature. Bussey et al. (1997) report that lesions of the medial frontal cortex created a profound impairment in reversal learning in a visual touch-screen based task, but only when the stimuli were more difficult to discriminate. Investigating this possible role of task difficulty in the contribution of the prefrontal cortex to cognitive flexibility, Kim and Ragozzino (2005) also report that inactivation of the OFC via infusion of muscimol caused a more profound impairment in an odour discrimination reversal learning task under the harder four-choice test than was seen under the easier two-choice condition. Such findings suggest that the OFC may be more fully engaged under difficult reversal conditions, possibly due to increased attentional demands; suggesting that SB242084 could gain its positive effects on reversal performance through actions within this region. Given the systemic manipulations employed in each of these prior tasks, it is also possible that these performance-enhancing effects are mediated by actions in different regions to the performance-impairing effects reported under easier task conditions. Examining the effects of localised infusions of SB242084 on performance of reversal learning tasks of differing degrees of difficulty would be necessary to further explore this possibility, which will require a more thorough understanding of the regions engaged by reversal learning tasks.

There was also evidence that drug-treatment specifically enhanced reversal performance under Learned Non-Reward, but not Perseverance conditions, despite evidence for a specific reduction in the number of incorrect and not correct trials to criterion during the full reversal test. This suggests that correct and incorrect responses are not accurate indicators of ability to overcome perseverance and learned non-reward, despite often being seen as proxy measures. This again points to a similarity of effect across deterministic and probabilistic feedback tasks, where this same effect on learned non-reward has previously been reported (Nilsson et al. 2012). The capacity for SB242084 to improve flexible cognition therefore appears underpinned by an improved ability to overcome learned non-reward at the previously incorrect (now correct) location.

Although the use of PRL tasks additionally enabled the exploration of affective processing, alongside the more purely cognitive function assayed in deterministic feedback tasks, there was no evidence that a drug-related change in reward or punishment sensitivity mediated the drug-related effect of performance on learned non-reward. There was evidence for a drug-related reduction in sensitivity to misleading feedback during the early stages of

reversal however, which was restricted to responses to probabilistic reward and not punishment. This ruled out general effects of drug-treatment on response inhibition or sensitivity to immediate trial outcomes, which would be expected to affect responses to misleading feedback of both valences. Though this could suggest a reduction in reward-sensitivity, this effect was not seen in the full-reversal test, nor was it seen in response to reward on accurate feedback trials. This effect therefore requires further exploration, but is potentially of great clinical relevance given the number of disorders believed to entail aberrant reward-sensitivity.

In sum, the experiments presented in this chapter provide evidence that PRL tasks can be successfully adapted for use in rodents, and produce behaviour that is consistent with human performance under similar conditions. A beneficial effect of SB242084 on PRL performance was also demonstrated, an effect that could not be attributed to a difference in sensitivity to misleading feedback more generally, or to reward/punishment sensitivity more specifically. The beneficial drug treatment effect was further localised to an improved ability to overcome learned non-reward at the previously incorrect location, rather than in overcoming perseverance at the previously correct location, an effect that extends findings previously reported under deterministic conditions. This drug-treatment effect, taken alongside prior findings, might demonstrate an interactive effect of 5-HT_{2C}R manipulations with reversal task difficulty, which could allow prior inconsistent findings to be reconciled.

CHAPTER 4

THE EFFECT OF 5-HT_{2C}R ANTAGONISM IN TESTS OF EXTINCTION AND LATENT INHIBITION

4.1. INTRODUCTION

Although the behaviour change associated with reversal learning could result from the loss of prior learning from the acquisition phase of the task, there is a variety of evidence to show that such simple 'unlearning' accounts are untenable. New learning is therefore believed to occur during the reversal stage, though the exact form this takes is debated. Reversal learning may therefore be more consistently conceived of as an example of an interference paradigm, where animals receive conflicting information about a CS in different stages of the task, resulting in two simultaneously available associations which act to hinder one another and must compete for behavioural expression (Bouton 1993; Todd et al. 2014). As such, it shares a broad likeness to several other interference paradigms which follow similar principles. Two which are of particular relevance here, encompassing the two interference aspects associated with reversal learning tasks (learning about the CS+ and the CS-), are extinction and latent inhibition.

Extinction paradigms examine responding to a CS which is presented without the US after previous CS-US pairings, and is often interpreted as an example of retroactive interference, because learning during Stage 2 of the task (that the CS no longer predicts reward), interferes with performance from Stage 1, reducing responding to the CS (Bouton 1993). Though reversal tasks are more often interpreted in terms of a proactive interference effect, extinction reflects the type of new learning that must occur to the CS- (previously the CS+) during Stage 2 of reversal learning, in order to overcome 'perseverative' responding at that location. By contrast, latent inhibition (LI) refers to the suppression of CS-US learning that occurs following repeated, non-reinforced presentations of the CS alone (Lubow & Moore 1959), when learning in Stage 1 causes proactive interference with performance during Stage 2. This shares a broad similarity with another crucial form of interference which must be overcome during Stage 2 of reversal learning, when new learning is required to occur to a CS+ which was previously not rewarded or irrelevant (previously the CS-).

The extinction and latent inhibition paradigms are clearly closely linked to the 'learned non-reward' and 'perseveration' conditions of reversal learning (see Table 4.1), and evidence

that $5HT_{2C}R$ antagonism affects the former but not the latter suggests that performance in these related paradigms might also be differently susceptible to serotonergic manipulation. Tests designed to model the 'perseverance' condition of a reversal learning task quite closely match extinction testing conditions, since a previously correct (rewarded) response becomes incorrect (non-rewarded), whilst the previously incorrect response is replaced with a novel correct response option. Under these conditions, there was limited evidence of impaired reversal performance in SB242084-treated animals relative to vehicle-treated controls in Experiment 3 of Chapter 3. Under 'learned non-reward' conditions of reversal, the conditions could be said to closely parallel those of latent inhibition tasks, since a previously non-rewarded response becomes rewarded. Within this task, $5\text{-}HT_{2C}R$ antagonism was found to significantly improve performance, potentially suggesting it may similarly enhance performance in latent inhibition tasks.

However, these forms of interference are unlikely to directly map onto those that occur during standard reversal learning tasks, since the simultaneous presentation of two stimuli can affect the relative learning that occurs to each, and the requirement to respond to just one stimulus on each trial necessarily impedes the expression of performance to the other. Furthermore, these paradigms are generally investigated in classical, rather than instrumental conditioning paradigms. This therefore provides an opportunity to further dissect the precise aspects of reversal learning affected by $5HT_{2C}R$ antagonism, to understand whether the effects observed in perseveration and learned non-reward conditions of reversal can extend to these related paradigms. The behavioural mechanisms, and the anatomical and neurochemical correlates of extinction and latent inhibition will therefore be examined in turn, in order to identify their potential relevance to reversal learning paradigms, and the possible involvement of serotonergic mechanisms in these phenomena.

Reversal condition	***Stage 1***	***Stage 2***	***Similar paradigms***
Perseverance	Rewarded (CS+)	Non-rewarded (CS-)	*Extinction*
	Non-Rewarded (CS-)	Novel rewarded (CS+)	
Learned Non-Reward	Rewarded (CS+)	Novel non-rewarded (CS-)	
	Non-Rewarded (CS-)	Rewarded (CS+)	*Latent Inhibition*

Table 4.1: *Representation of similarities between 'Perseverance' and 'Learned Non-Reward' conditions of reversal to extinction and latent inhibition paradigms, respectively. Note that in latent inhibition paradigms, the CS presented during stage 1 has no consequence (CS-nothing), rather than being a true CS-.*

4.1.1. EXTINCTION:

First characterised by Pavlov (1927), extinction is a complex phenomenon which despite being extensively studied since the 1970's, continues to defy simple explanation (Myers & Davis 2007). Extinction training refers to the process of repeatedly presenting a conditioned stimulus (CS) in the absence of the unconditioned stimulus (US) with which it was previously paired (Delamater 2004). Extinction refers to the decremental effect this procedure has on conditioned responding (CR) to the CS that was established during the initial phase, which can be measured both during the experimental procedure ('within-session extinction'), and at a later time ('extinction retention'), and which has been consistently observed across many different animal species (e.g. McNally & Westbrook 2003; Wellman et al. 2007), as well as in humans (e.g. Grady et al. 2016; Lerman et al. 1999; Lindberg et al. 2000). This decrease is also observed during instrumental conditioning, when a previously reinforced response stops being followed by the reinforcer (e.g. Nonkes & Homberg 2013; North & Stimmel 1960). Although research concerning the extinction of instrumental learning has only emerged more recently, the existing evidence suggests that a very similar set of principles may support both types of behaviour (Todd et al. 2014).

Many theories of Pavlovian conditioning and extinction adopt an associative framework, and one of the simplest associative mechanisms that can be proposed to govern extinction, which is a feature of some very influential theories (e.g. Rescorla & Wagner 1972; Wagner & Rescorla, 1972), is the destruction or 'unlearning' of the excitatory CS-US representations formed during acquisition. However, evidence that original learning is retained in extinction can be taken from several 'relapse' phenomena that display the recovery of the original conditioned behaviour. 'Reinstatement' refers to the recovery of conditioned behaviour observed following extinction training when the US is presented alone, independently of the CS (e.g. Rescorla & Heth 1975). 'Rapid reacquisition' refers to the swift return of responding to the CS observed when CS-US pairings are resumed following extinction (e.g. Napier et al. 1992; Ricker & Bouton 1996). 'Spontaneous recovery', first demonstrated by Pavlov (1927), is the observation that conditioned responding can also return with the simple passing of time; and greater time delays are associated with greater levels of spontaneous recovery (Robbins 1990). 'Renewal' refers to the restoration of an extinguished CR when extinction retention is tested in a context which differs from the one in which extinction training took place. There are several different renewal designs which can be employed, with the acquisition context, extinction context and testing (retention) context, all able to be altered. Renewal occurs not only when animals are tested in the original acquisition context (A) which differs from the extinction training context (B) in so-

called ABA renewal (e.g. Bouton & Peck 1989; Harris et al. 2000), but also when tested in an entirely novel context, either following acquisition and extinction training in the same (AAB renewal, e.g. Bouton & Ricker 1994) or different (ABC renewal, e.g. (Bouton & Bolles 1979) contexts.

Most of these 'relapse' effects have been demonstrated across a wide variety of tasks, including conditioned suppression, taste aversion, and appetitive conditioning (e.g. Bouton & King 1983; Bouton & Peck 1989; Rosas & Bouton 1997). Although the majority of the literature relates to Pavlovian extinction, there is evidence that these relapse effects also occur after instrumental extinction, though the evidence for the different forms of renewal was, until recently, less clear. Although ABA renewal has been consistently demonstrated using both food reward (e.g. Nakajima et al. 2000) and drug self-administration designs (Bossert et al. 2004; Crombag & Shaham 2002; Hamlin et al. 2008; Kearns & Weiss 2007), there are several reports that failed to demonstrate AAB (Crombag & Shaham 2002; Nakajima et al. 2000) and ABC renewal (Zironi et al. 2006), suggesting that instrumental extinction might not demonstrate the same context-specificity relative to conditioning as Pavlovian extinction. However, using a within-subjects design, Bouton et al. (2011) clearly demonstrated ABA, ABC and, for the first time in operant conditioning, AAB renewal effects following extinction of conditioned responding for food rewards. All animals were conditioned to lever press for food in context A, half were then extinguished in Context A and the other half in context B, then in a counterbalanced order all animals were returned to Context A as well as Context B. This same within-subjects design has since been successfully employed to demonstrate the existence of AAB renewal in extinction of operant responding for sucrose and high fat rewards (Todd et al. 2012).

Although these 'relapse' effects convincingly demonstrate that the CS-US association has not been completely destroyed during extinction, several researchers (Delamater 2004; Rauhut et al. 2001) have noted that renewal procedures often result in less CR in animals who underwent extinction training than in a control group who did not, even when animals were returned to the initial acquisition context (ABA renewal); suggesting that some unlearning may take place during extinction. Nevertheless, it is clear that unlearning cannot offer a complete account. Most associative theories of extinction therefore tend to propose that a second inhibitory association is formed to the US representation during extinction training, which directly opposes the original excitatory CS-US association formed during acquisition, thereby gradually leading to a loss of net activation. Support for these inhibitory association accounts of extinction can be taken from the similarities between extinguished CSs and conditioned

inhibitors: CSs that have a purely inhibitory association to the US (see Myers & Davis (2002) for a review). For example, 'overtraining' of extinction can produce a CS that passes summation and retardation tests for conditioned inhibition (Calton et al. 1996). However, evidence that reacquisition of conditioned fear to tone-shock pairings was faster in a group that had undergone extinction training than in a latent inhibition group that received multiple tone-alone exposures prior to the test phase, can pose a problem for inhibitory associative learning accounts, therefore the precise form of new learning that takes place during extinction is still debated. Though, as will later be discussed, the nature of learning during latent inhibition likely differs from that of conditioned inhibition.

Regardless of the exact from of learning that occurs, evidence that extinction performance may be lost with the passage of time (as seen in spontaneous recovery), or following removal from the extinction context (as in ABC and AAB renewal), suggests that the learning that occurs during extinction differs from the original excitatory learning. This has been explained in terms of inhibitory learning being more fragile and liable to disruption (Pavlov 1927), or as extinction learning being more context-dependent (Bouton 2007), only being retained when the context matches that which was present during learning. Evidence that extinction learning can also be readily retrieved under certain conditions tends to favour the latter explanation. For example, the degree of spontaneous recovery and renewal of conditioned responding can be dramatically reduced by the delivery of an extinction-related cue during testing (Brooks 2000; Brooks & Bouton 1993; Brooks et al. 1999).

This leads on to another now widely accepted key principle of Pavlovian and instrumental extinction; that context plays a fundamental role. Rather than posit that there is a weakening of stored information of either the acquisition or extinction test stage, differential retrieval hypotheses posit that both experiences remain equally intact, but that alterations that make the likelihood of retrieving one or other piece of information are responsible for performance differences (Bouton 1993; Miller et al. 1986). Although this could be proposed to be due to the direct formation of inhibitory learning between the context and the representation of the US during extinction (i.e. context-no US), there are several lines of evidence that appear to rule out this explanation (e.g. Bouton & Swartzentruber 1986; Brooks & Bowker 2001), and current theories propose that the context instead acts as an occasion-setter (Holland 1992), providing a cue that retrieves the meaning of an ambiguous CS. The context may be more important during extinction since the CS only becomes ambiguous at this stage, having two available meanings (Bouton 2007).

There is however a wealth of evidence which suggests excitatory representations (in aversive and appetitive preparations) are generally more stable across physical and temporal contexts than inhibitory associations (Bouton & King 1983; Bouton & Peck 1989; Bouton & Peck 1992; Gleitman & Holmes 1967). It may be that the higher adaptive value of excitatory learning necessitates its retrieval over time and physical contexts, whilst "the system seems to recognise that it is risky to accept the null hypothesis (i.e. that a CS means no US) on the basis of a single sample from a particular location or time" (Bouton 1993, p.92). This latter explanation is more consistent with evidence that the preexposure stage of learning is also more context-dependent in latent inhibition paradigms than the excitatory CS-US association formed during the conditioning stage, as will later be reviewed.

Context is not only provided by the specific environmental cues associated with the conditioning chambers however; and each of the 'relapse' phenomena discussed above can also arguably be understood as context effects (Bouton 1993; Bouton 2000). Reinstatement of conditioned responding by presentations of the US alone depends at least partly on the US conditioning the context, which is supported by evidence that reinstatement is weakened by delivering the US in a different context to CS testing (Bouton & King 1983; Bouton & Peck 1989). The CS-US pairings themselves form part of the conditioning context, therefore recent CS-US presentations following extinction allows for rapid reacquisition to occur. Furthermore, spontaneous recovery can be conceived of as a temporal contextual effect, where the passing of time provides an interoceptive cue that distinguishes the extinction context from the testing context (Todd et al. 2014). Animals have been shown to use time as a contextual cue to guide responding, with evidence that rewarded and non-rewarded trials can be discriminated on the basis of the relative length of the inter-trial interval (Bouton & Garcia-Gutierrez 2006; Bouton & Hendrix 2011). Other interoceptive cues have also been shown to guide behaviour, as seen in state-dependent learning where the drug effects themselves provide the context (e.g. Bouton et al. 1990). This however, points to a general problem with the ability to distinguish the effects of pharmacological manipulations on extinction from state-dependent learning, necessitating cautious interpretation of results when drug is introduced at either the extinction or retrieval stage of testing.

Context effects can also be used to explain the Partial Reinforcement Extinction Effect (PREE), which is the observation that partial reinforcement schedules (PRF) result in behaviour that is more resistant to extinction than continuous reinforcement schedules (CRF), which may be of particular relevance for assessing the role of extinction learning in probabilistic reversal tasks. This has been explained in terms of frustration theory, where the frustration of non-

reward has an arousing effect (Amsel 1958; Amsel 1962). In PRF schedules animals are reinforced for responding in the presence of frustration, and continue to respond for longer during extinction since this internal stimulus cue is still present. By contrast, sequential theory suggests that it is the memory rather than the frustration of non-reward that is responsible for the PREE (Capaldi 1967; Capaldi 1994). CRF schedules only ever reward subjects with a memory of recent reward, whilst PRF schedules reward subjects while remembering recent non-reward, a condition that is also met during extinction. Both explanations of PREE can therefore be conceived of in terms of context effects, with either frustration or memory of non-reward providing an internal contextual cue that makes the extinction context harder to distinguish from the acquisition context, thereby leading to persistence of behaviour. By contrast, the extinction context is much easier to discern under CRF schedules where non-reward is never experienced during acquisition.

Such advances in the understanding of the behavioural explanations of extinction have stimulated fresh interest in the underlying neurobiological mechanisms in recent years. The past decade has seen substantial advancements in the neurobiological study of extinction, though there remains a lag in the study of the neural mechanisms of appetitive extinction. It seems likely however that both forms of extinction learning, as with conditioning, are distributed across a network of structures, including the BLA, the periaqueductal gray, the hippocampus, and the mPFC; though these regions appear to be differentially involved in the acquisition, consolidation and retrieval of extinction, as well as in the contextual modulation of extinction memory (Quirk & Mueller 2008), and indeed, the PrL and IL regions of the PFC may even have opposing roles in extinction (Laurent & Westbrook 2009; Peters et al. 2009).

In line with evidence for the differential involvement of these brain regions in extinction learning, investigations into the neurochemical mechanisms have implicated several systems which also appear to be differentially involved in the various stages of extinction testing. Consistent with the belief that extinction comprises new learning, glutamatergic NMDA receptor activity is found to be important for the consolidation of extinction, since systemic administration of the partial NMDA receptor agonist D-cycloserine (DCS) is found to enhance extinction retrieval when administered prior to, or just after within-session extinction, both during fear extinction (e.g. Langton & Richardson 2010; Walker et al. 2002; Woods & Bouton 2009) and in non-fear related paradigms (e.g. Botreau et al. 2006; Groblewski et al. 2009; Myers & Carlezon 2010; Nunnink et al. 2007; Yang et al. 2013). However, evidence that DCS-treated animals still display renewal of extinguished behaviour when tested in the original conditioning context (Bouton et al. 2008; Woods & Bouton 2006) suggests that the context-dependence of

extinction learning is not affected by this manipulation, thus there is no qualitative change in the nature of learning. Although state-dependent explanations are possible when drug manipulations are administered prior to extinction training, evidence of consistent effects when DCS was administered post-training; as well as evidence that NMDA receptor antagonists are shown to exert the opposite effect, acting to impair extinction retrieval (e.g. Santini et al. 2001; Suzuki et al. 2004), tend to rule out these simpler explanations. Following such extensive pre-clinical interest in NMDA receptor mediated mechanisms of extinction, DCS treatment is now seen as an effective clinical adjunct to exposure therapy for the treatment of a number of disorders (e.g. Guastella et al. 2007; Hofmann et al. 2006; Smits et al. 2013).

Given the proposed inhibitory learning that occurs during extinction learning, the role of GABA has also been the focus of much research. In a conditioned freezing paradigm using clicker-shock pairings, Harris and Westbrook (1998) show that the $GABA_A$ receptor inverse-agonist FG 7142, which acts to antagonise the inhibitory effects of GABA, can slow both the acquisition and retention of extinction of fear relative to vehicle-treated controls, following systemic administration prior to within-session extinction, prior to extinction-retention, or both; ruling out state-dependent explanations. The authors suggest that this may be mediated by the drugs' effect on the contextual retrieval of extinction, since administration of FG 7142 only reversed extinction when rats were tested in the extinction context (ABB renewal), and not in a novel context (ABC renewal). Potentiation of GABA by the benzodiazepine agonist CDP has conversely been found to facilitate extinction of learned fear responses (Stowell et al. 2000). Importantly, this latter effect has also been replicated in positively-reinforced behaviour in an operant food-reward design using a variety of GABA potentiators (Leslie et al. 2004), showing that GABA does not simply mediate fear responding or behavioural suppression, and that it plays a role in both Pavlovian and instrumental extinction.

Though receiving considerably less attention, the role of the endogenous dopamine (DA) system has also been of interest in relation to extinction, due to its key role in modulating both appetitive and aversive learning (see Pezze & Feldon 2004 for a review). Clues to the potential involvement of DA in mediating extinction learning might also be taken from its likely involvement in signalling reward prediction-error (Montague et al. 1996; Schultz et al. 1997) and/or incentive-salience (Robinson & Berridge 1993; Berridge 2007). The role of DA in extinction processes is of particular importance here, since activity at $5HT_{2C}R$ is shown to exert a tonic inhibitory influence upon mesocorticolimbic DA transmission (V Di Matteo et al. 2001; Di Giovanni et al. 1999), therefore antagonism at these receptors is shown to disinhibit DA neurons within this pathway. Early studies examining the role of DA largely focussed on the role of

psychostimulants in mediating extinction learning. In a pair of studies, Cole (1967, 1970) showed that several doses of amphetamine (0.5/1.0/2.0 mg/kg) administered 15 minutes prior to testing led to depressed responding during extinction of an operant food-rewarded task, where short periods of reward availability were interspersed with non-reward. However, responding was also impaired during rewarded periods relative to controls, and caution must be taken in interpreting these outcomes given the known anorexic effect of amphetamine, which acts to decrease food intake under free-feeding conditions (Cole 1972). By comparison, when reward takes the form of direct electrical stimulation of the brain, a striking resistance of responding to extinction of lever-pressing behaviour has been demonstrated with pre-test administration of 2mg/kg of amphetamine, compared to saline-treated controls (Olds 1970). Furthermore, doses of amphetamine ranging from 1-2 mg/kg have been shown to cause a marked increase in responding during extinction in a negatively-reinforced conditioned avoidance task (Stone 1964). However, changes in responding were again not specific to extinction testing periods, leading to the possibility that amphetamine's well-documented effects on locomotor activation (e.g. Kuczenski & Segal 2001) could instead be responsible for these effects.

More recent evidence for the effects of enhanced DA transmission on extinction performance is also derived from psychostimulant administration, though the availability of more specific DA receptor ligands has aided investigations. In agreement with most prior evidence, Willick and Kokkinidis (1995) and Borowski and Kokkinidis (1998) demonstrated that systemic administration of cocaine, amphetamine, and the specific D_1 receptor agonist SKF 38393 all impaired fear extinction, observed as a potentiated startle response to a footshock-paired CS. SKF 38393 also impaired extinction retrieval in a separate group of fear-extinguished animals when administered prior to a retention test alone. However, Delamater (2004) argues that despite the authors' claims, state-dependent mechanisms have not been convincingly ruled out as an explanation for these findings. Similarly, Nader and LeDoux (1999) demonstrate that pre-extinction administration of the D_2 receptor agonist quinpirole impaired retrieval of fear extinction on a separate drug-free test day, suggesting that both D_1 and D_2 receptors may be similarly involved in mediating extinction retrieval; though no controls for state-dependent effects were employed in their design. On the contrary, systemic methylphenidate administration has been shown to enhance extinction learning and retention when administered either before or after extinction testing (Abraham et al 2012), though this drug acts to inhibit the reuptake of both dopamine and noradrenaline, so it is not clear to what extent dopaminergic processes are responsible. Contradictory evidence of enhanced extinction retrieval following a boost in DA transmission through administration of its biosynthetic precursor L-dopa has also

been reported however. Post-extinction L-dopa administration has been found to enhance retrieval of extinction learning, with evidence that it reduces spontaneous recovery, reinstatement, and ABA renewal of conditioned fear in both humans and mice (Haaker et al. 2013); suggesting there may be a qualitative change in the nature of extinction memory by this treatment, causing it to become context-independent.

There is less evidence concerning the effects of reduced DA receptor function on extinction performance, though there is some suggestion that it can have a facilitatory effect. Early work examining the role of neuroleptics, which broadly act to block DA receptors, have been hampered by their general inhibitory effects on locomotor activity (Mason 1984). However, whilst drugs such as haloperidol, pimozide and chlorpromazine have been shown to generally depress lever-press responding in a Sidman discriminated avoidance task, where responding has the effect of delaying shock presentations; this effect was significantly larger during extinction periods (Niemegeers et al. 1969), suggesting an extinction-enhancing effect of DA blockade. There have been several further reports that DA blockade can enhance extinction learning (e.g. Fowler 1974; Stolerman 1971), most critically in a negatively-reinforced taste aversion task, where extinction involved an increase in active responding, thereby overcoming the confound of general motor inhibition (Grupp 1977). Systemic pre-extinction administration of the D_2 receptor antagonist sulpiride has also been found to enhance extinction memory retention using a conditioned fear paradigm in mice, even when using spaced CS presentations which typically result in weak extinction retention (Ponnusamy et al. 2005). However, D_1 receptor knockout (KO) mice reportedly display abnormal persistence of fear responding during extinction retention tests compared to their heterozygote and wild-type siblings, persisting in tests given up to 90 days after the initial acquisition (El-Ghundi et al. 2001). Though there were significant discrepancies in task design between these two studies, others have also reported delayed extinction of a conditioned fear response (Morrow et al. 1999) as well as impaired extinction retention (Espejo, 2003) following 6-OHDA lesions of the dopaminergic fibres of the mPFC. This suggests that the impact of abolishing DA receptors, through lesioning or genetic inactivation, differs from the effects caused by temporary pharmacological inactivation, and compensatory mechanisms may instead account for differences in outcome. Espejo (2003) reports that mPFC DA loss led to a reactive enhancement of accumbal dopamine release and metabolism, therefore enhanced responding in subcortical DA neurons could be responsible for these impairment effects (Myers & Davis 2002). Overall, there is some debate over the effect of DA manipulations on extinction performance, though there is growing evidence that manipulations which serve to boost systemic DA generally result in extinction impairments,

though the evidence on retention is less clear; whilst DA depletion may act to enhance extinction learning and retention, at least in aversive tasks.

Relatively little work has been conducted in examining the specific involvement of 5-HT in extinction behaviour. Some early lesion studies of the medial raphe, which acts to deplete forebrain 5-HT, demonstrated increased resistance to extinction in lesioned animals compared to sham-operated controls in active avoidance tasks (Kovacs et al. 1976; Srebro & Lorens 1975), as well as faster running during extinction of a food-rewarded alleyway running task (Asin et al. 1979). Confirming the role of 5-HT depletion in this latter effect, and offering some evidence as to the receptor subtypes involved; the same effect on running speed during extinction has been observed following administration of cinanserin, a potent 5-$HT_{2A/2C}$ receptor antagonist (Rosen & Cohen 1973). Serotonin depletion by systemic administration of the 5-HT synthesis inhibitor PCPA is also shown to cause a marked resistance to extinction on both a passive step-down avoidance task, and a positively-reinforced instrumental task requiring animals to lever-press for food (Beninger & Phillips 1979). It should be noted that the latter effect was observed when using a continuous, but not a variable schedule of reward, which may offer a clue as to the psychological mechanisms involved; though no convincing argument has so far been offered (Mason 1983). These complementary findings in both active and passive response tasks are important, since they rule out an explanation in terms of the possible locomotor impairment effects of PCPA treatment; furthermore, they provide evidence of a role for 5-HT in controlling extinction of positively- and negatively-reinforced behaviours.

More recently, chronic administration of SSRIs have also been shown to affect extinction learning, though results are mixed. Pre-extinction administration of citalopram is shown to impair the acquisition of within-session extinction, though most likely through effects on the glutamatergic system, since this was accompanied by downregulation of the NR2B subunit of the NMDA receptor in the amygdala (Burghardt et al. 2013). By contrast, several experimenters report that chronic fluoxetine treatment facilitates the retention of extinction learning (Deschaux et al. 2011; Camp et al. 2012; Karpova et al. 2011); though differences in methodology, SSRI drug, and the time course of treatment might account for the discrepant findings. Though both drugs also act as direct antagonists at the 5-HT_{2C}R, fluoxetine shows higher occupancy levels compared to citalopram, which is a more potent 5-HT reuptake blocker (Pälvimäki et al. 1999). Furthermore, chronic SSRI treatment can cause upregulation of the 5-HT_{2C}R (Laakso et al. 1996), which might also account for a difference in outcome, and could suggest a critical role for this receptor subtype in extinction learning.

Conversely, elevations in brain 5-HT through systemic administration of 100mg/kg of the 5-HT precursor L-tryptophan, has been shown to slow responding during extinction of an active avoidance task (Mager & Klingberg 1973). However, baseline differences in the level of responding during acquisition between the L-tryptophan-treated and control group makes interpretation of these extinction effects difficult. Mice genetically modified for elevated 5-HT function, via the knockout (KO) of the 5-HT transporter (5-HTT), demonstrate normal acquisition and within-session extinction of conditioned freezing to a shock-paired CS, but exhibit a selective impairment in extinction retrieval (Narayanan et al. 2011; Wellman et al. 2007), again pointing to a role for 5-HT in extinction memory. Genetic variation in 5-HT signalling in mice, which parallels a common human polymorphism affecting serotonin transporter polyadenylation, has also been associated with persistent fear associations due to impaired retrieval of extinction learning (Hartley et al. 2012). Although the involvement of the different 5-HT receptor subtypes in extinction learning has received scant attention, Saito et al. (2013) report that systemic injections of the 5-HT_{1A} agonist tandospirone, both before and after within-session extinction, dose-dependently ameliorated the extinction deficit caused by developmental exposure to foot-shock stress in mice. However, this effect appears to be related to dopaminergic rather than serotonergic mechanisms, since 5-HT release in the mPFC was unaffected by treatment, whilst the extinction enhancing effect of treatment was associated with an increase in mPFC DA release. Coupled with evidence that chronic SSRI treatment can cause changes in glutamatergic signalling pathways (Burghardt et al. 2013), it seems likely that at least some of the observed effects of 5-HT are due to indirect actions in other circuits.

Evidence for the involvement of multiple other neurotransmitter systems in extinction learning, such as the noradrenergic, cholinergic, cannabinoid and peptide modulatory systems (see Fitzgerald et al. 2014), as well as the burgeoning interest in the molecular mechanisms, is testament to the diverse pharmacology of extinction, and of the interest this field of study has generated over the years. Although these different circuits, not to mention receptor subtypes, are likely to interact in ways that are currently not well understood, particularly due to their widespread and overlapping distribution in the brain, it is clear that pharmacological investigations are a fruitful avenue for future research, which will contribute to the general understanding of learning and memory processes, as well as in the development of clinical treatments for disorders as wide-ranging as phobias and schizophrenia.

In the current context, it seems clear that serotonergic manipulations are likely to affect extinction performance, and there is some indirect evidence to suggest that antagonism at $5HT_{2C}$ receptors may improve retrieval of extinction memory, though their role in the acquisition of

extinction is unclear. Prior evidence that 5-HT_{2C}R antagonism does not significantly affect performance under the 'perseverance' condition of a reversal learning task, presented in Experiment 3 of Chapter 3 and reported in Nilsson et al. (2012), further obscures predictions for the effect SB242084 might have in extinction tasks however. Therefore, the precise role of the 5-HT_{2C}R in this process warrants further attention, as this may be a critical aspect of its involvement in reversal learning.

4.1.2. LATENT INHIBITON (LI):

The literature concerning Latent Inhibition (LI), though showing many similarities to extinction learning in both design and performance effects, has developed largely independently, with many theories focussing on one phenomenon with little or no attempt to explain the other. This is largely due to the fact that the focus on extinction learning developed out of the interest in fear mechanisms, and exposure therapy treatment in humans; whilst the interest in latent inhibition (LI) stems from the observation that schizophrenic patients exhibit deficits in this task, providing the rationale for a useful model for testing the efficacy of antipsychotic drugs. The following section therefore seeks to highlight the similarities between extinction and LI learning, and to unify some of the theoretical and experimental literature, with a particular focus on evidence that each of these can be understood as an interference paradigm, in much the same way as reversal learning tasks.

When animals are repeatedly exposed to a stimulus without consequence prior to conditioning, they show a subsequent decrement in ability to learn that this stimulus predicts an important outcome. This interference effect of stimulus preexposure was first studied by Lubow and Moore (1959), who rather misleadingly termed it 'Latent Inhibition'. In standard classical conditioning designs, one group of animals is exposed to a CS that has no consequences, while a second control group receives no preexposure. Both groups are then exposed to the same CS which now predicts delivery of a positive (e.g. food) or negative (e.g. foot-shock) reinforcer US. Latent Inhibition (LI) is expressed as impaired conditioning in the preexposed group compared to the controls, and increasing the number of preexposures increases the degree of impairment observed (e.g. Siegal 1969). Although receiving slightly less attention than extinction, it too has been demonstrated in a range of both instrumental and classical conditioning paradigms, across a variety of species (e.g. Arwas et al. 1989; Chandra et al. 2010; (Konorski & Szwejkowska 1952; Lipp & Vaitl 1992; Lubow & Moore 1959; Rescorla 1971; Reiss & Wagner 1972), though in humans this typically requires an additional masking or distractor task

(Lubow & Gewirtz 1995). As with the phenomenon of extinction however, this seemingly simple task belies the complexity of processes involved; attested to by the fact that, some fifty years after its initial discovery, there is still little agreement on the nature of these processes, and no one account can readily explain the diversity of experimental findings associated with it.

Several experimenters have noted the similarities between habituation and LI. Both procedures involve the repeated presentation of a single stimulus, and the presence of each phenomenon is evidenced by a reduction in recorded behaviour. The former measures changes in unconditioned response (UCR) to the preexposed stimulus, whilst the latter examines the reduction in the subsequent development of a conditioned response (CR) to that stimulus, thereby measuring its associative strength (Lubow 1989a). It may be that a reduction in the UCR is therefore responsible for the reduced ability of the CS to elicit a CR; though the observation that similar principles may explain both phenomena goes no further to explaining the rules which govern them.

Despite similarities, there are multiple observations to suggest that the conditions which give rise to habituation are insufficient to generate LI. For example, Domjan and Siegel (1971) report that the number of tone presentations required to eliminate the UCR to the stimulus (5 preexposures) was significantly less than the number required to produce the LI effect (25 preexposures). Also, though particularly salient stimuli are believed to produce minimal habituation (Thompson & Spencer 1966), they are found to elicit rapid LI compared to less intense stimuli (Schnur & Lubow 1976). These different behavioural changes therefore appear to take place simultaneously; although there is some suggestion that common principles may govern LI and long-term habituation (e.g. Wagner 1976). Most critically, there is evidence to suggest that, unlike LI, habituation effects are context-independent. Habituation of the orienting response to a light stimulus presented in Context A is found to be unaffected by a switch to presentations of that stimulus in a distinctive context (Context B) in which it had not previously occurred (Channell & Hall 1983). However, in a subsequent appetitive conditioning stage where the light served as the CS signalling reward, animals showed significant LI when tested in Context A, where preexposure to the light had occurred, but no evidence of LI when tested in Context B. This context-specific nature of LI, in common with extinction, has been consistently demonstrated (e.g. Hall & Honey 1989; Bouton 1991), and is one of the key observations that any successful theory of LI must be able to account for.

One of the simplest theories, hinted at by the terminology its founders used to describe the phenomenon of 'Latent Inhibition', is the suggestion that through the course of preexposure

a CS - 'no US' relationship develops, and the stimulus comes to act as a conditioned inhibitor. Certainly, the ability for a stimulus to retard the development of a conditioned response (as is seen in LI) is one of two fundamental criteria for identifying a conditioned inhibitor (Rescorla 1969). However, latently inhibited stimuli do not pass the second criterion during summation tests, since they do not cause a deficit in conditioned responding when presented in compound with a previously-trained excitatory stimulus (e.g. Reiss & Wagner 1972; Rescorla 1971; Solomon et al. 1974). What is more, if a stimulus acts as a conditioned inhibitor it should function to facilitate inhibitory conditioning, but a preexposed stimulus is not more readily trained as a CS- in discrimination tasks, but is similarly impaired when it serves as either the CS+ or the CS- (e.g. Halgren 1974). Theoretically, it is not immediately obvious how simple non-reinforced preexposure would allow a stimulus to develop inhibition, as the most information such a stimulus could convey would be that of 'no event', unlike conditioned inhibition training where the CS comes to signal the absence of an expected event (a given US) (Hall 1991). It is therefore more consistent to view the form of learning that occurs during preexposure as 'learned irrelevance' or 'insignificance' of the CS.

Associative theories of LI, a group of theories based upon the Rescorla-Wagner model of classical conditioning (Rescorla & Wagner 1972; Wagner & Rescorla 1972), have had relatively more success in explaining the phenomenon, though still no one theory seems to sufficiently account for all observations. The change that occurs during pre-exposure has been conceptualised as a reduction in stimulus 'salience', 'attention' or 'associability' in different formulations, though these terms essentially explain the same process. There are two broad forms of associative explanations however, focussing on a reduction in CS-US processing which is caused either by the extent to which the CS is predicted by its antecedents, or by the extent to which the CS predicts its consequences. Mackintosh (1975) proposed that the associability of a CS is determined not solely by its intrinsic properties, but by past experience with it. This was an important modification of the Rescorla-Wagner model, and was a concept also taken up by Pearce and Hall (1980) in their general account of classical conditioning. Both theories proposed that the associability of a stimulus is determined by how well the consequences of that stimulus can be predicted, however, for Mackintosh a stimulus is only attended to when its consequences are consistent and predictable, "on the basis that the world is a reasonably stable place, if a stimulus has previously been a poor predictor of changes in reinforcement, it is unlikely to be the cause of future changes" (Mackintosh 1983, p. 230). However, this is unable to account for findings that partial reinforcement during preexposure, which has the effect of making the CS a poor predictor of outcome, causes an attenuation rather than an increase in LI (Pearce et al.

1982). The Pearce-Hall (1980) account of LI is able to deal with this observation, since it uniquely predicts that a stimulus is only processed when it is *not* an accurate predictor of its consequences, such that LI is produced by a decline in the associative strength of the CS due to it fully predicting its consequences during pre-exposure (i.e. no outcome). There are several other theories which similarly emphasise the importance of stimulus consequences (e.g. Conditioned Attention Theory, Lubow 1989; Lubow et al. 1976); however, none of these accounts readily deal with the existence of context effects, and many have had to introduce special terms to be able to explain them; arguably with little success (Hall 1991).

A second group of theories also contest that the associability of a stimulus is a function of the discrepancy between presented and expected events, but instead propose that it is determined by the extent to which the CS is predicted by its antecedents; and as such have more success in accounting for context effects. Wagner's priming theory (1976, 1978) provides an information-processing account of LI, and McLaren et al. (1989) a mix of an associative-connectionist model, but both propose that LI occurs because the CS comes to be predicted by other cues. These cues can be the presentation of the stimulus itself, which activates an internal representation of the CS; or those of the experimental context, which have entered into an associative relationship with the CS during preexposure. During conditioning, the presence of these cues means that the CS is fully predicted, thus its associability is low, preventing it from entering into an association with the US. Both theories therefore specifically predict that a context-change after preexposure will reduce the degree of LI observed during conditioning, since these additional context cues that prime the CS activation are removed. However, their difficulty comes in explaining why the extinction of contextual cue associations, by exposure to the context alone after preexposure, is not consistently found to reduce the magnitude of LI (Hall & Minor 1984; *cf* Baker & Mercier 1982). Nor are they able to deal with the plentiful evidence that LI depends at least partly on what the stimulus predicts (Hall 1991), as observed by the partial reinforcement effect.

Clearly, neither group of these predictive associative theories is successfully able to account for the full scope of experimental evidence relating to LI, though some modifications to each account may be able to rescue them. Perhaps more worryingly for all theories which emphasise that preexposure interferes with the formation of the CS-US relationship however, is evidence that a perfectly normal associative relationship can be revealed under certain conditions. For example, similar to the extinction effect, unsignalled presentations of the US between conditioning and a subsequent test stage can reveal a level of conditioned responding to the CS which almost matches that seen in control subjects who received no CS pre-exposure

(Kasprow et al. 1984). Furthermore, spontaneous recovery of the CS-US relationship can be observed, again paralleling that seen following extinction training. That is, when a longer delay is introduced between conditioning and test, less LI is observed than at shorter intervals (Kraemer & Roberts 1984). These effects have led some researchers to speculate that, rather than a failure of learning, LI reflects a failure of retrieval (e.g. Kasprow et al. 1984; Miller et al. 1986), though the specific mechanisms of interference are often not supplied.

As with extinction therefore, LI effects may be best understood in terms of context effects, with the context supplied either by the experimental chamber itself, the US, or the passing of time. However, retrieval failure hypotheses rest on the assumption that, following the first conditioning trial where CS-US are associatively paired, there will be two competing available CS associations, and the LI performance deficit results in the former association being initially stronger than the latter. However, poorer performance is typically seen in preexposed animals on the very first trial of conditioning, suggesting that this too may offer an incomplete account (Lubow 1989b). Furthermore, evidence from both extinction and latent inhibition designs suggests that the retrieval of the CS-US association is never complete, as compared to controls who have not undergone extinction or preexposure.

Taken together, it seems likely that a successful account of LI will require us to accept some aspects of each of these previous groups of theories. It is quite possible that preexposure to a stimulus results in both a retardation of new learning about that stimulus as well as forming a memory trace that interferes with any subsequently formed during conditioning (Hall, 1991). Bouton (1993) proposes an interference effect controlled by context, though additionally makes clear that interference could result from either a performance or an acquisition deficit. He was also one of the first researchers to note the similarity between LI and extinction, and describes them as examples of proactive and retroactive interference, respectively; and as such, provides an explanation for both phenomena without recourse to different theoretical underpinnings/ behavioural explanations.

Due to similarities between LI and the attentional deficits observed in schizophrenia (i.e. an inability to ignore irrelevant stimuli), and evidence that some schizophrenics show impaired LI (e.g. Baruch et al. 1988); much of the evidence relating to its pharmacological basis derives from the amphetamine-induced model of schizophrenia. Amphetamine, an indirect DA agonist, can cause psychotic symptoms in healthy individuals (e.g. Bell 1965), and exacerbate these symptoms in schizophrenic patients (e.g. Janowsky et al. 1973), which has also served as a useful model for testing the efficacy of antipsychotic drugs (APDs). Systemic amphetamine treatment

has been firmly established to disrupt LI in schizophrenic patients (Gray et al. 1992), rats (e.g. Joseph et al. 2000; Moran et al. 1996; Ruob et al. 1997; Russig et al. 2003; Weiner et al. 1997) and more recently, mice (Chang et al. 2007). These effects are found to be mediated by actions in the mesolimbic DA system (Gray et al. 1997; Solomon & Staton 1982) and are specific to the conditioning stage, with administration of amphetamine during preexposure alone producing no effect on LI (Weiner et al. 1984, 1988).

Conversely, most APDs have been found to potentiate LI under conditions which are insufficient to produce it in controls (e.g. Feldon & Weiner 1991; Moran et al. 1996) and to block the amphetamine-induced disruption of LI in both rats (e.g. Solomon et al. 1981; Warburton et al. 1994; Joseph et al. 2000; Moran et al. 1996) and in schizophrenic patients (e.g. Baruch et al. 1988), which suggests that APDs' effects on LI are mediated by their antagonistic action at DA D_2 receptors. As with manipulations which increase DA transmission and act to disrupt LI, the potentiating effect of reduced DA activity is also shown to critically depend upon administration during conditioning (Feldon & Weiner 1991; Shadach et al. 1999, 2000; Weiner et al. 1987; Weiner et al. 1996, 1997b), thereby ruling out simple state-dependent explanations for these effects. Furthermore, this suggests that DA plays a role not in mediating attention or salience to irrelevant stimuli, but in affecting the behavioural control these stimuli have on performance (Weiner & Arad 2009). If conditioning can be said to reflect the response-switching stage, when two different associations are available, it suggests reducing DA impairs the switch in responding toward current, rather than previous demands; which might explain why schizophrenic patients have such difficulty ignoring irrelevant stimuli.

However, the effects of typical and atypical APDs on LI have been found to differ, and since atypical APDs block 5-$HT_{2A/2C}$ receptors in addition to the typical action of antagonism at D_2 receptors, this can offer a clue as to the role of serotonergic mechanisms in LI. Shadach et al. (2000) tested the effects of typical (haloperidol) and atypical (clozapine) APDs, as well as the 5-HT_{2A} antagonist ritanserin, on LI when administered during preexposure, conditioning, or both test stages. They found that, under conditions which do not produce LI in controls, clozapine and haloperidol caused LI when administered during conditioning or both stages, but not when administered during preexposure, consistent with their D_2 receptor antagonising effects; whilst ritanserin was without effect. However, under conditions where LI is seen in controls, haloperidol was without effect, consistent with previous findings (e.g. Shadach et al. 1999; Weiner et al. 1987, 1997b); whilst clozapine only disrupted LI when administered during preexposure. Critically, ritanserin also disrupted LI during preexposure and not conditioning, but unlike clozapine, it also disrupted LI when given during both stages. The disrupting effect of

clozapine during preexposure is therefore most likely due to its 5-HT_{2A} antagonising effects, and the reason that no effect is observed when administered during both phases is due to its actions during conditioning ('typical' D_2 mediated) overriding its actions during preexposure ('atypical' 5-HT_{2A} mediated). This may explain previous reports that administration of clozapine is without effect on LI (e.g. Dunn et al. 1993).

Potentially complicating findings however, are reports that LI can be enhanced by administration of the 5-HT_{2A} receptor antagonists SR 46,349B and ICI 169,369, but only when given at both preexposure and conditioning stages (McDonald et al. 2003). However, unlike ritanserin (Moser et al. 1995) these drugs were also effective at reversing the amphetamine-induced attenuation of LI, suggesting some degree of dopaminergic action of these drugs, which might go some way to explaining this discrepancy. More consistently, the atypical APDs risperidone and olanzapine are, like clozapine, shown to disrupt LI when given during preexposure only (Mongeau et al. 2007; Weiner et al. 2003). Furthermore, dose-dependent differences in the effect of clozapine on LI have been reported in the literature (e.g. Trimble et al. 1998), consistent with its relative SB for D_2 and $5HT_{2A}$ receptors at high and low doses, respectively; seemingly confirming the difference in effect of DA and 5-HT manipulations on LI.

Manipulations which deplete brain 5-HT, and which have typically been administered prior to testing, are also shown to disturb LI. Depletion of 5-HT by systemic administration of PCPA prior to a single 100min stage of preexposure and conditioning has been found to abolish LI in an active avoidance task in rats, under conditions which produced LI in controls (Solomon et al. 1978). Consistent with evidence from selective DA manipulations, this effect is believed to be mediated by the mesolimbic serotonergic system, with depletion of 5-HT in the medial but not the dorsal raphe system via electrolytic lesions acting to impair LI in the same procedure (Solomon et al. 1980). Asin et al. (1980) confirmed this effect of medial raphe lesions on LI in the same active avoidance task, though it was without effect in a taste aversion task; and Cassaday et al. (1993b) further implicate the role of mesolimbic 5-HT terminals, with their demonstration that 5-7DHT lesions of the fornix-fimbria, which acts to significantly reduce hippocampal 5-HT levels, also attenuated LI of a conditioned suppression response.

The role of 5-HT in mediating LI has also been observed in a deficit model, where manipulations of 5-HT during the preexposure stage act to restore an impairment in LI caused by genetic deletion of the HPC-1/syntaxin 1A (STX1A) protein complex in mice (Fujiwara et al. 2010); a polymorphism for which has been linked to schizophrenia in humans (Wong et al. 2004). This attenuation of LI could be restored through administration of the SSRI fluoxetine and the 5-

HT_{2A}R agonist DOI, but not by the 5-HT_{2C}R agonist mCPP, the 5-HT_{1A}R agonist 8-OH-DPAT, or by a range of different dopaminergic and noradrenergic manipulations. This supports the assertion that 5-HT is preferentially involved in mediating LI during the preexposure phase, and that it may be specifically $5HT_{2A}$R mediated, though the role of $5HT_{2C}$R and 5-HT_{1A}R antagonists are yet to be investigated. Taken together, this suggests a role for serotonin in mediating stimulus salience or the associability of irrelevant stimuli during stimulus-nothing learning, with depletions of 5-HT and/or antagonism at (most probably) 5-HT_{2A}R acting to disrupt LI. This is consistent with evidence that 5-HT may be involved in the tuning out of irrelevant or non-reinforced stimuli in spontaneous alteration tasks. Therefore, depletions of 5-HT and increases in DA both act to disrupt LI, but they appear to do so at different stages, lending support to two-stage models of LI.

However, 5-HTT knockout (KO) rats, who show enhanced extracellular 5-HT levels, have demonstrated reduced LI compared to both non-preexposed controls and their wild-type counterparts (Nonkes et al. 2012). These animals also showed improved performance in the early 'perseverative' stage of an extra-dimensional set-shifting (EDSS) task, which the authors suggest may have been mediated by this LI effect, due to temporal similarities in occurrence. This is in line with evidence from the Experiment 2 of Chapter 3, that reversal benefits of 5-HT_{2C}R antagonism are seen early in testing, and might suggest a similar mechanism. However, once again 5-HTT KO animals appear to behave in a manner which contradicts the pharmacological evidence, suggesting a critical difference in genetic 5-HTT deletion and temporary pharmacological blockade. Adaptive changes in 5-HT homeostasis have been reported in 5-HTT KO mice, with a marked change in the expression and function of various 5-HT receptors as well as a depletion of 5-HT tissue stores (Bengel et al. 1998), which may not be adequately compensated for by increased 5-HT synthesis (Lesch & Mössner 2006); possibly explaining this anomalous finding.

Further complications arise from more recent evidence concerning the metabolism of 5-HT and DA in the rat brain during these different stages of LI testing however. Since changes in monoamine oxidase (MAO) activity are concomitant with changes in neurotransmitter levels in the synaptic cleft, Molodtsova (2002, 2003) took measures of MAO activity in the terminal regions of the dopaminergic and serotonergic systems (amygdala, striatum, hippocampus and prefrontal cortex) during the preexposure and conditioning stages of an active inhibition and passive avoidance LI task. Both 5-HT and DA metabolism were affected during preexposure, but in different directions and in different regions. The 5-HT-deaminating activity of MAO increased in the amygdala and striatum, while the DA-deaminating activity of this enzyme decreased in

the amygdala and hippocampus. However, during conditioning, high levels of 5-HT deamination were again observed in the amgygdala and striatum, with low levels additionally seen in the PFC; but DA metabolism was unaffected. This is consistent with evidence that DA and 5-HT exert opposing effects on LI, but contradicts pharmacological evidence which suggests that it is DA and not 5-HT which is critically involved in the conditioning stage. Instead, this suggests that enhanced 5-HT activity in subcortical regions produced by the preexposed stimulus is a principle biochemical mechanism underlying LI at both stages of testing (Molodtsova 2002). Clearly, further research is needed to fully understand the involvement of these systems, but it is clear that both DA and 5-HT are critically involved in regulating LI.

Though several other neurotransmitters have been studied in LI tasks, they have received far less attention. The role of the glutamatergic system, despite being of theoretical interest due to the emergence of the NMDA hypothesis of schizophrenia (Olney & Farber 1995), has received little attention due to evidence that acute, low-dose administration of NMDA receptor antagonists ketamine, PCP and the more potent and selective MK-801 leave LI intact (Aguado et al. 1994; Pålsson et al. 2005; Robinson et al. 1993; Weiner & Feldon 1992). However, there is evidence that the NMDA receptor antagonists can elicit abnormally persistent LI under conditions which disrupt it in controls (Gaisler-Salomon & Weiner 2003; Gaisler-Salomon et al. 2008; Lipina et al. 2005). This effect also occurs at the conditioning stage, consistent with a role for glutamatergic transmission in modulating attentional switching (e.g. Moghaddam et al. 1997).

4.1.3. COMPARISON OF EXTINCTION AND LI, AND RELEVANCE TO REVERSAL:

Several of the signature phenomena of LI are remarkably similar to those observed in extinction, with evidence for spontaneous recovery, contextual renewal, and reinstatement effects. Despite these two fields of research largely developing independently, it therefore seems likely that similar processes are responsible for both effects; therefore any successful theory must be able to account for both phenomena. Though 'unlearning' accounts have been widely disregarded due to extensive evidence that prior learning can be recovered, this recovery is rarely complete. Perhaps some of the most successful theories to date are therefore differential retrieval models which allow for the possibility for some amount of learning interference to occur, alongside interference at the performance/behavioural level (e.g. Bouton, 1993).

Within this conceptualisation, both LI and extinction can be viewed as interference paradigms, and in doing so, their relevance to reversal learning becomes particularly clear. Evidence that context effects can be seen in reversal learning can also provide support for such theories, since a change of context back to stage 1 leads not only to a renewal of stage 1 responding, but to a re-suppression of stage 2 responding (Bouton, 1993). Therefore, each of these phenomena may result from common underlying mechanisms, with interference (retroactive, proactive or both) occurring when conflicting information is available (Bouton 1993).

There is sufficient evidence to suggest a critical role for 5-HT and DA in both extinction and LI; though the exact nature of these effects, their possible interactions, and particular receptor subtypes involved, as well as the stage of learning that they exert their effects, are currently unclear. Though there is evidence that 5-HT_{2A}R may be more critical to both tasks than $5HT_{2C}$R, the effects of selective $5HT_{2C}$R antagonists are yet to be investigated. Given the inhibitory role $5HT_{2C}$R are known to play in mescocorticolimbic DA transmission (e.g. Alex et al. 2005), a system implicated as integral to both extinction and LI; it seems possible that antagonism at these receptors might affect performance in each of these tasks. Evidence from 'learned non-reward' and 'perseverance' conditions of reversal learning tasks might suggest a preferential role for 5-HT_{2C}R in modulating LI rather than extinction however (to the extent that these tasks can be said to measure similar mechanisms); since 'perseverance' condition performance was unaffected by systemic administration of SB242084, whilst 'learned non-reward' performance was considerably enhanced (Experiment 3, Chapter 3). This might suggest a specific role for SB242084 in allowing animals to overcome learned non-reward; an interpretation which would be supported by evidence that it is similarly involved in reducing the degree of LI that occurs to a preexposed stimulus. The following two experiments will therefore seek to clarify the role of 5-HT_{2C}R antagonism on performance in an extinction and Latent Inhibition (LI) task.

4.2. EXPERIMENT 1

THE EFFECT OF SB242084 IN EXTINCTION LEARNING

One possible mechanism through which SB242084 might support flexible cognition is by enhancing the extinction of responding to a previously rewarded location. Although a lack of drug treatment effects under perseverance conditions suggests this may be an unlikely explanation for the improvements seen in reversal, there is currently no evidence concerning the effect of 5-HT_{2C}R blockade on performance in extinction tasks. The effect of SB242084 treatment will be explored using a standard operant extinction design, where rewards are delivered following responses to a single stimulus presented during acquisition, but omitted following responses during extinction testing (e.g. Williams et al. 1990). Though most operant extinction procedures increase the ratio of the response requirement during training to encourage high levels of responding, the current study rewarded animals for every operant response made (fixed ratio 1 schedule), to maintain similarity to the reversal task.

In simultaneous serial discrimination tasks there is also the possibility that drug-treatment affects the likelihood of switching responding to the alternative location when reward is no longer received at the previous location, which might also serve to augment reversal learning. This possibility will therefore be additionally explored using a two-stimulus operant design, where rewards are delivered following responses to one of two stimuli presented during acquisition, but omitted following responses to either stimulus during extinction. Responses at the alternative, never-rewarded location will be measured to assess the extent of response switching during extinction. Consistent with the drug administration schedule employed in reversal learning tasks, SB242084 was administered during extinction testing but not during acquisition.

4.2.1. Materials and methods:

Animals:

Thirty-two male C57BL/6J mice were food restricted to 90% of their free-feeding weights for 2 weeks prior to behavioural testing. Animals weighed an average of 22.8g (SEM ± 0.4g) at the start of behavioural testing. Animals were pair-housed at the start of experimenting, in line with Home Office guidance, but persistent aggressive behaviour during acquisition testing resulted in one animal having to be culled due to injury, and all animals being single-housed, which occurred at least 5 days prior to any animal commencing the extinction phase.

Behavioural procedures:

Habituation

Animals received sugar pellets in their home cages for two consecutive days prior to testing. They then received two 45-minute habituation sessions in the testing chambers, with the lights off and the magazine loaded with 10 sugar pellets. Animals also received two sham injections prior to acquisition testing, to habituate them to the injection procedure. Animals were then randomly assigned to one of two conditions for acquisition testing (see Figure 4.1).

Acquisition:

One-hole condition (n = 15):

Sessions consisted of 60 trials initiated automatically on a random interval schedule. The random interval mean was initially set to 10s but after poor performance (high retrieval omissions), this was increased to 30s from session eight. Trials began with the onset of a single central nose-poke hole LED (the remaining nose-poke holes were covered with metallic plates). Responses into the central nose-poke hole within 30s of illumination (limited hold) were classed as 'correct', and resulted in the extinction of the nose-poke light and delivery of one sucrose pellet into the food magazine. This was followed by an inter-trial interval (ITI) of 11s (4s houselights off, 7s houselights on), to allow animals to retrieve the food reward prior to the start of the next trial. A reward retrieval omission was recorded if animals failed to retrieve the reward within this 11s ITI. Alternatively, if no response was made into the illuminated nose-poke hole within 30s limited hold period, the nose-poke LED was extinguished, a response omission was recorded, and the houselights were immediately turned on for the duration of the ITI (11s) (see Figure 4.2). Animals were required to reach a criterion of 90% 'correct' responses (i.e. fewer than 6 response omissions per 60-trial session) on two consecutive test days.

Two-hole condition (n = 16):

These sessions were identical to those in the one-hole condition, except that the central nose-poke hole was covered, and the left and right nose-poke holes were made available. Trials began with the illumination of both nose-poke hole LEDs, but only responses into one of these locations was correct (counterbalanced across left and right). Responses in the 'correct' location within 30s of nose-poke LED illumination resulted in extinction of both nose-poke lights, the delivery of one sucrose pellet into the food magazine, and the beginning of the 11s ITI (4s houselights off, 7s houselights on). A reward retrieval omission was recorded if animals failed to respond in the food magazine within the 11s ITI. Responses in the 'incorrect' location resulted

in LED lights extinguishing, no reward being delivered, and immediate onset of the houselights for the duration of the ITI (11s). Response omissions were recorded if no response was made at either location within the 30s limited hold period, and had the same consequences as an incorrect response (see Figure 4.2). Again, animals were required to reach a criterion of 90% correct responses over two consecutive test days. The response requirements in this condition were therefore higher, as animals were required to make fewer than 6 incorrect responses or response omissions in each 60-trial session.

***Extinction*:**

Once animals reached acquisition criterion they were assigned to a vehicle- or drug-treatment group, and proceeded to extinction conditions on the next test day. Ultimately, half the animals in each condition (one-hole/two-hole) were assigned to each treatment group, matched as closely as possible for performance during acquisition. All animals received injections of SB242084 or vehicle (0.5 ml/kg, *s.c.*) 30 minutes prior to each test session. In both conditions, the task parameters remained exactly the same as during acquisition, except that previously 'correct' responses no longer elicited reward delivery. All response measures taken were the same as during acquisition: 'correct' responses (responses at the previously rewarded location), 'incorrect' responses (responses at the previously non-rewarded location, two-hole condition only), 'response omissions' and 'retrieval omissions', as well as latency to make a 'correct' and 'incorrect' response, and latency to nosepoke into the food magazine (termed 'retrieval latency' for comparison to prior test stages, though rewards were no longer delivered). Responses at the 'correct' and 'incorrect' locations in the two-hole condition during extinction served to test the hypothesis that SB242084 treatment may enhance 'switching' behaviour, seen as a switch to the alternative ('incorrect') nose-poke location once the previously 'correct' response no longer elicits reward. Daily test sessions were received until animals responded on fewer than 20% of trials over two consecutive test sessions.

Statistics:

Independent measures t-tests were used to compare the number of incorrect responses and incorrect response latencies between drug treatment groups in the two-hole condition, since there were no 'incorrect' responses in the one-hole condition. The remaining measures were subjected to two-way independent-measures ANOVA with drug (Vehicle/SB242084) and condition (one-hole/two-hole) as factors. Performance was assessed during acquisition to ensure no baseline differences existed between drug-treatment groups, as well as during extinction.

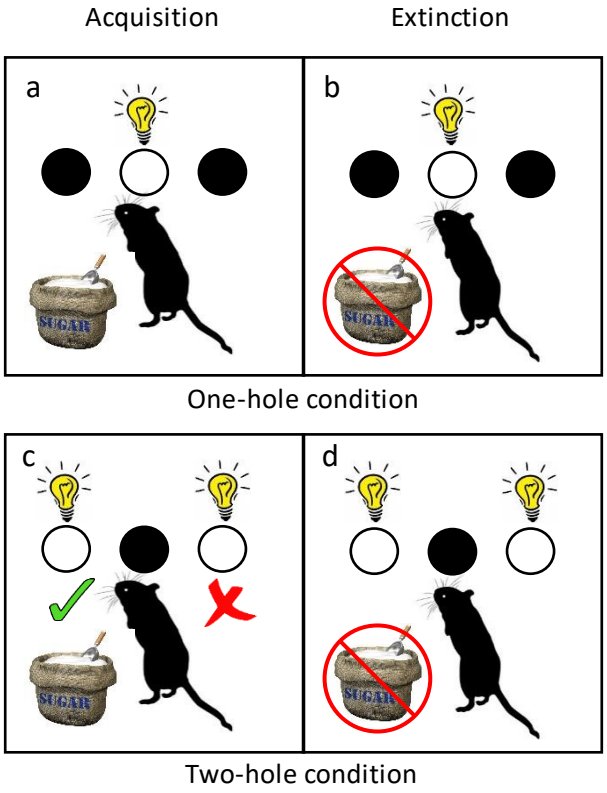

Figure 4.1. Representation of task requirements during acquisition and extinction test phases, under one-hole (a & b) and two-hole (c & d) conditions. *Trials were initiated automatically on a random interval schedule in both test phases and conditions. Failure to register a response in the nosepoke holes within 30s of trial initiation were recorded as 'response omissions', and failure to make a response in the magazine within the ITI were recorded as 'retrieval omissions' (during both acquisition and extinction stages). a) One-hole acquisition: A single central nosepoke hole LED is illuminated and responses made at this location within 30s of trial onset lead to delivery of a single sugar pellet reward. b) One-hole extinction: Trials are identical to acquisition stage, but responses at the central nosepoke location no longer result in reward delivery. c) Two-hole acquisition: Two nosepoke hole LEDs are illuminated but responses to only one of these locations is 'correct' (counterbalanced across left and right). Responses at the 'correct' location within 30s of trial onset result in reward delivery, and responses at the 'incorrect' location result in no reward. d) Two-hole extinction: Trials are identical to acquisition stage, but responses at the correct location no longer result in reward delivery.* ***Open circles = available nosepoke locations, filled circles = covered nosepoke locations.***

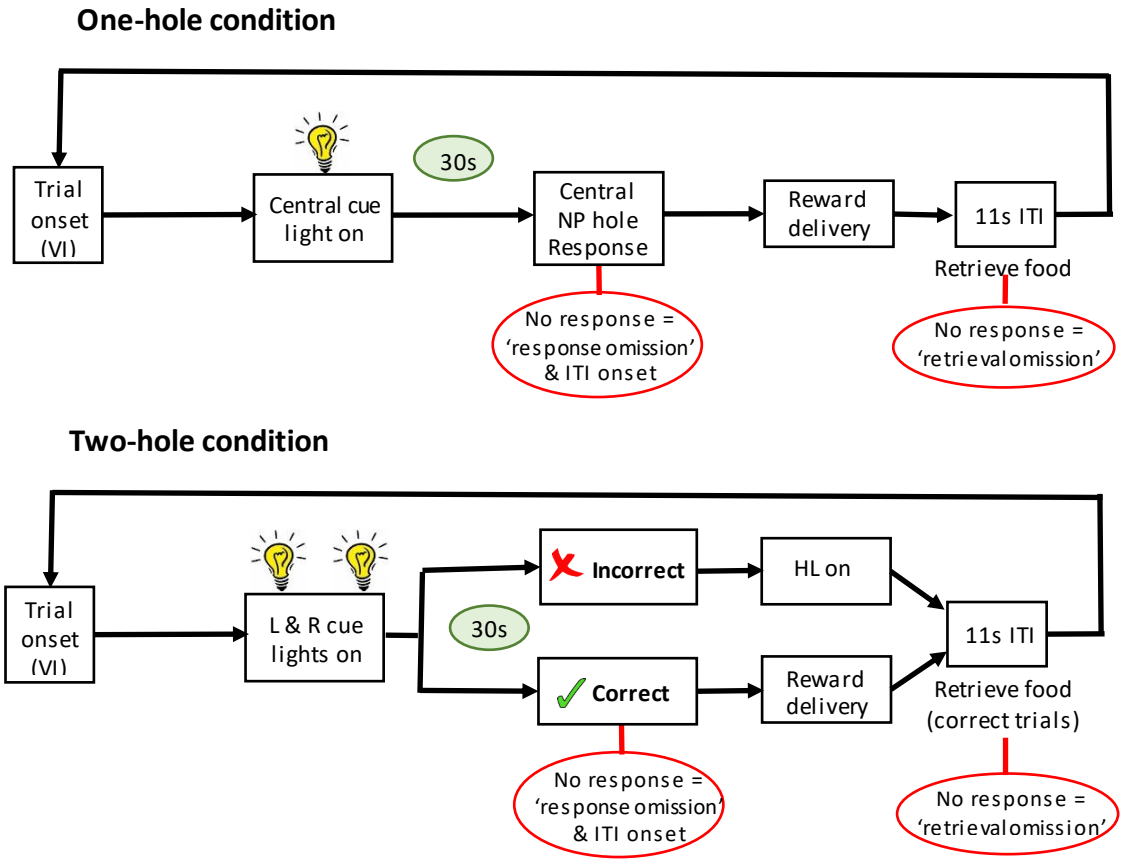

Figure 4.2. Representation of individual trial structure under one-hole and two-hole test condition prior to extinction. *Trials started automatically on a variable interval (VI) schedule. In the one-hole condition, responses in the central nosepoke location resulted in reward, in the two-hole condition, responses at the 'correct' response location (counterbalanced across left and right) resulted in reward, whilst 'incorrect' responses led to immediate onset of ITI. Animals must register a response in an illuminated nosepoke hole within 30s, or a 'response omission' is recorded. Following a rewarded trial, animals must make a response within the food magazine within the 11s ITI, or a 'retrieval omission' is recorded. Abbreviations: HL = houselights, ITI = Inter-trial interval, NP = Nosepoke, VI = Variable interval. Under extinction conditions, trials are identical except no rewards are delivered.*

4.2.2. Results:

Acquisition (drug-free):

Six animals failed to reach the acquisition criterion within 20 sessions of testing (3 from each condition). Rather than removing these animals from testing, they were allocated evenly across drug treatment groups, ensuring groups were matched for performance. Two-way independent measures ANOVA confirm there were no significant differences in performance between animals subsequently assigned to the vehicle or drug treatment groups, with no main effect of drug, or interaction with condition on any measure, either across all acquisition sessions (see Table 4.2) or across the final two days of testing prior to extinction (see Table 4.3). Although there was no main effect of condition (one-hole/two-hole) on most performance measures, animals in the one-hole condition made significantly more response omissions across the course of acquisition, and were slower to make a 'correct' response (see Table 4.2). The difference in response omissions most likely reflects the fact that animals in the two-hole condition could make 'incorrect' responses as well as omissions, since the number of 'correct' responses did not differ between groups. However, the slower speed of responding could

	One-hole condition		Two-hole condition		*Drug*	*Condition*	*Drug x Condition*
	Vehicle *(n = 7)*	**SB242084** *(n = 8)*	**Vehicle** *(n = 8)*	**SB242084** *(n = 8)*			
Sessions	16.0 ± 1.2	17.4 ± 1.1	16.4 ± 0.8	16.5 ± 0.9	*0.58*	*0.07*	*0.40*
Trials	959 ± 72	1037 ± 63	982 ± 47	989 ± 52	*0.52*	*0.05*	*0.37*
Correct	40.1 ± 1.1	34.4 ± 2.3	36.5 ± 1.5	37.1 ± 1.7	*2.08*	*0.05*	*3.29*
Incorrect	-	-	6.2 ± 0.4	6.5 ± 0.5	*-0.58*	-	-
Response omissions	19.9 ± 1.1	25.4 ± 2.2	17.3 ± 1.3	16.3 ± 2.0	*2.02*	***6.15****	*2.64*
Retrieval omissions	4.1 ± 0.5	4.0 ± 0.6	4.6 ± 0.5	4.4 ± 0.4	*0.14*	*0.50*	*0.01*
Response latency (correct) (s)	10.3 ± 0.3	10.8 ± 0.5	9.7 ± 0.3	9.7 ± 0.3	*0.40*	***5.40****	*0.45*
Response latency (incorrect) (s)	-	-	9.7 ± 0.5	9.6 ± 0.2	*0.08*	-	-
Retrieval latency (s)	3.0 ± 0.1	3.1 ± 0.1	2.9 ± 0.2	3.0 ± 0.1	*0.67*	*0.25*	*0.02*

Table 4.2: *Results of two-way independent measures ANOVA and independent measures t-tests comparing average performance (Mean ± SEM) during acquisition testing across conditions (one-hole/two-hole) and groups (Vehicle/SB242084). Independent measures t-tests were used to compare drug group performance for incorrect responses and latency to an incorrect response only (since incorrect responses were only possible in the two-hole condition). Significant effects are highlighted in bold.* * $p < .05$.*

	One-hole condition		Two-hole condition		*Drug*	*Condition*	*Drug x Condition*
	Vehicle *(n = 7)*	**SB242084** *(n = 8)*	**Vehicle** *(n = 8)*	**SB242084** *(n = 8)*			
Correct	54.7 ± 2.8	53.3 ± 2.9	53.7 ± 1.5	55.1 ± 1.1	*0.05*	*0.17*	*0.77*
Incorrect	-	-	3.2 ± 0.9	2.6 ± 0.4	*0.55*	-	-
Response omissions	5.3 ± 2.8	7.7 ± 2.9	3.1 ± 0.9	2.3 ± 1.0	*0.14*	*3.34*	*0.62*
Retrieval omissions	1.5 ± 0.5	3.2 ± 1.1	3.5 ± 1.5	2.4 ± 0.8	*0.08*	*0.34*	*1.63*
Response latency (correct) (s)	6.4 ± 0.7	6.8 ± 0.7	5.5 ± 0.6	5.6 ± 0.6	*0.15*	*2.74*	*0.02*
Response latency (incorrect) (s)	-	-	6.0 ± 0.6	6.4 ± 1.0	*-0.34*	-	-
Retrieval latency (s)	1.7 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	*0.09*	*0.07*	*0.26*

Table 4.3: *Results of two-way independent measures ANOVA and independent measures t-tests comparing average performance (Mean ± SEM) across the final two days of acquisition testing across conditions (one-hole/two-hole) and groups (Vehicle/SB242084). Results indicate no significant differences in performance between any of the four groups prior to extinction testing (all p's > .05).*

suggest that animals in the one-hole condition were less motivated to respond. Although differences were still evident in the final two sessions of acquisition testing prior to extinction, they no longer reached significance (see Table 4.3).

Extinction:

As observed during acquisition, animals in the one-hole condition made significantly more response omissions than those in the two-hole condition during extinction testing, leading to a significant main effect of test condition (see Table 4.4). The average number of 'correct' responses again did not differ between groups, therefore it is unlikely that animals in the one-hole condition were extinguishing responding at a faster rate, and in fact, the total number of response omissions in the one-hole condition was essentially equivalent to the number of response omissions plus incorrect responses in the two-hole condition, confirming that this effect was simply related to animals in the two-hole condition having a third response option (correct/incorrect/omission). There were no significant drug-related differences in performance on any measure, and no interaction effects (see Table 4.4).

Restricting analysis to the first 4 sessions of extinction testing, when all animals had yet to reach criterion, showed no performance differences between the one-hole and two-hole test conditions however, suggesting the difference in response omissions occurred later in testing, after this early 'perseverative' stage. There was also evidence of several drug-related differences in early extinction performance, with drug-treated animals making significantly more correct

responses, and making them faster than vehicle-treated animals, as well as making fewer response omissions (see Table 4.5). Independent measures t-tests show that drug-treated animals made no more incorrect responses than controls in the two-hole condition, but they did display a non-significant tendency to make faster incorrect responses. In fact, the difference in speed of responding between groups was larger at the incorrect than at the correct location in the two-hole condition, with this effect only just failing to reach significance (see Table 4.5).

	One-hole condition		Two-hole condition		*Drug*	*Condition*	*Drug x Condition*
	Vehicle *(n = 7)*	**SB242084** *(n = 8)*	**Vehicle** *(n = 8)*	**SB242084** *(n = 8)*			
Sessions	10.4 ± 1.9	10.5 ± 1.0	9.3 ± 1.1	10.1 ± 0.9	*0.15*	*0.39*	*0.11*
Trials	625 ± 114	630 ± 58	555 ± 67	608 ± 56	*0.15*	*0.39*	*0.11*
Correct	23.0 ± 1.2	24.6 ± 0.8	22.9 ± 1.0	22.8 ± 0.4	*0.71*	*1.18*	*0.89*
Incorrect	-	-	5.0 ± 0.4	4.9 ± 1.1	*0.14*	-	-
Response omissions	37.0 ± 1.2	35.4 ± 0.8	32.1 ± 1.0	32.4 ± 1.4	*0.37*	***13.00******	*0.71*
Retrieval omissions	17.5 ± 1.2	17.0 ± 0.7	16.5 ± 1.8	14.1 ± 0.9	*1.42*	*2.48*	*0.61*
Response latency (correct) (s)	12.6 ± 0.3	12.0 ± 0.5	12.0 ± 0.6	11.8 ± 0.4	*0.69*	*0.86*	*0.17*
Response latency (incorrect) (s)	-	-	12.4 ± 0.5	11.1 ± 0.6	*1.65*	-	-
Retrieval latency (s)	7.1 ± 0.3	6.6 ± 0.2	6.4 ± 0.4	6.5 ± 0.3	*0.61*	*1.33*	*1.22*

Table 4.4: *Performance of animals during extinction testing (Mean ± SEM) across conditions (one-hole/two-hole) and groups (Vehicle/SB242084). Independent measures t-tests compare performance between drug groups for incorrect responses and latency to an incorrect response only (since incorrect responses were only possible in the two-hole condition), and performance on the remaining measures was assessed with two-way independent measures ANOVA. Significant effects are highlighted in bold. *** p = .001.*

	One-hole condition		Two-hole condition		*Drug*	*Condition*	*Drug x Condition*
	Vehicle *(n = 7)*	**SB242084** *(n = 8)*	**Vehicle** *(n = 8)*	**SB242084** *(n = 8)*			
Correct	34.3 ± 3.2	40.8 ± 1.7	33.5 ± 2.1	36.6 ± 1.2	***5.25****	*1.39*	*0.68*
Incorrect	-	-	5.3 ± 0.6	4.8 ± 2.3	*0.50*	-	-
Response omissions	25.8 ± 3.2	19.2 ± 1.7	21.3 ± 1.8	18.7 ± 1.7	***4.68****	*1.41*	*0.09*
Retrieval omissions	24.1 ± 2.6	25.6 ± 1.6	22.3 ± 2.8	20.3 ± 1.4	*0.02*	*2.74*	*0.64*
Response latency (correct) (s)	11.2 ± 0.3	9.5 ± 0.6	10.1 ± 0.5	9.2 ± 0.3	***6.91****	*2.01*	*0.70*
Response latency (incorrect) (s)	-	-	10.5 ± 0.4	8.6 ± 0.8	*2.02~*	-	-
Retrieval latency (s)	6.7 ± 0.5	5.9 ± 0.3	6.3 ± 0.5	6.2 ± 0.2	*1.39*	*<0.01*	*0.64*

***Table 4.5**: Performance of animals during the first 4 days of extinction (Mean ± SEM) across conditions (one-hole/two-hole) and groups (Vehicle/SB242084). Independent measures t-tests compare performance between drug groups for incorrect responses and latency to an incorrect response only (since incorrect responses were only possible in the two-hole condition), and performance on the remaining measures was assessed with two-way independent measures ANOVA. Significant effects are highlighted in bold. *p < .05, **p < .02, ~p = .063.*

4.2.3. Discussion:

`The current study provides no evidence that 5-HT_{2C}R antagonism augments extinction learning, but instead offers evidence of a drug-related impairment in early extinction performance. Although drug treatment had no effect on the overall speed of extinction learning, with all animals extinguishing responding within a similar number of sessions and trials across both test conditions, there was evidence for a drug-related potentiation of responding during early extinction sessions relative to vehicle treated controls; though it is not clear to what extent this may reflect motor impulsivity effects. There was no evidence for a drug-related difference in the number of incorrect responses made during extinction in the two-hole test condition either, suggesting that drug treatment does not increase the likelihood of general 'switching' of behavioural responses. This study therefore rules out the possibility that SB242084 enhances reversal learning either by promoting extinction of a conditioned response or by promoting behavioural 'switching' more generally. There was an unexpected difference in responding under one-hole and two-hole test conditions however, possibly suggestive of reduced response motivation when animals are given fewer response options.

Evidence of a drug-related potentiation of conditioned responding in the early stages if extinction is consistent with prior evidence that 5-HT depletion causes increased resistance to

extinction (e.g. Kovacs et al. 1976; Srebro & Lorens 1975). During the first four sessions of extinction testing, drug-treated animals made fewer response omissions, made more responses at the location which previously delivered reward, and responded more quickly at this location that controls; seemingly demonstrating a tendency to perseverate. This latter effect is in line with prior evidence of faster running during extinction of a food-rewarded alleyway running task following 5-HT depletion by medial raphe lesions (Asin et al. 1979), or following administration of the 5-$HT_{2A/2C}$ receptor antagonist cinanserin (Rosen & Cohen 1973), possibly suggesting a specific role for 5-HT_{2C} receptors in this speeding effect. There was no concurrent increase in number or speed of responses made at the incorrect location in the two-hole condition during extinction, potentially ruling out an explanation centred on the general motor impulsivity effects of drug-treatment. However, the difference in speed of responding between groups was in fact larger at the incorrect than at the correct location in the two-hole condition, with this effect only just failing to reach significance. To effectively rule out the contribution of general motor effects, it may therefore be necessary to further examine the effect of SB242084 on extinction performance in a passive response task, such as a step-down avoidance task, where animals must learn to inhibit a prepotent step-down response to avoid receiving electric shocks. If drug-treated animals were to show similarly enhanced behavioural inhibition in early extinction sessions relative to controls, this would help to confirm a genuine extinction effect. Although it may not be possible to rule out the contribution of motor impulsivity to the extinction impairment seen following drug-treatment in the current experiment, it does nevertheless allow us to rule out the possibility that SB242084 improves reversal learning through an enhancement of extinction learning.

This possible drug-related impairment effect is inconsistent with prior evidence that SB242084 had no effect on responding during the 'perseverance' condition of a reversal learning task however. Under these conditions, where the previously correct response option was still available but no longer rewarded, drug-treated animals were no more likely to perseverate at the previously correct location. However, the existence of an alternative, rewarded response option under perseverance test conditions, as distinct from either no alternative response option (one-hole extinction), or a non-rewarded alternative response option (two-hole extinction), may have masked any potential drug-related effects on extinction learning. Although the two-hole condition demonstrated that SB242084 treated animals were no more likely to switch responding to an alternative location than controls, they might be equally as likely to continue responding at an alternative response location when these responses are

rewarded; obscuring any potential extinction impairment effect at the previously correct location.

There were subtle behavioural differences observed across testing conditions in the current experiment, with animals in the one-hole condition taking longer to make a response and making more response omissions than animals in the two-hole condition during acquisition. The difference in response omissions was most likely a simple reflection of the fact that animals in the two-hole condition had an additional response option (incorrect), since this effect was also observed during extinction, and the difference in omissions between drug-treated animals and controls almost perfectly matched the number of incorrect responses made in the two-hole group. The slowed response latency under one-hole conditions was no longer apparent during extinction however, and might have reflected reduced response motivation. Though animals in the one-hole condition were just as likely to receive reward when making a 'correct' response as animals in the two-hole condition, the additional uncertainty introduced by an extra response option ('incorrect') might have enhanced motivation to respond, in much the same way as intermittent schedules of reward are found to elicit enhanced responding relative to continuous schedules. Resultantly, there were no differences in speed of responding during extinction, when responses in both conditions were linked with uncertainty due to the surprising omission of rewards.

In summary, the current tests provide evidence that SB242084 does not improve reversal learning by enhancing the extinction of conditioned responding to a previously rewarded cue, or by promoting general behavioural switching. Conversely, there is evidence to suggest that 5-HT_{2C}R antagonism causes an early perseverative effect in extinction tests, though this is not reflected in the overall rate of extinction; an effect which might have been obscured in tests of perseverance due to the availability of a rewarded response alternative. This finding could be related to motor impulsivity effects of drug treatment however, given the general nature of the latency effects observed. Further tests will be needed to clarify the role of SB242084 in extinction using passive response tasks which require animals to overcome behavioural inhibition, rather than activation, during extinction.

4.3. EXPERIMENT 2

THE EFFECTS OF SB242084 IN LATENT INHIBITION:

The following experimental protocol is based upon a paper by Bonardi et al. (2010), as an example of a recent LI experiment in mice which employs tighter behavioural controls than most prior studies. A preexposure procedure deemed sufficient to produce LI under control conditions was employed, given evidence that the 5-HT_{2A} antagonist ritanserin is only shown to exert an effect under such conditions (Shadach et al. 2000). Consistent with the drug administration schedule employed in reversal learning tasks, SB242084 was administered during conditioning and not during preexposure.

4.3.1. Materials and methods:

Apparatus:

A clicker was delivered by a mechanical relay, and a tone by a 'Sonalert' (MedAssociates), set to deliver a 4.5KHz tone, both of which were mounted in the centre of the operant box on the ceiling of the chamber. The tone was programmed to be delivered intermittently (0.8s on, 0.2s off), to maintain similarity to the click stimulus. Both stimuli measured approximately 75dB.

Animals and behavioural procedure:

Twenty-four male C57BL/6J mice were food restricted to 90% of their free-feeding weights for 2 weeks prior to behavioural testing. Animals weighed an average of 21.8g (SEM ± 0.5g) at the start of behavioural testing. A repeated measures design was used, where animals were preexposed to one of two auditory stimuli, followed by a test of conditioning to both the preexposed (PE) and the non-preexposed (NPE) stimulus. See Table 4.6 for overview of task design.

Habituation:

Animals received sugar pellets in their home cages for two consecutive days prior to testing, and were given one 30 minute habituation session in the operant chambers, with the lights off. No sugar pellets were delivered to animals whilst in the operant chambers, since this might have interfered with subsequent preexposure effects.

PREEXPOSURE (7 SESSIONS)		***CONDITIONING (5 SESSIONS)***			
		VEHICLE *n = 12*		SB242084 *n = 12*	
N = 12	**TONE**	TONE+ (PE)	CLICK+ (NPE)	TONE+ (PE)	CLICK+ (NPE)
N =12	**CLICK**	TONE+ (NPE)	CLICK+ (PE)	TONE+ (NPE)	CLICK+ (PE)

Table 4.6: *Representation of task design. During the preexposure phase, half the animals were assigned to each stimulus type, and received unrewarded presentations of an intermittent tone (n = 12) or click (n = 12) for 7 sessions of 40 trials. In the conditioning stage, half the animals in each group were assigned to a drug-treatment group (Vehicle/SB242084), matched for performance during preexposure. Both stimuli were randomly presented to animals 15 times per session for 5 sessions, and offset of each stimulus was now followed by immediate delivery of one sucrose pellet into the food magazine. Magazine responding during presentation of the preexposed (PE) stimulus is compared to the non-preexposed (NPE) stimulus to assess latent inhibition effects; and across drug treatment groups to assess drug-related performance effects.*

***Preexposure phase*:**

Animals were preexposed to one of the two experimental stimuli: the intermittent tone (*n = 12*), or the clicker (*n = 12*). The pre-exposures were of 20s duration, interspersed by an inter-trial interval of 60s, plus a further variable interval with a mean of 30s, to prevent trial occurrence becoming predictable. No rewards were received during this phase. Responding in the magazine was measured during each 20s period of conditioned stimulus (CS) presentation ('during CS'), and in each 20s period immediately preceding CS presentation ('pre-CS'), to allow comparison of response rates. A further measure of responding in the period immediately following CS presentation was also taken ('post-CS'), to ensure there were no pre-existing differences in performance during this time-bin between animals that would later be assigned to different drug treatment conditions, since this will reflect the reward delivery period in the conditioning stage (see Figure 4.3). Additionally, a measure of latency to make a response in the magazine during CS presentation, and in the 20s immediately after CS presentation (which will constitute 'reward retrieval latency' in the conditioning stage) was taken. Each session consisted of 40 stimulus presentations, lasting approximately 90 minutes. All animals received 7 test sessions, with one session per day, deemed to be sufficient to produce LI in control animals.

Conditioning phase:

Half the animals in each stimulus-type group (click/tone) received SB242084 and the other half received vehicle (0.5 mg/kg, *i.p.*) 30 minutes prior to each conditioning test session. This created four different groups: Click–SB; Click–Vehicle; Tone–SB; Tone–Vehicle. These groups were counterbalanced for elevation scores during the preexposure phase for each stimulus type. Each session consisted of 30 trials: 15 presentations of the preexposed (PE)

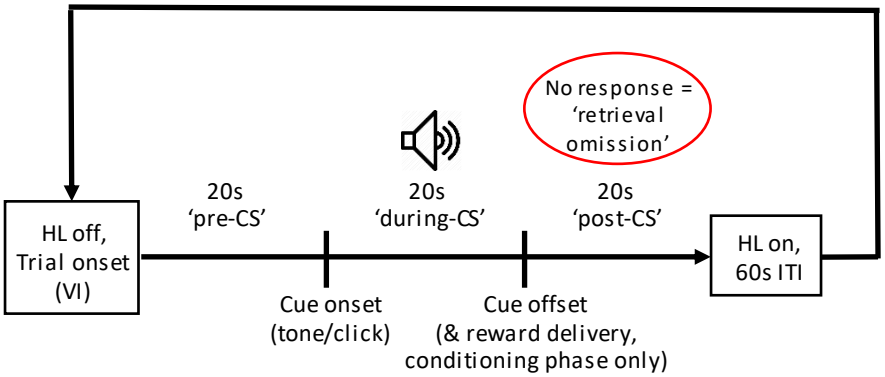

Figure 4.3. Representation of individual trial structure during pre-exposure and conditioning phase of latent inhibition task. *Trials started automatically on a variable interval (VI) schedule. Measures of responding in the food magazine (total responses, latency to first response) were taken during three 20s time-bins: 'pre-CS', a baseline measure of responding prior to cue onset; 'during CS', a measure of responding to an irrelevant cue (preexposure phase) or reward-predictive cue (conditioning phase); and 'post-CS', a measure of responding following reward delivery (conditioning phase). Failure to make a magazine entry during the 20s 'post-CS' phase results in a 'retrieval omission' being recorded. Note that during the preexposure phase animals receive 40 presentations of only one sound cue on every trial (either tone or click). In the conditioning phase, animals receive 15 presentations of this preexposed (PE) stimulus, interspersed randomly with 15 presentations of the non-preexposed stimulus (NPE). See Table 4.6 for details on how stimulus type (tone/click) was counterbalanced across drug treatment groups. Abbreviations: CS = conditioned stimulus, HL = houselight, ITI = inter-trial interval, VI = variable interval.*

stimulus, and 15 presentations of the non-preexposed (NPE) stimulus, presented on a pseudo-randomized schedule. The NPE stimulus was the click for those preexposed to the tone and the tone for those preexposed to the click. Offset of either stimulus was now followed by the immediate delivery of one sucrose pellet into the food magazine. As with the previous stage the ITI was set to 60s, but the additional variable interval was increased from 30s to 60s (in line with Bonardi et al. 2010), to ensure trial onset did not become predictable. Responses in the magazine were again measured during the 20s immediately prior to stimulus presentation ('pre-CS'), in the 20s during stimulus presentation ('during CS'), and during the 20s immediately following reward delivery ('post-CS'), see Figure 4.3. Latency measures were taken for responses in the magazine during the CS, and during reward delivery ('reward retrieval latency'). Additionally, the number of reward retrieval omissions were recorded, when no response was made into magazine within 11s of delivery. Animals received 5 successive daily test sessions.

Statistics:

Strength of conditioning was calculated as an 'elevation score'. Rate of responding (responses per minute, rpm) during the pre-CS period was subtracted from the rate of responding during the CS period, such that an elevation score of 0 reflects no difference in responding at baseline (pre-CS) compared to during stimulus presentation. Results from the

preexposure stage were analysed using three-way ANOVA with 'session' (1-7) as a within-subjects factor, and 'drug' (SB/Vehicle), and 'stimulus' (tone/click) as between-subjects factors, to ensure there were no pre-existing differences in responding to either stimulus-type or between groups of animals later assigned to either drug treatment condition. During conditioning, an elevation score was calculated for each trial type (PE or NPE stimulus presentation), pooling data over all trials of that type in the session, and results were analysed using three-way mixed ANOVA with 'drug' as a between-subjects factor, and 'session' (1-5) and 'preexposure' (PE/NPE) as within-subjects factors. Because pre-CS responding cannot be affected by the type of stimulus subsequently presented (PE/NPE), these responses were not separated by trial type, and were instead analysed using two-way mixed ANOVA with 'drug' and 'session' only as factors. Significant interaction effects were further explored using repeated measures t-tests, using Holms-Sidak method to control for multiple comparisons. Elevation scores were the primary measure of interest, but pre-CS responses were additionally analysed to check for baseline performance differences, as well as post-CS responses (made during reward delivery), and the latency to make a response and retrieve rewards.

4.3.2. Results:

Preexposure phase:

Elevation scores showed slightly more negative values early in pre-exposure, indicating lower response rates during CS presentation than in pre-CS periods; which was followed by a gradual recovery of responding over the course of the 7 test sessions (-.38, -.25, -.25, .08, -.11, -.08 and 0.22). This mirrors the effect reported by Bonardi et al. (2010) and could reflect habituation to the sound cues; though this difference over sessions did not reach significance ($F_{6, 120} = 1.99$, $p = .07$). There was a significant main effect of test session on magazine entries made pre-CS ($F_{6, 120} = 6.46$, $p < .001$), during CS ($F_{6, 120} = 4.08$, $p = .001$), and post-CS ($F_{6, 120} = 4.70$, $p < .001$), with a reduction generally observed across sessions compared to the higher response rates of the opening session. Despite animals having spent 30 minutes in the test chamber prior to preexposure testing, this most likely reflects habituation to the test apparatus and a general reduction in exploratory behaviour over time. There was no effect of test session on latency to make a response however, either during CS ($F_{6, 120} = 1.10$, $p > .05$) or post-CS ($F_{6, 120} = 1.50$, $p > .05$). There was no significant main effect of drug or stimulus type on any measure, nor were there any interaction effects; confirming that there were no pre-existing differences in performance between any groups prior to conditioning (data not shown).

Conditioning phase:

Elevation scores:

Elevation scores were significantly higher for the NPE stimulus than the PE stimulus during conditioning, leading to a significant main effect of preexposure ($F_{1, 22} = 7.44$, $p < .05$), confirming a Latent Inhibition effect. Elevation scores also differed significantly across test sessions ($F_{2.3, 51.1} = 6.18$, $p < .01$), with an increase observed in early test sessions, followed by a decline (see Figure 4.1). Treatment with SB242084 had no effect on the development of LI however, with no significant main effect of drug apparent on elevation scores ($F_{1, 22} = 0.11$, $p > .05$). There was a significant interaction between test session and preexposure ($F_{3.4, 74.0} = 7.73$, $p < .001$), but no further two-way or three-way interactions (data not shown). To further understand this interaction effect, post-hoc repeated measure t-tests were performed, comparing elevation scores to PE and NPE stimuli across each of the five conditioning sessions. A Latent Inhibition effect was observed early on in testing, with all animals displaying significantly lower elevation scores to the PE than the NPE stimulus during the opening three conditioning sessions ($t_{23} = -3.49, -3.10, -2.60$, respectively, all p's $< .05$), which was no longer evident in sessions 4 and 5 ($t_{23} = -0.36, -0.35$, respectively, all p's $> .05$). The absence of effect on days 4 and 5 of testing were not due to recovery of responding to the PE stimulus however, but due to an unexpected reduction in responding to both sets of stimuli, but which was particularly marked for the NPE stimulus (see Figure 4.4).

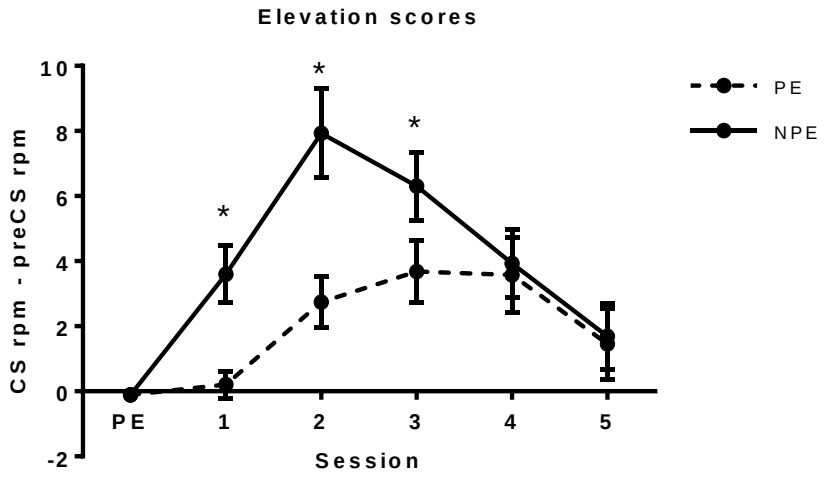

Figure 4.4 *Elevation scores during the pre-exposure stage (PE) and conditioning sessions 1-5 across drug-treatment groups. Dashed lines represent responding to preexposed (PE) stimuli, and solid lines to non-preexposed (NPE) stimuli. Mixed ANOVA reveal a significant interaction between pre-exposure and test session on performance, with post-hoc t-tests revealing responses were significantly higher to the NPE than the PE stimuli during sessions 1-3 across drug groups (*p < .05). Note the reduction in responding to both NPE and PE stimuli from session 3 onwards. until there is no difference in sessions 4 and 5.*

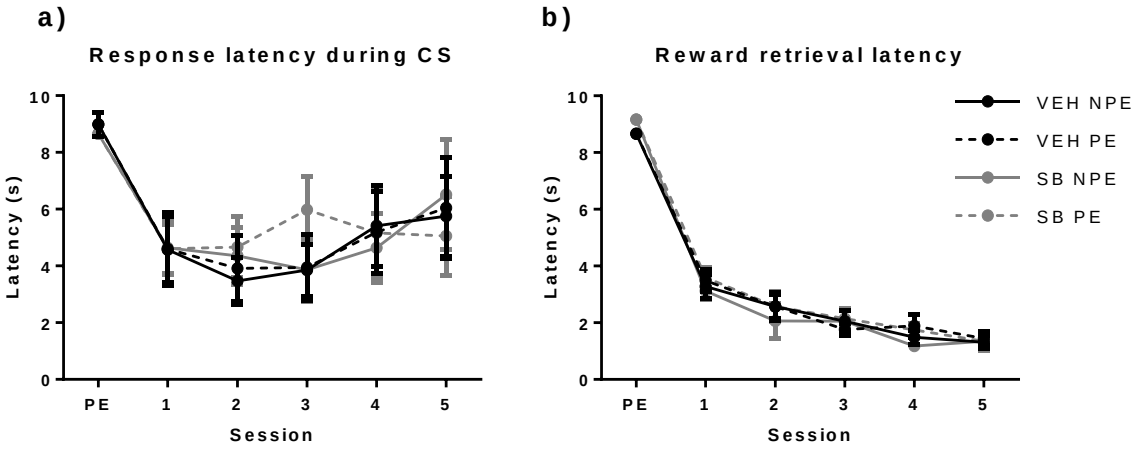

Figure 4.5 *Latency to make a response in the magazine during the CS (a) and to retrieve rewards from the magazine post-CS (b) during pre-exposure (PE) and the five sessions of conditioning. Mixed ANOVA reveal only a significant main effect of test session on these two measures; therefore no LI effect is evident for speed of responding. Though there is a speeding of responses during the CS in early conditioning sessions compared with pre-exposure (PE) stage levels, this is followed by a subsequent slowing of responding that mirrors the reduction seen in elevation scores in later test sessions. Pairwise comparisons confirm a significant increase in response latency between sessions 2-4 and 2-5 (all p's < .05). Reward retrieval latency is also much faster in the opening session of conditioning compared to PE levels. Unlike responding during the CS, this continues to reduce over later sessions, with pairwise comparisons confirming a significant latency reduction from sessions 1-3, 1-4, and 1-5 (all p's < .05). This suggests animals were continuing to reliably respond for food rewards despite reducing the speed and number of responses made during the CS in later conditioning sessions.*

Pre-CS (baseline) responses:

Two-way ANOVA show that baseline rates of responding in the 20s period immediately preceding CS presentation (pre-CS) did not differ across sessions ($F_{3.0, 66.5} = 1.54$, $p > .05$), and were unaffected by drug treatment ($F_{1, 22} = 0.15$, $p > .05$). Nor was there an interaction between session and drug on this measure ($F_{2.8, 66.5} = 0.01$, $p > .05$). The increase in elevation scores in early test sessions was therefore specific to an increase in responding during CS presentation.

Response Latency:

The LI effect seen in elevation scores was not reflected in latency to make a response during CS presentation. When animals made a response, they were just as fast to do so during presentation of the PE as the NPE stimulus ($F_{1, 21} = 0.05$, $p > .05$). Again there was an effect of test session on this measure ($F_{4, 84} = 5.75$, $p < .001$), with shorter response latencies observed in early test sessions, followed by a later increase (see Figure 4.5a). Pairwise comparisons show only this latter slowing of responding was significant, between sessions 2-4 and 2-5 (p's < .05).

This mirrors the drop-off in responding exhibited in elevation scores in the final two test sessions (see Figure 4.3), meaning that animals were both less likely to make a response during CS presentation and were slower to do so in these final sessions. There was no main effect of drug treatment ($F_{1, 21}$ = 0.01, p > .05), and there were no significant interaction effects (data not shown).

Retrieval Latency:

Animals were just as fast to retrieve rewards delivered following PE stimulus presentation as they were following the NPE stimulus ($F_{1, 22}$ = 2.76, p > .05), and vehicle-treated animals retrieved rewards at the same speed as drug-treated animals ($F_{1, 22}$ = 0.04, p > .05). Once again, there was a significant effect of test session ($F_{2.7, 59,7}$ = 20.97, p < .001), but here there was only evidence for a speeding of responding across testing, reflected in significant pairwise differences between sessions 1-3, 1-4 and 1-5 (all p's < .05) (see Figure 4.5b). There were no significant interaction effects (data not shown).

Retrieval omissions:

Reward retrieval omissions were very low throughout the task, but no significant effect of session ($F_{2.7, 60.0}$ =1.04, p > .05), preexposure ($F_{1, 22}$ = 1.36, p > .05), or drug treatment ($F_{1, 22}$ = 0.16, p > .05) were seen, and there were no interaction effects (data not shown). This shows animals continued to reliably and quickly retrieve rewards, even when responding to the CS had diminished in sessions 4 and 5, so this decline is unlikely to be explained by a loss of motivation.

Post-CS responses:

The total number of responses made in the magazine during reward delivery actually reduced over the course of testing ($F_{3.1, 68.5}$ = 6.98, p < .001), with pairwise comparisons demonstrating a significant decline in responding from sessions 1-3, 1-4, and 1-5 (all p's < .05). Magazine entries during this period also did not differ between drug groups ($F_{1, 22}$ = 2.00, p > .05), and was unaffected by preexposure ($F_{1,22}$ = 0.71, p > .05), and there were no significant interaction effects (data not shown). Taken alongside reward retrieval data, this suggests animals successfully acquired the reward contingencies of the task, acting to swiftly and efficiently collect rewards, and no longer anticipated further reward until the next trial.

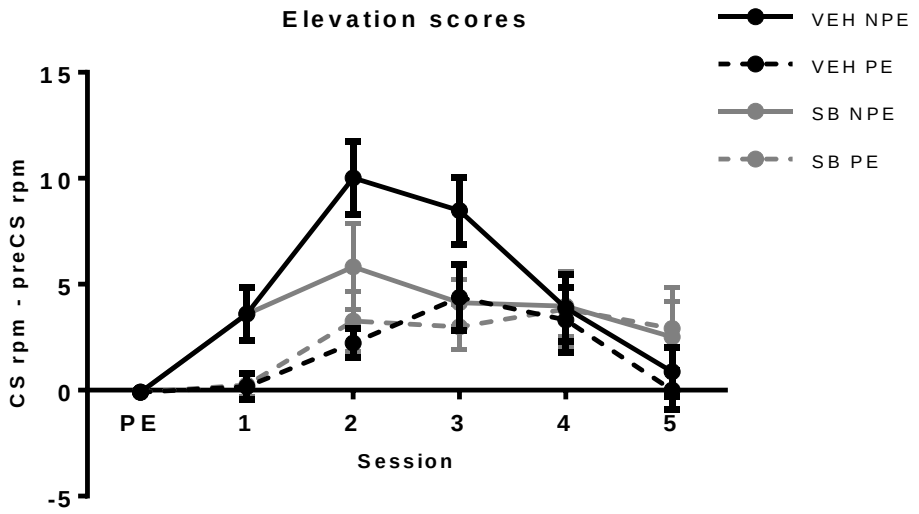

Figure 4.6 *Elevation scores during pre-exposure stage (PE) and conditioning sessions 1-5 for SB242084-treated animals and vehicle-treated controls. Dashed lines represent responding to preexposed (PE) stimuli, and solid lines to non-preexposed (NPE) stimuli. Note the latent inhibition effect is very similar for both drug groups in session 1, but SB242084 treated animals show a smaller increment in responding to the NPE stimulus compared to vehicle-treated controls in Sessions 2 & 3. Separate two-way mixed ANOVA were conducted for elevation scores to the PE stimulus and the NPE stimulus, respectively, with 'session' and 'drug' as factors. There was a significant interaction between session and drug for elevation scores to the NPE but not the PE stimulus, with post-hoc t-tests revealing reduced responding in drug-treated animals to the NPE stimulus in session 3 compared with controls, though this latter effect did not survive correction for multiple comparisons.*

Although there appear to be no significant effects of drug treatment on the development of LI, with SB242084-treated animals showing very similar rates of responding to the PE stimulus as vehicle-treated controls throughout testing; there was some evidence that drug-treated animals showed lower rates of responding to the novel (NPE) stimulus during sessions 2-3 (see Figure 4.6). Despite there being no significant interaction between drug treatment and preexposure on elevation scores, this effect was further explored because this is so contrary to prior evidence of increased responding (reduced omissions) and possible motor impulsivity effects of SB242084 treatment. Separate two-way mixed ANOVA were conducted for elevation scores to the PE stimulus and the NPE stimulus, respectively, with 'session' and 'drug' as factors. For the PE stimulus, there was a significant main effect of session on elevation scores ($F_{3.0,\ 66.1} = 4.02$, $p < .05$), with an increase seen from session 1-2 and 1-3, and a decrease observed from sessions 4-5 (all p's < .05), confirming the drop-off in responding previously noted. There was no effect of drug treatment ($F_{1,\ 22} = 0.24$, $p > .05$), and no interaction between session and drug ($F_{3.0,\ 66.1} = 1.12$, $p > .05$). For the NPE stimulus, there was also a significant main effect of session on elevation scores ($F_{2.3,\ 50.6} = 8.42$, $p < .001$), though the increase in scores was only evident from session 1-2, and a decrease was seen from sessions 3-4, 4-5, and 3-5 (all p's < .05).

Although there was also no main effect of drug treatment on elevation scores to the NPE stimulus ($F_{1, 22} = 0.80$, $p > .05$), there was a significant interaction between session and drug ($F_{2.3, 50.6} = 2.64$, $p < .05$). Post-hoc independent samples t-tests reveal that drug-treated animals exhibited significantly lower elevation scores to the NPE stimulus than vehicle-treated controls during session 3 ($t_{22} = 2.25$, $p = .025$). This suggests drug treatment could have suppressed responding to the novel NPE stimulus to some degree.

4.3.3. Discussion:

This study provides clear evidence of a LI effect during the early stages of testing across drug-treated animals and controls, with evidence of suppressed responding to the preexposed compared to the novel stimulus in the first three sessions of conditioning; an effect which was restricted to frequency and not latency of magazine responses made during stimulus presentation. Somewhat surprisingly, LI was no longer evident in later test sessions, which could not be explained by a recovery of responding to the preexposed stimulus, but was instead due to a drop in responding to both sets of stimuli; particularly marked for the novel stimulus. There was no evidence for a drug-related difference in responding to the preexposed stimulus however, suggesting that $5HT_{2C}R$ antagonism plays no role in mediating the development of LI. There was some suggestion of a difference in responding to the novel stimulus between drug treatment groups however, which requires further explanation.

The unexpected reduction in responding to both PE and NPE stimuli in later conditioning sessions, which was accompanied by a concurrent slowing of responding, was not previously observed by Bonardi et al. (2010) employing an appetitive food-rewarded procedure of almost identical design. The reason for this difference is not clear, but could suggest a general decline in motivation over testing. Analysis of behaviour at the magazine during reward delivery in these sessions however shows that animals were still reliably retrieving rewards, and were doing so even more rapidly than they had in earlier sessions, which means that this explanation can be discounted. Although the number of magazine entries made during reward delivery was also declining, these data could suggest that animals had begun to accurately predict the timing of reward delivery following offset of cue presentation, and to expect receipt of one reward per trial. Some aspect of the current task design may therefore have allowed for more rapid apprehension of this sequence, thereby reducing anticipatory responses during CS presentation. One of the only differences in the current design, compared with Bonardi et al. (2010) were the sound cues which served as the PE and NPE stimuli. Though both tasks used a clicker, the current

design employed an intermittent tone rather than continuous white noise, but in this preparation, both the intermittent tone and click cues were programmed to offset 0.2s prior to pellet delivery. This may have made delivery of the sucrose pellets particularly salient, since the noise the pellet dispenser creates could serve as an additional cue for reward; possibly rendering the sound cues somewhat redundant in the later stages of conditioning.

However, this response reduction is also consistent with the pattern of responding seen at the magazine during presentation of a CS in autoshaping tasks, for animals that display a sign-tracking (ST) rather than a goal-tracking (GT) tendency. For these animals, after an initial increase in responding at the magazine during delivery of a reward-predictive CS, there is a subsequent decline as the animal begins to direct behaviour towards the CS itself, rather than the magazine or 'goal'. It is therefore possible that the inconsistencies between the current results and those reported by Bonardi et al. (2010) are due to variations in the number of animals with a natural GT or ST tendency. Though reports of the distribution of GTs and STs in large populations of rats suggest a roughly even split between GTs, STs and those that show a mix of both behaviours (Meyer et al. 2012), the relative distribution is likely to vary considerably within small populations. Though it would seem unlikely to obtain a population sample formed entirely of STs, as the current data might suggest, there is some evidence of individual differences in responding, with one animal in particular displaying a consistent and large increase in magazine responses during presentation of the CS, consistent with a GT tendency. However, autoshaping tasks typically employ a discrete localisable stimulus as the reward-paired cue, such as a light or a lever, and there is some debate over whether auditory cues can elicit conditioned approach behaviour in the same manner, particularly in rodents (e.g. Davey & Cleland 1982). Though Holland (1977) reports that a localisable reward-paired sound cue does not elicit conditioned approach toward the location of sound delivery as a light CS does; he does report the development of a 'head jerk' orienting response to the cue in rats, which closely tracked the reduction in magazine-directed behaviour during tone presentation. One interpretation of the current data therefore might be that mice developed a ST-type response toward reward-paired cues, resulting in reduced responding at the magazine during cue presentation; though this tentative possibility will require further exploration.

The observation of reduced responding to the non-preexposed stimulus by drug-treated animals compared to controls is also notable, and requires further explanation. It is unlikely to reflect an increased fear response to the novel stimulus, since responding was very similar to vehicle-treated controls during session 1 of testing; and there is evidence that 5-HT_{2C}R antagonists have an anxiolytic rather than an anxiogenic effect in mice across a battery of

anxiety tests (e.g. Harada et al. 2006). Drug-treated animals might show increased generalisation between the two auditory cues, causing repressed responding to the non-preexposed stimulus; though again, evidence of an LI effect which matched that of controls during the first test session tends to argue against this explanation. If the reduction in conditioned responding to the CS in later test sessions across both groups could be said to reflect learning about the conditions associated with reward, leading to reduced pre-emptive responses to the cue, it is feasible that drug-treated animals learnt this association quicker, possibly reflective of increased attention to reward-related cues. Alternatively, if the reduction in conditioned responding reflects an increase in ST-type behaviour directed toward the cues, this could reflect faster (or stronger) development of a ST response in drug-treated animals.

The lack of drug effect on the development of LI to a preexposed, non-rewarded stimulus may seem at odds with evidence that 5-HT_{2C}R antagonism can support the ability to overcome 'learned non-reward' in reversal learning. Both tasks putatively measure proactive interference caused by presentation of a non-reinforced stimulus on performance of a conditioning task involving that stimulus, yet there are clearly relevant differences in task design. Firstly, the cues in the current LI task were auditory rather than spatial; therefore the relative saliency of cues might differ, altering the relative strength of learning that must be overcome in LI and 'learned non-reward' preparations. Secondly, LI tasks examine responding in successive conditioning trials to the PE and NPE stimuli following preexposure of a single stimulus, whereas during learned non-reward, two cues are present during both phases of the task. Whilst responses at the incorrect stimulus are not punished, the contrast against reward received following responses at the correct location could lead to the development of an inhibitory association to that cue (CS-'no US'), which is not reported to develop to the preexposed stimulus in LI tasks. This might allow a distinction in the involvement of 5-HT_{2C}R in the psychological processes subserving 'learned non-reward', as separate from those involved in LI, possibly through an involvement in the formation or expression of inhibitory associations (see Lister et al. 1996). The role of SB242084 in the development of inhibition to a CS- during simultaneous exposure to a CS+ in operant tasks would need to be further explored, by examining the ability for the CS- to act as a conditioned inhibitor in summation and retardation tests in drug-treated animals relative to controls.

Most notably however, the current LI task examined Pavlovian rather than operant conditioning, as is standard practice in reversal learning designs. LI is difficult to measure using instrumental procedures, since animals will make few responses at a location that does not result in reward before ceasing responding altogether. Although this necessitated use of a

Pavlovian LI procedure, this represents a significant deviation from the instrumental 'learned non-reward' task design. Given evidence that SB242084 has a significant impact on performance under the operant testing conditions of reversal in Chapters 2 and 3, as well as in the operant extinction task presented in Experiment 1 above, this lack of effect on LI could suggest that SB242084 exerts its effects on some specific aspect of instrumental performance, such as stimulus-response, response-outcome, or response selection processes. This possibility will need to be further explored by assesing the effect of SB242084 under Pavlovian reversal conditions.

It is important to note that this experiment does not rule out the possibility of 5-HT_{2C}R involvement in LI, it simply shows that antagonism at these receptors during the conditioning stage does not affect performance. As with evidence for the involvement of 5-HT_{2A}R in this task (Shadach et al. 2000), it may exert an influence during the original preexposure stage. The current experiment was only concerned with understanding the possible role of antagonism at these receptors during conditioning, since this is the stage at which drug is administered during reversal learning tasks (i.e. post-acquisition). As such, the current findings suggest that SB242084 does not improve reversal learning performance by reducing the degree of LI expressed to the CS+ following preexposure in the acquisition stage. An effect of 5-HT_{2C}R antagonism on specific aspects of instrumental performance is a possibility which warrants further exploration however.

4.4. GENERAL DISCUSSION:

The current chapter identifies a possible role for 5-HT_{2C}Rs in extinction learning, with evidence of an early perseverative effect of SB242084 when animals were required to inhibit responding to a previously rewarded stimulus. By contrast, drug-treatment was found to have no effect on the development of Latent Inhibition to a preexposed irrelevant stimulus. Although both effects appear to contradict evidence taken from the closely related 'perseverance' and 'learned non-reward' conditions of reversal learning, key task differences allow these discrepancies to be somewhat resolved, whilst also raising several important theoretical questions.

Evidence of perseverative behaviour in drug-treated animals during early extinction sessions appears at odds with evidence that it causes little impairment under 'perseverance' reversal tests. However, the presence of a novel, rewarded stimulus during testing in the latter design leads to the possibility that an extinction impairment was masked by intact responding

at the rewarded location. Evidence that LI is unaffected by drug treatment is also somewhat contrary to evidence for an SB242084 enhancement effect under 'learned non-reward' conditions of reversal. The difference could result from the nature of the conditioned association which develops to the preexposed (non-rewarded) stimulus in each design. The presence of a second rewarded stimulus during acquisition in 'learned non-reward' would likely cause an inhibitory CS-'no US' association to develop to the CS-; an association which does not occur to the preexposed stimulus in LI tasks. This could suggest a specific role for the 5-HT_{2C}R in mediating the expression of inhibitory associations. Alternatively, given the Pavlovian design of the LI task used, as compared to the operant design of extinction and reversal tests, this could suggest a specific effect of SB242084-treatment on instrumental response elements of reversal performance. This intriguing possibility should be further explored through the use of Pavlovian reversal tasks.

CHAPTER 5

THE ROLE OF 5-HT$_{2C}$R ANTAGONISM IN PAVLOVIAN CONDITIONED APPROACH AND REVERSAL

5.1. INTRODUCTION

Results from previous chapters point to the possibility that antagonism at 5-HT$_{2C}$ receptors might affect aspects of Pavlovian conditioned approach behaviour (sign-tracking/goal-tracking). During the latent inhibition (LI) task of Chapter 4 (Experiment 2), all animals demonstrated a similar LI effect on day one of testing, but a reduction in responding was observed to both the preexposed (PE) and non-preexposed (NPE) stimuli over later test days, an effect which was more pronounced for drug-treated animals. If this reduction in responding could be said to reflect the development of a sign-tracking response to the cues, which draws responding away from the magazine (or 'goal'), then SB242084 could be hypothesised to lead to faster development of a sign-tracking response. How this response might interact with performance during reversal learning is currently unclear.

The variance in reversal learning behaviour seen within the vehicle-treatment control group across different experiments (Chapter 2; Boulougouris et al. 2008, 2010; Nilsson et al. 2012; Pennanen et al. 2013) is also in need of explanation. The speed of reversal learning of vehicle-treated controls varied quite dramatically across these previous tests; an effect which could not be clearly explained by task differences. This performance difference could be suggestive a form of natural population variance, possibly in the degree of Pavlovian conditioned approach behaviour. One aim of this chapter is therefore to identify whether Pavlovian conditioned approach behaviour is related to performance in reversal learning tasks, and whether 5-HT$_{2C}$R antagonism can affect the development of this behaviour.

A third significant finding from previous experiments was that 5-HT$_{2C}$R antagonism exerted a significant effect on performance during instrumental conditioning tasks of reversal (Chapter 2 and 3) and extinction (Chapter 4, Experiment 1), but had no effect in the Pavlovian conditioning task of Latent Inhibition (Chapter 4, Experiment 2), despite the similarity of this task to the 'Learned Non-Reward' condition of reversal. Although this could be related to the difference in the conditioned associations formed to a non-rewarded as opposed to an irrelevant

stimulus, an intriguing possibility is that SB242084 exerts its effects specifically on aspects of instrumental performance. This possibility will therefore be explored by assessing the effects of SB242084 in a Pavlovian reversal task.

5.2. PAVLOVIAN CONDITIONED APPROACH

Broad individual variation can be observed in the type of response formed to a localisable conditioned stimulus (CS) that reliably signals an appetitive unconditioned stimulus (US). 'Autoshaping' was first noted by Brown and Jenkins (1968) in a procedure where pigeons were presented with brief illuminations of a key-light (CS) that signalled delivery of food (US). After several light-food pairings they noted that the pigeons began to approach and peck at the CS, despite no action being required to elicit food delivery. This propensity to engage with a CS that is positively correlated with an appetitive reinforcer, also termed 'sign-tracking' (Hearst & Jenkins 1974), contrasts with observations that some animals preferentially direct conditioned responding to the site of US delivery during CS presentation, termed 'goal-tracking' (Boakes 1977). Sign-tracking is now a well-documented phenomenon, shown to occur across a wide variety of species, including pigeons, cats, goldfish, monkeys, horses and humans (see Tomie et al. 1989 for a review).

The topography of the conditioned response elicited by a CS is often reported to resemble the behaviour produced by the reward itself. For example, the sipping response of pigeons to a CS paired with delivery of water is distinct to the pecking response observed when the same CS is paired with food (Jenkins & Moore 1973). Rats are often reported to show consummatory gnawing or chewing behaviour at a lever that has been paired with food reward (e.g. Davey & Cleland 1982), and male Japanese quail have even been reported to copulate with a terrycloth object that has been associated with the opportunity to copulate with a female (Koksal et al. 2004). This led researchers to speculate that the CS may be taking on the incentive properties of the reward through Pavlovian (stimulus-stimulus) learning processes (Berridge 2001), where the cues themselves become "attracted, desired, riveting incentives" (Berridge & Robinson 2003). Evidence that cues can acquire incentive salience for some individuals is not restricted to their ability to elicit Pavlovian conditioned approach (PCA) responses (sign-tracking). These cues can also come to powerfully control behaviour, acting as conditioned reinforcers where they are able to reinforce the learning of entirely new actions (e.g. Robinson & Flagel 2009); and produce maladaptive responses where animals continue to contact a CS even when doing so cancels reward delivery (e.g. Williams & Williams 1969). Recent findings

also suggest that the sign-tracking (ST) response, but not the goal-tracking (GT) response, is resistant to reward devaluation (Morrison et al. 2015), demonstrating how behaviour can become remarkably stimulus-bound.

Such findings have spurred significant interest in how individual differences in Pavlovian conditioned approach behaviour might affect how cues come to instigate and maintain maladaptive behaviours such as persistent drug-taking. This has led to a growing body of studies in this area with clear implications for understanding individual differences in susceptibility to addiction (e.g. Cunningham & Patel 2007; Flagel et al. 2008; Kearns & Weiss 2004; Tomie et al. 2002, 2003; Uslaner et al. 2006, 2008). However this natural form of variation in responding to cues signalling reward must also have a significant bearing on our understanding of any task that makes use of these seemingly simple CS-US (or stimulus-outcome, S-O) relationships, which are used to model a wide variety of behaviours. How such individual variation might affect tasks of cognitive flexibility such as reversal learning will be the focus of the current chapter.

There is evidence to suggest that simple Pavlovian associations can be formed to reward-associated cues during the normal course of instrumental learning, in addition to the more complex instrumental associations. Traditional stimulus-response (S-R) theories of instrumental learning (e.g. Hull 1943) proposed that the reinforcer (or 'outcome', O) itself does not become incorporated into the associative structure, but simply acts to strengthen the S-R relationship. Other theorists contend that the reinforcer does become encoded during the performance of instrumental tasks, forming associations with the antecedent response (R-O) (e.g. Mackintosh & Dickinson, 1979), and/or stimuli (S-O) (e.g. Rescorla & Solomon 1967) through Pavlovian processes. Evidence that a representation of the reinforcer is encoded in the course of instrumental learning can be taken from reinforcer devaluation tasks, as the instrumental response to earn a reinforcer is reduced following its devaluation by pairings with a toxin, or through satiation (Colwill & Rescorla 1985). That an association forms specifically between the stimulus and the reinforcer is evidenced by the transfer effect seen when the discriminative stimulus of an instrumental learning task (S-R-O) comes to control performance of a new instrumental response, if that response was previously associated with the same reinforcer (R-O) (Colwill & Rescorla 1988). The Pavlovian-to-Instrumental Transfer (PIT) effect also highlights how conditioned stimuli can come to exert control over instrumental behaviour, where a reward-paired CS can elicit and enhance instrumental responding for the same (or similar) reward (e.g. Estes 1943; Rescorla & Solomon 1967). Furthermore, Chudasama & Robbins (2003) point out that reversal learning tasks can combine both Pavlovian and instrumental learning, since classical conditioning processes can be sufficient to elicit the approach and

contact response to a CS+ needed to 'solve' a discrimination, without any need for the intervention of instrumental processes. This potential confound is of vital importance given that any individual variation in the degree of incentive value ascribed to Pavlovian cues during acquisition might affect the ability to subsequently reverse these learned associations, making it unclear to what extent reversal behaviour is mediated by instrumental learning principles.

The majority of reversal tasks discussed and presented in this thesis are based on simultaneous cue presentation, requiring animals to choose which of two cues to respond to on each trial. This is likely to result in weaker Pavlovian associations between cue and reinforcer than tasks where the CS+ is presented alone on discrete trials, since reward-linked cues cannot act as 'occasion-setters', telling an animal when to expect reward; and animals are likely to be aware of the presence of the CS- even while approaching and experiencing the CS+. However, the experiments presented in this thesis use two identical cues whose spatial location rather than physical appearance distinguishes where animals must direct responding. It is possible that an animals' location within the operant box at time of responding could serve as a discriminative cue that becomes associated with reward, much in the same way as a conditioned place-preference (CPP) develops to reward-paired environments (e.g. Ågmo et al. 1993; Spyraki et al. 1982), which itself has been described as a form of autoshaping (Newlin 1992). Given that the animal can only experience one physical location at a time, this may even provide a better discriminative stimulus than in visual discrimination tasks, where animals experience both the CS+ and CS- at once. There is currently no experimental evidence which demonstrates how a sign-tracking (ST) response directed towards such a discriminative stimulus might impact upon the subsequent ability to reverse instrumental responding.

Although the neurobiological and psychological processes supporting the attribution of incentive value to cues in Pavlovian tasks likely differ from those underlying instrumental learning (Cardinal et al. 2002), there is growing evidence that similar processes may be involved in Pavlovian conditioned approach and reversal learning. There is evidence of increased *cfos* mRNA expression in the OFC of sign-trackers compared to goal-trackers (Flagel et al. 2011); a region which has been strongly implicated in cognitive flexibility (e.g. Clarke et al. 2008; Dias et al. 1996, 1997; Fellows & Farah 2003). Lesions of the OFC which profoundly impair the acquisition of Pavlovian conditioned approach behaviour are also reported to increase perseverative responding during reversal of a learned instrumental discrimination (Chudasama & Robbins 2003; Jones & Mishkin 1972), and have been shown to impair reversal of a Pavlovian discrimination by preventing the development of normal responding to the previously unrewarded cue (Burke et al. 2009). This relationship is perhaps surprising given previously

presented evidence that sign-tracking (ST) can lead to maladaptive response patterns, seemingly bearing the hallmark of inflexibility. However, ST can also be interpreted as a form of heightened cue responsivity (Schulte et al. 2015), which might promote faster awareness of a switch in stimulus-reward relationships.

Further evidence that ST behaviour is associated with cognitive flexibility can be drawn from evidence of strain-dependent differences in both behaviours. Lewis rats make more conditioned responses to a lever paired with reward and acquire the ST response more rapidly than Fischer 344 rats, but also show a trend towards faster reversal of this response once the valence of the CS+ and CS- are switched (Kearns et al. 2006). Although this difference did not reach significance when examined across the full 12 reversal sessions, the authors point out that the superior learning of the initial discrimination by the Lewis strain could have masked some difference during reversal. The data do show a strain-related difference in performance during the early test sessions however, with Lewis rats displaying increased responding at the previously non-rewarded location (CS+), but no difference in their ability to suppress responding to the previously rewarded location (CS-) compared to Fischer rats.

This finding mirrors the effect described by Burke et al (2009) during reversal of a Pavlovian discrimination following OFC inactivation, where lesions caused impairments specifically in the ability to respond at the non-rewarded location; and is very reminiscent of the difference observed in SB242084-treated animals in our operant reversal tests. The two tests aimed at dissecting the role of perseveration versus learned non-reward revealed a specific benefit for drug-treated animals in overcoming learned non-reward, whilst not differing in perseveration at the previously correct location. The reversal benefit seen in our reversal tasks has also typically been observed early in the opening test sessions, before reducing so as to be absent on subsequent reversals. Although this seemingly contrasts with evidence of increased perseveration during reversal following OFC lesions (Chudasama & Robbins 2003), these 'perseverative' deficits simply reflect errors made early in reversal when animals' behaviour is below chance levels. Because CS+ and CS- stimuli were presented simultaneously, 'perseverative' errors cannot in fact differentiate between problems in overcoming learned non-reward versus perseveration at the previously correct location. The Pavlovian reversal tasks discussed above presented CS+ and CS- sequentially, so had the advantage of being able to assess responses to each type of stimulus at the point of reversal.

There is also evidence of a common serotonergic basis for these behaviours, since Lewis rats are shown to have lower levels of basal extracellular 5-HT in the nucleus accumbens and

PFC than Fischer rats (Selim & Bradberry 1996) and lower activity of tryptophan hydroxylase (Chaouloff et al. 1995), the rate-limiting enzyme in 5-HT biosynthesis. Furthermore, DBA/2 (DBA) mice, characterised by low 5-HT synthesis due to an allelic variation of tryptophan hydroxylase-2 (Zhang et al. 2004), show reduced basal extracellular 5-HT levels in the mPFC compared with C57BL/6J (C57) mice (Calcagno et al. 2007). Similar to the pattern of strain-related differences observed in rats, DBA mice exhibit higher asymptotic levels of ST behaviour and acquire the ST response faster than adult C57s (Campus et al. 2016), and are faster to learn an operant reversal (Izquierdo et al. 2006; Graybeal et al. 2014). Tomie et al. (2000) also report that 5-HIAA/5-HT turnover in the VTA is negatively correlated with acquisition of the Pavlovian conditioned response, where higher ratios are indicative of increased serotonergic activity. It is also interesting to note that exposure to stress in the forced swim test facilitated reversal learning in C57 but not DBA mice (Graybeal et al. 2011b; Graybeal et al. 2014), and that stress-induced corticosterone release has been linked to enhanced ST performance (Tomie et al. 2000). Evidence that reduced 5-HT activity is associated with increased ST behaviour seemingly contrasts with data that shows levels of 5-HT in mPFC tissue of outbred rats are increased following Pavlovian conditioned approach (PCA) training (Tomie et al. 2004); though levels were not compared between animals expressing a GT or ST response in this study, so these findings only relate to the PCA training procedure itself.

More direct evidence can be taken from studies which manipulate forebrain 5-HT. Campus et al. (2016) report that mPFC 5-HT depletions of approximately 85% via infusion of the neurotoxin 5-7,DHT in C57 mice led to an increase in ST responses compared to sham-operated controls, and resulted in faster acquisition of the ST response compared to non-operated controls. Winstanley et al. (2004) report that forebrain 5-HT depletions increase not only the number, but also the speed of conditioned responses during a PCA task. This is interesting given that one of the consistently reported effects of SB242084 in reversal learning tasks is to reduce response latencies, though this is most likely related to the motor impulsivity effects of serotonergic manipulations since this heightened response level is not specific to the CS+. Forebrain depletions of serotonin via 5-7,DHT have repeatedly been shown to impair performance on reversal learning tasks however (Clarke et al. 2004, 2005, 2007). Furthermore, although genetic inactivation of the serotonin transporter (5-HTT) is shown to improve reversal learning in mice (Brigman et al. 2010) and rats (Nonkes et al. 2013), there are no reported differences in sign-tracking behaviour in 5-HTT knockout rats compared to their wildtype counterparts (Nonkes et al. 2014). These findings suggest that specific strain-related differences

in serotonin function likely differ from those caused by widespread forebrain depletion and genetic manipulations affecting serotonin re-uptake.

Discrepancies in the effect of global 5-HT manipulations may not be surprising when considering evidence for the effects of systemic administration of serotonin agonists and antagonists on conditioned responding. The high-affinity 5-HT_{1A} agonist 8-OH-DPAT is found to increase conditioned responding (CR) in an autoshaping task (Meneses & Hong 1997; Meneses & Terrón 2001), which is amenable to antagonism with the silent 5-HT_{1A} receptor antagonists WAY100635 and S-UH-301 (Hong & Meneses 1995; Meneses & Terrón 2001). There is also evidence for involvement of 5-$HT_{2A/2C}$ receptors, since post-test injections of the 5-$HT_{2A/2C}$ receptor agonist DOI also increased CR in the same autoshaping task, which was reversed by administration of ketanserin and mesulergine (Meneses & Hong 1997), which both show affinity for the 5-$HT_{2A/2C}$ receptor subtypes despite acting as antagonists at several receptors. In addition to reversing the increase in conditioned responding caused by 5-HT agonists, administration of 5-$HT_{2A/2C}$ antagonists alone have been shown to reduce CR, as seen following administration of mesulergine (Meneses & Hong 1997) and mianserin (Neal & Sparber 1991).

However, in apparent contrast to these findings, post-test administration of a number of other 5-HT antagonists causes an increase in CR, such as the 5-$HT_{2A/2C}$ receptor antagonist ritanserin, the high-affinity 5-HT_{2A} receptor antagonist ketanserin, and the selective 5-$HT_{1B/1D}$ antagonist GR127935 (Meneses & Hong 1997; Meneses & Terrón 2001; Meneses et al. 1997a). Furthermore, the 5-HT_{1D} receptor agonist GR46611 (Meneses et al. 1997a), the 5-$HT_{2A/2C}$ receptor agonist TFMPP, and the 5-$HT_{2A/2B/2C}$ receptor agonist mCPP (Meneses & Hong 1997) are found to reduce, rather than increase, CR expression. The selective $5HT_{2A}$ receptor antagonist MDL100907 was able to abolish the effect of TFMPP on CR (Meneses et al. 1997a). However, it only moderately reduced the effect of mCPP, which was instead abolished by ritanserin and ketanserin administration (Meneses & Hong 1997) suggesting the involvement of 5-HT_2 receptors. mCPP is often used as a tool for evaluating 5-HT_{2C} receptor function, since many of its' effects are suppressed by 5-HT_{2C} receptor selective antagonists (Dalton et al. 2004), but these are yet to be evaluated for their effects on conditioned responding. It should be noted that the autoshaping task used in the majority of these studies differs from the technique used in PCA tasks. Although animals received reward whether they responded on the lever or not, responding led to earlier receipt of reward (see Meneses et al, 1997b), therefore it is not clear to what extent this method measures the development of Pavlovian conditioned approach. In addition, the drugs were administered post-test, therefore the reported results relate more to memory consolidation rather than learning effects. Nevertheless, taken together these results

suggest that serotonergic mechanisms are involved in conditioned responding, but as with the evidence supporting a role for serotonin in reversal learning they highlight the need for a clearer understanding of the role different receptor subtypes play, through the use of more receptor-specific ligands.

In sum, there are several lines of evidence to suggest a possible link between Pavlovian conditioned approach behaviour and cognitive flexibility, with one prior experiment revealing a strain-dependent difference in PCA behaviour which directly maps onto the ability to learn a reversal in a Pavlovian setting (Kearns et al. 2006). No experiment has to date explored whether performance on a Pavlovian conditioned approach task is correlated with performance in an operant reversal learning task however. Given that Pavlovian associations are thought to form to reward-paired cues during the normal course of instrumental learning, this could be a significant confound, with major implications for assessing the effects of experimental manipulations on reversal performance. The first experiment of this chapter seeks to address this question, by Identifying whether there is a relationship between Pavlovian conditioned approach behaviour (ST/GT) and performance in an instrumental reversal learning task. The second experiment will explore whether 5-HT_{2C} receptor antagonism using SB242084 affects the expression of Pavlovian conditioned approach behaviour, which could suggest a mechanism through which it impacts upon reversal learning. Finally, the third experiment investigates whether SB242084 can enhance the ability to reverse Pavlovian conditioned associations in a similar manner to its effects during reversal of an instrumental response, or whether its effects might be restricted to aspects of operant performance.

5.3. EXPERIMENT 1

INVESTIGATING THE RELATIONSHIP BETWEEN PAVLOVIAN CONDITIONED APPROACH (PCA) BEHAVIOUR AND REVERSAL LEARNING PERFORMANCE

5.3.1. Materials and methods:

Animals:

The animals used in this pilot study had previously been through a probabilistic reversal learning task (See Chapter 3, Experiment 2). Eight C57BL/6J mice from the vehicle control group of this study were subsequently trained on a Pavlovian Conditioned Approach (PCA) task, to establish if there was any relationship in performance between the two tasks. Animals were maintained on a food-restricted diet between testing on the two tasks, but average weights were steadily increased over this time, in line with averages for their strain and age. They weighed an average of 24.6g (SEM ± 0.7g) at time of starting the PCA procedure, aged approximately 20 weeks.

Behavioural procedures:

Probabilistic Reversal Task:

The probabilistic reversal learning task was a visuospatial discrimination task where animals learned to direct responses to one of two illuminated nose-poke locations which provided the most favourable schedule of reward (80% versus 20% of responses rewarded). The location of the schedule with highest density of reward was subsequently reversed, and behaviour monitored until animals learned to switch responding (See Chapter 3, Experiment 2 for details).

Pavlovian Conditioned Approach (PCA) task:

After a delay of 2 weeks following completion of the reversal task, animals received their first session of PCA testing. Due to prior reversal training animals already knew to expect reward delivery in the food magazine and required no magazine pre-training or habituation to the sugar pellets or test chambers. Each PCA test session consisted of 30 trials, and lasted approximately 30 minutes. The nose-poke holes that served as the discriminative stimuli in the reversal task were covered with metal plates. Trials were presented on a variable interval of 15s. The start of each trial took a 10s measure of baseline responding, where head entries into the magazine were recorded. This was followed by insertion of a lever (conditioned stimulus, CS) for 10 seconds. During this time both magazine head entries and lever presses were recorded, as well

as the latency to make the first response at the lever and magazine (on trials where a response was recorded). One sugar pellet was delivered into the magazine on a probabilistic 80/20 reward schedule when the lever was withdrawn, in keeping with the schedule animals had received during reversal testing. This was followed by a measure of magazine head entries in the 10s immediately following reward delivery, followed by a further 10s baseline measure of responding. Each trial therefore lasted 40s, followed by a variable ITI of 15s (see Figure 5.1 & 5.2). The location of the lever (left/right) was in the opposite location for each animal as the correct nose-poke had been during reversal testing. Although the levers were presented at the opposite end of the operant box to the nose-poke holes, this switch ensured that there was no generalisation of responding from the correct nose-poke location in the reversal task to the food-predictive cue in the PCA task, which might artificially boost ST responses. Animals were given repeated daily sessions until stable performance was evident over 3 consecutive sessions, defined as less than 10% variability in Pavlovian Conditioned Approach scores (see below).

***Extinction test**:*

One final session was given after completion of the PCA task, to check that animals had extinguished nose-poke responding from the previous reversal task. This session was identical to previous PCA sessions, except that the left and right nose-poke holes that served as the discriminative stimuli in the reversal task were uncovered, and nose-poke entries were recorded but had no outcome. This would indicate that any animals displaying low levels of lever press responding (indicative of goal-tracking) had not simply failed to learn a 'reversal', given that the reward-predictive lever was presented on the opposite side to the correct nose-poke hole during reversal.

Measures:

Because ST behaviour shows an inverse relationship to GT behaviour (Meyer et al, 2012), rate of conditioned lever pressing is typically used to classify degree of conditioned approach behaviour. However, not only is this relationship far from perfect, with a reported negative correlation of just r = -.058 between lever presses and magazine head entries made during the CS in a meta-analysis of a large sample of rats (Meyer et al, 2012), but in the current experiment three animals made no lever presses during any test session. This produced a distribution of scores that was not normal, but also meant any variance in the strength of GT response of these animals could not be accounted for by a measure of lever press rate alone. For this reason, an index score that incorporates measures of degree of both lever pressing and magazine entry behaviour was used to quantify conditioned approach behaviours. Meyer et al (2012) developed

Response Bias	= (Lever presses - Magazine entries)/(Lever presses + Magazine entries)
Probability Difference	= *p* Lever press - *p* Magazine entry
Latency Score	= (ẋ magazine entry latency - ẋ lever press latency)/length of CS duration
PCA Score(n)	= [Response Bias(n)+Latency Score(n)+Probability Difference(n)/3]

p = probability
ẋ = averaged latency
(n) = any particular test session

Table 5.1: *Adapted from Meyer et al (2012). Magazine entries refers only to responses made during 10s presentation of the CS lever.*

the Pavlovian Conditioned Approach (PCA) score to account for individual differences not only in the rate of lever pressing but also in the degree of interaction with the magazine (Response Bias), as well as the speed (Latency Score) and consistency (Probability Bias) of interaction with both (see Table 5.1 for information regarding how scores are calculated). To ensure Latency Scores remained reflective of performance in this study, animals that made no lever press responses within a session were given an average score of 10s for that session (the maximum latency score possible). The final PCA score was calculated by averaging Response Bias, Probability Difference and Latency scores. PCA scores range from -1 to +1, with more negative scores being indicative of GT behaviour, and positive scores of ST behaviour. Additionally, baseline level of magazine responding was used as a measure of general behavioural activity. This took an average measure of responding during the 10s immediately prior to cue onset ('pre-trial baseline'), and in the 10s post-US delivery ('post-trial baseline').

Statistics:

Animals' performance during the PCA task was used to identify them as sign-trackers (STs) or goal-trackers (GTs), on the basis of positive or negative PCA scores, respectively. Independent measures t-tests were conducted to compare the performance of these groups on several indices of reversal learning performance (sessions, correct and incorrect trials to criterion). During the extinction test, animals' responses at the nose-poke locations that served as the 'correct' and 'incorrect' stimuli during reversal were compared using a mixed ANOVA with PCA group (sign-tracker/goal-tracker) as a between-subjects factor and response type (correct/incorrect) as a within-subjects factor. Repeated-measures t-tests were also conducted to ensure there was no change in PCA behaviour between PCA testing and the extinction session.

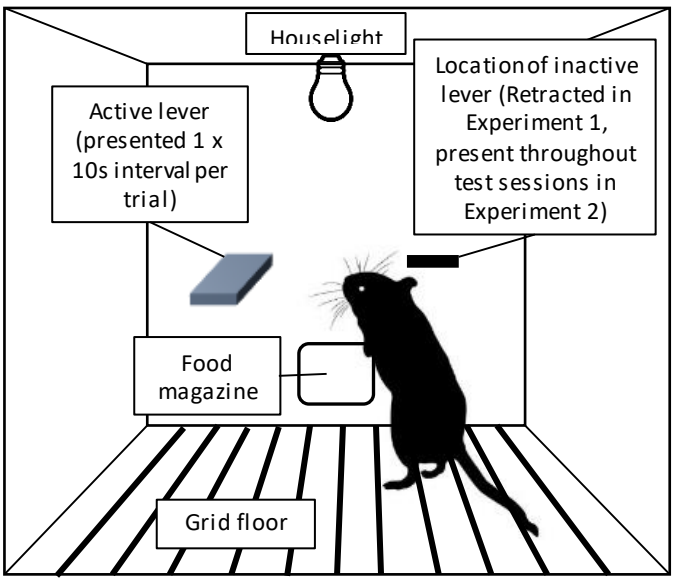

Figure 5.1. Diagram of operant box layout and task design of Pavlovian Conditioned Approach tasks (Experiment 1, 2 & 3). *During Experiment 1 a single 'active' lever (counterbalanced across left and right locations within groups) was presented on each trial for 10s intervals. Following retraction of the 'active' lever a single sugar pellet reward was delivered into the central food magazine. In Experiment 2, a second 'inactive' lever (retracted in the above diagram) was additionally inserted into the operant chamber at the start of each session, and remained present throughout to record general behavioural activity. In Experiment 3 (reversal test), the location of the 'active' and 'inactive' levers was switched, so the previously 'active' lever became 'inactive' and was present throughout testing, whilst the previously 'inactive' lever became 'active', was presented for 10s on each trial, and its retraction was now accompanied by reward delivery.*

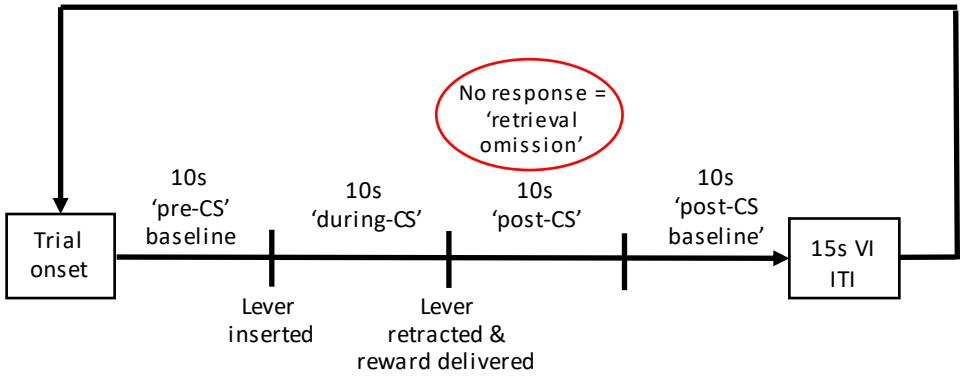

Figure 5.2. Representation of individual trial structure of Pavlovian Conditioned Approach test of Experiment 1. *Trials started automatically on a 15s variable interval (VI) schedule. Measures of responding in the food magazine (total responses, latency to first response) were taken during each of the four 10s time-bins, and measures of responding at the lever were taken during the 10s period of lever presentation ('during-CS'), providing a measure of relative responding to the 'sign' (lever) and 'goal' (magazine). Responses during the 10s 'pre-CS' and 'post-CS' time-bins were averaged to create a baseline measure of magazine responding, as a control for general behavioural activity. Responses in the magazine following reward delivery ('post-CS') were also recorded, and a retrieval omission was registered if an animal fails to make a response in the magazine during this time-bin. Note: trials in Experiment 2 were identical to that described, except that an additional 'inactive lever' was inserted at the start of each session and was present throughout the task, to provide an additional control for general behavioural activity. Abbreviations: CS = conditioned stimulus, ITI = inter-trial interval, VI = variable interval.*

5.3.2. Results:

Animals received 16 sessions of daily testing until stable performance could be identified in all animals. Performance was stable across the final three sessions of the task, with PCA scores highly correlated across these days (*r*'s > .96), and a repeated measures ANOVA demonstrating no significant effect of test day on scores ($F_{2, 14}$ = .05, p > .05). Subsequent analyses therefore focus on average scores taken across the final 3 sessions. Three animals made no active lever presses and registered negative PCA scores (*M* = -.78 *SEM* = .07). The remaining animals showed high levels of active lever pressing (*M* = 387.9, *SEM* = 61.2), and registered positive PCA scores (*M* = .45, *SEM* = .05). Conditioned approach measures are often used to divide animals into three groups: sign-trackers, goal-trackers, and an intermediate group which show signs of both. However, there were insufficient animals to create three groups in this pilot, as well as clear evidence of only two distinct groups (See figure 5.3). Animals were therefore divided into two PCA groups, with goal-trackers (GTs, n = 3) identified by negative, and sign-trackers (STs, n = 5) by positive PCA scores.

The performance of STs and GTs was compared for the number of sessions, correct, and incorrect trials taken to reach reversal criterion during the probabilistic reversal task. The groups were also tested for differences in general behavioural activity during the PCA task by comparing baseline magazine entries. Independent measures t-tests show there was a significant difference in the number of sessions, and incorrect, but not correct trials taken to reach criterion across reversals, with STs being faster to reverse and making fewer than half the number of incorrect responses as GTs (see table 5.2). Baseline measures of magazine responding showed no significant differences between groups (t_6 = -.73, p >.05).

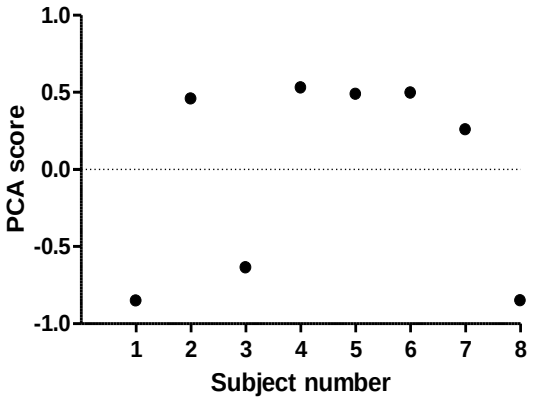

Figure 5.3: Individual variation in PCA scores after 16 days of Pavlovian training. *Average PCA scores for each individual subject across final 3 test sessions. Note the appearance of two distinct groups with positive (sign-trackers) and negative (goal-trackers) PCA scores. Dashed line shows cut off for group selection.*

	Sign-trackers n = 5	Goal-trackers n = 3	T_6
Sessions	4.9 ± 0.5	8.4 ± 1.0	*-3.80***
Correct	147.0 ± 9.9	139.0 ± 7.6	*0.56*
Incorrect	145.0 ± 25.7	367.7 ± 66.9	*-3.73***

Table 5.2: *Mean (±SEM) number of sessions, correct and incorrect trials required to reach criterion during probabilistic reversal learning for animals identified as goal-trackers and sign-trackers in a test of Pavlovian conditioned approach, and results of independent measure t-tests comparing performance across groups. **p ≤.01*

Because animals had previously been through a reversal test, it was necessary to ensure there were no group differences in the degree to which animals had extinguished previously learned responses which might interfere with PCA behaviour. A measure of responses at the left and right nose-poke holes previously used for reversal testing were taken. 'Correct' nose-poke entries (made into the rewarded location during reversal) were compared with 'incorrect' nose-poke entries (made into the non-rewarded location during reversal). A mixed ANOVA revealed no significant effect of PCA group on responding ($F_{1, 6} = 0.36$, $p > .05$), and no significant effect of response type ($F_{1, 6} = 0.26$, $p > .05$), with no interaction ($F_{1, 6} = 0.26$, $p > .05$). This confirms that the low number of nose-poke responses made throughout the session did not differ at the correct ($M = 29.62$ $SEM = 10.69$) or incorrect ($M = 21.5$ $SEM = 2.86$) location for either group of animals.

The large variance in responses at the correct location can be accounted for by one animal who made many more responses at the previously correct than incorrect location however, suggesting that this response had not been extinguished. This animal showed a ST tendency however, displaying the highest PCA index score of all animals (PCA = 0.53), suggesting there was little generalisation of response behaviour from the nose-poke holes to the levers between the two tasks. If prior learning were impacting on acquisition of a conditioned response to the levers, it would be to impair rather than enhance performance, since the reward-associated lever was presented on the opposite side of the chamber as the correct nose-poke hole during reversal. Nor did the endurance of this previous response affect this animals' PCA index score when the nose-poke holes were made available again. During the extinction test, this animal again displayed the highest PCA score of all animals (PCA = 0.56), and a repeated measures t-test confirms there was no significant change in PCA scores across animals during the extinction test ($t_7 = 0.28$, $p > .05$).

5.3.3. Discussion:

The results of this pilot study demonstrate that animals expressing a ST phenotype during PCA training required significantly fewer sessions to reach reversal criterion than those expressing a GT phenotype. Although the groups were small, these results lend support to the suggestion that Pavlovian conditioned approach behaviour is linked to cognitive flexibility, using a robust index measure to assess PCA behaviour which accounts for the relative expression of both ST and GT behaviours to distinguish phenotypes. This tentatively expands upon previous findings linking ST behaviour to faster reversal in a Pavlovian task in rats (Kearns et al. 2006) by demonstrating this same relationship within an operant reversal setting in mice. This finding suggests that Pavlovian associations develop to reward-paired cues during the normal course of instrumental learning. Experimenters may therefore need to be wary of this potential confound when drawing conclusions from operant tasks, particularly if there are reasons to believe that an experimental manipulation affects the attribution of incentive salience to such cues. The results of this experiment also highlight some difficulties with PCA tasks that must be considered for future experiments.

The specific pattern of reversal learning performance observed in the ST group of this study is similar to that observed in the drug-treatment group of the probabilistic reversal task (see Chapter 3, Experiment 2); with an almost two-fold reduction in the number of sessions and trials required to reach criterion, and which was specific to a reduction in incorrect but not correct trials. This adds weight to the suggestion that an alteration of the phenotypic expression of conditioned approach behaviour may be one of the mechanisms through which SB242084 exerts its influence on reversal performance, possibly by shifting animals in the direction of a ST tendency. This might also provide an account of the variance in performance of control groups across previous reversal experiments, since the relative distribution of GTs and STs is likely to vary considerably within small populations. However, it also suggests that any drug-treatment effect may be an artefact of this relative distribution across experiments, so warrants further exploration.

This specificity of effect is also similar to the strain-dependent difference observed by Kearns et al. (2006) during Pavlovian conditioned response reversal in Lewis and Fischer rats. Lewis rats, who showed stronger ST behaviour, made around twice as many conditioned responses at the CS+ than Fischer rats in early test sessions, whilst not differing in their responses to the CS-. Although this pattern differs from the current findings, where STs made fewer incorrect, rather than more correct responses during reversal, differences in task design

must be considered. In the Pavlovian reversal design of the Kearns et al. (2006) experiment, responses to the CS+ and CS- were without effect. Animals could therefore begin to respond on the new CS+ lever whilst continuing to make responses at the lever previously associated with reward, without having any impact on delivery of that reward. However, in the operant reversal learning task described above, incorrect responses led to cue lights being immediately extinguished and the absence of an anticipated reward. Evidence that STs made fewer incorrect responses during reversal in the current study suggests they switched their responding to the newly correct location more rapidly than did GTs. This is the same interpretation given for Lewis rats making more responses on the CS+ during reversal of conditioned associations in the Kearns et al. (2006) study.

Some difficulties associated with Pavlovian approach tasks have emerged from this pilot study that can inform the design of subsequent experiments however. Rank order splits of lever pressing rates are often used to divide animals into groups. This pilot highlights how measures that focus on only one aspect of performance, with the assumption that ST is inversely related to GT behaviour, are insufficient to account for behavioural variation when no/few ST responses are registered. Furthermore, rank split methods suffer from the inherent problems of working only on within-experiment variation and thus being a rather arbitrary way of grouping animals. There can be considerable variation in the prevalence of STs and GTs within and across small populations, which can lead to erroneous identification of animals on the basis of their relative difference to others within the sample. The PCA index (Meyer et al. 2012), which was developed on the basis of a large sample of rats (N = 1,878), increases the reliability of classification of animals, and is less affected by differences between samples due to the use of standardised scoring created from difference scores. Nevertheless, there remain issues with drawing arbitrary boundaries to divide animals into groups (which are noted by the authors, who suggest that these boundaries can be flexible in terms of where they are drawn, depending on the experiment). Such difficulties with separating animals into meaningful groups based on performance leads to the conclusion that repeated measures designs, where animals' performance under different conditions need only be compared to its' own previous performance, will be the best way to execute further experiments in following up this pilot.

The low levels of lever-pressing observed in the present task may in fact be species or strain-related, since prior tests of PCA performance in C57BL/6J (C57) mice have resulted in conditioned approach behaviour being exclusively directed at the food magazine over the course of 7-10 test sessions (Parker et al. 2010), or in such low levels of lever-directed responding over the course of 15 sessions so as to necessitate video analysis of 'approach'

behaviours (Tomie et al. 2012). Animals in the current task received a similar number of sessions and trials as that reported by Tomie et al. (2012), therefore it may be the probabilistic nature of the current task that was sufficient to induce stronger ST behaviour here, since uncertain reward schedules have been shown to potentiate ST responding in rats (Anselme et al. 2013; Robinson et al. 2014). Either way, this possible species-related difference points to the particular need for measures that focus on relative levels of both GT and ST behaviours when testing in mice, and highlights the potential utility of probabilistic tasks as a more sensitive measure for registering ST performance where it might otherwise appear absent.

This pilot study did not use a control CS- lever to measure levels of general behavioural activity. Whilst a baseline measure of magazine responding was taken to ensure that any group differences could not be attributed to general activity levels, this only measures behaviour as directed toward the magazine. An additional 'inactive' lever may provide a better measure of general activity, given that it protrudes into the operant box and can be unintentionally pressed while climbing and jumping. Whilst the existence of a link between PCA scores and speed of reversal suggests this is not the case, it is possible that a general increase in behavioural activity (particularly exploratory-type behaviours) could also facilitate reversal learning, since this would enhance the likelihood of inadvertently making a correct response, which once reinforced is more likely to be repeated. This control is also important given the motor impulsivity effects of SB242084, which has been consistently shown to reduce response latencies and omissions.

In summary, these pilot data extend previous findings linking a ST tendency to faster reversal in a Pavlovian task by suggesting that it may also be linked to faster reversal in an operant setting. This could suggest that Pavlovian associations are forming during the acquisition of instrumental responses to the CS+ and CS- which come to affect how readily these associations are reversed; or it could simply suggest that the two behaviours have common underlying mechanisms, particularly given evidence that both the ST response and reversal performance may be enhanced by stress (Graybeal et al. 2011; Graybeal et al. 2014; Tomie et al. 2000). Given these links, a specific test of whether 5-HT_{2C}R antagonism can affect the expression of ST behaviour is required, since this represents a potential mechanism through which drug treatment can affect reversal. Given prior research indicating a possible species-related difference in ST lever-press responses in mice compared to rats, alongside evidence that reliable lever-press performance could be induced in a subset of animals in this task by using a probabilistic reward schedule, the same 80/20 reward schedule was employed in subsequent experiments, as was the PCA index system of scoring for the relative expression of ST and GT behaviour. However, due to concerns over separating animals into meaningful groups based on

performance, a repeated-measures design was employed in Experiment 2, so that each animals' performance under drug treatment could be directly compared to its own performance during vehicle treatment.

5.4. EXPERIMENT 2

THE EFFECT OF SB242084 ON DEVELOPMENT OF PAVLOVIAN CONDITIONED APPROACH (PCA)

The current experiment addresses the question of whether antagonism at 5-HT_{2C} receptors can affect the relative expression of ST and GT behaviour in mice, since this is a potential mechanism through which it could affect reversal, given findings that ST behaviour is linked with faster reversal in both Pavlovian (Kearns et al. 2006) and operant tasks (Experiment 1), and that both behaviours have a common serotonergic basis (e.g. Izquierdo et al. 2006; Campus et al. 2016). Prior use of a broad range of 5-HT agonists and antagonists in autoshaping tasks has revealed the complex role different receptor subtypes play in this behaviour, and highlights a need to use more receptor-specific ligands to further understand this interplay. This is one of the first experiments to explore the effect of a receptor-specific ligand on Pavlovian conditioned approach (rather than autoshaping) behaviour, and will be the first, to my knowledge, to explore the effect of antagonism at 5-HT_{2C}Rs on this behaviour.

5.4.1. Materials and methods:

Animals and behavioural procedures:

Thirty-two male C57BL/6J mice were food restricted to 90% of their free-feeding weights for 2 weeks prior to behavioural testing. Animals weighed an average of 22.2g (SEM ± 0.4g) at the start of behavioural testing. This experiment employed a repeated measures design to assess drug-related impacts on performance within the same animal, in order to minimise animal numbers and to overcome issues of variance in PCA behaviour within small groups.

Habituation and pre-training:

Animals received sugar pellets in their home cages for two consecutive days prior to testing. Two pre-training sessions were given, during which the levers remained retracted and nosepoke holes covered. Thirty food pellets were delivered on a variable interval (VI) 15-s schedule, to determine that mice were reliably retrieving pellets.

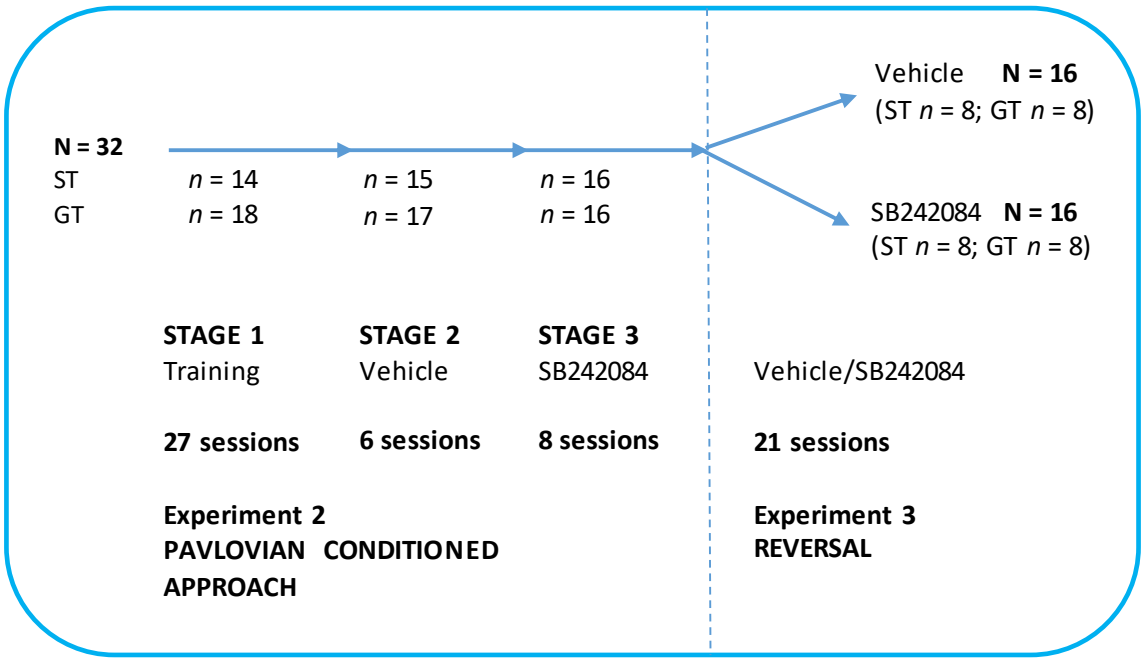

Figure 5.4: *Representation of the experimental design of Experiments 2 and 3. Following a training period, Experiment 2 uses a repeated measures design to assess changes in Pavlovian conditioned approach behaviour during vehicle and drug testing (SB242084). Experiment 3 uses the same animals in a between-subjects design to assess their ability to reverse these learned associations, assigning half the animals to each treatment group (Vehicle/SB242084), counterbalanced for number of signtrackers (ST) and goaltrackers (GT) identified during Stage 3 of Experiment 2.*

Stage 1 Pavlovian training:

One lever was designated the CS+ or 'active' lever (counterbalanced across left or right between subjects), and was presented for 10 seconds on a variable interval (VI) 15-s schedule for 30 trials, as during the pilot. Upon withdrawal of the active lever, one sugar pellet was dispensed into the magazine. The other lever, the 'inactive lever' CS- was present throughout the session, to measure general activity and lever pressing. Active and inactive lever presses and magazine entries were recorded in 4 bins: those made during the 10 sec pre-trial baseline, the 10 sec during CS+ presentation, the 10 sec during/following US delivery, and the 10 sec post-trial baseline. Latency to first lever press and to first magazine head entry during presentation of the lever on each trial was also recorded on trials where responses were made. It is particularly important in a repeated-measures design to ensure that any change in performance in subsequent test stages is specific to the experimental manipulation rather than being time- or experience-related. It was initially planned to allow animals 15 sessions of training, in line with that required for performance to stabilise in the pilot study. Stable responding took a longer time to emerge however, and animals required 27 sessions of testing before moving on to the

subsequent test stage. This was the first stage at which all animals could be said to have reached asymptote, as evidenced by a lack of significant change in PCA index scores over three consecutive test sessions.

***Stage 2 Vehicle testing*:**

Once reliable performance was reached on the autoshaping task, animals received vehicle injections (0.5 ml/kg, *s.c.*) 30 minutes prior to each day of testing, for 6 days, to ensure that the stress of the injection procedure itself was not affecting signtracking behaviour. Once performance during this phase was stable, with no significant change in PCA scores over three consecutive test sessions, animals proceeded to testing under drug conditions.

Stage 3 Drug testing:

All animals received injections of SB242084 (0.5 ml/kg, s.c.) 30 minutes prior to each day of autoshaping testing for 8 days, until stable performance was reached.

The order of drug/vehicle treatment was not counterbalanced in this design as it was possible that drug treatment might have carry-over effects, therefore any animals receiving drug-treatment first would not receive a sufficient control test of performance. Animal numbers would need to have been doubled to ensure sufficient power from the 'vehicle-drug' group had the 'drug-vehicle' group data been unusable. Instead, a further vehicle-test stage was planned to follow on from drug treatment, to see if any drug-related effects were still observable once drug was removed, and to control for time- or experience-dependent effects.

Measures:

Pavlovian conditioned approach behaviour was assessed by calculating PCA index scores for each animal, as described in Experiment 1. A baseline measure of magazine entries per session was calculated by averaging pre-trial and post-trial baseline entries. This served as a control for general behavioural activity at the magazine. The number of inactive lever presses made whilst the CS+ (active lever) was present was used as a control for general activity as directed toward the levers. Additionally, number of magazine entries made during reward delivery was analysed, to assess possible motivational effects of drug treatment.

Statistics:

As with the pilot data, there was no evidence of an intermediate group of animals, and a clear bimodal distribution of scores was apparent for all performance measures. Non-parametric tests were therefore required to compare performance between conditions across

animals, since the assumption of normal distribution was violated. Friedman tests were used to check PCA index scores were stable over the final three days of testing during each stage, and Spearman's rank tests checked this performance was highly correlated over days. Wilcoxon signed-rank tests were used to check there was no significant difference in performance during vehicle testing compared to training, and to assess drug-related effects on performance across all measures, using Holms-Sidak correction for multiple comparisons. In order to check there was no interactive effect of PCA phenotype on performance, animals were subsequently divided into ST and GT groups on the basis of their PCA scores. Because scores within these groups were normally distributed, parametric tests could be used to compare performance of these groups across conditions, using mixed ANOVA with PCA group (ST/GT) as a between-subjects factor and Drug (Vehicle/SB242084) as a within-subjects factor. Post-hoc independent measure t-tests were used to further explore any significant effects. Violations of equality of variance were dealt with using square root or $\log_{10}$ transformations. Where they could not be corrected for, individual repeated measures t-tests were used to compare performance under each treatment condition for STs and GTs separately, using Bonferroni correction for mutliple comparisons.

5.4.2. Results:

Training:

Due to the repeated measures design employed, it was important to stabilise performance during the initial test phase. Stable performance took a long time to emerge, with a sign-tracking response occuring in 6 sessions for one animal, but taking up to 25 sessions for others (*M* = 15.64 sessions, *SEM* = 1.73). Histograms of individual PCA scores for each session clearly demonstrate a strong skew towards negative values (GT) on Day 1 (Figure 5.5a), but the emergence of two different subpopulations of GTs (n = 18) and STs (n = 14) by the final day of training (Figure 5.5b). This early bias for responding in the magazine was most likely because animals were pretrained to retrieve pellets from the magazine for 2 days prior to testing. Performance was stable over the final three days of training, with PCA scores highly correlated across days (r_s's > .90), and Friedman test confirming no significant effect of test day ($x^2_{2, 32}$ = 3.81, p > .05).

Vehicle/SB242084 testing:

There was no significant change in performance across the six days of vehicle testing compared to the final three days of training on any measure of interest (data not shown). However, because one animal began to express ST behaviour during vehicle testing, as evidenced by a change from a negative to a positive PCA index score, the final 3 days of vehicle

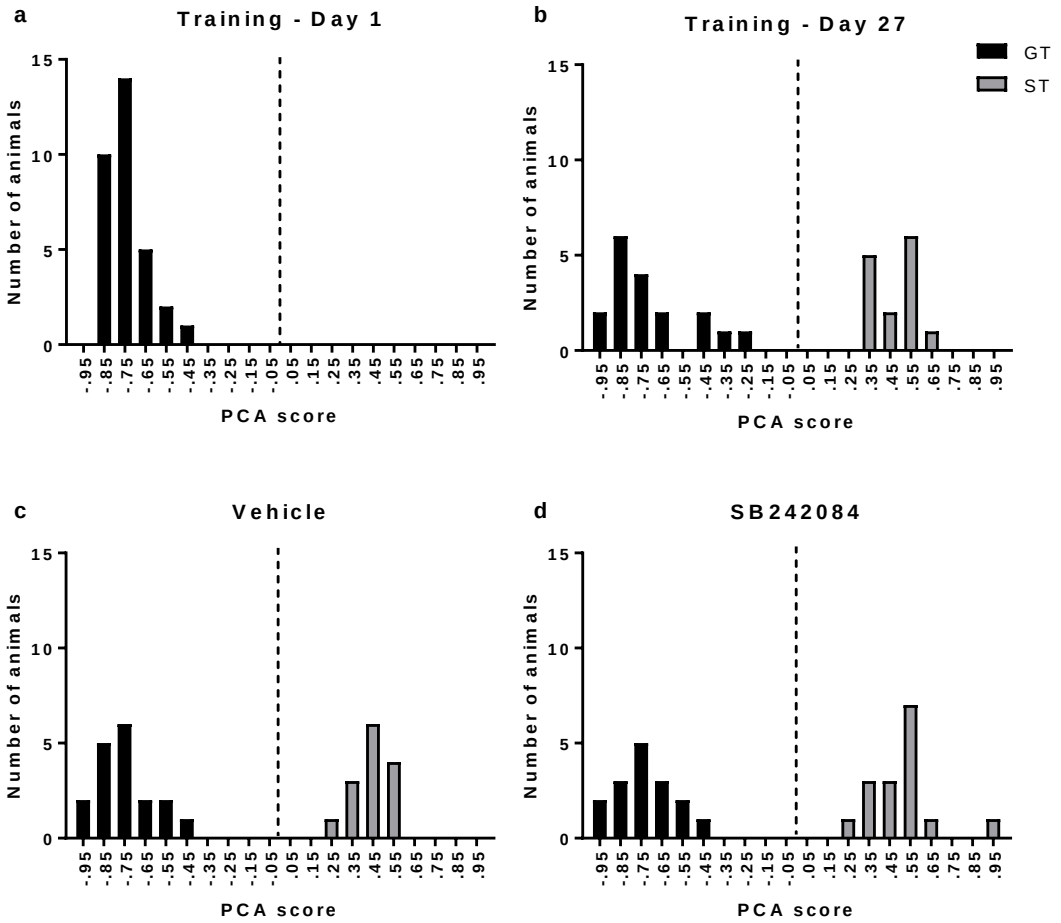

Figure 5.5: *Distribution of PCA scores from first (a) to last (b) session of Stage 1 training, and for last three days under Vehicle (c) and SB242084 (d) testing. The number of mice with a given PCA score are binned into 20 bins of equal size (0.1) according to their score, which ranges from a possible -1 to +1. The vertical axis shows number of mice in each bin. Dashed line denotes PCA score of zero, the cut-off point for group selection. Note the development of two subpopulations by Day 27 of Stage 1 training.*

testing only were compared to the final three of SB242084 testing to assess drug-related effects, where performance was stable for all animals. Friedman tests confirm stable performance across the final three days of testing in both treatment conditions, with no significant effect of test day on PCA scores (Vehicle: $x^2_{2, 32} = 2.69$, $p > .05$; SB242084: $x^2_{2, 32} = 4.75$, $p > .05$); and PCA scores showing high correlations across days (Vehicle: r_s's > .90; SB242084: r_s's > .89).

Wilcoxon signed-rank tests reveal there was no significant change in general behavioural activity for animals under drug-treatment relative to vehicle, either in the number of baseline magazine entries ($z = -.97$, $p > .05$) or inactive lever presses ($z = -.91$, $p > .05$) across conditions. There was a significant increase in the number of magazine entries made following reward delivery however ($z = -3.47$, $p = .001$, $r = -.43$), with animals making more entries during drug treatment (*Median* = 56.0) than vehicle (*Median* = 50.8). There was also a significant change in PCA scores ($z = -2.32$, $p = .02$, $r = -.29$) with evidence of a shift towards increased ST behaviour

under SB treatment (*Median* = -.09) relative to vehicle (*Median* = -.48) (see Figure 5.5c & d). However, only one animal showed a behavioural switch from GT to ST behaviour, as evidenced by a change in PCA index score from a negative (-0.50) to a positive value (0.43).

Although a repeated measures design was employed in an attempt to overcome some of the issues of defining GT and ST groups, it was important to check there was no interactive effect of this behaviour on performance. Animals were therefore divided into groups based on their average PCA index score for the final 3 sessions of vehicle testing. Because PCA scores displayed a bimodal distribution (see figure 5.3), with no clear evidence for an 'intermediate' group of animals, animals were designated GTs (N = 17) if they registered a negative PCA score (*M* = -.76, *SEM* = .03) and STs (N = 15) if they had a positive PCA score (*M* = .45, *SEM* = .03). These groups were used in mixed ANOVA with 'PCA Group' (ST/GT) as a between-subjects factor and 'Drug' (Vehicle/SB242084) as a within-subjects factor for all performance measures.

Results confirm a significant drug-related impact on magazine entries made during reward delivery (US), but only a trend for an effect on PCA index scores (p = .059), and there were no interactive effects for any measure (see Table 5.3 & Figure 5.6). This suggests a possible shift towards ST-type behaviour under drug treatment, but with no evidence of a difference in effect for animals depending on their ST or GT tendency. Additionally, there was a significant effect of group on PCA index scores (as expected given that PCA index score was the criterion used to classify animals into ST and GT groups), as well as on baseline magazine entries, with GTs displaying significantly higher baseline rates of responding than STs (see Table 5.3).

This higher rate of baseline responding could be indicative of a nonspecific elevation of magazine activity in GTs relative to STs. To ensure GTs were learning a conditoned association to the reward-paired stimulus, two mixed ANOVA were performed to compare baseline magazine entries to those made during the CS+ under vehicle and SB242084 treatment, with 'time bin' (baseline/during CS+) as a within-subjects factor and 'PCA group' (ST/GT) as a between-subjects factor (see Table 5.4). Data were square-root transformed to correct for equality of variances violations. Again, there was a main effect of group on magazine responding during vehicle- and drug-treatment, with GTs making more magazine entries than STs overall (both at baseline and during CS+). There was no significant main effect of time bin on magazine entries, but there was a significant interaction with PCA group during vehicle testing. Post-hoc repeated measures t-tests confirmed a significantly higher number of magazine entries were made during the CS+ than at baseline by GTs (t_{16} = -2.51, p < .05), but not STs (t_{14} = 0.03, p > .05) under vehicle treatment. This interaction was not significant during SB242084 treatment

however, possibly due to the small reduction in magazine entries made during the CS+ by GTs and during baseline by STs, relative to vehicle rates (see Table 5.4).

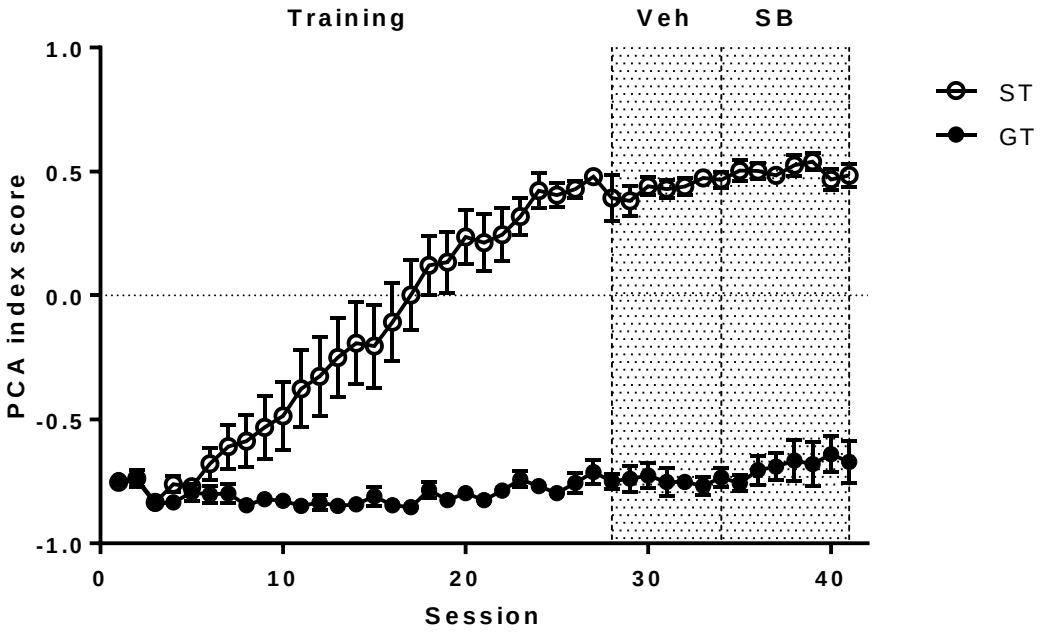

Figure 5.6: *Development of PCA scores over testing. Horizontal dashed lined represents cut-off point for ST/GT grouping. Shaded areas denote vehicle- and drug-treatment testing stages. Animals were grouped as STs or GTs based on performance during final 3 days of training. Note, one animal in the ST group originally identified as a GT during training, causing high variance in the ST group during training stage.*

	Sign-trackers (n = 15)		Goal-trackers (n = 17)		*Group*	*Drug*	*Group x Drug*
	Vehicle	**SB242084**	**Vehicle**	**SB242084**	$F_{1, 30}$	$F_{1, 30}$	$F_{1, 30}$
Inactive lever during CS+	2.0 ± 0.8	2.3 ± 1.2	0.8 ± 0.5	0.6 ± 0.3	*2.77*	*0.01*	*0.11*
Magazine entries (baseline)	18.2 ± 2.3	14.5 ± 2.5	31.0 ± 4.4	30.1 ± 4.1	***9.12****	*1.42*	*0.55*
Magazine entries (during US)	52.6 ± 2.8	57.4 ± 2.3	51.5 ± 2.1	58.6 ± 2.3	*.001*	***15.12****	*0.60*
PCA index score	0.45 ± 0.03	0.50 ± 0.04	-0.76 ± 0.03	-0.66 ± 0.08	***376.9******	*3.85~*	*0.28*

Table 5.3: *Results of mixed ANOVA comparing performance of GTs and STs across drug conditions. Inactive lever presses and baseline magazine entries assess changes in general behavioural activity levels. Magazine entries during US can be seen as a measure of motivation for food reward. PCA index scores measure changes in Pavlovian conditioned approach behaviour. ***p ≤ .001, **p ≤ .01, ~p < .06.*

Because PCA scores are based on derived scores and contain latency measures known to be affected by SB242084 treatment, it was important to understand which elements of behaviour may have contributed towards the possible drug-related effect. The three derived measures that were averaged to create the PCA index score (see Table 5.1) were therefore explored in isolation, to deduce the impact of each measure on overall scores. Scores for each measure used to derive the PCA index (Response bias, Probability difference, Latency score) were analysed using mixed ANOVA with 'PCA Group' (ST/GT) as a between-subjects factor and 'Drug' (Vehicle/SB242084) as a within-subjects factor.

Response bias:

Response bias scores showed the biggest difference between STs and GTs of the three measures, with a mean difference of 1.74 points (*SEM* = .07) between groups ($F_{1, 30} = 683.25$, $p < .001$, see Figure 5.7b). This measure reflects the relative difference in number of lever presses and magazine entries made during the CS+, and indicates that GTs made considerably more magazine entries than lever presses, and STs more lever presses than magazine entries. There was no significant effect of drug treatment ($F_{1, 30} = 1.30$, $p > .05$) or interaction with PCA group ($F_{1, 30} = 0.54$, $p > .05$) on response bias.

Probability difference:

Probability difference scores show a similar, yet smaller degree of difference between ST and GT groups ($F_{1, 30} = 143.58$. $p < .001$; $M = 1.13$, *SEM* = .09, Figure 5.7c), indicating the groups differed more in the number, than the probability of interaction with the 'goal' or 'sign'. Again, there was no significant effect of drug ($F_{1, 30} = 0.48$, $p > .05$) or interaction with PCA group ($F_{1, 30} = 0.32$, $p > .05$) on these scores.

	Magazine entries				*Time bin (Baseline/ during CS+)*	*Group (ST/GT)*	*Time bin x Group*
	Baseline		During CS+		$F_{1, 30}$	$F_{1, 30}$	$F_{1, 30}$
	ST	GT	ST	GT			
Vehicle	18.2 ± *2.3*	31.0 ± *4.4*	18.1 ± *3.5*	45.4 ± *9.6*	*1.58*	***9.96****	***4.23****
SB242084	14.5 ± *2.5*	30.1 ± *4.1*	18.5 ± *4.3*	41.2 ± *9.0*	*1.39*	***10.45****	*0.59*

Table 5.4: *Results of mixed ANOVA with group (ST/GT) as between-subjects factor and drug (Vehicle/SB242084) as within-subjects factor, comparing magazine entries made during baseline and during CS+ (timebin). Results show GTs made more magazine entries than STs, but GTs made more entries specifically during the CS+ than at baseline under vehicle treatment, but not drug treatment. Statistics are based on square-root transformed data; means and standard errors presented are untransformed. *p < .05.*

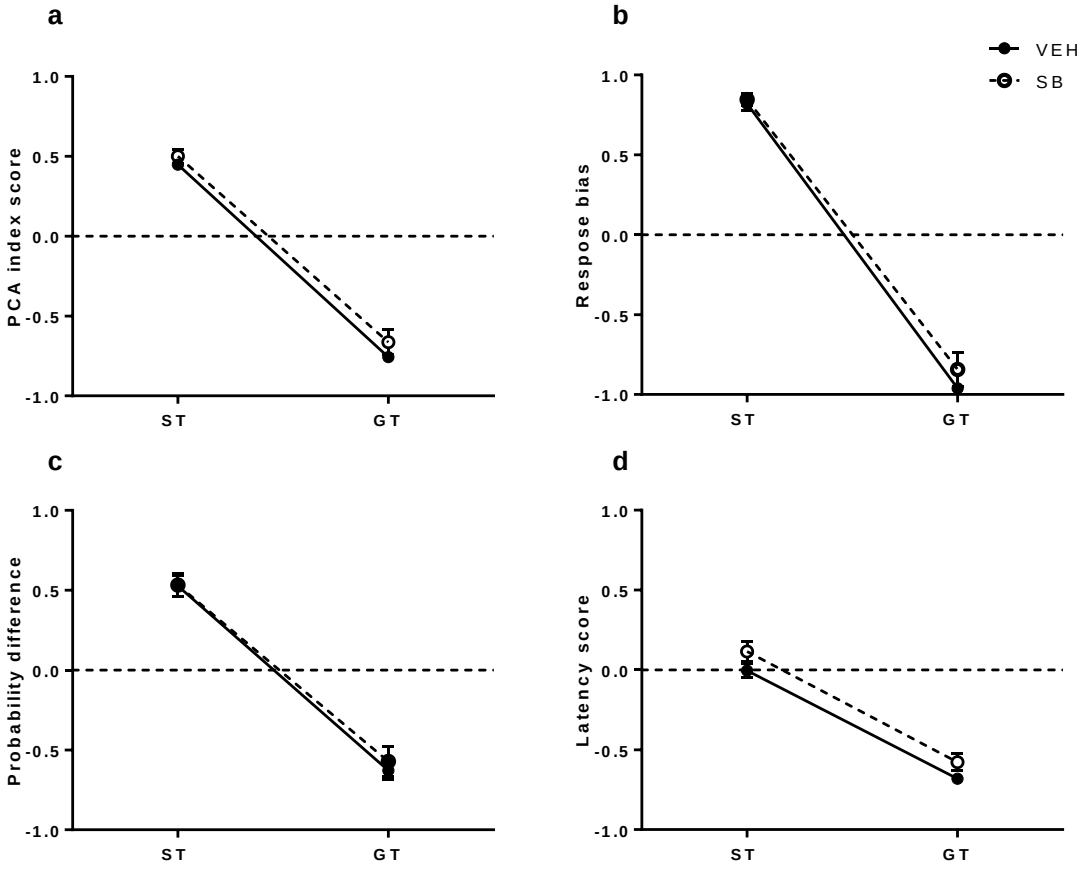

Figure 5.7: *Change in PCA index scores, and the three measures used to derive them, under SB242084-treatment relative to vehicle treatment for animals displaying a signtracking (ST, n = 15) or goaltracking (GT, n = 17) tendency. (a) Overall PCA index scores; (b) Response Bias score, measures the relative degree of interaction with the lever and magazine; (c) Probability Difference Score, measures the relative probability of interacting with the lever or magazine; (d) Latency Score, reflects the relative difference in speed of responding to the lever or magazine whilst the lever is active. See Table 1 for full details of how these scores are calculated. Note that latency score statistics are based on log10 transformed data, but data presented are untransformed means and SEM. Horizontal dashed line represents a score of zero where there is no difference in degree of goal- or sign-tracking behaviour.*

Latency scores:

There was a far smaller group difference on the latency score component between goal-trackers and sign-trackers, realtive to the other two measures ($F_{1, 30} = 131.74$, $p < .001$, $M = 0.69$, $SEM = .06$). This is because, unlike GTs, who responded more quickly at the magazine than at the lever, STs show no difference in latency to respond at either location, as evidenced by their average latency score of approximately zero (see Figure 5.7d). It is for this reason that the overall PCA index scores for STs is actually quite low, at around 0.5 (see Figure 5.7a), despite the very high group average on the response bias component. This is a cutoff often used to denote an 'intermediate' group of animals who show neither a strong ST or GT tendency but a mixture of

the two (between a PCA score of -0.5 to 0.5, Meyer et al, 2013). The reason for this is not clear, but these animals do not show generally heightenened activity levels since they make significantly fewer baseline magazine entries than GTs and make a similarly low number of inactive lever presses (see Table 5.4). There was also a significant main effect of drug treatment on latency scores ($F_{1,30} = 9.15$, $p = .005$) which was not clearly evident from analysis of overall PCA index scores. This reveals a small shift to more positive scores under drug treatment, reflective of increased speed of responding to the lever relative to the magazine, and is clearly what is driving the borderline significant effect of drug treatment on overall PCA index scores. The interaction effect was not significant ($F_{1, 30} = 0.12$, $p > .05$).

The three individual measures that consitute the PCA index score were further broken down into their two constituent ST and GT elements (i.e. responses at the lever and magazine, respectively), to explore the relationship between GT and ST measures, and to see if both are affected the same way during drug treatment:

Response Bias:

The two measures used to calculate a response bias score (number of lever presses/magazine entries during CS+) show an inverse relationship (see Figure 5.8a & b). Mixed ANOVA with 'PCA group' and 'drug' as factors confirm a significant effect of group on magazine entries made during the CS+ ($F_{1, 30} = 8.93$, $p < .01$), with GTs making significantly more entries than STs (see Figure 5.8b). There was no effect of drug treatment on magazine responding however ($F_{1, 30} = 0.67$, $p > .05$) and no interaction effect ($F_{1, 30} = 0.25$, $p > .05$). Because GTs made almost no lever presses under either treatment condition (Veh: $M = 0.5$, $SEM = 0.3$; SB242084: $M = 4.8$, $SEM = 4.2$), the lever press data violated assumptions of equality of covariance which could not be corrected for. Repeated measures t-tests were therefore performed for each group separately. The results indicate no effect of drug treatment on lever press responses for either group (GTs: $t_{16} = -1.02$, $p > .05$; STs: $t_{14} = -0.67$, $p > .05$).

Probability difference:

An inverse relationship is also seen between the two measures used to calculate the probabiliy difference score (percentage of trials with active lever press/magazine response, see Figure 5.8c & d). There was a large, signficant effect of group on percentage of trials with an active lever press ($F_{1, 30} = 691.77$, $p < .001$); and a smaller effect on percentage of trials with a magazine entry during the CS+ ($F_{1, 30} = 9.36$, $p = .01$). There were no drug-related differences however (Active lever: $F_{1, 30} = 1.95$, $p > .05$; Magazine entries: $F_{1, 30} = 0.01$, $p > .05$), and no interaction effects (Active lever: $F_{1, 30} = 0.21$, $p > .05$; Magazine entries: $F_{1, 30} = 0.01$, $p > .05$).

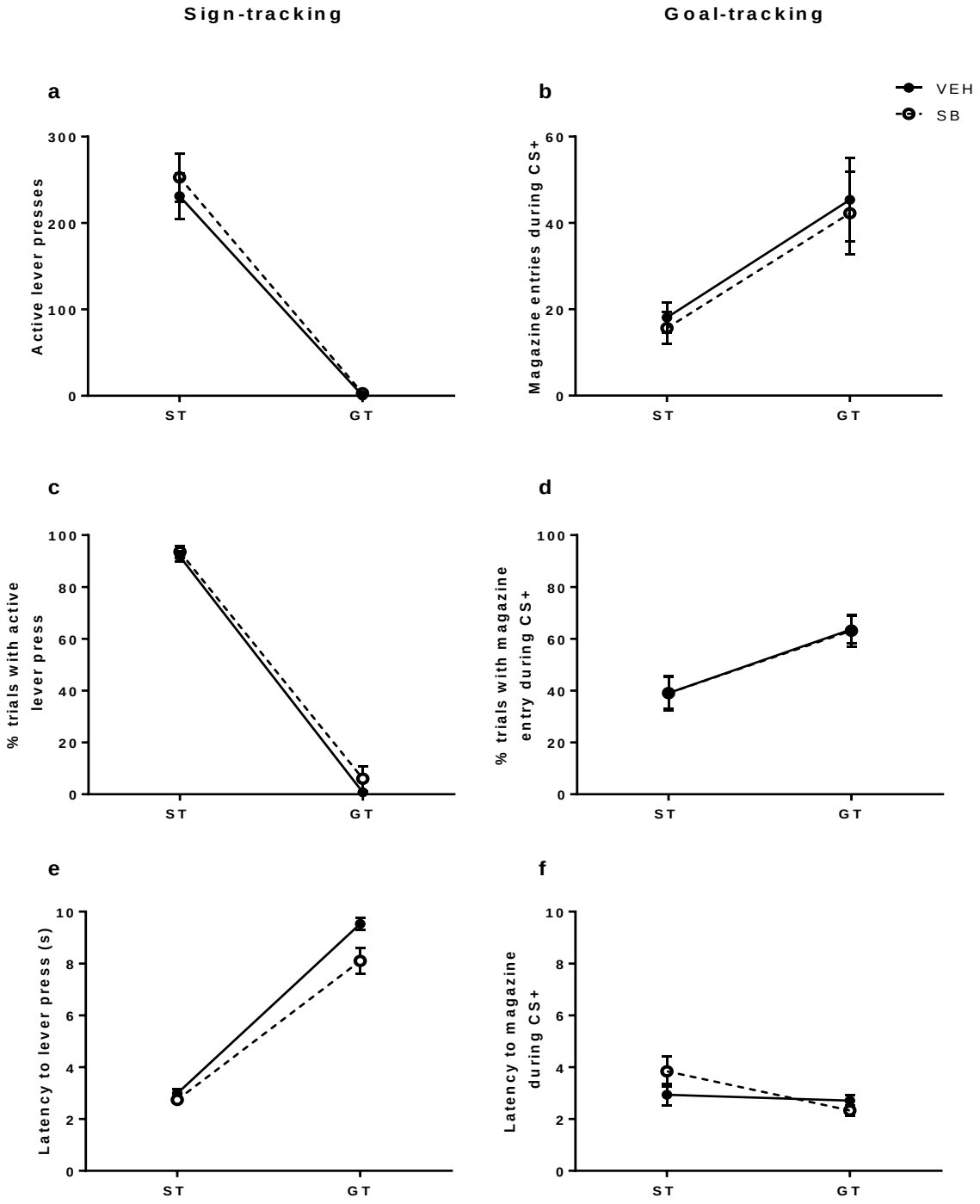

Figure 5.8: *Left panel - sign-tracking (ST) behaviours, right panel - goal-tracking (GT) behaviours. Raw data used to calculate response bias (a & b), probability difference (c & d) and latency scores (e & f). Latency data include maximal scores (10s) for any animals registering no responses in any one session. Due to violations of assumptions, active lever press data were analysed using repeated measure t-tests for each group separately (ST/GT), magazine entries during CS+ and percentage of trials with active lever press were SQRT transformed, and all latency data were log10 transformed. There was a significant main effect of drug and a borderline interaction between drug and PCA group on latency to lever press, (e), and post-hoc tests show a significant latency reduction for GTs under drug treatment not seen in STs. There was a borderline significant interaction effect for latency to magazine (f), but post-hoc tests revealed no significant change for either group under drug treatment.*

Latency:

The two measures which create the latency score (latency to first active lever press/magazine entry during CS+) show a less distinct inverse relationship. On average, GTs were significantly slower to respond to the lever compared with STs ($F_{1, 30}$ = 273.51, p < .001; see Figure 5.8e), but only marginally faster at responding in the magazine during CS+ presentation ($F_{1, 30}$ = 4.43, p < .05; see Figure 5.8f). There was no main effect of drug on latency to respond in the magazine ($F_{1, 30}$ = 0.38, p > .05), but there was a trend for a significant interaction ($F_{1, 30}$ = 3.54, p = .07), with STs showing slower responding and GTs faster responding to the magazine under drug treatment. Post hoc repeated measures t-tests for each group (ST/GT) revealed neither of these effects to be significant however (STs: t_{14} = -1.25, p > .05; GTs: t_{16} = 1.95, p > .05). There was a significant effect of drug treatment on latency to lever press ($F_{1, 30}$ = 11.83, p = .002), as well as a trend for a significant interaction ($F_{1, 30}$ = 3.76, p = .062). Repeated measures t-tests identify a significant increase in speed of responding to the lever under drug-treatment for GTs (t_{16} = 3.15, p <.01) but not for STs (t_{14} = 1.70, p > .05). It should be noted that one ST animal failed to make any magazine head entries during the CS+ under drug treatment and was given a maximal latency score of 10s for analysis. Under vehicle treatment 13 GTs failed to make a single lever press response, whilst under drug treatment only 8 failed to do so. These animals were scored a maximal 10s lever-press latency. These results are supported by analysis of latency data for only those animals that registered a lever press or magazine response during both vehicle and SB242084 testing, and therefore cannot solely be explained by the number of animals failing to make a response (data not shown).

5.4.3. Discussion:

This study expands on previous experiments exploring the effects of various non-selective serotonergic drugs on autoshaped conditioned responding by testing the effect of the selective 5-HT_{2C} receptor antagonist SB242084 on performance in a Pavlovian conditioned approach task. However, unlike the effect of post-test administration of various other 5-HT ligands on conditioned responding, the current study found little evidence of an effect of 5-HT_{2C} receptor antagonism, as administered pre-test. Although initial analyses showed a significant effect of drug treatment on PCA index scores, further breakdown of these scores identified no significant effect of drug treatment on the overall number or probability of making a lever-press or magazine entry during the CS+. There was an effect of drug treatment on latency scores and magazine responding during reward delivery however, which could suggest a small drug-related

impact on conditioned responding, though drug-related motor impulsivity effects may explain these findings.

Initial analyses revealed that drug-treatment caused a general shift in PCA index scores toward more positive scores, indicative of a stronger ST response. This is in-line with prior evidence that conditioned responding is increased following post-test administration of the 5-HT_2 receptor antagonist ritanserin (Meneses & Hong 1997), and could provide evidence of 5-HT_{2C} receptor involvement in this behaviour. However, only one animal displayed a switch in behaviour from a GT to a ST phenotype, and the relevance of this finding is questionable given that one animal had previously displayed this switch during vehicle testing. This could instead point to stress-related effects of the injection procedure, given previously documented links between stress and the ST conditioned response (Tomie et al. 2000). Although animals received several sham injections prior to the initial test day, the procedure is still likely to be stressful. The effect of drug treatment on PCA scores also failed to reach significance when animals were grouped for analysis according to the direction of scores (GT/ST), and provided no evidence for a selective or contrasting impact of drug treatment on these two forms of conditioned responding.

Further breakdown of PCA index scores also revealed no impact of drug treatment on either the total number of responses or the likelihood of making a response at the lever or magazine during the CS+ . However, there was a significant effect on latency scores, with further dissection of this effect revealing a significant drug-related reduction in the average latency to respond on the active lever. This could reflect a general motor impulsivity effect of drug treatment, particularly given evidence of a general drug-related increase in the number of magazine responses made during delivery of reward, which was not specific to either PCA group. However, there was no corresponding increase in speed of responding at the magazine during the CS+, with STs even showing a (non-significant) slowing of responses under drug treatment. There was also some evidence for an interaction effect on latency to respond to the active lever, with post-hoc tests revealing that only GTs were significantly faster to respond to the lever under drug treatment; and ST response latencies, at an average of 3s, were unlikely to be at floor levels. Although caution must be taken in interpreting this effect, since the interaction only showed a trend towards significance, the specificity of this effect challenges the conclusion that it was a drug-related motor effect, or a more general experience- or stress-related effect. Just under a third of animals from the GT group who made no lever presses during vehicle testing also showed at least one response under drug treatment. Taken together, this could suggest that 5-HT_{2C}R antagonism affects the expression of Pavlovian influences, acting to increase the saliency

or draw of the reward-paired lever. Even if this effect were to be confirmed in future tests, the effects are small and seemingly take a long time to emerge, suggesting this is of limited value in explaining the effects of SB242084 on reversal learning.

The length of training required to stabilise performance in this task was surprising, taking nearly twice as many sessions as required during Experiment 1. This most likely reflects the addition of an 'inactive' lever (CS-), introduced as a control for general behavioural activity, which likely increased attentional demands and made conditioned associations to the active lever harder to develop, with animals having to deduce which of two levers was presented reward-contingently. Once lever-pressing behaviour emerged, it typically took several days for a ST response to reach asymptote. During these delays, several animals that previously displayed very stable GT-type performance also started to lever-press and later identified as STs. In rats, this behaviour seems to emerge much more rapidly (e.g. Meyer et al. 2012), therefore experimenters typically test for far fewer days prior to any experimental manipulation. These data suggest that, at least for mice, results based on shorter testing periods may not adequately capture experience-dependent changes in ST behaviour.

The length of training might also have obscured any drug-related effects, if conditioned responding had already reached maximal levels prior to SB242084 administration. Scores observed among STs on the response bias component of the PCA index were nearly maximal (1.0), leaving little room for an increase in number of responses made at the lever relative to the magazine. However, far from maximal scores were observed for STs on the probability difference component, and there was plenty of scope for increased conditioned approach behaviour to be observed in GT animals across all three composite measures; suggesting it is unlikely that extended training resulted in ceiling effects which obscured the influence of drug-treatment.

Such extended training does lead to the possibility that responding had become habitual however. Overtraining of the same response routine is known to result in behavioural autonomy, where goal-directed behaviour, under the control of prefrontal cortical (particularly prelimbic) regions, switches to habitual responding, thought to be supported by dorsal striatal regions and modulated by the infralimbic cortex (Daw et al. 2005). It is possible that overtraining alters the balance of control of behaviour between cortical and subcortical regions, and renders PCA behaviour differentially sensitive or even insensitive to manipulation. It has been postulated that a corticostriatal network involving the OFC, Anterior Cingulate Cortex (ACC) and Nucleus Accumbens core regions is responsible for controlling the expression of PCA behaviour (Robbins

& Everitt 2002). The precise effects overtraining might have on the relative balance of behavioural control within these different regions is yet to be elucidated, although the available evidence suggests that overtraining leads to increased rather than decreased control of behaviour by Pavlovian cues, at least with respect to control over instrumental responding in the Pavlovian-to-Instrumental Transfer (PIT) task (Holland 2004).

In summary these results show that SB242084 treatment had little impact on the expression of Pavlovian conditioned approach responses in mice, save for a small latency effect in GT animals. This latency effect was solely responsible for driving the overall drug-related effect on PCA index scores, which highlights the need for caution when using derived measures, particularly where drugs have a known effect on speed of responding. Although differences in speed of acquisition of conditioned responding cannot be ruled out by the current design, there is little evidence of a dissociation between speed of acquisition and asymptotic levels of performance in previous sign-tracking tasks (e.g. Campus et al. 2016; Kearns et al. 2006; Tomie et al. 1998) and the neurochemical correlates of early and late autoshaping show a high degree of congruence (Tomie et al. 2000). While extended training might have obscured some effects, the overall lack of significant findings suggests that altering the incentive value of cues associated with reward is an unlikely mechanism through which the SB242084 exerts its effects on reversal learning, particularly given how slowly this behaviour emerged.

5.5. EXPERIMENT 3

THE EFFECT OF SB242084 IN PAVLOVIAN REVERSAL

Despite 5-HT_{2C}R seemingly playing a minimal role in Pavlovian conditioned approach behaviour, there is still the question as to how it impacts upon the ability to reverse learned Pavlovian associations. Reversal learning tasks often combine both Pavlovian and instrumental elements, and can arguably be solved without the need for the intervention of instrumental processes (Y Chudasama & Robbins 2003), therefore it is important to understand what contribution 5-HT_{2C}R might play during reversal of Pavlovian associations in the absence of an instrumental response confound. The OFC, a region which receives dense serotonergic innervation (Lewis 1990), is recognised to play an important role in reversal learning, and inactivation of this region is shown to affect reversal of a Pavlovian association (Burke et al. 2009) in the same way as it impairs instrumental reversal (e.g. Chudasama & Robbins 2003; Jones & Mishkin 1972). This suggests that the role of the OFC might be in encoding associations between cues and outcomes rather than actions and outcomes (Ostlund & Balleine 2007). It is not clear what role serotonin plays in these processes, since reversal tasks exploring such manipulations tend to exclusively employ instrumental tests. However, one experiment examining strain-related performance differences in rats suggests a benefit of lowered serotonin function during a Pavlovian reversal task (Kearns et al. 2006), which replicates strain-related differences observed during instrumental reversal in mice (Izquierdo et al. 2006; Graybeal et al. 2014; Elias 1970). Whether the beneficial effect of SB242084 seen in instrumental reversal tasks also extends to Pavlovian contexts is yet to be determined. If SB242084 guides reversal learning by affecting signalling of associations between cues and outcomes, it should similarly support reversal in a Pavlovian task. If however, it affects some aspect of instrumental learning, either in encoding action-outcome associations or in guiding action selection, SB242084 should not affect Pavlovian reversal learning.

5.5.1. MATERIALS AND METHODS

Behavioural procedure:

Because SB242084 had little impact on performance in the Pavlovian conditioned approach task (Experiment 2), animals could be assigned to a drug or vehicle treatment group in a between-subjects design to subsequently test whether SB242084 impacts upon ability to reverse Pavlovian associations in the same way as it does operant responses (see Figure 5.2). During this experiment the test sessions remained exactly the same as in Experiment 2, but the CS+ and CS- contingencies were switched, so that the previously inactive lever became the

'active' reward-predictive lever (CS+) and the previously active lever was now 'inactive', present throughout the task, and no longer contingent with reward (CS-). Animals were assigned to a vehicle or SB242084 treatment group on the basis of their Pavlovian conditioned approach performance, to ensure that both groups were matched evenly for degree of signtracking/goaltracking behaviour. Groups were therefore counterbalanced for PCA index scores during the final 3 days of testing in Experiment 2, and for the total number of GT and ST animals per group (see Figure 5.2 for group compositions). Animals received 16 daily test sessions and received vehicle or SB242084 injections (0.5ml/kg, *s.c.*) 30 minutes prior to testing each day.

Measures:

Because successful reversal learning can only be identified by a switch in the location of lever press responding, analysis of active and inactive lever press behaviour is necessary, with reversal behaviour likely to be less evident from PCA index scores. 'Active' lever presses refers to responses at the previously inactive location, which now predicts reward (CS+), and 'Inactive' lever presses to responses at the previously reward-predictive but now inactive (CS-) location. Given that the inactive lever was previously reward-paired but is now inactive and constantly available, total levels of inactive lever pressing needed to be monitored alongside presses made specifically when the CS+ lever was inserted. Successful reversal performance is harder to assess in goaltracking animals, given the lack of lever press responding, but baseline magazine entries and head entries made during the CS+ were monitored to assess whether a change in the location of the reward-predictive cue affected behaviour. The 21 sessions of reversal performance were assessed in four blocks of sessions to assess the relative change in performance over test days.

Statistics:

Though drug-treatment groups were matched for PCA behaviour during assignment, two-way independent measures ANOVA were used to compare baseline performance levels between drug-treatment groups and PCA groups (final 3 test days prior to reversal), to ensure there were no pre-existing performance differences. Three-way mixed ANOVA were used to compare baseline and reversal performance across the 4 blocks of reversal between drug-treatment and PCA groups. Baseline active lever presses (during final 3 days of SB testing) were log transformed, and total inactive lever presses during reversal were square root transformed to correct for violations of normal distribution.

5.5.2. Results:

Baseline performance checks:

Performance during the final three days of testing of Experiment 2 were compared between animals that were assigned to vehicle and SB242084 treatment groups during reversal, to ensure there were no pre-existing differences in performance on any measure of interest. Pavlovian conditioned approach behaviour needed to be accounted for in these analyses, to provide a baseline control for any differences that might be seen in performance of STs or GTs during reversal. This required the grouping of animals according to their PCA index scores during the three test days prior to reversal. Two-way ANOVA with 'PCA Group' (ST/GT) and 'Reversal Drug' (Vehicle/SB242084) as between-subjects factors were used to compare baseline performance across groups. There were significant differences in performance between GTs and STs, in the direction expected, with STs making significantly more active lever presses and GTs more magazine entries (both at baseline and during CS+), and the groups showed significantly different PCA index scores. There were no significant baseline differences between the Vehicle and SB242084 treatment groups however, and no interaction effects on any measure (see Table 5.5).

	Sign-trackers (n = 16)		Goal-trackers (n = 16)		*Group*	*Drug*	*Group x Drug*
	Vehicle (n = 8)	SB242084 (n = 8)	Vehicle (n = 8)	SB242084 (n = 8)	$F_{1, 28}$	$F_{1, 28}$	$F_{1, 28}$
PCA index scores	0.46 ± 0.04	0.53 ± 0.07	-0.73 ± 0.05	-0.74 ± 0.04	***544.98******	*0.32*	*0.50*
Active lever presses	207.1 ± 36.6	256.2 ± 35.1	0.3 ± 0.1	1.0 ± 0.5	***836.44*****	*3.47*	*0.10*
Inactive lever presses during CS+	2.0 ± 1.5	1.7 ± 1.0	0.4 ± 0.2	2.0 ± 1.0	*0.39*	*0.30*	*0.81*
Total inactive lever presses	30.7 ± 10.0	29.1 ± 10.0	7.5 ± 1.8	22.7 ± 7.6	*3.22*	*0.72*	*1.08*
Baseline magazine entries	19.0 ± 3.8	10.2 ± 1.8	32.2 ± 8.2	29.9 ± 3.2	***11.37****	*1.29*	*0.44*
Magazine entries during CS+	20.8 ± 6.6	14.5 ± 5.2	41.1 ± 16.9	45.8 ± 9.0	6.09*	*0.01*	*0.27*

Table 5.5: *Results of two-way ANOVA with PCA Group (Sign-tracker/Goal-tracker) and Reversal Drug (Vehicle/SB242084) as between-subjects factors. Results confirm no significant baseline drug-related differences in performance for animals with a goaltracking or signtracking tendency prior to reversal. Note that results are based on performance during the final 3 test sessions of Experiment 2, where all animals were receiving SB242084. Means and SEM are untransformed, but results for active lever presses are based on log10 transformed data and total inactive lever presses on square root transformed data. ** p < .01; ***p < .001.*

Reversal:

Performance across reversal (blocks 1-4) was compared to baseline performance (scores on the final three days of testing in Experiment 2). Three-way mixed ANOVA with 'session block' (5 levels: Baseline/Reversal block 1-4) as a within-subjects factor, and 'PCA group' (ST/GT) and 'Reversal Drug' (Vehicle/SB242084) as between-subjects factors were used to assess performance for each measure of interest (see Figures 5.9 & 5.10). Drug treatment had no effect on reversal performance within this Pavlovian reversal design, with no significant main effect of SB242084 evident on any measure, and no significant two- or three-way drug interaction effects (data not shown). The following results section therefore refers to the effects of 'PCA group', 'session block' and 'PCA group x session block' effects only.

Active lever presses:

GTs made as many active lever presses as STs during reversal, with no main effect of PCA group on this measure (see Table 5.6). This was surprising given the large, significant baseline differences in performance between groups prior to reversal (see Table 5.5). Inspection of the data reveals that this was not driven entirely by a failure of STs to learn the reversal and to begin responding on the now-active lever; but also indicates an increase in ST-type behaviour among animals previously identified as GTs (see Figure 5.9a & b).

There was also a significant effect of session block and a significant interaction between session block and PCA group (see Table 5.6). Post-hoc tests were run to further understand this interaction effect. Separate repeated measures ANOVA assessing the effect of session block for STs and GTs demonstrate a significant effect of session block for both groups (STs: $F_{2.4, 36.6}$ = 75.35, $p < .001$; GTs: $F_{1.4, 20.5}$ = 9.24, $p < .005$). Pairwise comparisons show that GTs significantly increased active lever pressing between the first and final reversal session block ($p = .05$), and responses were significantly higher than baseline levels by the final reversal block ($p = .02$). STs showed no significant change in responding across session blocks however (p's > .05), and levels were significantly lower than baseline during every stage of reversal (p's < .001). Therefore, although both groups increased 'correct' active lever presses over the course of reversal, the increase was only significant for GTs, and ST performance was still significantly lower than pre-reversal levels even by the end of testing.

To ensure the increase in lever-pressing behaviour in GTs was not due to side-bias effects resulting in increased responding once the active lever switched sides, a mixed ANOVA was performed with active-lever location (Left/Right) and reversal session block (1-4) as factors. Results confirm there was no significant effect of lever location on number of active lever-

presses made during reversal ($F_{1, 14} < 0.01$, $p > .05$), and no interaction with session block ($F_{1.6, 21.6} = 1.11$, $p > .05$).

Inactive lever presses during CS+:

STs made significantly more inactive lever presses during the CS+ than GTs, despite no differences in this behaviour observable at baseline, resulting in a significant main effect of PCA group on this measure (see Table 5.6 and Figure 5.9e & f). This is most likely because the previously reward-paired active lever (now the inactive lever) was now present throughout the task, which allowed for continued responding at this lever throughout the session.

There was also a significant effect of session block, and an interaction between session block and PCA group (see Table 5.6). Post-hoc tests revealed a significant effect of session block for both STs ($F_{2.3, 35.0} = 43.51$, $p < .001$) and GTs ($F_{3.8, 57.1} = 2.64$, $p < .05$). Pairwise comparisons show that STs made significantly more inactive lever responses during the CS+ compared with baseline levels across all session blocks of reversal (p's < .001), but significantly decreased responding across the reversal test as a whole, from block 1 to 3 ($p = .04$) and from 1 to 4 ($p = .003$). Therefore, STs showed a reduction in inactive lever presses over reversal, but still significantly differed from baseline levels by the final reversal block. GTs showed no significant pairwise differences in inactive lever performance, either compared to baseline levels, or across any stage of reversal (p's > .05).

	PCA group	*Session block*	*PCA group x session block*
Active lever presses	$F_{1, 28} = 2.54$	$\mathbf{F_{1.9, 52.5} = 20.98***}$	$\mathbf{F_{1.9, 52.5} = 44.48***}$
Inactive lever presses during CS+	$\mathbf{F_{1, 28} = 50.47***}$	$\mathbf{F_{3.2, 88.2} = 34.57***}$	$\mathbf{F_{3.2, 88.2} = 18.34***}$
Total inactive lever presses	$\mathbf{F_{1, 28} = 23.76***}$	$\mathbf{F_{3.0, 85.2} = 14.79***}$	$\mathbf{F_{3.0, 85.2} = 11.66***}$
Baseline magazine entries	$\mathbf{F_{1, 28} = 11.63**}$	$\mathbf{F_{2, 50} = 19.53***}$	$\mathbf{F_{2, 50} = 6.46**}$
Magazine entries during CS+	$\mathbf{F_{1, 28} = 6.80*}$	$\mathbf{F_{2, 54} = 10.85**}$	$F_{2, 54} = 3.09$~

Table 5.6 *Results of three-way mixed ANOVA of reversal performance with 'Drug', 'PCA group', and 'Session block' as factors. Only results for PCA group, Session block, and PCA group x Session block interaction are shown. There were no effects of drug treatment and no further interaction effects (see Appendix for F and p values for these effects). Session blocks include baseline levels of responding and responses during each block of reversal (1-4). Huyn-Feldt correction is applied to df where sphericity was violated. Significant effects highlighted in bold. *p < .05, **p < .01, ***p < .001, ~p < .06.*

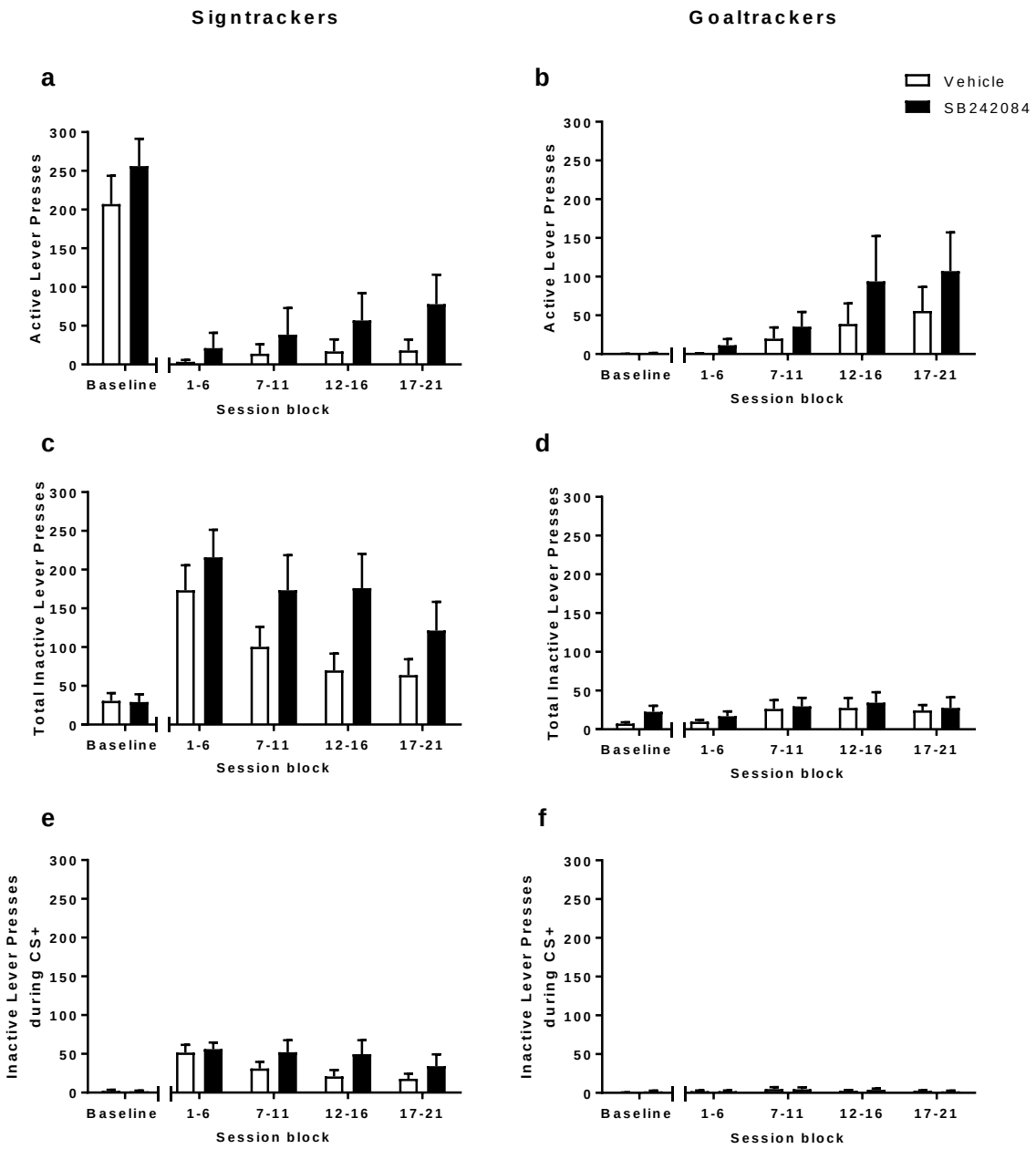

Figure 5.9: *Change in lever-press behaviour from baseline (3 day average from final sessions of Experiment 2) to reversal testing, across the four blocks of test sessions. Left panel reflects behaviour of signtrackers, and right panel of goaltrackers: (a & b) Responses to 'active' lever presentations; (c & d) Responses to the 'inactive' lever, present throughout the task; (e & f) Responses to the 'inactive' lever specifically during presentation of the 'active' lever (CS+).*

Total inactive lever presses:

As with inactive lever presses made during the CS+, there was a significant effect of PCA group and session block, and an interaction between the two (see Table 5.6 and Figure 5.9c & d). Although post-hoc tests revealed a significant effect of session block for both groups (STs: $F_{2.4,\ 35.3} = 19.61$, $p < .001$; GTs: $F_{4,\ 60} = 3.19$, $p < .05$), GTs again showed no significant pairwise differences in performance across any stage of testing, either compared to baseline levels or

across reversal session blocks (*p*'s > .05). STs showed a reduction in total inactive lever presses in each session block of reversal compared to the first (*p*'s < .05), but responses were significantly higher than baseline in all but the final block (*p*'s < .05). So, there was a general decrease in responding for STs over the course of reversal, until responses did not differ from baseline levels by the final testing block.

Baseline magazine entries:

There was a significant effect of PCA group, with GTs continuing to make more magazine entries during baseline than STs (see Table 5.6 and Figure 5.10a & b), as they had prior to reversal (see Table 5.5). There was also a significant effect of session block and an interaction between PCA group and session block. Post-hoc tests show that the effect of session block was significant for both groups (STs: $F_{2.3, 33.8} = 3.42$, $p < .05$; GTs: $F_{1.4, 20.9} = 16.84$, $p < .001$). However, pairwise comparisons failed to reach significance for any contrasts within the ST group (*p*'s > .05). GTs demonstrated significantly lower levels of responding at every stage of reversal compared to baseline levels (*p*'s < .01), but also showed no change in responding across reversal blocks (*p*'s

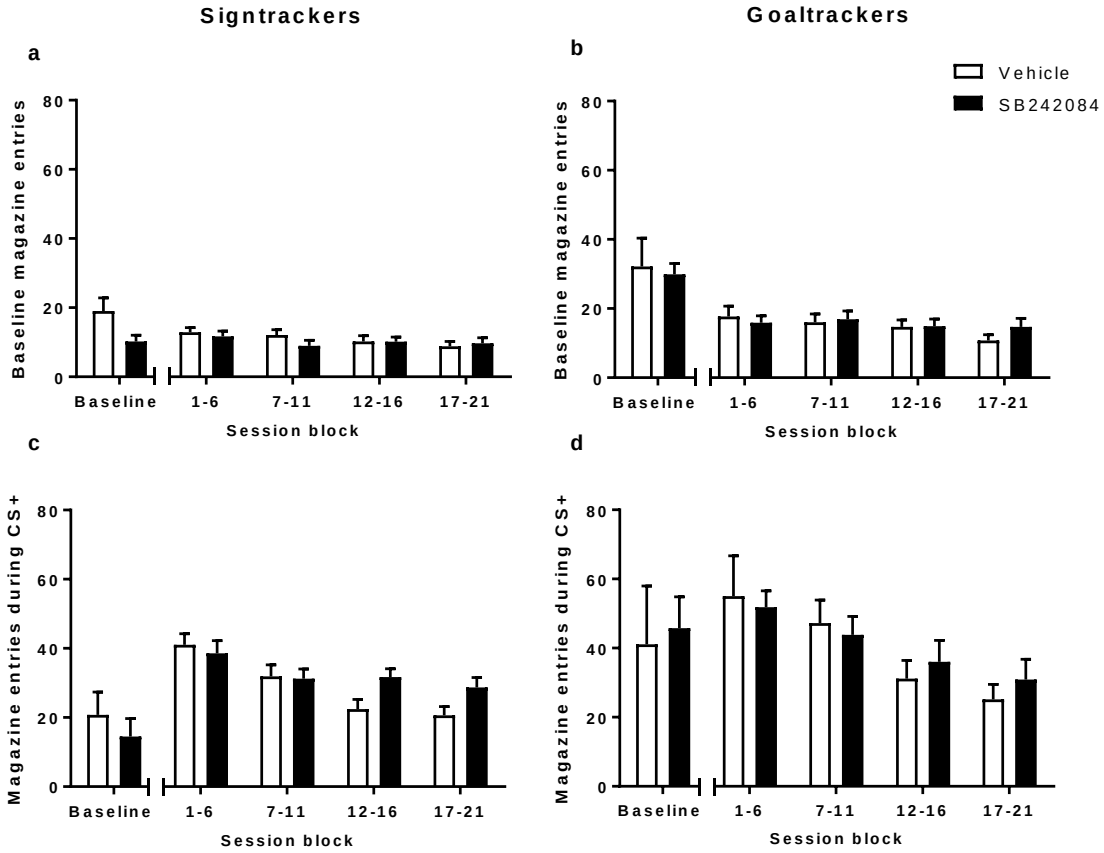

Figure 5.10: *Change in magazine head-entry behaviour from baseline (3 day average from final sessions of Experiment 2) to reversal testing, across the four blocks of reversal test sessions. Left panel represents responses of signtrackers, and the right panel of goaltrackers, during either baseline (a & b) or during presentation of the CS+ (c & d).*

>.05). Therefore, neither group showed a change in general activity at the magazine across reversal, but GTs showed a decline compared to pre-reversal levels.

Magazine entries during CS+:

As for baseline magazine entries, there was a significant effect of PCA group and session block, but there was only a borderline significant interaction between the two (p = .055, see Table 5.6 and Figure 5.10c & d). Pairwise comparisons made across groups show there was a significant increase in responding during the first reversal session block compared to baseline levels (p = .001), followed by a significant reduction in responding between every stage of reversal except from 3-4 (p's < .01). Due to the near significant interaction effect, post-hoc tests were carried out to better understand the nature of this possible interaction. There was a significant effect of session block for both groups of animals (STs: $F_{2.6, 39.9} = 11.35$, $p < .001$; GTs: $F_{1.6, 24.0} = 5.69$, $p < .05$). STs showed more magazine entries during the opening two blocks of reversal compared to baseline levels (p's < .05), but a significant reduction from the first block compared to the final three (p's < .02). GTs showed no difference in responding compared with baseline levels (p's > .05), but also showed reduced responding between reversal blocks 1-3 and 1-4 as well as 2-3 and 2-4 (p's < .05). Therefore, STs showed an increase in GT-type behaviour in the early sessions of reversal compared to pre-reversal levels, followed by a reduction in this behaviour as active lever pressing made a (non-significant) recovery. Conversely, GTs responding was initially unaffected by the reversal switch, but also reduced over testing as active lever pressing increased, showing a shift towards more ST-type behaviour for this group.

Some of the results surrounding magazine entry behaviour during the early stages of reversal were somewhat surprising. Given that the new CS+ (active lever) was previously an irrelevant stimulus (inactive lever), a significantly lower level of magazine responding during its presentation might be anticipated during opening sessions compared to pre-reversal levels. However, GTs showed no change in responding in the first session block, and STs showed an increase compared to pre-reversal levels, which could not be explained by a general increase in magazine directed behaviour as assessed by baseline responding. Furthermore, despite the fact that the inactive lever which previously signalled reward became present throughout the session, GTs showed a reduction, rather than an increase, in baseline levels of magazine responding during reversal. The fact that there is a change in behaviour at the point of reversal shows that animals have noticed a change in task parameters, but the pattern of responses at the magazine suggests a *better* awareness of reward contingency in both groups, rather than any confusion arising from the switch.

It is possible that animals learned the reversal switch very quickly, and that learning effects were obscured through grouping the opening six sessions together. Although a switch in responding at the appropriate lever took a long time to occur (and in fact did not occur for the majority of animals), this effect likely requires the accumulation (and attenuation) of incentive value attached to cues. Given how long it took for lever pressing behaviour to initially emerge in Experiment 2, it could be that animals noticed a change in the reward associations (directed at the 'goal') far before they actually acquired a new conditioned approach response (directed at the 'sign'). STs might therefore show increased activity at the magazine during CS+ because they had learned the switch in contingency, but were yet to acquire the competing ST response to the active lever. For this reason, animals' responses during the first reversal test session only were broken down into 10-trial bins, and performance compared to the final test session prior to reversal (see Figure 5.11). All data were square root transformed to correct for unequal variances, as assessed by Levene's tests.

Baseline magazine entries in first reversal session:

Consistent with data taken across reversal, GTs made more magazine entries than STs during the opening reversal learning session, leading to a significant main effect of PCA group ($F_{1, 28} = 4.82$, $p < .05$). There was also a significant effect of time-bin ($F_{4.3, 121.0} = 14.67$, $p < .001$), and a significant interaction between group and time-bin ($F_{4.3, 121.0} = 2.67$, $p < .05$). Post-hoc tests employing separate repeated measures ANOVA for STs and GTs revealed a significant effect of time-bin on baseline magazine entries for both groups (STs: $F_{5, 75} = 7.59$, $p < .001$; GTs: $F_{3.8, 57.7} = 10.14$, $p < .001$). However, pairwise comparisons failed to show any significant differences in performance during any 10-trial bin of reversal compared with baseline levels for either group (p's > .05).

Magazine entries during the CS+ in first reversal session:

There was a significant main effect of time-bin ($F_{3.3, 93.6} = 17.50$, $p < .001$), and of PCA group ($F_{1, 28} = 4.78$, $p < .05$), with GTs showing higher overall levels of responding than STs. There was also a significant interaction effect ($F_{3.3, 93.6} = 3.36$, $p < .05$). Post-hoc tests show a significant effect of time-bin for both groups (STs: $F_{2.6, 38.2} = 17.38$, $p < .001$; GTs: $F_{3.7, 55.8} = 4.82$, $p < .01$), with pairwise comparisons indicating a significant increase in responses during reversal compared to baseline levels in every 10-trial bin for STs (p's < .05), but no change in performance for GTs (p's > .05). This indicates that STs increased responding immediately, and therefore this could not be argued to be a 'learning' effect. Similarly, the lack of change in responding by GTs during the opening session of reversal shows no evidence of a learning effect.

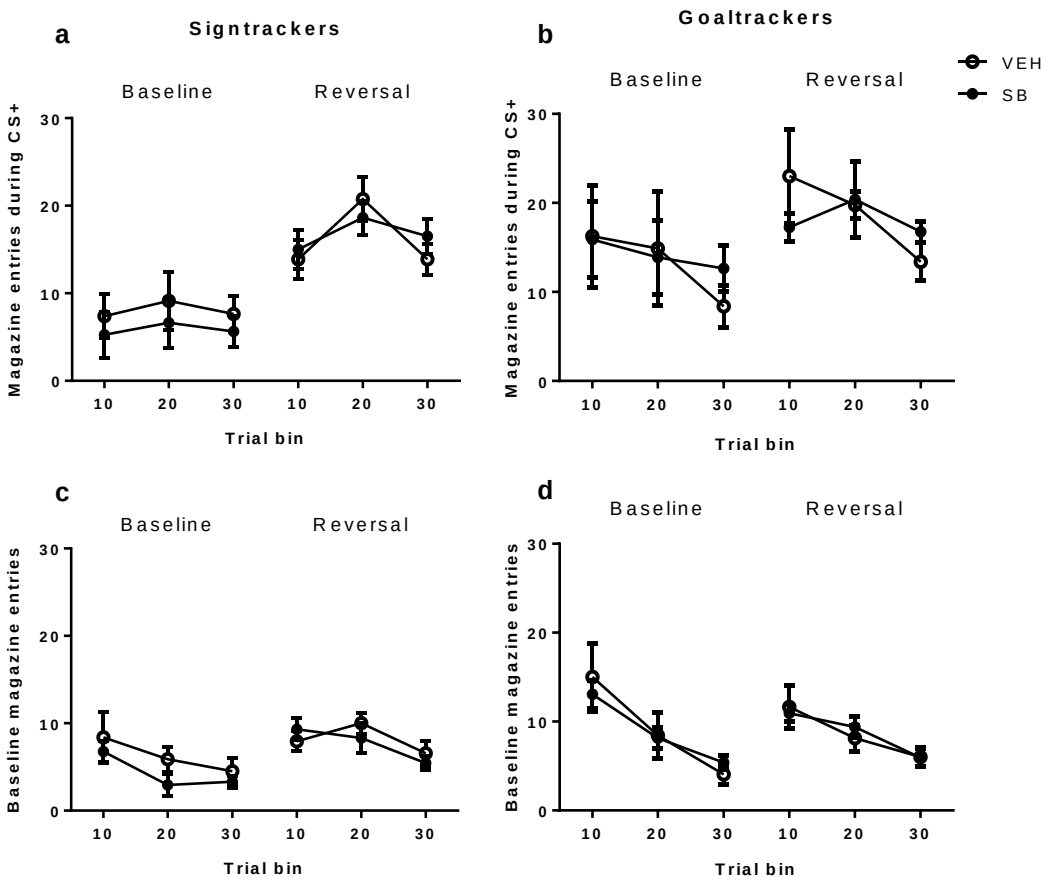

Figure 5.11: *Performance of sign-trackers (left panels) and goal-trackers (right panels) during the first session of reversal as compared to the final session of training, broken down into three 10-trial bins. Sign-trackers show an immediate increase in magazine entries during CS+ (panel a), but no other differences are observed.*

5.5.3. Discussion:

This study provides the first description of how blockade of 5-HT_{2C}R affects reversal of Pavlovian associations in the absence of an instrumental response confound, and produces a number of relevant findings. Although the majority of animals failed to demonstrate successful reversal over the course of testing, at least as evidenced by responding at the levers, drug treatment was found to have no effect on the ability to reverse Pavlovian (stimulus-stimulus) associations. There was evidence of the reversal shift itself causing a change in behaviour for animals who previously identified as goal-trackers, with these animals expressing increased lever-press behaviour coupled with a reduction in magazine-directed responding over the course of testing. In fact, though both groups increased 'correct' active lever-pressing over the course of reversal, only for goal-trackers was this increase significant. Finally, analysis of

responding in the magazine at the point of reversal shift revealed a pattern of behaviour that was not consistent with evidence of learning, necessitating further explanation.

The failure of most animals to switch responding away from the 'incorrect' (previously active) and toward the 'correct' (previously inactive) lever over the course of 21 sessions of reversal is perhaps surprising, given that rats have been reported to learn a similar reversal within 12 sessions (Kearns et al. 2006), and experiments reported here and in previous work (e.g. Nilsson et al. 2012, 2013; Pennanen et al. 2013) show that mice very quickly learn to reverse instrumental associations. However, unlike instrumental reversal tasks which drive behaviour change by the absence of anticipated reward following incorrect responses, Pavlovian reversal tasks do not necessitate a change in behaviour in order to elicit continuing reward. In the previous Pavlovian reversal task (Kearns et al. 2006), the CS- was also presented on discrete, intermittent trials, so only one lever could be in the chamber at a time. Consequently, CS- presentation led to an absence of expected reward during reversal, rather than becoming irrelevant to reward delivery; an effect which is also likely to have accelerated behaviour change. Moreover, because the inactive lever was present throughout the session in the current task, animals could continue to respond at this location, even when a competing response began to develop to the active lever. The response competition that occurs when both levers are present explains why inactive lever responses made during presentation of the active lever (CS+) were far lower than the total number of incorrect responses made, and why only incorrect responses made during the CS+ returned to baseline levels by the end of testing. This response competition effect might also explain the lack of increased responding at the active lever amongst STs, which persisted far longer than any reported latent inhibition effects (e.g. Bonardi et al. 2005; Bonardi et al. 2010), or learned non-reward effects seen during instrumental reversal (e.g. Nilsson et al. 2012, 2013). By contrast, goal-trackers were able to successfully increase active lever pressing during reversal, presumably due to the lack of constraint on responding caused by a competing response at the previously active lever.

Aside from differences in task design, the insensitivity sign-trackers displayed during reversal is also likely to be related to the length of training animals required in order to initially establish PCA behaviour. A sign-tracking response is proposed to develop as the reward-paired cue acquires incentive value, which increases with repeated pairings (Berridge 2001). Given that this response was trained to asymptote levels during acquisition, following which animals were subject to 14 additional days of vehicle- and drug-treatment testing where this behaviour remained largely stable (Experiment 2), it is highly likely that the lever had acquired maximal incentive value prior to reversal, which was subsequently difficult to overcome. By contrast,

acquisition of an operant response pattern in instrumental tasks typically takes only a few test sessions, therefore any incentive value that might be ascribed to a reward-paired cue during training would be minimal, also possibly enabling faster reversal learning in instrumental over Pavlovian tasks.

The inability of a number of animals to successfully reverse lever-press responding in this task led to large error variances, and it is tempting to speculate that this distorted drug-related differences in outcome. Although not significant, drug-treated animals did display a faster increase in responding at the active lever compared to controls, both for sign-trackers and goal-trackers, which fits with prior evidence of a drug-related enhancement in overcoming learned non-reward. However, for sign-trackers this was coupled with (non-significant) higher rates of inactive lever responding compared to controls, which seemingly contradicts evidence reported in Chapter 3, Experiment 3 that drug treatment does not affect responding in the perseveration condition of an operant reversal task, though this is consistent with the extinction impairment reported in Chapter 4, Experiment 2. However, the response competition that emerges during simultaneous presentation of the active and inactive levers in this task means that, as with instrumental simultaneous discrimination reversal tasks, the present design is unable to fully segregate the effects of learned non-reward and perseveration, since effects on one likely impact directly upon the other. Overtraining is likely to have had a larger impact on preventing the acquisition of active lever pressing than the extinction of inactive lever pressing however, given that overtraining has been shown to enhance speed of extinction (e.g. North & Stimmel 1960; Tombaugh 1965). Nevertheless, these effects were not significant, and aside from the small possibility that overtraining led to an insensitivity to reversal that obscured drug treatment effects, there is no evidence to suggest that antagonism at 5-HT_{2C}R affects the reversal of Pavlovian associations in the same way as it has been shown to facilitate operant reversal learning. This raises the interesting possibility that blockade at these receptors affects some aspect of instrumental responding, either by impacting upon action selection or stimulus-response and response-outcome associations. This might explain the lack of drug-treatment effect on the expression of the conditioned approach behaviour in experiment 2, and in the latent inhibition task of chapter 4, Experiment 2.

The unexpected change in behaviour observed during reversal in animals that had previously identified as goal-trackers also requires explanation. These animals had previously displayed almost no active lever pressing behaviour during acquisition, yet demonstrated a significant increase over the course of reversal, which was tracked by a similar reduction in responding at the magazine during CS+ presentation. This shift toward more ST-type behaviour

could not be accounted for by side-bias effects, which might have led to increased lever pressing once the active lever switched sides. Therefore, some aspect of the reversal shift itself must be responsible for this behaviour change. Expectancy violation can elicit measurable frustration in animals, eliciting increased aggression, and stress as measured by plasma corticosterone levels (Dantzer et al. 1980). Given evidence of a significant correlation between the acquisition of the sign-tracking response and post-session corticosterone levels (Tomie et al. 2000), the stress induced by reversal of conditioned associations could provide an explanation for the increase in ST behaviour observed. However, there is some uncertainty as to the direction of this relationship. Rats receiving paired presentations of the lever (CS) and food (US) exhibit higher corticosterone levels than those in an unpaired or random CS-US group (Tomie et al. 2000; Tomie et al. 2004), suggesting that it is the autoshaping procedure itself that induces stress. Furthermore, in the Tomie et al. (2000) study, corticosterone levels were measured after 20 test sessions, but the difference in behaviour between high and low responders was seen in speed of acquisition within the first 10 sessions. Interestingly though, corticosterone levels after the very first autoshaping session are found to be higher in animals that later identify as sign-trackers (Flagel et al. 2009). It is not clear how much stress is likely to be elicited during Pavlovian reversal tasks however. Though a rapid increase in plasma corticosterone levels is observed during extinction following both instrumental and Pavlovian reinforcement schedules (Coe et al. 1983), these effects relate to the unexpected omission of rewards, and not to a change in reward-predictive cues where reward continues to be received. Future work will be needed to determine if this expectation violation is sufficient to elicit stress during reversal, and indeed whether such stress can induce changes in the expression of sign-tracking behaviour.

Expectancy violation has also been shown to induce measurable new learning, and it might be that the uncertainty caused by a reversal shift allowed for renewed attention to reward-paired cues that enabled new learning to occur. Anselme's (2010) uncertainty processing theory conceives of motivation as an information-processing system which collects information about the world in an attempt to optimise the reaching of goals. Seeking behaviours (approach and avoidance) are postulated to be uncertainty-reducing behavioural strategies induced and controlled by motivation. Uncertainty, about the location or availability of food in a given environment for example, can be a threat to survival; therefore whenever uncertainty arises, animals are motivated into seeking behaviours in a bid to collect further information about the conditions associated with this event. When cues signalling reward availability change, animals must start to use 'bottom-up, sensory-induced cues' instead of 'top-down, expectation-driven' information (Anselme 2010). Therefore, the uncertainty that occurs during reversal can

cause an increase in motivation and attention to relevant cues, which subsequently lead to new learning.

Despite the use of a variable interval and a probabilistic reward schedule in the current design, the conditions associated with reward are likely to have become very predictable for animals over the course of so many sessions, reducing the need to collect further information from the environment, and resulting in a loss of motivation. This is supported by evidence from Experiment 2 of this Chapter, which shows a decline in magazine responding during the CS+ for GTs over the course of training, until it no longer differed from baseline levels. During a reversal shift however, animals are motivated to reduce uncertainty about the relevance of environmental cues, and begin to attend to and approach relevant cues once more, providing another opportunity for the active lever to acquire incentive value. In this way, responding is re-invigorated which may allow a proportion of goal-trackers to acquire a new sign-tracking response. Evidence that uncertainty over the probability and/or magnitude of reward increases conditioned responding in autoshaping tasks (Anselme et al. 2013) provided the initial rationale for using a probabilistic reward schedule in the current experiments, given the problems caused by low levels of lever-press behaviour in autoshaping tasks in mice. Taken alongside evidence that such uncertainty can even extend the sign-tracking response to normally unattractive cues (Robinson et al. 2014), this provides powerful evidence that uncertainty enhances the ability of the CS to act as an attractive motivation magnet, even when it previously had no incentive value. Importantly though, this uncertainty does not need to be about the US itself (i.e. surprise omission of reward or change in size, quantity of reward); it can simply be a change in the relationship between a CS and US, since these relationships allow animals to try to reduce the uncertainty associated with reward availability in the environment (Anselme 2010).

The uncertainty processing theory of motivation (Anselme 2010) might also be called upon to explain the unexpected effects of reversal on magazine response behaviour. Baseline levels of responding in the CS+ were expected to initially increase following reversal, at least for goal-trackers, since the stimulus that previously predicted reward became present throughout the session. However, goal-trackers showed a significant and early (session 1-6) drop in baseline levels of responding compared to pre-reversal levels, which could also be explained by an increase in motivation caused by the uncertainty of reversal. If goal-trackers started to explore more in an attempt to understand the relevance of environmental cues to reward availability, behaviour might no longer be directed exclusively at the magazine. Although sign-trackers showed no change in baseline levels of magazine responding, these levels were already low prior to reversal, and the reduction seen on this measure in goal-trackers during reversal matched the

levels displayed by sign-trackers both prior to and during reversal, suggesting that this may have been a floor effect.

More difficult to reconcile is evidence of an early increase in magazine entries during presentation of the CS+ compared to pre-reversal levels in sign-trackers, and the lack of change observed on this measure in goal-trackers. Given that the reward-predictive cue had changed, it was anticipated that, at least for goal-trackers, there would be an initial drop in responding during the CS+, followed by a gradual recovery as animals learned to associate the new lever with reward; much in the same way as was seen for active lever-pressing. Given how long it took for lever-press behaviour to initially emerge, it is plausible that a 'cognitive' reversal switch occurred at a much faster rate than changes could be ascribed to the incentive value of the reward-paired levers. This would allow for the appropriate magazine response to develop at a faster rate; an effect which might have been obscured by grouping together several reversal sessions in analysis. In the absence of a competing lever press response at the active lever, which is slower to develop, this would also explain why sign-trackers showed an early increase in responding on this measure compared to pre-reversal levels, followed by a subsequent decline once responding to the active lever developed. However, analysis of the opening session of reversal showed that these effects were seen immediately, during the very first 10-trial bin of reversal, and could therefore not be described as learning effects. One explanation of these effects might be that animals were using alternative cues to guide responding during reversal. Insertion of the lever into the chamber is accompanied by a distinct and loud noise, which likely served as an additional reward-predictive cue during acquisition. For goal-trackers, magazine directed behaviour would be unaffected by a change in the specific lever predicting reward if animals were using this additional sound cue to guide responding. For sign-trackers, who have yet to develop a competing sign-tracking response at the active lever, magazine entry behaviour during the CS+ was in effect 'unmasked', with animals also using sound cues to guide magazine responding. The ability for reversal to elicit a behaviour change in goal-trackers shows that animals were not responding exclusively to the sound of lever insertion prior to reversal however, and that they were paying continued attention to the specific lever being presented.

In summary, alterations in responding to the active and inactive levers following a change in the location of the reward-paired lever took a long time for animals to acquire, or did not occur at all, possibly due to overtraining effects. This delay in acquiring a competing active lever-press response may have allowed for 'goal-tracking type' behaviour to be unmasked in sign-trackers, seen as an increase in the number of magazine entries made during presentation of the active lever, and which gradually reduced for both groups as the competing lever-directed

response developed. The sound associated with lever insertion likely served as an additional reward-predictive cue however, which continued to guide responding at the magazine during reversal, explaining the lack of 'learning' effects on magazine directed performance. The change in which lever predicted reward was likely sufficient to increase uncertainty, and therefore motivation, which could explain the switch to lever-directed responding observed in goal-tracking animals. However, antagonism at 5-HT_{2C}R had no significant impact on any of these performance measures, suggesting these receptors are not involved in reversal of Pavlovian (stimulus-stimulus) associations, and may be more critically involved in aspects of instrumental responding.

5.6. GENERAL DISCUSSION:

Due to a confound of classical and instrumental response requirements in most reversal learning designs, there is the potential for these tasks to be solved through development of Pavlovian conditioned approach responses to reward-paired stimuli, without any need for the involvement of instrumental processes (Chudasama & Robbins 2003). Given prior evidence that serotonergic manipulations can affect conditioned approach behaviour in autoshaping tasks (e.g. Meneses & Hong 1997; Meneses & Terrón 2001), the current chapter aimed to identify whether previously reported reversal learning benefits observed following antagonism of 5-HT_{2C}R might be sub-served by simple Pavlovian, rather than instrumental learning effects.

The first experiment of this chapter suggested a benefit of a sign-tracking phenotype to the ability to solve an operant reversal task; an advantage that replicated the order of effects seen following treatment with SB242084. This was consistent with the hypothesis that antagonism at 5-HT_{2C}R might alter aspects of conditioned approach behaviour that confer a reversal learning advantage, possibly by making animals more likely to approach and contact reward-paired stimuli. However, subsequent experiments investigating the effect of SB242084 treatment on the development and subsequent reversal of Pavlovian conditioned approach provided little evidence for the involvement of 5-HT_{2C}R in these processes. There was evidence for a small drug-related effect on response latencies, making goal-tracking animals faster to contact the lever 'sign' while not affecting approach to the food magazine 'goal', which might explain some of the drug-related latency effects repeatedly observed during reversal tests. However, these effects are unlikely to account for the advantage drug treatment confers during reversal, both because the reversal benefit is not limited to speed of responding, and because of how long these effects took to emerge.

Previous experimenters have also reported difficulties in eliciting the sign-tracking conditioned response in mice; with some having to perform video analysis of lever approach behaviour due to a lack of contact (Tomie et al. 2012). Use of a probabilistic reward schedule likely accounts for the greater ability to induce lever-press behaviour in the current series of experiments, given evidence that reward uncertainty can increase the sign-tracking response (Anselme et al. 2013); and is a design alteration that should be considered by future experimenters wishing to assess Pavlovian conditioned approach behaviour in mice. Nonetheless, a large number of sessions were required to elicit and stabilise lever-press responding, particularly when the task increased in difficulty with the addition of an inactive lever; leading to the possibility that drug-treatment effects were obscured through overtraining and development of habitual responding. Mice may not therefore be the ideal species in which to study these effects, and future experiments in rats may be needed to deduce the precise role of 5-HT_{2C}R in this behaviour. Despite this, evidence that reversal learning is fast and efficient in mice means it is unlikely that alterations in conditioned approach to reward-paired cues directly affect reversal performance. Nevertheless, animals that performed well in an operant reversal task were subsequently shown to develop a sign-tracking orientation. Therefore, the neural circuitry underlying a sign-tracking phenotype may be similar to that required for efficient reversal and cognitive flexibility, though there may be a different role for 5-HT_{2C}R in these two behaviours.

The discovery of phenotypic variance in reversal performance in the current chapter is consistent with prior reports of strain-related differences in both reversal and Pavlovian conditioned approach behaviour in rats (Kearns et al. 2006), and mice (Campus et al. 2016; Graybeal et al. 2014; Izquierdo et al. 2006), where strains that display increased sign-tracking behaviour also show improved reversal performance. This association might explain the variance in performance observed in vehicle-treatment control groups across prior reversal experiments reported here and by others (Nilsson et al. 2012; Pennanen et al. 2013), since the distribution of phenotypes can vary considerably within small populations (Meyer et al. 2012). Therefore, any effect of drug treatment on reversal might be obscured by random variations in the ST/GT phenotype composition of vehicle control groups. Evidence from this chapter, taken alongside previous work, suggests thast the sign-tracking phenotype is associated with increased cognitive flexibility, indicating a possible need to screen animals for conditioned approach behaviour prior to tests of reversal, or to make use of repeated-measures designs, in order to isolate this potential confound from experimental effects in future studies.

Evidence that a reversal switch can itself affect the expression of conditoned approach behaviour, eliciting sign-tracking behaviour in animals previously expressing a goal-tracking phenotype, was an unexpected finding of the current chapter. Evaluated within an uncertainty processing perspective however, this effect becomes understandable. A change in the environmental conditions that give rise to reward can motivate renewed seeking behaviour in order to obtain further relevant information (Anselme 2010), thus enabling new learning to occur. Just as a probabilistic reward schedule was employed to increase sign-tracking behaviour in a species known to show low response levels, the uncertainty associated with a reversal switch can elicit conditioned approach behaviour in animals who previously demonstrated none. Evidence that uncertainty can elicit sign-tracking to previously unattractive cues, that are normally too distal from reward or too risky to elicit sign-tracking (Robinson et al. 2014), provides further support for this effect. This reveals the importance of seemingly small adaptations of task design; and despite evidence of inherent genetic differences in Pavlovian conditioned approach (e.g. Kearns et al. 2006), the phenotypic expression of this behaviour is in fact highly sensitive to specific environmental conditions. Sign-tracking and goal-tracking tendencies are often referred to as behavioural traits (e.g. Flagel et al. 2014; Robinson & Flagel 2009b), and often implicitly assumed to be stable over time. Despite an awareness that environmental factors such as stress (Tomie et al. 2000) and early-life social experience (Lomanowska et al. 2011) can impact upon the relative expression of these tendencies, the evidence presented here suggests that even small, short-term environmental fluctuations can alter the expression of this behaviour, and it is therefore far less 'trait'-like than previously suggested.

That 5-HT_{2C}R antagonism was found to have no effect on the ability to reverse Pavlovian conditioned assocations, when it is shown to support reversal in instrumental tasks, suggests that these receptors may be more critically involved in a 'response' aspect of learning. Which particular aspect of instrumental responding is affected is unclear however, with stimulus-response, response-outcome, or response selection processes all possibly involved. Further research will be required to fully understand the role $5HT_{2C}$R play in these related processes. Though the OFC has been implicated as a critical region for flexible cognition, its role appears to be related to supporting the encoding of associations between cues and outcomes, rather than actions and outcomes (Ostlund & Balleine 2007), since inactivation of this region affects both Pavlovian (Burke et al. 2009) and instrumental (e.g. Chudasama & Robbins 2003; Jones & Mishkin 1972) reversal performance. Current evidence for an effect of $5HT_{2C}$R antagonism on instrumental but not Pavlovian reversal performance seems to suggest that receptors located in

regions other than the OFC may therefore be responsible for this effect. Further research using selectively localised drug administration techniques will be needed to delineate the precise regions responsible for these effects; which will require a full understanding of the various cortical and subcortical regions activated during instrumental reversal tasks.

CHAPTER 6

EXPLORING THE NEUROANATOMICAL BASIS OF REVERSAL LEARNING IN THE MOUSE BRAIN

6.1. Introduction:

Through the use of systemic manipulations, experiments described in the current thesis, along with other recent research, has identified a possible role for the 5-HT_{2C}R in mediating reversal learning performance, possibly through a specific effect on the ability to overcome learned non-reward. However, evidence of variability in outcomes across tasks; as well as the diverse effects 5-HT_{2C}R manipulations have been found to exert on other behavioural measures – such as motor impulsivity (omissions and latency data), behavioural impulsivity (5CSRTT), and reward-responsivity (win-stay/lose-shift responding) – make it difficult to distinguish a clear role for brain systems containing these receptors in mediating flexible cognition. Given the widespread distribution of 5-HT_{2C}Rs throughout the rodent and mammalian brain, as well as their recognised functional interaction within the mesolimbic dopaminergic system, it seems likely that the multiplicity of effects reported following global manipulations may be attributable to actions within different brain regions. Some of these effects (e.g. impulsivity) may even work to counteract any gains in flexible cognition, which could explain the existence of discrepant findings across tasks of seemingly minimally different design. Targeting manipulations to regions known to be involved in flexible cognition should therefore allow a clearer picture to emerge of the role of 5-HT_{2C}Rs in this behaviour. Identifying regions specifically engaged by reversal learning tasks will therefore be the focus of the current chapter.

6.1.1. Role of the prefrontal cortex in flexible cognition: functional heterogeneity and species-related differences

The prefrontal cortex (PFC) has long been known to play a critical role in tasks of cognitive flexibility (e.g. Dias, Robbins, & Roberts, 1996; Jones & Mishkin, 1972; Milner, 1963; Nonneman, Voigt, & Kolb, 1974). However, consistent with the range of functional deficits observed in humans with frontal lobe damage, an understanding of the considerable functional heterogeneity of regions within the PFC has more recently been developed, refining our understanding of the role these regions play in different aspects of flexible behaviour. The

prefrontal cortex (PFC), located in the most rostral area of the frontal lobes, consists of a number of interconnected neocortical areas which sends and receives projections from virtually all cortical sensory systems and motor systems, as well as many subcortical structures (Pandya & Yeterian 1990), making it well-placed for coordinating a flexible behavioural repertoire (Miller & Cohen 2001). Historically, there has been some debate as to the existence of a comparable PFC in rodents, largely due to the absence of a frontal granular region, which early cytoarchitectonic researchers used to distinguish the PFC in primates (e.g. Brodmann, 1909). However, equivalent areas have been identified through the examination of similar neuroanatomical connections across species, with Rose and Woolsey (1948) suggesting this region could be discerned by its dense innervation from the mediodorsal thalamic nucleus (MD). Using similar connectivity criteria, but further emphasising the reciprocity (Nauta 1962) and relative strength (Uylings & van Eden 1990) of MD connections, alongside the development of more sensitive and reliable tracing and immunohistochemical techniques, it has since been possible to identify PFC in a wide variety of mammals. What is more difficult to discern however, is the existence and location of functionally homogenous sub-regions across species, given the increasing size, segregation and specialization of cortical areas in primates relative to rodents.

Based on cytoarchitectural and connectional characteristics, the primate PFC can be divided into three broad sub-divisions, including a dorsolateral (dlPFC), a medial (mPFC) and an orbital (OFC) region of the frontal lobe, rostral to the precentral motor cortex (Fuster 1997; Ongür & Price 2000). The dlPFC has been implicated in higher-order cognitive processes as well as motor planning and regulation, whilst the mPFC and OFC regions are believed to be more strongly involved in the conduct of emotional behaviour. However, these broad regions can be further divided into a large number of distinct architectonic sub-regions, which may be

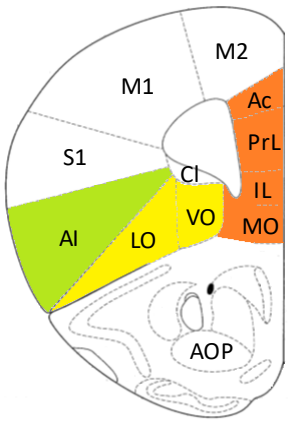

Figure 6.1 *Diagram of mouse prefrontal cortex illustrating medial (orange), orbital (yellow) and lateral (green) subdivisions, based on structural and connectional differences. Adapted from Paxinos & Franklin, 2004. Abbreviations: Ac, Anterior cingulate cortex; AI, Agranular insular cortex; IL, Infralimbic cortex; LO, Lateral orbital cortex; MO, Medial orbital cortex; M1, Primary motor cortex; M2, Secondary motor cortex; PrL, Prelimbic cortex; S1, Primary somatosensory cortex; VO, Ventral orbital cortex.*

differentially involved in the cognitive and emotional processes sub-served by the PFC (Carmichael & Price 1994, 1996; Fuster 1997). The rodent PFC can also be divided into three topographically different regions – a medial, an orbital and a lateral division (see Figure 6.1). The medial prefrontal region (mPFC) comprises of the anterior cingulate (Ac), infralimbic (IL), and prelimbic (PrL) cortical areas, as well as the medial orbital (MO) area (Heidbreder & Groenewegen 2003; Steketee 2003; Uylings & van Eden 1990). The orbital prefrontal (OFC) region, which appears remarkably similar to the primate OFC, consists of the ventral (VO) and lateral orbital (LO) cortex (Krettek & Price 1977b; Reep et al. 1996). Finally, the lateral region of the PFC contains the dorsal and ventral agranular insular (AI) cortices (Leonord, 1969; Kretteck & Price, 1977a). Due to differences in rodent and primate cytoarchiture and connectivity, there is some debate as to the existence of a homologous dlPFC region in the rodent (see Preuss, 1995), though functional and anatomical evidence suggests that regions of the rodent mPFC, particularly dorsal regions including the Ac and PrL, most closely resemble characteristics of the primate dlPFC (Uylings et al. 2003).

Comparing the interactions of the PFC with neuromodulatory systems provides further evidence of cross-species similarities. Both the rodent and primate PFC receive cholinergic innervation from the basal forebrain and laterodorsal tegmental nuclei, and there is a similar pattern of projection back to these basal forebrain and brainstem nuclei across species, with the PFC (in rodents mainly the mPFC, and in primates the mPFC and OFC) being the only cortical area to project directly back to these areas (Ghashghaei & Barbas 2001; Zaborszky et al. 1997). There are also reasonable anatomical similarities with respect to dopaminergic circuitry, with the mPFC of both rodents and mice receiving input from the ventral tegmental area (VTA), whilst the dorsal anterior cingulate in rats, and the dlPFC in primates receives dopaminergic input from the more lateral VTA and substantia nigra (Ongür & Price 2000; Uylings et al. 2003; Williams & Goldman-Rakic 1998). In both rodents and primates, the mPFC also demonstrates a dense projection back to the VTA (Frankle et al. 2006). Patterns of noradrenergic and serotonergic innervation are also widely distributed across the entire PFC of both species, yet in the rodent, cortical projections to the serotonergic raphe nuclei and noradrenergic locus coeruleus derive predominantly from the mPFC, whilst in the primate, these are found to arise from the dlPFC (Uylings & van Eden 1990). Once again the PFC is the only cortical area found to project back to these nuclei, allowing the PFC, via excitatory glutamatergic projection neurons, to regulate the input received from these regions; highlighting the unique integrating or 'gating' position of the PFC (Uylings & van Eden 1990).

6.1.2. Distinct connectivity of orbital (OFC) and medial prefrontal (mPFC) networks

Based on patterns of intrinsic and extrinsic cortico-cortical and cortico-subcortical connectivity, the orbital and medial regions of the PFC have been proposed to consist of two largely distinct networks (Carmichael & Price 1996; Ongür & Price 2000), though these cortical areas appear to be less segregated in the rat than in higher primates (Uylings & van Eden 1990). Injections of axonal tracers into mPFC regions leads to cell-labelling within medial and ventromedial cortical areas, but very few orbital regions. By contrast, injection of tracers into orbital areas has been shown to label many other orbital areas, but very few in the medial wall (Carmichael & Price 1996). Using anterograde and retrograde axonal tracing techniques, the mPFC has been identified as the major source of cortical output to the visceromotor regions of the brain stem and hypothalamus (An et al. 1998; Floyd et al. 2000), as well as containing a greater number of projections to striatal regions than the OFC (Ongür & Price 2000), making it likely to play an integral role in the guidance of behaviour. This medial network also demonstrates strong connections with the hippocampus, a region known to play a role in long-term and contextual memory (Maren & Holt 2000; Whitlock et al. 2006), as well as with associated areas of the cingulate, retrosplenial and entorhinal cortices and the anterior thalamus (Mesulam et al. 1983; Pandya et al. 1981; Vogt & Pandya 1987). Within the rodent brain, the major input to dorsal regions of the mPFC (Fr2, Ac and PrL) comes from sensorimotor and thalamic regions (Gabbott et al. 2005; Jay et al. 1989), consistent with suggestions that these areas are most similar to the dlPFC of primates; whilst the ventral mPFC shows stronger connections with the hippocampal formation and amygdala (Heidbreder & Groenewegen 2003).

Unlike the mPFC, the OFC has few connections to motor regions, though it does show strong connections with the nucleus accumbens core (Haber et al. 1995). Although the mPFC also receives a large amount of sensory input, these projections are strongest to the lateral structures of the PFC (Carmichael & Price 1995a), and the OFC is unique in that it receives input from all sensory modalities, including visual and sensory related areas (Seltzer & Pandya 1989; Webster et al. 1994), as well as from somatosensory (Seltzer & Pandya 1989), gustatory and olfactory cortex (Carmichael & Price 1994, 1995a). Many areas also receive converging inputs from several sensory modalities (Rolls & Baylis 1994); thus the OFC appears to play a major role in sensory representation and integration. The orbital PFC network is further characterised by its strong connections with the basolateral amygdala (Carmichael & Price 1995b; Ongür & Price 2000; Krettek & Price 1977b, McDonald 1991), a region heavily implicated in mediating emotional responses and in building associations between stimuli and outcomes (Balleine & Dickinson 1998; LeDoux 1992).

These medial and orbital networks, though somewhat distinct, are found to share communication pathways (Carmichael & Price 1996), providing a basis for sensory-motor linkage (Ongür & Price 2000), and together form part of a wider circuit which includes reciprocal connections with the striatum. Both the rodent and primate PFC is shown to have a special relationship with the basal ganglia, projecting via the cortico-striatal circuit to these structures as well as receiving highly organised projections from the basal ganglia via the thalamus, which project in a parallel segregated manner to different prefrontal regions, both in the primate and the rodent brain (Alexander et al. 1986; Middleton & Strick 2002; Ongür & Price 2000). Although projections from other cortical areas, including the parietal, temporal and occipital lobe, also reach the basal ganglia, with the exception of area TE of the inferotemporal cortex these other cortical areas do not demonstrate reciprocal connections with basal ganglia (Middleton & Strick 1996). The PFC is therefore a component of several parallel, functionally segregated cortical-subcortical networks believed to perform cognitive, emotional/motivational, sensorimotor and visceral functions, making it well positioned to exert control over many complex cognitive and behavioural processes. Perhaps unsurprisingly then, these largely distinct, yet overlapping circuits of the PFC are believed to mediate different aspects of flexible cognition.

6.1.3. Functional dissociation of OFC and mPFC networks in tasks of cognitive flexibility

Consistent with their distinct patterns of connectivity, different sub-regions of the PFC are found to play functionally specific and dissociable roles in tasks of cognitive flexibility. Animal variants of behavioural tasks used to measure prefrontal damage in humans (e.g. Wisconsin Card Sort Task, WCST) have been instrumental in helping to elucidate the differential functional involvement of distinct neuroanatomical areas of the rodent and primate PFC in supporting flexible cognition. Set-shifting tasks tap into several different forms of cognition, including discriminative learning, reversal learning (sometimes referred to as 'affective-shifting'), formation of an attentional set, and shifting of attention within the same perceptual dimension (intra-dimensional shift, IDS*)* or between different perceptual dimensions (extra-dimensional shift, EDS). With the use of these tasks, researchers have been able to fractionate the pattern of deficits observed into distinct abnormalities related to different regions of the PFC. Lesions of the OFC (typically LO, sometimes including VO and AI) have long been known to impair reversal performance (Butter 1969; Jones & Mishkin 1972; Teitelbaum 1964), but more recent reports demonstrate that such lesions have no effect on the ability to shift attentional set between different perceptual dimensions (Bissonette et al. 2008; Ghods-Sharifi et al. 2008; McAlonan & Brown 2003). Conversely, lesions to the mPFC (typically IL/PrL) in rodents (Birrell & Brown 2000; Bissonette et al. 2008; Floresco et al. 2008), and the dlPFC in primates (Dias et al. 1996a; 1996b;

1997) and humans (Hornak et al. 2004; Owen et al. 1991), have been found to cause deficits in attentional set-shifting, whilst leaving reversal of stimulus-reward contingencies within a single perceptual dimension intact. On this basis, it has been proposed that, in rodents, the mPFC mediates the implementation of 'higher-order' cognitive rules governing selective attention and/or strategy selection, whilst the OFC is involved in 'lower order' functions of responding to changes in the affective significance of stimulus exemplars.

However, it is possible that different sub-regions within the mPFC and OFC are differentially involved in flexible cognition. Sub-regions of the mPFC have occasionally been studied in isolation, and although the PrL appears to be specifically involved in higher-order processing of set-shifts (Churchwell et al. 2009), there is some evidence that lesions to the IL can impair reversal of a visual discrimination task in rats (Y Chudasama & Robbins 2003), though they are without effect in a spatial version of this task (Boulougouris et al. 2007). Given the proposed role of the IL region in supporting the development of habitual behaviour, it could be that these lesions impair reversal by preventing the development of a new, stable choice pattern. Although this form of impairment is inconsistent with early nature of the reversal effects seen following administration of SB242084; the role of the IL region of the mPFC in reversal, particularly across different modalities, is still unclear, and requires further investigation. The specific role of the MO in flexible cognition has also been difficult to discern, given that it has sometimes been lesioned in conjunction with LO/VO regions as part of the OFC (e.g. Young & Shapiro, 2009), and sometimes with the PrL/IL regions as part of the mPFC (e.g. Ferry et al. 2000). A recent study has explored the effects of specific inactivation of MO and LO regions of the OFC on reversal performance however, and implicates the LO more strongly in this behaviour, since inactivation of the MO impaired performance during both acquisition and reversal of a probabilistic task, whilst the deficit caused by LO inactivation was specific to the reversal stage (Dalton et al. 2016). Though the majority of studies exploring the role of OFC regions in tasks of flexible cognition have tended to target ventrolateral regions (e.g. McAlonan & Brown 2003; Schoenbaum et al. 2000; Schoenbaum et al. 2002), the VO has rarely been specifically targeted, and there is currently more evidence for the specific involvement of LO in reversal (e.g. Churchwell et al. 2009; Ghods-Sharifi et al. 2008; Klanker et al. 2013). Evidently, there is a need for more research identifying the specific contribution of each of these frontocortical sub-regions to tasks of cognitive flexibility.

Given that attentional set-shifts are also more difficult than reversals, typically requiring more trials to reach criterion, it is also possible that mPFC and OFC regions are differentially recruited according to level of task difficulty. This possibility gains support from evidence that

lesion or inactivation of mPFC circuitry (IL/PrL) causes deficits in reversal learning tasks when using hard but not easy to discriminate stimuli (Brigman & Rothblat 2008; Bussey et al. 1997), as well causing greater deficits in set-shifting tasks when animals are habituated to the originally irrelevant stimulus dimension prior to testing, thus reducing saliency of the new dimension during a shift (Floresco et al. 2008). In contrast to the pattern of deficits reported above under standard deterministic conditions, there is also evidence to suggest a role for PrL in reversing associations under more difficult probabilistic conditions, whilst IL inactivation has no effect in this task (Dalton et al. 2016). Imaging studies in healthy human subjects also suggest the recruitment of the dorsal anterior cingulate (dACC) and dlPFC in addition to the OFC under probabilistic feedback conditions (Cools et al. 2002; O'Doherty et al. 2003; Remijnse et al. 2005); though patients with focal frontal lesions of the dACC show no such impairment on this task (Tsuchida et al. 2010). Specific regions of the mPFC may therefore be more critically involved in both forms of flexible cognition under conditions of increased task complexity, which could be of relevance to findings in the current thesis for the differential impact of SB242084 treatment under probabilistic and deterministic reversal conditions; though there is also evidence that the OFC is more critical to reversal performance under harder task conditions (Kim & Ragozzino 2005). Nevertheless, interpretations of these findings are largely consistent with the 'supervisory' role proposed for the mPFC in maintaining attention to relevant stimulus dimensions, with increasing stimulus complexity presumably requiring greater attentional resources; and evidence for the specific involvement of OFC regions in reversal learning but not set-shifting tasks suggests these two measures of flexible cognition are supported by largely distinct frontocortical networks.

In sum, regions of the mPFC have most often been implicated with higher order functions such as attentional set-shifting, but are potentially recruited by reversal learning tasks within certain modalities, and under conditions of increased difficulty. There is a particular need to more clearly distinguish the involvement of PrL and IL in these forms of flexible cognition, given evidence that their effects appear to be dissociable. By contrast, the majority of evidence clearly implicates the OFC in reversal learning, with the evidence cited above taken across tasks employing multiple stimulus modalities (e.g. visual, tactile, olfactory, spatial) and with varied motor response requirements (e.g. nose-poking, lever-pressing, bowl-digging). Given the seemingly crucial role of the OFC in supporting reversal learning, it is to the functions of the OFC to which we will now turn.

6.1.4. Broad functional role of the OFC

The effect of OFC lesions on reversal performance has previously been explained in terms of a deficit in response inhibition, given evidence that lesioned animals perseverate longer with the previously correct response pattern than controls (Dias et al 1996a; 1996b; 1997). However, OFC lesions are found to have no effect on task acquisition prior to reversal, where an animal must initially learn to inhibit inappropriate responses to a non-rewarded stimulus (Meunier et al. 1997; Rolls et al. 1994; Schoenbaum, Nugent, et al. 2002); and lesioned animals demonstrate normal inhibition of a prepotent response (Chudasama et al. 2007). Nor would such a deficit clearly explain the dissociation discussed above for the role of the OFC in attentional set-shifting and reversal. The OFC therefore does not appear to be necessary for response inhibition, and more recent theories centre on the role of the OFC in the flexible updating of stimulus-reward contingencies. Electrophysiological recording studies have linked the firing of neurons in the OFC to the innate or acquired significance of stimuli (Schoenbaum & Eichenbaum 1995a; Schoenbaum & Eichenbaum 1995b); and cue-selective activity in these neurons has been found to track changes in reward value (of both reward and punishment). For example, neurons in the OFC begin to fire selectively to a reward-paired cue in a two-choice discrimination task, but stop demonstrating this selectivity when outcomes are reversed; when a largely separate group of OFC neurons begin to fire selectively to the now-rewarded stimulus (Schoenbaum et al. 1999). Human functional magnetic resonance imaging (fMRI) studies demonstrate that feeding subjects to satiety, a form of reinforcer devaluation, reduces activation in the area of the OFC which responds to that food or a cue predictive of its delivery when hungry (Gottfried et al. 2003; O'Doherty et al. 2000). This is consistent with studies conducted in non-human primates (Butter et al. 1963) and rats (Gallagher et al. 1999) showing that lesions of the OFC prevent the normal reduction in responding to a reward-predictive cue seen following its selective devaluation in sham lesioned controls. Therefore, neurons in the OFC do not only encode stimulus identity, but appear to reflect their current incentive value; consistent with the strong connections observed between the OFC and regions involved in sensory representation, limbic regions, and the hypothalamus, which provides motivational state information.

However, the OFC does not appear to simply be involved in linking stimuli to the incentive value of rewards. As previously noted, OFC lesions have no effect on discrimination performance prior to reversal; and OFC lesioned animals show intact development of response-outcome associations in reward devaluation tasks, despite being unable to modify responses to reward-paired cues following a change in reinforcer value (e.g. Gallagher et al. 1999). This

mirrors a commonly reported effect of OFC lesions in human subjects, where a change in a stimulus-outcome rule, either during reversal and extinction (Rolls et al. 1994), or in a complex gambling task (Bechara et al. 1997) is acquired and can be reported, but subjects are unable to modify responding to reflect this knowledge. These data appear to suggest that the OFC does not simply represent the current incentive value of stimuli, but is required for using these values to guide choice performance (Schoenbaum & Setlow 2001). This is consistent with evidence that selective encoding by OFC neurons in rats occurs very late in discrimination learning, only once performance has actually been altered to reflect the information provided by the cues (Schoenbaum et al. 1999; 2000), and with fMRI evidence in humans which demonstrates greater signal change in the ventrolateral PFC during the final reversal error, immediately preceding a shift in response to the newly relevant stimulus (Cools et al. 2002). It has therefore been proposed that the OFC might function to modulate the expression of previously acquired associative information in downstream areas, to guide response choices.

Consistent with the direct reciprocal interconnections observed between the OFC and basolateral amygdala (BLA) complex (Krettek & Price 1977b; McDonald 1991), Schoenbaum and colleagues (1999, 2000) have proposed a model of amygdala-frontal function in which incentive value information is encoded by the BLA, which conveys this information to the OFC in order to guide the execution of an appropriate behavioural strategy. Accordingly, performance in reversal learning tasks can be disrupted by lesion or inactivation of the amygdala (Clarke et al. 2008; Jones & Mishkin 1972; Schwartzbaum & Poulos 1965), as well as following contralateral disconnection of the BLA and OFC (Churchwell et al. 2009), demonstrating that the amygdala also contributes to reversal and interacts with the OFC during normal performance. The exact role the amygdala plays in cognitive flexibility is under debate however, particularly given that improved (Rudebeck & Murray 2008) and intact (Izquierdo & Murray 2007; Kazama & Bachevalier 2009) reversal performance has also been reported following amygdala lesions, though this has been explained in terms of compensation by other supporting neural structures (Holland & Gallagher 2004; Kazama & Bachevalier 2009), or differences in the extent of aspirative versus axon-sparing excitotoxic lesions (Baxter & Murray 2000; Izquierdo & Murray 2007; Murray 1992). Lesions to the ventral striatum, which has strong topographical connections with the ventral PFC (Alexander et al. 1986), are also found to disrupt reversal learning (Annett et al. 1989; Stern & Passingham 1995; Taghzouti et al. 1985), and single-cell recordings of neuronal responses in the dorsal striatum appear to reflect the output of OFC neurons (Rolls et al. 1983), possibly representing the integration of motivational information from the limbic to the motor system (Mogenson 1987). It is evident that complex interactions exist between

connected structures within the OFC circuit, which also act to modulate performance in reversal tasks, but further evidence concerning the precise role different cortical and subcortical regions play in supporting cognitive and behavioural flexibility is needed.

In sum, evidence suggests that the OFC is critically involved in reversal learning due to its role in representing incentive value within the context of ongoing behaviour; but activity in connected subcortical regions, particularly the basolateral amygdala, are also likely to mediate aspects of performance. Given that the OFC appears to be selectively involved in the modulation of behaviour following changes in incentive value, and given the lack of direct connections of the OFC to regions involved in motor control, connections between PFC regions and subcortical striatal structures are also likely to be important in guiding behavioural flexibility. Thus, rather than functioning in isolation, a large network of cortical and subcortical regions are implicated alongside the OFC in the maintenance, updating, selection and/or expression of reward-associations, any number of which could be critically involved in mediating the effects of 5-HT_{2C}R antagonism on reversal learning performance.

6.1.5. Neurochemical dissociation of 5-HT and DA function within the OFC and mPFC in tasks of flexible cognition

As well as showing an anatomical dissociation, there is evidence for a neurochemical double dissociation within the mPFC and OFC in tasks of cognitive flexibility. In a series of experiments in marmosets, Clarke et al. (2004, 2005, 2007) report that 5,7-DHT induced reductions in 5-HT within the ventral PFC impair performance in a reversal learning task by increasing the number of early errors to criterion (Clarke et al. 2004), but have no effect on an ED set-shifting task (Clarke et al. 2005). This effect was further localised to the OFC (Clarke et al. 2007), whereas selective 6-OHDA induced depletion of DA in the OFC had no effect on reversal. By contrast, prefrontal 6-OHDA lesions (Crofts et al. 2001), as well as lesions of the dorsal noradrenegeric bundle (Tait et al. 2007), the main source of NA input to the mPFC, have been found to impair ED set-shifting without affecting reversal. Administration of the catechol-o-methyltransferase (COMT) inhibitor tolcapone, found to increase extracellular DA within the mPFC, is also found to improve ED shift performance in rats (Tunbridge et al. 2004). Taken together, this points to the specific involvement of catecholamines at the level of the mPFC in mediating ED set-shifting, and of 5-HT within the OFC in mediating reversal performance. This latter assertion is further supported by evidence that extracellular levels of 5-HT within the OFC of freely moving rats are increased during reversal learning (Lapiz-Bluhm et al. 2009) and that individual differences in reversal performance can be predicted from 5-HT and 5-HTT levels in

the OFC in rodents (Barlow et al. 2015; Stolyarova et al. 2014), and in vervet monkeys (Groman et al. 2013); thus identifying the OFC as a particularly critical region for studying the effects of serotonergic manipulations on reversal.

The 5-HT_{2C}R has been implicated as one of the receptors through which 5-HT may exert its effects on reversal learning in the OFC, though few studies have to date explored the effects of such targeted manipulations. Localised administration of the 5-HT_{2C}R antagonist SB242084 into the OFC, mPFC or nucleus accumbens of rats prior to reversal of a two-lever spatial discrimination test reveals that the improvement in performance seen following systemic administration of this drug (Boulougouris et al. 2008) may be mediated within the OFC, since administration into this region led to a dose-dependent reduction in the number of trials and early errors to criterion, but was without effect in other regions (Boulougouris & Robbins 2010). By contrast, 5-HT_{2A}R antagonists, which are also found to affect reversal performance when administered systemically, but which have a detrimental effect on performance (Boulougouris et al. 2008), have no effect following targeted infusion into these regions (Boulougouris & Robbins 2010). Thus, whilst 5-HT_{2A} and 5-HT_{2C} receptors bi-directionally modulate reversal learning, only the latter appear to exert their effects within the OFC. These findings are consistent with evidence of reduced compulsive responding following intra-OFC infusions of the 5-HT_{2C}R antagonist RS102221 in a signal attenuation task, which is used as an animal model of OCD, where a 5-HT_{2A}R antagonist was without effect (Flaisher-Grinberg et al. 2008). Alsiö et al. (2015) also report an improvement in early reversal performance during a visual touchscreen task in rats following both systemic and intra-OFC administration of SB242084. They additionally identified a late reversal impairment following systemic administration which was absent following targeted OFC micro-infusions. It is possible that these late errors were linked to impulsivity effects, since the speeding of response latencies observed following systemic administration, and which has been consistently reported in the literature, was also absent following intra-OFC infusions. This highlights how both disruptive and beneficial effects might be simultaneously conferred by systemic manipulations, through actions in different brain regions; the relative balance of which might tip in either direction. This might explain the diversity of previous findings when employing systemic manipulations of 5-HT_{2C}R function, and firmly indicates a need for more selective investigations. Further targeted manipulations will be necessary to clearly identify the locus of these different effects and their relative impact on cognitive and behavioural flexibility. Being able to identify the various regions activated by reversal learning tasks, as well as where most co-localisation of activity occurs with 5-HT_{2C}R expression, should clearly identify targets for focussed manipulations.

6.1.6. Distribution of 5-HT_{2C}Rs in the rodent brain

Although the use of in-situ hybridization techniques has demonstrated that 5-HT_{2C}R mRNA is abundantly expressed throughout the human and rat CNS (Hoffman & Mezey 1989; Molineaux et al. 1989; Pompeiano et al. 1994), establishing a functional role for these receptors has been more difficult, due to the lack of a high affinity ligand selective for this receptor subtype which would allow precise anatomical mapping through use of autoradiographic techniques. However, Clemett et al. (2000) have made progress in this regard, by raising polyclonal antibodies against the rat 5-HT_{2C}R protein which allows for immunohistochemical characterisation. In general agreement with the distribution determined by in-situ hybridisation, they report that 5-HT_{2C}R-like immunoreactive (IR) cells were distributed widely throughout the cerebral cortex, in all cortical forebrain regions, the parietal and cingulate cortices and the piriform cortex; as well as in subcortical regions, throughout the basal ganglia, as well as in the septum, hippocampal formation and all amygdaloid nuclei, the thalamus, hypothalamus and subthalamus. 5-HT_{2C}R-IR cells were also observed in all regions of the mesencephalon and metencephalon, particularly the dorsal raphe nucleus, with the single exception of the cerebellar cortex. The highest levels of immunoreactive cells were reported in the piriform cortex, intercalated and medial amygdaloid nuclei, hippocampal pyramidal cells, and most regions of the cerebral cortex and thalamus, followed by the olfactory bulb and caudate-putamen.

Such a distribution is consistent with evidence of a role for these receptors in anxiogenesis, in feeding behaviour and neuroendocrine function, and in locomotor activity, as well as being consistent with a potential role for these receptors in executive function. Though potential confounds of motor impulsivity and feeding-related changes have been discussed and largely discounted as explanations for the reported behavioural effects, such a broad distribution of 5-HT_{2C}Rs throughout the CNS further points to the need for more localised manipulations. Although these receptors appear to be post-synaptic in relation to serotonergic neurons, given the high levels of 5-HT_{2C}R-like IR observed in serotonergic neuronal projection areas, detection of 5-HT_{2C} positive neurons in the raphe nuclei of the brain stem, particularly the dorsal raphe, suggests these receptors may be pre-synaptically located in some regions. Within the PFC, the 5-HT_{2C}R appears to have an inhibitory function on neuronal activity, since these receptors are found to be present on GABAergic, primarily parvalbumin-containing, interneurons (Liu et al. 2007), whilst decreased 5-HT_{2C}R function elevates DA-dialysate levels in the PFC (Millan et al. 1998; Gobert and Millan 1999; Gobert et al. 2000) and potentiates glutamatergic AMPA-receptor transmission in the OFC (Rueter et al. 2000). Whilst 5-HT_{2C}R antagonists have no effect on basal 5-HT cell firing, they do have an indirect effect on dorsal

raphe activity, enhancing activity by blocking the inhibitory effects of 5-HT_{2C}R stimulation (Boothman et al. 2006; Liu et al. 2000). In sum, 5-HT_{2C}Rs show a broad distribution throughout the brain, and are able to modify function in several neurochemical systems. Determining where neurons activated by a reversal learning tasks also express 5-HT_{2C}Rs may therefore allow future research to better target manipulations of 5-HT_{2C}R function to those regions specifically involved in reversal.

6.2. EXPERIMENT 1

CHARACTERISATION OF REVERSAL-SPECIFIC C-FOS IMMUNOREACTIVITY AND 5-HT_{2C}R CO-LOCALISATION IN THE MOUSE BRAIN

Although a substantial amount of research has gone in to examining the role of different brain regions in behavioural adaptation, there remains uncertainty as to the precise contributions made by these regions to distinct aspects of flexibility, and it is not clear exactly how 5-HT_{2C}Rs contribute to these processes. Whilst event-related fMRI studies in humans and non-human primates have demonstrated differences in activity within the PFC during reversal and set-shifting tasks, complementing existing lesion data, most have not distinguished between brain activation associated with the control processes specific to reversal learning versus those that are generally involved in feedback-driven learning, and patterns of activation in rodents during performance of these tasks remains relatively unexplored. By measuring expression of *c-Fos*, an immediate early gene used as a marker of neural activity and plasticity (Dragunow et al. 1989; Sagar et al. 1988), it should be possible to identify the neuroanatomical basis of reversal learning. As an additional aim, examining co-localisation of *c-Fos*-positive cells with those demonstrating 5-HT_{2C}R immunoreactivity may allow better visualisation of the functional role these receptors play within these circuits, allowing future researchers to target manipulations to these areas.

Although measures of gene expression in relation to the ID and ED elements of set-shifting tasks have previously been conducted (DeSteno & Schmauss 2008; Glickstein et al. 2005), only one previous study to date has examined activity specifically in relation to the reversal stage of this task (Burnham et al. 2010), as far as I am aware. This study compared activity levels in behaviourally naïve animals with animals completing either a reversal or a repeat of the previous ID-shift stage. In contrast to evidence derived from lesion studies, they report that activity within both the OFC and mPFC was elevated relative to behaviour-negative controls, but that it did not differ across animals in either of the behaviour-positive groups.

However, Fos levels within the BLA and striatum were not examined; regions which have also been strongly implicated in reversal performance. One further study has employed in-situ hybridization analysis of regional Fos expression after completion of the ID/ED set-shifting task within these cortical regions as well as within the dorsal and ventral striatum; which demonstrated significantly greater activity in the OFC, but not the mPFC, compared to behaviour-negative controls (Egerton et al. 2005). Since all behaviour-positive animals completed every stage of the ID/ED task prior to tissue collection, activity could not be specifically tied to performance of any individual element of the task in this design. However, the authors do report that performance during the first reversal stage of the task was associated with levels of Fos expression in the dorsolateral striatum, the nucleus accumbens shell, and the lateral OFC.

In order to localise regional Fos activity to performance of the reversal shift, three groups of animals were employed, similar to the design employed by Burnham et al. (2010), with the exception that the behaviour-negative group received an identical training history and received reward in their home cages on the final test day, to control for Fos-activity levels relating to reward consumption. All animals were trained to criterion on a two-choice spatial discrimination task, followed by a single test day which differed for each of the three groups. A low behaviour control (LBC) group received sugar pellet rewards in their home cages and no behavioural test; a high-behaviour control (HBC) group received a repeat session of spatial discrimination, to control for activity relating to the acquisition/maintenance of a two-choice discrimination as well as motor response requirements of the task; and the experimental (REV) group received a single reversal test session, where the response contingencies from the spatial discrimination stage were reversed. Because Burnham et al. (2010) also identified a link between the number of trials performed during reversal task and the amount of Fos activity expressed in cortical regions, animals in each behavioural group were tested for the same length of time in the final session, rather than being stopped once a criterion performance was attained. Pilot research has confirmed that animals in both groups complete a similar number of trials within a 40 minute test session (data not shown). On the basis of prior research suggesting a potential involvement of these regions in some aspect of flexible behaviour, activity within regions of the lateral and ventral OFC (LO, VO), infralimbic and prelimbic regions of the mPFC (IL, PrL), the caudate putamen (CP), nucleus accumbens shell and core (NacS, NacC), and the basolateral amygdala (BLA) were examined.

6.2.1 Materials and methods:

Animals and behavioural procedures:

Thirty male C57/BL6-J mice (Charles River) weighing an average of 22.4g (SEM ± 0.4g) at the start of behavioural testing were pair-housed, and placed on a restricted feeding schedule until reaching 85-90% of free-feeding weight, which was maintained throughout testing. Habituation and stages 1 & 2 of training were identical to those previously described in Chapter 2, Experiment 1.

Stage 3: Spatial Discrimination:

Animals were assigned to one of three experimental groups (Low Behaviour Controls (LBC), n = 7; High Behaviour Controls (HBC), n = 9; Reversal (REV), n = 14), counterbalanced across left and right 'correct' response locations, and matched for number of sessions and trials to reach criterion during the previous training stage. To minimise animal numbers, fewer animals were allocated to LBC and HBC relative to REV group, since the REV group was likely to show greatest performance variance. Because perfusion of *n* = 30 animals is not possible in a single test day, animals were allocated to one of three test groups (*n* = 10), counterbalanced for number of LBC, HBC and REV animals, and tested on alternate days (Group 1 – Monday, Thursday; Group 2 – Tuesday, Friday; Group 3 – Wednesday, Saturday, see Figure 6.3). This schedule ensured all animals received an equal number of test-free days between test sessions and the final experimental session (Stage 4), and could go on to be perfused on three separate test days (Group 1 – Monday; Group 2 – Tuesday; Group 3 – Wednesday). It is for this reason that animals were assigned to experimental groups prior to training on the spatial discrimination, despite all animals receiving identical testing during this stage (see Figure 6.2).

As in previous experiments, left and right nosepoke holes were lit and the central nosepoke hole covered. A nosepoke response into the correct hole led to nosepoke lights being extinguished, delivery of one sucrose pellet reward, and beginning of the ITI (4 s houselight off, 4 s houselight on, 3 s delay to next trial), whilst an incorrect response led to nosepoke lights being extinguished and immediate onset of the ITI (8 s houselight on, 3 s delay to next trial). Animals had 20 s to initiate a trial, and 30 s to make a response; with failure to do either resulting in immediate onset of the ITI, and an initiation omission or response omission being recorded, respectively. Animals received 7x10-trial blocks per session, and were required to respond correctly in 9 out of 10 trials within any single block, after which the session terminated. Animals received a minimum of three test sessions, and needed to reach a criterion of 9 out of 10 correct trials in any 10-trial block in at least two consecutive test sessions. Animals failing to reach this

criterion received a fourth and final test session. All animals received sugar pellets in their home cages 2 days after their final test session of each week, to reduce any surprise associated with receiving pellets in the home cage during Stage 4 for animals assigned to LBC condition.

Stage 4: Test day

LBC group:

Animals received some handling (consistent with animals being placed into operant chambers), and received 50 sucrose pellets in their home cages. The number of pellets given was determined by the average number of rewards earned by animals in a single 40 minute spatial discrimination session during a pilot study, following the same training history. As such, the number of rewards consumed in this group was expected to closely match that of the HBC group.

HBC group:

Animals received a further session of spatial discrimination testing identical to the previous stage, with the exception that the session lasted 40 minutes and did not terminate when criterion responding was reached.

REV group:

Animals in this group received a single 40 minute session of reversal testing. Reinforcement contingencies were switched, with responses to the previously incorrect nosepoke location rewarded and to the previously correct location punished (non-rewarded).

After completion of the task, all lights were extinguished in the operant chambers, and animals were left undisturbed for 60 minutes before being killed by barbiturate overdose and transcardial perfusion.

Immunofluorescence:

Animals were sacrificed with a terminal dose of sodium pentobarbital (200 mg/kg *i.p.*, Cliffe Veterinary Group, Lewes, UK) and transcardially perfused with saline followed by 4% paraformaldehyde (PFA). One animal from group REV experienced respiratory problems and was culled prior to perfusion, and one further REV animal was incorrectly perfused, making the final group numbers LBC = 7; HBC = 9; REV = 12. Brains were removed and post-fixed overnight (22 hrs) in 4% PFA at 4˚c, then suspended in a 30% sucrose in 0.1M phosphate buffered saline

(PBS) solution for 5 days (4˚c). Brains were snap frozen in crushed dry ice for 1 hour and stored at -80 ˚c until sectioning. Serial coronal sections (20μm) from the orbitofrontal cortex to the cerebellum were obtained with a cryostat and collected in PBS-Azide (0.1M PBS with 0.02% sodium Azide) and stored at 4˚c until processing for immunofluorescence. Sections from the cerebellum were taken in addition to the primary regions of interest to provide a negative control for 5-HT_{2C}R localisation prior to double-labelling within the regions of interest, since the cerebellum is known to contain very few 5-HT_{2C}Rs whilst all other regions of interest show dense population.

Double-labelling immunofluorescence was used to determine whether reversal-related *c-fos* immunoreactivity (Fos-IR) could be identified, and whether Fos-IR co-localised with immunoreactivity for 5-HT_{2C}Rs (Fos/2C-IR). Free-floating brain sections containing the orbitofrontal cortex (including ventral (VO) and lateral (LO) divisions), medial pre frontal cortex (mPFC) (containing Infralimbic (IL) and Prelimbic (PrL) subdivisions); the ventral and dorsal striatum (including the nucleus accumbens core (NAcC) and shell (NAcS) sub-divisions, and caudate-putamen (CP)), and the basolateral amygdala (BLA), as well as the cerebellum (as a negative control for 5-HT_{2C}R expression) were identified using Paxinos and Franklin's (2004) Brain Atlas (Academic Press, San Diego, CA, USA). Two sections from each of the OFC, mPFC, striatum, BLA and cerebellum were exposed to 3 x 10 minute washes in 1 x Tris-buffered saline (TBS), followed by 30 minutes incubation in a blocking serum (10% normal goat serum (NGS) in 0.2% TBS-TX to permeabilise the tissue). Sections were incubated in anti-*c-fos* sc-52 rabbit polyclonal (1:1000 dilution, Lot #A2194, Santa Cruz, CA, USA) and anti-SR-2C goat polyclonal (1:15000 dilution, Lot #D114, Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies in a 3% NGS solution in 0.2% TBS-TX overnight (14-16 hours at 4˚c with gentle agitation). The following day, sections were washed a further 3 x 10 mins in TBS, and incubated in secondary antibodies Alexa Fluor 488 donkey anti-rabbit (*c-fos*) and Alexa Fluor 568 donkey anti-goat (2C) (1:200 dilution, Life Technologies, Paisley, UK) in 0.2% TBS-TX for 1 hr at room temperature in the dark, with gentle agitation. Slices were washed a further 3 x 10 minutes in in TBS and mounted onto Super Frost Plus histology slides (Fisher Scientific, Loughborough, UK). Following air-drying, slides were cover-slipped using PermaFluor mounting medium (Thermo Fisher Scientific, Loughborough, UK).

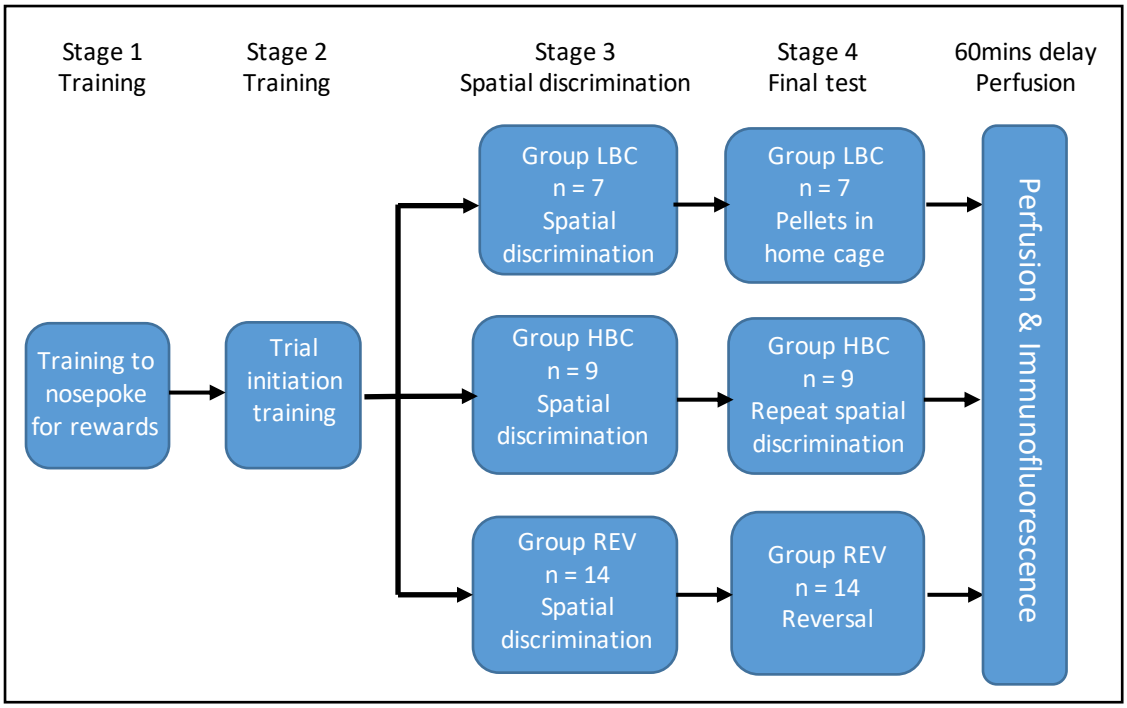

Figure 6.2. Overview of experimental design. *Following training, animals were allocated to one of three experimental groups: Low-Behaviour Controls (LBC) who received sugar pellets in their home cages on the final test day; High-Behaviour Controls (HBC) who received a 40min repeat session of the previously acquired spatial discrimination on the final test day; and a Reversal group (REV) who received a 40min reversal test on the final test day (correct and incorrect response locations were reversed). After completion of the final test, animals were left undisturbed for 60 mins prior to transcardial perfusion and processing for immunofluorescence.*

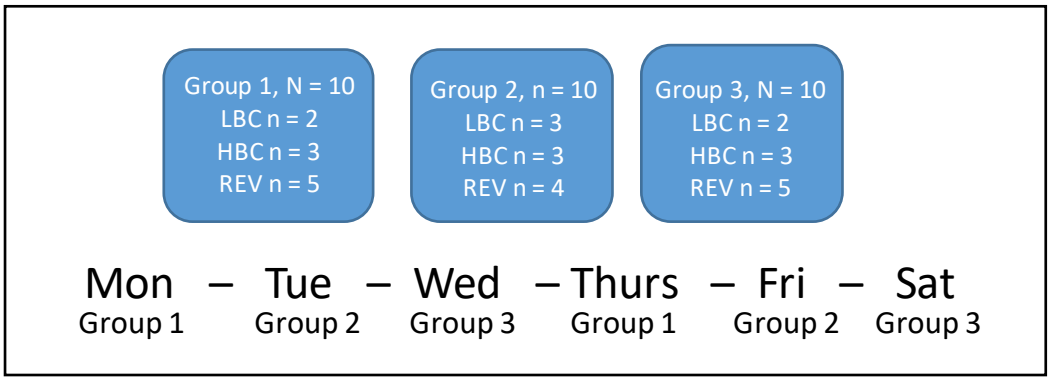

Figure 6.3. Timeline of behavioural testing during stage 3 & 4 of testing. *To allow sufficient time for perfusion on the final test day, animals were divided into three groups of N = 10 for behavioural testing, counterbalanced as best as possible for number of LBC, HBC and REV animals per group, and tested on alternate days. This pattern of testing was repeated for three sessions of spatial discrimination testing, and the single final test day occurred on Monday for Group 1, Tuesday for Group 2, Wednesday for Group 3.*

Image analysis:

Images were captured from brain sections using a Zeiss Axioskop 2 fluorescent microscope with a Zeiss AxioCam HRc fluorescent imaging camera interfaced to a computer running AxioVision 4.8.2 software. Images were taken at 20x magnification. For each section, two identical images of the same area were captured, one for each filter set detecting immunoreactivity (IR) for each antibody, and these two images were overlaid. Counts of Fos-positive nuclei and Fos/2C double labelled cells were made manually by an observer blind to experimental group. Two bilateral sections for each brain region were counted per animal (see Figure 6.2), and averaged to give number of Fos-positive cells and double-labelled cells per mm^2 of tissue. Counts were verified for a sub-section of slices by a second blind observer, and showed less than 10% variability in outcome.

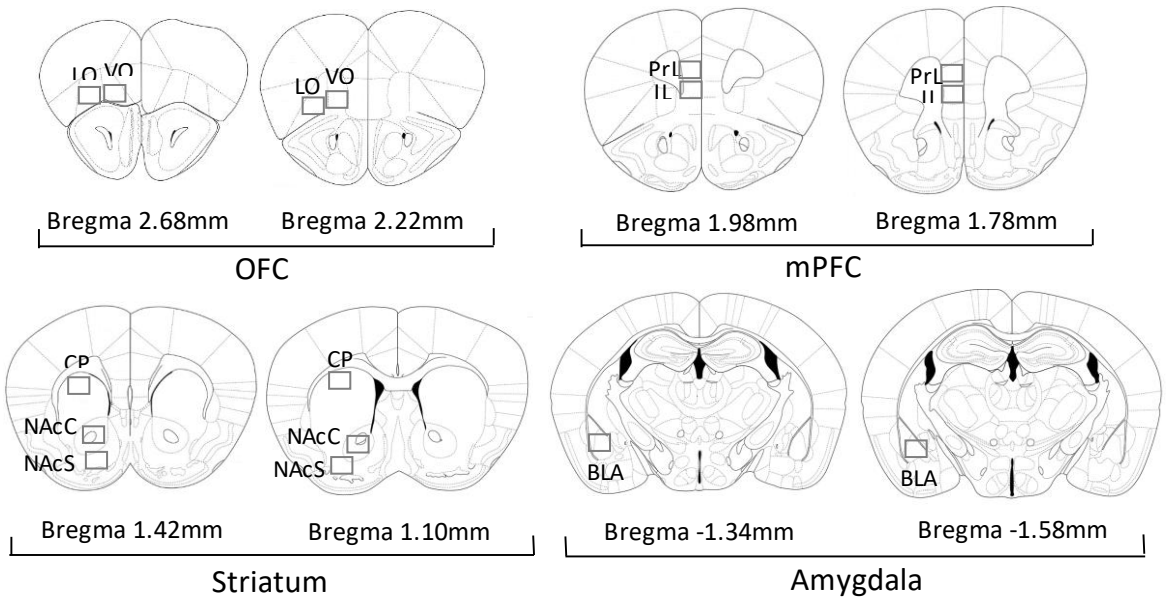

Figure 6.2 *Diagrammatical representation of regions in which number of Fos-positive cells and Fos/2C double-labelled cells were counted. Abbreviations: BLA, Basolateral amygdala; CP, Caudate putamen; IL, Infralimbic cortex; LO, Lateral orbital cortex; NAcC, Nucleus accumbens core; NAcS, Nucleus accumbens shell; VO, Ventral orbital cortex. Adapted from Paxinos & Franklin, 2004.*

Statistics*:*

Independent measures t-tests explored the differences in behavioural performance of animals in the HBC and REV groups, according to the number of correct, incorrect and total trials completed within the test session, as well as the number of omissions made (initiation, response, reward retrieval), and latency data (initiation, response, reward retrieval). Because the total number of trials completed within a 40 minute test session does not bear a clear relationship to performance accuracy (since animals were not stopped from testing upon reaching a criterion threshold), performance of animals in the HBC and REV groups up to a set criterion was also analysed. Data was taken from each animal for the number of 10-trial bins required to reach a criterion of 9/10 correct responses, with further trials excluded from analysis, which was also subjected to an independent measures t-test.

Total number of Fos-positive cells and Fos-2C double-labelled cells in eight regions (VO, LO, IL, PrL, NAcC, NAcS, CP, BLA) were analysed according to experimental group (LBC, HBC, REV) using two-way repeated measures ANOVA (between subjects factor: experimental group; within-subjects factor: brain region). Simple effects analyses were additionally employed where significant interactions were identified. The relationship between regional Fos activation and performance (total number of trials and incorrect responses, number of 10-trial bins to criterion) within each behaviour-positive group (HBC, REV) was further explored by correlational analysis, employing Sidak-Holm correction to control for multiple comparisons. All cell-count data was SQRT transformed to correct for violations of equality of variance (untransformed means and SEMs are reported). A Huyn-Feldt correction was applied where data violated the assumption of sphericity.

6.2.2. Results:

Behaviour:

Animals in the HBC and REV groups did not differ in the total number of trials completed within the 40 minute test session (t_{19} = -1.12, p > .05), but animals in the REV group made significantly fewer correct (t_{19} = 2.82, p = .01) and more incorrect ($t_{11.8}$ = -5.42, p < .001) responses than HBC controls (see Figure 6.3). Group REV also required significantly more 10-trial bins to reach a criterion threshold of 9/10 correct responses than group HBC ($t_{13.8}$ = -5.25, p < .001). There was no difference in the number of omissions made between groups either for trial initiation, response execution, or reward retrieval (see Table 6.1). There was also no difference

in latency to initiate a trial or make a response between groups, but REV animals were slightly but significantly slower than HBC animals to retrieve rewards (see Table 6.1).

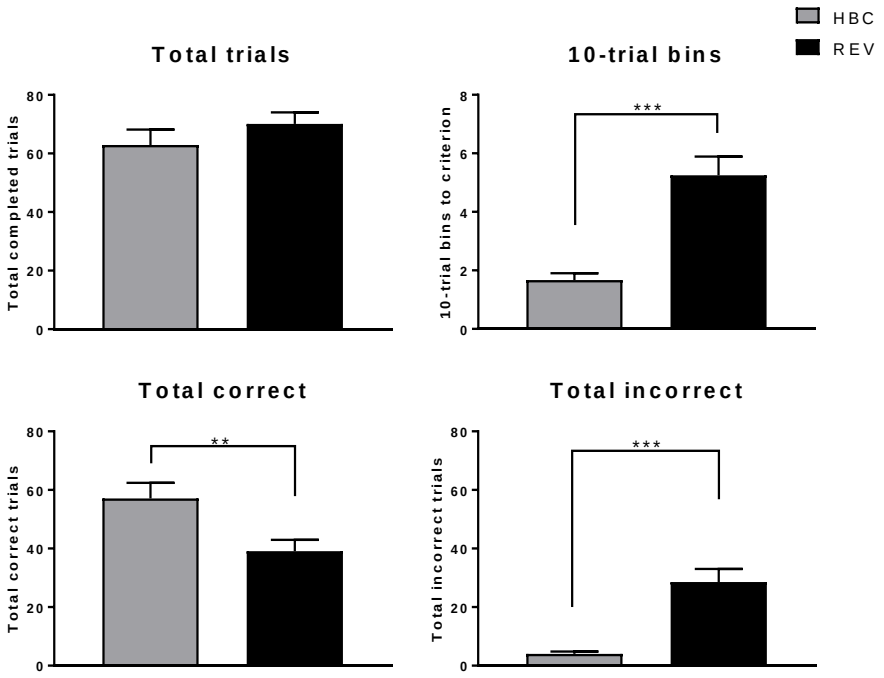

Figure 6.3 *Performance of group HBC and REV during behavioural testing. Total number of trials, correct and incorrect responses (errors) within the 40 minute test session are shown, as well as total number of 10-trial bins required to meet a criterion of 9/10 correct responses. **p < .01, ***p < .001.*

		HBC (n = 9)	REV (n = 12)	t_{19}
Omissions	Initiation	28.0 ± 4.6	22.4 ± 8.4	1.16
	Response	1.8 ± 0.6	2.4 ± 0.9	-0.54
	Reward retrieval	1.7 ± 0.9	0.3 ± 0.2	1.75
Latency (s)	Initiation	6.6 ± 0.3	6.0 ± 0.2	1.52
	Response	6.7 ± 0.5	7.0 ± 0.6	-0.34
	Reward retrieval	1.8 ± 0.1	2.1 ± 0.1	**-2.20***

Table 6.1 *Omissions and latency data for animals in group HBC and REV during behavioural testing. Independent measures t-tests reveal a small but significant difference in speed of reward retrieval between groups. * p < .05.*

Fos-Immunoreactivity:

Behavioural performance of the spatial discrimination task (Group HBC) or reversal learning task (Group REV) led to greater *c-Fos*-like immunoreactivity (Fos-IR) in most brain regions tested compared to low-behaviour controls (Group LBC), who received rewards in their home cages (see Figure 6.4 & 6.5a). This difference was not related to number of reinforcers consumed, which was not found to differ across groups ($F_{2, 25}$ = 2.56, p > .05), although Group REV did consume slightly fewer rewards than Group HBC (REV: M = 42.0, SEM = 13.4; HBC: M = 54.7, SEM = 16.5). There was little evidence for a difference in number of Fos-IR cells in animals completing a reversal relative to a repeated spatial discrimination test however, suggesting that regions previously implicated in flexible cognition were not differentially engaged by the present reversal learning task (see Figure 6.4 & 6.5a).

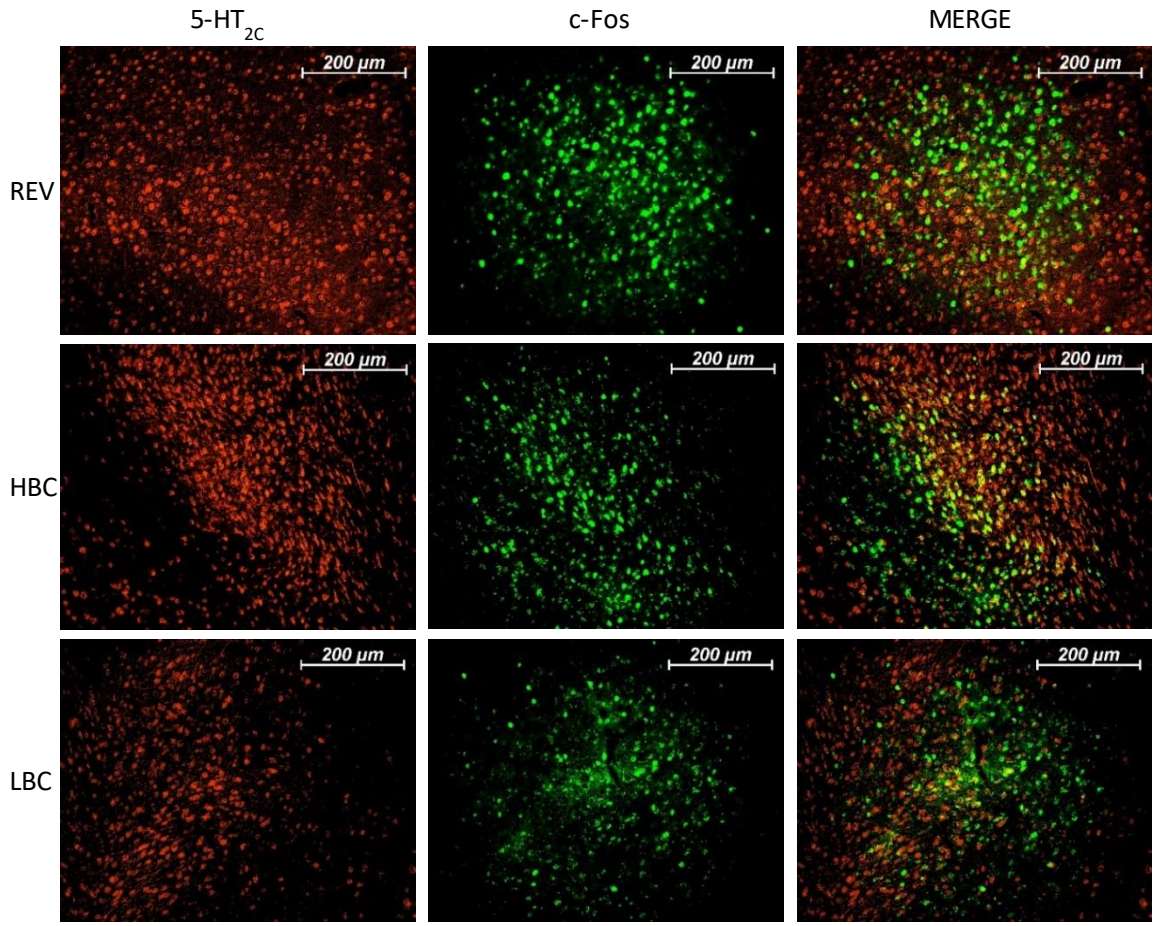

Figure 6.4 *Representative lateral orbital (LO) prefrontal cortex sections of mice completing a reversal test (REV, n = 12), a repeat spatial discrimination test (HBC, n = 9) or no behavioural test (LBC, n = 7), stained with immunofluorescence, showing 5-HT$_{2C}$R (red) and c-Fos (green) neuronal expression.*

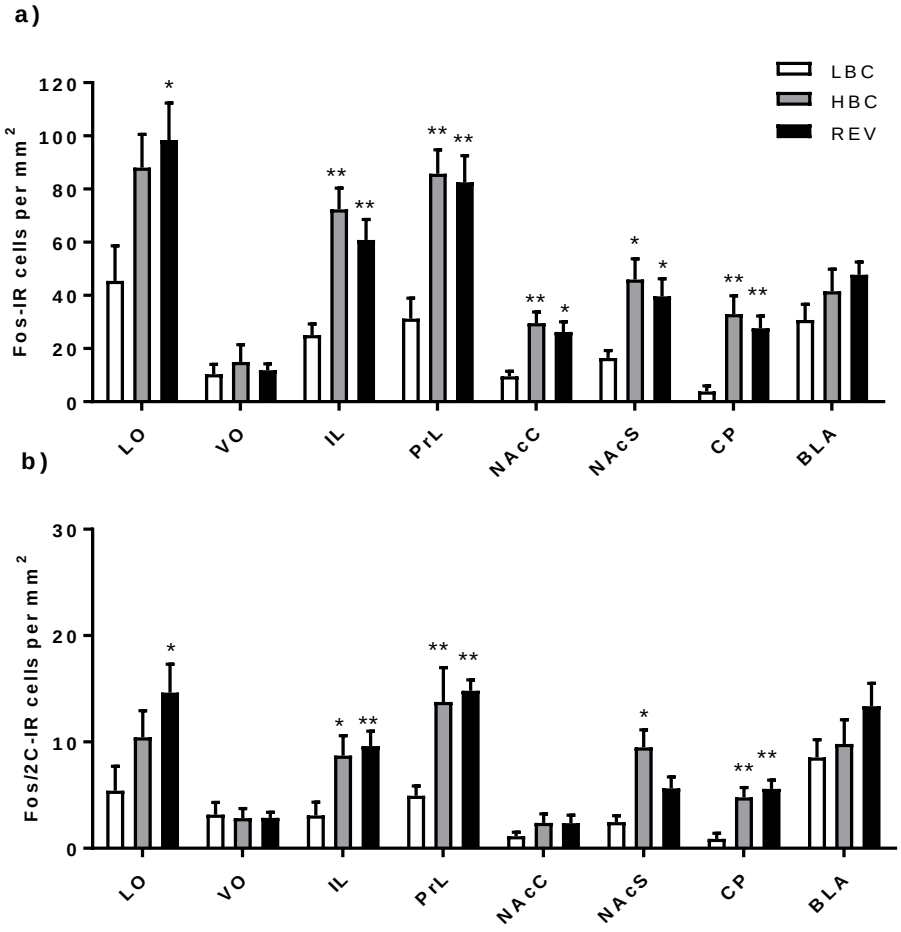

Figure 6.5 *The mean number of Fos-positive cell counts (a) and Fos/2C receptor double-labelled cells (b) per mm2 of tissue in the LO, VO, IL, PrL, NAcC, NAcS, CP, BLA from animals which had performed a reversal test (REV, n = 12), a repeat spatial discrimination test (HBC, n = 9), or no behavioural test (LBC, n = 7). Data expressed as mean ± SEM. *p < .05, **p < .01 significantly different from LBC (simple effects analysis with pairwise contrasts).*

Mixed ANOVA exploring levels of Fos-IR revealed a significant main effect of group ($F_{2, 25} = 14.44$, $p < .001$), region ($F_{6.3, 156.6} = 41.76$, $p < .001$), and a significant group x region interaction effect ($F_{12.5, 156.6} = 2.16$, $p < .05$). Pairwise comparisons of average activity across all regions show that the mean difference in number of Fos-IR cells between the LBC controls and both behaviourally active groups (HBC, REV) was significant (p's < .001), however there was no difference in the number of Fos-IR cells following reversal (REV) as compared to repeated spatial discrimination (HBC) testing ($p > .05$). Greatest Fos-IR was evident in the LO, as well as in both regions of the mPFC measured (PrL and IL), where there were significantly more Fos-positive cells than in any other region (p's < .05), but activity between these regions did not significantly differ (p's > .05). Somewhat surprisingly, the number of Fos-IR cells in the VO, as well as in the NAcC and CP regions of the striatum, was significantly lower than in any other region measured

(p's < .01); whilst the BLA and NAcS region of the striatum showed an intermediate level of activity relative to other regions (see Figure 6.5a).

Simple effects analyses were used to further explore the group x region interaction effect. The difference in Fos-IR across groups was significant in all regions (p < .05) except the VO and BLA, where LBC controls showed similar IR levels to the other two groups (p > .05). In the VO, this was seemingly due to low levels of activation across groups, but in the BLA this appeared to be driven by higher levels of activation in the LBC group compared to that seen in other sub-cortical regions. Pairwise group contrasts within each brain region additionally demonstrated that, in the LO, the difference between LBC and HBC groups did not reach significance (p > .05), whilst there was a significant difference between the LBC and REV groups (p < .05); providing some evidence of a trend for greater involvement of this region during reversal as compared to spatial discrimination, despite there being no difference in Fos-IR between HBC and REV groups (p > .05). However, overall, there was little evidence of selective involvement of any region during reversal.

Correlational analyses:

In order to explore whether relative levels of Fos-IR could be linked to reversal performance, correlational analyses were performed between total number of trials and errors (incorrect trials) made during behavioural testing for the HBC and REV groups and levels of Fos-IR in each brain region. Results identified no significant correlation between Fos-IR and number of trials or errors to criterion in any brain region for animals undergoing repeated spatial discrimination testing (Group HBC) (all p's > .05). However, for animals completing the reversal test (Group REV), there was a significant positive correlation between Fos-IR in the LO (r_{12}= 0.69, p < .05) and NAcS (r_{12} = 0.75, p < .01) and the total number of trials completed. There was also a significant negative correlation between number of errors made during reversal and number of Fos-IR cells in the BLA (r_{12} = -0.76, p < .05). (see Figure 6.6a). Animals making more errors during reversal showed significantly less activity in this region than those that performed more optimally. However, none of these findings survive controls for multiple comparisons.

Because the total number of trials completed does not bear a clear relationship to performance accuracy (since animals were not stopped from testing upon reaching a criterion threshold), correlational analyses were also performed between Fos-IR and the number of 10-trial bins required to reach a criterion threshold of 9/10 correct responses. Results confirmed there was no significant correlation within any region for Group HBC (all p's > .05). There was also no longer a significant correlation between Fos-IR activity in the LO (r_{12} = 0.33, p > .05) or

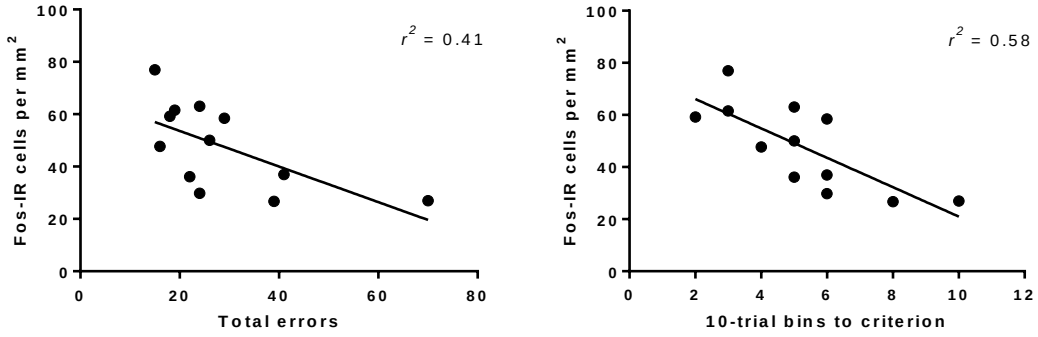

Figure 6.6 *Negative correlation between Fos-positive cell counts in the basolateral amygdala (BLA) and number of errors (incorrect responses) made (a) or 10-trial bins required (b) to reach criterion for animals completing a reversal test (Group REV, n = 12).*

NAcS (r_{12} = 0.22, p > .05) and performance during reversal (Group REV), as measured by number of 10-trial bins to criterion. This suggests that activity in these regions was more closely linked to overall number of trials completed than performance accuracy during reversal. However, there was evidence of a significant negative correlation between Fos-IR in the BLA and 10-trial bins to criterion (r_{12} = -0.64, p < .001) (see Figure 6.6b), in line with the negative correlation observed between Fos-IR in this region and the total number of errors made during reversal. Taken together, this shows that animals who performed poorly during reversal - making more errors and taking longer to reach criterion - showed less activity within the BLA than those demonstrating superior reversal performance, with the important caveat that only this latter finding survived correction for multiple comparisons.

Fos/2C receptor co-localisation:

5-HT_{2C}R labelling was not restricted to cell bodies, but appeared to label neuronal processes such as dendrites; consistent with previous reports (Bubar et al. 2005). Abundant 5-HT_{2C}R expression was observed in all regions of interest, but was particularly abundant in the caudate-putamen, with little to no staining in the cerebellum, also consistent with previous findings (Clemett et al. 2000). Co-localisation of 2C receptors to Fos-positive cells averaged 18.5% of the total number of Fos-IR cells across regions. The pattern of Fos/2C double-labelled cells across groups and regions was fairly consistent with that observed for Fos-IR cells. There was a significant main effect of group ($F_{2, 25}$ = 13.38, p < .001), region ($F_{6.1, 65.1}$ = 20.34, p < .001) and a group x region interaction ($F_{12.1. 65.1}$ = 2.02, p < .05). Pairwise comparisons between groups again demonstrate that the significant group effect was driven by the difference between LBC controls and the behaviourally-active HBC and REV groups (p's < .001), whereas the number of

double-labelled cells following reversal (REV) did not significantly differ from that observed following repeated spatial discrimination (HBC) testing ($p > .05$). The number of double-labelled cells in the LO, mPFC (IL and PrL), NAcS and BLA was significantly greater than that seen in all other areas (p's < .001), but did not differ significantly from each other (p's < .05). The presence of double-labelled cells was lowest in those regions which showed the least Fos-IR, in the VO, NAcC and CP (see Figure 6.5b). Simple effects analyses show that the difference in Fos/2C co-localisation between groups was restricted to the LO, mPFC (IL and PrL regions), NAcS and BLA (p's < .05). As with overall Fos-IR, pairwise comparisons between groups in each of the regions of interest additionally revealed a trend for a difference in the number of double-labelled cells across groups within the LO, with evidence for a significant difference between the LBC and REV group ($p < .05$) which was not evident between the LBC and HBC group ($p > .05$), though again, there was no significant difference between the HBC and REV group ($p > .05$). There was also a difference in co-localisation across groups within the NAcS, with greater Fos/2C-IR in Group HBC relative to LBC ($p = .001$), but no significant difference between Groups REV and LBC ($p > .05$).

6.2.3. Discussion:

Results of the present experiment demonstrate that a broad network of interconnected cortical and subcortical structures was activated by performance of a spatial discrimination or reversal learning task. In contrast to evidence derived from lesion studies, there was little evidence for the specific involvement of any of these anatomical regions during reversal; though there was evidence for slightly greater activation of the lateral region of the OFC (LO). Regions of the mPFC and the LO were most strongly activated by behavioural performance of the discrimination task and reversal, as compared to low behaviour controls (LBC), but regions of the dorsal and ventral striatum were also activated by behavioural testing. Somewhat surprisingly, the ventral portion of the OFC (VO) appeared to play no role in performance of either behavioural test, possibly pointing to a dissociation of function between ventral and lateral regions of the OFC. Despite a lack of difference in activity in the BLA across groups, partly driven by relatively higher activity in this region in low-behaviour controls, correlational analyses nevertheless identified this region as being of possible significance to cognitive flexibility, since Fos activity levels in this region were found to correlate with performance during the reversal but not the repeat discrimination test; though care must be taken in interpreting these effects given the number of comparisons made in this analysis. Examining patterns of co-localisation for cells demonstrating Fos activity with 5-HT_{2C}R immunoreactivity suggested that a proportion

of cells in all regions activated by behavioural testing expressed 5-HT_{2C}Rs, and thus are a potential focus for future targeted manipulations, with the possible exception of the nucleus accumbens, where the number of cells expressing co-localised immunoreactivity in the reversal group (shell) or in both behavioural groups (core), did not differ from that of LBC controls, despite evidence of greater behaviour-induced Fos activity in these regions.

Animals undergoing behavioural testing displayed significantly greater Fos-positive cell counts than LBC controls in all regions except the VO and the BLA. This helps to demonstrate the successful induction of behaviourally relevant Fos activity in this study, as well as allowing for identification of regions most strongly recruited by performance of these tasks. There was evidence that activity within the LO was marginally higher during reversal as compared to repeated discrimination testing, and only animals in the REV group showed significantly greater activation in this region relative to LBC controls. However, on the whole, activity did not differ between the two behaviour-positive groups. The similarity of activity in striatal regions could be related to the comparable motor requirements of the behavioural tasks, consistent with previous reports by Egerton et al. (2005) that levels of Fos expression in the dorsolateral striatum and nucleus accumbens demonstrated a positive correlation with performance (as measured by trials to criterion) during both the compound discrimination and reversal stages of the ID/ED task. Interestingly, they report a dissociation between the acquisition and reversal stages with respect to the accumbal subdivisions involved, with only the core engaged during acquisition, and the shell during reversal performance. Although this does not appear to fit with evidence that the OFC, the region most strongly implicated in reversal performance, shows stronger links to the core than the shell division of the nucleus accumbens (Haber et al. 1995), evidence for such a functional distinction does gain support from the present study. Whilst there was no evidence for a relationship between performance and Fos activity levels in the striatum for animals performing the repeat discrimination test in this study, we also report a significant positive relationship between number of trials performed during reversal and Fos activity in the striatum, specifically within the shell division of the nucleus accumbens (NacS). The difference in striatal involvement during discrimination testing in the present study as compared to that observed by Egerton et al. (2005) could be due to the introduction of a novel stimulus dimension in their compound discrimination test, relative to the well-practiced nature of the task currently employed. Although this could suggest a more critical role for the NacS for reversal learning as compared to other striatal regions, this relationship was not observed for the number of trials to criterion, therefore it is unlikely that activity was related specifically to reversal ability. Furthermore, these findings did not survive controls for multiple comparisons.

Comparable levels of activity in the mPFC and OFC between the two behaviour-positive groups in the present study suggests these prefrontal regions are both involved in maintaining performance on a spatial discrimination as well as reversing it. This is consistent with evidence reported in the one prior study to examine the relative expression of Fos activity during reversal testing relative to a comparable task where no shifting was required. Burnham et al. (2010) also report that the mPFC (IL and PrL) and OFC were more strongly activated following performance of a reversal shift when compared to behaviour-negative controls, but that activity in these regions did not differ from animals completing a repeat of the previous ID-shift stage. Given the apparent wealth of prior studies suggesting a dissociation of function between the mPFC and OFC in tasks of set-shifting and reversal, respectively (e.g. Birrell & Brown 2000; Bissonette et al. 2008; Dias et al. 1996a, 1996b, 1997; Floresco et al. 2008; Ghods-Sharifi et al. 2008; McAlonan & Brown 2003; Owen et al. 1991), it may seem surprising that the OFC was not specifically or preferentially activated by reversal in these studies. However, in contrast to lesion or inactivation studies, measures of Fos-IR suggest only the involvement, and not the necessity of brain regions in performance of a given task. Evidence taken from single-unit and ensemble cell recordings within the OFC demonstrates that neurons in this region are activated during performance of a discrimination task, displaying firing patterns which encode the physical attributes and assigned reward contingencies of cues, as well as the expectation of upcoming reward (Schoenbaum & Eichenbaum 1995a; Schoenbaum & Eichenbaum 1995b). During reversal learning, although a new population of neurons is shown to be recruited in the OFC, which fire selectively to the new stimulus associations, this coincides with a reduction in selective firing in neurons which previously signalled the old reward contingencies, which return to baseline levels; whilst a smaller number of individual OFC neurons also show full reversal of responding from the old to new contingencies (Schoenbaum et al. 1999). Overall activity levels within the OFC might therefore be similar in animals performing a discrimination and a reversal test, but this does not preclude the possibility that activity in this region is more crucial to the reversal stage of the task. There is also evidence the mPFC is more generally involved in representing behaviourally relevant information, in addition to its role in attentional shifting. Recordings taken from neurons within this region in rats during performance of a set-shifting task demonstrate that they maintain an online representation of a behavioural strategy even once this rule has been learnt and the information is no longer relevant to responding (Durstewitz et al. 2010). Thus, neurons in both the mPFC and OFC may be activated by the rule-monitoring requirements of both the discrimination task and its subsequent reversal, perhaps functioning to maintain a reference point to allow detection of rule or reward expectancy violations. Given that lesions of the mPFC and OFC reportedly have no effect on discrimination

learning prior to affective or attentional shifts, it seems likely that some of these functions can be supported by other regions. In fact, evidence that lesioned animals can complete affective and attentional shifts at all, albeit more slowly than sham lesioned controls, suggests neither of these regions are entirely necessary for cognitive flexibility, thus other regions must also be capable of supporting flexible behaviour.

One region of particular interest to reversal may be the basolateral amygdala (BLA). Although activity in this region was not found to differ across groups, this might have been due to the relatively high levels of activation seen within the LBC group compared to other regions, also apparent within cortical regions; and levels of activity in this region were found to relate to key performance measures in reversal. Although the reason for the higher levels of PFC and BLA activity in the LBC group cannot be explicitly determined, and could simply reflect higher baseline activity or reward consumption-related activity, it seems likely that the behavioural training background of these LBC animals might also have had an impact. In order to control for the effects of physical handling on Fos expression, animals in the LBC were handled on the final test day prior to being returned to their home cages to receive food rewards. Given that these animals had a fairly extensive history of being handled over several weeks of training prior to test, it is possible that this induced an expectation for behavioural testing on the final test day, perhaps sufficient to generate elevated levels of Fos expression in task-associated regions. Although the training backgrounds were matched to ensure as much similarity between groups as possible, the use of fully behaviourally naïve animals may therefore be preferable for future work of this nature. Despite the possibility of this confound obscuring behaviour-related differences in Fos expression across groups, there was evidence that activity levels within the BLA correlated to performance during reversal, which was not seen in animals repeating the spatial discrimination test. Although a relationship was also observed between Fos levels in the LO and reversal performance, as measured by total number of trials performed, there was no correlation with the number of trials taken to reach a set criterion - the better measure of performance accuracy. This is consistent with evidence reported by Burnham et al. (2010) that cortical activity levels are more closely linked to number of trials performed than the cognitive demands of the tasks, demonstrating the need for close controls over this potential confound. By contrast, activity within the BLA was not related to total trials completed, but was negatively correlated with the overall number of errors made, as well as the number of 10-trial bins taken to reach criterion. This could suggest that individual differences in neural activity within the BLA are linked to efficiency of reversal learning; though it is again important to point out that the only correlational analysis to survive correction for multiple comparisons was between BLA

activity and number of 10-trial bins to criterion. Nevertheless, this points to the BLA as a potential target for future localised manipulations to further explore its contribution to reversal.

Evidence for a lack of task-related activity in the VO in the present study was somewhat surprising given the current lack of experimental evidence to suggest a differentiation of function for lateral and ventral divisions of the OFC in tasks of associative learning and/or reversal. However, given that the VO has typically been lesioned or inactivated in conjunction with the LO (e.g. Bissonette et al. 2008; McAlonan & Brown 2003; Schoenbaum et al. 2002), the unique contribution of this region to performance in such tasks is not currently clear. Several studies have examined the effect of targeted LO inactivation on performance and attributed a role to this region in reversal learning (e.g. Churchwell et al. 2009; Ghods-Sharifi et al. 2008), thus the deficits observed following more widespread lesioning could relate to functions sub-served specifically within the LO. Although Burnham et al. (2010) also measured activity only within the lateral division of the OFC, Egerton et al. (2005) took measurements from both the VO and LO following completion of the ID/ED task, and report a similar outcome. Whilst levels of Fos activity within the LO were significantly greater in behaviour-positive than behaviour-negative controls, activation in the VO was low in both groups, and was not found to differ, suggesting that none of the tasks involved in the ID/ED test battery, including reversal, activated this region of the OFC. Although studies of OFC function have so far identified a mediolateral distinction of function, with the medial region more strongly implicated in the monitoring of reward value and the lateral region to the evaluation of punishers (O'Doherty et al. 2001); as well as posterior-anterior distinction, with abstract reinforcers represented more anteriorly to simple reinforcers such as taste (De Araujo et al. 2003; O'Doherty et al. 2001); a distinction in ventrolateral function is so far lacking. Evidence from the current study, in conjunction with reports by Egerton et al. (2005) suggest there may be a key difference in function across these regions, which may be vitally important for more clearly characterising the functions of the OFC.

Finally, explorations of Fos/2C co-localisation demonstrated that a proportion of cells activated by behavioural performance in each region were found to express 5-HT_{2C}Rs, but this proportion did not appear to be particularly pronounced in any specific region. The pattern of co-localised expression across regions was similar to that observed for Fos activity, with the exception of the nucleus accumbens core, where co-localisation was particularly low and no difference in the number of Fos/2C positive cells was demonstrable in behaviour-positive animals as compared to LBC controls. Additionally, despite animals in the reversal condition demonstrating greater Fos activity levels in the nucleus accumbens shell region than LBC controls, they showed no such difference in extent of co-localisation, suggesting that very few

neurons recruited in the nucleus accumbens shell during reversal learning expressed 5-HT_{2C}Rs. Regions of the ventral striatum might therefore be an unlikely focus for future targeted manipulations of 5-HT_{2C}R function. However, given that 5-HT_{2C}Rs are found to mostly be located on GABAergic, primarily parvalbumin-containing, interneurons, and are believed to have an inhibitory function on neuronal activity (Liu et al. 2007), it might be expected that few active neurons express these receptors. A comparison of co-localised Fos/2C activity between SB242084-treated animals and vehicle-treated controls might therefore provide a better indication of the regional importance of 5-HT_{2C}Rs to reversal, by demonstrating in which regions 5-HT_{2C}R antagonism most enhances neural activity.

6.3. GENERAL DISCUSSION

The aim of this study was to identify the neuroanatomical systems activated by performance of a reversal learning task, through the use of immediate early gene (IEG) expression as a marker of neural activity. Contrary to results reported following ablation or inactivation of specific cortical regions, the present results indicate that regions of the OFC and mPFC are both involved in the acquisition of a spatial discrimination, as well as its subsequent reversal. Thus, the OFC does not remain inactive prior to reversal, and the mPFC is not only active when a shift of attention is required. These cortical regions might therefore play a greater role in behaviour than previously suggested, highlighting the need to complement lesion data with explorations of normal function in intact behaving animals. In line with previous evidence, the BLA was identified as a potentially key region for supporting reversal, with higher activity in this region linked to faster learning. In fact, this was the only region to show a significant relationship with reversal performance, as distinct from activity which could be attributed to the number of trials performed during behavioural testing; identifying the BLA as a key focus for future targeted manipulations. Evidence that a subpopulation of neurons in several areas recruited during reversal performance demonstrated 5-HT_{2C}R immunoreactivity, with the exception of the nucleus accumbens, points to a possible role for any of these regions in mediating the effect of 5-HT_{2C}R antagonism on performance. Further identification of regions most critical to 5-HT_{2C}R mediated reversal performance effects could be made through comparison of IEG activity and 5-HT_{2C}R co-localisation in animals administered a 5-HT_{2C}R antagonist prior to testing as compared to vehicle-treated controls. Future studies may also benefit from exploring the expression of different IEGs in relation to reversal performance, particularly those more closely linked to neural plasticity such as *arc* (Bramham et al. 2010).

CHAPTER 7

DISCUSSION AND CONCLUSIONS

In chapter 1, three main aims of this thesis were identified:

1. **To investigate the role of task differences in mediating the effect of 5-HT_{2C}R manipulations upon reversal learning, as identified from prior studies reporting discrepant outcomes.**
2. **To dissect reversal learning tasks into their key constituent components, and identify the effect of reduced 5-HT_{2C}R activity in these tasks.**
3. **To provide preliminary identification of areas of neuronal activation during the performance of an operant spatial discrimination and reversal task.**

The following chapter will review the results of experiments presented in Chapters 2-6 of this thesis and discuss the implications of these results for the issues highlighted in Chapter 1. It will consider the implications of these results for understanding the role of 5-HT systems in reversal learning, as well as the limitations of the paradigms used, and future directions for resolving outstanding questions regarding the role of 5-HT_{2C}Rs in cognitive flexibility.

7.1. INTRODUCTION

Previous research has highlighted a potentially significant role for 5-HT_{2C}Rs in mediating flexible cognition, but the effect of modulating activity at these receptors has been found to differ (Boulougouris et al. 2008; Boulougouris & Robbins 2010; Nilsson et al. 2012; Pennanen et al. 2013). The evidence reviewed in Chapter 1 showed that the effects of 5-HT manipulations can vary depending on task modality and differences in response requirements across otherwise similar reversal tasks, and highlighted the potential significance of small design differences. Furthermore, although reversal learning tasks have been widely used for assessing cognitive flexibility in both humans and experimental animals, the cognitive and behavioural mechanisms involved in adapting performance are not well understood. Though seemingly simple, reversal learning tasks involve numerous, often concurrent processes; any number of which may be affected by 5-HT_{2C}R manipulations. The experiments described in this thesis have explored some of these key processes in more detail using adaptations and refinements in design described earlier, and the effect of 5-HT_{2C}R antagonism within these tasks has been explored.

7.2. OVERVIEW OF RESEARCH CONTRIBUTIONS:

Addressing the first key aim of this thesis outlined in Chapter 1, Chapter 2 undertook a systematic exploration of design differences of prior visuospatial reversal tasks where 5-HT_{2C}R function was manipulated, and where discrepant outcomes have been reported (Boulougouris et al. 2008; Boulougouris & Robbins 2010; Nilsson et al. 2012). This highlighted a possible key difference in trial initiation requirements across tasks which, it was hypothesised, could alter the nature or degree of impulsive responding induced by 5-HT_{2C}R antagonism, and which might act to obscure any effects on cognitive flexibility. A comparison of automatic versus self-initiated trial conditions revealed no impact of this manipulation on performance however, and a general impairment was instead seen following 5-HT_{2C}R antagonism relative to vehicle-treated controls in both trial initiation conditions. This is consistent with reports of retarded reversal learning in 2CKO mice reported by Pennanen et al. (2013), but contradicts several other accounts of a beneficial effect of pharmacological or genetic inactivation of 5-HT_{2C}Rs (Boulougouris et al. 2008; Boulougouris & Robbins 2010; Nilsson et al. 2012). Although this task difference could not explain discrepant outcomes in previous tasks, this chapter highlighted a potentially significant role for task difficulty in mediating the effects of 5-HT_{2C}R manipulations, as explored in the Discussion of Chapter 2.

Consistent with this observation, Chapter 3 presented evidence that SB242084 significantly improved performance relative to controls when employing a more difficult probabilistic reversal learning (PRL) task. Since all other task parameters matched that of the previous deterministic reversal task, the probabilistic element of the design was likely the critical manipulation producing this difference in outcome. This suggests that the ease with which subjects solve a reversal task needs to be taken into consideration, since this can have a significant bearing on the effect of at least some experimental manipulations of the underlying neurochemistry. It might also suggest the recruitment of different brain regions during more challenging reversal tasks, and the Introduction of Chapter 6 highlights evidence that the mPFC (Brigman & Rothblat 2008; Bussey et al. 1997), and particularly the PrL (Dalton et al. 2016), may be a key region mediating performance under taxing conditions, despite evidence that these regions are not normally important for reversal. However, there is also evidence to suggest that the OFC is more critically involved under increasing task demands, with lesions to this region causing greater impairments in a four-choice as compared to two-choice odour reversal test (Kim and Ragozzino, 2005).

Previous evidence taken from within-session PRL tasks in rodents appears to question the translational value of these tasks, since rats and, particularly, mice appeared to adopt a model-free 'win-stay/lose-shift' strategy to solve the task, in a manner very different from that observed in humans and non-human primates. Another key contribution of the studies described in Chapter 3 was the establishment of a PRL task that could be readily solved by mice, with evidence that they were adopting a model-based response strategy that allowed them to disregard occasionally misleading feedback. This was attributed to the use of a between-session reversal design, which has never previously been used in probabilistic visuospatial reversal tasks in rodents, as far as I am aware. This Chapter therefore provides evidence that a between-session variant of a PRL task can be useful for producing model-based response strategies in rodents, which at least appear similar to the strategies employed by humans and primates. Although the processes involved in solving this task may differ from those recruited by within-session reversals, where prediction or expectation of reversal shifts becomes a factor, between-session tasks arguably provide a more ecologically valid model too, since stimulus-outcome associations in the real-world typically demonstrate at least a moderate degree of stability over time.

The final experiment of Chapter 3 (Experiment 3) is one of several experiments designed to address the second main thesis aim, that of dissecting reversal tasks into their constituent components in order to better characterise the effects of 5-HT_{2C}R antagonism. This experiment examined the effects of SB242084 on two forms of behavioural interference which occur concurrently during reversal learning, and which cannot normally be dissociated using standard reversal tasks – continued responding at the previously rewarded location ('perseverance') and avoidance of the previously non-rewarded (or punished) location ('learned non-reward'). Through use of tasks which isolate the influence of each form of interference on responding it was possible to localise the beneficial effect of SB242084 treatment in reversal to a specific improvement in the ability to overcome learned non-reward. This is consistent with the only prior study to examine the effects of 5-HT_{2C}R manipulations on these separable components of reversal (Nilsson et al. 2012), and extends these findings to probabilistic conditions. This finding is also useful for ruling out explanations centred on general motor impulsivity or response inhibition effects of treatment, which would be expected to exert an impact upon both perseverance and learned non-reward conditions. Therefore, at least one of the effects of reducing 5-HT_{2C}R activity appears to be to reduce the influence of learned non-reward on responding during reversal.

Another benefit of establishing a PRL task for use in rodents is the ability to identify potential effects of experimental manipulations on reward and punishment sensitivity by examining reactions to spurious feedback. This is important since any change in reactivity might mediate performance in reversal, and could be another way in which 5-HT_{2C}R manipulations affect performance, particularly given the strong links between 5-HT and punishment sensitivity (Dayan & Huys 2009). The between-session amendment of the PRL task allowed the inclusion of both punished correct response (PCR) trials and rewarded incorrect response (RIR) trials, whereas previous experimenters have omitted the latter in the belief that it places too high a demand on cognition in rodents (Ineichen et al. 2012). Although there were problems related to insufficient data, this did allow some exploration of the immediate effects of accurate and spurious feedback, of both a positive and negative valence, on subsequent responding. Evidence taken from the full reversal test of Chapter 3 (Experiment 2) provided no evidence for an effect of drug treatment on reward or punishment sensitivity, or on the ability to inhibit inappropriate responses to misleading feedback more generally, which might be indicative of an effect of drug treatment on response inhibition processes. However, inspection of early reversal data during learned non-reward and perseverance conditions revealed that SB242084 did reduce the influence of misleading wins (RIR trials) on subsequent responding, with drug-treated subjects more often shifting to the correct response location. This could be indicative of a drug-related reduction in reward-sensitivity. This effect is unlikely to mediate reversal learning performance however, since it was apparent across both test conditions, whilst SB242084 only improved performance in the learned non-reward test. Nevertheless, potential impacts on reward-sensitivity could be of significant clinical value, and will need to be considered in future experiments as this could act as a significant treatment confound.

Despite the evidence discussed in Chapter 3 that SB242084 improves the ability to overcome learned non-reward without affecting perseverance, these findings did not extend to the closely related tasks of extinction and latent inhibition, explored in Chapter 4. Instead, SB242084 caused an impairment in early extinction learning, reducing the number of response omissions and speeding responses to the previously rewarded stimulus relative to controls. As explored in Chapter 4 Discussion, the lack of an impairment effect under perseverance test conditions could be related to the existence of an alternative, novel response option that is rewarded. A perseverative effect of SB242084 may therefore have been obscured under Perseverance test conditions, demonstrating the importance of further breaking down and exploring the constituent elements of reversal learning tasks. Drug treatment also had no effect on the development of latent inhibition to a preexposed non-rewarded stimulus, with

SB242084-treated subjects making just as many responses in the food magazine as non-preexposed controls during presentation of this stimulus when paired with food reward. Chapter 4 Discussion considered how the nature of learning that occurs to an irrelevant stimulus presented alone, as compared to a non-rewarded stimulus concurrently presented alongside a rewarded stimulus, might differ. Therefore, SB242084 appears to play a specific role in reducing the influence of inhibitory CS-'no US' associations, which do not develop during presentation of irrelevant stimuli; further refining our understanding of the nature of the improvement observed in reversal learning.

Alternatively, since latent inhibition tasks have no operant response requirements, this could suggest a specific involvement of SB242084 treatment in modulating some aspect of response learning/execution. During acquisition of a discrimination task subjects learn many associations between cues, responses and outcomes, which each require modification during reversal, and it is not possible to pinpoint the effect of drug treatment to any one of these processes using standard operant tasks. Despite this, very few reversal tasks have, to date, employed classical conditioning procedures. The main aim of experiments presented in Chapter 5 was therefore to explore the effects of SB242084 in Pavlovian conditioning and reversal. These experiments demonstrated that SB242084 had no effect on the development of Pavlovian Conditioned Approach (PCA) ('sign-tracking') to a reward-paired stimulus, or on the subsequent reversal of this Pavlovian conditioned association. This further suggests the involvement of 5-HT_{2C}Rs in aspects of flexible response execution, rather than in flexibly updating S-O associations, which is a novel research finding.

Another significant contribution of the experiments presented in Chapter 5 was to demonstrate that a tendency toward sign-tracking/goal-tracking behaviour may not be the stable trait previously assumed, since a switch in reward contingencies during reversal generated sign-tracking behaviour in animals previously identified as goaltrackers. Therefore, despite evidence of inherent genetic differences in Pavlovian conditioned approach (e.g. Kearns et al. 2006), the phenotypic expression of this behaviour appears to be sensitive to specific environmental conditions in a way that has not been reported previously. This could have a significant bearing on the understanding of this behaviour in relation to conditions such as addiction.

Although OFC-basal ganglia circuitry has been most often implicated in reversal learning, there remains uncertainty as to the precise contributions made by different regions to distinct aspects of cognitive and behavioural flexibility, particularly given evidence that mPFC regions

might be recruited under increased reversal task demands. Furthermore, future exploration of the role of 5-HT_{2C}Rs in these processes will greatly benefit from studying the effects of targeted CNS infusions. It was therefore the aim of Chapter 6 to provide preliminary identification of areas of neuronal activation during the performance of an operant spatial discrimination and reversal task, which satisfies the third key aim of this thesis. Most human imaging studies, and the few immunochemical studies conducted in experimental animals to date, have not distinguished between brain activation associated with the control processes specific to reversal learning versus those that are generally involved in feedback-driven learning. Experiment 6.1 was designed to more clearly dissociate these processes, which additionally included controls for neural activity related purely to receipt/consumption of reward. Employing these tighter controls, the results demonstrated little reversal-specific neural activity, with regions of the OFC, mPFC, and dorsal and ventral striatum all active during both acquisition of a spatial discrimination and its subsequent reversal. Therefore, similar regions appear to support performance during both acquisition and reversal. There was a significant correlation between activity in the basolateral amygdala and reversal performance however, pointing to the potential significance of this region as a target for future localised manipulations.

Evidence for a lack of neural activity specific to reversal learning conditions does not rule out the possibility that distinct neural populations were activated during the acquisition of a spatial discrimination and its subsequent reversal however. Future tests for reversal specific c-fos activity may therefore benefit from exploiting recently developed methods which allow for selective manipulations of populations of neurons that have been active during a particular task. The Daun02-inactivation method (Koya et al. 2009) involves micro-injecting Daun02 into *c-fos–LacZ* transgenic rats, in order to selectively inactivate or kill Fos-expressing neurons. In *c-fos–lacZ* transgenic animals, neural activity stimulates the *c-fos* promoter in the transgene, driving expression of the bacterial *lacZ* gene that encodes the protein β-galactosidase. Daun02 is an inactive prodrug which is converted by β-galactosidase to the cytotoxic agent daunorubicin, which putatively causes cell death (Ghosh et al. 2000) and/or blockade of voltage-dependent calcium channels (Santone et al. 1986), thus selectively inactivating only strongly activated (Fos-positive) neurons (Cruz et al. 2013). This technique could therefore be used to examine the role of putative neuronal ensembles in reversal learning in the future, by examining whether Daun02 injections administered after reversal testing decrease the ability for reversal conditions to subsequently reactivate the same neuronal ensemble and impair reversal performance on test day; and whether Daun02 injections administered after acquisition of the spatial discrimination

inactivate a distinct set of neurons from those affected by post-reversal Daun02 administration, and which should therefore have no effect on reversal test performance.

Two further techniques which could be used to identify and selectively target neuronal ensembles activated by reversal learning would be to combine the use of c-fos-tTA transgenic mice with optogenetic or DREADD methods; techniques which have been used to investigate the causal role of neuronal ensembles in fear conditioning (Garner et al. 2012; Liu et al. 2012). In c-fos-tTA-transgenic mice (Reijmers et al. 2007; Reijmers & Mayford, 2009), neuronal activity can activate the c-fos promoter to drive expression of a tetracycline transcriptional activator (tTA or TET-off) protein. In the absence of doxycycline, a drug which binds to and represses tTA, the tTA protein can bind to a tet operator in the promotor of a second transgene to induce expression of a marker gene, only in neurons that were activated (Fos-positive) during the learning task (Cruz et al. 2013). Doxycycline can be added to the mouse diet to block expression of the marker gene, and can be removed from the diet prior to a first learning session in order to identify expression of the marker gene (e.g. lacZ, histone2B-green fluorescent protein (GFP)) in neurons that were activated during that selected time window. The identification of neurons that were active during a second test session can then be made through immunohistochemical labelling of the protein products of c-fos (Cruz et al. 2013). Removing doxycycline from the diet prior to acquisition and reversal of a spatial discrimination test, respectively, would therefore allow comparison of the neural populations activated by these different test conditions. Combining this technique with the use of optogentic or DREADD genetic constructs as the second transgenes, as has been used in previous tests of conditioned fear, could further allow for the manipulation of these putative neuronal ensembles, and the effects of selective reactivation or inhibition of neurons that were activated by reversal learning could be explored.

In sum, the experiments presented in this thesis have identified that differences in task difficulty might be responsible for previous inconsistencies reported following reduced activity at 5-HT_{2C}Rs, an effect which warrants further exploration. Further dissection of reversal tasks into their constituent processes has also identified that a beneficial effect of 5-HT_{2C}R antagonism observed in a probabilistic reversal setting is specific to an improved ability to overcome learned non-reward, and that this effect is most likely localisable to an improvement in flexible responding, rather than the flexible updating of stimulus-outcome associations. Several other possible explanations of drug-treatment effects on reversal have additionally been ruled out, by tasks exploring the contribution of changes in reward and punishment sensitivity, or the development of sign-tracking and goal-tracking tendencies. Greater dissection of reversal learning components has also allowed the identification of a possible detrimental effect of

SB242084 on extinction learning, alongside its beneficial effect on learned non-reward; an effect which is likely to have been obscured using perseverance tests. This might explain contradictory reports of impaired and improved reversal performance following reduced 5-HT_{2C}R activity, if the relative influence of these effects can be altered by variations in task demands or conditions.

7.3. CLINICAL RELEVANCE:

Considering the large investments made in clinical drug development, as well as the advances that have been made in recent years in identifying potential new drug targets, success rates for drugs during clinical development have remained persistently low, including in the area of psychiatry and mental health (Hay et al. 2014). One potential explanation for this is flawed preclinical research, and it is clear that the selection of sufficiently validated and predictive animal models is essential in bridging the translational gap to the clinic (Denayer et al. 2014), in addition to the development of more sophisticated early-stage preclinical human models. Using a design amendment of between-session rather than within-session reversal, work presented in this thesis demonstrates, apparently for the first time, that mice are able to solve probabilistic reversal learning tasks that are standard practice in tests of human cognitive flexibility. Furthermore, they appear to employ similar problem-solving strategies in doing so, demonstrating an ability to integrate reinforcement history over multiple trials as well as to regulate responding to local reinforcement, instead of adopting the trial-by-trial 'win-stay/lose-shift' strategies previously demonstrated in rodents under the more stochastic conditions created by within-session reversal. This is a significant advance in the development of a translationally valid model of cognitive flexibility, and should be useful for developing treatments with improved clinical efficacy. Extending this model for use in mice also has advantages in terms of the wide array of molecular genetic techniques not currently available in rats, which can be used to create custom-made mouse models for a wide array of specific diseases.

As discussed in Chapter 1, there are diverse neuropsychiatric conditions and neurodegenerative diseases in which deficits of flexible cognition are displayed, and developments in pro-cognitive treatments are of potentially immensely significant clinical value. However, there is currently little understanding of the specific impairments which underlie the reversal deficits observed in these diverse clinical populations. As discussed throughout this thesis, reversal learning tasks are fairly crude measures of cognitive function, since several processes occur simultaneously during testing, and it is likely that different conditions give rise

to different specific deficits. Research using the ID/ED task has started to fractionate these deficits more clearly for some conditions. For example, schizophrenic patients appear to display a perseverative tendency in attentional shifting tasks, without demonstrating an impairment in learned irrelevance (Elliott et al. 1995; Elliott et al. 1998). By contrast, participants with autistic spectrum disorder (ASD) display deficits in attentional shifting that appear not to be caused by either enhanced perseveration or reduced learned irrelevance, but which are attributable to reduced novelty processing (Maes et al. 2011).

Without the relevant dissociation of reversal deficits across clinical populations, it is difficult to assess the clinical value of 5-HT_{2C}R antagonists. Studies in animal models suggest they will be of most clinical value in the treatment of conditions for which excessive learned non-reward (but not latent inhibition) is a feature, but of limited value for the treatment of perseverative deficits, for which this may even cause further impairments, given their effects on extinction learning. There is therefore a great need for tasks, in both non-human and human models, which enable a more fine-grained decomposition of processes involved in reversal, of the type presented in this thesis, in order to develop more clinically effective treatments. The additional motor impulsivity effects of 5-HT_{2C}R antagonists, and their potential impact on reward sensitivity must also be considered when assessing clinical potential however, particularly since many of the conditions which display cognitive impairments also report impulsivity and affective/reward processing dysfunctions; and effective treatments for one class of symptoms may have unintended effect on others.

7.4. LIMITATIONS AND FUTURE DIRECTIONS:

The visuospatial reversal learning tasks presented in this thesis demonstrate a significant potential confound of stimulus generalisation. Through use of a spatial dimension, employing left and right nosepoke response locations, the use of a novel central response location in tests of perseverance and learned non-reward might simply present a 'less-left' or 'less-right' response alternative, and stimulus generalisation across locations might therefore make these tests identical to the full reversal condition. This confound could be removed through the use of a non-spatial discriminative stimulus domain, using visual, olfactory or tactile cues for example. However, such tasks typically carry their own confounds. In pilot tasks not presented in this thesis, use of textured runways were used to discriminate the location of the rewarded arm of a T-maze. This task solved potential problems of stimulus generalisation, but created issues in controlling for the use of allocentric and odour cues, and I was never satisfied

that the controls for such confounds were adequate. Such problems might also be overcome through use of the rodent bowl-digging reversal task, where different digging mediums or odours serve as the discriminative stimuli, a task which also holds high ecological validity. However, as with maze-based tasks, this is a low-throughput method, and introduces potential issues with experimenter handling in between trials. The use of visual cues in a touchscreen format might also reduce stimulus generalisation, but the ecological validity of these tasks has been questioned, as they require particularly extensive training in rodents (Horner et al. 2013; Mar et al. 2013). Given evidence for a difference in SB242084 treatment effects across perseverance, learned non-reward and full reversal conditions, it seems likely that stimulus generalisation did not present a significant confounding factor in the studies presented in this thesis however, and since alternative stimulus modalities arguably present more significant potential confounds, I believe visuospatial operant tasks remain one of the most suitable method for assessing cognitive flexibility in the rodent.

A second potential confound in tests of perseverance and learned non-reward was created through the use of a novel response alternative in place of the previously correct or incorrect response location, since differences in novelty attraction or recognition could affect performance in these tasks. However, previous work has demonstrated that SB242084 has no effect on the number of entries or amount of time spent in the novel arm of a radial maze relative to vehicle treated controls (Nilsson et al. 2013). Furthermore, an internal control for these effects is present in the design, since a general effect on novelty attraction or recognition would be expected to produce opposing effects in tests of perseverance and learned non-reward, whilst there is an absence of novelty in the full reversal condition. The pattern of effects reported following SB242084 treatment, of improved reversal, reduced learned non-reward, and unaffected perseverance, suggests novelty was not a significant confounding factor.

Potentially more significantly, due to the limited number of available stimuli in visuospatial reversal learning tasks, pre-training occurred to the same stimuli that were used in the subsequent spatial discrimination and reversal tests. The learning and habituation that occurs to these stimuli during training could be a source of additional interference during reversal. Most reversal learning studies reported in the literature demonstrate differences in pre-training, which is often assumed to be of little relevance for later reversal performance. It is even possible that the interactive effects of SB242084 treatment across reversal task difficulty could be linked to differences in training history, given that more difficult tasks, such as PRL tasks, also typically involve more protracted pre-training schedules. Future exploration of this 'task difficulty' effect might therefore benefit from the use of visual cues in touchscreen-based

tasks, or tactile/olfactory cues in the bowl-digging task, where a wider array of stimulus exemplars means that different stimuli can be used during pre-training and testing. However, given the issues highlighted above, it may be better to simply employ tighter controls over the extent of pre-training given across visuospatial reversal studies, whilst systematically varying the complexity of the task. This could be achieved by adding an extra 'distractor' item during testing, by utilising all three available nosepoke ports. The training history for animals under 'easy' and 'hard' task conditions could therefore be matched, right up until the distractor item is added during acquisition of the spatial discrimination.

In Chapter 5, I presented evidence that SB242084 treatment had little effect on reversal of a Pavlovian conditioned response, as distinct from its effects on operant reversal, and suggest that this could provide evidence that the drug exerts its effects on some aspect of flexible responding, rather than in flexibly updating S-O associations. However, design differences between these tasks must also be taken into account. Firstly, the task was not probabilistic, and contradictory results have been reported when using deterministic tasks. However, the evidence presented in Chapter 2 suggests that drug-treatment might be expected to impair performance, rather than be without effect. Animals in the Pavlovian task also required extensive training before they demonstrated reliable Pavlovian conditioned approach (PCA) to the food-predictive lever, and some animals never displayed this behaviour ('goal-trackers'). Establishing PCA is necessary for determining whether animals go on to successfully reverse these conditioned associations, but the length of acquisition training required may have considerably altered the nature or strength of the associations which must be overcome during reversal, which could also be responsible for the lack of drug treatment effect. Future research might therefore benefit from exploring the effect of SB242084 treatment on Pavlovian reversal learning in the rat, to confirm the effects reported in mice, since the length of training required to establish this behaviour in rats is considerably shorter, and even goal-tracking rats typically demonstrate a degree of lever-press behaviour to the reward-paired lever, making reversal of conditioned associations easier to detect.

Another avenue for future research should be to explore role of serotonin in modulating the neural circuitry that supports cognitive flexibility. The results reported in Chapter 6 suggest that there is substantial overlap in the CNS structures and circuits that support the expression of a learned discrimination and that are involved in reversing or otherwise modifying the performance of that discrimination. This is not altogether surprising but it does mean that the standard techniques of lesioning or drug microinjection may not be adequate to identify the mechanisms particularly involved in cognitive flexibility. In the last decade a series of techniques

that use genetically manipulated mice have been developed in order to allow targeted interventions not just of a particular neural structure, but to cells within the CNS that have a particular neurochemical profile, and even to subsets of neurons within a structure. Methods that are now available include Designer Receptors exclusively activated by Designer Drugs (DREADDs) to modulate GPCRs (Urban & Roth 2015), and optical technologies that rely on the activation of channel rhodopsins expressed in the target cell type (Smith & Graybiel 2013). Each of these techniques has been applied to the study of serotonergic systems, especially in the contexts of feeding and appetite, and anxiety and depression (Hainer et al. 2015). They also have potential, in combination with the behavioural models described in this thesis, to further elucidate the role of serotonin in cognition.

In summary, the main findings of this thesis are:

1. Task differences can alter the effect of 5-HT_{2C}R antagonism on reversal learning, with evidence that SB242084 impairs performance under deterministic conditions, but improves reversal under probabilistic schedules of reward; potentially demonstrating an interactive effect of task demands.
2. SB242084 treatment specifically improves the ability to overcome learned non-reward without affecting perseverance, but additionally impairs extinction without affecting latent inhibition. This pattern of effects could suggest SB242084 exerts its effects specifically on operant aspects of behaviour, particularly since it does not affect the reversal of a Pavlovian discrimination. Additionally, drug treatment may serve to reduce reward sensitivity, but this effect does not mediate reversal performance.
3. The basolateral amygdala may be a particularly important region for modulating reversal learning performance, and targeted manipulations of 5-HT_{2C}R function within this region should be a key focus of future research, as well as work targeting specific neural ensembles recruited under different test conditions.

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