Too little and too much: investigating the role of balanced prefrontal neural activity in behavioural flexibility

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Abstract

A consistent deficit of schizophrenia, among numerous neuropsychiatric disorders, is impaired cognitive flexibility, the prefrontal-dependent ability to overcome pre-existing behavioural strategies to adjust behaviour in line with changing environmental demands. Prefrontal gamma-aminobutyric acid (GABA) dysfunction has been linked to these cognitive impairments and there is evidence that tasks requiring cognitive flexibility require the medial prefrontal cortex (mPFC) and local GABA-mediated transmission. In this thesis, I report five experiments in which an operant strategy shifting task was used to test whether both increased and decreased GABA inhibition in the mPFC impair strategy shifting in Lister Hooded rats. In experiment 1, prefrontal hypo-activation or functional disinhibition was induced by micro-infusing the $GABA_A$ agonist muscimol (62.5ng/side) or antagonist picrotoxin (PTX) (300ng/side) respectively, into the mPFC; these manipulations had markedly impaired sustained attention in a 5-choice serial reaction time (5CSRT) test during previous research. Results showed that neither prefrontal hypo-activation nor disinhibition affected shifting from a spatial response to a visual light-cue-based response, although disinhibition impaired expression of the spatial response and increased trial omissions. Remarkably, all rats required up to three times the number of trials to shift the responses as compared to previous studies in other rat strains. Two additional behavioural studies without infusions followed; experiment 2 showed removal of pre-training cue-light pre-exposure and reduction of training trials for the initial spatial response strategy did not facilitate shifting to cue-based strategy; experiment 3 showed rats could acquire the cue strategy and then shift to the spatial

response strategy, and also perform spatial response reversals, within a similar number of trials as in previous studies. Ultimately, however, there was always a substantially higher shift cost for response-to-cue than for the cue-to-response shift (~ 200 trials). Experiment 4 investigated effects of mPFC disinhibition and hypo-activation on shifting from cue to response strategies, and there is evidence to suggest prefrontal picrotoxin disrupted shifting ability, impaired expression of rule retrieval following rule acquisition, and response latency. Additionally, experiment 5 found that administering the same prefrontal disinhibition, prior to initial rule acquisition, as well as prior to strategy shifting, increased perseveration. Finally, a Bayesian analysis approach was used to infer the types of learning strategies that rats were using during task performance, with preliminary results suggesting that rats predominantly use a lose-shift strategy during the first trials of shifting to a spatial response. From these data one conclusion is that when present during initial rule learning prefrontal disinhibition may augment reward-association, and impair expression of newly learnt shift rules, thus increasing perseveration.

Publications based on this thesis

- BNA2019 Abstract Book, April 2019; Too little and too much: impact of functional inhibition and disinhibition of the medial prefrontal cortex on behavioural flexibility assessed using an operant strategy-shifting task

- FENS Forum 2018 Abstract; Too little and too much: impact of functional inhibition and disinhibition of the medial prefrontal cortex on behavioural flexibility assessed using an operant strategy-shifting task

Awards

- BBSRC In Vivo Strategic Skills Award 2016

Declaration

I declare that this thesis is the result of my own work which has been undertaken during my period of registration for this degree at The University of Nottingham. I have complied with the word limit for my degree (as stated in the Quality Manual).

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- Behavioural Neuroscience Group seminar (School of Psychology, University of Nottingham) 16/03/2018
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Poster presentations

- BNA Festival of Neuroscience 2019
- FENS Forum 2018 (Berlin)
- Neuroscience at Nottingham Symposium 2018
- Psychology Postgraduate Conference 2017
- DTP Spring School 2017
- Neuroscience at Nottingham Symposium 2017

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List of abbreviations

Acronym	Definition
5-HT	5-hydroxytryptamine
ED	extra-dimensional
FR1	fixed-ratio 1
GABA	gamma-aminobutyric acid
ID	intra-dimensional
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
OFC	orbital frontal cortex
PTX	picrotoxin
RTC	responses to criterion
SST	set or strategy-shifting task
VTA	ventral tegmental area

Keywords

Cognitive Flexibility; Behavioural Flexibility; Strategy Shifting; GABA; Disinhibition; Operant Strategy Set-Shifting Task (operant SST); Attentional Set-Shifting Task (non-automated SST); medial Prefrontal Cortex (mPFC).

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Chapter 1: Introduction

The role of GABA-mediated transmission in shaping cortical activity

Healthy brain activity requires balance of two opposing neuronal events: excitatory and inhibitory neurotransmission; indeed, synaptic mechanisms that drive these neuronal states are considered to be linked in terms of co-occurrence, whether generated by sensory stimuli or spontaneous cortical activity (Isaacson & Scanziani, 2011). The major inhibitory system comprises neurons that release the neurotransmitter gamma-aminobutyric acid (GABA) to inhibit post-synaptic neurons. In the cortex, GABAergic interneurons account for approximately 20% of the neuronal population (Meinecke & Peters 1987). They form reciprocal interactions with excitatory glutamatergic principle cells that form the basis of feedback inhibition (Isaacson & Scanziani 2011).

Clinical links between aberrant GABA-mediated transmission and cognitive impairment

Evidence from clinical and pre-clinical studies of cognitive impairments associated with age-related cognitive decline, schizophrenia and autism, suggests that this neuronal balance is disturbed in brain regions mediating cognitive function, including the prefrontal cortex (PFC) (Bast, Pezze & McGarrity, 2017; Huang & Mucke 2012; Marín 2012). Such imbalances

possibly interfere with the processes underlying cognitive functions such as cognitive flexibility and attention (PFC-mediated).

Disinhibition, resulting from reduction of GABAergic neurotransmission, has been consistently implicated in patients with cognitive disorders such as schizophrenia, as evidenced both by post-mortem GABA-marker alterations in the frontal lobes and by aberrant PFC gamma oscillations during performance of cognitive tasks (Lisman , 2008; Tse, Piantadosi and Floresco, 2015). Recent preclinical work has begun to manipulate the GABA-mediated neurotransmission of the PFC and connected sites (such as the hippocampus and striatal regions), to investigate the role of balanced prefrontal activity in cognitive functions such as decision making, attention and working memory (Auger and Floresco, 2014; Pezze *et al*, 2014; Paine *et al*., 2015; McGarrity *et al.*, 2016). Such work has shown that both disinhibition and hypo-activation within the prefrontal cortex cause cognitive deficits (Pezze *et al*, 2014).

Studying cognitive and behavioural flexibility

Building on the previously mentioned work, the aim of this project was to investigate the role of balanced activity within the prefrontal regions on strategy shifting in an automated task of behavioural flexibility (Brady & Floresco, 2015). Cognitive flexibility is a prefrontal dependent executive function which allows behavioural adaptation in response to changing environmental demands; as a covert cognitive function it is not directly measurable, but can be inferred by observing overt behavioural flexibility (Brown & Tait 2015). Defects in various aspects of this ability are observed in a substantial range of psychiatric disorders, including autism,

(Ozonoff, South & Miller, 2000), Alzheimer's disease (Guarino *et al*. 2019), bipolar depression (Clark, Sarna & Goodwin, 2005; Martínez-arán *et al*. 2004), Parkinson's disease (Cools *et al.* 2001) and schizophrenia, and is sensitive to PFC GABAergic perturbation (Ceaser *et al*. 2008; Tse, Piantadosi & Floresco, 2015).

Cognitive flexibility requires coordination of various cognitive processes, including rule generation, inhibition of previously relevant (now irrelevant) responses, working memory and attention (Dajani & Uddin 2015; see Fig 1A). Successful performance of these processes therefore involves a host of extensively investigated neural substrates, including anatomical sub-regions of the PFC, dorsal striatum and amygdala and their interconnectivity with areas such as the VTA and hippocampus, as well as various neurotransmitters systems including GABA, 5-HT, acetylcholine and dopamine (Nilsson *et al,* 2015; Tse, Piantadosi & Floresco, 2015; Floresco, Zhang & Enomoto, 2009; Cools & D'Esposito, 2011; See Fig 1B). The key prefrontal sub regions investigated for their involvement in behavioural flexibility include the medial PFC (mPFC), deemed crucial in set-shifting, and the orbital frontal cortex (OFC) which appears to be necessary for reversal learning (McAlonan & Brown, 2003).

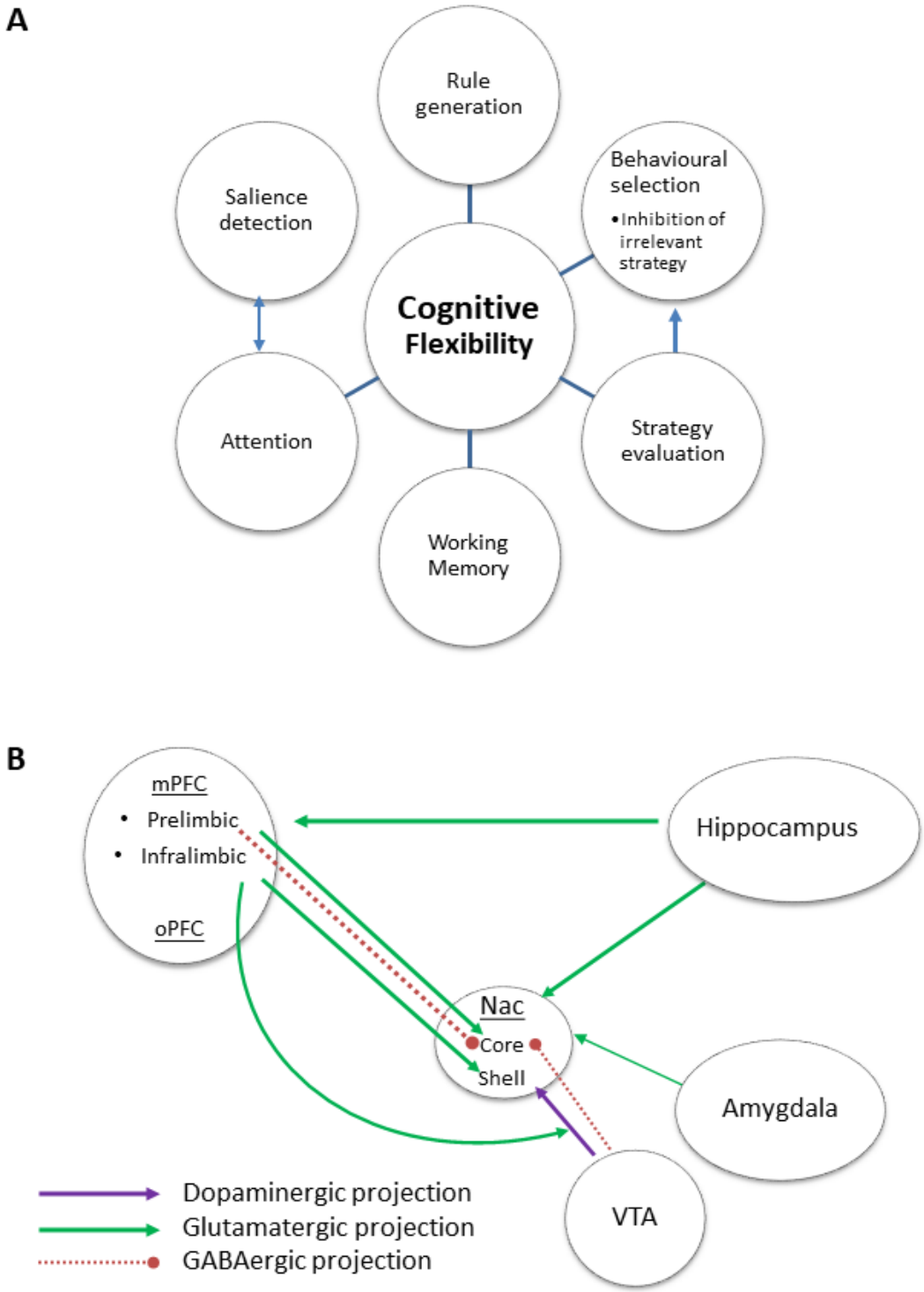


Figure 1.1 – A) Coordination of various cognitive processes is required to produce cognitive flexibility B) Thus various neural substrates (brain regions and neurotransmission systems) are implicated in the function of these cognitive processes, with the projection sites of the PFC underpinning key underlying processes such as reward learning and motivation, involving the nucleus accumbens (NAc); see Nilsson *et al,* 2015; Tse, Piantadosi & Floresco, 2015 Floresco, Zhang & Enomoto, 2009).

Behavioural flexibility in humans can be measusred by performance of strategy shifting tasks involving intra- (ID) and extra-dimensional (ED) shifting, such as the ID/ED component of the Cambridge Neuropsychological Automated Testing Battery (CANTAB) which can also be used in primates, or the Wisconsin Card Sorting Test (Berg, 1948; Sahakian and Owen, 1992)). These tests incorporate two aspects of cognitive flexibility, attentional set shifting and reversal learning. These two processes both require inhibition of a previously-rewarded response behaviour (reflecting formation of an initial "attentional set"), which is replaced with responding to a new, relevant (previously irrelevant) response rule. This involves the subject initially learning to discriminate one strategy wherein responses to one particular stimulus are rewarded at a fixed ratio of 1 and thus forming an attentional set. As attentional sets are described as hypothetical "stores" which maintain a stimulus' reward-predicting aspects, the contents of the set should be updated under changing task requirements (Tait, Chase & Brown, 2014). Therefore, once the initial set has been acquired, the rule changes from rewarding responses to the initially reward-relevant stimulus to rewarding responses to a previously irrelevant stimulus. In set shifting, a rule shift involves the sensory dimension of the relevant stimulus changing, such as in the ED component of the CANTAB which requires subjects to discriminate between pictorial stimuli of line segments superimposed onto abstract shapes; a shift in this case could involve rewarding one of the presented line-segment stimuli when initially a stimulus of the abstract shape dimension was relevant. The alternative ID component requires adaptation of behaviour toward *novel* relevant stimuli within the same sensory

dimension as the previously learnt rule, for example responding to novel abstract shapes which are rewarded at initial discrimination and set shifting, with the line-segment stimuli remaining irrelevant. If an attentional set is formed, ID shifting should be easier than ED-shifting, as shifting attention to an extra-dimensional stimulus requires updating of the attentional set's content (Tait, Chase & Brown, 2014). Ultimately, the ease (speed of completion or fewness of errors) with which ED shifts can be learnt (with ID shift-performance remaining relatively constant) can reflect the cognitive flexibility of experimental groups.
Reversal learning, on the other hand, differs from set shifting in that the initial reward-relevant stimuli are switched with the *familiar* stimuli that were previously presented as reward-irrelevant; the reward contingencies are simply reversed within the same dimension (Birrell & Brown 2000). Reversal learning paradigms are also extensively used across species to test cognitive flexibility and are applied in clinical research of psychiatric disorders (Izquierdo *et al*. 2017).

Rodent models of behavioural/cognitive flexibility

There are various rodent analogues of behavioural flexibility tasks, including the non-automated attentional set-shifting tasks (SSTs); the "bowl digging task" (Birrel &Brown, 2000) and cross-maze tasks (Ragozzino *et al*, 1999; Floresco*,* Zhang & Enomoto*,* 2009; Floresco *et al*. 2006). There are also automated tasks such as the operant-based strategy shifting task (operant SST)(Brady & Floresco 2015) (See Table 1).

These automated and non-automated tasks have been used to demonstrate that rats can form attentional sets, and that lesions and pharmacological manipulations of prefrontal regions can selectively impair performance of certain task stages (Birrel & Brown, 2000; Floresco, Block & Tse, 2008; Enomoto, Tse & Floresco, 2011).

Both non-automated SSTs and automated SSTs involve measuring reversal learning and set-shifting. The rodent mPFC (analogous to the primate dorsolateral PFC) has been shown to be specifically crucial in the set-shifting phase of the non-automated SST, while the orbitofrontal cortex (OFC) appears to be necessary in reversal learning, with some cases of OFC lesions producing reversal learning impairments while sparing attentional set-shifting (McAlonan & Brown 2003).

There are many advantages of the non-automated tasks mentioned above; in the bowl-digging task the rats' natural exploratory and foraging behaviours are exploited in that they must learn to dig in bowls to find a food reward. Similarly in the cross-maze task, the rats' exploratory behaviours are exploited as they must navigate through the arms of the maze to reach a food reward. In the bowl-digging task, the stimuli consist of two sensory dimensions (modalities), odour (added to bowls) and tactile (texture of digging media) which are suitable for rats to quickly learn to discriminate. As a result of the large variety of stimulus exemplars available, one can test for set formation via comparison of ID/ED shift costs. It is possible to acquire multiple (typically seven) choice

discriminations within a few hours, as opposed to the hundreds of trials that rat visual discrimination tasks might require (Fig2A).
In the cross-maze task, stimuli modalities can be visual (involving visual cues indicating food-baited or reward-irrelevant arms) or directional (requiring the rat to choose an arm to the left or right, depending on the rule). Strategy shifting occurs across modalities, e.g. from visual to directional or vice versa. Reversal learning can be trained using intra-modal shifts from the initial rule to the opposite rule; in the directional modality, an example would be changing the initially learnt rule from, say, "turn left" to "turn right" (Ragozzino *et al*. 1999; Floresco *et al*. 2009).

The reasons for using the automated operant SST described in this thesis were to avoid limitations of non-automated tasks in general, including 1) methodological variations such as equipment, and stimulus choice (such as digging medium, odour and the combination of these), salience and placement, which are all variables that may affect performance (Tait, Chase & Brown, 2014); 2) the time-consuming and labour-intensity of the non-automated task, and 3) to reduce the opportunity for experimenter bias and error (such as forgetting to bait an arm in the cross-maze or mixing up the placement of digging-bowls). Automated operant tasks are designed to remove experimenter bias and standardise stimuli presentation (Brady & Floresco, 2015).

Publication	Behavioural Tasks	Brain regions	Drug	Drug Behavioural Effect	Lesion	Lesion Effect
Enomoto, Tse, Floresco – 2011 – Biol Psychiatry	1. OSST: a. Visual Cue-Response b. Reversal (separate group to a) 1. Working Memory	PFC	Bicuculline (12.5-50ng) (GABAA blocker)	1. Impaired set shifting 1. Increased response latencies(WM 1. Increased locomotor activity		
Placek et al, 2013 - Behav Brain Research	OSST: Response-to-Visual Cue shift (lesion)	mPFC	N/A	N/A	Neonatalventral hippocampal	Impaired strategy shift- mainly perseverative errors. Unimpaired in reversal or set task (Response-to-Cue)
Brady and Floresco, 2015 - JoVE	OSST: Visual Cue – Response Shift	mPFC	D2 antagonist Haloperidol (0.2mg/kg) prior to shift	Impaired reminder trials (visual cue) rule) - Improved shift to response – fewer perseverative errors		
Floresco, Zhang, Enomoto 2009 – Behav Brain Research – Review	Cross Maze OSST	Circuits – PFC – subcortical regions – cortico-thalamic-striatal	D1/D2 blockers – mPFC D4 agonist D4 antagonist	PFC suppression: 1. increased perseverative errors (e.g. D1/2 antagonism) D4 agonist – impaired set shift D4 antagonist – improved shift (DA over-activation impedes performance?)	Orbitofrontal PFC lesions	Reversal learning impairment

Abbreviations: OSST (Operant-based Strategy Shift Task); (m)PFC ((medial) prefrontal cortex); D1-4 (Dopaminergic receptor 1-4), PPI (Pre-pulse Inhibition), E-phys (Electro-physiology), LMA(locomotor activity; WM (Working Memory)

Floresco, Block, Tse (2008), Behavioural Brain Research	1. Operant Set Shifting Task 2. OSST with pre-exposure to visual-cue stimulus lights 3. OSST with Response Reversal learning	mPFC–(prelimbic sub-region)	Bupivacaine hydrochloride (0.75%, 0.5µl) – Na Channel blocker	mPFC inactivation: OSST 1: - No impairment of initial learning (either Visual-Cue or lever Response). - Impaired visual cue-response set-shift. - No impairment of response-visual cue shift. OSST 2 (stimulus lights pre-exposure): - Impaired Response-Visual-cue set shift - Fewer trials to criterion for initial Response learning compared to OSST1 OSST 3 (Reversal learning) - No significant effect of mPFC inactivation.	N/A	N/A

Table 1 - Studies have found mPFC GABA-egic manipulations and lesions impair aspects of pre-frontal cognitive function, including strategy shifting.

Publication	Experiment/ Behavioural Task	Brain regions	Drug	Drug Behavioural Effect
Pezze, McGarrity et al, (2014) J. Neurosci	5 Choice serial reaction time (5CSRT) PPI Electro-physiology. In vivo	mPFC	GABA-$_A$ antagonist – Picrotoxin (75,150,300ng) – does per side* GABA-$_A$ agonist – muscimol (62.5, 125, 250ng)*	Impaired attention: - Reduced accuracy - Increased omissions Picrotoxin – locomotor hyperactivity Muscimol – reduced LMA Muscimol: increased premature responses (impaired response control) E-phys. - Muscimol; inhibited PFC firing - Picrotoxin: - increased PFC firing (bursts) - increased LFP power PPI – no effect: particularly insensitive PPI in Lister-hooded rats
McGarrity et al, (2016), Cerebral Cortex	5-CSRT (PFC-dependent) Water maze delayed matching to place test (Hippocampal dependent) PPI, LMA E-phys	Ventral Hippocampus	GABA-$_A$ antagonist - Picrotoxin	Hippocampal disinhibition: 1. impaired water maze performance (memory) 2. impaired 5-CSRT (attention) 3. slight reduction of startle, PPI unaffected 4. Moderate increase of LMA 5. E-phys: enhanced burst firing of HPC neurons

Table 2- Studies have found effects of $GABA_A$ antagonist picrotoxin and $GABA_A$ agonist muscimol in Lister Hooded rats performing prefrontal dependent behavioural tasks.

The operant SST is conducted within an operant conditioning chamber equipped with two response levers and a stimulus light above each (Brady & Floresco, 2015, Floresco, Block & Tse, 2008) (Fig1B). The two modalities in this set-up are, like with the cross-maze, visual and directional ("spatial"). The difference is that to learn the spatial rule, the rodent must learn to respond on one specific lever for every trial (either left or right lever) and ignore the location of the illuminated cue light (spatial response strategy). In the following shift stage of the task, the rodent must suppress the spatial strategy and learn to respond correctly to the light cues by pressing the lever beneath which the cue light illuminates, in order to receive a food reward (response-to-visual-cue strategy shift) (Brady & Floresco 2015). The order of these two tasks can be reversed (visual-cue-to-response shift); this test sequence has been used to demonstrate that rats undergoing pharmacological inactivation of the mPFC using bupivacaine infusions have required significantly more trials than controls to perform this set shift (Floresco *et al*. 2008).

One of the main benefits of the automated operant SST is that data collection is relatively rapid; despite requiring hundreds of training trials, training and testing takes 30-60 minutes per day, with multiple rodents being tested simultaneously. Additionally, the computer-control provides consistency to the timing of trials, inter-trial intervals, and stimuli presentation, as well as ensuring that rewards are always delivered correctly. Precise data-collection, including

exact recording of response latencies, is also an advantage, as well as the ability to carry out detailed analysis of the different error types (e.g. perseverative or never-reinforced) made during shifts or reversals, which lend detail to data interpretation (Brady & Floresco, 2015). Additionally, the different aspects of behavioural flexibility can be tested with the operant SST: reversal learning is possible; for instance, with rats trained to learn the initial response discrimination of pressing the lever opposite their bias must in the reversal stage switch to pressing the other lever to be rewarded. However Brady & Floresco report that it is virtually impossible to train rats to perform reversals of the visual cue discrimination (ignoring the light and responding to the unlit cue), presumably due to the cue lights being more salient when lit than unlit (Brady & Floresco, 2015). It is possible to incorporate "retrieval" trials, which are the same as those of the initial discrimination, before strategy shifting allows for testing and comparison of flexibility. Finally, the switching of attention between sensory dimensions is preserved for the ED shift.

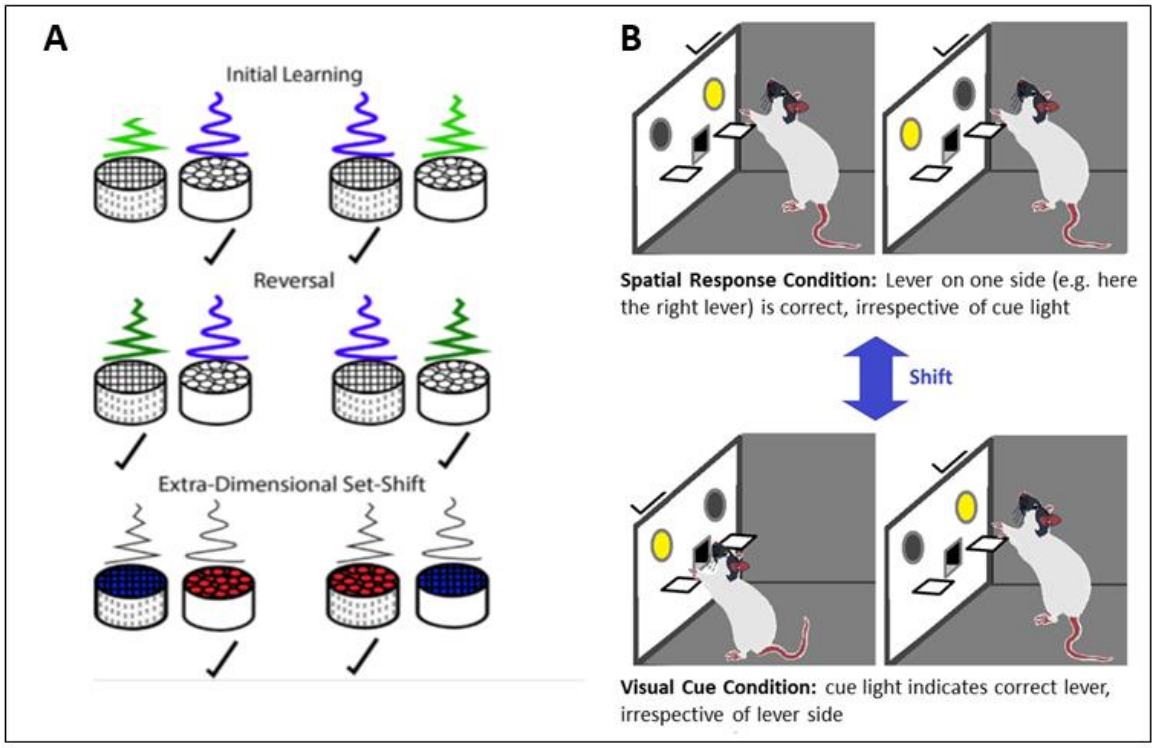


Figure 1.2- Schematic illustration of (A) the bowl digging attentional set-shifting task (non-automated SST) in which pairs of pots are presented with distinguishable odour and tactile stimuli (Adapted from Hurtubise and Howland, 2016) and (B) the operant- based strategy shifting task (operant SST), in which cue lights and lever location form the distinct attentional stimuli for learning the two types of strategy in the automated strategy-shifting procedures conducted in an operant chamber. During spatial discrimination (top), rats are required to always press one of the levers (e.g. right) regardless of the position of the cue light. Visual cue light discrimination (bottom), requires rats to always press the lever that has a stimulus (cue) light illuminated above it.

The components of behavioural flexibility shown in Figure 1A are required to successfully perform the automated SST. Salience detection of the

relevance of the left vs right position of the lever and illuminated lights is required to associate these as cues for performing food-rewarded responses. Subsequent attention to these cues allows for reinforced rule generation, such as during the first strategy acquisition, when one set of exemplars (e.g. position of one lever) indicates correct (rewarded) responses and the remaining set of exemplars (position of illuminated cue) is assigned irrelevance. Inhibition of irrelevant responses (behavioural selection) is required when implementing any new strategy. This occurs while learning to consistently perform the first strategy (e.g. position of one lever) and inhibiting spontaneous (non-reinforced) responding, or during the ED shift to the second reinforced strategy while suppressing the initially-learnt strategy. Failure to adopt the second strategy during the shift stage and instead implementing the initial (and irrelevant) strategy is referred to as perseveration of the first strategy. In the experimental protocol described in this thesis, perseveration is measured on an individual trial basis as a percentage of overall responses made by an individual rat during a shift session. Infusion studies reported here examined initial acquisition of response strategy and shifting to cue strategy or the reverse sequence of strategies.

Project objectives

In this project, the operant SST was employed initially to test the hypothesis that balanced pre-frontal neural activity is important for shifting between spatial response-to- visual cue strategy-shifting (see chapter 2). Local mPFC microinfusions of $GABA_A$ antagonist picrotoxin or agonist muscimol respectively were used to temporarily reduce prefrontal

GABAergic inhibition (causing local disinhibition) or to temporarily reduce mPFC neural activation (hypo-activation) at doses that were found to show effect in other PFC-dependent tasks such as the 5CSRT (See Table 2- Pezze *et al*. 2014). Our initial findings (experiment 1– chapter 3) led to four further studies. Experiments 2 and 3 (chapter 4) tested modifications of our training procedures on 1) cue-strategy learning and 2) spatial response-to-cue shifting performance. The results of these experiments led to the final two studies discussed in this thesis (chapters 5-6), which focussed on using the reverse sequence of acquiring the visual cue response and shifting to the spatial response, with and without prefrontal GABAergic manipulation prior to first rule acquisition. Finally, Bayesian analysis to infer which strategies (go cue/win-stay/shift or lose-shift/stay) rats were implementing during task sessions was conducted (chapter 7).

END OF CHAPTER 1

Chapter 2: General Methods & Materials

The experiments described in this thesis followed a standard protocol as described in this chapter, with any variations further detailed in the individual experiment chapters.

2.1 Subjects

Experimentally naïve adult male Lister-Hooded rats (Charles River Laboratories) were used for all experiments to allow for comparison with previous research into disruption of GABA transmission on cognitive function (Bast, Pezze & McGarrity, 2017; Pezze *et al*. 2014; McGarrity *et al*. 2016). Animals were aged 9 weeks (body weight ~320g) at surgery (12 weeks at start of pre-training) as maturation of GABAergic transmission to the PFC is suggested to be complete by postnatal days 65-85 (Caballero *et al*. 2015). Rats were housed in groups of four in two level 'Double Decker', individually ventilated cages (462 mm X 403 mm X 404 mm; Tecniplast, UK) under temperature- and humidity controlled conditions (21±1.5°C; 50±8%) and alternating 12 hour light/ 12 hour dark cycles (all procedures were conducted during the light-phase). Rats initially had ad libitum water and food access (Teklad Global 18% Protein Rodent Diet 2018C; Envigo) but received a restricted amount of food (~15-22g food per rat daily) one week before and during training and testing. This aimed to keep rats at 85-90% of their free-feeding projected bodyweight during behavioural testing (estimated according to a pre-established weight growth curve). Rats which received surgical cannula implantations were allowed at least 5 days to recover from surgery and

regain pre-surgical bodyweight prior to any food restriction. All rats were handled by the primary experimenters prior to the start of any procedures, during at minimum 7 days' acclimatisation period to the housing in the animal unit. All procedures were conducted in accordance with the requirements of the United Kingdom (UK) Animals (Scientific Procedures) Act 1986. Experiments 1, 4, and 5 combined intra-mPFC drug infusions with behavioural testing on the operant task. Experiments 2 and 3 were purely behavioural studies, testing the effect of changing different parameters in the testing procedure. A summary diagram of general testing procedures is shown in Figures 2.1.

2.2 Experiments 1, 4 and 5: Impact of prefrontal muscimol and picrotoxin on operant task of cognitive flexibility

Experiments 1, 4 and 5 were each run in two batches, each with N=8 per group. This totalled at maximum 48 rats per experiment (N=16 per group). The three groups were the saline control, picrotoxin (300ng) or muscimol (62.5ng) drug groups. These experiments were conducted to examine the effect of mPFC hypo-activation and disinhibition on strategy shifting ability as described below (See Fig2.1). A between-subjects design was used to avoid the confounding effect of repeated testing conducted in within-subjects designs which has been suggested to render aspects of the pre-frontal dependent cognitive flexibility independent of the PFC (Rich & Shapiro 2007). Previous studies used group sizes of n=8 to reveal the impact of prefrontal inactivation by a sodium channel blocker, which had a very large effect size (d>1) (Floresco *et al*., 2008). Our final group sizes of n=16 were chosen to give us a power of about

80% to detect effect sizes of d = 1. Rats were randomly assigned subject identification numbers and systematically allocated to one of the 3 prospective infusion groups in a counterbalanced manner across cages, across operant boxes and across both experiment batches, so that each group had equal numbers of rats tested in each of the eight operant boxes. After collecting the initial pre-infusion spatial response acquisition data, these group allocations were adjusted to match if required, to even out response to criterion (RTC) average scores of the initial strategy acquisition across the 3 groups, whilst retaining counterbalanced numbers of groups across cages. Apart from the first series of experiment 1, all experiments were run with other primary experimenters who administered infusions, ran behavioural testing or analysed data blinded to the drug group allocation.

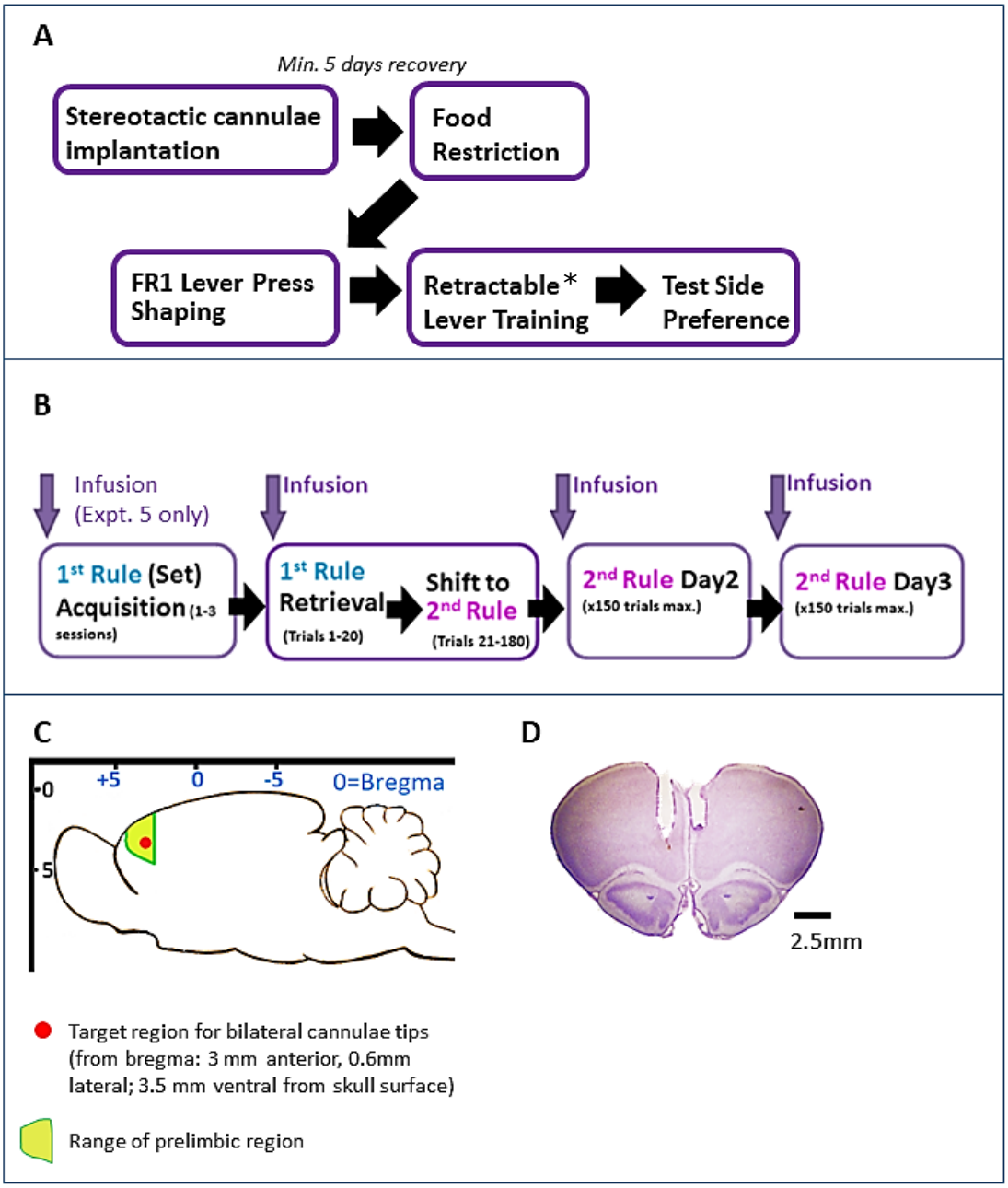


Figure 2.2 – **Protocol for experiments 1, 4 and 5**: A) Summary flow-diagram of pre-training procedures (* with cue pre-exposure) and B) testing sequence, with infusions administered 10 minutes prior to shift sessions; in experiment 5, an additional infusion carried out 10 minutes prior to 1st rule acquisition. C) Sagittal view of rat brain schematic with target site for cannulae located in pre-limbic mPFC, with coordinates based on Paxinos & Watson (1998). D) Cresyl-violet stained coronal

section of rat brain showing cannulae tracts with tips located in pre-limbic region.

2.2.1 Stereotactic implantation of guide cannulae into the medial prefrontal cortex

Implantation and infusion followed procedures detailed in Pezze *et al*, 2014. Rats were anesthetized with isoflurane delivered in oxygen (induction: 5%; maintenance; 1-3%) and were secured in a stereotactic frame. To minimize risk of pain, rats received perioperative analgesia (Rimadyl® (carprofen) 0.1 ml/200g; Zoetis) and EMLA cream 5% (lidocaine 2.5%, prilocaine 2.5%; AstraZeneca) was applied to the ear bars, and eye-lubricant (Lubrithal™, Dechra) applied to prevent open eyes drying out during surgery. With skull exposed, bregma was located. Bilateral infusion guide cannulae ("mouse" model C235GS-5-1.2; Plastic Ones, Bilaney, UK), consisted of a 5mm plastic pedestal that held 2 26 gauge metal tubes, 1.2mm apart and projecting 4.5mm from the pedestal. These were implanted through small holes drilled in the skull. The guide cannulae tips were aimed 0.5 mm above the injection site in the pre-limbic prefrontal cortex at the following coordinates: 3 mm anterior and 0.6mm lateral from bregma and 3.5 mm ventral from the skull surface (Pezze *et al*. 2014). Cannulae were secured to the skull with dental acrylic and stainless steel screws. Double stylets (33 gauge; Plastic Ones) were inserted into the guides (with no protrusion) and the guides were closed with a dust cap. After surgery, the rats were allowed at least 7 days of recovery before food restriction commenced. During the recovery period, rats were checked and habituated to the manual restraint necessary for the drug microinfusions, and injected daily with antibiotic

suspension (ceporex^{tm} (18% w/v Cefalexin; Schering-Plough Animal Health) in experiment 1, batch 1, or synulox^{tm} (14% Amoxicillin; Zoetis) in experiment 1, series 2 and all subsequent infusion experiments) prophylactically against possible meningitis. The choice of these different antibiotics was based on the availability of the antibiotic at the time of the experiment.

2.2.2 Micro-infusion procedure and drugs

Rats were gently restrained while 33 gauge injectors (Plastic Ones) were inserted into the guides. The injector tips extended 0.5mm below the guides into the mPFC while the injector ends were connected via polyethylene tubing to 5 µl syringes mounted on a micro-infusion pump. Bilateral infusions of 0.5 µl/side of either 0.9% sterile saline (control), or of solutions of picrotoxin (to cause disinhibition) or muscimol (causing functional inhibition) (Sigma-Aldrich; 300ng or 62.5ng in saline, respectively), were administered over 1 minute. Included in the tubing was an air-bubble, the movement of which was monitored to verify the successful infusion of solution into the brain. After the initial 1 minute, the injector remained in place for another 1 minute to allow tissue absorption of the infusion bolus. Then the injectors were removed and the stylets replaced. Testing began 10 minutes after the infusion.

2.3 Experiments 2-3: behaviour without mPFC manipulations

Experiments 2 and 3 did not involve surgical nor drug manipulations of the subjects, but followed the sequence of acclimatisation, food restriction, pre-training and testing. Following completion of behavioural testing, rats

were euthanized with rising concentration CO_2 and cervical dislocation to confirm death. Histology was not conducted due to the absence of intracerebral manipulations.

2.3.1 Operant task: cognitive flexibility testing

2.3.1.1 Apparatus

All training procedures were adopted from Brady & Floresco (2015). All training and testing was conducted in eight standard operant boxes (30.5 cm x 24.1 cm x 21.0 cm; Med-Associates, St. Albans, VT, USA), which were enclosed in sound-attenuating boxes. Extraneous noise was masked by a fan in each box. Operant boxes were equipped with two LED cue lights (4-7 lux), each located above one of two retractable response levers positioned on the left and right side of a receptacle into which the reward pellets (5TUL-45mg; Testdiet, UK) were dispensed, and a single 100-ma house-light located in the top centre of the wall opposite to the levers and cue lights. All experimental data was recorded by a Dell (OS: Windows XP) computer connected via an interface to the operant boxes.

2.3.2 Pre-Training:

On the day prior to initial exposure to the operant boxes, rats were given approx. 20 reward pellets in their home cage to reduce neohypophagia during training. Prior to placing the rat in the box for the first training session of each lever, a small pinch of crushed pellet powder was placed on the extended lever.

2.3.2.1 Fixed ratio-1 lever- press training

On the first day of pre-training, rats were trained to press one extended lever to obtain a reward pellet under a fixed-ratio 1 schedule, to a

criterion of 50 lever presses in 30 minutes (50% of rats trained first on left lever, 50% on right lever (counterbalanced between groups and housing cages)). Once rats achieved criterion on the first lever, they progressed to pressing the other lever on the following day. If rats failed to reach this criterion during lever-press training, they repeated the session the following day on the same lever. Rats required 2 to 3 sessions of training per lever.

2.3.2.2 Retractable lever-press training

Following completion of fixed lever-press training, rats were familiarised with insertion of the levers into the box, undergoing 90 trials per daily session of retractable lever training for 5 consecutive days. In each trial, the levers where initially retracted from the chamber interior, and the house-light switched off. After 20 seconds, the house-light was illuminated and one of the levers extended into the chamber. Rats had 10 seconds to press the lever and receive a reward pellet. A lever press, or failure to press the lever in time (omission), would cause the lever to retract and the house-light to switch off, ending the trial. In the fifth session of training, rats were expected to make fewer than 5 omissions. During each session of this stage of pre-training, rats were pre-exposed to illumination of both cue lights during each of the trials- totalling 450 trials over 5 sessions. There was an exception of one group in experiment 2 which were not pre-exposed to determine the effect on rats' ability to shift to the cue strategy. The rationale for pre-exposing the rats to the cue lights was to increase the difficulty of the later shift task, based on findings by Floresco, Block and Tse (2008) that cue light pre-exposure at this pre-training stage decreased the difficulty of spatial response strategy acquisition and increased the difficulty of spatial-response-to-cue shifting,

with suggestions that this was due to the cue lights becoming less salient with pre-exposure. No infusions were administered during the pre-training.

2.3.2.3 Side preference testing

Directly after the fifth session of retractable lever training, the rats' lever side – preference or bias was determined in a single session, in order to test rats on the opposite lever to their bias. In this procedure, both levers were extended into the chamber but the cue lights were not illuminated during trials. On the first trial, the rat could press either of the two extended levers to obtain a reward; on the following trial, the rat had to press the opposite lever to whichever lever he had pressed in the first trial, in order to be rewarded (pressing the same lever yielded no reward and extinguished the house-light, with trials continuing until the rat pressed the lever opposite to the initial lever). The rat had to complete 7 pairs of trials like this. Thus, these pairs of trials, in which two opposite levers were pressed, allowed for side preference to be determined by whichever lever the rats pressed most in the first trials.

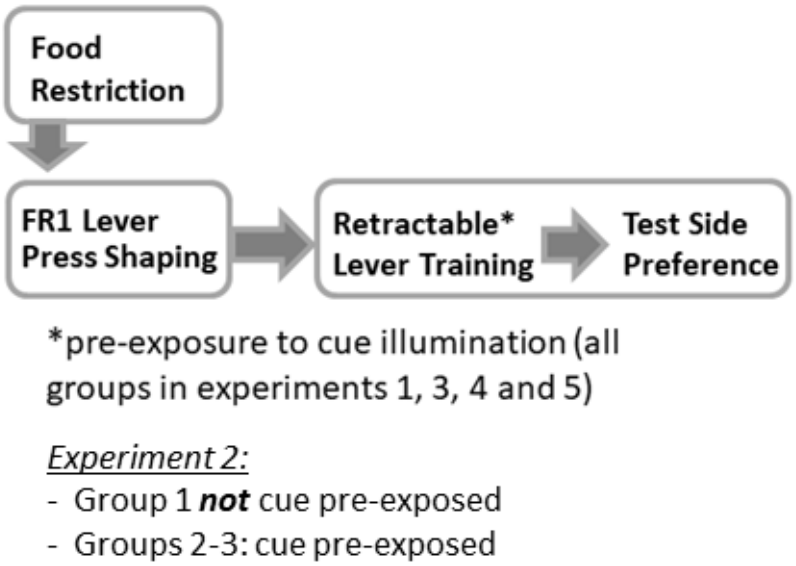


Figure 3.2 – pre-training protocol: asterisk indicates cue light pre-exposure during retractable lever training sessions. The exception of experiment 2, group 1 was not pre-exposed as the effect of cue light pre-exposure vs non-pre-exposure was tested

2.3.3 Testing Sequence:

2.3.3.1 Acquisition of initial response strategy

The initial response strategy was varied according to the study objectives. For experiments 1 and 2, the initial discrimination was the spatial response strategy, whereas experiments 3-5 started with the visual cue strategy.

A session began with both levers retracted and all lights switched off (the inertial state). Every 20 seconds, a trial began with one of the cue lights illuminating and 3 seconds later both levers extended into the chamber. The rat had 10 seconds to make a choice of lever to press. For the spatial response strategy, the rat was required to respond on the lever opposite to the rat's side bias, and in this case the location of the illuminated cue

light was irrelevant (see chapter 1, Fig1.2B). For the visual cue strategy, the rat was required to press whichever lever had the cue light above it illuminated, which varied across trials.

A response on the correct lever resulted in the retraction of both levers, extinguishing of the cue light and delivery of one reward pellet, with the house light remaining on for 4 seconds after reward delivery. An incorrect response resulted in the chamber returning to the inertial state. Failure to respond on either lever resulted in the chamber returning to its inertial state and recording of the trial as an omission. In every pair of trials, each of the two cue lights was illuminated once, and the order of left or right within the pairs of trials was random. For each trial, the rat's choice of lever was recorded and when ten consecutive correct choices (criterion of 10) were made, a "streak" was recorded. This criterion was chosen based on some previous work (Brady & Floresco 2015). In the infusion experiments, the trials totalled 150, continuing regardless of the rat reaching criterion, to enforce the initial strategy.

2.3.3.2 Shift from first strategy to second strategy

On the day following the initial response acquisition, rats were tested on their ability to cease using the first response strategy and shift to the second strategy to obtain the reward (Fig2.1B). Rats in the mPFC infusion study received their respective drug or saline infusions ten minutes prior to beginning the session. The first 20 trials repeated the response strategy of the initial acquisition to test the animal's retrieval of this task (retrieval trials). The subsequent 160 trials were shift trials, in which the second rule was relevant and they carried on performing shift-trials over 2 additional sessions (maximum 150 trials per session) during the subsequent 2 days, performing a

maximum of 460 shift trials over the 3 sessions. Each session of shift trials ended once the rat achieved criterion of 10.

2.3.4 Performance measures & analysis

As with previous research (Brady & Floresco, 2015), the number of trials which the rat required before making ten consecutive correct responses (not counting omissions) was the performance measure to compare the speed of strategy acquisition (responses to criterion (RTC)), calculated for the first strategy acquisition and the shift or reversal tasks. In addition, analysis of "%correct" ([correct responses/ (correct responses + incorrect responses)]* 100%), reflected errors of commission independent from errors of omission; percentage omissions ([omissions/ (correct responses incorrect responses + omissions)] * 100%). For first strategy-to-second strategy shift sessions, percentage perseveration was calculated ([perseverative errors/ (correct responses incorrect responses)] * 100%). A perseverative error was scored during the shift when a rat responded according to what would have been a correct response in the first strategy task but was incorrect in the second strategy shift. For instance, during cue shift trial, a perseverative error was counted as a response on the lever which did not have the cue light illuminated above it. Results of each experiment were analysed with one-way ANOVA using (drug or training) group as the between-subjects factor. Due to the experimental design of the shifting stages that involved 3 sessions of trials, 2-way ANOVA was used for percentage correct responding, omissions and perseverative errors over cue shift sessions, with drug group as the between-subjects

factor and cue session as the within-subjects factor. Main effects from the ANOVA were further investigated, using Fisher's LSD test.

Correlational analysis of relationships between performance measures, such as whether a relationship existed between %omissions and %correct responses, or bodyweight and %correct, were carried out using two-tailed Spearman's ρ.

2.4 Perfusions and Histology

Verification of cannula placements

After the completion of the experiments, rats were anesthetized with a lethal dose of sodium pentobarbitone (1–1.5 ml Euthatal; sodium pentobarbitone, 200 mg /ml; Genus Express) and transcardially perfused with 0.9% saline followed by 4% formaldehyde solution in saline. Brains were then extracted from the skull, and post-fixed in 4% formaldehyde, before being cut into 80μm coronal sections on a vibratome, targeting the area encompassing pre-frontal cortex. These sections were then mounted on slides and stained with cresyl violet. Placements of the injector were determined using a light microscope and mapping onto coronal sections of a rat brain stereotaxic atlas (Paxinos and Watson, 1998).

END OF CHAPTER 2

Chapter 3: Experiment 1 – Effect of prefrontal GABAergic manipulation on spatial-response-to visual cue shifting

Introduction

The aim of this first experiment was to test whether prefrontal functional inhibition or disinhibition would impair shifting performance in the automated strategy shifting task. The protocol described was adopted based on the rationale that a combination of cue light pre-exposure during pre-training with shifting from the spatial strategy to the cue based strategy would render the task difficult enough to reveal differences in performance between controls and drug groups (see Floresco, Block & Tse, 2008).

Methods

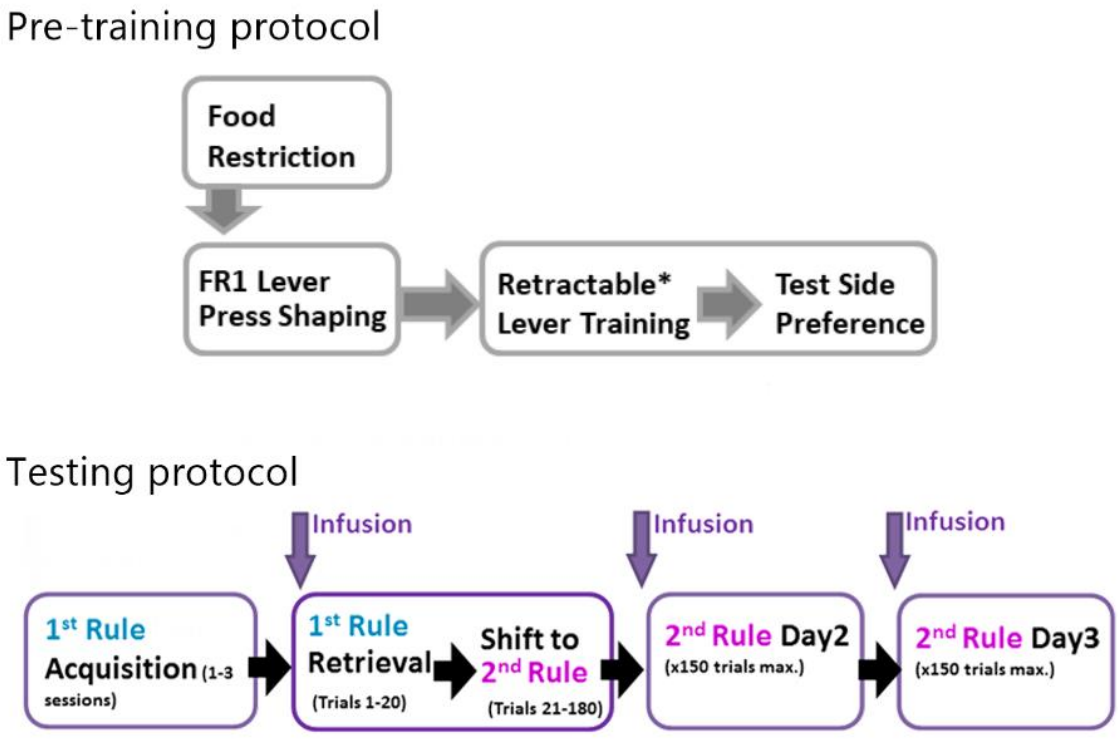


Figure 3.1 – Experiment 1 pre-training and testing procedure. Asterisk indicates cue light pre-exposure.

The general protocol for this experiment was as described in chapter 2, with the following specifications (Fig3.1):
Rats (N=16 per group) were food restricted to reach ~85% of their free-fed bodyweight. During pre-training, they were pre-exposed to illumination of both cue lights. Rats were initially trained on the spatial response as the first strategy, but were given 150 trials in these sessions even if they met the criterion of 10 in fewer trials. This was intended to train them thoroughly on the first task so that the subsequent shift would be challenging enough but achievable within comparable RTC (e.g. ~80 responses) to previous research (Brady & Floresco 2015).Rats were shifted to the visual cue strategy. The cue trials were delivered over 3 sessions (shift day 1 included 20 spatial trials (first strategy retrieval) followed immediately by 160 cue trials at maximum; days 2-3, included 150 trials at maximum). At this stage, a session ceased once the rat reached criterion, though the rat would still undergo all three sessions regardless of whether it reached criterion in an earlier session. The aim was to obtain a %correct measure across shift sessions, rather than only a response to criterion measure. Intra-cerebral microinfusions were administered 10 minutes prior to each of the 3 shift sessions, but not prior to first strategy acquisition. The experiment was run in two parts such that sample sizes aimed for N=16 per group in total.

Results

Spatial response first strategy acquisition

One control group rat had a blocked cannula and could not receive saline infusions in subsequent testing but was kept for further testing. Another control rat was excluded from analysis due to receiving incorrect response-acquisition procedures (in which it underwent 16 more trials than the other rats), resulting in final group sizes of control N=15, picrotoxin N =16, muscimol N=16 for the following tasks described. 5 rats were found to have cannulae tips in the border of the pre-limbic and cingulate cortex regions and 7 rats had tips bordering the pre-limbic-medial orbital cortex (Fig3.2). No animals were excluded on histological grounds.

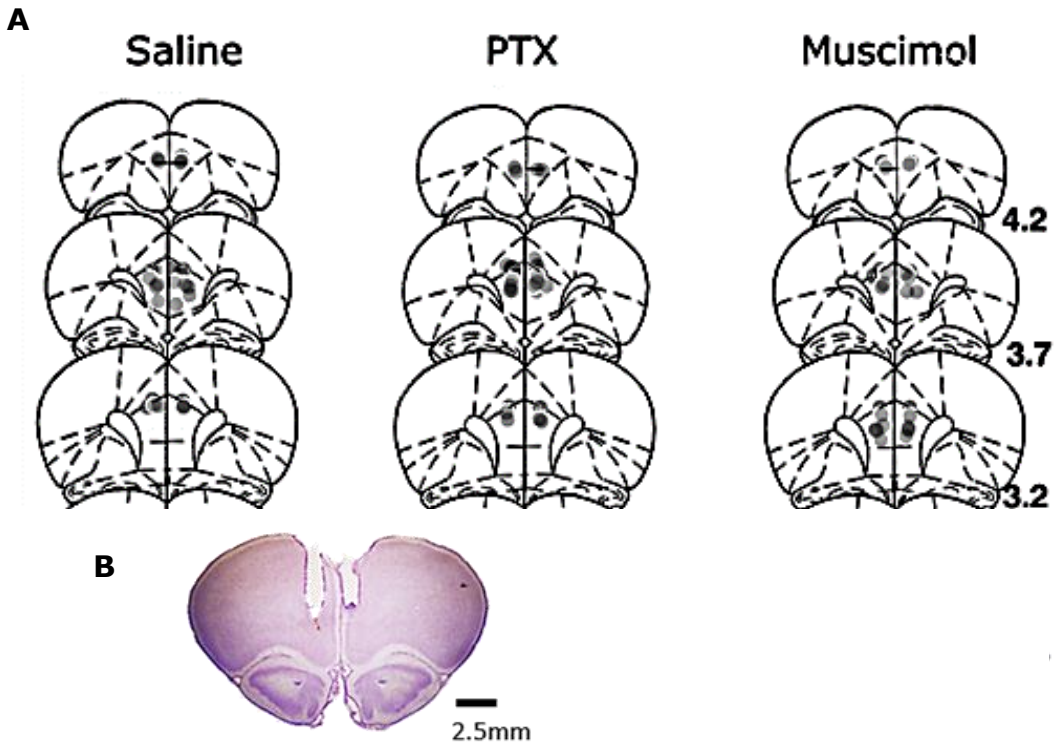


Figure 3.2 - Histology. **A)** Schematic showing coronal rat brain sections, showing the placements of the cannulae tips within pre-limbic mPFC for all rats in Experiment 1. Sections are adapted from the atlas by Paxinos and

Watson (1998), with numbers indicating the distance from bregma (mm). **B)** Cresyl-violet stained coronal section of rat brain showing cannulae tracts with tips located in pre-limbic region.

Rats managed to achieve criterion within an average number of responses (RTC) of 71.81 (muscimol group), 72.2 (saline group), and 78.31 (PTX group), though these numerical differences were statistically not significant (one-way ANOVA: $F(2, 44) = 0.1767$, $P=0.8386$ (Fig 3.1). Percentage of correct responses made were also not significantly different between groups (Ordinary one-way ANOVA: $F(2, 45) = 0.627$ $P=0.5388$), with average correct performance at 77.93% (muscimol), 75.79% (saline), and 77.44% (PTX).

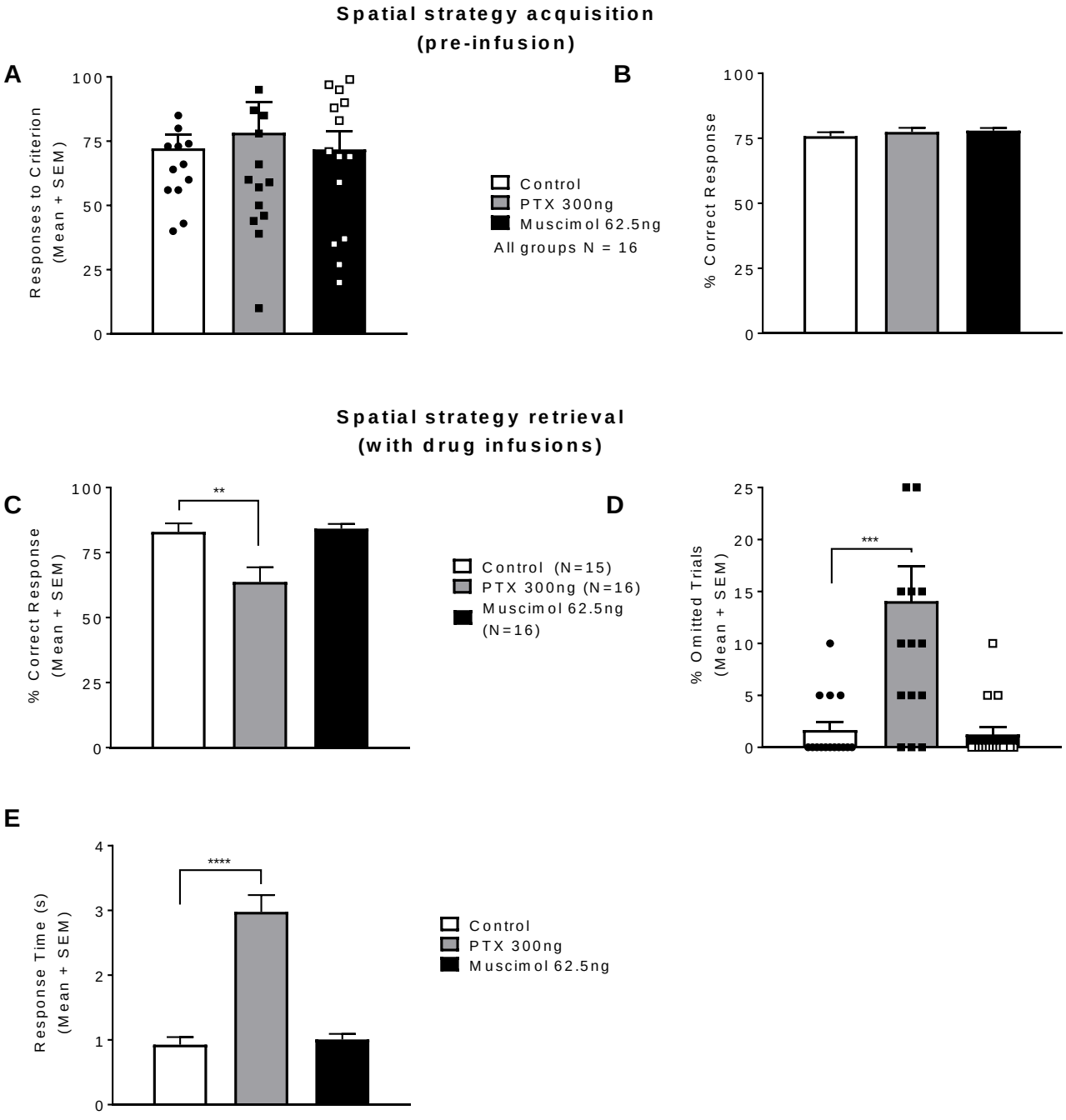


Figure 3.3- Experiment 1: performance of spatial strategy (Mean + SEM) Pre-infusion strategy acquisition, A – B) and post-infusion strategy retrieval; C) picrotoxin (300ng) impairs retrieval accuracy (% correct responses = [correct responses / (correct responses + incorrect responses)] * 100 %), and D) increases omission rate (omissions = [total trials – (correct responses + incorrect responses *)] 100 %), and E) PTX

increases response time. Asterisk indicates significance relative to control ** = P≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001.

Prefrontal disinhibition impairs spatial strategy retrieval

Prefrontal PTX significantly reduced percentage of correct responses compared to control and muscimol F (2, 44) = 8.781, P=0.0006; *post hoc* comparisons control vs PTX p= 0.0012, muscimol vs PTX p= 0.0005), performing correctly in 63.72% of responses versus 82.99% (saline) and 84.22% (muscimol). Additionally, PTX markedly increased omission rates to an average of 14.06% of trials, significantly higher than the other groups which had omission rates of 1.67% (saline) and 1.25% (muscimol)(one-way ANOVA: F (2, 44) = 12.24, P<0.0001; post hoc comparisons, control vs PTX p= 0.0001, muscimol vs PTX p= <0.0001). However, individual omission levels were considerably variable within the PTX group, with rates varying from 0% (N=3) to 45%. Correlation analysis (two-tailed Spearman r) between omission rates and %correct responding within the PTX group showed no significant correction; *rs*(14) = -0.46, p=0.074. Response latencies were also increased by PTX (F (2, 45) =45.69, P<0.0001, *post hoc* comparisons for both control vs PTX and muscimol vs PTX, p<0.0001), with PTX-infused rats responding at an average of 2.05 seconds longer than controls (Fig3.3D). Correlation analysis (two-tailed Spearman r) between omission rates and response latencies within the PTX group showed significant correction; *rs*(14) = -0.64, p=<0.05.

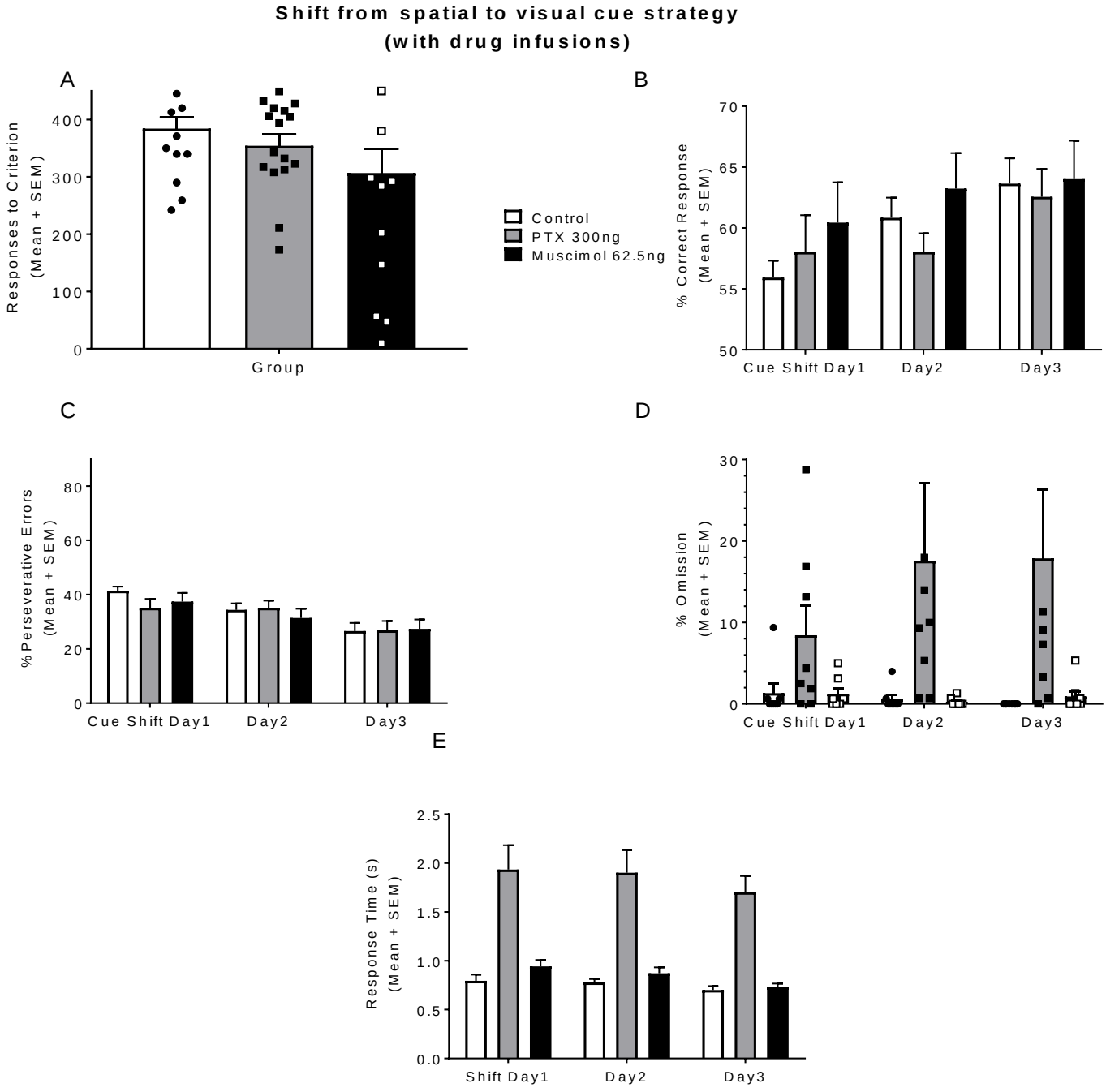
Shifting from spatial to visual cue strategy unaffected by prefrontal manipulations

No statistically significant difference in RTC was found between drug groups (one-way ANOVA: F (2, 44) = 1.752, P=0.1853). RTC values indicated that rats were slow to shift, with mean RTC ranging between 306.8 (muscimol) and 384.1 (saline), which corresponds to a minimum of 3 sessions before average criterion was reached (Fig 3.4A). Re-analysis of data when reducing the criterion from a streak of 10 to streaks of 8 or 6 (Floresco *et al*. 2008; Birrell & Brown 2000) did not reveal any significant differences either between groups, and all groups still required more than one session (~200 trials) to reach a criterion of 6 (data not shown).

Analysis was conducted to test if a relationship existed between bodyweight and performance: there was no significant correlation either between bodyweight and %correct responding during shift day 1; $r(45) = -0.04$, p=0.77, or between body weight and shift RTC; $r(45) = -0.16$, p=0.29. This indicates that motivation to perform the food-rewarded task was not significantly influenced by variation in bodyweight. Percentage of correct responses in all groups were non-significantly different between drug groups across shift sessions (two-way ANOVA, effect of drug: F (2, 44) = 0.6854, P=0.5092), all three groups showed a session effect of improved performance with the two subsequent shift days (F (2, 88) = 5.654, P=0.0049), and there was no drug x session interaction (F (4, 88) = 0.6429, P=0.6333) (Fig 3.4B).

Correlation analysis (two-tailed Spearman r) between omission rates and %correct responding showed no significant correction; shift days 1-3: $rs(46) = >-0.19$, $p>0.1$). The number of perseverative-type errors was also not significantly different between drug groups (two-way ANOVA, drug effect: F (2, 44) = 0.1887, P=0.8287). All three groups showed a

session effect of reduced perseveration with increased sessions (F (2, 90) = 18.24, P<0.0001) and no interaction (F (4, 90) = 0.7539, P=0.5580)



(Fig3.4C).

Figure 3.4 - Experiment 1: performance of shift to cue strategy with drug infusions (Mean+SEM). A)) When shifting from response to cue strategy, there is no significant drug effect on responses to criterion (RTC) measure

B-C) Significant session effects but no drug effects were found on increasing accuracy (% correct responses = [correct responses / (correct responses + incorrect responses)] * 100 %) and decreasing perseverative errors across successive cue trial sessions. D-E) PTX increases omission rate (omissions = [total trials – (correct responses + incorrect responses *)] 100 %) and response times.

As with first strategy retrieval, PTX-induced increases in response times (two-way ANOVA, effect of drug: $F(2, 135) = 61.74$, $P<0.0001$; *post hoc* comparisons of both control vs PTX and muscimol vs PTX: $p <0.0001$), with no drug vs session interaction ($F(4, 135) = 0.09517$, $P=0.9838$), nor session effect ($F(2, 135) = 1.497$, $P=0.2274$). PTX-induced increased omissions ($F(2, 21) = 4.368$, $P=0.0259$: *post hoc* comparisons for both control vs PTX and muscimol vs PTX, $p<0.05$) with no drug vs session interaction ($F(4, 42) = 1.571$, $P=0.1997$)) or session effect ($F(2, 42) = 0.8727$, $P=0.4253$) held true for the shift task (Fig 3.4D-E).

Discussion

The aim of this experiment was to investigate the effect of prefrontal disinhibition and functional inhibition on behavioural flexibility as assessed in the automated strategy shifting task. The main finding was that neither prefrontal PTX nor prefrontal muscimol affected strategy shifting, as would have been reflected by differing numbers of responses required to reach criterion or changes in %correct responding relative to controls. A possible reason for this lack of observable effect is the remarkably slow shifting shown by these rats. None of the infusion groups (including controls) reached criterion within the standard first session of 160 cue trials

reported in the current literature; all infusion groups required up to three sessions of cue trials, after which point training on this stage was ceased. Acute prefrontal disinhibition by $GABA_A$ antagonist picrotoxin did produce three significant effects on general performance; reduced accuracy in spatial response retrieval, as well as increased errors of omission and increase in response times in both the first strategy-retrieval- and cue-shift- tasks of the response-to-cue shift. One suggestion for this contrast with reported studies is that the strain of rats used here (Lister Hooded) may have an impact compared with, for example, the use of Long-Evans or Sprague Dawley in the current literature (Placek *et al*. 2013; Floresco *et al*. 2008). This suggestion was made given that our experiments adopted the training protocols recommended by the authors (Brady & Floresco 2015). One point to be considered is that by impairing strategy retrieval, PTX infusions may have

From these findings, the next step was to clarify whether the disproportionately high shift RTC made by these Lister hooded rats was due to the training parameters described (cue light pre-exposure or forced completion of 150 spatial trials) having rendered the cue shift task too difficult for this strain, or whether these rats struggled specifically to perform the shift sequence of spatial-to- cue strategy. The following chapters will describe behavioural experiments which investigated the effects of protocol modifications; modulating training parameters and task sequences to test their effect on rat's ability to perform shifts.

END OF CHAPTER 3

Chapter 4: Experiments 2 and 3; did training parameters affect shifting performance?

Introduction

The findings of experiment 1 led to the design of behavioural experiments 2 and 3 (without invasive manipulations) to examine if changes to the training protocol would result in more rapid shifting from one response rule to another. Experiment 2 investigated the effects of a) removing cue light pre-exposure during pre-training and of b) reducing the number of spatial response trials rats received by terminating training once a rat had reached criterion. Additionally, spatial reversals were performed, in order to compare performance with strategy shift performance and examine if this provided evidence for a specific shift cost. Experiment 3 investigated how a separate batch of rats performed when acquiring the cue based strategy as the initial discrimination, shifting to the spatial response, and finally shifting back to the cue strategy.

Experiment 2: testing effect of variation in training parameters on spatial response-to-visual cue shift

The general protocol for experiment 2 was as described in chapter 2, with the following specifications:

this experiment used 3 groups of rats without cannula implants (all n=8), which differed with respect to the training parameters used (Fig4.1):

group 1 was not pre-exposed to illumination of the cue lights during the retractable lever phase of pre-training. Because pre-exposure may reduce

the salience of the cue lights (see Floresco *et al*., 2008), it was thought that removal of the pre-exposure may facilitate learning about cue light and, thereby, reduce the number of trials required to shift from the spatial to the cue strategy. As in experiment 1, chapter 3, this group received 150 trials per spatial acquisition session regardless of whether they met criterion in fewer trials. Group 2 was pre-exposed to cue lights as in experiment 1, but the spatial response acquisition sessions were terminated once rats had reached criterion and training progressed to the next stage the following day. Group 3 (the controls) was pre-exposed to the cue light during pre-training and also received 150 trials during spatial acquisition session, regardless of when they reached criterion, as in experiment 1.

Rats in this study were food restricted to reach 90% of their free fed bodyweights.Following testing on the first spatial strategy-to-cue strategy shift, rats were tested further to examine: 1) performance of the reverse cue strategy-to-spatial strategy shift, involving cue-retraining sessions to ensure acquisition; 2) performance of 3 successive spatial response reversals; 3) performance of a second (final) spatial response-to-cue response shift. This would allow us to compare the difficulty of these different response changes, potentially providing evidence concerning differential 'costs' of ED response shift compared to ID response reversals (Floresco *et al*. 2008; McAlonan & Brown 2003). Thus, rats received the following sequence of tasks after pre-training:

1. initial spatial response-strategy acquisition
2. spatial retrieval and spatial-to-visual cue strategy shift
3. cue strategy acquisition

4. cue-to-response reverse shift
5. three reversals of spatial response
6. second cue shift

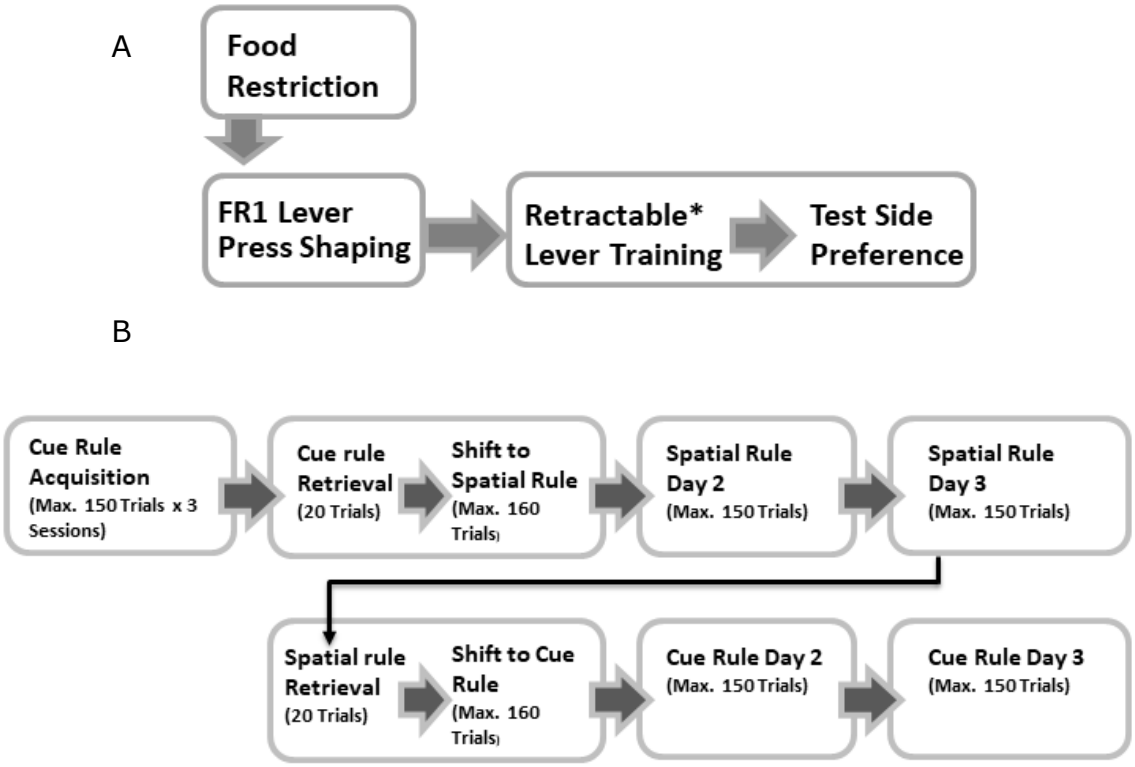


Figure 4.1 – Flow chart of training protocol for experiment 2 and 3: A) Pre-training with * indicating absence of cue light pre-exposure for experiment 2 group 2 during retractable lever training. B) Experiment 3: sequence of testing sessions.

Experiment 3 – Cue-strategy acquisition followed by shift to spatial strategy

Experiment 3 examined whether the poor performance of the cue strategy was due a difficulty to shift away from the initial spatial response, or due to an inherent difficulty in acquiring the cue strategy at all. Thus the general protocol for this experiment was as described in chapter 2, with the following specifications: 16 rats (N=16 to give a power of about 80% to detect effect sizes of d = 1) were food restricted to maintain 90% of free-fed bodyweight. During pre-training, rats were pre-exposed to illumination of both cue lights. Rats were initially trained on the cue strategy acquisition task (given at maximum 3 sessions of 150 trials each). Upon reaching criterion cue trials ceased and rats were tested the following day on their ability to shift to the spatial response strategy. If a rat underwent 3 cue acquisition sessions without meeting criterion, it was excluded from further testing. The cue-to-response shift included 20 cue retrieval trials preceding 160 spatial response trials. Once they had reached criterion on the spatial response shift, rats were tested on their ability to shift back from spatial response strategy to cue response strategy. As with our preceding studies, the final cue shift was delivered in 3 sessions (shift day 1 included 20 spatial strategy retrieval trials immediately preceding at maximum 160 cue shift trials; days 2-3, included at maximum 150 cue trials).

Results

Experiment 2: Removing pre-exposure and reducing spatial response acquisition trials do not facilitate spatial response- to- cue-strategy shifting

As in experiment 1, all groups acquired the spatial response readily, but again were slow to shift performance to the cue response (Fig. 4.2.).

During initial response acquisition, all groups reached criterion in ~73±5 responses (RTC), with no significant difference between groups ($F(2, 21) = 0.462$, $P=0.6363$), however a significant reduction in %correct responding was found for group 1 ($F(2, 21) = 8.097$, $P=0.0025$; *post hoc* tests group 1 vs 2 and 1 vs 3, $p < 0.05$; see Figure 4.2 A and B respectively). However, the groups showed no difference in % correct responding during retrieval trials ($F(2, 21) = 0.4267$, $P=0.6582$; Figure 4.2C). At the shift stage, there was also no significant difference between RTC scores for cue strategy acquisition between groups ($F(2, 21) = 0.4854$, $P=0.6222$; Figure 4.2D). As with the infusion studies, these rats were not able to shift from spatial response to cue strategy in one session, and were put through two additional cue trial sessions. A session effect ($F(2, 42) = 24.15$, $P<0.0001$) for increased %correct responding was found, however no significant group effect was found ($F(2, 21) = 1.798$, $P=0.1903$), nor group X session interaction ($F(4, 42) = 0.5914$, $P=0.6707$) (Figure 4.2E). Perseveration was not significantly different between groups, with all groups similarly reducing perseverative errors across sessions ($F(2, 42) = 10.26$, $P=0.0002$) and no significant group x session interaction ($F(4, 42) = 0.04654$, $P=0.9958$) (Figure 4.2F). Subsequent cue strategy retraining performance revealed a significant difference between groups (overall 1-way ANOVA; $F(2, 21) = 7.59$, $P=0.0033$); group 1 had significantly higher RTCs (average 281 trials) than both group 2 (average 52 trials; group 1 vs 2, $P=0.0010$), and group 3 (average 126 trials; group 1 vs 3, $P=0.0177$) (Figure 4.3A). Shifting from cue-to-response strategy was achieved in <75 trials, with no significant difference between groups ($F(2, 21) = 0.2704$, $P=0.7657$).

There was a significant increase in the RTC scores (~120 ± 5 RTC) for the subsequent first response reversal (response shift vs reversal 1; $F(1, 21) = 15.37$, $P=0.0008$), but again no significant difference between groups ($F(2, 21) = 0.2005$, $P=0.8199$) (Figure 4.3B). All three spatial reversals revealed a significant decrease of RTCs from first to third reversal (2-way ANOVA; session effect of $F(2, 42) = 11.22$, $P=0.0001$), but no effect of group ($F(2, 21) = 0.9462$, $P=0.4042$) or group X session interaction ($F(4, 42) = 0.2914$, $P=0.8819$) (Figure 4.3B). Compared to the third response reversal, the final response-to-cue shift shows a significantly increased RTC for all three groups ($F(1, 20) = 77.36$ $P<0.0001$), with no significant difference between groups ($F(2, 20) = 1.238$, $P=0.3113$).

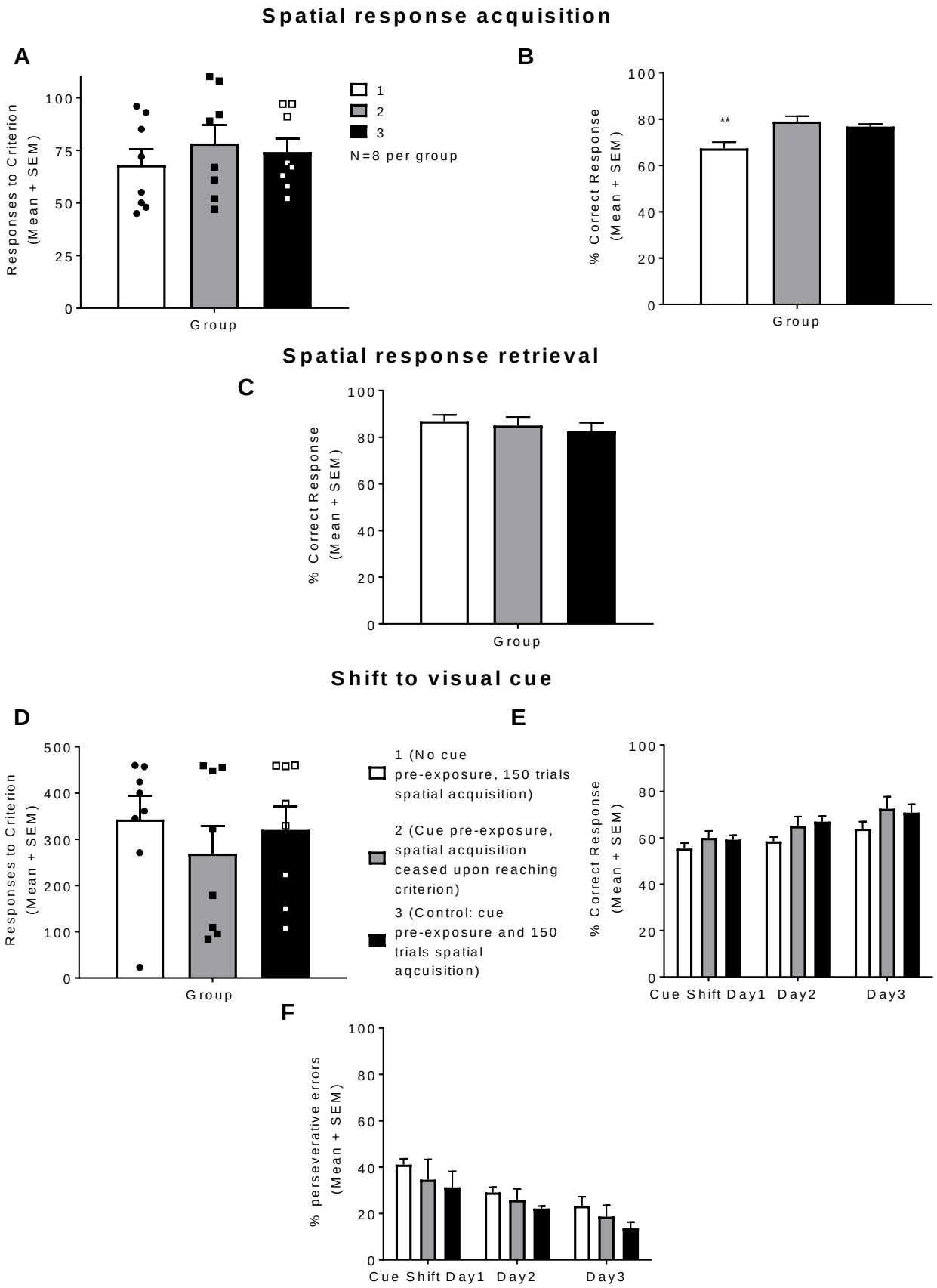


Figure 4.2 - Experiment 2: performance of spatial strategy (Mean + SEM) A) no group difference in acquisition RTC; B) ceasing spatial acquisition

trials upon meeting criterion (group 1) decreased acquisition %correct responding (% correct responses = [correct responses / (correct responses + incorrect responses)] * 100 %), asterisk indicates significance relative to control ($P \leq 0.01$); C) no group differences in spatial retrieval accuracy; D) shift to visual cue strategy with no group differences in either RTC or E) %correct responding, with significant effects of session (shift day) on increasing %correct responding and on F) decreasing perseveration. No group differences for perseveration.

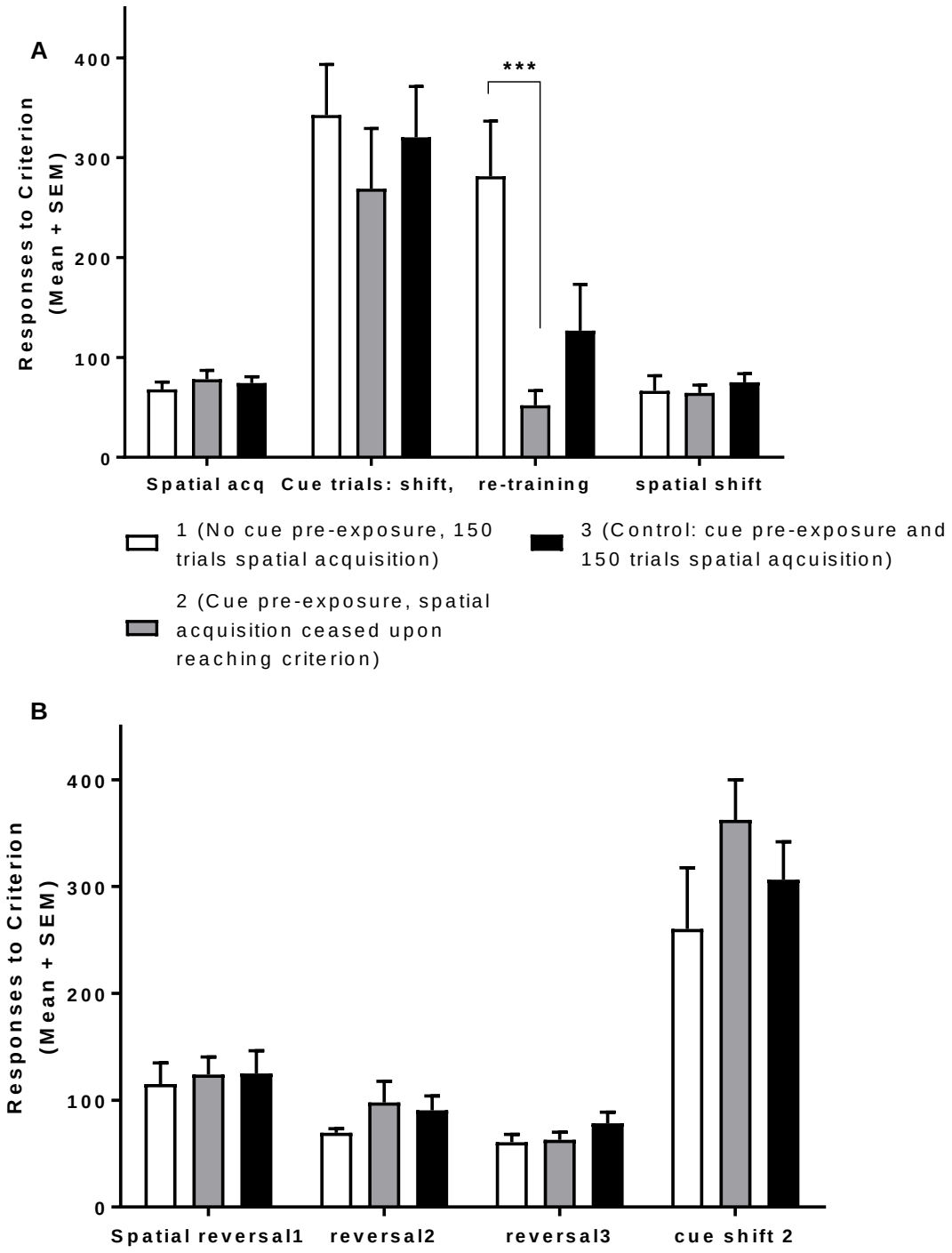


Figure 4.3 – RTC (Mean+SEM) for all task stages in chronological order left to right; A) removing cue pre-exposure (group 2) or ceasing spatial acquisition trials upon meeting criterion (group 1) had no significant effects compared to control group 3 (cue pre-exposed with enforced spatial training of 150 trials) on task performance on shifting to cue strategy, but post shift cue re-training showed increased RTC scores for

group 1 ($P \leq 0.001$). B) First spatial reversal showed significant increase in RTC versus preceding spatial shift (see Figure A) but no group differences. The second shift from spatial (reversal 3) to cue strategy proved as difficult as the initial (naïve) cue shift in Figure A, with no group differences.

Experiment 3: Lister Hooded Rats can acquire cue strategy pre-shift, but struggle to shift from spatial to cue strategy.

16 rats were initially trained on the cue-strategy acquisition task. One of these 4 rats failed to reach criterion within 3 sessions, and was therefore excluded from further testing, while the 3 rats who achieved criterion within 450 trials were excluded due to human error resulting to exposure to incorrect subsequent testing procedures.

The remaining 12 rats which acquired the cue strategy underwent the cue-to-response shift task, and achieving criterion in ~80 trials on average, but then struggled again to shift back from the spatial response to the cue response, requiring on average nearly 300 trials to reach criterion (Fig. 4.4C). RTCs during the shift back from the spatial to the original cue response were substantially increased as compared to the RTCs during the acquisition of the cue response and during the shift from the cue to the spatial response (both $p<0.0022$), which did not differ from each other ($p=0.57$) (Fig 4.4C).

Percentage of correct responses (out of total responses made) was calculated for each task showing significant differences overall ($F(3, 48) = 7.356$, $P=0.0004$), specifically a significant decrease in the shift tasks vs

cue acquisition; (spatial shift vs cue acquisition (p= 0.0475); cue acquisition vs. cue shift (p= 0.0035). No significant difference was found in %correct responding between initial cue acquisition and cue retrieval (p= 0.2248). Omissions for each task were <2% of total trials (Figure 4.4D). For individual cue-shift sessions (not depicted), there was an increase in %correct responding across sessions (F (1.579, 17.37) = 6.308, P=0.0125), specifically between cue shift Day1 vs Day2 (p=0.0086), and Day1 vs Day3 (p=0.0092).

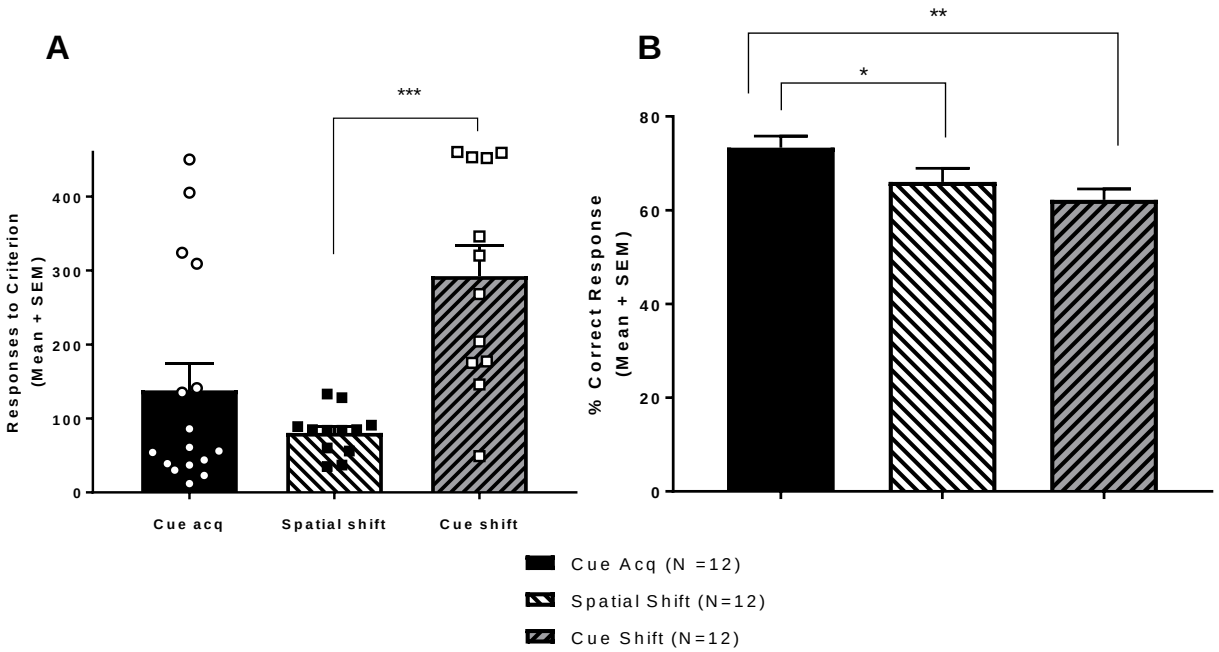


Figure 4.4 - Experiment 3: cue to spatial and back to cue shifts. A) RTC measures and B) percentage of correct responses, show rats can acquire and retrieve cue rule as an initial strategy, and shift to spatial strategy but struggle to shift back to the cue strategy once trained on the spatial strategy. Asterisk indicates significant difference: * = P≤0.05; ** = P≤0.01; *** = P≤0.001.

Discussion

Both experiments 2 and 3 confirmed that a large shift cost was specifically involved for the spatial-to cue strategy shift

These two behavioural experiments confirmed that a very large shift cost was always involved for specifically the spatial-to-cue strategy shift. Moreover, they showed that modifying training procedures to increase cue light saliency (removal of pre-training cue-light exposure) or prevent engraining of the spatial strategy (reduction of trainings trials for the spatial response) did not facilitate shifting to the cue-based response, and did not affect response reversal performance. Reversal performance was not affected by modifying training parameters, and the first reversal showed a small cost (~120 RTC) similar to previous studies, which became smaller with successive reversals (Floresco *et al*. 2008). Interestingly, it was found that rats could acquire an initial cue strategy and then shift to a spatial response strategy, requiring a similar number of trials to reach criterion as in previous experiments, but showed the same difficulty in reaching criterion for the subsequent spatial-to-cue shift as in the infusion studies.

Removal of cue pre-exposure and reduced spatial acquisition trials do not facilitate spatial to cue response shift

When findings from experiment 1 revealed that all infusion groups including controls performed significantly worse when having to suppress the spatial response strategy and acquire a new, visual cue-based strategy, it was assumed that these infusion results reflected that training parameters designed to make the cue shift difficult enough to involve prefrontal executive control had in fact made the task too difficult; by

either eliminating saliency of the cue lights with pre-exposure during pre-training, or by engraining the spatial strategy during the initial strategy acquisition. However, in experiment 2, removing visual cue pre-exposure during pre-training and limiting spatial response training to meeting criterion did not significantly improve cue-shift performance to allow shifting, and rats still required two additional cue sessions to reach criterion, as with experiment 1. This contrasts previously published work which found that pre-exposure was a significant factor that increased responses to criterion for shifting to the visual cue, and also reduced the RTCs for spatial response acquisition, suggesting that task-irrelevant cue light illumination during pre-training might, via latent inhibition, reduce attention to the cue light during the two testing stages (Floresco *et al*. 2008). However, once our rats had been trained to acquire the cue strategy (requiring three additional cue discriminations after the first cue-shift), these rats were able to shift back to the initially learned spatial strategy at similar levels to control rats of other strains which performed spatial-to-cue shifts in previous studies (Floresco *et al*. 2008; Placek *et al*. 2013). Interestingly, during the three additional cue discriminations, the only significant group differences were found for RTC scores, with the non-pre-exposed group acquiring the cue task within the least number of trials, and the group trained on fewer spatial response trials requiring the most cue trials. If these cue acquisition sessions were to be treated as extensions of the preceding cue-shift sessions, one might argue that pre-exposure may result in improved performance relative to the other groups of this experiment, however cue strategy shifting was still excessively slow

(requiring three-times the number of trials) compared to the standard performance seen in other research.

Lister Hooded Rats can acquire cue strategy pre-shift, but struggle to shift from spatial to cue

The next hypothesis tested was that our Lister-Hooded rats struggled with the cue-shift because this particular strategy was intrinsically difficult to acquire as a strategy (meaning that initial acquisition of the cue strategy, prior to any exposure to the spatial response strategy, would be more difficult than spatial response strategy acquisition). Interestingly, the majority of these rats did manage to acquire the cue-based strategy within one session of 150 trials (apart from 25% of the group which required additional cue acquisition sessions), and for this majority, shifting to the spatial strategy occurred at similar performance levels as seen for spatial acquisition and shifting in our previous experiments. Additionally, it is to be noted that there was no significant difference between RTC scores for the rats (N=12) that performed both cue strategy acquisition and spatial shift tasks, but when performing the subsequent reverse shift from the spatial-to-cue task, the same exceedingly large shift cost (>160% increase in trials) was observed as in our previous experiments. These findings seem to indicate that in these Lister Hooded rats the spatial response seems to be more prepotent and difficult to overcome than the cue strategy, contrasting performance by other strains (Long-Evans or Sprague Dawley) in the current literature (Placek *et al*. 2013; Floresco *et al*. 2008), given that our experiments adopted the training protocols recommended by the authors (Brady & Floresco 2015).

With these findings, it was decided that the spatial-to-cue shift sequence may produce a floor effect and not be suitable for divulging effects of prefrontal infusions. The following chapters describe experiments that investigated whether cannulated rats could repeat the performances of subjects in experiment 3 and show effects of mPFC disinhibition and hypo-activation on shifting from cue to spatial strategies. Previous work by Floresco has found that mPFC inactivation in Long-Evans rats resulted in impairments of this shift-sequence, therefore one would expect to see an increased shift cost with PFC muscimol infusions.

END OF CHAPTER 4

Chapter 5: Experiment 4 – Effect of prefrontal GABAergic manipulation on cue-to-spatial response shifting

Introduction

Lister Hooded rats in experiment 3 were in fact able to acquire the visual cue strategy if training was *not* preceded by the spatial response task, and shifted reliably within one session to the spatial response. The experiment described in this chapter aimed to test if picrotoxin-induced disinhibition or muscimol-induced inhibition of the mPFC would affect this shift sequence.

Methods

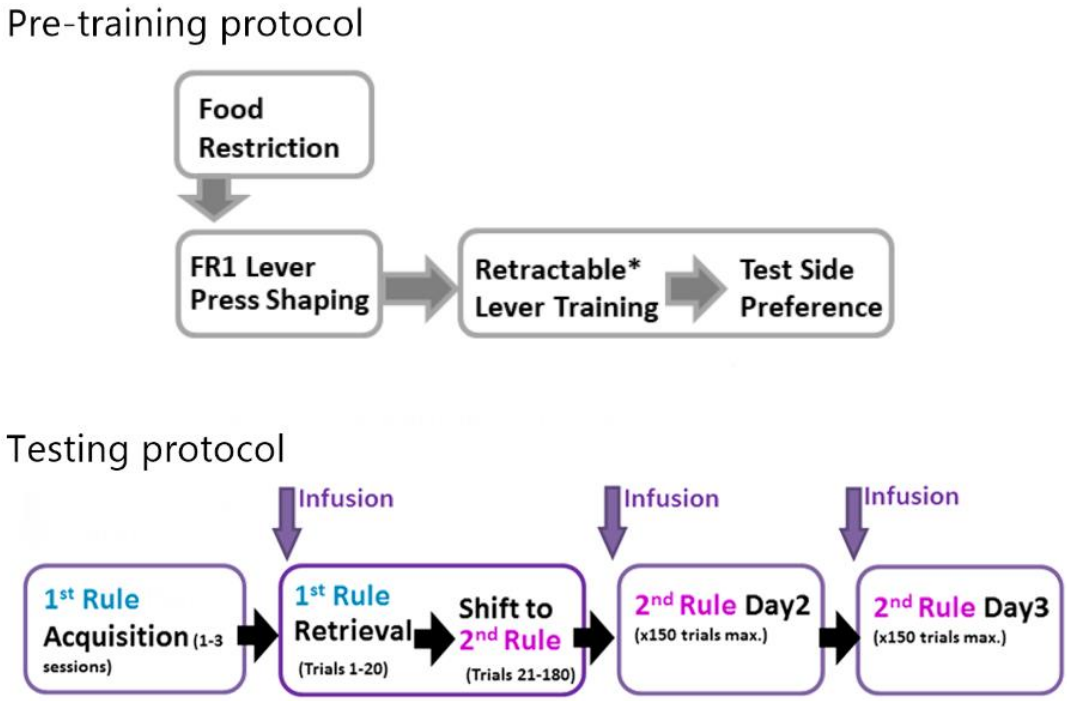


Figure 5.1- Experiment 4 pre-training and testing procedures. Asterisk indicates cue light pre-exposure

The general protocol for this experiment was as described in chapter 2, with the following specifications (Fig 5.1): the experiment was run in two batches, with each batch including n=8 rats per group and overall target group size of n=16 across the two batches. Rats were food restricted to maintain 90% of free-fed bodyweight. During pre-training, they were pre-exposed to illumination of both cue lights during retractable lever training (5 sessions of 90 pre-exposed trials per session). Rats were initially trained on the visual cue as the initial strategy and, if they met the criterion of 10, were progressed to the cue retrieval-shift to spatial response the following day. If a rat failed to reach criterion, it repeated the strategy acquisition session the following day, and was given a maximum of 3 sessions to learn the cue strategy. If a rat underwent 3 sessions without meeting criterion, it was excluded from further testing. Intra-cerebral microinfusions were administered 10 minutes prior to each of the 3 shift sessions. The cue-to-response shift included 20 cue retrieval trials preceding 160 spatial response trials. The spatial response (shift) trials were delivered over 3 sessions (shift day 1 included 160 trials at maximum; days 2-3, included 150 trials at maximum). At this stage, a session ceased once the rat reached criterion, though the rat would still undergo the second and third session regardless of whether it reached criterion in an earlier session. The experiment was run in two parts such that group sizes aimed for N=16 in total.

Results

Visual cue strategy acquired as in behavioural experiment

Similar patterns of cue acquisition performance were found in this study as with experiment 3. Seven rats (3 in batch 1, 3 in batch 2) failed to reach criterion within 3 sessions and were excluded from further testing and analysis. One rat from batch 2 was culled prior to training due to illness, and one rat was excluded due to erroneously receiving the wrong training procedure during cue acquisition training, resulting in final group sizes of control N=12, picrotoxin N =12, muscimol N=15 for the following tasks described. All rats included in the final groups had their cannulae placed within the mPFC, mainly in the pre-limbic region; 2 rats were found to have cannulae tips in the border of the pre-limbic and cingulate cortex regions, while 6 rats had tips bordering the pre-limbic-medial orbital cortex (Fig5.2). No animals were excluded on histological grounds.

Rats which achieved criterion managed to do so within an average number of responses (RTC) of 140 (muscimol group), 125 (control group), and 155 (PTX group), though these numerical differences were not statistically significant (one-way ANOVA: $F(2, 37) = 0.1821$, $P=0.8343$ (Fig 5.3A)). Percentage of correct responses made were very similar between groups (one-way ANOVA: $F(2, 35) = 0.1429$, $P=0.8674$), with average correct performance at 72.96% (muscimol), 71.78% (control), and 71.28% (PTX) (Fig 5.3B)), and response time was also not significantly different between groups ($F(2, 39) = 1.249$, $P=0.2979$) (Fig 5.3C).

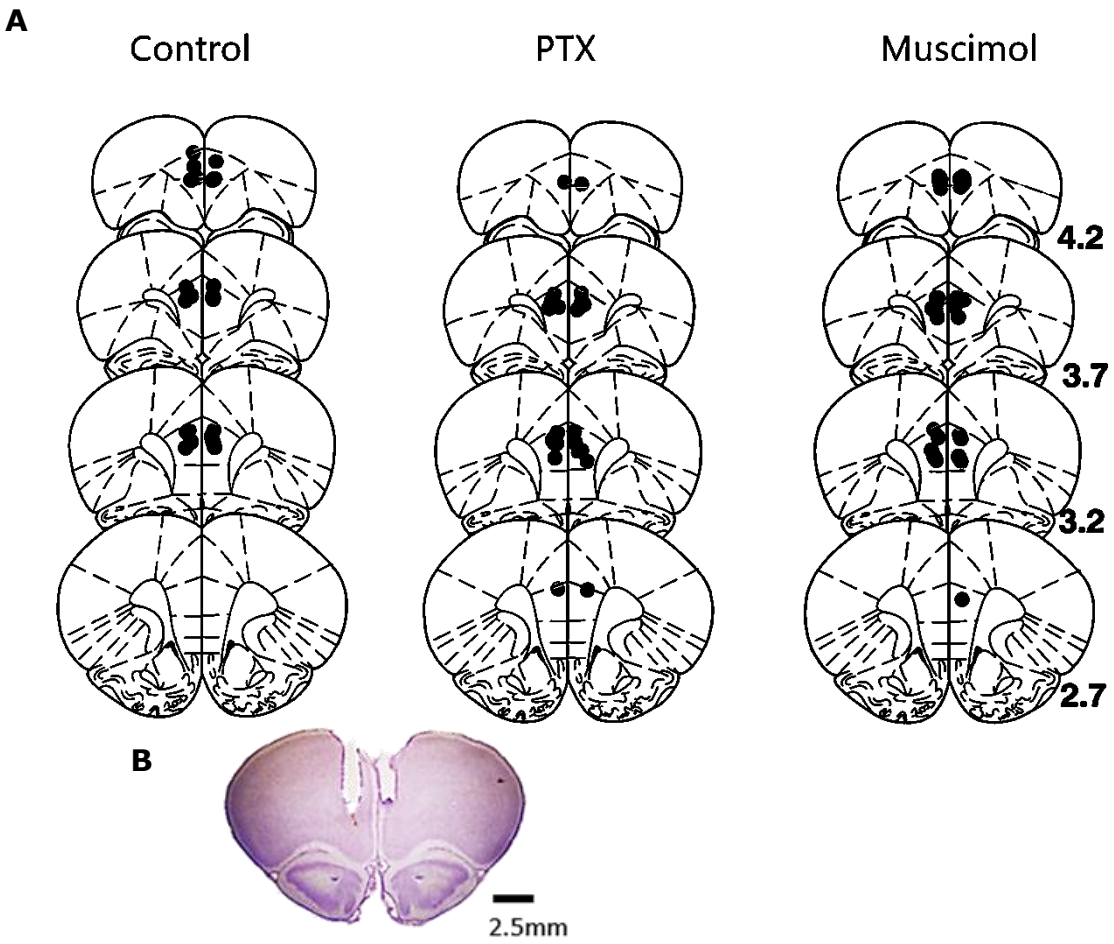


Figure 5.2 - Histology. **A)** Schematic showing coronal rat brain sections, numbers indicating distance from the bregma (mm), showing the placements of the cannulae tips within pre-limbic mPFC for all rats in Experiment 4. Sections correspond with Paxinos G, Watson C, 1998. **B)** Cresyl-violet stained coronal section of rat brain showing cannulae tracts with tips located in pre-limbic region.

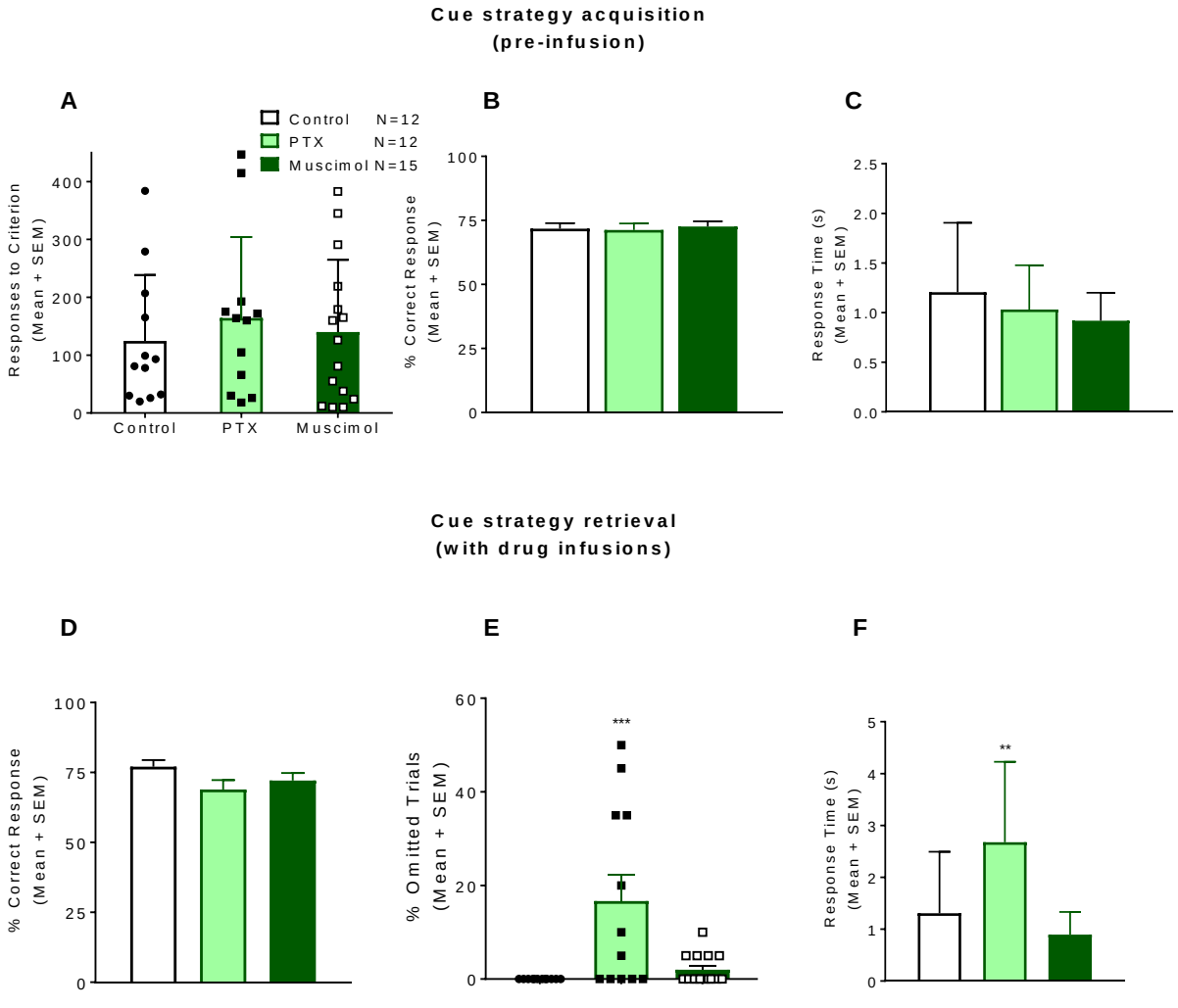


Figure 5.3- Experiment 4: Pre-infusion performance of cue strategy (A – C) and post-infusion expression of cue response (retrieval). A-C) Acquisition showed no significant differences between groups (Mean + SEM); D) Post-infusion strategy retrieval; no significant difference in retrieval accuracy between drug groups (% correct responses = [correct responses / (correct responses + incorrect responses)] * 100 %), E) PTX 300ng increases omission rate (omissions = [total trials – (correct responses + incorrect responses *)] 100 %). F) PTX increases response time. Asterisk indicates significant difference from control, (** = $P \leq 0.01$; *** = $P \leq 0.001$)

Impact of prefrontal disinhibition on cue strategy retrieval

ANOVA did not indicate a significant main effect of infusion group on %correct responses during the 20 trials of cue strategy retrieval ($F(2, 36) = 1.961$, $P=0.1555$); however, based on the significant impairment in the retrieval of the original spatial strategy by prefrontal PTX in experiment 1, we performed planned comparisons between PTX- and saline-infused rats, which indicated a strong trend for a PTX-induced impairment ($p=0.0574$). PTX-infused rats performed correctly in 68.9% of responses versus 77.08% (control), while muscimol-infused rats performed 72.11% responses correctly (Fig5.3D).

PTX markedly increased omission rates to an average of 16.67% of trials (one-way ANOVA: $F(2, 36) = 8.513$, $P=0.0009$), significantly higher than the other two groups (*post hoc* tests for both = $p \leq 0.0001$). Individual omission levels were considerably variable within the PTX group (Fig5.3E). There was no significant correlation between %omissions vs %correct ($r_s(37)= -0.12$, $p=0.46$).

Response latencies were also increased by PTX ($F(2, 37) = 9.037$, $P=0.0006$), with PTX-infused rats responding more slowly than the other groups (*post hoc* tests for control vs PTX, $p=0.0040$ and muscimol vs PTX, $p= 0.0002$) (Fig5.3F).

PTX disrupted shifting to spatial response strategy

No statistically significant difference in RTC was found between drug groups (one-way ANOVA: $F(2, 36) = 0.9108$ $P=0.4113$), with mean RTC ranging from 73.67(muscimol) to 94.83 responses (control) (Fig5.4A).

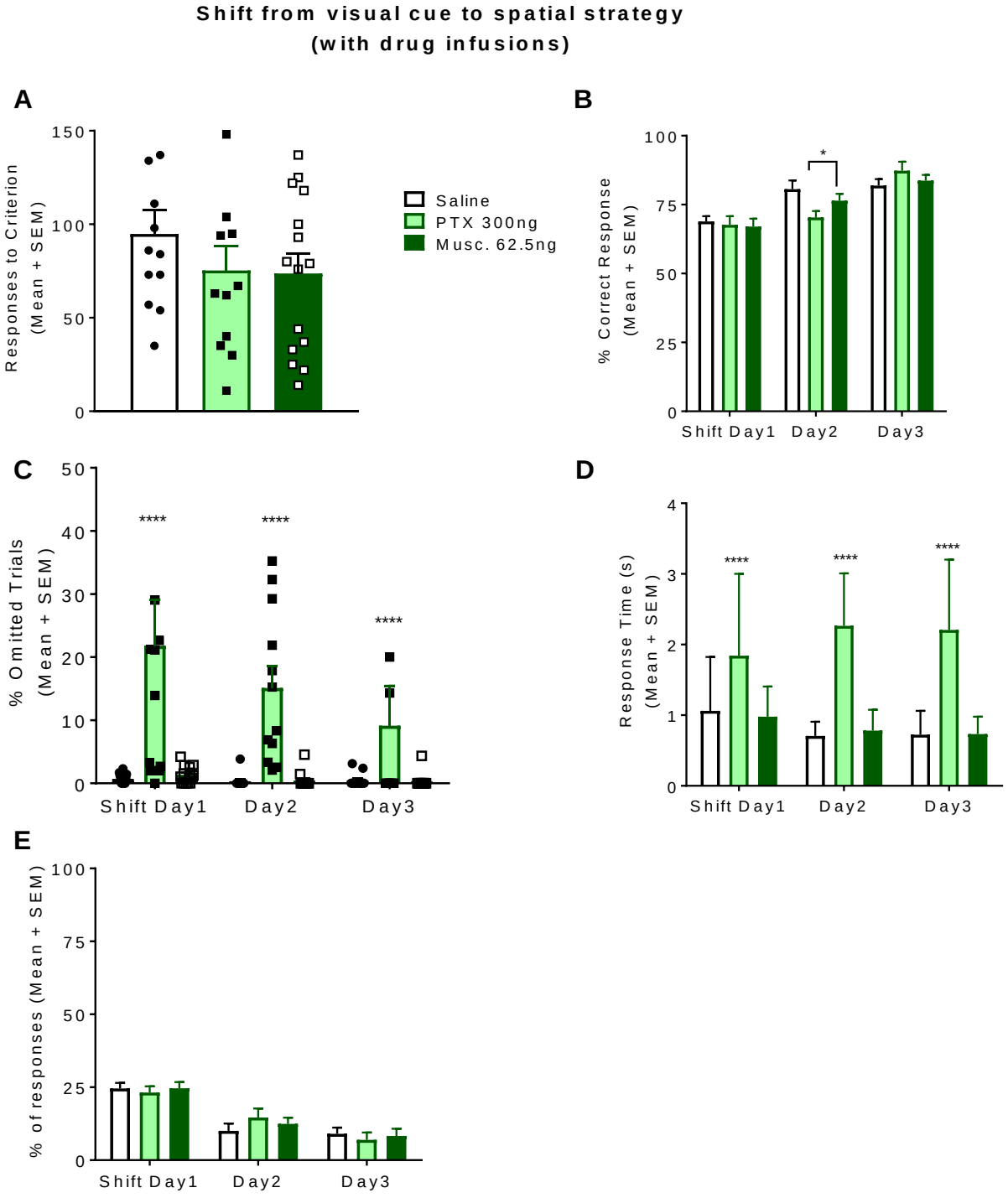


Figure 5.4 - Experiment 4: performance of shift to spatial strategy with drug infusions (Mean+SEM). A) When shifting from cue to spatial strategy, there is no significant drug effect on responses to criterion (RTC) measure. B) Drug effects on increasing accuracy were not significant over three

days but PTX showed significantly decreased accuracy on day 2 (Control vs PTX, p=0.0112) (% correct responses = [correct responses / (correct responses + incorrect responses)] * 100 %). C) PTX increases omission rate (omissions = [total trials – (correct responses + incorrect responses *)] 100 %) and D) response times. E) Significant decrease in perseverative errors across successive cue trial sessions (% perseverative responses = [perseverative responses / (correct responses + incorrect responses)] * 100 %).

All groups shifted to the spatial response, as reflected by an increase of %correct responses across the 3 shift days. Interestingly, PTX-infused rats tended to shift to the spatial response slightly less efficiently than the other groups, reflected by lower %correct responses on day 2 (one-way ANOVA, $F(2, 35) = 3.631$, $P=0.0369$; *post hoc:* control vs PTX, $p=0.0112$) (Fig5.4B). This was supported by an ANOVA of %correct responses, which revealed a significant main effect of day ($F(2, 70) = 29.73$, $P<0.0001$), no significant main effect of infusion ($F<1$), and a trend towards a significant drug X day interaction ($F(4, 70) = 2.232$, $P=0.0743$).PTX markedly increased omission rates to an average of 15% of trials (two-way ANOVA: $F(2, 35) = 12.71$, $P<0.0001$, no drug vs session interaction: $F(4, 70) = 1.555$, $P=0.1961$), significantly higher than the other groups (*post hoc* tests for PTX vs control or muscimol; both = $p\leq0.0001$) (Fig5.4C). There was no significant correlation between %omissions vs. %correct during shift days 1-3; $rs(36)= -0.31$ to 0.07, $p>0.2$.

The number of perseverative-type errors was also not significantly different between drug groups (two-way ANOVA, drug effect: $F(2, 35) = 0.04352$, $P=0.9575$, drug vs session interaction: $F(4, 70) = 0.6324$, $P=0.6411$). All three groups showed a session effect of reduced perseveration with increased sessions ($F(2, 70) = 40.61$, $P<0.0001$) (Fig5.4D). Response latencies were also increased by PTX (two-way ANOVA, drug effect: $F(2, 35) = 32.21$, $P<0.0001$, drug vs session interaction: $F(4, 70) = 2.011$, $P=0.1024$), with PTX-infused rats responding longer than the other groups (*post hoc* tests both for control vs PTX and muscimol vs PTX, p=<0.0001) (Fig5.4E).

Discussion

The aim of this experiment was to investigate whether PTX-induced prefrontal neural disinhibition or muscimol-induced inhibition would impair shifting from the visual cue to the spatial response strategy. The main finding was that, while rats generally shifted quickly from cue to the spatial rule and improved over the course of three sessions of spatial strategy trials, this tended to be slightly disrupted by prefrontal PTX, as reflected by fewer correct responses compared to the other groups on day 2. In spite of this PTX-induced impairment apparent on day 2, all infusion groups required a similar number of trials to reach the criterion of 10 consecutively correct responses. In the first experiment reported (chapter 3), the lack of observable effects on RTC and correct responding was possibly attributable to the very slow shifting (>300 trials) from spatial to visual cue strategies, reflecting either a floor effect or the possibility that this type of slow shifting was not prefrontal-dependent enough to be

sensitive to the manipulations. Conversely, the rats in experiment 4 performed a comparatively rapid shift from visual cue to spatial strategies, reaching criterion within one session with markedly higher %correct responses and shift RTC values actually below the RTCs for the initial strategy acquisition, and it was expected that there would be some drug-dependent impact on RTC and/or correct responding. When considering that PTX impaired correct responding specifically during sessions when a strategy had to be recalled after meeting RTC the previous day (the trend level impairment of cue retrieval and the significantly impaired spatial strategy performance during the second shift day), it may be that prefrontal disinhibition was specifically affecting expression of the newly learnt rule. This may be via prefrontal disinhibition causing aberrant firing in ventral tegmental dopamine projections to the nucleus accumbens (NAc); disruption of NAc mechanisms have been implicated in impairments of maintaining newly-learnt shifting strategies (Enomoto, Tse & Floresco, 2011; Smith-Roe & Kelley 2000); Floresco *et al*. 2006; Jackson, Frost & Moghaddam, 2001). The fact that perseveration was not significantly increased by PTX on the second shift day would support this by suggesting that the impaired performance was not due to failure to suppress the cue-based response.

Other notable effects of prefrontal PTX once again included increased omissions, a possible sign of impaired attention which would correspond with previous findings that prefrontal PTX markedly impairs attentional performance (Paine, Slipp & Carlezon Jr, 2011; Pezze *et al*. 2014), and consistent with the first experiment of this project. Likewise, PTX again decreased the general speed of responding. In the 5-choice serial reaction

time (5CSRT) test using the same prefrontal PTX manipulation, Pezze *et al*. (2014) differentiated response latencies from food retrieval latencies, and also investigated PTX effects on locomotor activity. They found that response latencies but not food retrieval latencies were increased by PTX, while locomotor activity was increased, which indicated that decreased speed of responding was not due to motivational nor sensorimotor impairments respectively.

One procedural aspect that so far was not addressed in experiments 1-4 was the potential impact of these prefrontal manipulations on strategy performance when given prior to the acquisition sessions of the initial strategy. Previous research into manipulations of prefrontal GABA transmission has described impairments in set-shifting as dependent on whether $GABA_A$ blocker bicuculline was administered before initial strategy acquisition (Enomoto, Tse & Floresco, 2011).The following chapter will describe the final experiment which aimed to confirm and extend the present findings, first by replicating the result that PTX disrupted shift performance as reflected by reduced %correct on day 2. Secondly, this last experiment was to assess if the prefrontal manipulations affected acquisition of the initial (visual cue) strategy. Prefrontal disinhibition has been shown previously not to affect initial response acquisition, but to impair subsequent shifting to a new response strategy (Enomoto, Tse & Floresco, 2011). Therefore it is possible that the shift impairment caused by PTX administered prior to shift sessions may be exacerbated by PTX administered prior to initial strategy acquisition. Thus, experiment 5 by investigated the effects of prefrontal PTX and muscimol when

administered prior to initial strategy acquisition, retrieval, and shifting stages.

END OF CHAPTER 5

Chapter 6: Experiment 5 – Effect of prefrontal GABAergic manipulation during both cue response acquisition and spatial response shifting

Introduction

Lister Hooded rats in experiments 3 and 4 could acquire the visual cue strategy if training was *not* preceded by the spatial response task, and shifted reliably within one session to the spatial response. PTX-induced disinhibition of the mPFC administered prior to 3 shift days tended to disrupt performance of the shift strategy on the second day of shift trials. Therefore, experiment 5 was conducted to confirm and extend the findings of experiment 4. Firstly, the aim was to confirm that prefrontal disinhibition by PTX infusion prior to each of the 3 shift days disrupts shifting performance, as reflected by decreased correct responses on shift day 2 (as in experiment 4). Secondly, in addition to infusing rats prior to shift sessions, rats were also infused prior to the acquisition sessions for the first rule. This was done to i) examine if prefrontal disinhibition affects acquisition of the cue response; ii) test the possibility that prefrontal disinhibition during acquisition of the first rule (shown to increase perseveration during subsequent shifting (Enomoto, Tse & Floresco, 2011) would exacerbate the shifting impairments caused by disinhibition during subsequent shifting by increasing perseverative errors.

Methods

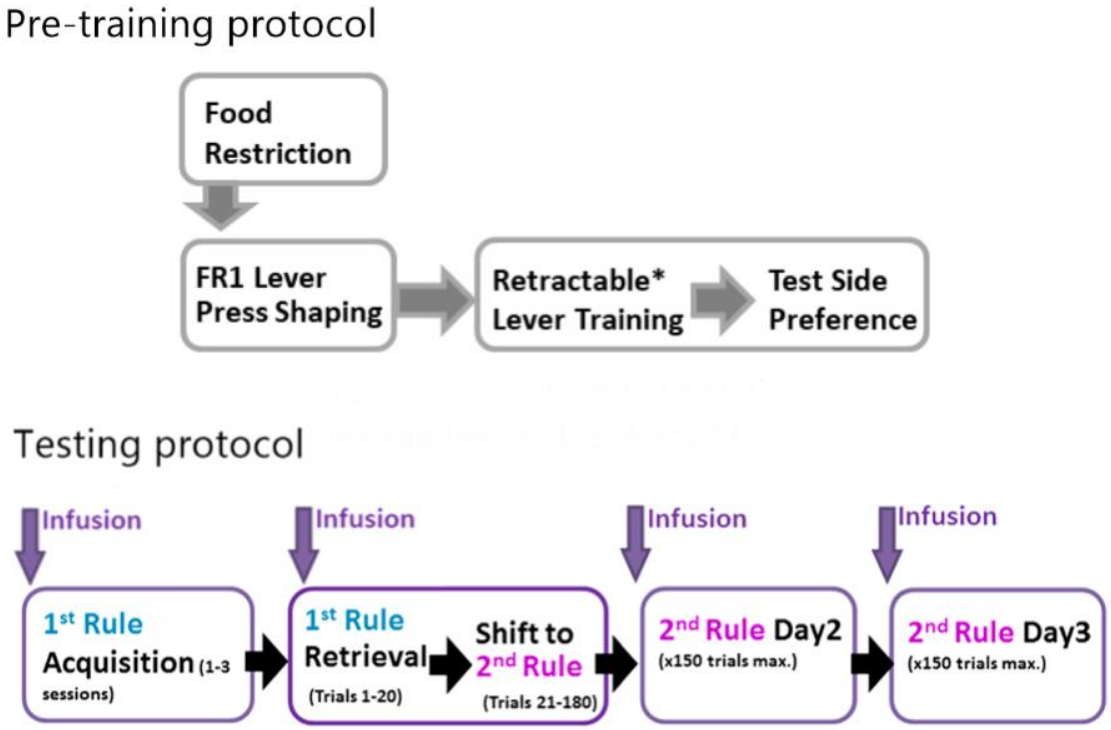


Figure 6.1- Experiment 5 pre-training and testing procedures. Asterisk indicates cue light pre-exposure.

The general protocol for this experiment was as described in chapter 2, with the following specifications (Fig 6.1): the experiment was run in two batches, with each batch including n=8 rats per group and overall target group size of n=16 across the two batches. Rats were food restricted to maintain 90% of free-fed bodyweight. During pre-training, they were pre-exposed to illumination of both cue lights during retractable lever training (5 sessions of 90 pre-exposed trials per session). Intra-cerebral microinfusions were administered 10 minutes prior to the acquisition sessions of the first strategy as well as prior to each of the 3 shift sessions. Rats were initially trained on the visual cue as the first strategy

and, if they met the criterion of 10, were progressed to the cue retrieval-shift to spatial response the following day. If a rat failed to reach criterion, it repeated the strategy acquisition session the following day, and was given a maximum of 3 sessions to learn the cue strategy. If a rat underwent 3 sessions without meeting criterion, it was excluded from further testing.The cue-to-response shift included 20 cue retrieval trials preceding 160 spatial response trials. The spatial response (shift) trials were delivered over 3 sessions (shift day 1 included 160 trials at maximum; days 2-3, included 150 trials at maximum). At this stage, a session ceased once the rat reached criterion, though the rat would still undergo the second and third session regardless of whether it reached criterion in an earlier session.

Results

Prefrontal disinhibition and inhibition did not impair rule 1 acquisition

Similar patterns of cue acquisition performance were found in this study as with experiment 3-4. Several outliers (rats which did not achieve criterion within 3 sessions) were found in the control (N=4) and muscimol (N=2) groups and were excluded from subsequent testing. One rat from the PTX group was excluded from all analysis after being given the wrong testing program before it was able to reach criterion in the rule 1 acquisition stage. All rats included in the analysis had their cannulae placed within the mPFC, mainly in the pre-limbic region; 4 rats were found to have cannulae tips in the border of the pre-limbic and cingulate cortex regions, 12 rats had tips bordering the pre-limbic-medial orbital cortex, and 5 rats had tips in the region bordering pre-limbic-ventral orbital

cortices (Fig6.2). No animals were excluded on histological grounds.

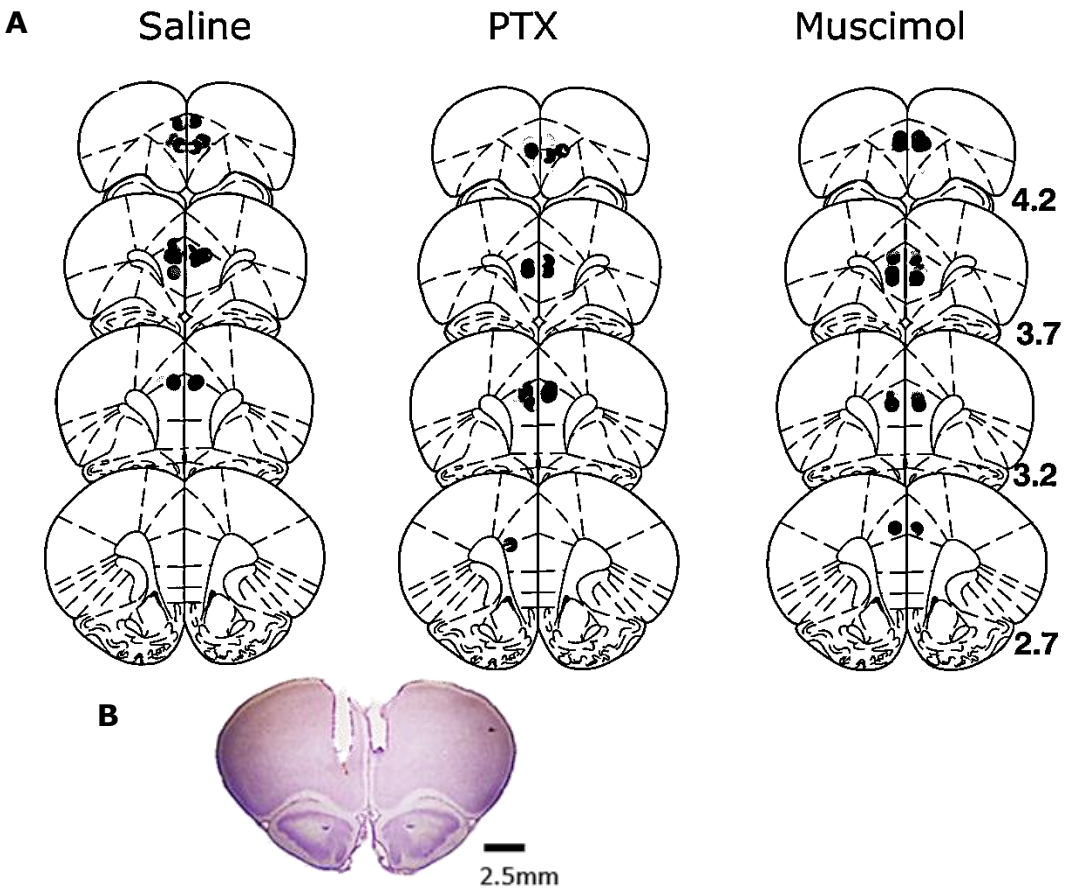


Figure 6.2 - Histology. **A)** Schematic showing coronal rat brain sections, numbers indicating distance from the bregma (mm), showing the placements of the cannulae tips within pre-limbic mPFC for all rats in Experiment 4. Sections correspond with Paxinos G, Watson C, 1998. **B)** Cresyl-violet stained coronal section of rat brain showing cannulae tracts with tips located in pre-limbic region.

Rats which achieved criterion did so within a similar number of responses (RTC), regardless of infusion group (one-way ANOVA: F (2, 36) = 0.6636, P=0.5212) (Fig6.3A). Percentage of correct responses made were not significantly different between groups (one-way ANOVA: F (2, 44) =

2.377, P=0.1046), with average correct performance at 72.97% (muscimol), 68.78% (saline), and 65.96% (PTX) (Fig6.3B).

Prefrontal PTX markedly increased omissions, with an average of 19.7% of trials omitted, while omission rates for the other two groups remained low at 1.28% (saline) and 1.13% (muscimol) (one-way ANOVA: F (2, 44) = 12.64, P<0.0001) (Fig6.3C). PTX also significantly increased response times (F (2, 44) = 26.99, P<0.0001; *post hoc* tests for control and muscimol vs PTX, p<0.0001) (Fig6.3D). Correlational analysis revealed a significant positive correlation between %omissions and response times, both within the PTX group (rs(11)= 0.71, p= 0.009), and across groups (rs(37)= 0.59, p= <0.0001). No correlation was found between subjects' body weights and performance, indicating that motivation to perform the food-rewarded task was not significantly influenced by variation in bodyweight: bodyweight vs %omissions (rs (37)=-0.01, p=0.96); bodyweight vs %correct (rs(37)=-0.096, p=0.56).

Impact of prefrontal disinhibition on cue strategy retrieval

ANOVA indicates a significant main effect of infusion group on %correct responses during the 20 trials of cue strategy retrieval (F (2, 36) = 5.196, P=0.0104); *post hoc* comparisons between PTX and control rats show a PTX-induced impairment (p=0.0091). The PTX group performed correctly in 58.76% of responses versus 73.46% (saline) and 73.27% (muscimol). (Fig6.3E).

Once again, PTX markedly increased omission rates to an average of 21.54% of trials, significantly higher than the other groups (F (2, 36) = 12.73, P<0.0001; *post hoc* comparisons for both control vs PTX and muscimol vs PTX, p <0.0001) (Fig6.3F). Response times were also once

again increased by PTX ($F(2, 36) = 34.55$, $P<0.0001$), with PTX-infused rats responding more slowly than the other groups (*post hoc* tests for both control and muscimol vs PTX, $p<0.0001$, with control and muscimol not differing from each other; $p= 0.7743$) (Fig6.3G). While correlational analysis revealed that %omissions negatively correlated with %correct responses when including all groups, ($rs(37)= -0.41$, $p= 0.009$), there was no significant correlation between these measures within the PTX group, itself responsible for the increase in omissions ($rs(11)=-0.39$, $p=0.19$).

Correlational analysis revealed a positive correlation between %correct responses during rule 1 acquisition and retrieval across groups ($rs(37) = 0.39$, $p=0.01$). A negative correlation was found between the retrieval session's %omissions and %correct responses across all groups ($rs(37)= -0.41$, $p= 0.009$), however this was not found to be significant within the PTX group ($rs(11)= -0.27$, $p= 0.37$), in which these two performance measures were respectively significantly increased and decreased compared to the other groups.

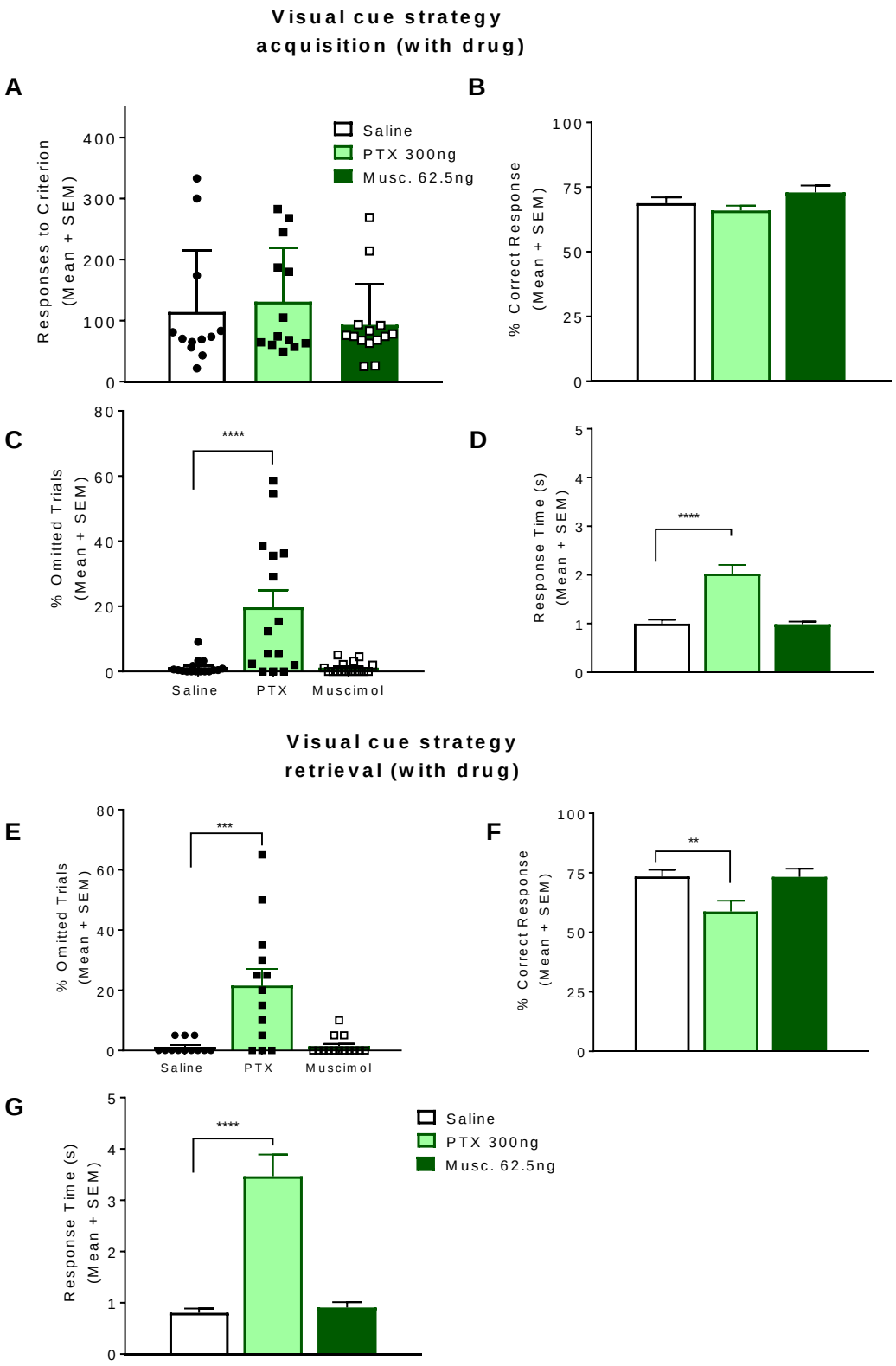


Figure 6.3- Experiment 5: Performance of cue strategy post-infusion (A – C) and post-infusion expression of cue response (retrieval). A-D) Acquisition show no significant differences between groups apart from

PTX-induced increase of C) omissions and D) response times (Mean + SEM); E) Post-infusion strategy retrieval; PTX 300ng increases omission rate (omissions = [total trials – (correct responses + incorrect responses *)] 100 %); F) PTX reduces retrieval accuracy (% correct responses = [correct responses / (correct responses + incorrect responses)] * 100 %); G) PTX increases response time. Asterisk indicates significant difference from control, (** = P ≤ 0.01; *** = P ≤ 0.001; **** = P ≤ 0.0001)

PTX disrupted shifting to spatial response strategy

No statistically significant difference in RTC was found between drug groups (one-way ANOVA: F (2, 36) = 2.159, P=0.1302), with mean RTC ranging from 57.07 (muscimol) to 84.33 (saline).) (Fig6.4A).

All groups shifted to the spatial response, as reflected by an increase of %correct responses across the 3 shift days. A two-way ANOVA revealed a significant main effect of day (F (2, 70) = 25.83, P<0.0001), but no significant main effect of infusion (F (2, 35) = 1.91, P=0.1632). Although there was no drug X day interaction (F (4, 70) = 1.621, P=0.1785), data were analysed separately for each shift day, based on the findings of experiment 4. Separate ANOVAs of %correct responses on each shift day indicated that PTX reduced correct responses on day 2 (F (2, 35) = 3.221, p=0.052), as compared to saline and muscimol (*post hoc* test*,* control vs PTX, p=0.0167) which did not differ (*post hoc* test, control vs muscimol, p= 0.2857). Percentage of correct responses was similar across groups on day 1 and 2 (both (F<0.5, p>0.6) (Fig6.4B). No correlation was found between %correct vs %omissions for each shift day: days 1 to 3; rs(36) =-0.05 to -0.19, p>0.2).

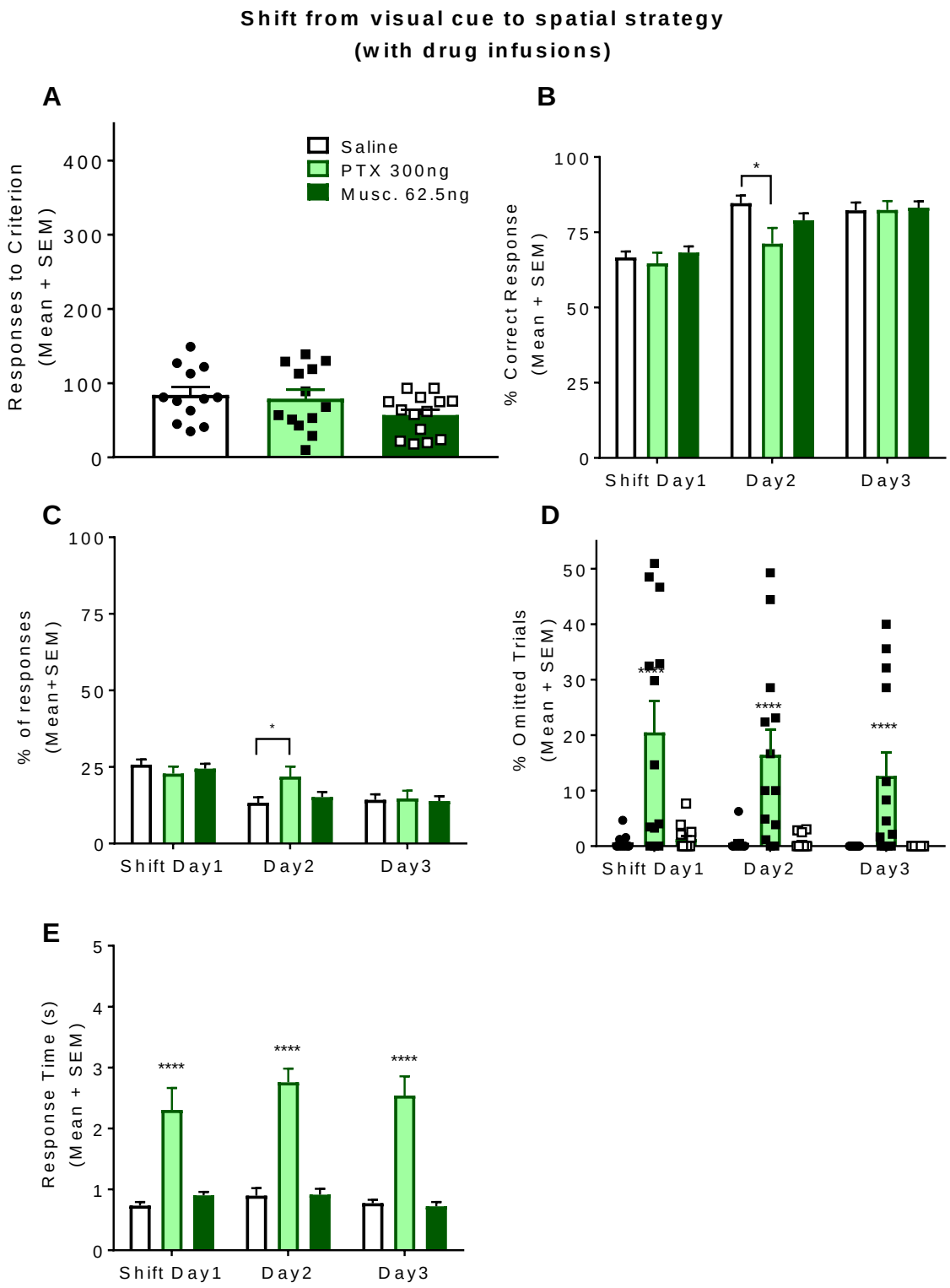


Figure 6.4 - Experiment 5: performance of shift to spatial strategy with drug infusions (Mean+SEM). A) When shifting from cue to spatial strategy,

there is no significant drug effect on responses to criterion (RTC) measure. B) Drug effects on increasing accuracy were not significant over three days but PTX showed significantly decreased accuracy on day 2 (Control vs PTX, p=0.0112) (% correct responses = [correct responses / (correct responses + incorrect responses)] * 100 %). C) Significant decrease in perseverative errors for all groups across successive cue trial sessions (% perseverative responses = [perseverative responses / (correct responses + incorrect responses)] * 100 %). D) PTX increases omission rate (omissions = [total trials – (correct responses + incorrect responses *)] 100 %) and E) response times. Asterisk indicates significant difference from control (* = P ≤ 0.05; **** = P ≤ 0.0001).

The pattern of %perseverative errors was the mirror image of the pattern of correct responses. Two-way ANOVA revealed a significant main effect of day ($F(2, 70) = 18.29$, $P<0.0001$), but no significant main effect of infusion (two-way ANOVA, drug effect: $F(2, 35) = 0.9407$, $P=0.4000$), or drug X day interaction ($F(4, 70) = 2.03$, $P=0.0995$). However, the analyses of separates days revealed that on day 2 PTX significantly increased perseveration (one-way ANOVA: $F(2, 35) = 3.526$, $P=0.0403$; *post hoc:* control vs PTX, p= 0.0185) (Fig6.4C).

PTX markedly increased omission rates to an average of 17% of trials (two-way ANOVA drug effect: ($F(2, 35) = 18.35$, $P<0.0001$)). There was no main effect of day ($F(2, 70) = 1.434$, $P=0.2453$) or drug vs session interaction ($F(4, 70) = 0.7225$, $P=0.5795$). PTX-induced omissions were significantly higher than the other groups (*post hoc* tests for PTX vs control or muscimol; both = $p<0.0001$) (Fig6.4D).

Response latencies were also increased by PTX (two-way ANOVA, effect of drug: $F(2, 35) = 51.91$, $P<0.0001$). There was no main effect of day ($F(2, 70) = 1.378$, $P=0.2588$) or drug vs session interaction $F(4, 70) = 0.63$, $P=0.6427$). PTX-infused rats responded longer than the other groups (*post hoc* tests both for control vs PTX and muscimol vs PTX, p=<0.0001) (Fig6.4E).

As with the cue acquisition, no correlation was found between subjects' body weights and shift performance, indicating that motivation to perform the food-rewarded task was not significantly influenced by individual variation in bodyweight: e.g. bodyweight vs shift day 2 %omissions ($rs(36)=0.19$, $p=0.26$); bodyweight vs shift day 2 %correct ($rs(36)=-0.02$, $p=0.91$).

Discussion

The aim of this experiment was to investigate whether PTX-induced prefrontal neural disinhibition or muscimol-induced inhibition would impair shifting from the visual cue to the spatial response strategy when infusions were given before visual cue and spatial strategy sessions, and whether findings of PTX-induced disruption of shifting to the spatial response could be replicated or would be exacerbated by pre-cue infusions. The main finding indeed confirmed that of experiment 4, that while rats generally shifted quickly from cue to the spatial rule and improved over the course of three sessions of spatial strategy trials, this tended to be slightly disrupted by prefrontal PTX. This was reflected by fewer correct responses compared to the other groups on the second day, although all infusion groups required a similar number of trials to reach

the criterion of 10 consecutively correct responses. Additionally, in this last experiment there were an increased number of perseverative errors committed by PTX-infused rats on the second day of spatial shift trials, accounting for the decrease of correct responses. This differs from experiment 4, in which perseverative errors were not found to be increased. Therefore the increased perseveration seen in experiment 5 suggests that infusions of PTX prior to both cue strategy acquisition and spatial shift sessions induced some impairment in suppressing the initial cue strategy. This finding corresponds with previous research, in which blockade of prefrontal $GABA_A$ receptors with bicuculline selectively increased perseveration during cue-to-spatial strategy shifting if applied prior to initial rule learning (Enomoto, Tse & Floresco, 2011). One suggestion is that prefrontal disinhibition augments reward-association with the initial (visual cue) rule, thus making perseveration more likely (Enomoto, Tse & Floresco, 2011).

Once again, PTX infusions markedly increased omissions and response latencies, regardless of session type, corresponding with previous findings on prefrontal GABAA receptor antagonism and suggestive of increased distractibility (Enomoto, Tse & Floresco, 2011; Pezze *et al*. 2014). Another finding that has remained consistent throughout all the experiments reported here is a lack of effect by the muscimol infusions. The dosage of 62.5ng caused significant impairments in other prefrontal dependent tasks, such as the 5CSRT test for attention, while not causing sensorimotor effects (Pezze *et al*, 2014), and for these reasons was chosen for the strategy shifting tasks. It was expected that some impairment in shifting would be observed, as in other research into mPFC

inactivation and strategy shifting, though these used different and less specific pharmacological manipulations such as the sodium channel blocker bupivacaine (Floresco, Block and Tse, 2008). The lack of effect by muscimol (62.ng) may reflect either that the visual cue-to-spatial shifting task described here is not as sensitive to mPFC inactivation as in the 5CSRT. On the other hand, it must be noted that the performance measures thus far described provide a general indication of task learning, but they do not yield more sensitive information about how the subjects learnt the correct rule via adopting various other potential choice strategies. These other strategies include win-stay/lose-shift or win-shift/lose-stay (Reed, 2016). It may be that more subtle drug effects of either PTX or muscimol would be better investigated using the more strategy-sensitive approach of Bayesian analysis described in the following chapter.

END OF CHAPTER 6

Chapter 7: Trial-by-trial analysis of response strategies using Bayesian estimates

Introduction

This thesis has thus far described the rule learning behaviour in terms of whether rats, through trial and error, performed and switched between two operant "strategies"; either repeatedly pressing the lever associated with an illuminated visual cue ("visual cue strategy"), or pressing the lever located left or right, depending on the individual rat's pre-determined side bias ("spatial strategy"). Furthermore, the rats' flexibility was assessed according to measures of number of responses required to reach criterion (rule 1at 10 consecutive correct responses, excluding omissions), as well as percentage of correct responses and perseverative errors. However, although these approaches provide a general indication of task learning, they do not yield more sensitive information about how the subjects learnt the correct rule via adopting various other potential choice strategies, and whether there was an effect of either PFC functional inhibition or disinhibition on the types of choice strategies implemented. These other strategies include win-stay/lose-shift (where a subject chooses to "stay" with the same response as long as this results in a reward or "win", such as pressing the same lever across trials, but will "shift" to a different response if the action is unrewarded ("lose"), such as switching to the opposite lever). Alternatively, rodents have also been observed to pursue win-shift/lose-stay strategies (attempting a new response after being rewarded for a previous response) (Reed 2016).

A new analysis approach is being developed to uncover the specific type of choice strategies that subjects could learn and attempt to use during the shifting tasks, analysed at the resolution level of discrete trials. This is

being done in collaboration with Silvia Maggi and Mark D. Humphries, who have developed a probabilistic approach to estimate trial-by-trial use of strategies based on a Bayesian accumulation of evidence (see Fig7.1). Recent studies have applied similar approaches to examine trial-by-trial the choice strategies rats use in the ID/ED task (Wang *et al*. 2019) and primates use on a reversal task (Jang *et al*. 2015). This chapter will present first preliminary outcomes from applying this approach to the lever press data from rats performing the operant strategy shift task.

Overview of trial-by-trial Bayesian analysis of lever press responses

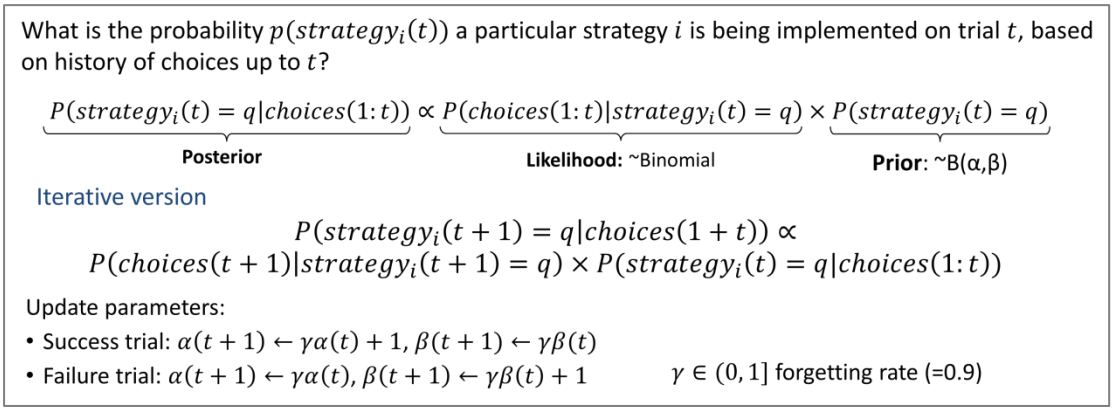


Figure 7.1 –Model used to estimate trial-by-trial use of strategies by rats, based on Bayesian accumulation of evidence (rats' choices) over time. Posterior is the estimate of the distribution of possible P(strategy$_i$(t)) given the choices made up to trial t. Likelihood is consistency of the choices, given the strategy. Prior is the estimate that strategy i is occurring with specific probability q (Maggi *et al*. 2019)

The model used here estimates a probability distribution of having that specific strategy_*i* at trial *t*. This estimation can be updated on a trial-by-trials bases by using an iterative version of the model, where the parameters of the posterior distribution are updated based on the success or failure in applying the strategy. (Fig 7.2A). The posterior (the estimate of the distribution of possible P(strategyi(t)) given the choices made up to trial *t*) and prior (the initial estimate of strategy *i* occurring with probability *q*) are both Beta distributions $B(\alpha;\beta)$ (defined in the range [0; 1] to describe the full range of *q* in $P(\text{strategy}_i(t))$). From the Beta distribution one measures the maximum a posteriori (MAP) estimate ($B(\alpha(t),\beta(t))$) for (for a particular strategy i on trial t, which is the probability corresponding to the peak of the beta distribution. (Fig7.2).

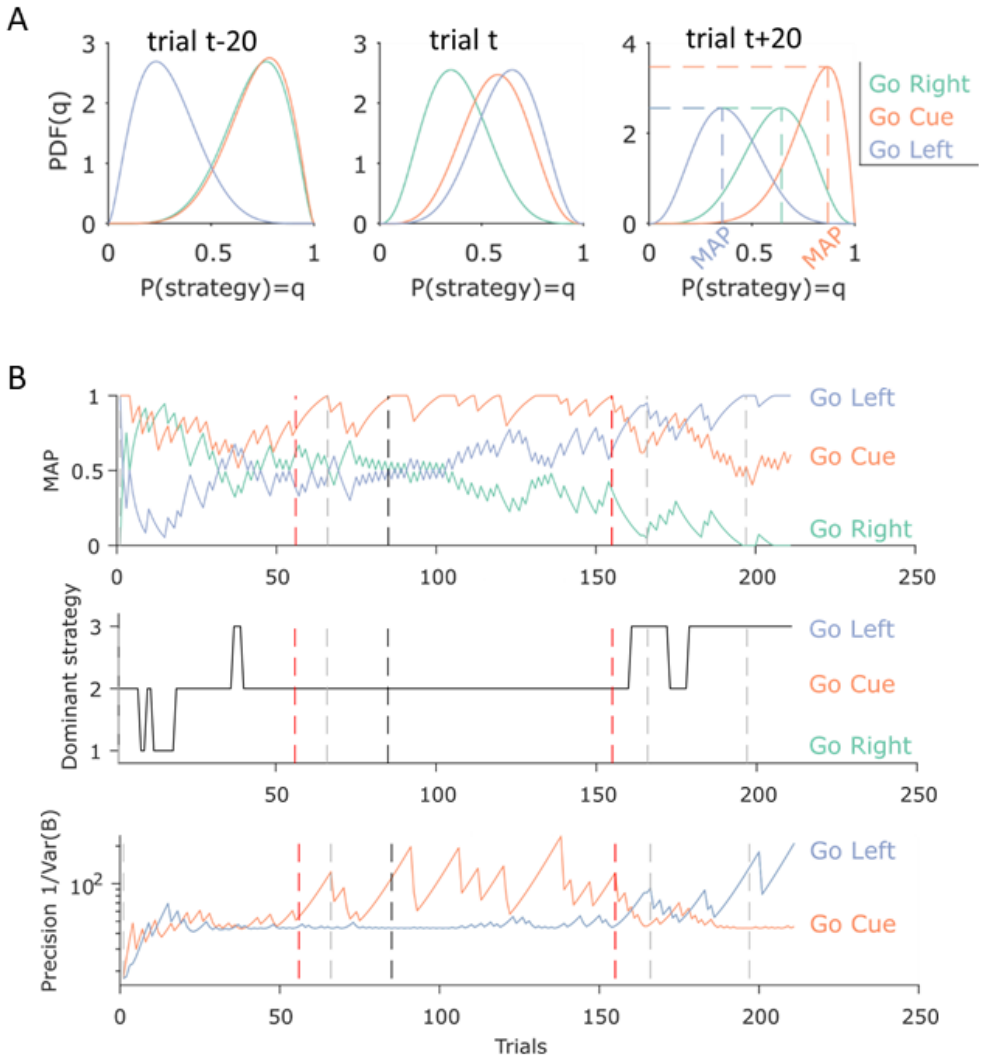


Figure 7.2 – A) examples of Beta distributions for 3 trials (t-20, t, t+20), for strategies "go right", "go left" and "go cue". Dotted lines indicate from where MAP estimates are derived. B) Examples of MAP values, from which dominant strategy per trial is derived, and measure of strategy precision $1/\mathrm{Var}(B)$ (Adapted from Maggi . 2019).

From these MAP estimates, the strategy with the highest probability is derived (the dominant strategy) (Fig7.2B). Furthermore, the corresponding precision (1/variance) of the probability distribution P(strategy *i*) is measured, where variance of the Beta distribution is $Var(B) = \alpha\beta/(\alpha + \beta)^2(\alpha + \beta + 1)$. (Fig7.2B). Precision is a measure of how confident one can be are that a specific strategy has been implemented in any given trial.

Finally a fragmentation value can be calculated as the number of times the dominant strategy is switched along the session.

This model was tested on data from experiments 1 and 5, aiming to allow inference of the most likely strategy being used at each trial during visual cue and spatial rule sessions and to infer when the learning of a rule congruent association occurred. Six implicit and explicit strategies were hypothesised to describe behavioural patterns (Fig. 7.3.)

Go right	Choice of right lever
Go left	Choice of left lever
Go cue	Choice of lever associated with illuminated cue
Win-stay	Choice of lever that was rewarded in previous trial
Lose-shift	Choice of opposite lever to previously unrewarded choice
Alternate	Choice of opposite lever to previous trial

Figure 7.3 – Six potential strategies to describe behavioural patterns observed in the cue and spatial rule sessions.

Results

Dominant strategies

The dominant strategies performed by each group in experiment 5 were analysed for the 48 trials preceding the rule shift (rule 1) and subsequent 48 trials (rule 2), which was the maximum number of trials which all rats performed. Results suggest that the rats appeared to implement the learning strategy for the cue rule before the behavioural learning emerged (meeting the RTC criterion of 10); the cue rule was learnt mainly by applying the 'go cue' (explicit) strategy. Following the rule change, the spatial rule was eventually learnt by following the 'lose-shift' (implicit) strategy, with a tendency for the PTX group to follow the lose-shift strategy from the onset of the rule change, sooner than the other two groups (consistent with our finding that prefrontal PTX disrupts expression of the first rule). By contrast, the saline and muscimol groups appeared to persevere with the go-cue strategy until the 16^{th} and 20^{th} spatial trials, respectively (Fig7.4).

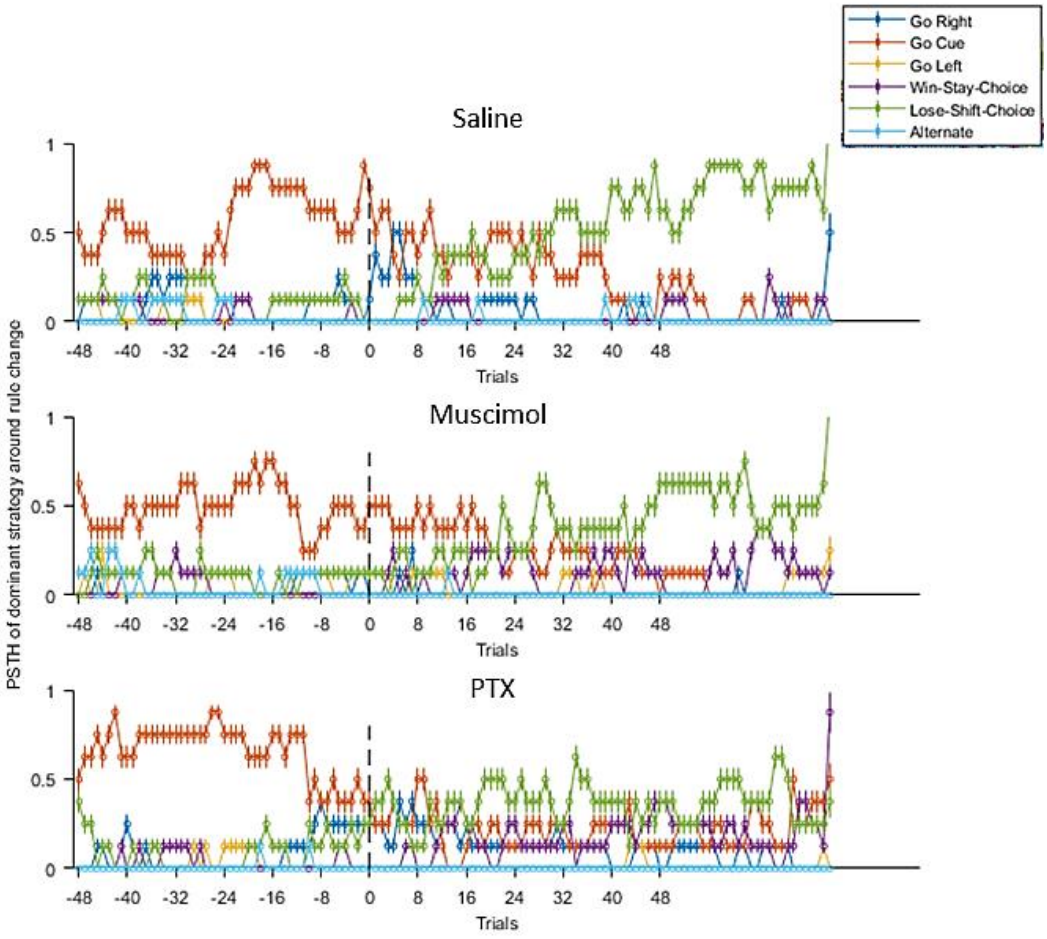


Figure 7.4 – Proportion of subjects with dominant strategy (mean±SEM) preceding and after the rule change at trial 0 (adapted from Maggi *et al* (2019), unpublished).

Strategy fragmentation

Preliminary results suggest that all groups in both experiments 1 and 5 showed higher fragmentation (number of switches between dominant strategies) during visual cue sessions than spatial sessions (Fig7.5).

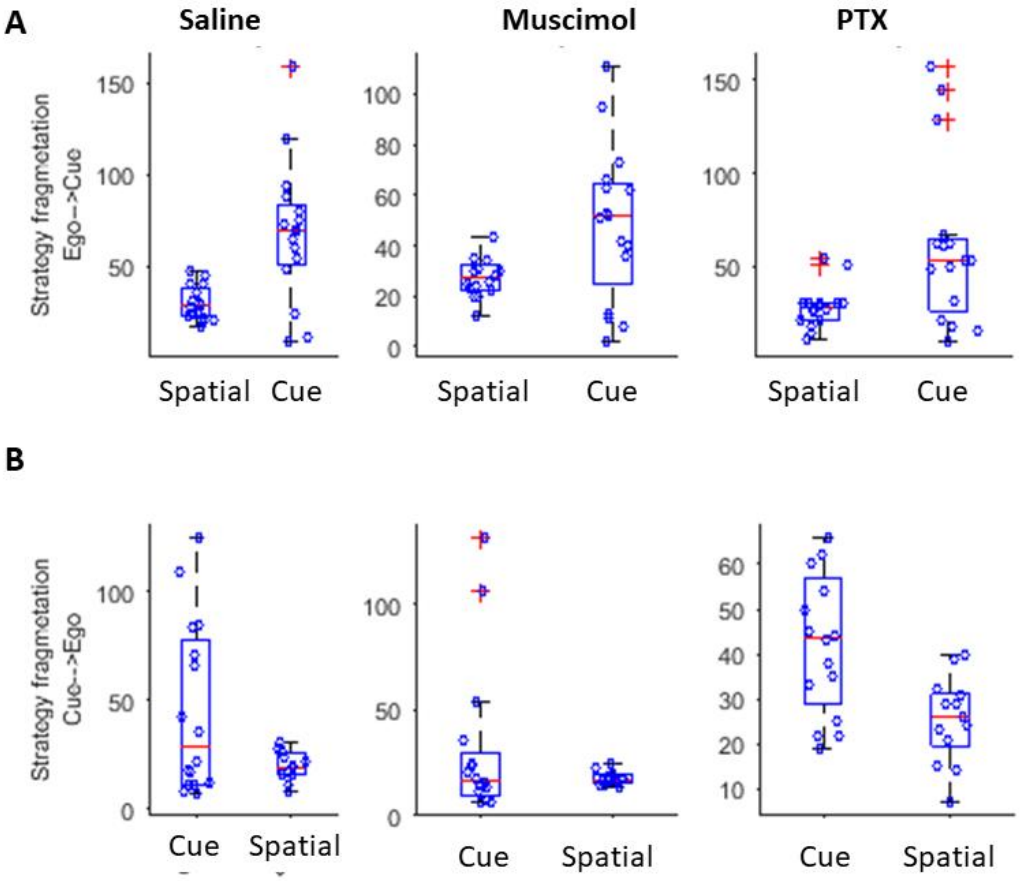


Figure 7.5 – Higher fragmentation of strategies preformed during cue- rule sessions than spatial rule sessions A) Experiment 1: number of strategy switches rats performed during initial (pre-infusion) spatial-rule sessions and (post-infusion) cue-rule shift sessions. B) Experiment 5: post-infusion performance of strategy switches during initial spatial-rule and shift cue-rule trials. Each point represents one animal (adapted from Maggi (2019) *unpublished*).

The muscimol group performed fewer switches compared to controls during the cue rule session in experiment 1(Fig7.5A), and during both cue and spatial sessions in experiment 5 (when infusions preceded both rules)

(Fig7.5). During the cue shift sessions (experiment 5) the PTX group performed more switches compared to controls (Fig7.5B)

Discussion

These preliminary findings demonstrate the feasibility of a trial-by-trial Bayesian analysis of the response strategy the rats followed on the operant behavioural flexibility task. They indicate interesting possibilities to uncover more detailed information about the learning behaviour of the rats in the rule–shifting task, and potential variances between drug groups. For example, PTX-infused rats, which showed increased perseveration during the second day of spatial shift trials (experiment 5 – chapter 6), showed less of the perseverative-type strategy (go-cue) during the first 48 trials of the first shift day, consistent with their impairment in the expression of the first rule during the 20 retrieval trials revealed by the analysis of %correct responses. Instead, their most dominant strategy during these trials appeared to be lose-shift. In fact, an apparent switch from the previously implemented go-cue strategy to a range of other strategies began to occur during the last 10-12 trials of the visual cue task (Fig7.4). This would imply that even as these rats were performing the streak of 10 consecutive correct responses required to meet the original learning criterion, their actual strategies became more explorative, without any particularly one dominating at this point. Performance of the lose-shift strategy would suggest an intact sensitivity to negative feedback (unrewarded incorrect responses) in the early stage of shifting from cue to spatial rules.

One issue with the present analysis is that the six hypothesised strategies do not include one of the two explicit rules, namely the 'spatial rule', in

which the individual subject's strategy is in accordance with the lever side (left or right) allocated to that particular subject's task. So far, the "go left/go right" choice strategies analysed here only analyse whether a rat's lever choice is left or right, not whether the rat's choice is congruent with the spatial rule. This is to be amended in the future.

END OF CHAPTER 7

Chapter 8 –General Discussion

Aims of project

This PhD project aimed to investigate the impact of prefrontal neural disinhibition and functional inhibition on behavioural flexibility in Lister Hooded rats using an automated strategy shifting task. It was hypothesised that manipulation of GABAergic transmission via acute bilateral micro-infusions of picrotoxin (300ng) or muscimol (62.5ng) would impair shifting performance, as would be observable if the numbers of responses rats made before reaching the learning criterion was increased, or the overall proportion of correct responses on shift trials was decreased, relative to controls.

Summary of findings

1. Lister Hooded rats took substantially longer to shift to a visual cue strategy (2-3 days) than other rat strains in many previous studies (1 day) (Brady & Floresco, 2015), struggling to overcome the spatial response strategy. In contrast, they readily shifted in the reverse direction, from the visual cue response to the spatial response.
2. The slow shift from spatial response to cue-based responding was unaffected by prefrontal functional inhibition or disinhibition. In contrast, our experiments provide some evidence that the rapid shift from visual cue-based to spatial responding was impaired by PTX-induced prefrontal disinhibition, consistent with previous

findings that prefrontal disinhibition by bicuculline impaired cognitive flexibility, as assessed on the operant task (Enomoto, Tse & Floresco, 2011).

3. PTX decreased retrieval of the first strategy and consistently increased response latencies and omissions. This is consistent with previous studies and, in light of impaired attention on the 5CSRT task in the absence of motivational impairments, possibly reflects distractibility (Enomoto, Tse & Floresco, 2011; Pezze *et al*., 2014; see Tables 1-2, *Chapter 1*).

In experiment 1, the rats were trained on the spatial response as the first rule and the visual cue strategy as the second rule. While capable of acquiring the spatial rule at comparable performance levels to previously published work the shift performance was remarkably slow in comparison (Enomoto, Tse & Floresco, 2011; Floresco *et al*. 2008; see Table 1, *Chapter 1*). None of the infusion groups (including controls) reached criterion within the standard first session of 160 cue trials reported in previous studies (Placek *et al*. 2013; Enomoto, Tse & Floresco, 2011; Floresco *et al*. 2008); all infusion groups required up to three sessions of cue trials, after which point training on this stage was ceased. This finding is not unprecedented however, as other studies report similarly high trials to criterion for specifically visual cue responses (Beas *et al,* 2017; Beas, Setlow & Bizon, 2016). This difficulty in shifting rapidly may have produced a floor effect, resulting in an absence of observable effects on shift performance by the prefrontal GABAergic manipulations. Alternatively, this very slow shifting may not have relied much on

prefrontal mechanisms, but more on alternative mechanisms linked to habitual behaviour, such as the dorsal striatum (Balleine, Delgado & Hikosaka, 2007).

To test whether this slow shifting was due to training parameters used, additional behavioural experiments 2 and 3 were conducted, involving reversals of the shift sequences (spatial-to-cue and cue-to-spatial). They confirmed that a large shift cost was always involved for specifically the spatial-to-cue strategy sequence. Moreover, experiment 2 showed that modifying training procedures to increase cue light saliency (removal of pre-training cue-light exposure) or prevent engraining of the spatial strategy (reduction of trainings trials for the spatial response) did not facilitate shifting to the cue-based response, and did not affect response reversal performance. Reversal performance was not affected by modifying training parameters, and the first reversal showed a small cost similar to previous studies, which became smaller with successive reversals (Boulougouris, Dalley & Robbins, 2007; Enomoto, Tse & Floresco, 2011).

Experiment 3 showed that, interestingly, rats could acquire an initial cue strategy and then shift to a spatial response strategy, requiring a similar number of trials to reach criterion as in previous studies, but showed the same difficulty in reaching criterion for the subsequent spatial-to-cue shift as in the infusion studies. Therefore it was decided that the spatial-to-cue shift sequence was not suitable for studying effects of prefrontal infusions in these rats, and the infusion experiments (4-5) which followed were conducted using the cue-to-spatial sequence. Experiment 4 suggested that PTX infusions prior to spatial shift sessions disrupted performance of the

spatial rule by significantly decreasing percentage of correct responding on the second day of trials, while experiment 5 confirmed this finding with a significant reduction of correct responses and, furthermore, found that PTX, when infused prior to both first cue rule and second spatial rule learning sessions, increased perseveration during the second shift day. Additional effects across all experiments of PTX included increased omissions, impaired retrieval of 1^{st} rule despite not affecting 1^{st} rule acquisition, and increased response latencies. No significant correlations were found for task performance (%correct or RTC) vs. either bodyweight or omission rates, thus suggesting that these were not significant extraneous factors causing the impairments observed in PTX-infused groups.

Evidence for prefrontal PTX disrupting shift to spatial strategy

Experiments 4 and 5 both found decreased correct responding in the PTX group on the second day of shifting to the spatial rule. While rats which did not receive infusions prior to 1^{st} rule (cue) acquisition did not show increased perseveration to account for this impairment, the rats which received PTX prior to both 1^{st} rule acquisition and 2^{nd} rule shifting sessions did show increased perseveration during the second shift day.

This finding corresponds with previous research, in which blockade of prefrontal $GABA_A$ receptors with bicuculline selectively increased perseveration during cue-to-spatial strategy shifting if applied prior to initial rule learning (Enomoto, Tse & Floresco, 2011). One suggestion is that prefrontal disinhibition augments reward-association with the initial

(visual cue) rule, thus making perseveration more likely (Enomoto, Tse & Floresco, 2011). It is notable that in both experiments 4 and 5, PTX-induced reduction in correct responding did not occur on the first day of shift trials, but appeared during sessions when retrieval of the previous day's strategy was required (such as during 1st rule (cue) retrieval and 2nd rule (spatial shift) day 2). Thus, it may be that prefrontal disinhibition was specifically affecting expression of the newly learnt rule; in the case of rats which had prefrontal disinhibition during 1st rule acquisition, they formed a stronger reward-association with the 1st rule. Therefore they defaulted back to this 1st strategy during shifting on the 2nd day when they failed to retrieve/express the second strategy. The rats which did not receive infusions prior to learning the 1st rule 1) did not form a strong reward-association with it and thus did not show increased perseveration during shift days, and 2) struggled to retrieve the 2nd rule on shift day 2, so they made more non-perseverative errors. Impaired rule expression may have been caused by aberrant firing in ventral tegmental dopamine projections to the nucleus accumbens (NAc); disruption of NAc mechanisms have been implicated in impairing the maintenance of newly-learnt strategies and appetitive instrumental learning (Jackson, Frost & Moghaddam, 2001; Floresco *et al*. 2006; Enomoto, Tse & Floresco, 2011; Smith-Roe & Kelley 2000).

PTX increases omissions and response latencies

Regardless of rule or session type, prefrontal PTX markedly and consistently increased the omissions and general response latencies. This

corresponded with previous findings on prefrontal $GABA_A$ receptor antagonism and is suggestive of increased distractibility (Enomoto, Tse & Floresco, 2011; Pezze *et al*., 2014). Pezze *et al* (2014), who used the same PTX manipulation during the prefrontal-dependent 5-CSRT task of sustained attention, differentiated response latencies from food retrieval latencies, and also investigated PTX effects on locomotor activity. They found that response latencies but not food retrieval latencies were increased by PTX, while locomotor activity was increased, which indicated that decreased speed of responding was not due to motivational nor sensorimotor impairments respectively. Nevertheless, in hindsight it may have been useful to replicate these findings, differentiating between response and food retrieval latencies in the strategy shifting task.
Given that there was no significant correlation between the effects of PTX on increased latencies/omissions and task performance (reduced % correct responding) during strategy retrieval and shift stages, it seems unlikely that increased omission rates and latencies were masking performance effects for measures such as RTC.

Lack of effect by prefrontal muscimol

While PTX consistently produced the effects mentioned above, another consistent finding was the *lack* of observable effects by prefrontal muscimol at 62.5ng. This manipulation was expected to cause some impairment of strategy shifting performance via prefrontal hypo-activation, as pharmacological and lesion studies which reduced mPFC activation found this impaired prefrontal-dependent shifting performance (Floresco *et al*. 2008; Birrell & Brown 2000). It might be argued that the

$GABA_A$ antagonist muscimol was not as effective at inactivating the mPFC as lesions or less specific pharmacological manipulations (such as sodium channel blockers) used in other work (Floresco *et al*. 2008; Birrell & Brown 2000; Tait *et al*. 2009). However, the same dose of muscimol did cause marked impairments of sustained attention in the 5-CSRT (Pezze *et al*. 2014). One possible explanation is that the strategy shifting task used here was not as sensitive to this specific GABAergic prefrontal inactivation as the 5-CSRT task (Pezze *et al*. 2014). Another possibility is that the lack of effects may reflect partial inactivation or compensation of the functional inhibition. Thus, in hindsight, it may have been worth testing higher doses of muscimol; 125ng/side (double the dosage used in our experiments) was shown to have noticeable effects on various prefrontal-dependent tasks (Pezze *et al,* 2014; Pezze *et al,* 2017). A potential risk of using this higher dosage would have been the potential sensorimotor side-effects such as reduced locomotor activity that would interfere with performance, as observed by Pezze *et al*, 2014, which was the initial reason for avoiding the dosages of 125ng and 250ng. With this in mind, however, experimenting with an intermediate dose of, for instance, 94ng (rounding up 93.75ng, 75% of 125ng) may have been useful if it proved sufficient to produce observable cognitive impairments while being low enough to avoid causing sensorimotor side-effects. Another useful addition to the experiment would be a positive control for muscimol. However, the batches of muscimol used in this study were shared in the laboratory and intra-cranial infusions at higher doses (125ng-250ng) were found to cause effects in a range of other behavioural experiments, suggesting that the batches were of useable quality.

Strategy-sensitive Bayesian analysis

Another possible explanation is that the performance measures mainly used in the automated strategy shifting task may have provided too general an indication of task learning, describing relative rates of learning rather than giving direct information about patterns of learning unfolding across trials. Thus, drug effects may have emerged when using a more choice strategy sensitive approach at a trial-by-trial resolution. For example, other studies have used Bayesian analyses to reveal that impairments in discrimination learning caused by prefrontal damage may specifically reflect reduced sensitivity to negative feedback (Wang *et al*. 2019).

For this reason, preliminary work was begun on developing a probabilistic Bayesian model to uncover with greater sensitivity the specific types of choice strategies, such as win-shift vs lose-shift, on a trial-by-trial basis. Preliminary results suggest that rats predominantly adopted a "go cue" rule when about to achieve criterion on the visual cue task, while predominantly adopting the lose-shift strategy during spatial shift sessions. Further modifications of the analysis model are in progress to improve detection of spatial-rule specific choices and produce statistical analyses.

Assessing prefrontal function using ED shifting

One point that must be addressed is the fact that with an automated chamber-based shifting task such as this, there are only 2 exemplars per stimulus dimension: either illuminated light or not illuminated for the visual cue dimension, or left- versus right-side lever for the spatial location dimension. This precludes the possibility of running an intra-dimensional (ID) shift, and therefore an ID vs ED shift cost cannot be established (Tait *et al*. 2018). Consequently one cannot prove set formation occurs during the 1st rule training (Tait *et al*. 2018). Therefore, to examine directly the role of prefrontal function in ED shifts, other tasks may be more suitable, such as the rodent ID/ED task (Birrell & Brown 2000).

Conclusion

The findings of this project provide some evidence that prefrontal disinhibition impairs rapid strategy shifting from visual cue-based to spatial responding, consistent with previous findings that prefrontal disinhibition by bicuculline impairs these aspects of cognitive flexibility (Enomoto, Tse & Floresco, 2011). Given that PTX impaired retrieval of the initial rule, and also increased perseveration during later shift stages (day 2) specifically when the prefrontal cortex was disinhibited during initial rule learning, it may be that prefrontal disinhibition specifically affected expression of a newly learnt rule, both in the case the initial rule or the second (shift) rule. This could partly be explained by prefrontal disinhibition driving aberrant firing in ventral tegmental dopamine projections to the nucleus accumbens (NAc) (Jackson, Frost &

Moghaddam, 2001; Floresco *et al*. 2006; Enomoto, Tse & Floresco, 2011; Smith-Roe & Kelley 2000). The lack of observable effect by prefrontal muscimol may require reconsideration of the assumed sensitivity of the operant shift task used to assess the effect of prefrontal inactivation; more sensitive performance analysis via use of trial-by-trial Bayesian analysis may reveal more details about strategy learning during these tasks.

END OF CHAPTER 8

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Appendix

PIP Report Summary

Leibniz Institute für Neurobiologie, Magdeburg, Germany
9th April – 5th July 2018

Placement Title: SPECT imaging of the brain wide activation changes

Summary

This project involved using a new single-photon-emission-computed-tomography (SPECT) method to measure the brain-wide metabolic activation patterns induced by local brain stimulation in freely moving rats. The metabolic brain activation markers are an important bridge to clinical imaging studies. 12 rats were implanted with bilateral cannulae targeting the ventral hippocampus, and after a recovery period, were implanted with a jugular vein catheter. In a within-subjects design, rats were on separate days infused with picrotoxin (150ng) or saline into the v. Hippocampus, 10 minutes prior to injection of radioactive tracer Tc-HMPAO into the jugular vein catheter. Rats were then anaesthetised with isoflurane and scanned for the accumulation of the tracer using SPECT.
Main finding: significant increases in blood flow were detected at infusion sites for PTX infusions compared to controls, as well as a decrease in blood flow in the dorsal hippocampus.
Results provide a translational bridge to clinical studies, allowing comparison of findings with clinical imaging studies and translation into specific hypotheses for future imaging studies in patients.

New skills acquired:

Experimental techniques

- SPECT scanning procedure

- Free-hand drilling
- Cryofixation for brain histology
- Jugular vein catheter implantation surgery (rat)

Analysis Techniques

- Brain ROI mapping with OsiriX (software)
- SPECT data analysis:
    - Scan preprocessing
    - MRI-CT-SPECT image realignment (MPI tool)
    - Conversion and rescaling processes to prepare image files for t-testing

Presentation skills

(I gave a presentation on our findings at LIN, 01.06.2018 at the departmental seminar)

- SPECT data figures prepared and presented on powerpoint slides
- Communication of scientific concepts and experimental details to wider neuroscience audience

Additional Activities:

- Observed Optogenetic: virus injection, optical fibre implantation (ventral hippocampus)
- Observed: MRI scanning and optogenetic stimulation procedure

End of thesis