# The Effects of Repeated Dopamine $D_2$ Receptor Antagonism on $D_2$ Receptor Expression and Behavioural Inflexibility

by

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## Abstract

**Background.** Reversal learning has been shown to improve as a function of dopamine $D_2$ receptor expression, and manipulations that downregulated $D_2$ expression also impaired behavioural flexibility. The possibility remains that an upregulation may facilitate reversal learning. Further, $D_2$ receptor expression and reversal learning were compromised following repeated methamphetamine exposure. It stands to reason that restoration of receptor expression following methamphetamine exposure might prevent this impairment from occurring.

**Objectives.** Develop a procedure for measuring reversal learning and determine whether repeated $D_2$ receptor antagonism increases $D_2$ receptor expression and improves behavioural flexibility in rats. Further, the possibility that this treatment would ameliorate behavioural inflexibility produced by prior methamphetamine exposure was examined.

**Methods.** A task was developed to measure reversal learning. I systematically replicated an existing procedure, but then developed a novel procedure that focused on identifying a reasonable criterion for visual discrimination. To do this, male Sprague Dawley rats completed 25 days of visual discrimination. Various criteria requiring different levels of accuracy and persistence were retrospectively applied to the data and a single criterion was selected from these. The procedure was then extended to include three reversal tasks. In the next study, male Sprague-Dawley rats received repeated daily pretreatment with the dopamine $D_2$ antagonist, eticlopride (0.0 or 0.3 mg/kg/day, IP, 14 days). Three days after treatment, whole-brain (minus olfactory bulbs and cerebellum) dopamine $D_2$ receptor expression was measured using flow cytometry. Another cohort was exposed to the same regimen between the second and third reversal task to determine whether this treatment improved reversal learning. The final study was then conducted, the only difference from the previous study being that methamphetamine (0.0mg/kg or 2.0mg/kg, SC, 4 injections, 2 hours apart) was administered the day before eticlopride treatment commenced in order to produce behavioural inflexibility.

**Results.** The criterion developed for the acquisition of visual discrimination was adopted for several reasons. First, the entire sample was able to meet the criterion. Second, the number of days to acquisition was equivalent irrespective of which visual cue served as the discriminative stimulus. Finally, accuracy was persistent both within and between test sessions. Importantly, therefore, these criteria eliminated confounds that might otherwise

have been expected to impact reversal learning performance. The adjunct reversal tasks were typically completed within 2-5 test days, which was important since neuroadaptations can revert over time, and individual differences in performance were consistent across tasks. As expected, eticlopride treatment increased $D_2$ receptor expression and improved reversal learning. Further, this improvement was predicted by baseline behavioural flexibility measures, such that, the improvement produced by repeated eticlopride treatment preferentially impacted rats that were less behaviourally flexible prior to treatment. Methamphetamine treatment impaired reversal learning, but this impairment was prevented by repeated eticlopride treatment.

**Conclusions.** Choice of criterion impacts behavioural performance in visual discrimination learning. $D_2$ receptor expression is also a critical determinant of behavioural flexibility, irrespective of whether the discriminative stimulus is visual or spatial. Further, pre-existing, or experimentally produced behavioural inflexibility is sensitive to a treatment that upregulated $D_2$ expression. Collectively, these findings support the critical role of $D_2$ expression in reversal learning and suggest it is an effective target for facilitating behavioural flexibility.

## List of Abbreviations

| | |
|---|---|
| 6-OHDA | 6-hydroxydopamine |
| ANOVA | Analysis of Variance |
| $CS^+$ | Stimulus associated with reward |
| $CS^-$ | Stimulus not associated with reward |
| DLS | Dorsolateral Striatum |
| DMS | Dorsomedial Striatum |
| DTC | Days to Criterion |
| FR | Fixed Ratio |
| ID/ED | Intradimensional/Extradimensional Set-Shift Test |
| IL | Infralimbic Cortex |
| IP | Intraperitoneal Injection |
| MFI | Mean Fluorescence Intensity |
| NAcc | Nucleus Accumbens |
| OFC | Orbitofrontal Cortex |
| PBS | Phosphate Buffered Saline |
| PFC | Prefrontal Cortex |
| PL | Prelimbic Cortex |
| RPE | Reward Prediction Error |
| $S^+$ | A discriminative stimulus that signals reinforcement |
| $S^-$ | A discriminative stimulus that does not signal reinforcement |
| SC | Subcutaneous Injection |
| SN | Substantia Nigra |
| SRL | Spatial Reversal Learning |
| TOST | Two One-Side Test |
| TTC | Trials to Criterion |
| VRL | Visual Reversal Learning |
| VTA | Ventral Tegmental Area |
| WCST | Wisconsin Card Sorting Test |

**Table of Contents**

# CHAPTER 1: GENERAL INTRODUCTION

*Parts of this chapter have been adapted, with permission, from previously published work in the Journal of the Royal Society of New Zealand (Highgate & Schenk, 2021).*

## Introduction

Imagine that you have just started to learn to drive. One of the many rules that you must learn is to drive on the correct side of the road. You must process and store this as knowledge and draw upon that knowledge when you begin practising. Initially, you draw on this frequently, but following extended practice, driving on the appropriate side of the road becomes habitual. However, what happens when you must now drive a car in a country where you have to use the opposite side of the road? Faced with this, you must suppress the behaviours that you typically use, pay close attention to all the changes in your driving environment, develop new mental strategies for adapting to this change, and produce the appropriate behavioural change. This process of adaptively shifting thoughts, strategies, and actions is referred to as cognitive flexibility (Dajani & Uddin, 2015; Diamond, 2013; Eslinger & Grattan, 1993; Rende, 2000).

Cognitive flexibility is often described as being an executive function (Dajani & Uddin, 2015; Diamond, 2013; Rende, 2000). Cognitions are mental processes that are used to interpret the information related to physical sensations, thoughts, knowledge, and experiences (Sathyanarayana Rao & Tandon, 2016). Executive functions, on the other hand, are a type of cognition that is actively engaged so that information can be systematically processed, interpreted, and manipulated in a way that aligns with the organism's current contextual goals (Dajani & Uddin, 2015; Diamond, 2013). To behave in a way that can be considered flexible, pre-existing cognitive, emotional, behavioural, and environmental interference must be inhibited or suppressed, attention must be actively and selectively guided to the contextual features that have changed, and existing knowledge must be manipulated so that an alternative strategy can be developed that will allow the environment to be successfully navigated once again (Dajani & Uddin, 2015; Diamond, 2013). In the driving example used earlier, to be successfully positioned on the opposite side of the road, previously used mental strategies (e.g., how you gauged the distance from the curb) and behaviours (e.g., automatically positioning yourself in the correct lane after turning) must be suppressed and the processing of less relevant

information limited (e.g., ignoring rear-view mirror checks). The aspects of the environment that have changed must be identified and attended to (e.g., all the road signs are now on the curb or footpath on the opposite side of the road) and new strategies developed ("I will keep close to the centre lane to maintain my distance from the curb") that are updated as required ("using the centre lane was dangerous because I was too close to oncoming traffic, I will try to position myself in the middle of my lane instead"). Despite the environment changing, many fail to rapidly shift to a more adaptive strategy. The extent to which they engage in this perseverative behaviour may be indicative of how cognitively flexible they are (Eslinger & Grattan, 1993; Rossi et al., 1997).

**Measuring Cognitive Flexibility in Human Participants**

Many tasks have been developed to measure cognitive flexibility in the laboratory. The objective of these tasks is to determine how readily a learned response strategy can be changed after environmental contingencies are altered. These measures of cognitive flexibility are typically categorised as set-shifting, reversal learning, or task-switching.

In set-shifting tasks, participants first learn to select a stimulus containing a specific physical feature (e.g., shape or colour). Positive feedback ("correct"; monetary gain) and negative feedback ("incorrect"; monetary loss) follow correct and incorrect selection, respectively. Once the target stimulus is accurately selected, the relevant physical feature is changed. Now the previous rule or strategy must be discontinued and a new one acquired (V. J. Brown & Tait, 2016; Dajani & Uddin, 2015; Diamond, 2013). The *Wisconsin Card Sorting Test* (Berg, 1948; WCST; Grant & Berg, 1948; Heaton et al., 1993) and the *Intradimensional/Extradimensional Set-Shift Test* (ID/ED; Downes et al., 1989; Owen et al., 1991) are the most widely used set-shifting tasks.

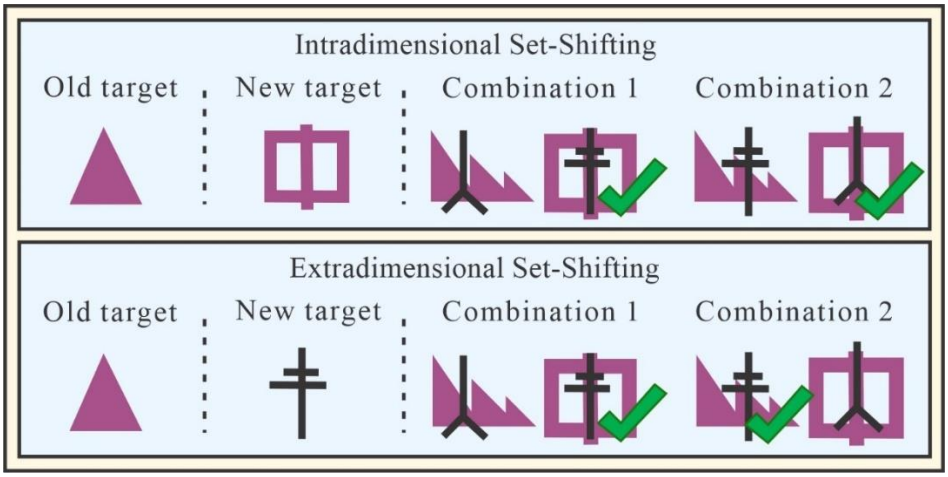
In the WCST four *template cards* are presented, each with a unique shape, colour, and number of shapes. For example, if one template card had 4 blue triangles, the other three would not have four shapes, blue shapes, or triangles. Instead, they might have 3 red squares, 2 yellow circles, or 5 green hexagons. An *exemplar card* is drawn from a pack which includes the shape of one template card, the colour of another, and the number of shapes as a third. The exemplar card must be matched to one of the template cards based on a rule of colour, shape, or number. Once the template cards are reliably matched to the correct card each trial, the rule is inconspicuously changed, and behaviour must be

adjusted to reflect the new rule. For example, if the previous rule was to match based on 'colour', the new rule would require matching based on 'shape' or 'number'. A shortened version of the WCST was developed and is particularly useful for participants suffering from clinical conditions that limit their ability to complete the original version of the task (WCST-64; Kongs et al., 2002).

In the ID/ED task compound stimuli are presented onscreen, each made by overlapping a line stimulus and a shape stimulus. Two 'line' and two 'shape' stimuli are combined to create four unique shape-line compound stimuli. For example, if the shapes were 'triangle' and 'square' and the lines were 'fork' and 'plus', the four compound stimuli would be triangle/fork, triangle/plus, square/fork, and square/plus. However, only two compound stimuli are presented in each trial, and each pattern and line stimulus only appear once. Participants must select the compound stimulus containing a specific line or shape on each trial. If the target stimulus was 'triangle', selecting compound stimuli including the 'triangle' shape would result in positive feedback. After the participant reliably selects the correct compound stimulus, attentional set-shifting is assessed. Now participants must identify a new target stimulus in four previously unencountered compound stimuli. The new compound stimuli are created from two new shapes and two new lines and the previous compound stimuli are no longer used. If the new target belongs to the same physical dimension (shape or line) as the previous target, the task is measuring *intradimensional set-shifting*, and if it belongs to the previously irrelevant dimension, the task is measuring *extradimensional set-shifting*. For example, if the previous target was a triangle, the relevant dimension would have been 'shape'. If the new target is also a shape stimulus, the task is measuring intradimensional set-shifting, but if the new target is a line stimulus, the task is measuring extradimensional set-shifting (see, Figure 1.1 for an illustration of intradimensional and extradimensional set-shifting).

**Figure 1.1**

*An Example of Intradimensional and Extradimensional Set-Shifting*




*Note.* This figure shows an example of intradimensional (top panel) and extradimensional (bottom panel) set-shifting in the ID/ED task. Ticks indicate the correct response during set-shifting. Only one of the two combinations of stimuli is presented in each trial, and each combination is presented in a pseudorandomised order from trial to trial.

Reversal learning tasks have become an increasingly popular measure of cognitive flexibility in recent decades. In these tasks, participants must correctly identify a target stimulus presented alongside other irrelevant stimuli. In its simplest form, two stimuli are presented onscreen, and the participant selects one of these stimuli each trial. For example, a blue square and an orange square may be presented side-by-side, but the location of these stimuli would randomly swap between trials. If the blue square is the target stimulus, selecting the blue square would produce positive feedback and selecting the orange square would produce negative feedback. Once selection accuracy reaches some criterion level, the outcomes associated with the stimuli reverse. Now, selecting the previous target produces negative feedback and selecting the previously irrelevant stimulus produces positive feedback. Continuing with the previous example, selecting the blue square would now produce negative feedback and selecting the orange square would now produce positive feedback.

Various parts of this general procedure for measuring reversal learning differ between studies. In the example used above, feedback is deterministic as positive feedback always follows a correct response and negative feedback always follows an incorrect response (Fellows & Farah, 2003; Javadi et al., 2014; Tsuchida et al., 2010).

However, feedback can also be probabilistic, such that correct selection produces positive feedback more often than negative feedback (80% vs 20%) and incorrect selection has the opposite feedback rates (20% vs 80%) (Cools et al., 2002; D'cruz et al., 2013; Mehta et al., 2001; Swainson et al., 2000). The stimuli presented can also vary along more than one physical property. In the example used above, the stimuli vary according to a single physical dimension (colour), but the presented stimuli can also vary according to multiple physical dimensions (e.g., colour and shape; Downes et al., 1989; Rogers et al., 1999). Many reversal learning tasks have been developed by varying these procedural details.

The third cognitive flexibility task is task-switching. These procedures require participants to complete two tasks, one at any given time. Both tasks are explicitly detailed in the instructions so that the response contingencies do not need to be learned. For example, they might be shown a letter-number combination (e.g., "B3") and must identify if the letter is a vowel or consonant (task 1) or if the number is even or odd (task 2). The current task on any given trial is signalled by another stimulus. In this case, if the letter-number combination is coloured green the vowel-consonant task is completed and if it is coloured red the odd-even task is completed (Sohn et al., 2000). One task is completed over many trials to form a prepotent response and then the task-signalling stimulus changes. If the letter-number combinations were previously green, they would now be red, and the participant would need to switch to the odd-even task. The time required to complete trials after the signalling stimulus changes are usually longer than those completed before it changes (i.e., switch-cost) (Gopher et al., 2000; Meiran, 1996; Sohn et al., 2000). It should be noted that the appropriate response to produce during task switching is explicitly signalled and known before the response is made, and so, these tasks measure how flexibly behaviour can be changed in the absence of new learning. This differs fundamentally from set-shifting and reversal learning tasks which require the current behavioural contingency to be acquired through trial-and-error responding. Nonetheless, many consider task-switching an important measure of cognitive flexibility.

Cognitive flexibility is operationalised by measuring how readily behaviour is altered in response to changes in task conditions during the critical set-shift, reversal, and switch stages of each task. For set-shifting and reversal learning, the most common approach is to measure the number of trials required to accurately respond according to a predetermined criterion (Downes et al., 1989; Greve, 2001; Pantelis et al., 1999; Sánchez et al., 2017). The number of incorrect responses is also often reported and whether these

errors are due to perseveration or rule maintenance is examined (Fellows & Farah, 2003; Pantelis et al., 1999; Woicik et al., 2011). Measures of sensitivity to positive and negative feedback (Cools et al., 2009; van der Schaaf et al., 2014), the number of consecutive set-shifts (Greve, 2001; Sánchez et al., 2017) or reversals (Tsuchida et al., 2010) completed, and how many subjects fail to complete a task (Downes et al., 1989; Pantelis et al., 1999) have also been reported. These measures typically cannot be measured in task-switching procedures since they do not require new learning, and instead, cognitive flexibility is indexed by switch cost (Gopher et al., 2000; Meiran, 1996; Sohn et al., 2000). The specific task adopted to measure cognitive flexibility largely determines which of these measures can be attained. These tasks have been widely used over the last half-century to determine the underlying neural circuitry contributing to cognitive flexibility.

### *Neuroanatomical Basis of Cognitive Flexibility*

Many different methods have been adopted to determine the brain regions relevant to cognitive flexibility. The earliest approach was to determine whether impaired task performance could be explained by pre-existing lesions. In these studies, participants with known brain damage, often following a traumatic head injury or medical complication, completed the cognitive flexibility tasks described above. Impaired performance has been regularly observed in samples with damage to the *prefrontal cortex* (PFC) (Drewe, 1974; Eslinger & Grattan, 1993; Hornak et al., 2004; Kehagia et al., 2014; McDowell et al., 1998; Milner, 1963, 1982; Nelson, 1976; Owen et al., 1993; Pantelis et al., 1999; A. L. Robinson et al., 1980; Rolls et al., 1994; Stuss et al., 2000) and *striatum* (Bellebaum et al., 2008; Benke et al., 2003; Cools, Ivry, et al., 2006; Eslinger & Grattan, 1993). Others have employed functional neuroimaging techniques to identify which regions are active at a given point. Consistent with reports from lesion studies, coincident frontostriatal activation is observed during cognitive flexibility tasks, although this activation is often localised in the lateral PFC (Cools et al., 2002, 2004; Hampshire & Owen, 2006; Langley et al., 2021; Monchi et al., 2001; Rogers et al., 2000; Wilmsmeier et al., 2010), orbitofrontal cortex (OFC) (Dang et al., 2012; Hampshire & Owen, 2006; Kringelbach & Rolls, 2003), and striatum (Cools et al., 2002, 2004; Dang et al., 2012; Langley et al., 2021; Monchi et al., 2001).

Further support for the role of frontostriatal neurocircuitry comes from studies conducted in clinical populations. For example, abnormal frontostriatal metabolic activity

(Chamberlain et al., 2008; De Ruiter et al., 2009; Lao-Kaim et al., 2015; Menzies et al., 2008; Remijnse et al., 2006; Vaghi et al., 2017; Verdejo-Garcia et al., 2015; Zastrow et al., 2009) was observed in participants with psychiatric illnesses characterised by cognitive inflexibility, such as those with eating disorders (Lao-Kaim et al., 2015; Zastrow et al., 2009), obsessive-compulsive disorders (Remijnse et al., 2006; Vaghi et al., 2017), a history of pathological gambling (De Ruiter et al., 2009; Verdejo-Garcia et al., 2015), and substance misuse problems (Cunha et al., 2010; Hoff et al., 1996; van der Plas et al., 2009; van Holst & Schilt, 2011). Further, participants with diseases that result in marked neuronal degeneration within the frontal and/or striatal neurocircuitry, like Parkinson's Disease (see, Kordower et al., 2013) and Huntington's Disease (see, Gil & Rego, 2008), were cognitively inflexible (Cools, 2001; Cools et al., 2001, 2003; Cronin-Golomb et al., 1994; Downes et al., 1989; Josiassen et al., 1983; Langley et al., 2021; Lawrence et al., 1996, 1998; Marié et al., 1999; Peterson et al., 2009). The results of these lesion and neuroimaging studies suggest that the integrity of the prefrontal-striatal neurocircuitry is of critical importance to cognitive flexibility.

The research described thus far implicates disturbances in frontostriatal neurocircuitry in cognitive inflexibility, but these studies lack the experimental control required to unequivocally determine whether this relationship is, in fact, cause-and-effect. Nonetheless, this research continues to provide important information concerning the potential role of neurobiological mechanisms in cognitive flexibility. It is also noted some techniques can be used to manipulate electrical signalling in the human brain (e.g., transcranial electrical stimulation), but the application of these techniques is limited, and lacks the specificity and power afforded by manipulations used in non-human subjects. As a result, experimental approaches have been adopted for use in non-human subjects so that the specific role of brain mechanisms in cognitive flexibility can be determined.

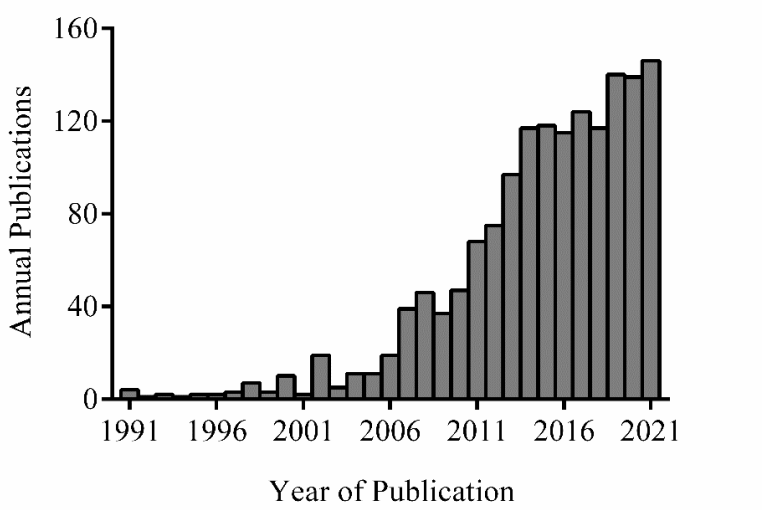
**Measuring Cognitive Flexibility in Non-Human Subjects**

Many tasks have been developed to measure how readily behaviour can change in laboratory animals and these are analogous to the cognitive flexibility tasks described above (Bari et al., 2010; Birrell & Brown, 2000; Boulougouris et al., 2007; Brady & Floresco, 2015; Bussey et al., 1997; Chudasama & Robbins, 2003; Nilsson et al., 2015; Ragozzino, Wilcox, et al., 1999). These tasks are typically referred to as measures of *behavioural flexibility*, and so, I will refer to these tasks as such. Behavioural flexibility

has received considerable interest in recent years (see, Figure 1.2) and procedures have been developed for use with *primates* (Groman et al., 2012; Kamigaki et al., 2011; Millan et al., 2010; Moore et al., 2005; Rygula et al., 2010; Zeamer et al., 2011), *canines* (Boutet et al., 2005; Brucks et al., 2019; Chan et al., 2002), *felines* (Irle & Markowitsch, 1984; Sherman et al., 2013), *marsupials* (Bonney & Wynne, 2002, 2004; Masterton et al., 1974), *alpaca* (Abramson et al., 2018), *sheep* (Morton & Avanzo, 2011), *pigs* (Bolhuis et al., 2004), *rodents* (Boulougouris et al., 2007; Boulougouris & Tsaltas, 2008; Butts et al., 2013; Delatour & Gisquet-Verrier, 2000; Floresco et al., 2008; Floresco, Ghods-Sharifi, et al., 2006; Nikiforuk & Popik, 2014; Ragozzino, Detrick, et al., 1999; Scheggia & Papaleo, 2016), *lizards* (Gaalema, 2011; Leal & Powell, 2012), *birds* (Gonzalez et al., 1967; MacDonald & Roberts, 2018), *fish* (Kuroda et al., 2017; Miletto Petrazzini et al., 2017; Parker et al., 2012), and *invertebrates* (Morrow & Smithson, 1969). Regardless of the specific paradigm used, they all measure how well behaviour adapts in response to changing reward contingencies.

**Figure 1.2**

*Annual Publications Mentioning Behavioural Flexibility as a Function of Year*




*Note.* The number of publications mentioning "behavioural flexibility" or "behavioral flexibility" as a function of the year from 1991 until 2021. This search was conducted using PubMed (https://www.ncbi.nlm.nih.gov) on July 4th, 2022.

To avoid deviating from the scope of the present thesis, models of reversal learning will be the primary focus henceforth. However, it is noted that behavioural flexibility is not a homogenous construct, and in fact, there is evidence that different examples of behavioural flexibility (i.e., reversal learning VS set-shifting) rely on distinct neurocircuitry and neurochemical systems. As such, reversal learning, the literature subsequently reviewed on this topic, and the reversal learning studies outlined in the present thesis should only be considered one example of behavioural flexibility. Additionally, it is noted that the use of the term behavioural flexibility beyond this point will be referring to reversal learning unless otherwise stated, and any use of this term should not be taken as a suggestion of generalisation to other behavioural flexibility tasks.

### *Reversal Learning*

Reversal learning procedures are frequently used with non-human subjects. The procedures comprise an initial discrimination task and a subsequent reversal learning task. During the initial discrimination task, one discriminative stimulus predicts reinforcement ($S^+$), and another discriminative stimulus does not ($S^-$). Trials continue until *discrimination* (i.e., behaviour is more likely to occur in response to the $S^+$ than the $S^-$) reaches some criterion level. At this point, the operant contingencies reverse. Now, the $S^-$ from the discrimination task predicts reinforcement (new $S^+$), and the $S^+$ from the discrimination task does not (new $S^-$). Reversal learning sessions continue until discrimination reaches some predetermined criterion level.

When the contingencies initially reverse, a high level of behaviour continues to be produced in response to the previous $S^+$ despite it no longer resulting in reward presentation (i.e., negative feedback), but this perseverative behaviour reduces following repeated instances of receiving negative feedback. One explanation of this is that the previously reinforced behaviour must be inhibited and that fewer perseverative errors are indicative of greater inhibition. Another interpretation that has become very popular is that behavioural change requires learning from prediction errors (Klanker et al., 2015, 2017; Schoenbaum et al., 2009). A prediction error occurs whenever an expected outcome and an actual outcome differ, and this discrepancy adjusts stimulus-outcome expectations. In this case, future expectations produced by the stimulus should "reduce" since it failed to predict reward. If these expectations are not updated, the previous $S^+$ would continue to be predictive of reward, and the likelihood of perseverative behaviour occurring would

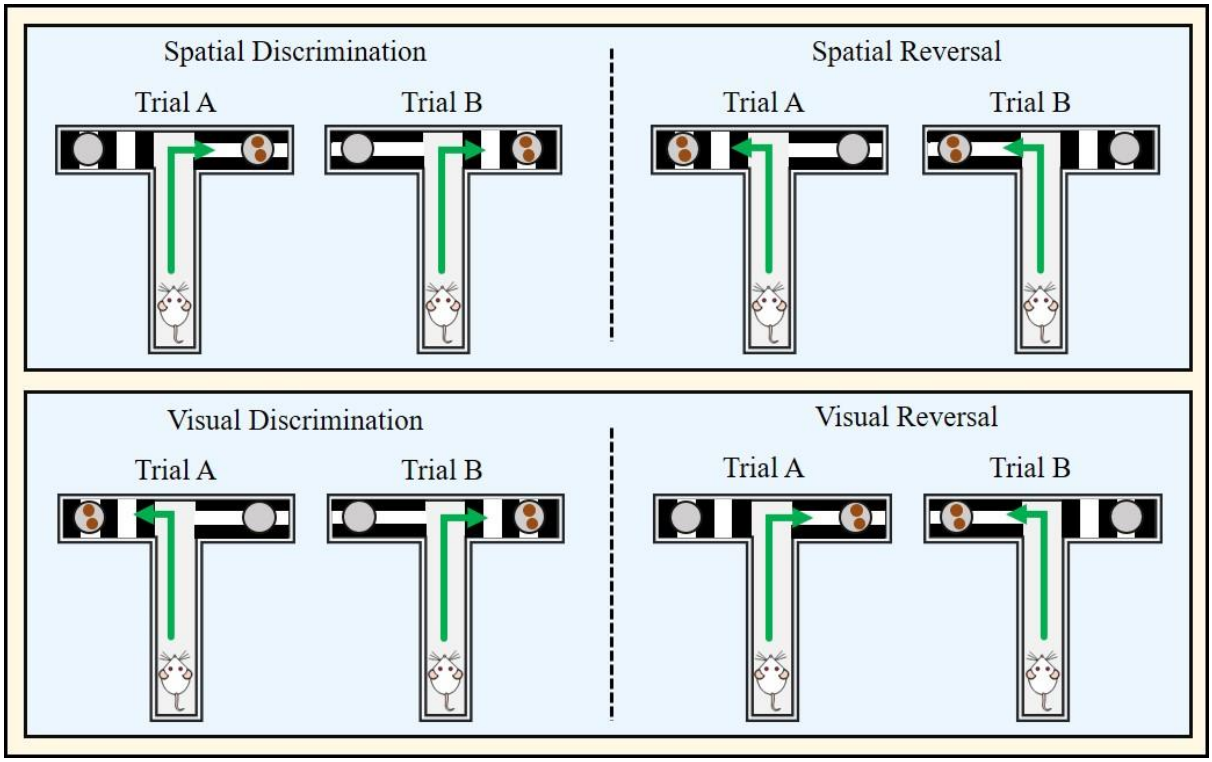
remain high. As these expectations are updated, the association between this stimulus and the reinforcer decreases, and as a result, perseverative behaviour will be less likely to occur.

To meet the criterion during reversal learning, an association between the new $S^+$ and the reinforcer must also be acquired. Initial encounters with this reinforcement contingency will occur sporadically, but with each instance of reinforcement, the association between the new $S^+$ and reinforcement increases. According to prediction error theory, each time behaviour is unexpectedly reinforced, the actual outcome (i.e., reinforcer) has a greater value than the expected outcome (i.e., no reinforcer), and behaviour must be changed to reflect this prediction error. Once this new $S^+$ comes to predict reward availability to some degree, correct behaviour must stabilise ("strategy maintenance") for the criterion to be eventually met. As a result of these overlapping learning processes, behaviour becomes increasingly less likely to occur in response to the previous $S^+$, and instead, behaviour becomes increasingly more likely to occur in response to the new $S^+$.

The first apparatus to be widely used to measure reversal learning was the 'T-maze' (W. F. Hill, Cotton, et al., 1962; W. F. Hill, Spear, et al., 1962) and this has since been adapted for use with other maze apparatuses (e.g., Ragozzino, Detrick, et al., 1999; Ragozzino, Ragozzino, et al., 2002). The T-maze is comprised of three sections: the starting arm, the left arm, and the right arm. Discriminable visual stimuli (e.g., a sheet with vertical stripes vs a sheet with horizontal stripes) are placed in the left and right arms and one of these arms is baited with a reinforcer. This arm must be located by exploring the maze and successfully discriminating between arm locations (i.e., spatial discrimination) or the two visual stimuli (i.e., visual discrimination). Once the reinforcer is reliably located, the stimulus-outcome contingencies reverse, and testing continues until the reinforcer is reliably located again (see, Figure 1.3).

**Figure 1.3**

*An Example of T-maze Reversal Learning Tasks Using Spatial or Visual Cues*




*Note.* This figure shows an example of spatial discrimination (right arm exclusively baited) and the reversal of this contingency (left arm exclusively baited) in the top panel. The bottom panel shows an example of visual discrimination (vertical striped arm exclusively baited) and reversal of this contingency (horizontal striped arm exclusively baited). Green arrows indicate the arm to enter to access the reinforcer and the brown dots represent this reinforcer (e.g., chocolate drops). Trial A and B indicate the two possible setups for each trial. These are typically presented in a pseudorandomised order from trial to trial.

Other procedures have utilised digging tasks (see, Birrell & Brown, 2000). The simplest version of these tasks requires two odour stimuli to be discriminated. For example, two digging bowls are presented that contain a common digging substrate like 'newspaper'. One bowl is scented (e.g., cinnamon; $S^+$) and contains a buried reinforcer, and the other bowl has a different scent (e.g., rosemary; $S^-$) but does not contain a reinforcer. Once the bowl containing the reinforcer is reliably dug in, the contingencies reverse. Now, the rosemary-scented bowl contains the reinforcer, and the cinnamon-scented bowl does not (see, Figure 1.4). This task can also utilise more complex stimuli

that include an additional stimulus property. For example, the bowls might also contain a unique texture so that compound stimuli can be formed in a way analogous to the ID/ED task described earlier.

**Figure 1.4**

*An Example of Reversal Learning Using Bowl-Digging Apparatus*


*Note.* This figure shows an example of odour discrimination (middle) and reversal of this (right). The discriminative stimulus during each stage is shown ($S^+$). Green ticks indicate which bowl contains the buried reinforcer. See text for details.

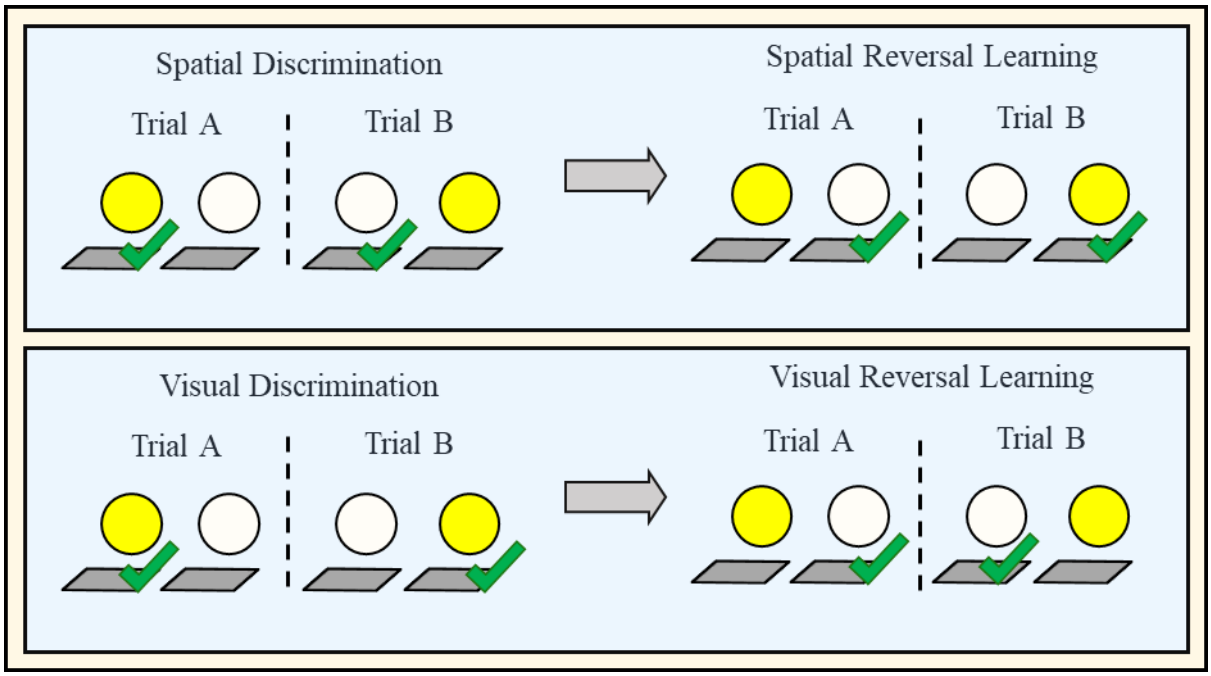
One major disadvantage to using mazes or digging apparatus is that they are low throughput. Each subject is tested individually, and the apparatus and stimuli arrangements must be manually set up and changed between successive trials. Each subject must be placed in the testing apparatus prior to each trial and removed from the testing apparatus after each trial. As a result of these procedural requirements, a high level of involvement is required by the experimenter, and this can result in a relatively small amount of data being collected. Additionally, experimenter demand (i.e., unconscious systematic alterations in the animal's behaviour as a result of experimenter) and increased stress to the animal are of concern when such presence is required.

Procedures have been developed that make use of operant chambers which considerably increase throughput and eliminate experimenter error. In these operant tasks, the chambers are equipped with two operanda and two visual stimuli. Thus, the chamber is an analogue of the T-maze, equipped with two spatially distinct response locations and two distinct visual stimuli. This allows for initial spatial discrimination or visual discrimination to be learned, and to be followed by a subsequent reversal (see, Figure 1.5 for illustration). Modern operant chambers are controlled by computer programmes and

behavioural measures are collected automatically. This allows many subjects to be tested simultaneously, and typically, only requires the experimenter to be present momentarily at the start and end of each test session. As a result, many trials can be conducted during each test session (Brady & Floresco, 2015). These high throughput tasks have become increasingly used to measure reversal learning and are well-suited to overcome the limitations inherent to more manual tasks.

**Figure 1.5**

*An Example of Operant Chamber Reversal Tasks Using Spatial or Visual Cues*




*Note.* This figure shows an example of spatial discrimination (left lever depression reinforced) and spatial reversal learning (right lever depression reinforced) in the top panel. The bottom panel shows an example of visual discrimination (lever depression below the illuminated light reinforced) and visual reversal learning (lever depression below the unilluminated light reinforced). Ticks indicate which response is currently reinforced. Trial A and B indicate the two possible light-lever setups for each trial. These are typically presented in a pseudorandomised order from trial to trial.

Behavioural flexibility is operationalised analogously to cognitive flexibility. It is very common for trials to criterion and errors to criterion to be reported. In addition to these, errors might be separated, for example, into perseverative and strategy maintenance

errors (e.g., Ragozzino, Detrick, et al., 1999) or early, mid, and late errors (e.g., Alsiö et al., 2015; Sala-Bayo et al., 2020). Others report sensitivity to types of feedback and win-stay/lose-shift scores (e.g., Alsiö et al., 2019). As it was with cognitive flexibility, the behavioural flexibility tasks used are an important determinant of which measures can be recorded.

A considerable number of studies have used reversal learning procedures to identify the neurobiological systems critical to behavioural flexibility and many neurochemical systems have been implicated in behavioural flexibility (M. N. Hill et al., 2006; Ragozzino, Jih, et al., 2002; Stefani et al., 2003; Totah et al., 2015; Young & Shapiro, 2009). In particular, the dopamine system has received considerable attention. However, before discussing this I will first provide an overview of the role of dopamine in associative learning and reward.

**Dopamine and Associative Learning**

Dopamine is likely to be the most extensively investigated neurochemical within the associative learning literature and many excellent reviews have covered this topic in detail (see, Peters et al., 2021; Salamone et al., 2022; Schultz, 2016; Speranza et al., 2021; Starkweather & Uchida, 2021). Dopamine cells of the mesolimbic pathway that project from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) are highly responsive to reward, and as a result, this so-called 'reward circuitry' has been thoroughly investigated for its role in associative learning. Specifically, it is well-established that natural rewards transiently increase both the firing rate of dopamine cells projecting from midbrain nuclei and the release of dopamine within the terminal regions of these dopamine projections (Bassareo et al., 2017; Bassareo & di Chiara, 1997; H. D. Brown et al., 2011; Norgren et al., 2006; Roitman et al., 2008; Schultz et al., 1993, 1997). Early explanations of the role of dopamine in reward processing suggested that changes in central dopamine, specifically within the brain's 'reward circuitry', signalled the subjective pleasure produced by reward (Wise, 1980, 1982), but this account is not consistent with various subsequent findings. For example, if the primary role of dopamine is to signal pleasure, then such a signal might be expected to occur each time reward is encountered, however dopamine cells no longer fire in response to a reward once it is predictably delivered (see, Schultz, 2016). Additionally, extensive central dopamine depletion in rats with 6-OHDA left pleasure-related responses intact (i.e., orofacial

reactions), and instead, profoundly impaired motivation to access and consume food rewards (Berridge et al., 1989; Berridge & Valenstein, 1991). These observations suggest that dopamine may not be necessarily involved in encoding reward or pleasure but may instead play a more general role relevant to reward-related learning and motivation.

Considerable research has implicated dopamine in stimulus-outcome learning. This is commonly examined by measuring conditioned approach towards stimuli or contexts that are ($CS^+$) or are not ($CS^-$) associated with imminent reward, and typically, increased responding is observed towards the $CS^+$. However, 6-OHDA dopamine lesions of the NAcc markedly disrupted the acquisition and maintenance of this conditioned response (Dalley et al., 2002; Parkinson et al., 2002) and transgenic mice unable to synthesise dopamine only acquired conditioned approach after dopamine synthesis was virally restored in the ventral, but not dorsal striatum (Darvas et al., 2014). Optogenetic techniques have also been used to modulate light-sensitive ion channels that are expressed on these dopamine cells in transgenic animals (see, Tsai et al., 2009), and importantly, these techniques have shown that stimulating dopamine cells in a way that mimics tangible reward was sufficient for conditioned responding to emerge (Saunders et al., 2018; Tsai et al., 2009). Further, photoinhibition coincident with food reward receipt prevented the emergence of conditioned responding (Chang et al., 2017). Taken together, these observations suggested that dopamine transmission is important for reward-mediated associative learning.

There are two large families of G protein-coupled dopamine receptors, the $D_1$-like ($D_1$, $D_5$) and $D_2$-like ($D_2$, $D_3$, $D_4$) families (Andersen et al., 1990; Niznik & van Tol, 1992), and manipulation of these receptors has been shown to impact stimulus-outcome learning. For example, infusions of the $D_1$-like/$D_2$-like dopamine receptor antagonist α-flupenthixol into the NAcc disrupted both the acquisition and maintenance of conditioned responding (di Ciano et al., 2001). Further, in a classical conditioning task, post-training infusions of the $D_1$-like antagonist, SCH 23390, prevented the development of conditioned responding across sessions, but the $D_2$-like antagonist, sulpiride, did not, implicating NAcc $D_1$-like mechanisms in the consolidation of appetitive classical conditioning (Dalley et al., 2005).

A stimulus that is repeatedly paired with a reward can develop incentive salience. In fact, the conditioned approach behaviour described earlier is one such example of stimulus gaining incentive salience. A stimulus that acquires incentive salience can

capture the attention of an organism, trigger desires and motivation ('wanting') to access the reward, and promote behaviour towards it (see, Berridge & Robinson, 2016). Consistent with this idea, measures of self-reported 'wanting' for food rewards were associated with changes in central dopamine levels (Volkow et al., 2002). Further, attentional bias produced by reward-predicting but task-irrelevant stimuli (i.e., $CS^+$) was associated with how much dopamine levels increased in response to the presentation of the $CS^+$ (Anderson et al., 2016). Thus, these studies implicate dopamine in the processing of incentive salience in humans.

Incentive salience has also been examined in laboratory animals by examining sign-tracker and goal-tracker phenotypes. In autoshaping tasks conducted in a similar way to the conditioned approach tasks discussed earlier, how behaviour is distributed between the location of the $CS^+$ and the food magazine is examined; sign-trackers interact with the $CS^+$ and do so vigorously prior to reward delivery, whereas goal-trackers spend more time waiting at the food magazine for reward delivery. In this context, dopamine does appear to play a specific role in sign-tracking. In one study, selectively bred rat strains that preferentially display sign-tracking or goal-tracking phenotypes were subject to an autoshaping task. In the sign-tracking strain, changes in dopamine release produced by the $CS^+$ steadily increased as sign-tracking behaviour developed across sessions. In contrast, the goal-tracking strain did not interact with the $CS^+$ and dopamine release remained at baseline levels throughout testing (Flagel et al., 2011). Administration of the $D_1/D_2$ antagonist, α-flupenthixol, also selectively disrupted sign-tracking (Flagel et al., 2011; Fraser & Janak, 2017). This likely reflects the $D_1$-like actions of α-flupenthixol since $D_1$-like, but not $D_2$-like blockade produced comparable effects to α-flupenthixol (Chow et al., 2016). Further, the acquisition of sign-tracking was impaired following temporary inhibition of $D_1$-, but not $D_2$-mediated signalling within the NAcc (Macpherson & Hikida, 2018). These studies point to an important role of the $D_1$ receptors in sign-tracking behaviour, and by extension, the development of incentive salience.

Reinforcement learning is another form of associative learning driven by dopamine mechanisms. Enhanced dopamine levels, as measured by radioligand displacement, were observed in human subjects that met criterion during a probabilistic reinforcement task (Calabro et al., 2020). Using laboratory animals, optogenetic studies have also shown that (i) response-contingent photostimulation of the mesolimbic pathway reinforced operant responding (Covey & Cheer, 2019; Kim et al., 2012; van der Merwe

et al., 2021; Witten et al., 2011), (ii) food reward with coincident photostimulation was more reinforcing than food reward alone (Adamantidis et al., 2011; Stauffer et al., 2016) and (iii) the number of dopamine cells that expressed the light-sensitive ion channels was positively correlated with the number of responses made during behavioural testing (Kim et al., 2012). Further, responding went into extinction when (i) response-contingent photostimulation of dopamine neurons was omitted (Kim et al., 2012; Witten et al., 2011), (ii) dopamine neurons were inhibited during response-contingent food reward receipt (Fischbach & Janak, 2019), and (iii) $D_1$ or $D_2$ receptor antagonists were locally infused into the NAcc (Steinberg et al., 2014). Interestingly, reinforcement learning impairments observed in dopamine deficient mice were ameliorated following the viral restoration of dopamine synthesis in the dopamine pathway projecting to the dorsal striatum (Darvas & Palmiter, 2009, 2010; S. Robinson et al., 2007), a separate pathway from the one discussed above. Related to this, terminal stimulation of dopamine afferents in either the ventral striatum or dorsal striatum was sufficient for instrumental responding to be acquired (see, van der Merwe et al., 2021). The results of these studies suggest that the acquisition of an instrumental response can occur through multiple dopamine pathways.

Studies administering lesions or dopamine receptor ligands in reinforcement learning tasks have also suggested that mesolimbic dopamine may be particularly important for effort-related decision-making. For example, antagonism of the $D_1$ or $D_2$ receptors (Nicola et al., 2005; Yun et al., 2004) and 6-OHDA lesions (Aberman & Salamone, 1999; Cousins et al., 1996; Salamone et al., 2001) failed to impact instrumental responding in two-choice spatial discrimination tasks requiring a low-level of effort (i.e., fixed ratio 1). However, the impact of lesions did significantly reduce responding when a greater response cost was required, such that a preference to respond to low-cost low-reward options over high-cost high-reward options emerged (Aberman & Salamone, 1999; Cousins et al., 1996; Salamone et al., 2001). This bias to switch to a low-effort low-reward option has also been observed following local infusions of $D_1$ or $D_2$ antagonists (Farrar et al., 2010; Nowend et al., 2001; Salamone et al., 1991). These data highlight the importance of dopamine in regulating motivational processes.

Insofar, instrumental behaviour has been discussed from the perspective of behaviour produced to access a specific outcome. This type of decision-making is flexible and capable of adapting in response to circumstantial changes but is also more taxing as a result, and when decision-making occurs in this volitional manner, it is said to be goal-

directed. In other circumstances, where reinforcement history is extensive, the stimuli in an environment may be capable of automatically initiating the appropriate behavioural response in a relatively low effort manner but this behaviour is less amenable and relatively insensitive to changes in outcome. When behaviour is consistent with this relatively rigid pattern, it is considered habitual. Two manipulations are commonly used to differentiate between goal-directed and habitual learning behaviour. The first is to devalue the outcome produced by a behaviour, either by manipulating motivation (e.g., pre-feeding the animal in a food-related task) or by pairing it with a noxious stimulus. The second is to degrade the contingency between the behaviour and outcome by non-contingently presenting the outcome. If the behaviour is goal-directed, then these manipulations should reduce behaviour oriented towards the outcome that has been subject to devaluation or contingency degradation. In contrast, if the behaviour is habitual and is driven by prior reinforcement as opposed to the current outcomes, then responding should remain relatively stable following either of these manipulations (Balleine & Dickinson, 1998; Dickinson, 1985). Using this approach, electrolytic or excitotoxic lesions to the dorsomedial striatum (DMS) or prelimbic cortex (PL) made previously goal-directed behaviour insensitive to devaluation or degradation, whereas similar lesions to the dorsolateral striatum (DLS) or infralimbic cortex (IL) rendered previously habitual behaviour sensitive to these manipulations (Balleine & Dickinson, 1998; Corbit, 2018; Killcross & Coutureau, 2003; Yin et al., 2004, 2005), implicating the PL-DMS pathway in goal-directed actions and the IL-DLS pathway in habitual processes.

All four of these structures are innervated by midbrain dopamine, and so, dopamine manipulations might account for some of these effects. Consistent with this idea, 6-OHDA dopamine lesions to the DMS rendered goal-directed behaviour insensitive to contingency degradation but responding remained sensitive to outcome devaluation (Lex & Hauber, 2010). Similar findings were observed following 6-OHDA lesions and local infusions of the $D_1/D_2$ antagonist α-flupenthixol to the PL (Naneix et al., 2009). These results indicate that dopamine in the DMS and PL may be particularly relevant to representing and/or updating the relationship or contingency between actions and outcomes, but less involved in representing the current value of contingent outcomes.

Habitual behaviour is also sensitive to dopamine manipulations. For example, 6-OHDA dopamine lesions to the DLS in extensively well-trained rats resulted in behaviour becoming sensitive to devaluation and degradation manipulations (Faure, 2005; Faure et

al., 2010). Interestingly, opposing effects of $D_1$ blockade and $D_2$ stimulation were observed on habitual behaviour in the IL, such that $D_1$ blockade and $D_2$ stimulation shifted decision-making to be goal-directed and sensitive to contingency degradation (Barker et al., 2013). As such, dopamine does appear to play a role in both goal-directed and habitual learning, but it is unlikely to be the only transmitter molecule relevant to them.

One of the most popular accounts as to how dopamine promotes associative learning proposes dopamine functions as the neural substrate for reward prediction errors. The earliest demonstration that dopamine neurons fire in this way came from the seminal studies conducted by Wolfram Schultz (Schultz, 1993; 1997; 1998). In these studies, the firing activity of midbrain dopamine cells was measured in monkeys during an instrumental learning task. The facing wall was equipped with two levers that could be depressed and two images were presented in each trial, one above each lever. Touching the lever positioned below one of these images resulted in the delivery of fruit juice (i.e., reward) and touching the lever below the other image did not. When training first commenced, correct lever responses unexpectedly produced reward, and so, a positive reward prediction error (RPE) was experienced (the outcome was more rewarding than expected). At the time of reward presentation, a transient burst of activation was recorded from these dopamine cells. However, by the time the task was completed, this transient activation occurred in response to the reward-predicting cue and not the reward itself. Further, when the previously reinforced behaviour unexpectedly failed to produce a reward, a *negative* RPE was experienced (the outcome was *less* rewarding than expected), and the firing of these cells was temporarily suppressed shortly after the time juice would normally be delivered. These seminal studies provided important evidence that dopamine cells fire in a way that is consistent with a neural signal for reward prediction errors, and not simply in response to reward presentation.

To determine whether these positive RPE signals do drive subsequent learning, Steinberg and colleagues (2013) mimicked positive RPE signals using optogenetics in a context where learning is typically blocked. The blocking effect refers to the situation where the presentation of a neutral stimulus alongside a conditioned stimulus that is already fully predictive of imminent reward prevents the neutral stimulus from acquiring conditioned properties (Kamin, 1968). From the perspective of RPE theory, no RPE should occur in this situation since the reward is fully expected/predicted. However, if RPEs do drive subsequent learning, then mimicking a positive RPE under these

conditions by stimulating dopamine cells during reward delivery would be expected to overcome the blocking effect and result in the development of conditioned approach. In fact, this is exactly what they found (Steinberg et al., 2013). These results indicate that the firing of these dopamine cells is both consistent with an RPE signal and that positive RPEs (i.e., increased dopamine firing) do drive subsequent learning.

For the above account, it appears that the dopamine system plays a critical role in both classical and instrumental conditioning processes and is a key modulator of motivational processes relevant to reward. Further, it may serve as the neural substrate for RPEs, both in signalling expectations and driving subsequent learning. Given its importance in the acquisition and maintenance of flexible goal-directed action and inflexible habitual action, it may also be involved in situations where behavioural adaptation is required. This raises a question integral to the present thesis; what is the role of dopamine in reversal learning?

**Dopamine and Reversal Learning**

Several approaches have been taken to determine the role of dopamine in reversal learning. Some have examined how differences in dopamine levels are related to behavioural performance. In one study, dopamine synthesis capacity was determined by measuring uptake of the synthesis tracer 6-[18F]fluoro-L-m-tyrosine with positron emission tomography (PET). It was shown that dopamine synthesis positively correlated with reversal learning accuracy following unexpected positive feedback (Cools et al., 2009). Ventral, but not dorsolateral, striatal dopamine release was also shown to reduce and increase in response to negative and positive RPEs in rats, respectively, following contingency reversal, and the rate at which dopamine activity returned to pre-reversal firing patterns predicted task performance (Klanker et al., 2015, 2017). Further, photoinhibition coincident with unexpected reinforcement (i.e., positive RPE) impaired reversal learning (Radke et al., 2019). Others have also shown that aged rats with reduced dopamine synthesis performed poorly on a reversal learning task (K. Mizoguchi et al., 2010) and that dopamine levels were elevated during reversal learning, compared to discrimination learning (Van Der Meulen et al., 2007). These studies point to an important role of dopamine levels in reversal learning.

If dopamine is important to reversal learning, then depletion of dopamine should lead to reversal learning impairments. PD is a neurodegenerative disease characterised by

extensive nigrostriatal dopamine denervation (see, Kordower et al., 2013) and patients with PD have been shown to have marked reversal learning impairments (Peterson et al., 2009; Swainson et al., 2000). However, the dopamine replacement medication prescribed to these individuals may be the cause of these cognitive impairments since temporary treatment cessation improved reversal learning (Cools, 2001; Cools, Altamirano, et al., 2006). Interpretation of these results is further complicated by the fact that repeated use of dopamine replacement treatments would be expected to lead to widespread neuroplastic changes in the dopamine system (see, de la Fuente-Fernandez, 2004), and so, an unequivocal determination as to how dopamine depletion impacts reversal learning is difficult to achieve in PD patients.

The idea that dopamine depletion impacts reversal learning is supported by the results of experimental depletion studies. Acute depletion produced by administration of the tyrosine hydroxylase inhibitor, alpha-methyl-paratyrosine, increased the number of errors committed during a probabilistic reversal learning task without impacting performance during the initial discrimination task in humans (Hasler et al., 2009). In laboratory animals, dopamine terminal region was a critical determinant of whether reversal learning was impacted by dopamine lesions. Lesions of the DLS did not impact reversal learning (Seip-Cammack et al., 2017) and is consistent with the observations that optogenetic stimulation of DLS dopamine terminals did not support reversal learning (van der Merwe et al., 2021). These reports may not be surprising, given the important role of DLS dopamine in supporting actions that are insensitive and inflexible to changes in outcome value and contingency (i.e., habitual behaviour). NAcc dopamine lesions that impaired reversal learning also markedly impaired discrimination learning making it difficult to determine the nature of this impairment (Taghzouti et al., 1985). In contrast, dopamine lesions of the DMS markedly impaired reversal learning without impacting initial discrimination learning (Clarke et al., 2011; Grospe et al., 2018; O'Neill & Brown, 2007; Tait et al., 2017). Of interest, it was suggested in these studies that DMS dopamine lesions disrupted how accurately the new reinforcement contingency was used. This pattern may not be surprising, given the DMS is required to adapt goal-directed behaviour in response to changes in contingency as discussed in the previous section on dopamine and learning. The results of depletion studies suggest that DMS dopamine plays a critical role in reversal learning.

The $D_2$-like receptor class has received considerable attention for its potential role in cognitive and behavioural flexibility. Human studies are primarily conducted using within-subject designs. In these studies, subjects are tested repeatedly with each test session being separated by several days or weeks, and the treatment conditions are counterbalanced. Some studies have reported that dopamine $D_2$ ligands impaired reversal learning, but only if they were administered during the first reversal learning session. If participants had already completed a reversal learning session, then reversal learning remained intact (Mehta et al., 1999, 2001). Therefore, repeated testing might obscure any effect and explain why other studies failed to observe effects (Dodds et al., 2008; van der Schaaf et al., 2013). The doses used in these studies may have also been insufficient to reliably alter brain activity relevant to reversal learning. Indeed, the highest dose of sulpiride used in these studies only produced 28% occupancy of striatal $D_2$ receptors (Mehta et al., 2008) and did not alter central metabolic activity during reversal learning (Dodds et al., 2008). Greater receptor occupancy may be required to reliably impact reversal learning which could be achieved by exploring a full dose-effect curve.

The impact of pharmacological manipulations is further complicated by existing individual differences in dopamine function. Carriers of the Taq1A polymorphism had reduced $D_2$ receptor density (see, Jönsson et al., 1999) and impairments in reversal learning (Jocham et al., 2009) and the effects of the $D_2$-like agonist, cabergoline, on reversal learning differed between carriers and non-carriers of this polymorphism (Cohen et al., 2007). The $D_2$-like agonists, bromocriptine, also improved reward-based reversal learning, but only in participants with lower baseline dopamine levels (Cools et al., 2009). These studies raise the important question of whether the impact of ligands can be examined without accounting for existing individual differences.

Although genetic and environmental factors are often exquisitely controlled in laboratory animals, variability in the effects of pharmacological manipulations of $D_2$ receptor function has also been observed. Systemic administration of dopamine $D_2$ antagonists has produced mixed effects on reversal learning (Alsiö et al., 2019; Boulougouris et al., 2009; Lee et al., 2007; Marino et al., 2022), whereas the $D_2$-like agonist, quinpirole, dose-dependently impaired reversal learning (Alsiö et al., 2019; Boulougouris et al., 2009). Local infusions also impacted reversal learning, but the infusion locus was a critical determinant of this effect. Equivocal results have been observed following local infusions of $D_2$-like antagonists into the NAcc core (Haluk &

Floresco, 2009; Sala-Bayo et al., 2020), whereas local infusions of the $D_2$-like agonist, quinpirole into the NAcc produced a similar dose-dependent impairment to what is observed following systemic administration (Alsiö et al., 2019). A reduction of NAcc $D_2$ receptors binding within the NAcc is proposed to be particularly important to the processing of negative RPEs, and so, this impairment may be the result of quinpirole preventing the typical negative feedback response from occurring under reversal learning conditions. Consistent with this idea, quinpirole doses that impaired reversal learning did so by completely blocking learning from negative feedback (Alsiö et al., 2019). It is important to note that the acute effects of full $D_2$ agonists in the NAcc are unlikely to mimic ordinary changes in dopamine activity which phasically increases and decreases due to task demands, and so, these effects may be better conceptualised as a model of aberrant dopamine function (see, Haluk & Floresco, 2009).

The impact of local infusions has also been examined in the DMS. Local infusions of the $D_2$-like antagonist, raclopride, impaired reversal learning and increased errors that occurred later in the test session (Sala-Bayo et al., 2020). This effect was comparable to those observed following DMS dopamine depletion, which resulted in increased errors during later testing. Further support for the idea that DMS $D_2$ mechanisms are important comes from a relatively recent study that locally infused various doses of the $D_2$-like agonist, quinpirole, into the DMS. Instead of analysing the impact of the drug by studying each dose, which is conventionally done, the authors fitted a dose-effect curve for each subject and observed a tri-phasic dose-response pattern. However, the dose-effect function varied considerably for each subject. Lower doses impaired reversal learning compared to vehicle conditions presumably via presynaptic mechanisms, whereas moderate doses improved reversal learning above vehicle levels, and higher doses were severely disruptive and prevented testing completion (Horst et al., 2019). These studies suggest the $D_2$ receptor is closely tied to reversal learning, but that the effects of acute drug challenges are likely to be nuanced by individual differences.

An alternative approach that overcomes these potential issues is to examine whether relatively static differences in receptor function contribute to individual differences in reversal learning. To examine this, Groman and colleagues (2011) conducted reversal learning in non-human primates and measured dopamine $D_2$ expression. Trials to criterion were fitted as a function of $D_2$ expression and two particularly important findings were reported. First, behavioural flexibility increased as a

function of DMS $D_2$ expression. Second, a non-linear curve best explained this, such that changes in trials to criterion were greatest between subjects that had the lowest levels of $D_2$ expression and performance reached asymptote much earlier than $D_2$ expression did. This study further stresses the importance of individual differences at both the neurochemical and behavioural level.

A critical role of $D_2$ expression has been reported by other studies. $D_2$ receptor knockdown impaired reversal learning in mice (Linden et al., 2018) and non-human primates (Takaji et al., 2016). Others have implicated $D_2$ downregulation in reversal learning by observing how repeated exposure to drugs that are misused impacted reversal learning. Reduced $D_2$ density was reported in subjects with a history of stimulant misuse (Ballard et al., 2015; Kohno et al., 2021; Volkow et al., 2001), as were reversal learning impairments (Ersche et al., 2011; Pilhatsch et al., 2020). In non-human primates, a 31-day methamphetamine regimen where the dose and number of daily injections were systematically increased resulted in concomitant impairments in reversal learning and $D_2$ expression which were correlated (Groman et al., 2012). Consistent with this report, others have also shown that repeated methamphetamine treatment impaired reversal learning (B. M. Cox et al., 2016; Groman et al., 2012, 2018; Izquierdo et al., 2010; Kosheleff et al., 2012; Perez Diaz et al., 2019) and reduced $D_2$ expression (Thanos et al., 2017). These studies implicate $D_2$ downregulation as a mechanism capable of impairing reversal learning, and collectively with the literature already reviewed in the present thesis, indicate that dopamine $D_2$ receptor expression is a critical determinant of behavioural flexibility (Groman et al., 2011; Jocham et al., 2009; Laughlin et al., 2011).

**Objectives of the Present Thesis**

An interesting question remains as to how upregulation of the $D_2$ receptor would impact reversal learning. Given that individual differences are an important determinant of drug effects, any changes in behaviour following upregulation might also be expected to relate to other $D_2$-dependent measures, such as pre-treatment behavioural flexibility. These ideas form the scope of the present thesis which aimed to determine whether an upregulation of the dopamine $D_2$ receptor impacted reversal learning, and in particular, whether this treatment would restore compromised behavioural flexibility (pre-existing or manipulated).

Reversal learning had not been measured in my laboratory group before the present thesis, so, the first objective was to identify a suitable measure for examining reversal learning. This required an initial literature review and the development of a programme capable of conducting the chosen procedure. The initial procedure used (detailed in Chapter 3) was a systematic replication of a previously published procedure. However, several issues emerged in my replication of this, and the follow-up study. It was decided that the most optimal path forward was to develop a novel procedure, and so, the first objective changed to developing a reliable measure of performance within a reversal learning task. A visual discrimination procedure was developed first. The acquisition criterion for this procedure was determined through a retrospective analysis of the data. After a reliable criterion was identified, a reversal learning procedure was developed. The purpose of these two studies was to develop a visual discrimination and reversal learning measure that was reliable, that accounted for individual differences in performance and resulted in relatively stable performance across serial reversals.

The next objective was to determine a treatment capable of upregulating dopamine $D_2$ receptors. This has been previously achieved through repeated administration of $D_2$ antagonists (Burt et al., 1977; LaHoste & Marshall, 1991; Memo et al., 1987; Samaha et al., 2007). Therefore, daily injections of the dopamine $D_2$-like antagonist, eticlopride, were administered (0.0mg/kg vs 0.3mg/kg) for 14 consecutive days and a brain-wide examination of $D_2$ expression was conducted using flow-cytometry. I expected that repeated eticlopride injections would upregulate dopamine $D_2$ receptors (Hypothesis 1, see Chapter 4).

The third objective was to determine whether reversal learning was impacted by a treatment that upregulated $D_2$ receptors and determine whether these changes were related to individual differences in behavioural flexibility. To do this, two reversal tasks were conducted prior to treatment, and another following repeated eticlopride treatment. This treatment was expected to improve reversal learning (Hypothesis 2, see Chapter 4) and based on the results of Groman (2011) discussed above, I expected that these might preferentially impact subjects that performed poorly on pre-treatment measures (Hypothesis 3, see Chapter 4).

The final objective was to determine whether experimentally produced behavioural inflexibility could be ameliorated by the same eticlopride treatment. Prior to eticlopride treatment, 4 methamphetamine injections (0.0mg/kg vs 2.0mg/kg) were

administered, 2 hours apart, in a single day. Methamphetamine was expected to impair reversal learning in the present study (Hypothesis 4, see Chapter 5). Further, the results of the prior study led to the prediction that repeated eticlopride treatment would improve reversal learning (Hypothesis 5, see Chapter 5). Lastly, these combined treatments were expected to counteract one another to some extent during reversal learning (Hypothesis 6, see Chapter 5).

# CHAPTER 2: GENERAL METHODS

These general methods were developed in Studies 3 and 4 (see, Chapter 3). Any deviations from these general methods are explicitly detailed.

## Subjects

All rats were male Sprague Dawley and were bred in the Te Toki a Rata vivarium at Victoria University of Wellington. Rats were pair-housed in individually ventilated odour- and allergen-free polycarbonate cages (C83510M, Optirat® GenII, Animal Care Systems Inc.). Cages contained a polycarbonate rectangular prism to serve as a shelter (ASRRR-PC, Able Scientific Pty Ltd, Australia) and a PuraTM aspen chew block (ASAEBM-A Able Scientific Pty Ltd, Australia). Temperature (20.5°C ± 0.5°C) and humidity (55% ± 5%) were controlled in housing and testing rooms. Water and food were available *ad libitum* prior to the start of experimental procedures. Once rats were approximately 8 weeks old, a food deprivation schedule was imposed to maintain them at 85-90% of their free-feeding weight. Initially, 8g-10g of chow was provided each day for 3-5 days to decrease weights within this range. Thereafter, 12-14g of chow was provided daily and target ranges were adjusted periodically to allow for ordinary weight changes.

## Apparatus

Standard operant chambers (ENV-008-VP; Med Associates Inc., USA) were equipped with two retractable levers (ENV-112CM) located on one wall of the chamber, one on the left side and one on the right side. An illuminable stimulus light (ENV-221M) was above each lever and a liquid dipper (ENV202M-S) that delivered 0.1mL of sweetened condensed milk was fixed between the two levers. Condensed milk (Nestlé Highlander, Sweetened Condensed Milk) was diluted with drinking water to produce a 27.5% volume/volume solution. The diluted condensed milk was refrigerated when not in use and made fresh every second day. MED-PC IV (Med Associates Inc., USA) was used to control the operant chamber and to collect all behavioural data.

## Procedure

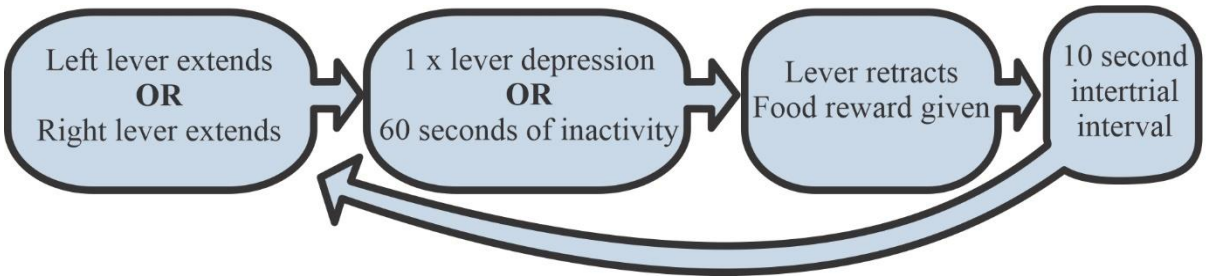
All behavioural testing was conducted during the dark phase of the day/night cycle (7:00 am - 7:00 pm). Each subject was assigned to a specific operant chamber and exclusively tested in this chamber throughout each study. Housing cages were transported to the testing room and remained there until the session ended. At this point, rats were returned to their housing cages, and the housing cages were returned to the colony.

### *FR-1 Lever Training (Autoshaping)*

Either the left or right lever was extended into the chamber at the start of each trial. Lever order was pseudorandomised, and each lever was presented twice every four trials. Following a single lever depression or 60s, whichever came first, the lever retracted, and the liquid dipper was activated. There was a 10s inter-trial interval and the session concluded when 100 trials were completed or 45 minutes had elapsed, whichever occurred first. This initial training was considered complete once a minimum of 25 responses were made on each lever during two consecutive daily sessions. See Figure 2.1 for a schematic of an FR-1 lever training trial.

**Figure 2.1**

*A Schematic of an FR-1 Lever Training Trial*




### *FR-3 Lever Training*

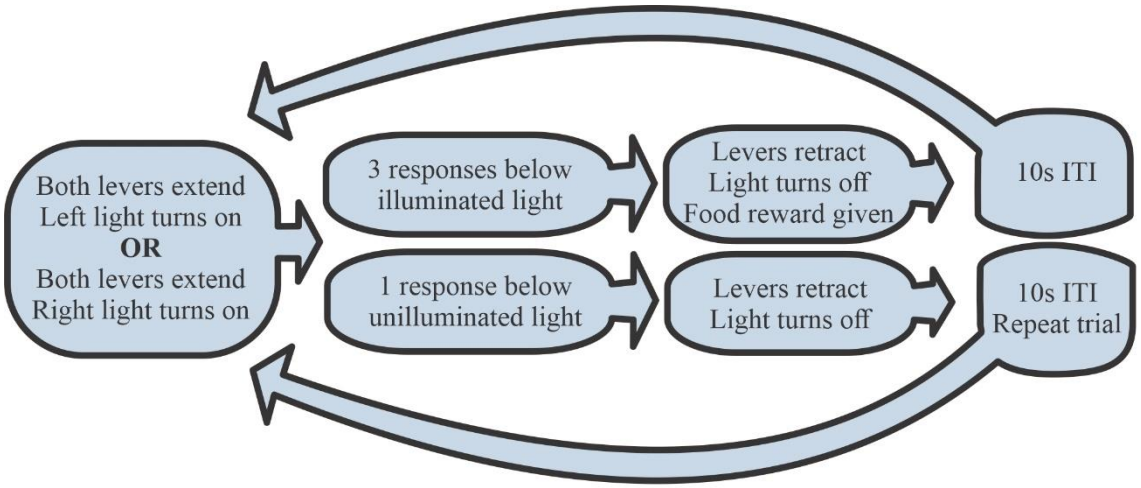
During this next phase of training, three lever depressions were required for the dipper to be activated. Failure to respond within 60s was recorded as “inactivity” but had no programmable consequence. This phase of training was considered complete once 45 or more FR-3 responses were made on each lever during two consecutive daily sessions.

***Visual Discrimination Training***

During visual discrimination training, both levers were extended into the chamber at the start of each trial and a stimulus light was illuminated above one of the levers. The order of presentation of the illuminated light was pseudorandomised such that either light was illuminated twice every four trials. All subjects were exclusively reinforced for depressing the lever below the illuminated light. After 3 consecutive depressions of the appropriate lever the stimulus light turned off, the two levers retracted, the liquid dipper presented sweetened condensed milk, and the trial ended. Following an incorrect response, the stimulus light was turned off, both levers retracted, and the trial immediately ended. If an incorrect response was made, the same light was illuminated on the following trial. This was repeated until a correct response was made. Thereafter the pseudorandomised presentation of the light stimulus recurred. Every 60s period during which no lever responses occurred was recorded as inactivity. There was a 10s intertrial interval. Each session lasted until 100 trials were completed or 45 minutes elapsed, whichever occurred first. Data from the 100 trials were analysed in blocks of 10 trials. Testing continued until 80% of responses made in 8 of the 10 blocks were on the correct lever in a single day. See Figure 2.2 for a schematic of a visual discrimination trial.

**Figure 2.2**

*A Schematic of a Visual Discrimination Lever Training Trial*



***Reversal Learning***

Three serial reversal learning tasks were conducted. Two tasks were conducted before treatment to establish a baseline measure of reversal learning and to ensure that performance for each rat was relatively stable. A third reversal task was conducted following the treatment period. During the first reversal task, the response rule was the opposite of the one that had been used in visual discrimination learning; depressing the lever below the unilluminated light was exclusively reinforced according to an FR-3 schedule. A maximum of 500 trials were conducted daily. These trials were organised into blocks of 10 trials and light presentation was pseudorandomised, as in visual discrimination testing. The session ended when either all 500 trials were completed, 120 minutes elapsed, or there were five consecutive minutes of inactivity once 200 or more correct trials had been completed (i.e., satiation). Daily sessions continued until accuracy[1] in each block reached at least 80%, for 8 of 10 consecutive blocks, in a single session.

Prior to starting the second reversal task, depression of the lever below the unilluminated light continued to be reinforced but the session was reduced to 10 blocks of 10 trials. These "retention" tests were conducted to ensure that responding had stabilised and was comparable to the initial visual discrimination before the subsequent reversal task. These sessions continued until the criterion of 80% accuracy in 8 out of 10 blocks had been achieved. The second reversal task started the following day. Now depression of the lever below the illuminated light was exclusively reinforced. The number of trials to criterion (TTC) was determined as the number of trials completed immediately prior to the 100 trials in which the criterion was met for each subject. Testing was conducted 7 days a week for the entire duration of each study.

Drug treatments commenced the day after the criterion was met in the second reversal learning task. Subjects were assigned to the treatment groups by ensuring that the range of TTC scores and the average TTC scores for the first two reversal tasks were comparable for each drug condition. All treatments were followed by two rest days to ensure all treatment compounds were eliminated prior to retention testing.

[1] Accuracy was calculated using the following formula: $100\% \times \frac{Correct\ Trials\ in\ Current\ Block}{10\ (total\ trials\ in\ each\ block)}$

The following day, a retention test was conducted in which the depression of the lever below the illuminated light continued to be reinforced for 10 blocks of 10 trials. Daily sessions were conducted to ensure the criterion of 80% accuracy in 8 out of 10 blocks was achieved and that accuracy had stabilised. The final reversal task was then conducted in which responding on the lever below the unilluminated light was reinforced. The lever response that is reinforced and the number of daily trials conducted during each stage of testing are summarised in Table 2.1.

**Table 2.1**

*Lever Responses Reinforced and Maximum Daily Trials in Each Stage of Behavioural Testing*

| Stage of Testing | Reinforced Lever Response | Maximum Trials |
|---|---|---|
| First Visual Discrimination | Below illuminated light | 100 |
| First Reversal Learning Task | Below unilluminated light | 500 |
| Retention of the First Reversal | Below unilluminated light | 100 |
| Second Reversal Learning Task | Below illuminated light | 500 |
| Drug Treatment Followed by Two Rest Days | | |
| Retention of the Second Reversal | Below illuminated light | 100 |
| Third Reversal Learning Task | Below unilluminated light | 500 |

*Note.* This table shows the lever response that was reinforced and the maximum number of trials available during each stage of testing. Drug treatment commenced the day after the criterion was met in the second reversal learning task.

### Data Analysis

The analyses were specific for each study, and so, the data analysis for each study is detailed within the corresponding chapter. In all cases, the assumptions for any statistical test were tested and the results of these are discussed in each chapter. Any instances where these assumptions were violated are detailed, as well as the transformation(s) applied. The results of these assumptions tests for the raw data and any transformed data subject to statistical analysis are presented in the appendices of this

thesis and will be referred to in the text using the following format: “Table A1” refers to “Table 1” presented in “Appendix A”. All analyses were conducted using SPSS Version 27 (IBM Corporation, Armonk, NY), Prism version 6.1 (GraphPad, La Jolla, CA), or Jamovi version 2.3 (The Jamovi Project). The level of significance for all testing was set to $\alpha = .05$ level. All corrections used are detailed in the appropriate chapters. Tables were created with Microsoft Excel or Word (Microsoft Corporation), graphical figures were created using Prism 6.1 (GraphPad, La Jolla, CA) and illustrative figures were created using CorelDRAW 2021 (Corel Corporation).

**Drugs**

Eticlopride (S-(−)-Eticlopride hydrochloride, Sigma-Aldrich Castle Hill, Australia) was dissolved in 0.9% saline solution and the solution was prepared fresh every 1-2 days. Methamphetamine (N-methylamphetamine hydrochloride, BDG, Porirua, New Zealand) was dissolved in a 0.9% saline solution and the solution was prepared fresh as was required and no solution was used for more than 2 days. Both solutions were administered in a volume of 1ml/kg. The drug weight described in chapters 4 and 5 refers to the salt.

**Ethics Approval**

All experimental procedures conducted in this thesis were in accordance with animal ethics applications that were approved by the Animal Ethics Committee at Victoria University of Wellington prior to testing commencing (applications: 26249, 27156).

# CHAPTER 3: DEVELOPING A REVERSAL LEARNING TASK WITH RATS

*Parts of this chapter have been adapted, with permission, from previously published work in Behavioural Brain Research (Highgate et al., 2022).*

## **Study 1: Systematic Replication of Boulougouris and Colleagues** (2007)

### Introduction

Reversal learning had not been measured by our laboratory group prior to the present thesis, and so, an existing procedure that had been previously reported with spatial discrimination was systematically replicated (Boulougouris et al., 2007). This procedure was chosen because it utilised equipment already accessible in our laboratory (i.e., retractable levers, stimulus lights) and it was produced by a reputable research group. The aim of the following study was to systematically replicate this procedure and explore the behavioural profile produced by it. This study was purely exploratory, and so, there were no *a priori* hypotheses.

### Methods

All information relating to subjects, housing conditions, and testing apparatus is detailed in the general methods section. Any deviations from these are explicitly stated in the text below and are the result of this study being a replication of methods that differ from those outlined in the general introduction. The sample size was 13.

#### Procedure

The start of all trials was signalled by the illumination of a house light. FR-1 and FR-3 lever training were conducted as described in the general methods, but all sessions ended after 50 daily trials were completed or 15 minutes elapsed, whichever occurred first. Further, during FR-3 training, failure to respond within 15 seconds resulted in the termination of that trial, retraction of the lever, and the trial was scored as an omission. The criterion for completion of FR-1 and FR-3 training was completing all 50 trials in a single test session.

Visual discrimination sessions also commenced with the illumination of a house light, followed by the extension of both levers and pseudorandom illumination of one of the stimulus lights. Depressing the lever below the illuminated light resulted in retraction of both levers, the light turning off, and reinforcement delivery. Incorrect lever depression or failure to respond within 15 seconds (omission) also resulted in both lever retracting and the stimulus light turning off. Daily tests continued until 5 blocks of 10 trials were completed or the criterion of 90% accuracy (correct trials divided by 10) in a single block of 10 trials was met, whichever occurred first. If this criterion was met, the test session closed immediately, and all equipment was turned off. Reversal learning sessions started the following day.

All reversal learning sessions contained two stages: *retention testing* and *reversal testing.* During retention testing, the previous contingency continued to be reinforced (i.e., depressing the lever below the illuminated light) for up to 5 blocks of 10 trials, 15 minutes, or until the criterion of 90% accuracy in a single block of 10 trials was met, whichever occurred first. Failure to meet the criterion during retention testing resulted in the session terminating immediately. If this criterion was met, the contingencies were immediately reversed, and *reversal testing* started. During reversal learning, up to 5 blocks of 10 trials were completed and the criterion for completing reversal learning was 90% accuracy in a single block during the reversal test. Failure to do so resulted in the session being repeated the subsequent day. In summary, to complete reversal learning, the criterion was required to be met during the retention test and reversal test in a single day.

The procedure detailed here differed in two ways from the original procedure used by Boulougouris and colleagues (2007). First, in the original procedure, a head-entry response was required to be used after the house light was illuminated. This resulted in the extension of the levers and the illumination of the stimulus light. Effectively, this serves as a trial-initiating response. I did not have access to the equipment required to include this, so it was omitted from the procedure. Second, my replication utilised visual discrimination. In the original study, a light was pseudorandomly presented during spatial discrimination trials so that the procedure could be easily adapted to examine visual discrimination in the future, but to date, these methods have not been published.

## Data Analysis

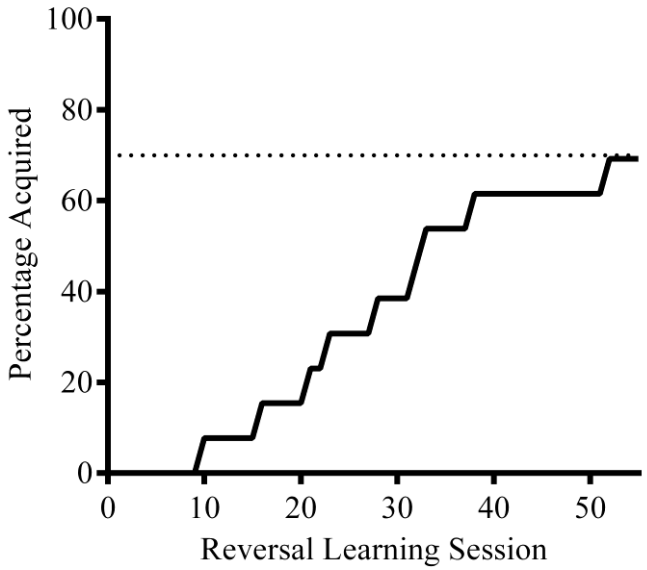
The purpose of this study was to determine the general parameters for measuring reversal learning in rats. As such, no inferential data analysis was conducted. The initial FR-1, FR-3, and visual discrimination tasks were readily completed within 2-3 weeks of testing by most rats, as expected based on the original report (Boulougouris et al., 2007). The focus of this analysis was to explore the reversal learning data and determine how readily a single reversal task could be completed and what the typical behavioural profile is under these reversal learning conditions.

## Results

An initial examination of the behavioural data indicated that this task was much more difficult to complete than expected. 30% of the sample failed to reverse within 55 days of testing, at which point, testing stopped. Further, of those that did learn the reversal, the mean number of days required to complete the single reversal task was high ($M$ = 28.11; range: 10 days – 52 days). The acquisition data are shown in Figure 3.1.

**Figure 3.1**

*Cumulative Percentage of Sample to Reach Criterion as a Function of Session*




*Note.* The dashed line indicates the percentage of the sample that completed reversal learning within 55 days of testing.

As noted in the methods above, each reversal learning session included both retention testing and reversal testing (provided the retention criterion was met). A closer examination of the data indicated the inclusion of retention testing likely contributed to this protracted acquisition since approximately one-third (range for individual subjects: 14% - 54%) of all reversal learning sessions were terminated because the criterion was not met during retention testing. Consequently, reversal learning did not occur on these days and exposure to the reversal contingencies was highly intermittent (there tended to be several consecutive test days between sessions where the criterion was met during retention testing). This confounds the reversal learning data. The second study of this thesis adapted this procedure to mitigate this issue.

## Study 2: Systematic Replication of Study 1

### Introduction

One of the aims of the present thesis was to determine how experimenter-produced neuroadaptations impact reversal learning. However, these changes can revert to pre-treatment levels over time. As such, it was imperative that reversal learning be completed much faster than it was in Study 1. One possibility is that increasing the number of daily trials would facilitate reversal learning. Increasing the number of retention trials would provide additional opportunities to progress to reversal learning each day, even if responding was relatively inaccurate initially. Further, the increased reversal learning trials also provide a greater opportunity to reach the reversal criterion during the reversal learning test. The aim of this study was to facilitate reversal learning by adapting the procedure detailed in Study 1.

### Methods

This study was conducted according to the methods detailed in Study 1, except up to 10 blocks of 10 trials were conducted during visual discrimination testing, retention testing, and reversal testing. The sample size was 12.

## Data Analysis

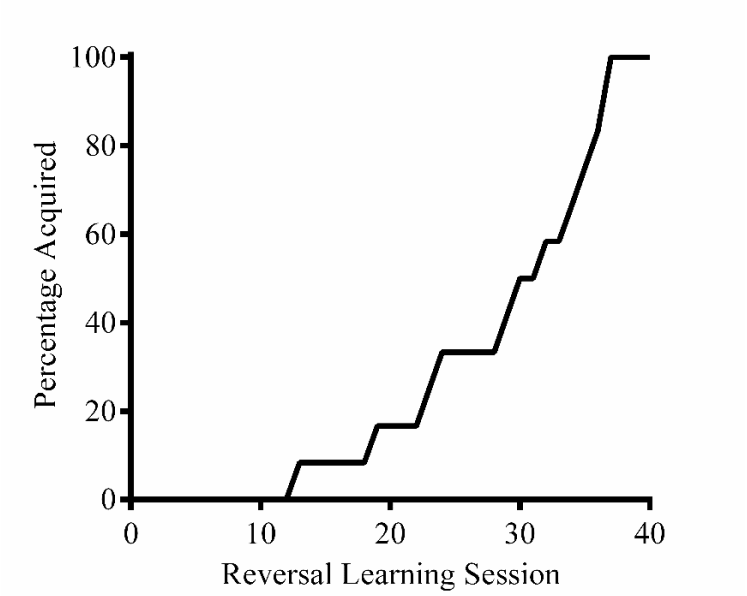
The number of days to criterion was determined, as well as the percentage of days where reversal training did not commence because the retention criterion was not met.

## Results

Increasing the number of daily blocks did facilitate reversal learning to some extent. In the first study, 30% of the sample failed to acquire the reversal within 55 days of testing, whereas the entire sample was able to acquire the reversal learning behaviour in the present study within 37 reversal learning sessions. However, the days to criterion remained long (*M* = 29.08; range: 13 days – 37 days; acquisition data are shown in Figure 3.2.). Contrary to my expectations, increasing the number of daily trials decreased the likelihood that the retention criterion was met. The retention criterion was not met during 42.44% of the reversal learning sessions (range for individual subjects: 23% - 75%) which prevented reversal learning from being conducted during these sessions. The acquisition data are shown in Figure 3.2.

**Figure 3.2**

*Cumulative Percentage of Sample to Reach Criterion as a Function of Session*



Another striking feature observed in the data was the tendency for responding to be relatively inaccurate during the first retention test before subjects had any experience with the reversal learning rule. 75% of the sample failed to meet the retention criterion during the first block of retention testing and the overall accuracy was low during this first block ($M$ = 67%). Further, the proportion of the sample that failed to meet the criterion required another 40 trials, on average, to do so.

## Discussion: Study 1 and Study 2

The aim of Study 1 was to determine the standard behavioural profile of subjects that completed my adaptation of a previously published reversal learning task. The observed behavioural profile was problematic for the aims of the present thesis, and these issues were not remedied by the changes employed in Study 2, despite them facilitating reversal learning. The original study reported that 87.25 retention trials and 124.42 reversal trials were required during the first spatial reversal before the reversal learning criterion was met, on average. In my initial replication, 458 retention trials and 512 reversal trials were required to complete the first reversal, on average. Possible reasons for this protracted acquisition are discussed below.

One possibility is that the use of a visual discrimination task instead of a spatial discrimination task negatively impacted behavioural performance. However, a direct comparison between these two versions of the procedure detailed here, has to my knowledge, not been reported. Some have suggested that the acquisition of visual discrimination is somewhat protracted compared to the acquisition of spatial discrimination (e.g., required approximately twice as much training; Wright et al., 2019), but this contrasts with the 5-fold increase in trials observed in Study 1 relative to those reported in the original paper (see, Boulougouris et al., 2007). As such, this exceedingly longer acquisition is unlikely to be exclusively due to the use of visual discrimination.

It may also be the case that omitting the trial-initiating response from this procedure negatively impacted performance. Indeed, the rate at which visual discrimination was acquired in an automated touchscreen procedure was reduced in a sample of Sprague-Dawley rats not required to produce a trial-initiating response compared to a sample that was required to produce this trial-initiating response. However, another interesting finding was reported in this same study. Namely, mean accuracy in both samples was below 90%, and appeared to asymptote between 75% and 85%, even

after 20 consecutive visual discrimination sessions (Bussey et al., 2008). Therefore, both the omission of the trial-initiating response and the high criterion requirement in the first two studies (90% accuracy) may have contributed to this prolonged acquisition.

It was also noted there was a tendency for responding to be inaccurate following the 24-hour delay that separated the last visual discrimination session and the first retention test. It is not clear as to why this occurred, but it may indicate that responding was relatively unstable when the criterion was met or that criterion was being met by chance. As such, it may be that persistence of accuracy needs to be considered when using visual discrimination (i.e., more than 9 accurate trials are needed).

The inclusion of retention testing was highly problematic in both studies and undoubtedly contributed to the prolonged reversal learning testing. The fact the retention criterion was so infrequently met resulted in highly intermittent reversal learning, and for some subjects, many test sessions separated subsequent reversal tests. It may be that this was also, at least in part, the result of the other factors discussed since there were no issues reported in the paper these studies were based on (Boulougouris et al., 2007). Faced with these mounting concerns, the current procedure was tabled, and the focus shifted towards the development of a procedure and discrimination criterion that would allow me to assess the aims outlined in the General Introduction.

## Study 3: Developing a Visual Discrimination Procedure

### Introduction

Several problems were observed in Studies 1 and 2 which indicated the procedure used would not be suitable for meeting the aims of the present thesis. Consequently, the focus shifted towards developing a visual discrimination reversal learning procedure. One of the concerns discussed in the previous section was that the criterion may not have been suitable for indicating when the behaviour was being reliably produced. Of interest, choice of criterion is also likely to be the most variable aspect of reversal learning procedures and this might be expected to impact task performance considerably. Overly lenient criteria may be met before discrimination is reliably observed, as was suggested in the previous discussion section, whereas overly stringent criteria may not be met within a reasonable timeframe. This raises an important question relevant to the present thesis, namely, what constitutes discrimination?

An argument for discrimination can be made when the reinforced behaviour occurs more often than the non-reinforced behaviour (i.e., more than 50% of the trials). However, the caveat with this definition is that responding on one lever is expected to fluctuate, both above and below 50%, if a subject is operating at chance-level responding under two-choice discrimination conditions (see, Fields, 1932). To overcome this, more stringent accuracy requirements are typically applied. Accuracy requirements typically range from 70% up to 100%, but most studies require the stimuli to be correctly discriminated to a level between 80% and 90% (e.g., Alsiö et al., 2015; Boulougouris et al., 2007; Chudasama & Robbins, 2003). There is currently no accepted gold standard as to the level of accuracy that should be used.

The number of trials and days of testing this level of accuracy is required to persist also varies across studies. In terms of within-session persistence, some studies require 100% accuracy during a relatively small number of consecutive trials. For example, discrimination may be considered acquired once there are 8 consecutive trials of accurate responses regardless of the total number of trials in a test session (e.g., Floresco et al., 2008). Other studies require a lower level of accuracy but over a greater number of trials. For example, discrimination may be considered acquired when there is 80% accuracy in 100 trials (e.g., Roebuck et al., 2020). In terms of persistence across sessions, some studies require accuracy to persist for 2 (e.g., Bryce & Howland, 2015) or 3 (e.g., Amodeo et al., 2017) consecutive days whereas others only require a single day of accuracy (e.g., Boulougouris et al., 2007). To determine an appropriate discrimination criterion, careful consideration of these persistence variables is also required.

A discrimination criterion in these tasks serves to delineate the point at which the discrimination has truly been acquired, yet a determination of whether this is the case is rare. One approach to determining whether a criterion reflects this is to collect an extensive amount of visual discrimination data and then retrospectively apply criteria that vary according to different accuracy and persistence requirements. This approach is not well documented but was recently used in a study of wild avian species (Reichert et al., 2020). To do this, wild birds were fitted with a tag that allowed them to activate a visually distinct feeder. Visual discrimination sessions were conducted for eight days. Two different accuracy criteria (80% or 90%) and 3 different persistence criteria (10, 20, or 30 consecutive trials) were retrospectively applied to determine how they impacted the acquisition of visual discrimination. The number of birds that would be described as

having acquired the visual discrimination was dependent on both criteria variables. Perhaps unsurprisingly, the number of subjects that met criterion decreased as accuracy and/or persistence requirements became more stringent. The criterion of 80% accuracy during 20 consecutive trials was met by 92% of the sample, and so, this criterion was suggested to be reasonable. To determine whether accuracy persisted after this criterion was met, it was determined how many subjects responded at the correct location on 80% or more of the remaining trials. 75% of the total sample displayed this post-acquisition persistence, and so, this criterion was suggested to be a reasonable indication of acquisition. Unfortunately, between-session persistence was not examined for the other criteria (Reichert et al., 2020). The authors suggested 75% of the sample maintaining accuracy was sufficient evidence of persistence, but a greater proportion, or all of the sample, might be expected to remain accurate if a criterion truly delineates subjects that have acquired the discrimination from those that have not.

In the following study, criteria with varied accuracy and persistence requirements were retrospectively applied to visual discrimination data obtained over 25 days of testing in Sprague Dawley rats. I used criteria that required 80% or 90% accuracy since these are commonly used levels of accuracy. The criteria required accuracy to persist for various numbers of trials within each session and over a varying number of days. I determined how readily each criterion was met, the amount of subject attrition associated with each criterion, and persistence of accuracy. The aim of this study was to determine a reliable criterion for measuring visual discrimination.

## Methods

All information relating to subjects, housing conditions, and testing apparatus is detailed in the general methods section. Any deviations from these are explicitly stated in the text below. The sample size was 36.

### Visual Discrimination Training

After completing FR-3 training, subjects were randomly assigned to one of two groups. They were exclusively reinforced for depressing the lever below the illuminated (n = 18) or below the unilluminated light (n = 18). Visual discrimination testing continued for 25 consecutive days, irrespective of performance, and no reversal learning was conducted.

**Data Analysis**

Various criteria for acquisition were applied to the visual discrimination. Each of the daily 100 trials was divided into blocks of 10 and the percentage of correct responses within each of the 10 daily blocks was determined. I set a criterion of 80% or 90% accuracy in either 8 of 10 blocks or all 10 blocks during each daily session. These criteria were required to be met for 1, 3, or 5 daily consecutive testing sessions. The number of days required to meet each criterion was the dependent measure in this study.

I first ensured no extreme scores were present in the data using a rule of greater than three standard deviations away from the mean. Then I determined the percentage of the sample that acquired each criterion. Failure to acquire discrimination is seldom reported, and so, any criterion that was not acquired by the entire sample within 25 test days, which I consider to be a long testing period for a single discrimination task, was deemed overly stringent and not considered further.

I planned to conduct serial reversal learning in future studies. To avoid introducing any noise related to the behaviour being reinforced, it was also imperative that both visual discrimination behaviours could be acquired within a comparable time frame. As such, days to criterion for the remaining criteria were subject to TOST (two one-sided tests) tests to determine whether the two groups were equivalent. My sample sizes were relatively small, and so, to determine the bounds of the test, a power analysis was conducted and the minimum effect size that would be detected with confidence was calculated (see, Lakens, 2017). The resulting value was $d = 1.318$, and so, the lower bound was set to -1.318 and the upper bound set to 1.318.

The skewness of the distribution is seldom addressed in studies relevant to the present thesis, but some have suggested it may be positively skewed (e.g., Barlow et al., 2015; Miletto Petrazzini et al., 2017). Positive (or rightward) skew can be produced because a disproportionate number of subjects produce higher scores on these measures than expected according to a normal distribution[2]. It's possible that the magnitude of this skew is, at least in part, dependent on the criteria applied for learning and the application

[2] It is noted that this is not the only cause of distributional skew. For example, skew may also be the result of a scale or measure being bounded (i.e., the limits of the measure are finite).

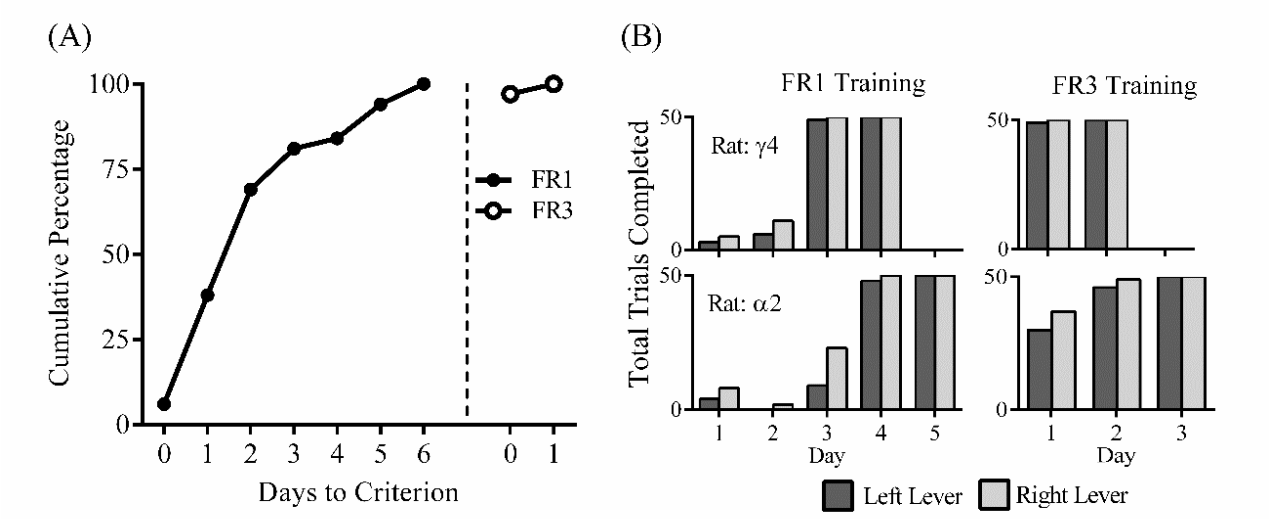
of more lenient or stringent criteria will impact the extent of the skew observed. The remaining criteria were subject to examination of skewness and one criterion was selected as most appropriate. Overall session accuracy was also examined in this chosen criterion over the two sessions immediately following acquisition to determine whether accuracy persisted once the criterion was met.

## Results

FR-1 lever training was rapidly completed with most rats only requiring 3-5 sessions before meeting the criterion (Figure 3.3, Panel A). Responding remained high when the FR was increased, and no more than 2 additional training sessions were required before the FR-3 criterion was met. The data from two randomly selected representative rats are shown alongside the sample acquisition curve in Figure 3.3 (Panel B).

**Figure 3.3**

*Acquisition of the Initial FR-1 and FR-3 Lever Training*




*Note.* This figure shows the cumulative percentage of the sample to meet the FR-1 and FR-3 criteria in Panel A. Days to criterion did not include the day criterion was met, and so, the 0 values on the x-axis indicate that the criterion was met on the first day of testing. Panel B shows the number of trials completed by two randomly selected representative rats as a function of test day during FR-1 and FR-3 lever training.

The number of days to criterion was more than three standard deviations greater than the sample means for one of the rats on all criteria. The data from this rat were therefore excluded from any analysis. Data from three other rats had to be excluded as ongoing lever malfunction prevented them from completing testing. The final sample size was 32 (n=16, illuminated; n=16, unilluminated). Days to criterion and the proportion of each group that met each criterion is presented in Table 3.1.

**Table 3.1**

*Percentage of Rats and Latency to Meet Various Criteria for Acquisition of the Visual Discrimination*

| Block Accuracy | Accurate Blocks | Days Criterion Must be Met | Percentage to Meet Criterion | | Mean [Range] Days to Criterion | |
|---|---|---|---|---|---|---|
| | | | Illuminated | Unilluminated | Illuminated | Unilluminated |
| 80% | 8 of 10 | 1 | 100% | 100% | 5.5 [3-10] | 6.4 [3-10] |
| | | 3 | 100% | 100% | 5.7 [3-10] | 6.6 [3-12] |
| | | 5 | 100% | 100% | 5.8 [3-12] | 8.9 [5-15] |
| | All 10 | 1 | 100% | 100% | 8.1 [4-15] | 10.6 [7-17] |
| | | 3 | 88% | 82% | 12.7 [8-21] | 16.5 [11-22] |
| | | 5 | 82% | 44% | 16.0 [10-20] | 17.3 [10-20] |
| 90% | 8 of 10 | 1 | 100% | 100% | 6.4 [3-13] | 8.6 [4-14] |
| | | 3 | 88% | 88% | 6.8 [3-12] | 10.0 [4-18] |
| | | 5 | 88% | 81% | 8.6 [3-16] | 11.5 [5-16] |
| | All 10 | 1 | 94% | 81% | 10.3 [6-18] | 11.8 [7-20] |
| | | 3 | 56% | 37% | 15.0 [8-18] | 19.0 [17-22] |
| | | 5 | 41% | 0% | 17.3 [10-20] | - |

*Note.* This table shows the percentage of the sample to meet each criterion and the number of consecutive days required (both mean and range) to do so for each combination of block accuracy, within-session persistence (number of blocks), and between-session persistence variable.

When the most stringent criterion of 90% accuracy in all 10 blocks was applied to the data, attrition was observed, irrespective of the number of days the criterion needed to be met and this became markedly worse as the number of consecutive days required to meet criterion increased. These three criteria were deemed too stringent and not considered further. When the criterion was relaxed by reducing the number of accurate blocks in each session (90% accuracy in 8 of 10 blocks) or by reducing the number of correct responses required in each block (80% accuracy in 10 blocks), the entire sample was able to meet criterion for a single day indicating that these may be appropriate. However, there was also a progressive reduction in the percentage of subjects that met these criteria as the number of consecutive days increased. As such, only the 1-day criteria were subject to further testing. When a criterion was applied that reduced the accuracy and number of blocks (80% accuracy in 8 of 10 blocks), all subjects met the criterion for 1, 3, and 5 consecutive days. Based on these observations, five criteria remained: 80% accuracy in 8 out of 10 blocks for 1, 3, or 5 consecutive days, 80% accuracy in all 10 blocks for 1 day, and 90% accuracy in 8 out of 10 blocks for 1 day.

The remaining criteria were subject to TOST testing to determine whether the mean days to acquisition were equivalent between the two groups (illuminated vs unilluminated). The data did not meet the assumptions for the TOST test when the criterion '80% accuracy in 8 of 10 blocks for 5 consecutive days' or '80% accuracy in all 10 blocks for 1 day' were applied but did following a reciprocal transformation ($x^{-1}$ or 1/x). These assumptions were met when the three other criteria were applied (see, "Table A1" and "Table A2" for all assumptions results). The results of the TOST testing suggested that the two groups were only equivalent when the criteria requiring '80% accuracy in 8 of 10 blocks for 1 day' and '80% accuracy in 8 of 10 blocks for 3 days' were applied (see, "Table A3"). Based on this analysis, either of these criteria appeared suitable for use.

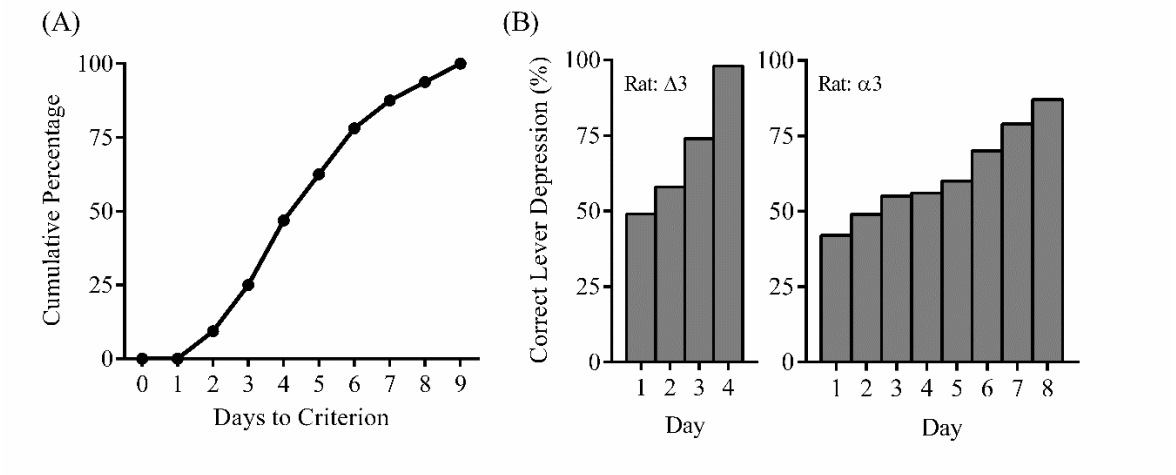
The distribution of data was examined in the remaining two criteria, and consistent with others, positive skew was observed. However, the distribution was less skewed when I applied the criterion requiring 1 [$W$ (32) = .95, $p$ = .113; Skew = 0.42], compared to 3 [$W$ (32) = .94, $p$ = .093; Skew = 0.73] consecutive days of accurate responding. As such, the criterion requiring 80% accuracy in 8 of 10 blocks for 1 day was considered the most reasonable criterion for acquisition of the visual discrimination.

Importantly, accuracy remained above 80% during the two days immediately following acquisition for all subjects in the final sample after meeting this criterion.

The acquisition data are displayed in Figure 3.4 (Panel A). The percentage of rats that acquired the visual discrimination steadily increased over days once three training sessions had been completed. Lever responding accuracy from two representative rats, a "fast learner" and a "slow learner", is shown in Figure 3.4 (Panel B). In both cases, accuracy progressively develops as a function of days up until acquisition, consistent with previous reports (Jonkman et al., 2009).

**Figure 3.4**

*Acquisition of the Visual Discrimination Using the Selected Criterion*




*Note.* This figure shows the cumulative percentage of the sample to meet the criterion of 80% accuracy in 8 of 10 blocks for a single day. Days to criterion did not include the day criterion was met, and so, the zero values on the x-axis indicate that criterion was met on the first day of testing. Panel B shows lever accuracy for a relatively "fast" and "slow" acquiring rat.

## Study 4: Extending the Procedure to Include Reversal Learning

### Introduction

The previous study determined a criterion that would be appropriate for indicating when visual discrimination was acquired. Study 4 extended this procedure to

include serial reversal learning tasks. As noted in the discussion of Studies 1 and 2, requiring a retention criterion to be met at the start of every reversal learning session was highly problematic, and so, this was not included in the procedure.

One of the advantages of measuring behavioural flexibility in non-human subjects is that behavioural flexibility scores can be determined before manipulations are imposed. This allows for a determination of whether pre-existing differences in behavioural flexibility contribute to the effect(s) of the manipulation of interest. However, this also assumes that these pre-existing differences are relatively stable. As such, the aim of this study was to determine whether a reversal learning task that utilised a reliable discrimination criterion would result in stable reversal learning data.

## Methods

This procedure was identical to those described in the general methods, except no drug regimen was imposed. As a result, the retention test following the second reversal rule started the day after the criterion was met in the second reversal learning task. The sample size was 20.

### Reversal Learning

Reversal learning was expected to require more daily sessions to complete than the initial visual discrimination, as has been previously reported (Chudasama & Robbins, 2003). Because these preliminary pilot studies aimed to provide methods for eventually measuring reversal learning following experimental manipulations, it was important to be able to measure reversal learning in as short a time as possible so that both transient and persistent effects of manipulations could be measured. I, therefore, increased the total number of daily trials to 500 to reduce the number of daily sessions required. The session ended after all 500 trials were completed or 120 minutes had elapsed, whichever occurred first.

During the first reversal tests, I observed that many subjects stopped responding once 200-250 correct responses had been produced, possibly due to satiation. We, therefore, imposed this additional criterion for termination of a test session: if there were five consecutive minutes of inactivity following 200 or more correct trials, the session was terminated. As the sessions often closed before all 500 trials were conducted, the

number of trials to criterion (TTC) was measured as the dependent variable, rather than days to criterion.

### Data Analysis

The study was exploratory, and so, it included a limited data analysis. The distribution of the reversal learning data was examined for normality and skewness. In my future studies, I intend to determine how behavioural flexibility changes following various manipulations by measuring reversal learning before and after these manipulations are imposed. This approach requires that individual differences in behavioural flexibility are stable, even following successive reversals. This was achieved by conducting a Spearman's correlation on the rank-ordered individual scores across all three reversal measures (see, Logan, 2016).

## Results

The TTC measure from two rats was more than three standard deviations away from the mean TTC of all three reversals. These data were therefore considered outliers and were excluded from any analysis. The remaining sample size was 18.

I planned to match subjects prior to imposing manipulations based on their performance during the first two reversal tasks and required any changes between these tasks to be within a reasonable range. To determine how much TTC changed between these reversal tasks, reversal 1 TTC was subtracted from reversal 2 TTC for each subject. Those that improved in reversal 2, did so by up to 462 trials. When performance declined, it did so by up to 171 trials. For two rats, however, 1194 and 1516 additional trials were required to meet criterion during reversal 2, compared to reversal 1. These data were outliers according to Tukey's criterion and the data were not analysed further. It was also observed that these data were 2.3 and 2.9 standard deviations away from the average change. An additional criterion for indicating an unstable baseline was therefore imposed on the data: any subject that changed more than 2 standard deviations away from the average change in TTC between reversals 1 and 2 for the group was considered unstable and was not included in further analysis. This led to the exclusion of data from these 2 rats. The final sample size was 16. The reversal learning data are shown in Figure 3.5.

**Figure 3.5**

*Average TTC (+SEM) as a Function of Reversal Learning Task for the Final Sample*

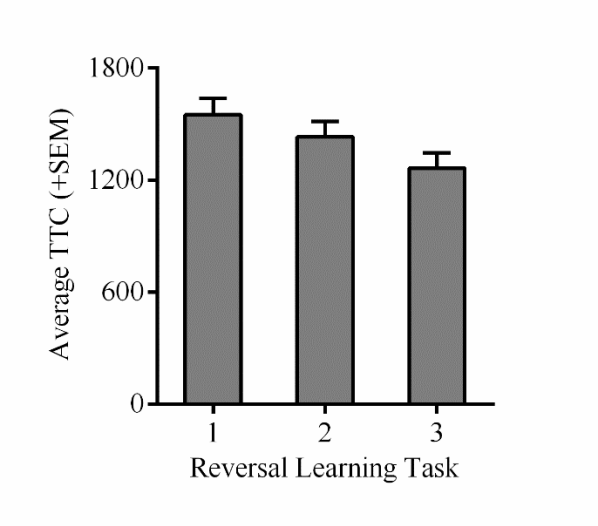
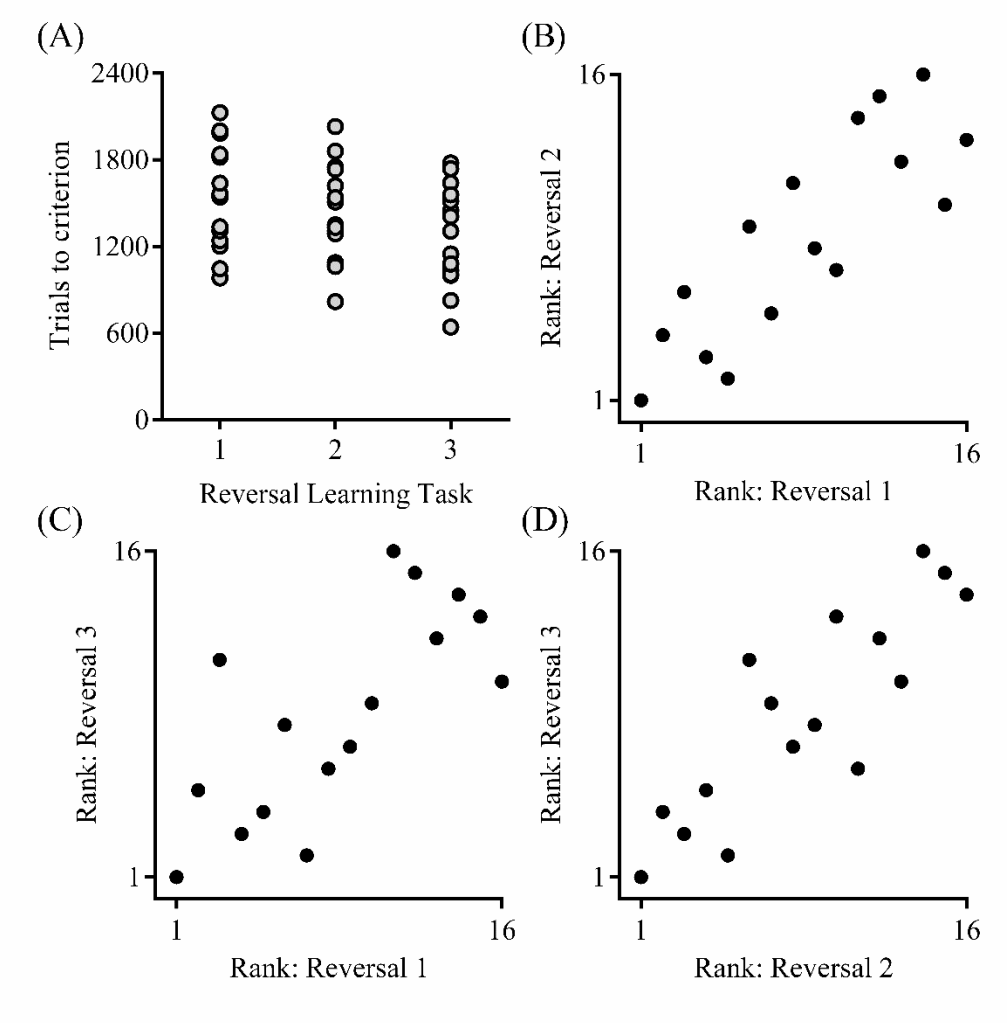



Figure 3.6 (Panel A) shows the individual TTC scores for each of the 3 reversal tests. There was considerable variability in TTC during each reversal task. I determined whether the rank order of the TTC score was relatively constant as a function of the reversal test, as would be expected if a stable cognition is being measured (Boogert et al., 2018). Strong-positive Spearman correlations were found between TTC for reversals 1 and 2 [$r_s$ = .84, $p$ < .001; Figure 3.6, Panel B], reversals 1 and 3 [$r_s$ = .72, $p$ < .01; Figure 3.6, Panel C], and reversals 2 and 3 [$r_s$ = .86, $p$ < .001; Figure 3.6, Panel D]. Thus, while TTC appears to reduce during successive reversals, individual differences in behavioural flexibility appear to be stable. TTC from each reversal task were also subject to a Shapiro-Wilk test of normality. TTC for reversal 1 [$W$ (16) = .97, $p$ = .802), reversal 2 [$W$ (16) = .97, $p$ = .818], and reversal 3 [$W$ (16) = .92, $p$ = .144] were normally distributed and had minimal skew (reversal 1 = 0.03; reversal 2 = -0.02; reversal 3 = -0.07). Thus, the distribution of data was relatively stable across reversal tasks, despite the gradual decline in TTC as a function of reversal number.

**Figure 3.6**

*Individual TTC Scores Across Serial Reversals and Rank-Order Plots*




*Note.* Panel A shows the individual TTC scores as a function of reversal learning task and the distribution of these scores gradually declines. Panel B-D plots the rank scores between the three reversal learning tasks. Ranks are in ascending order, such that, rank 1 required the fewest TTC on a given reversal (i.e., most behaviourally flexible).

## Discussion: Study 3 and Study 4

In these studies, a visual discrimination reversal learning task was developed that would be suitable for addressing the aims of the present thesis. The initial objective was to determine an optimal criterion for acquisition of a visual discrimination that would then guide the development of a procedure for measuring behavioural flexibility. In separate groups of rats, I first measured the number of days of testing required to learn to

either depress the lever below an illuminated light or to depress the lever below an unilluminated light. 100 trials were conducted daily, and I retrospectively applied various criteria to the data to determine the latency to the acquisition of the visual discrimination. More stringent criteria resulted in longer latencies to acquisition of the discrimination, extensive subject attrition, and increased rightward skew in the distribution of the data. Group differences also emerged as increasingly stringent criteria were applied to the data. Specifically, the group required to depress the lever that was not below the illuminated light became increasingly less likely to meet criterion and required significantly more trials to do so, as has been reported by another group (Fisken & Ward, 2019).

The group differences in latency to acquisition of the visual discrimination might be due to the spatial contiguity effect (Polidora & Fletcher, 1964). The illuminated light is likely to be the discriminative stimulus for both groups since this is the only stimulus to be both absent when reinforcement is unavailable and present when reinforcement is available. Accordingly, in one group, depression of the lever below the illuminated light is exclusively reinforced, and in the other group, depression of the lever not below the illuminated light is exclusively reinforced. The spatial contiguity effect predicts that increased distance between the illuminated light and the lever that leads to reward would disrupt visual discrimination learning, consistent with what I observed. No group differences were observed when more lenient criteria were applied which indicates the detriment produced by decreased spatial contiguity might depend on accuracy requirements, and by extension, task difficulty.

There were 100 trials per daily test session, and I broke these into 10 blocks of 10 trials each. Based on accuracy, low subject attrition and stability I adopted the criterion for acquisition of the visual discrimination to be the first day that 80% accuracy in 8 out of 10 blocks was achieved. These criteria were then applied to a reversal learning task to obtain an index of behavioural flexibility. I conducted three serial reversal learning tasks and determined the stability of the behavioural measures obtained from them. There was substantial variability in the number of trials to criterion for reversal learning. Trials to criterion decreased with repeated reversal tasks, but rank-order performance in each of the three reversal tasks was strongly correlated, supporting the idea that this task is measuring a relatively stable cognition (see, Matzel et al., 2003). This provides a method to confidently assess the impact of $D_2$ receptor manipulations on behavioural flexibility.

# CHAPTER 4: THE EFFECTS OF REPEATED $D_2$ ANTAGONIST ADMINISTRATION ON $D_2$ EXPRESSION AND REVERSAL LEARNING

*Parts of this chapter have been adapted, with permission, from previously published work in Behavioural Brain Research (Highgate et al., 2022).*

## Introduction

Dopamine $D_2$ receptor expression is a critical determinant of reversal learning performance and downregulated $D_2$ receptor expression is associated with impairments in reversal learning (see, "Dopamine and Reversal Learning" in the General Introduction). It was also noted in the General Introduction of the present thesis that I aimed to determine whether an upregulation of dopamine $D_2$ receptor expression would improve reversal learning. The following two studies addressed this objective by determining a treatment regimen capable of upregulating $D_2$ receptor expression (Study 5) and applying this treatment regimen to the reversal learning procedure developed in the previous chapter (Study 6). It was hypothesised that repeated $D_2$ antagonist administration would upregulate $D_2$ expression (Hypothesis 1), consistent with previous reports (Burt et al., 1977; LaHoste & Marshall, 1991; Memo et al., 1987; Samaha et al., 2007). It was also hypothesised that this treatment would improve reversal learning (Hypothesis 2). Given that performance was reported to asymptote before $D_2$ expression did and that poorer performance was observed in subjects with the lowest $D_2$ expression (Groman et al., 2011), it was also hypothesised that the amount reversal learning improved would increase as pre-treatment behavioural flexibility scores decreased (Hypothesis 3).

## Study 5: Effects of Repeated Eticlopride on $D_2$ Receptor Expression

### Methods

#### Subjects

All methods related to subjects, housing, and food deprivation were conducted as described in the general methods. No behavioural testing was conducted in Study 5.

## Drug Treatment

Rats were randomly assigned to the eticlopride (n=14) or saline (n=9) groups. Eticlopride (0.3mg/kg, IP) or an equal volume of the saline vehicle was administered daily for 14 consecutive days. The 14-day treatment period was used because it is intermediate to the number of days (3-28) of administration where upregulation of $D_2$ receptors has been observed following repeated dopamine treatment in rats (Braun et al., 1997; Burt et al., 1977; Hashimoto et al., 2018; Samaha et al., 2007; Seeman et al., 1978; Varela et al., 2014) and because a minimum of 14-days of antagonist treatment was required to upregulate dopamine receptors in the nucleus accumbens (Prosser et al., 1988). I chose this dose of eticlopride because repeated administration had previously been shown to result in behavioural sensitisation (Mattingly et al., 1998; van de Wetering & Schenk, 2017) which has been suggested to reflect receptor upregulation (Kimura et al., 2021; Servonnet & Samaha, 2020).

## Flow Cytometry

A number of techniques have been developed in order to measure receptor expression directly or indirectly. For example, expression can be estimated by measuring the amount of messenger ribonucleic acid that has been transcribed from a gene of interest (i.e., Northern Blotting) or homogenate receptor concentrations can be directly measured (i.e., Western Blotting). Others utilise receptor autoradiography to measure absolute receptor density or use semi-quantitative immunohistochemistry methods to estimate protein levels. Indeed, a number of these would have been suitable to address the aims of this study, and each technique comes with its' own advantages and disadvantages (see, Benoit et al., 2018; Elliott et al., 2014).

In order to determine which technique to use, the School of Biological Sciences at Victoria University of Wellington were consulted and advised the use of flow cytometry. Flow cytometry has not been traditionally used to measure receptor expression, but advancements in this technique have resulted in it being capable of measuring membrane-bound receptor expression in single intact cells. In fact, a procedure for measuring $D_2$ receptor expression in rodents was recently optimised and published by the consulted research group (Robichon et al., 2021). Additionally, this group generously offered to provide both the technical support and laboratory supplies required to conduct

flow cytometry. For all of the above reasons, flow cytometry was used to measure dopamine $D_2$ receptor expression in the present thesis.

***Brain Extraction***

Three days following the last injection, rats were deeply anesthetized with sodium pentobarbital (100mg/kg, IP). Approximately 100mL of phosphate-buffered 0.85% saline (10mM PBS) was transcardially perfused at a rate of 14mL/minute with a perfusion pump (EYLA micro tube pump MP-3, Tokyo Rikakikai Co., Ltd, Tokyo, Japan). Brains were immediately extracted, the olfactory bulbs and cerebellum were removed, and the remaining tissue was submerged in a 5mL Vulcan tube filled with PBS and then stored on ice.

***Cell Preparation***

A single-cell suspension of brain tissue was generated using a 70 μM cell strainer. 10mL of PBS was added to each sample. The samples were then centrifuged at 760xg for 5 minutes. The supernatant was discarded, and the cells were resuspended in 10mL of 37% Percoll™ (Sigma-Aldrich; Saint Louis, MO), and centrifuged at 760xg for 30 min with low acceleration and no deceleration. The myelin was removed from each sample using an autopipette and the cells were resuspended in FACs buffer (1xPBS, 2% fetal calf serum, 0.1% 1M sodium azide). Duplicates from each sample were plated in a U-bottom 96-well plate. An additional well contained a randomly selected sample from a saline-treated rat that would eventually serve as the isotype control. The plate was centrifuged at 400xg for 4 minutes then the supernatant was discarded. Each well was stained with 50μL of Zombie NIR™ (BioLegend; San Diego, CA) and the plate was incubated on ice in the dark for 15 minutes. 150μL of PBS was added to each well, the plate was centrifuged at 400xg for 4 minutes, and then the supernatant was discarded. Cells were resuspended in 50μL of Fc receptor binding inhibitor (2.4G2, BD Biosciences) before incubating in the dark on ice for 15 minutes. 150μL of PBS was then added to each well. Rabbit polyclonal anti-dopamine $D_2$ receptor antibody (Abcam, Cambridge, UK) was diluted into a 1:100 solution with FACs buffer, and 50 μL was added to each well, except for the isotype control, and the samples were incubated on ice for 20 minutes. Fluorescein isothiocyanate (FITC) goat anti-rabbit IgG antibody was diluted into a 1:50 solution with FACs buffers, 50μL of the solution was added to each well and the samples

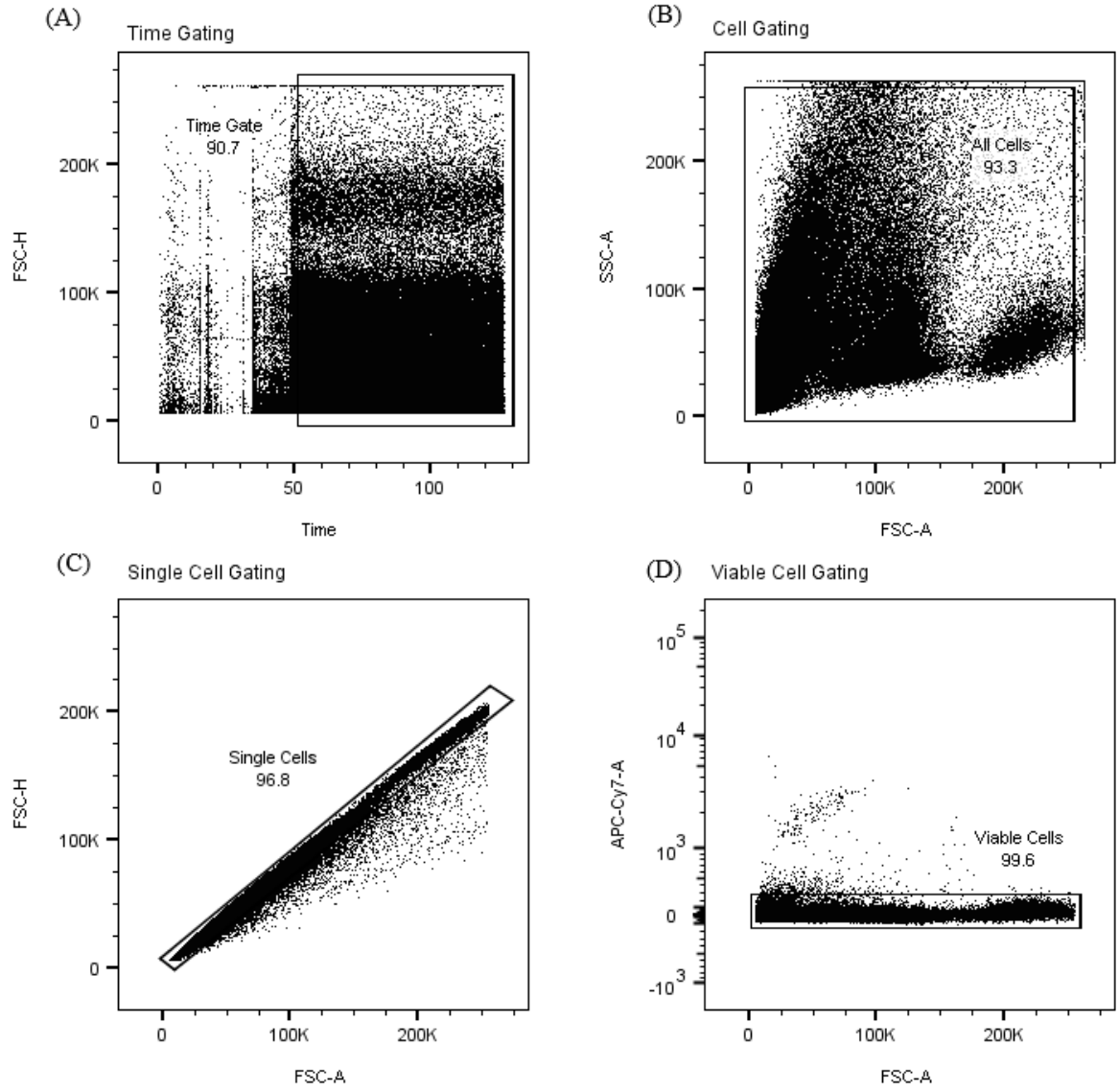
were incubated for a further 20 minutes on ice. Flow cytometry was immediately performed on a BD FACS Canto II (BD Biosciences) and analysed using FlowJo software version 10.6 (Treestar Inc., Ashland, OR, USA). This general procedure has been previously reported (Robichon et al., 2021).

***Gating Procedure***

The gating strategy consisted of time gating to select cells that had flown through whilst the flow stream was stable (Figure 4.1, Panel A). I was not looking for a particular cell type, so all cells were gated (Figure 4.1, Panel B). The samples were then gated for single cells to exclude doublets or clumps of cells (Figure 4.1, Panel C). Lastly, the samples were gated for viable cells using Zombie NIR™ dye, which permanently binds to cells that are not intact (Figure 4.1, Panel D). As a result of this procedure, $D_2$ expression was only measured in singular cells that were intact.

**Figure 4.1**

*Results of the Gating Procedure Used to Identify Viable Single Cells*




*Note.* This figure shows the time (Panel A), cell (Panel B), single-cell (Panel C), and viable cell (Panel D) gating procedures described in the text. FSC = forward scatter, SSC = sideways scatter, APC-Cy7-A = amount of fluorescence associated with the viability dye.

## Data Analysis

The mean fluorescence intensity (MFI) of viable cells was measured in each well. The MFI values from the duplicate wells for each subject were averaged so that

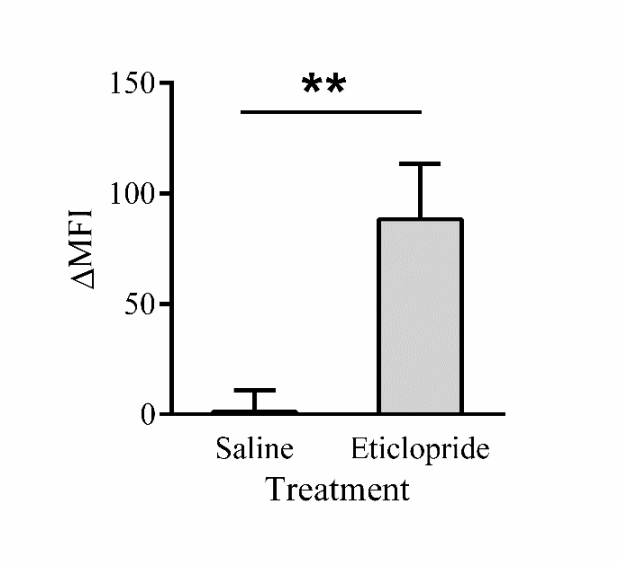
there was a single aggregate MFI score for each subject. Each aggregate MFI score was transformed into a percentage change of the isotype MFI control to measure fluorescence exclusively due to $D_2$ expression in each subject (ΔMFI). ΔMFI values were subject to an independent samples t-test with the variable drug treatment (eticlopride, saline).

## Results

ΔMFI values were normally distributed [saline: $W$ (9) = .93, $p$ = .441; eticlopride: $W$ (14) = .95, $p$ = .575], but the assumption of homogeneity was violated. A natural log transformation could not be applied as some of the ΔMFI values were negative, and instead, a Welch's t test was conducted which is robust to violations of homogeneity. As shown in Figure 4.2, ΔMFI values were greater in the group that received repeated eticlopride treatment [t (16.65) = 3.27, $p$ = .005, Hedge's $G$ = 1.118].

**Figure 4.2**

*Mean Difference in $D_2$ expression (ΔMFI + SEM) as a Function of Treatment Condition*




*Note.* Asterisks indicate significant differences (** $p$ < .01).

## Study 6: Effects of Repeated Eticlopride on Reversal Learning

### Methods

All procedures were conducted according to the general methods. The treatment regimen used in Study 5 was used immediately following completion of the second reversal task. The original sample size was 30. Five subjects from the original sample did not complete behavioural testing. The electrical wiring of one of the operant chambers was damaged which prevented complete data collection from two rats that had been allocated to this chamber. The liquid dipper in another chamber began malfunctioning during testing which prevented the collection of data from two subjects allocated to this chamber. In addition to these, the change in TTC between reversals 1 and 2 for one subject was more than two standard deviations away from the average change for the sample. As a result, the baseline data from this subject was considered unstable and was excluded from further analysis. The final sample size was 25 (eticlopride = 14; vehicle = 11).

### Data Analysis

#### *Baseline Reversal Measures*

To determine whether individual differences in baseline behavioural flexibility scores were stable, TTC from reversals 1 and 2 were converted into rank scores and subjected to a Spearman's Rank-Order Correlation. To determine whether the TTC of the two treatment groups was comparable, a mixed-measures analysis of variance (ANOVA) was conducted on the TTC as a function of reversal number (reversal 1, reversal 2) and treatment group (eticlopride, saline).

#### *Effects of Eticlopride on Reversal Learning*

Three analyses were conducted to determine the effects of eticlopride treatment. The first analysis determined whether eticlopride treatment impacted the retention of the previously learned rule. For this test, TTC for the retention test were subject to an independent-samples t-test (treatment: saline, eticlopride).

The second test determined whether repeated exposure to eticlopride improved reversal learning. To do this, an improvement score was first calculated by subtracting TTC for reversal 3 from TTC from reversal 2 and was denoted as ΔTTC (a positive score

indicated an improvement and a negative score indicated decrement in performance in the third reversal task). This analysis was chosen over a mixed ANOVA (i.e., treatment (eticlopride, saline) x reversal task (reversal 2, reversal 3)) for several reasons. First, the use of difference scores further controls for noise related to individual differences on the baseline behavioural flexibility tests not accounted for by matching and allows for a simplified examination of improvement. Second, multiway ANOVAs include additional terms which ultimately reduce power. This may not be a problem in the present chapter (two-way ANOVA, sample sizes 11-14), but it would have been problematic for the analysis in the subsequent chapter (three-way ANOVA, sample sizes 6-8). Third, inclusion of additional interaction terms increases the number of assumption tests that must be met in order to conduct parametric analysis; this would have required 4 and 8 normality tests to be non-significant in the following two chapters, respectively. Further, transformations where these are not met are unlikely to be successful if only a small number of the conditions fail to meet these assumptions. Indeed, this becomes evident in the subsequent chapter during the baseline analysis. In sum, in order to reserve power, minimise the number of assumption tests required to be met, simplify the analysis, and avoid using non-parametric tests where possible, ΔTTC was examined in both this chapter and the following chapter.

The third test determined whether ΔTTC was dependent on pre-existing differences in behavioural flexibility. To do this, ΔTTC was plotted as a function of baseline behavioural flexibility (reversal 2 TTC) and a simple linear regression was fitted to the data for each treatment group.

## Results

### Initial Training and Visual Discrimination

The initial lever training stage was completed in an average of 5.92 days (*SEM:* 0.83). The mean TTC for the initial visual discrimination was 745.92 (*SEM:* 49.06). These results are comparable to the results in Chapter 3.

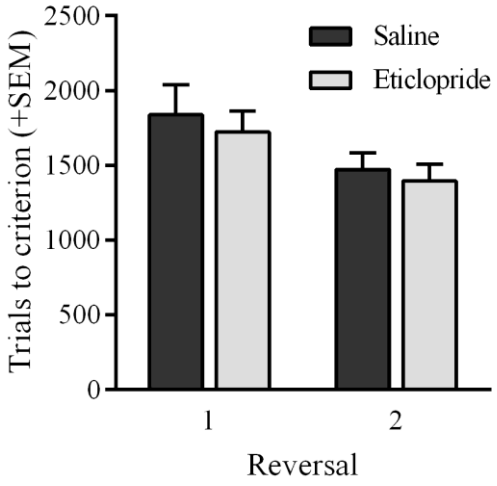
### Baseline Reversal Measures

There was a strong positive correlation between ranked TTC scores from reversal 1 and 2 [$r_s = 0.84$, $p < .001$]. Figure 4.3 shows the TTC for the groups that were

to receive saline or eticlopride during the first two reversal tasks. Assumptions of normality and homogeneity were met for reversal 1 and reversal 2 TTC (see, "Table B1"). There was no interaction between reversal number and drug treatment [$F$ (1,23) = .08, $p$ = .784, $\hat{\eta}_p^2$ = .003] or main effect of drug treatment [$F$ (1,23) = .215, $p$ = .647, $\hat{\eta}_p^2$ = .009]. There was a significant main effect of reversal number [$F$ (1,23) = 21.99, $p$ < .001, $\hat{\eta}_p^2$ = .489], indicating that TTC was reduced for both groups in the second reversal task (see, Figure 4.3 for pretreatment reversal learning performance).

**Figure 4.3**

*Mean TTC (+SEM) as a Function of Treatment Condition and Reversal Task*




## Effects of Eticlopride Treatment

### *Retention Test*

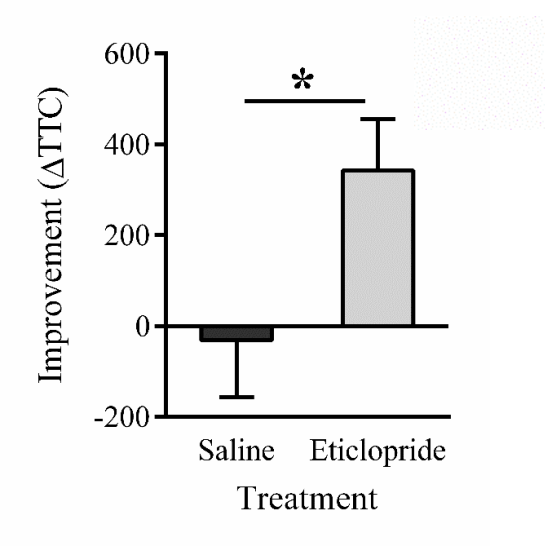
The raw retention data did not meet the assumptions for conducting an independent samples t-test but did following a natural log transformation (see, "Table B2"). Retention testing did not differ significantly [t (23) = .42, $p$ = .678, Hedge's $G$ = .164] between eticlopride- (raw: $M$ = 178.57, $SD$ = 204.48; *ln(x)*: $M$ = 5.42, $SD$ = 0.67) and saline-treated groups (raw: $M$ = 119.09, $SD$ = 86.08; *ln(x)*: $M$ = 5.32, $SD$ = 0.40).

***Reversal Learning***

The ΔTTC data met the assumptions of normality and homogeneity (see, "Table B3"). As can be seen in Figure 4.4, eticlopride treatment ($M$ = 342.60, $SD$ = 422.00) resulted in a significant improvement in reversal learning [t (23) = 2.22, $p$ = .036, Hedge's $G$ = 0.866] relative to vehicle controls ($M$ = -31.90, $SD$ = 413.00).

**Figure 4.4**

*Mean Improvement (ΔTTC + SEM) as a Function of Treatment Condition*

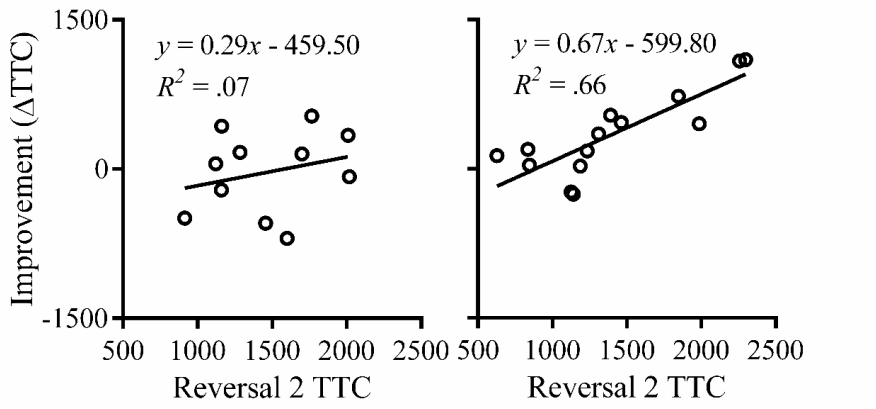



*Note.* Positive scores on the y-axis indicate an improvement and negative scores indicate a decrement. Asterisks indicate significant differences (* $p$ < .05).

The final analysis examined whether ΔTTC could be explained by individual differences in baseline behavioural flexibility (reversal 2 TTC). The assumptions of homogeneity and normality were met (see, "Table B4"). As shown in Figure 4.5, the linear model explained a significant amount of the variance in ΔTTC in the eticlopride group [$F$ (1, 14) = 27.16, $p$ < .001, $R^2$ = .66], but not in the saline group [$F$ (1, 9) = 6.67, $p$ = .433, $R^2$ = .07]. Further, the standardised coefficient was significant for the eticlopride group [$\beta$ = .83, $p$ < .001], but not the saline group [$\beta$ = .26, $p$ = .433]. These data support the idea that eticlopride-produced improvement increased as baseline behavioural flexibility decreased.

**Figure 4.5**

*Improvement in Reversal Learning as a Function of Baseline Reversal Learning*




*Note.* This figure shows the regressions for the saline (left) and eticlopride (right) treatment conditions. Positive scores on the y-axis indicate an improvement and negative scores indicate a decrement. Baseline behavioural flexibility is inversely related to the values displayed on the x-axis (greater TTC = less behaviourally flexible). $R^2$ values are adjusted and indicate model fit.

## Discussion: Study 5 and Study 6

The two studies in this chapter aimed to determine the effects of repeated eticlopride administration of dopamine $D_2$ receptor expression (Study 5) and reversal learning performance (Study 6). Flow cytometry was used to measure $D_2$ receptor expression. Flow cytometry, like autoradiographic or immunohistochemical techniques, is used to measure protein expression (see, Benoit et al., 2018) and is capable of detecting changes in $D_2$ expression in rodents (Robichon et al., 2021). Flow cytometry allows unfixed tissue to be extracted, processed, and analysed within a single day. Further, non-viable cells, which increase the likelihood of observing false positives, are excluded by viability gating (see, Adan et al., 2017). Using this technique, repeated administration of the dopamine $D_2$ antagonist, eticlopride, for 14 consecutive days was shown to increase expression of central dopamine $D_2$ receptors. This supports Hypothesis 1 and is consistent with previous reports following repeated antagonist administration (Burt et al., 1977; Hurley et al., 1996; Kimura et al., 2021; Lévesque et al., 1995; Memo et al., 1987; See et al., 1990).

Repeated eticlopride also improved reversal learning (Hypothesis 2) and I was able to show that this improvement was dependent on individual differences in behavioural flexibility prior to treatment (Hypothesis 3). The latter finding raises an interesting question, namely, if repeated $D_2$ receptor antagonism improved reversal learning in behaviourally inflexible subjects, can it also restore experimentally produced behavioural inflexibility?

# CHAPTER 5: REPEATED ETICLOPRIDE TREATMENT PREVENTED METHAMPHETAMINE-PRODUCED REVERSAL LEARNING DEFICITS

*Parts of this chapter have been adapted, with permission, from previously published work in Behavioural Brain Research (Highgate et al., 2022).*

## Introduction

In the previous study it was shown that a repeated $D_2$ antagonist treatment that upregulated $D_2$ receptor expression also improved reversal learning, preferentially so in subjects that were less behaviourally flexible. The aim of this study was to produce a model of behavioural inflexibility to determine if this drug treatment could also restore experimenter-produced impairments.

Several studies have suggested that a history of methamphetamine misuse is associated with moderate impairments in cognitive flexibility in humans (Ballard et al., 2015; Pilhatsch et al., 2020; Simon et al., 2001; van der Plas et al., 2009). However, duration of drug misuse, polydrug use, period of abstinence, age, and various other factors can vary quite markedly, and these factors may preclude the ability to attribute cognitive deficits specifically to drug exposure (Dean et al., 2013; Hart et al., 2012; Potvin et al., 2018). Additionally, it is difficult to differentiate impairments in cognitive flexibility that are due to methamphetamine misuse from pre-existing cognitive inflexibility (see, Dean et al., 2018). However, a number of preclinical studies have been able to demonstrate a casual role for methamphetamine in behavioural inflexibility, reporting that repeated self- (Bernheim et al., 2016; B. M. Cox et al., 2016; Perez Diaz et al., 2019) or experimenter- (Groman et al., 2012, 2018; Izquierdo et al., 2010; Kosheleff et al., 2012) administered methamphetamine impaired reversal learning. These studies implicate repeated methamphetamine as a means by which cognitive and behavioural inflexibility may emerge.

Dopamine and the $D_2$ receptor have been repeatedly shown to play a critical role in reversal learning throughout the present thesis, both in the literature reviewed and in the results of the previous study. Of relevance, the $D_2$ receptor has also been shown to be downregulated in humans who misused methamphetamine (Ballard et al., 2015; Lee et al., 2009; Volkow et al., 2001) and in laboratory animals exposed to methamphetamine

(Groman et al., 2012; Thanos et al., 2017). Further, repeated methamphetamine administration resulted in concomitant impairments in reversal learning and $D_2$ expression which were correlated (Groman et al., 2012). This raises an interesting possibility that methamphetamine-produced impairments in reversal learning are the result of the opposing neuroadaptation described in the previous chapter (i.e., receptor downregulation).

If a downregulation of the $D_2$ receptor is an important mechanism underlying this methamphetamine-produced impairment, then manipulations that restore $D_2$ mechanisms may also improve behavioural flexibility. Consistent with this idea, acute administration of the $D_2$-like agonist, pramipexole, transiently restored reversal learning performance in humans with a history of stimulant misuse (Ersche et al., 2011). Therefore, the eticlopride treatment used in the previous chapter, which was shown to upregulate $D_2$ receptors, might also be expected to ameliorate these deficits. The aim of the present study was to answer this question. It was hypothesised that the repeated methamphetamine would impair reversal learning (Hypothesis 4), repeated eticlopride would improve reversal learning (Hypothesis 5), and that these two treatments would counteract one another (Hypothesis 6).

## Methods

All methods were conducted in accordance with the general methods unless otherwise stated. The sample size was 30.

### Drug Treatment

Methamphetamine (2.0 mg/kg, SC) or an equal volume of the saline vehicle was administered 4 times, spaced 2 hours apart. This treatment regimen was administered because it impaired reversal learning in rats (Izquierdo et al., 2010). During the following 14 days, eticlopride (0.3mg/kg, IP) or an equal volume of the saline vehicle was administered, once daily.

## Procedure

All reversal learning procedures were conducted in accordance with the general methods. Subjects were matched and assigned to the saline/saline (sal/sal; n=6),

saline/eticlopride (sal/etic; n=7), methamphetamine/saline (meth/sal; n=8), or methamphetamine/eticlopride (meth/etic; n=8) condition.

## Data Analysis

The analyses below were based on the analyses conducted in Study 6.

### *Baseline Reversal Measures*

To determine whether baseline behavioural flexibility scores were stable, TTC from reversals 1 and 2 were converted into rank scores and subjected to a Spearman's Rank-Order Correlation. To determine whether the groups assigned to drug treatment were comparable, a mixed measures ANOVA was conducted on TTC as a function of reversal number (reversal 1, reversal 2), methamphetamine exposure (methamphetamine or saline) and eticlopride treatment (eticlopride, saline).

### *Effects of Methamphetamine and Eticlopride Treatment on Reversal Learning*

Two analyses were conducted to determine the effects of methamphetamine and eticlopride treatment. The first analysis determined whether these treatments impacted the retention of the previously learned rule by subjecting the TTC data to a 2 (methamphetamine treatment) x 2 (eticlopride treatment) ANOVA. The second ANOVA test determined whether these treatments impacted reversal learning. To do this, an improvement score was first calculated by subtracting TTC for reversal 3 from TTC from reversal 2 and was denoted as ΔTTC (a positive score indicated an improvement and a negative score indicated decrement in performance in the third reversal task). ΔTTC were subject to the same ANOVA as above.

# Results

## Initial Training and Visual Discrimination

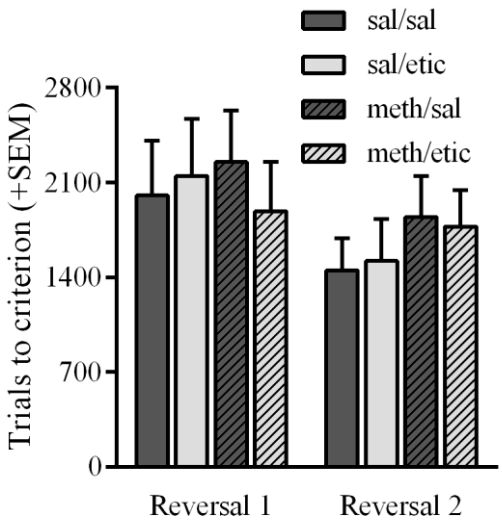
The initial lever training stage was completed within an average of 4.93 days (*SEM:* 0.24). The mean TTC for the initial visual discrimination was 833.42 (*SEM:* 66.00). These results are comparable to the results in Chapters 3 and 4.

**Baseline Reversal Measures**

There was a strong positive correlation between ranked TTC scores for reversal 1 and 2 [$r_s$ = .73, $p$ < .001]. Figure 5.1 shows the raw TTC data for the drug conditions during the first two reversal tasks prior to treatment. The assumption of normality was not met for the methamphetamine/saline condition in both reversal tasks (see, "Table C1"), and the entire data set was not normalised following conventional transformations, such as, the reciprocal function (1/x), root function (square, cube), and log function (base 2, ln). It is noted that the natural log function did normalise the reversal 1, but not reversal 2 data for this condition without violating these assumptions for the other conditions (see, "Table C2"). The complex design (repeated measures with two between-subject factors with 2 levels) precluded this data from being subject to non-parametric analysis. Instead, two analyses were conducted. The first, was the planned ANOVA using the natural log-transformed data, despite the assumption for normality not being met by the methamphetamine/saline condition for reversal 2. The main reason for conducting this analysis was to confirm the groups were equivalent prior to treatment, and so, a 2 (methamphetamine treatment) x 2 (eticlopride treatment) robust ANOVA with 20% trimmed means was also conducted within each reversal task to further determine whether the conditions did, in fact, differ.

**Figure 5.1**

*Mean TTC (+SEM) as a Function of Treatment Condition and Reversal Learning Task*



The three-way interaction [$F$ (1,25) = 1.81, $p$ = .191, $\hat{\eta}_p^2$ = .067] was not significant. The two-way interactions between reversal task and eticlopride treatment [$F$ (1,25) = 0.32, $p$ = .575, $\hat{\eta}_p^2$ = .013] and between methamphetamine treatment and eticlopride treatment [$F$ (1,25) = 0.22, $p$ = .643, $\hat{\eta}_p^2$ = .009] were not significant. There were no main effects of methamphetamine treatment [$F$ (1,25) = 0.41, $p$ = .523, $\hat{\eta}_p^2$ = .016] or eticlopride treatment [$F$ (1,25) = 0.18, $p$ = .736, $\hat{\eta}_p^2$ = .005]. There was a significant main effect of reversal number indicating that TTC reduced as a function of reversal task [$F$ (1,25) = 19.86 $p$ < .001, $\hat{\eta}_p^2$ = .443]. There was also a two-way interaction between methamphetamine treatment and reversal task [$F$ (1,25) = 4.70, $p$ = .040, $\hat{\eta}_p^2$ = .158] and was the result of the reduction in TTC between reversal 1 and 2 reaching significance for saline-treated [$t$ (12) = 4.21, $p$ = .001, Cohen's $d$ = 1.17], but not methamphetamine-treated rats [$t$ (15) = 1.80, $p$ = .092, Cohen's $d$ = 0.450]. Importantly, this interaction did not reflect significant differences between methamphetamine- and saline-treated groups in the first [$t$ (27) = 0.03, $p$ = .973, Cohen's $d$ = 0.013] or second [$t$ (27) = 1.16, $p$ = .258, Cohen's $d$ = 0.431] reversal task. The results of the robust ANOVA were consistent with these results, such that, there was no significant main effect of methamphetamine (Reversal 1: Q = .04, $p$ = .839; Reversal 2: Q = .05, $p$ = .826) or eticlopride (Reversal 1: Q = .03, $p$ = .868; Reversal 2: Q = 1.25, $p$ = .285), nor a two-way interaction between the eticlopride and methamphetamine factors (Reversal 1: Q = 0.34, $p$ = .573; Reversal 2: Q = .04, $p$ = .851).

**Effects of Methamphetamine and Eticlopride Treatment**

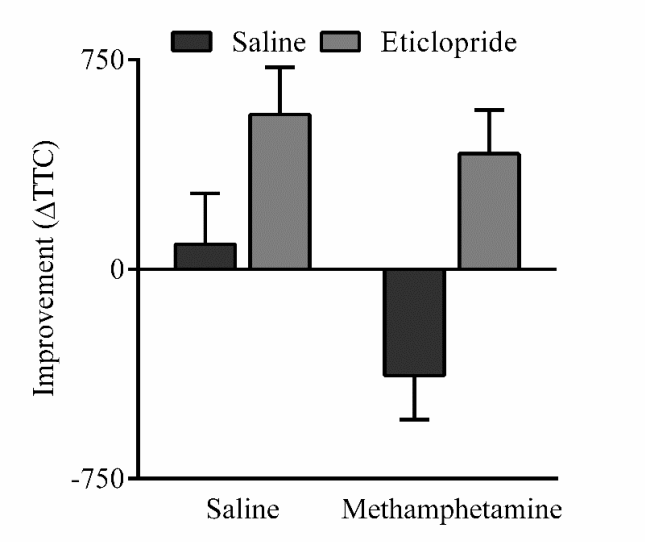
The raw retention data did not meet the ANOVA assumptions, even following the various transformations mentioned in the previous section (see, "Table C3"). As such, these data were subject to a two-way (eticlopride treatment X methamphetamine treatment) robust ANOVA with a 20% trim applied. There was no interaction between the variables (Q = 1.65, $p$ = .224), and no effects of eticlopride (Q = .03, $p$ = .863) or methamphetamine (Q = 1.89, $p$ = .196) indicating that neither treatment impacted retention of the behaviour learned prior to treatment.

The raw ΔTTC data are shown in Figure 5.2. These data meet the assumptions of normality and homogeneity (see, "Table C4"). The two-way interaction was not significant [$F$ (1,25) = 0.15, $p$ = .703, $\hat{\eta}_p^2$ = .006], but the main effects of

methamphetamine treatment [$F$ (1,25) = 4.70, $p$ = .040, $\hat{\eta}_p^2$ = .158] and eticlopride treatment were [$F$ (1,25) = 15.13, $p$ <.001, $\hat{\eta}_p^2$ = .377]. These effects were the result of methamphetamine impairing performance relative to controls and eticlopride improving performance relative to controls.

**Figure 5.2**

*Mean Improvement (ΔTTC + SEM) as a Function of Treatment Condition*




*Note.* Positive scores on the y-axis indicate an improvement and negative scores indicate a decrement in TTC. The x-axis refers to the methamphetamine treatment variable and the legend refers to the eticlopride treatment variable. As two main effects were reported, these statistical differences cannot be identified using asterisks in this figure.

## Discussion

This study aimed to determine whether repeated eticlopride treatment would restore behavioural flexibility that had been disrupted by exposure to a regimen of methamphetamine that impaired reversal learning. It was hypothesised that methamphetamine treatment would impair reversal learning (Hypothesis 4), eticlopride treatment improve reversal learning (Hypothesis 5), and that these treatments would oppose each other (Hypothesis 6).

The hypothesis that methamphetamine treatment would impair reversal learning was supported by the results of this study. These results also support the conclusions

drawn by other research that repeated methamphetamine exposure impairs the cognitive and behavioural processes required to successfully adapt behaviour in response to changes in environmental contingencies (Bernheim et al., 2016; B. M. Cox et al., 2016; Groman et al., 2012, 2018; Izquierdo et al., 2010; Kosheleff et al., 2012; H. Mizoguchi & Yamada, 2019; Perez Diaz et al., 2019; Potvin et al., 2018). This methamphetamine-produced impairment was relatively persistent since reversal learning tests started between 17- and 25-days following methamphetamine treatment[3]. Others have reported that reversal learning was not impaired following a long delay between treatment and reversal testing, but this delay was more than 40 days following methamphetamine exposure, indicating that this deficit might be expected to recover over more extended time frames (Daberkow et al., 2008; Izquierdo et al., 2016).

It is noted that one of the treatment groups (methamphetamine/saline) improved, but not significantly so, between the first two reversal measures. However, this is unlikely to have impacted the critical ΔTTC measures for two reasons. First, there were no differences between any of the conditions in the first or second reversal measure according to both analyses conducted. Second and most importantly, ΔTTC was determined by how much behaviour changed between pre-treatment and post-treatment reversal measures, and so, this would be expected to account for any differences in baseline scores prior to treatment.

The present study measured the effects of experimenter-administered methamphetamine under conditions that do not emulate patterns of methamphetamine self-administration observed in the laboratory. However, it is noted that both self-administered (Bernheim et al., 2016; B. M. Cox et al., 2016; Perez Diaz et al., 2019) and experimenter administered (Groman et al., 2012, 2018; Izquierdo et al., 2010; Kosheleff et al., 2012) methamphetamine impaired reversal learning. Nonetheless, it will be important to determine whether repeated eticlopride exposure can also restore behavioural flexibility that had been disrupted by methamphetamine self-administration.

[3] Note: These numbers vary because they are the sum of the 1 day of methamphetamine treatment, the 14 days of eticlopride treatment, the 2 rest days that followed, and the number of test sessions required to complete the post-treatment retention test.

The results of this study also support Hypothesis 5 and 6. Repeated eticlopride treatment improved reversal learning, consistent with the results of the previous chapter. Further, the reversal learning impairments produced by methamphetamine were not present in subjects that received repeated eticlopride treatment, suggesting that the eticlopride regimen prevented the development of behavioural inflexibility. Thus, upregulation of the dopamine $D_2$ receptor may be an effective means of restoring substance-produced behaviourally inflexibility.

# CHAPTER 6: GENERAL DISCUSSION

*Parts of this chapter have been adapted, with permission, from previously published work in Behavioural Brain Research (Highgate et al., 2022).*

## Discussion

### Summary of the Present Thesis

The purpose of the present thesis was to further our current understanding of the underlying neurochemical processes relevant to behavioural flexibility. I first aimed to operationalise behavioural flexibility by measuring reversal learning in rats. The focus then shifted towards identifying a treatment regimen capable of upregulating dopamine $D_2$ receptor expression and assessing the impact of this treatment regimen on reversal learning. Additionally, the impact of this treatment on subjects with pre-existing or experimentally produced behavioural inflexibility was determined. The overarching aim of this thesis was to expand upon the proposed role of the dopamine $D_2$ receptor in reversal learning.

To address my aims an existing procedure from another laboratory group was systematically replicated (Boulougouris et al., 2007), however, I was unable to produce timely reliable data with this method. Consequently, I worked towards the development of a new reversal learning procedure and emphasised the need for the discrimination criterion to be 'reasonable'. This was determined by applying various criteria requiring different levels of responding accuracy, stability, and persistence to a large collection of visual discrimination data. Several behavioural indices were then examined so that I might have confidence in this criterion. The selected criterion required at least 80% of the responses made in each block to be on the correct lever in 8 of 10 consecutive blocks. Subsequently, three serial reversal tasks were added to the procedure. These reversal tasks resulted in relatively stable measures between individual subjects, consistent with what others have observed in discrimination tasks utilising non-human subjects (Matzel et al., 2003). Each reversal task was also completed within a relatively small number of test sessions. Critically, this allowed me to address the overarching aim of the present thesis while reducing the likelihood that any experimentally produced neuroadaptations would revert before testing was completed.

The focus then shifted to examining the role of the dopamine $D_2$ receptor in behavioural flexibility. The first step in doing this required a treatment regimen capable of upregulating dopamine $D_2$ receptor expression to be determined. It was hypothesised that repeated systemic administration of the dopamine $D_2$-like receptor antagonist, eticlopride (0.3mg/kg), over 14 consecutive days, would upregulate $D_2$ expression (Hypothesis 1). This hypothesis was supported by the flow cytometry analysis conducted in Study 5 and was expected since $D_2$ receptor upregulation has been frequently observed following repeated $D_2$ antagonist treatment (Burt et al., 1977; Hurley et al., 1996; Kimura et al., 2021; Lévesque et al., 1995; Memo et al., 1987; See et al., 1990). Some have employed more protracted dosing regimens (e.g., 21 days, Memo et al., 1987), but I unequivocally show that the shorter dosing regimen reported here was sufficient to upregulate $D_2$ receptor expression. As such, this treatment may be a relatively efficient means of producing an upregulation of $D_2$ receptor expression.

Behavioural flexibility has been shown to improve as a function of $D_2$ receptor expression (Groman et al., 2011, 2012; Jocham et al., 2009; Linden et al., 2018) and following acute administration of dopamine $D_2$ receptor agonists but only in subjects with compromised dopamine function (Cools et al., 2009; Ersche et al., 2011; Kimberg et al., 1997). These ideas formed the basis of the next two hypotheses tested, those being that repeated eticlopride administration would improve reversal learning (Hypotheses 2 and 5) and that this treatment would preferentially improve reversal learning in subjects with pre-existing behavioural inflexibility (Hypothesis 3). The behavioural results from the present thesis supported these hypotheses and present at least three interesting findings that have not been previously reported. First, a treatment regimen that upregulated $D_2$ expression also facilitated reversal learning. Second, this treatment preferentially improved reversal learning in behaviourally inflexible subjects. This may reflect a "ceiling effect", such that performance cannot improve any further in subjects that are highly behaviourally flexible prior to treatment, or that upregulation in subjects with putatively higher levels of $D_2$ expression does not produce a functional change in performance. The third novel finding is that individual differences in a behavioural measure related to dopamine function (i.e., pre-treatment reversal learning) were a critical determinant of a manipulation that produces a relatively long-term change in $D_2$ function (i.e., receptor upregulation). This provides support for the idea that individual differences should be considered when determining the impact of neurochemical manipulations on

reversal learning (Cools et al., 2009), irrespective of whether these manipulations result in acute or long-term changes in neurochemical function.

I then determined whether a methamphetamine regimen would impair behavioural flexibility (Hypothesis 4) and whether the eticlopride regimen used in the previous studies would prevent the emergence of this (Hypothesis 6). The results of Study 6 supported Hypothesis 4 and concur with a previous report showing this same regimen impaired reversal learning (Izquierdo et al., 2010). Repeated methamphetamine exposure downregulated $D_2$ expression (Groman et al., 2012; Thanos et al., 2017) and others have shown acute restoration of $D_2$ function restored reversal learning (Ersche et al., 2011; Kanen et al., 2019). Importantly, the results of the present study add to these and support Hypothesis 6 by showing a treatment that upregulated $D_2$ receptor expression also prevented the emergence of a methamphetamine-produced impairment. These results indicate that the $D_2$ receptor is an effective target for acutely or chronically restoring behavioural flexibility.

**The Importance of a Reasonable Criterion**

The criterion used in the first two studies required 90% accuracy in a single block of 10 trials, but it seemed behaviour was relatively unstable after meeting it. The test session terminated immediately after this criterion was met which precluded an examination of how stable behavioural responding was immediately after meeting the criterion (e.g., if this criterion was met in the second block of 10 trials, no additional trials were conducted), although I did previously note that accuracy tended to be low the day after meeting criterion. For this reason, and others related to the latency of testing, I suggested that this was not suitable for the purposes of the present thesis.

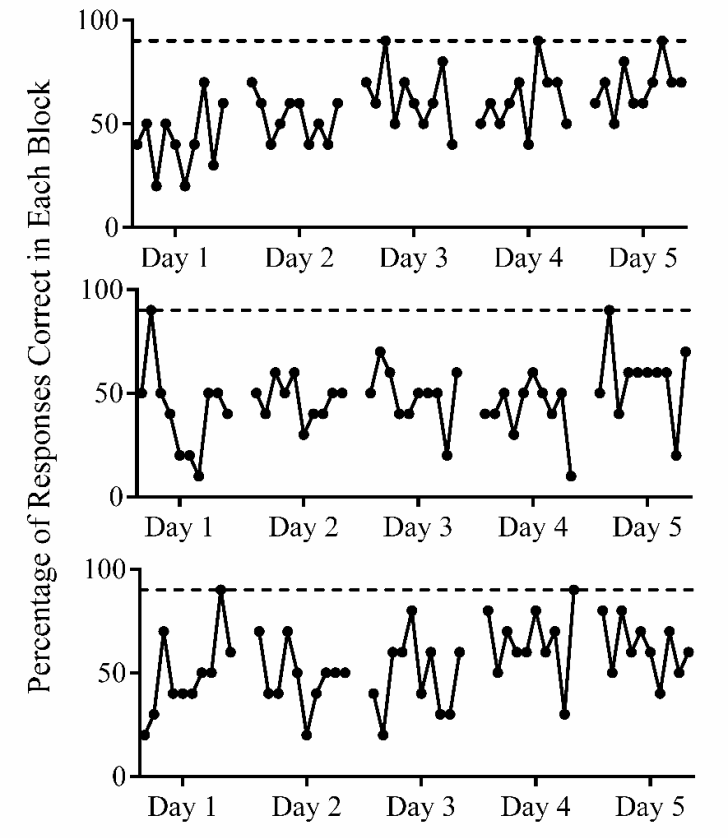
In the original paper, it was argued that this criterion was reasonable from a probability standpoint, that is, it is unlikely to be met by chance alone[4]. However, an informal examination of my visual discrimination data would suggest otherwise. The data

[4] It was reasoned, that if accuracy is currently fluctuating around chance-level (i.e., probability of a correct response is 0.5), as we might expect it to be when a discrimination task commences, then the binomial probability of any subject reaching 90% accuracy or above in a single block is $p=.011$.

from three randomly selected representative rats from Study 3 is shown in Figure 6.1 to illustrate this. It can clearly be seen that lever accuracy was highly inconsistent both within and between sessions, despite this criterion being met on multiple instances by all three rats. These observations support the concerns detailed in Chapter 3 and necessitated the development of a more suitable criterion for visual discrimination.

**Figure 6.1**

*Accuracy as a Function of Test Session for Three Representative Rats*




*Note.* This figure shows block accuracy over the first five days of discrimination training from three randomly selected representative rats. Each subject is displayed in a single graph (top: α-9; middle: γ-12; bottom: Δ-11). Each point represents the percentage of responses in a single block of 10 trials that were correct. Each set of points connected by a line represents all 10 blocks from a single day of training. The dashed line indicates 90% accuracy. Overall, accuracy was relatively low (relative to 90%) despite accuracy reaching criterion level (according to Studies 1 and 2) on more than one occasion.

To address this, I focussed on developing a procedure that utilised what I consider to be a 'reasonable' discrimination criterion. This was determined by ensuring the selected criterion did not result in sample attrition, the two alternative behaviours (i.e., which lever to depress) were acquired within a comparable amount of testing, and that accuracy was persistent after meeting the criterion. A comparison of differing criteria is seldom examined in reversal learning procedures, yet the result of Study 3 clearly showed discrimination criterion was a critical determinant of behavioural performance, consistent with previous reports (see, Reichert et al., 2020). A test of reversal learning presupposes that the initial discrimination has truly been acquired (see, Boulougouris et al., 2007), and so, we must first have some confidence that our criterion delineates subjects that have acquired the initial behaviour of interest from those that have not. These results stress the importance of ensuring the criterion used for behavioural measures in laboratory animals supports such a distinction.

**Repeated Eticlopride Treatment Upregulated Dopamine $D_2$ Receptor Expression**

I hypothesised that repeated injections of eticlopride would upregulate dopamine $D_2$ receptor expression (Hypothesis 1). The results of Study 5 unequivocally support this hypothesis and are consistent with other reports that repeated $D_2$ antagonism is an effective means of upregulating $D_2$ receptor expression (Burt et al., 1977; Hurley et al., 1996; Kimura et al., 2021; Lévesque et al., 1995; Memo et al., 1987; See et al., 1990). This study was critical to the current thesis as this treatment regimen had not been shown to upregulate dopamine $D_2$ expression.

I examined $D_2$ expression using whole-brain samples (minus the olfactory bulb and cerebellum), and so, several questions remain as to the nature of this receptor upregulation, one being whether it was preferentially expressed in specific brain regions. However, it has been repeatedly shown that this upregulation is particularly pronounced within the dorsal and ventral striatal regions (Hurley et al., 1996; Lévesque et al., 1995; See et al., 1990). Further, dorsal striatal $D_2$ expression is considerably higher than other $D_2$-expressing brain regions (see, Camps et al., 1989) and dorsal striatal $D_2$ expression was closely tied to performance in a reversal task (Groman et al., 2011). As such, alterations of dorsal striatal $D_2$ expression would be expected to impact reversal learning in a way consistent with these observed results. If this upregulation is primarily occurring within these regions, then the data obtained from the whole brain analysis may be

underestimating how much $D_2$ receptor expression increased within them. However, it should not be assumed that $D_2$ expression in other regions remains unchanged. In fact, I would still expect $D_2$ receptor expression to be upregulated within them. Instead, the critical question with relevance to the current work is whether the change in expression within a specific region was associated with the improvement in reversal learning. Such a question requires $D_2$ expression to be assayed both prior to and following treatment, which cannot be achieved using a within-subject design that relies on *ex vivo* tissue analysis, but it does present an interesting idea to be tested by future research.

Another important question to address regarding $D_2$ receptor upregulation is whether this change is driven by presynaptic and/or postsynaptic $D_2$ receptor mechanisms. Indeed, it is possible that repeated eticlopride treatment led to an upregulation of $D_2$ receptors at either synaptic locus since the $D_2$ receptor is expressed as both an autoreceptor and postsynaptic receptor (see, Ford, 2014). To my knowledge, this idea has not been directly addressed.

A determination of how upregulation impacts motor activity may provide some insight into whether this upregulation is driven by presynaptic or postsynaptic changes in receptor expression since opposing roles of these receptors have been suggested. Evidence for this comes from studies that have examined how dopamine ligands or deletion of the genes putatively responsible for expressing $D_2$ autoreceptors or postsynaptic receptors impacted locomotor activity. Low doses of $D_2$ agonists, which preferentially stimulate autoreceptors and reduce synaptic dopamine (see, Ford, 2014) also reduced locomotor activity in rats (Johnson et al., 1976). Further, when $D_2$ autoreceptor expression was genetically knocked out in mice, locomotor activity was increased under drug-free conditions (Bello et al., 2011). In contrast, higher agonist doses, which would also be expected to stimulate postsynaptic $D_2$ receptors also facilitated locomotor activity (Frantz & van Hartesveldt, 1995; Johnson et al., 1976) and mice lacking postsynaptic $D_2$ receptors had markedly reduced locomotor activity under drug-free conditions (Wang et al., 2000). As such, $D_2$ autoreceptor mechanisms appear to regulate locomotion, and when stimulated, inhibit it, whereas postsynaptic $D_2$ receptors are implicated in the generation of locomotor activity.

Based on these studies, we can predict that postsynaptic upregulation would facilitate locomotor activity and presynaptic upregulation would attenuate locomotor activity. Consistent with the hypothesis that these changes are due to postsynaptic

mechanisms, repeated $D_2$ antagonist treatment that upregulated $D_2$ expression in rats also increased the amount of voluntarily produced locomotor behaviour (Kimura et al., 2021) and potentiated the locomotor-producing effects of methamphetamine (Oda et al., 2015). The idea that improvement in reversal learning is driven by changes in postsynaptic mechanisms is also consistent with the finding that putative postsynaptic, but not presynaptic, $D_2$ receptor stimulation in the dorsal striatum facilitated reversal learning (Horst et al., 2019). Therefore, it is tentatively suggested that at the very least, the treatment used in the present thesis resulted in an upregulation of postsynaptic $D_2$ receptors in the dorsal striatum. While the results of the present thesis are unable to ascertain this, it does present a second interesting idea to be addressed by future research.

### A Critical Role of Dopamine $D_2$ Receptor Expression in Reversal Learning

The present thesis also determined whether an eticlopride regimen that upregulated dopamine $D_2$ expression would improve reversal learning in rats (Hypothesis 2 & Hypothesis 5). The behavioural results of Studies 6 and 7 supported this and it is tempting to suggest that this behavioural effect is due to the upregulation of dopamine $D_2$ receptors since this treatment was clearly shown to upregulate receptor expression. Indeed, such a suggestion would concur with the works of others showing that reversal learning improved as a function of dopamine $D_2$ expression (Groman et al., 2011, 2012; Jocham et al., 2009; Linden et al., 2018).

Eticlopride also has a high affinity for the dopamine $D_3$ receptor (Shaik et al., 2021), and so, changes in behavioural flexibility might reflect antagonist-produced upregulation of this receptor. However, this is unlikely to be the case for two reasons. First, repeated exposure to dopamine $D_2/D_3$ antagonists selectively upregulated $D_2$ receptors in most brain sites (Hurley et al., 1996; Lévesque et al., 1995). Second, dopamine $D_2$ and dopamine $D_3$ receptor expression are related to reversal learning in opposing directions. While reversal learning has been shown to improve as a function of $D_2$ expression, the opposite pattern was reported between $D_3$ expression and reversal learning, such that reversal learning performance decreased as a function of $D_3$ receptor expression (Groman et al., 2016). As such, an upregulation of the dopamine $D_3$ receptor might be expected to impair reversal learning, and therefore, cannot explain the observed results. Instead, I propose the observed results are most likely a consequence of eticlopride-produced upregulation of dopamine $D_2$ expression.

I also determined whether the improvement in reversal learning was dependent on pre-existing differences in behavioural flexibility. It was previously shown that reversal learning improved as a function of $D_2$ expression, but critically, the magnitude of this improvement was greatest between subjects with lower $D_2$ expression (Groman et al., 2011). I reasoned, that because of this pattern, an upregulation would be expected to preferentially benefit subjects with lower baseline behavioural flexibility scores taken as an index of reduced $D_2$ receptor expression (Hypothesis 3). Indeed, the amount reversal learning improved following eticlopride treatment was negatively related to baseline behavioural flexibility. These findings provide support for the idea that the impact of dopaminergic manipulations on reversal learning are dependent on individual differences related to dopamine function (Cools et al., 2009).

The impact of this treatment regimen on an experimental model of behavioural flexibility associated with dopamine $D_2$ receptor expression was then examined. To induce behavioural inflexibility, a methamphetamine regimen shown to produce such a deficit was administered (Izquierdo et al., 2010) and was followed by the eticlopride regimen. Based on previous findings, methamphetamine was expected to impair reversal learning (Hypothesis 4) and eticlopride to protect against this deficit (Hypothesis 6). The results of the seventh study supported both hypotheses.

It might not come as a surprise that methamphetamine impaired reversal learning since reduced dopamine $D_2$ expression appears to be a functional consequence of repeated methamphetamine exposure (Groman et al., 2012; Thanos et al., 2017). As such, it is possible the deficit produced by the methamphetamine treatment and the prevention of this deficit following repeated eticlopride administration are the result of reciprocal changes in the same neurochemical target (i.e., opposing changes in the expression of the dopamine $D_2$ receptor). However, the impairment produced by methamphetamine may not exclusively reflect changes in $D_2$ expression. The dopamine $D_2$ receptor is not directly bound by methamphetamine, and instead, methamphetamine acts on other dopamine proteins, like the dopamine reuptake transporter. As such, $D_2$ receptor downregulation caused by methamphetamine is a downstream consequence and unlikely to be the only functional consequence. For example, repeated methamphetamine has also been shown to alter the expression of other dopamine proteins and is neurotoxic to dopamine cells more generally, especially within the striatum (for review, Gibson & Keefe, 2021). Given the critical role of dopamine in reversal learning (see, General Introduction), it may be

the case that any one of these effects, or some combination of them is producing this impairment. However, it is noted that the extent to which methamphetamine downregulated $D_2$ expression was related to the severity of the reversal impairment produced by it (Groman et al., 2012). Nonetheless, the reversal learning impairment produced by methamphetamine was prevented by a treatment that upregulates $D_2$ receptor expression, indicating that this neuroadaptive response is also restorative of or protective against experimentally produced behavioural inflexibility.

$D_1$ receptor expression was not measured or manipulated in the present thesis, but I will briefly touch on this receptor in order to provide a more holistic account of how the dopamine system contributes to associative learning. Generally speaking, the dopamine $D_1$ receptor is implicated in the processing of positive feedback and promoting approach towards rewarding-predicting stimuli (Alsiö et al., 2019; Chow et al., 2016; S. M. L. Cox et al., 2015; Dalley et al., 2005; Verharen et al., 2019) whereas the dopamine $D_2$ receptor is implicated in the processing of negative feedback, behavioural adaptation, and promoting avoidance behaviour (Alsiö et al., 2019; S. M. L. Cox et al., 2015; Verharen et al., 2019). The idea that $D_1$ and $D_2$ receptor mechanisms have functionally opposing roles in associative learning has received a great deal of interest and specific pathways have been identified that predominantly express $D_1$ or $D_2$ receptors (i.e., the go-pathway and no-go pathway, respectively; for review, Macpherson et al., 2014). Accordingly, $D_1$-expressing neurons promote the acquisition of behaviour via positive feedback and $D_2$-expressing neurons promote behavioural adaptation via negative feedback (see, S. M. L. Cox et al., 2015). Based on this framework, we might predict that the dopamine $D_1$ receptor is particularly important during the initial discrimination training stage, but not the subsequent reversal learning stage. However, this distinction is not supported by the literature, and instead, a number of studies have reported that $D_1$ receptor ligands failed to disrupt both discrimination and reversal learning (Floresco, Magyar, et al., 2006; Haluk & Floresco, 2009; Lee et al., 2007; Marino et al., 2022; Ragozzino, 2002; Sala-Bayo et al., 2020).

It is tempting to suggest that the $D_1$ receptor is unimportant for discrimination learning, but there are some possible caveats that must be considered. First, in order to discriminate between two stimuli, a subject must learn the relevance of the $S^+$ in signalling reinforcer availability and learn the $S^-$ is irrelevant (i.e., it does not predict reinforcer availability). As such, even a simple discrimination between two stimuli will be, at least

in part, dependent on the acquisition of stimulus irrelevance and this might not be expected to be dependent on $D_1$ receptor mechanisms since it is not driven by positive feedback. This raises the possibility that these tasks rely on other participating dopamine receptors and/or neurochemical systems. Second, there is a tendency to use broader behavioural measures (e.g., trials to criterion) during the initial discrimination stage which may limit how sensitive these tasks are to changes in feedback sensitivity. As such, the role of the dopamine $D_1$ receptor remains unclear in reversal learning tasks, but this may be a result of paradigmatic issues. In contrast, the idea that the dopamine $D_2$ receptor is selectively implicated in behavioural adaptation is consistent with the results of behavioural studies. For example, genetic ablation of $D_2$ expressing projecting spiny neurons left discrimination learning intact but markedly impaired reversal learning (Matamales et al., 2020), administration of $D_2$ receptor antagonists that impaired reversal learning spared discrimination learning (Bailey & Lee, 2007; Marino et al., 2022), and perseveration following contingency reversal decreased as a function of $D_2$ receptor expression (Groman et al., 2011). These findings, those presented earlier in the present thesis, and the results of the studies I have conducted provide mounting support for the idea that $D_2$ receptor mechanisms are critically involved in modifying already-established behaviour.

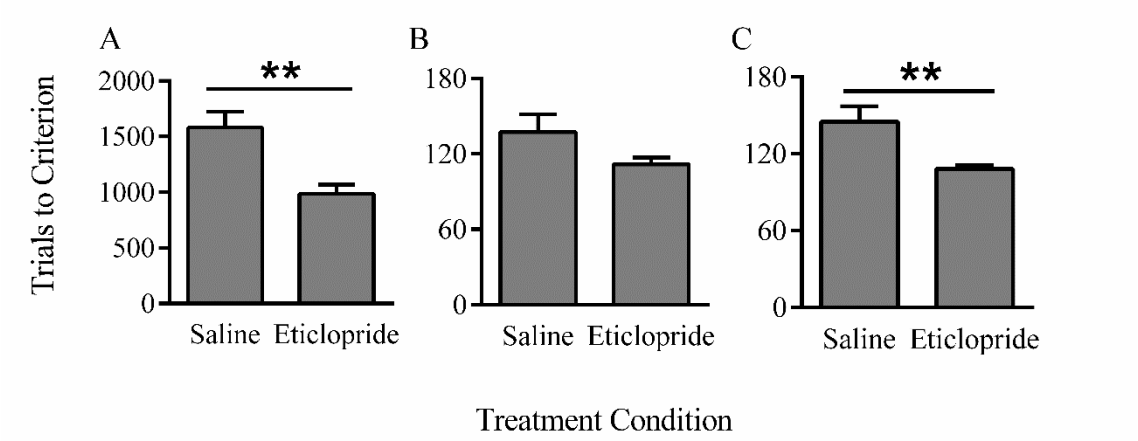
**Is Visual Discrimination More Difficult than Spatial Discrimination?**

In Chapter 3 the idea was raised that it may be easier to acquire behaviours that require the use of spatial cues compared to ones that require the use of visual cues. While I was unable to examine this idea in the studies detailed in that chapter, I was able to address this question to some extent using supplementary findings. To do this, a set of additional tests were conducted immediately after the third reversal task was completed by the subjects in Study 6 (Chapter 4, "Effect of Repeated Eticlopride on Reversal Learning"). In order, these tests included retention of the third reversal task, a set-shift task where depression of the left lever was exclusively reinforced, a retention test of the set-shift task, and finally, a spatial discrimination reversal where depression of the right lever was exclusively reinforced. This testing could not be conducted with the entire sample due to equipment constraints, and so, only 19 subjects were able to complete all stages of this testing (eticlopride: $n = 11$; saline: $n = 8$). Additionally, I lacked the pre-treatment spatial reversal learning data required to calculate a spatial TTC difference

score, as done in Chapter 4. For these reasons, TTC during the third visual reversal task (VRL), the set-shift task, and the spatial reversal task (SRL) were examined, but not included in Chapter 4, and instead, discussed here as supplementary findings (see, Figure 6.2).

**Figure 6.2**

*TTC (+SEM) for the VRL, Set-Shift, and SRL task*




*Note.* This figure shows the TTC from the VRL (Graph A), Set-Shift (Graph B), and SRL (Graph C) tasks. The data displayed only includes that from the 19 subjects that completed all three behaviour tests shown here. It is also noted that the y-axis range is different for the VRL task (Graph A). ** $p < .01$

Perhaps the most striking observation is that the criterion was met within considerably fewer trials in the set-shift and SRL tasks, which required the use of spatial stimuli, compared to the VRL which required discrimination between visual stimuli[5]. I am not the first to suggest that additional training is required to acquire visual discrimination (e.g., Wright et al., 2019), but to my knowledge, such extensive differences have not been previously reported. It may be the case that the use of visual stimuli impacted behaviour more than originally speculated in the discussion section of Studies 1 and 2. However, it is also noted that the criterion used in Study 5 imposed a much higher persistence requirement than the one used in Studies 1 and 2. As such, it

[5] I did not see any reason to subject these data to statistical analysis since there was approximately a 10-fold difference in TTC between the visual and spatial tasks.

may be the case that this difference in criteria is also exaggerating any differences attributable to the type of cue used. Nonetheless, these data suggest that when conducted under comparable testing conditions, much more extensive training was required to meet the criterion when the discriminative stimulus was a visual cue.

The fact discrimination between visual stimuli required such extensive training to acquire begs the question as to whether the data obtained from the VRL and SRL tasks measure the same underlying processes. To examine this idea, the TTC data for these two tasks (VRL and SRL) were subject to correlational analysis and a moderate-to-strong positive correlation was observed between these measures ($r$ = .61, $p$ = .005). While this does mean that these measures were not perfectly aligned, it is also noted that the correlation values between the various visual reversal tasks discussed in the present thesis also ranged from $r$ = .71 to $r$ = .86. Given the vast difference in trials required to complete the SRL and VRL, and the use of a different stimulus type, this small reduction in strength is not unexpected. However, I still suggest that this correlation does provide reasonable evidence for consistency in what is being indexed by the measures obtained from the VRL and SRL task. That is, these tasks are likely to be measuring a similar construct.

A final important observation that can be drawn from this supplementary analysis is the eticlopride treated group required significantly fewer trials to complete the SRL task, but this did not reach significance in the set-shift task (see, "Table D1" for assumptions and "Table D2" for analysis of group differences). This indicates two important findings. First, the improvement in reversal learning produced by $D_2$ upregulation appears to be restricted to reversal learning. Second, the effect of eticlopride was highly persistent, lasting several weeks beyond the initial treatment regimen. Of interest, the advantage produced by eticlopride during the VRL (approximately 35% reduction in TTC compared to saline) appears to be greater than it was during the SRL task (approximately 26% reduction in TTC compared to saline), indicating that this treatment may more effectively impact VRL performance. However, it may also be the case that dopamine $D_2$ receptor expression had begun reverting towards pre-treatment expression levels by the time SRL testing began, and this may instead be the source of this apparent reduction in effectiveness. Nonetheless, this treatment facilitated reversal learning, irrespective of whether the discriminative stimulus had spatial or visual properties. These findings further support the critical role of $D_2$ receptor expression in

reversal learning, even under conditions where exceedingly different amounts of testing are required to meet the criterion.

**Additional Considerations**

All studies detailed in the present thesis utilised male test subjects, so my observations may not generalise to female subjects. Indeed, sex differences in the expression of $D_2$ expression have been observed in juvenile rats (Orendain-Jaime et al., 2016), although these differences were not observed in older rats that had comparable performance in a reversal task (Izquierdo et al., 2016). However, a treatment regimen that targeted the dopamine transporter did produce sex-dependent effects on reversal learning (Izquierdo et al., 2016), and so, it is possible that the treatment regimen used in my studies may differentially impact female rats. The decision to not include both male and female subjects was largely due to time and equipment constraints. In short, daily testing required the occupancy of shared operant chambers for approximately half a day. I could not extend these hours since the equipment was in use outside of this, and so, the inclusion of a sex variable would have required each study to be effectively conducted as two separate studies, one after the other. This would have considerably extended the data collection period for each study, effectively turning the 6 behavioural studies detailed here into 12 behavioural studies. This was considered beyond the scope of the present thesis. Nonetheless, the possibility of sex difference remains an important question for future research.

$D_2$ expression was not examined at any other stage of the testing beyond Study 5. As noted above, a critical question remains as to whether changes in $D_2$ expression were responsible for the improvement in reversal learning, but that this also requires $D_2$ expression to be measured prior to and following treatment, which is not possible with an *ex vivo* technique like flow cytometry. However, it is also noted that flow cytometry was only conducted once, and this was three days following eticlopride treatment. While the behavioural data indicates that the improvement produced by eticlopride was persistent, I cannot ascertain whether the changes in $D_2$ expression were. As such, this remains a critical question to address.

## Conclusion

To better understand the underlying neurochemical processes relevant to behavioural flexibility I developed a means of measuring reversal learning in rats. From here, I was able to clearly demonstrate that a repeated antagonist treatment that upregulated dopamine $D_2$ receptor expression also facilitated reversal learning and ameliorated impairments in behavioural flexibility, irrespective of whether they were pre-existing or experimentally produced. By conducting two reversal tasks prior to treatment, I was able to determine that this measurement of behavioural flexibility was reliable. Baseline behavioural flexibility scores were variable and individual differences were maintained across the two pre-treatment tests for all rats included in the final samples of each study. I also ensured that baseline behavioural flexibility was comparable between treatment groups to rule out pre-existing differences as an explanation of any observed effects and used a change in TTC score to further control for this. A retention phase was also included to ensure that the treatment did not interfere with the memory of the behaviour that had been learned prior to treatment. Collectively, this allowed me to confidently attribute the observed changes in behavioural flexibility in the present thesis to the treatments used. Thus, the studies detailed in the present thesis support the critical role of $D_2$ receptor expression in reversal learning. Further, they indicate that the dopamine $D_2$ receptor is a highly effective target for enhancing reversal learning in subjects with compromised behavioural flexibility.

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## Appendix A

### Results of Assumption Tests from Chapter 3

**Table A1**

*Results of the Assumption Tests from Criterion Requiring 80% Accuracy in 8 of 10 Blocks*

| Assumption Tested | Details About the Assumption Test Conducted: 1-Day Criterion | | | |
|---|---|---|---|---|
| | Group | Data | Test | Result |
| Normality | Illuminated | Raw | Shapiro-Wilk | $W$ (16) = .92, $p$ = .156 |
| Normality | Unilluminated | Raw | Shapiro-Wilk | $W$ (16) = .93, $p$ = .214 |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 30) = 0.00, $p$ = .982 |
| Assumption Tested | Details About the Assumption Test Conducted: 3-Day Criterion | | | |
| | Group | Data | Test | Result |
| Normality | Illuminated | Raw | Shapiro-Wilk | $W$ (16) = .93, $p$ = .267 |
| Normality | Unilluminated | Raw | Shapiro-Wilk | $W$ (16) = .91, $p$ = .137 |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 30) = 0.36, $p$ = .553 |
| Assumption Tested | Details About the Assumption Test Conducted: 5-Day Criterion | | | |
| | Group | Data | Test | Result |
| Normality | Illuminated | Raw | Shapiro-Wilk | $W$ (16) = .89, $p$ = .046* |
| Normality | Unilluminated | Raw | Shapiro-Wilk | $W$ (16) = .85, $p$ = .014* |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 30) = 8.73, $p$ = .006* |
| Normality | Illuminated | $x^{-1}$ | Shapiro-Wilk | $W$ (16) = .91, $p$ = .103 |
| Normality | Unilluminated | $x^{-1}$ | Shapiro-Wilk | $W$ (16) = .89, $p$ = .053 |
| Homogeneity | Entire sample | $x^{-1}$ | Levene's | $F$ (1, 30) = 2.65, $p$ = .114 |

*Note.* This table shows the results of the assumption tests. It details the assumption required to conduct the test mentioned in the text of this thesis, whether the assumption was examined within a specific group or across all conditions, whether the data were transformed or raw, the test used to examine whether the assumption was met, and the appropriate statistical output of the test. *Assumption not met with data used.

**Table A2**

*Results of the Assumption Tests for the Remaining Criteria in Chapter 3*

| Assumption Tested | Criterion: 90% accuracy in 8 out of 10 blocks for 1 day | | | |
|---|---|---|---|---|
| | Group | Data | Test | Result |
| Normality | Illuminated | Raw | Shapiro-Wilk | $W$ (16) = .92, $p$ = .143 |
| Normality | Unilluminated | Raw | Shapiro-Wilk | $W$ (16) = .97, $p$ = .790 |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 30) = 0.02, $p$ = .898 |
| Assumption Tested | Criterion: 80% accuracy in all 10 blocks for 1 day | | | |
| | Group | Data | Test | Result |
| Normality | Illuminated | Raw | Shapiro-Wilk | $W$ (16) = .89, $p$ = .075 |
| Normality | Unilluminated | Raw | Shapiro-Wilk | $W$ (16) = .82, $p$ = .005* |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 30) = 2.22, $p$ = .147 |
| Normality | Illuminated | $x^{-1}$ | Shapiro-Wilk | $W$ (16) = .90, $p$ = .073 |
| Normality | Unilluminated | $x^{-1}$ | Shapiro-Wilk | $W$ (16) = .90, $p$ = .079 |
| Homogeneity | Entire sample | $x^{-1}$ | Levene's | $F$ (1, 30) = 1.14, $p$ = .294 |

*Note.* This table shows the results of the assumption tests. It details the assumption required to conduct the test mentioned in the text of this thesis, whether the assumption was examined within a specific group or across all conditions, whether the data were transformed or raw, the test used to examine whether the assumption was met, and the appropriate statistical output of the test.

*Assumption not met with data used.

**Table A3**

*Results of TOST testing for Group Equivalence*

| Criterion | Lower Bound Test | Upper Bound Test |
|---|---|---|
| 80% Accuracy/8 of 10 blocks/1 day* | $t$ (30) = 1.93, $p$ = .031 | $t$ (30) = -4.74, $p$ < .001 |
| 80% Accuracy/8 of 10 blocks/3 days* | $t$ (30) = 2.10, $p$ = .022 | $t$ (30) = -4.58, $p$ < .001 |
| 80% Accuracy/8 of 10 blocks/5 days | $t$ (30) = 6.29, $p$ < .001 | $t$ (30) = -0.38, $p$ = .352 |
| 80% Accuracy/All 10 blocks/1 day | $t$ (30) = 5.85, $p$ < .001 | $t$ (30) = -0.83, $p$ = .207 |
| 90% Accuracy/8 of 10 blocks/1 day | $t$ (30) = 1.28, $p$ = .105 | $t$ (30) = -5.40, $p$ < .001 |

*Note.* This table shows the results of the TOST analysis. For groups to be considered equivalent, both the lower bound and upper bound tests must be statistically significant.

* When the criterion was applied, days to the criterion were equivalent between groups.

## Appendix B

### Results of Assumption Tests from Chapter 4

**Table B1**

*Results of the Assumption Tests from the Baseline Reversal Learning Measures in Chapter 4*

| Assumption Tested | Details About the Assumption Test Conducted: Reversal 1 Data | | | |
|---|---|---|---|---|
| | Condition | Data | Test | Result |
| Normality | Saline | Raw | Shapiro-Wilk | $W$ (11) = .96, $p$ = .929 |
| Normality | Eticlopride | Raw | Shapiro-Wilk | $W$ (14) = .89, $p$ = .108 |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 23) = 0.37, $p$ = .547 |
| Assumption Tested | Details About the Assumption Test Conducted: Reversal 2 Data | | | |
| | Condition | Data | Test | Result |
| Normality | Saline | Raw | Shapiro-Wilk | $W$ (11) = .93, $p$ = .334 |
| Normality | Eticlopride | Raw | Shapiro-Wilk | $W$ (14) = .93, $p$ = .513 |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 23) = 0.36, $p$ = .553 |

*Note.* This table shows the results of the assumption tests. It details the assumption required to conduct the test mentioned in the text of this thesis, whether the assumption was examined within a specific condition or across all conditions, whether the data were transformed or raw, the test used to examine whether the assumption was met, and the appropriate statistical output of the test.

**Table B2**

*Results of the Assumption Tests from the Retention Measures in Chapter 4*

| Assumption Tested | Details About the Assumption Test Conducted: Retention Test Data | | | |
|---|---|---|---|---|
| | Condition | Data | Test | Result |
| Normality | Saline | Raw | Shapiro-Wilk | $W$ (11) = .87, $p$ = .073 |
| Normality | Eticlopride | Raw | Shapiro-Wilk | $W$ (14) = .81, $p$ = .006* |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 23) = 3.72, $p$ = .066 |
| Normality | Saline | *ln* (x) | Shapiro-Wilk | $W$ (11) = .87, $p$ = .096 |
| Normality | Eticlopride | *ln* (x) | Shapiro-Wilk | $W$ (14) = .91, $p$ = .160 |
| Homogeneity | Entire sample | *ln* (x) | Levene's | $F$ (1, 23) = 3.55, $p$ = .072 |

*Note.* This table shows the results of the assumption tests. It details the assumption required to conduct the test mentioned in the text of this thesis, whether the assumption was examined within a specific condition or across all conditions, whether the data were transformed or raw, the test used to examine whether the assumption was met, and the appropriate statistical output of the test.

*Assumption not met with data used.

**Table B3**

*Results of the Assumption Tests from the ΔTTC Measures in Chapter 4*

| Assumption Tested | Details About the Assumption Test Conducted: ΔTTC | | | |
|---|---|---|---|---|
| | Condition | Data | Test | Result |
| Normality | Saline | Raw | Shapiro-Wilk | $W$ (11) = .95, $p$ = .453 |
| Normality | Eticlopride | Raw | Shapiro-Wilk | $W$ (14) = .94, $p$ = .612 |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 23) = 0.01, $p$ = .941 |

*Note.* This table shows the results of the assumption tests. It details the assumption required to conduct the test mentioned in the text of this thesis, whether the assumption was examined within a specific condition or across all conditions, whether the data were transformed or raw, the test used to examine whether the assumption was met, and the appropriate statistical output of the test.

**Table B4**

*Results of the Assumption Tests for the Regression Analysis in Chapter 4*

| Assumption Tested | Details About the Assumption Test Conducted: Regression Analysis | | | |
|---|---|---|---|---|
| | Condition | Data | Test | Result |
| Normality | Saline | Raw | Shapiro-Wilk | $W$ (11) = .84, $p$ = .134 |
| Normality | Eticlopride | Raw | Shapiro-Wilk | $W$ (14) = .90, $p$ = .112 |
| Homoskedasticity | Saline | Raw | Breusch-Pagan | $\chi^2$ (1) = 1.01, p = .314 |
| Homoskedasticity | Eticlopride | Raw | Breusch-Pagan | $\chi^2$ (1) = 0.11, p = .742 |

*Note.* This table shows the results of the assumption tests. It details the assumption required to conduct the test mentioned in the text of this thesis, whether the assumption was examined within a specific condition or across all conditions, whether the data were transformed or raw, the test used to examine whether the assumption was met, and the appropriate statistical output of the test.

## Appendix C

### Results of Assumption Tests from Chapter 5

**Table C1**

*Results of the Assumption Tests from the Baseline Reversal Learning Measures in Chapter 5*

| Assumption Tested | Details About the Assumption Test Conducted: Reversal 1 Data | | | |
|---|---|---|---|---|
| | Condition [a] | Data | Test | Result |
| Normality | Sal/Sal | Raw | Shapiro-Wilk | $W$ (6) = .91, $p$ = .453 |
| Normality | Sal/Etic | Raw | Shapiro-Wilk | $W$ (7) = .90, $p$ = .346 |
| Normality | Meth/Sal | Raw | Shapiro-Wilk | $W$ (8) = .73, $p$ = .005* |
| Normality | Meth/Etic | Raw | Shapiro-Wilk | $W$ (8) = .88, $p$ = .193 |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (3, 25) = 0.03, $p$ = .994 |
| Assumption Tested | Details About the Assumption Test Conducted: Reversal 2 Data | | | |
| | Condition [a] | Data | Test | Result |
| Normality | Sal/Sal | Raw | Shapiro-Wilk | $W$ (6) = .85, $p$ = .143 |
| Normality | Sal/Etic | Raw | Shapiro-Wilk | $W$ (7) = .91, $p$ = .370 |
| Normality | Meth/Sal | Raw | Shapiro-Wilk | $W$ (8) = .75, $p$ = .008* |
| Normality | Meth/Etic | Raw | Shapiro-Wilk | $W$ (8) = .86, $p$ = .132 |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (3, 25) = 0.44, $p$ = .728 |

*Note.* This table shows the results of the assumption tests. It details the assumption required to conduct the test mentioned in the text of this thesis, whether the assumption was examined within a specific condition or across all conditions, whether the data were transformed or raw, the test used to examine whether the assumption was met, and the appropriate statistical output of the test.

[a] Sal = Saline; Meth = Methamphetamine; Etic = Eticlopride

* Assumption not met with data used.

**Table C2**

*Results of the Assumption Tests from the Baseline Reversal Learning Measures in Chapter 5*

| Assumption Tested | Details About the Assumption Test Conducted: Reversal 1 Data | | | |
|---|---|---|---|---|
| | Condition [a] | Data | Test | Result |
| Normality | Sal/Sal | *ln* (x) | Shapiro-Wilk | $W$ (6) = .94, $p$ = .677 |
| Normality | Sal/Etic | *ln* (x) | Shapiro-Wilk | $W$ (7) = .98, $p$ = .939 |
| Normality | Meth/Sal | *ln* (x) | Shapiro-Wilk | $W$ (8) = .86, $p$ = .106 |
| Normality | Meth/Etic | *ln* (x) | Shapiro-Wilk | $W$ (8) = .98, $p$ = .961 |
| Homogeneity | Entire sample | *ln* (x) | Levene's | $F$ (3, 25) = 0.26, $p$ = .853 |
| Assumption Tested | Details About the Assumption Test Conducted: Reversal 2 Data | | | |
| | Condition [a] | Data | Test | Result |
| Normality | Sal/Sal | *ln* (x) | Shapiro-Wilk | $W$ (6) = .92, $p$ = .528 |
| Normality | Sal/Etic | *ln* (x) | Shapiro-Wilk | $W$ (7) = .97, $p$ = .879 |
| Normality | Meth/Sal | *ln* (x) | Shapiro-Wilk | $W$ (8) = .80, $p$ = .027* |
| Normality | Meth/Etic | *ln* (x) | Shapiro-Wilk | $W$ (8) = .92, $p$ = .411 |
| Homogeneity | Entire sample | *ln* (x) | Levene's | $F$ (3, 25) = 0.56, $p$ = .649 |

*Note.* This table shows the results of the assumption tests. It details the assumption required to conduct the test mentioned in the text of this thesis, whether the assumption was examined within a specific condition or across all conditions, whether the data were transformed or raw, the test used to examine whether the assumption was met, and the appropriate statistical output of the test.

[a] Sal = Saline; Meth = Methamphetamine; Etic = Eticlopride

* Assumption not met with data used.

**Table C3**

*Results of the Assumption Tests from the Retention Measure in Chapter 5*

| Assumption Tested | Details About the Assumption Test Conducted: Retention Data | | | |
|---|---|---|---|---|
| | Condition [a] | Data | Test | Result |
| Normality | Sal/Sal | Raw | Shapiro-Wilk | $W$ (6) = .82, $p$ = .091 |
| Normality | Sal/Etic | Raw | Shapiro-Wilk | $W$ (7) = .72, $p$ = .006* |
| Normality | Meth/Sal | Raw | Shapiro-Wilk | $W$ (8) = .86, $p$ = .119 |
| Normality | Meth/Etic | Raw | Shapiro-Wilk | $W$ (8) = .84, $p$ = .067 |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (3, 25) = 9.05, $p$ < .001 |

*Note.* This table shows the results of the assumption tests. It details the assumption required to conduct the test mentioned in the text of this thesis, whether the assumption was examined within a specific condition or across all conditions, whether the data were transformed or raw, the test used to examine whether the assumption was met, and the appropriate statistical output of the test. These data could not be transformed to meet the assumptions (see, chapter 5 for details), and so, only the results of the raw data are presented for these data.

[a] Sal = Saline; Meth = Methamphetamine; Etic = Eticlopride

* Assumption not met with data used.

**Table C4**

*Results of the Assumption Tests from the ΔTTC Measure in Chapter 5*

| Assumption Tested | Details About the Assumption Test Conducted: ΔTTC Data | | | |
|---|---|---|---|---|
| | Condition [a] | Data | Test | Result |
| Normality | Sal/Sal | Raw | Shapiro-Wilk | $W$ (6) = .89, $p$ = .308 |
| Normality | Sal/Etic | Raw | Shapiro-Wilk | $W$ (7) = .94, $p$ = .599 |
| Normality | Meth/Sal | Raw | Shapiro-Wilk | $W$ (8) = .94, $p$ = .587 |
| Normality | Meth/Etic | Raw | Shapiro-Wilk | $W$ (8) = .87, $p$ = .136 |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (3, 25) = 1.40, $p$ = .267 |

*Note.* This table shows the results of the assumption tests. It details the assumption required to conduct the test mentioned in the text of this thesis, whether the assumption was examined within a specific condition or across all conditions, whether the data were transformed or raw, and the test used to examine whether the assumption was met, and the appropriate statistical output of the test.

[a] Sal = Saline; Meth = Methamphetamine; Etic = Eticlopride

## Appendix D

### Statistical Analysis of Supplementary Findings Reported in General Discussion

**Table D1**

*Results of Assumption Tests for Analysis of TTC*

| Assumption Tested | Details About the Assumption Test Conducted: Visual Reversal Task | | | |
|---|---|---|---|---|
| | Condition | Data | Test | Result |
| Normality | Saline | Raw | Shapiro-Wilk | $W$ (8) = .93, $p$ = .539 |
| Normality | Eticlopride | Raw | Shapiro-Wilk | $W$ (11) = .98, $p$ = .936 |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 17) = 1.11, $p$ = .306 |
| Assumption Tested | Details About the Assumption Test Conducted: Set-Shift Task | | | |
| | Condition | Data | Test | Result |
| Normality | Saline | Raw | Shapiro-Wilk | $W$ (8) = .86, $p$ = .108 |
| Normality | Eticlopride | Raw | Shapiro-Wilk | $W$ (11) = .73, $p$ = .001* |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 17) = 7.35, $p$ = .015* |
| Assumption Tested | Details About the Assumption Test Conducted: Spatial Reversal Task | | | |
| | Condition | Data | Test | Result |
| Normality | Saline | Raw | Shapiro-Wilk | $W$ (8) = .84, $p$ = .081 |
| Normality | Eticlopride | Raw | Shapiro-Wilk | $W$ (11) = .73, $p$ = .001* |
| Homogeneity | Entire sample | Raw | Levene's | $F$ (1, 17) = 28.92, $p$ < .001* |

*Note.* This table shows the results of the assumption tests. It details the assumption required to conduct the test mentioned in the text of this thesis, whether the assumption was examined within a specific condition or across all conditions, whether the data were transformed or raw, the test used to examine whether the assumption was met, and the appropriate statistical output of the test. The spatial set-shift and reversal tasks could not be transformed to meet assumptions for conducting a Student's or Welch's t-test.

*Assumption not met with data used.

**Table D2**

*Results of Inferential Analysis of TTC*

| Behavioural Task | Results of the Supplementary Data Analysis | | |
|---|---|---|---|
| | Statistical Test | Statistical Output [A] | Effect Size [B] |
| Visual Reversal Task | Student's t-test | $t$ (17) = 3.86, $p$ = .001* | *Cohen's d* = 1.79 |
| | Mann-Whitney U | U (19) = 6.00, $p$ < .001* | *RBC* = .86 |
| Set-Shift Task | Mann-Whitney U | U (19) = 26.00, $p$ = .131 | *RBC* = .41 |
| Spatial Reversal Task | Mann-Whitney U | U (19) = 11.00, $p$ = .006* | *RBC* = .75 |

*Note.* This table shows the results of the supplementary data analysis in the general discussion. When the assumptions were not met to conduct Student's or Welche's t-test, the data were subject to a Mann-Whitney U test. The only variable in any test was drug treatment (saline, eticlopride). For consistency, a Mann-Whitney U test is also reported for the Visual Reversal Task despite these data meeting the assumptions.

[A] The degrees of freedom and sample size are in the parenthesis for the Student's t-test and Mann-Whitney U-test, respectively.

[B] RBC refers to the Rank Biserial Correlation effect size estimation.

*Significant effect of eticlopride treatment.