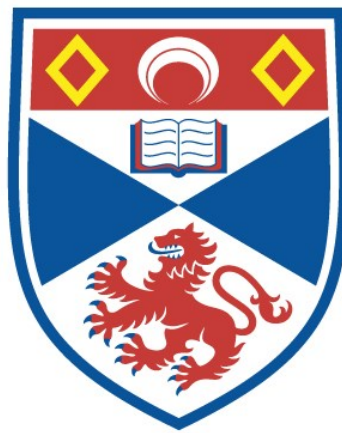


LEARNING TO FOCUS AND FOCUSING TO LEARN:  
MORE THAN A CORTICAL TRICK

Sandeep S. Dhawan

A Thesis Submitted for the Degree of PhD  
at the  
University of St Andrews



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Learning to focus and focusing to learn: more than  
a cortical trick

Sandeep S. Dhawan



University of  
St Andrews

This thesis is submitted in partial fulfilment for the degree of

Doctor of Philosophy (PhD)

at the University of St Andrews

March 2018

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# Abstract

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The consequence of many psychiatric and neurodegenerative disorders, such as Parkinson's disease and schizophrenia, is an impairment in 'executive functioning'; an umbrella term for several cognitive processes, including the focussing and shifting of attention and the inhibition of responding. The ability to form an 'attentional set' involves learning to discriminate qualities of a multidimensional cue, and to subsequently learn which quality is relevant, and therefore predictive of reward. According to recent research, the subthalamic nucleus (STN) and possibly the adjacent zona incerta (ZI) may mediate the formation of attentional set. Dysregulation of the STN as a result of Parkinson's disease contributes to characteristic motor symptoms, and whilst deep-brain stimulation of this region may treat gross motor impairments, it may also impair cognition. The work in this thesis aimed to expand our understanding of the mechanisms of attentional set-formation, and the role of the STN in this process.

This thesis evaluates new methods for examining set-formation in the attentional set-shifting task; rather than inferring this behaviour solely from the cost of shifting set, modifications to the task design in Chapters 3 & 4 explored several hypotheses designed to exploit a deficit in this behaviour. Chapter 6 revealed that inhibition of this region with designer receptors leads to a disruption in attentional selectivity, which compromises the ability to form an attentional set. This manifested as an inability to parse relevant information from irrelevant, and instead, animals learned the stimuli holistically. The findings in this thesis also suggested that reversal and attentional shifting processes do not operate independently, but rather in a hierarchy, and that consequently, the STN is a region that may be crucial in selecting appropriate responses during associative learning that leads to the formation of an attentional set.

# Abbreviations

---

5CSRTT	5-Choice Serial Reaction Time Task
5-HT	5-hydroxytryptomine
6-OHDA	6-hydroxydopamine
AAV	Adeno-associated virus
ADHD	Attention-deficit/hyperactivity disorder
ADS	Antibody diluting solution
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ASST	Attentional set-shifting task
BG	Basal ganglia
CaMKII	Ca <sup>2+</sup> /calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CANTAB	Cambridge Neuropsychological Automated Test Battery
CD	Compound discrimination
CeA	Central nucleus of the Amygdala
CNO	Clozapine <i>n</i> -oxide
CRT	Choice Reaction Time
CT	Circadian time
cZI	Caudal Zona Incerta
DAB	Sigma Fast 3.3-Diaminobenzidine
DAT	Dopamine transporter
DBS	Deep-brain stimulation
dIPFC	Dorso-lateral prefrontal cortex
DMS	Dorsomedial striatum
DREADDs	Designer Receptors Exclusively Activated by Designer Drugs
DRN	Dorsal Raphe Nuclei
ED	Extradimensional acquisition stage

EEG	Electroencephalogram
Ef1a	Human elongation factor-1-alpha
EP	Entopeduncular nucleus
FF	Fields of Forel
GABA	$\gamma$ -aminobutyric acid
GAD	Glutamic acid decarboxylase
GFAP	Glial-fibrillary acidic protein
GFP	Green fluorescent protein
GIRK	G-protein inward-rectifying potassium channel
Glu	Glutamate
GPe	External globus pallidus
GPi	Internal globus pallidus
HA	Human influenza haemagglutinin
HFS	High-frequency stimulation
hsyn	Human synapsin
ID	Intradimensional acquisition stage
IGT	Iowa Gambling Task
LHA	Lateral hypothalamic area
LMA	Locomotor activity
LTP	Long-term potentiation
mGluR	Metabotropic glutamate receptor
MGP	Medial globus pallidus
MPEP	2-methyl-6-(phenylethynyl)-pyridine
mPFC	Medial prefrontal cortex
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSNs	Medium spiny neurons
NA	Noradrenaline
NCD	Novel compound discrimination
NMDA	<i>N</i> -methyl- <i>D</i> -aspartate
OFC	Orbital prefrontal cortex

ORE	Overtraining Reversal Effect
ORG	ORG49209
OT	Overtraining
PAM	Positive allosteric modulator
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PFC	Prefrontal cortex
PSD	Postsynaptic density
Psth	Posterior subthalamic area
REV	Reversal learning stage
rGT	Rat Gambling Task
RNAP	Ribonucleic acid polymerase
RT	Reaction time
SD	Simple discrimination
SEM	Standard error of the mean
SNc	Substantia nigra compacta
SNr	Substantia nigra reticulate
SRT	Simple Reaction Time
SSRT	Stop-signal reaction time
STN	Subthalamic nucleus
WCST	Wisconsin Card-Sorting Task
WGTA	Wisconsin General Testing Apparatus
ZID	Dorsal zona incerta
ZIV	Ventral zona incerta



# Chapter 1

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## General Introduction

Executive functions are exercised readily in daily life, facilitating a variety of tasks, such as maintaining focus in a noisy environment or planning and executing a goal. Disruption of these processes is found in many psychiatric/neurodegenerative disorders and is also associated with healthy ageing. One such process is the ability to form an attentional set, and there is recent evidence that the STN – a key input structure of the basal ganglia – and possibly the ZI, might be implicated in this process. In this chapter, I will outline a brief history of behavioural tasks that led to the conception of the attentional set-shifting task used in this thesis, along with a theoretical framework which accounts for it. Information regarding the connections and neurochemistry of the STN/ZI will also be briefly explored, along with examining the current role of the STN in cognition.

It was the summer of 2014. As a first-year PhD student, I eagerly anticipated my first summer ‘vacation’ in picturesque Scotland, and decided to hire a car for the purpose of driving to the Highlands. I ventured westward and was staying the night in Ballachulish, near Glencoe, which has been lauded for its striking, natural beauty. However, there was an ‘obstacle’ which loomed over me and required addressing before setting off from St Andrews – as a Canadian who has never driven in the UK before, I have never operated a right-hand drive car.

I hesitantly departed from the east coast with this consideration in mind, and being a motoring enthusiast I felt confident in my ability to not become ‘one with nature’ by ending up in a ‘loch’. As the journey progressed, I gradually became more accustomed to driving on the left side of the road, whilst concurrently operating the gear stick with my left hand. I also kept a watchful eye out for signs for the A85, to guide me toward the Trossachs National park, whilst equally ignoring signs that would take me north, into the Cairngorms National park. Despite not having driven since leaving Canada over a year prior to this trip, I managed to arrive at my destination unscathed, and fortunately, so did the hired car. Whilst this adaptation in driving behaviour does not seem overly arduous, there existed a myriad of cognitive processes which facilitated the journey, each intersecting with harmonious complexity. In order to make this cross-Scotland expedition, I needed to:

1. remember how to operate a car, including mechanical skills such as operating a clutch pedal
2. attend to visual cues in my environment consistent with my goal, such as directional signs, and signs indicating the speed limit
3. retain navigational directions for my destination, and continually update these directions with passing road signs
4. extrapolate relevant memories from navigating Canadian roads to Scottish roads (*i.e.*, landmark X is roughly 10 miles from the upcoming turn onto road Y)
5. remember the rules for the direction of traffic in Canada (look left first, then right when proceeding)

6. manipulate and apply these rules to driving in the UK (look right, then left when proceeding)
7. respond flexibly to sudden changes in my internal (*e.g.*, sudden motion sickness) or external (*e.g.*, deer in the road) environment
8. avoid distraction from extraneous activities in my environment (*e.g.*, a kilted bagpiper on the roadside)

The listed steps illustrate fundamental cognitive requirements for everyday life, including the ability to identify goals and organise them into a plan, retain information in memory, access and integrate relevant memories with current environmental information, all whilst remaining flexible to changes in one's environment. These processes exemplify what is known as 'executive control'; an umbrella term used to describe cognitive functions such as working memory, planning, mental flexibility, and the initiation or inhibition of action (for review see Chan, Shum, Touloupoulou, & Chen, 2008). Inhibition of action can be further divided based on whether the cognitive demands arise from a required change in the internal ('self-control': resisting temptation or impulsive action) or external ('interference control': selectively attending to relevant information/inhibiting irrelevant information) environment (Diamond, 2013). As in my trip to Ballachulish, the roles of many executive functions intersect, or are dependent on one another, which gives rise to whether a 'central executive' or an attentional-controlling system may govern executive resources (Atkinson & Shiffrin, 1971; but see: Baddeley & Hitch, 1974).

Research by Atkinson & Shiffrin (1968) posited that a 'short-term memory store' held the role of controlling the executive system, and introduced a formalised model which maintained that sensory information was stored in a temporary buffer until pertinent qualities of the information were encoded into 'long-term memory'. The authors claimed that the role of the central executive included coordinating and monitoring complex subroutines and mediating novel information, whilst retrieving old information from storage (Atkinson & Shiffrin, 1971). Subsequent research by Baddeley & Hitch (1974) critiqued the role of Atkinson & Shiffrin's central executive by claiming they focussed too much on it being a memory system, especially since Atkinson & Shiffrin's model failed to

reconcile competing evidence for non-memory executive tasks, along with selective memory impairments in one sensory modality and not another (*e.g.*, auditory and not vision; see Warrington & Shallice, 1969). Baddeley & Hitch (*ibid*) proposed a ‘working memory’ system which simultaneously provides storage and processing of information via the central executive and two slave systems: the visuospatial sketchpad (which manipulates visual images), and the phonological loop (which stores and rehearses speech-based information; Baddeley, 1992; 1996).

Research attempting to identify the neurological substrate of the central executive has revealed that the prefrontal cortex (PFC) is the most likely contender for this role (Duncan, 2001; Miller & Cohen, 2001). Evidence which spurred early experimental research mostly stemmed from observations of aberrant behaviour after some form of trauma, irrespective of direct aetiology (*i.e.*, tumour, injury, cerebrovascular occlusion, etc.). Increased admission rates of brain-damaged patients – typically to the frontal area – during World War II (Goldstein & Scheerer, 1941), and in some case studies, as early as the end of World War I (Gelb & Goldstein, 1920, reviewed in Goldstein & Scheerer, 1941), present as some of the earliest formalised research of executive functioning impairment. Whilst this exploration of covert cognition by examining overt behaviour paved the road for researchers in the generations to come, these experiments were predominantly qualitative case studies, with a general diagnosis of “dementia” or “amnesia”, and in the case of the World War veterans, symptoms resembling “dementia praecox” (*i.e.*, schizophrenia; *ibid*). Based on this account, there appeared to be a functionally similar consequence of frontal brain damage and psychiatric symptoms.

### **1.1 Impairments in executive functioning**

Patients with schizophrenia who experience cognitive symptoms, typically exhibit impairments in executive functioning, including difficulty sustaining attention, trouble focussing, and varying degrees of impairment in working memory (National Institute of Mental Health). Since schizophrenia affects several distinct brain regions, generally resulting in a decrease in brain volume (Wright *et al.*, 2000), the brain region of origin for the pathology of schizophrenia is still under debate. One specific region that experiences

reduction of volume is the PFC (see: Takayanagi *et al.*, 2010), resulting in its dysfunction, and likely contributing to the observed cognitive symptoms (Meyer-lindenberg *et al.*, 2005). Furthermore, aberrant PFC morphology can also be used as a predictor for symptom onset, and potential development of schizophrenia amongst high-risk groups (Chakirova *et al.*, 2010). In addition to schizophrenia, PFC dysregulation has been implicated in a plethora of psychiatric illnesses and conditions resulting in a variety of executive impairments, including attention-deficit/hyperactivity disorder (ADHD) (Bush, Valera, & Seidman, 2005), depression (Mann *et al.*, 1996; but see: Stanley, Virgilio, & Gershon, 1982), Alzheimer's disease (Donix, Small, & Bookheimer, 2012), frontotemporal dementia (Rohrer & Warren, 2011), Parkinson's disease (Taylor, Saint-Cyr, & Lang, 1986), along with participating in a reward circuit facilitating drug abuse (Volkow & Fowler, 2000). In patients with PFC tumours, the degree of insult varies by case, which creates heterogeneous symptomology. For example, in one case a patient scored highly in language ability, memory proficiency, visual perception, ethical reasoning and computational calculation, but was impaired in decision-making, and despite being able to solve decision-making problems in abstract circumstances (*i.e.*, in a test or battery), the patient could not apply problem-solving solutions to 'real-life' situations (Eslinger & Damasio, 1985).

Given the involvement of the PFC in the mediation of executive functioning, and that its dysregulation leads to impairment in said functioning, it is apparent why it has been ascribed the role of the 'central executive'; however, it is pertinent to note the important connections the PFC shares with other regions of the brain, many of which actually present as the origin for the pathology of the aforementioned psychiatric illnesses. Inherently, the frontal cortex does not operate independently, and participates in conjunction with other cortices, subcortical structures, and with certain sectors of the sensory and motor apparatus (see: Fuster, 2015). PFC dysregulation then, in many instances, arises as a result of a downstream effect from a distal brain region or group, such as the basal ganglia (BG), with which the PFC forms a circuit. For example, Parkinson's disease and Huntington's disease – two neurodegenerative disorders – are marked by dysfunction in the striatum, which consequently results in dysregulation of the fronto-striatal pathway (Obeso *et al.*, 2008; Ross & Margolis, 2001). Furthermore, there is evidence that ADHD patients present with

dysfunction of the caudate nucleus of the dorsal striatum (Bush *et al.*, 2005), emphasising the importance of the fronto-striatal pathway, and more broadly the fronto-BG circuitry, in the maintenance of executive functioning.

Given this intimate connectivity, it is essential to investigate how the subcortex contributes to the maintenance of ‘processes’ typically considered executive functions. These contributory processes can be isolated and studied across a range of species to better understand how psychological concepts (*e.g.*, working memory, inhibition, etc.) are influenced by neural substrates. It is therefore pertinent to explore how quantifiable neuropsychological tasks in humans, non-human primates and rodents have developed to probe such processes in order to shift away from qualitative case studies.

## **1.2 Neuropsychological tests of executive function: sorting and shifting**

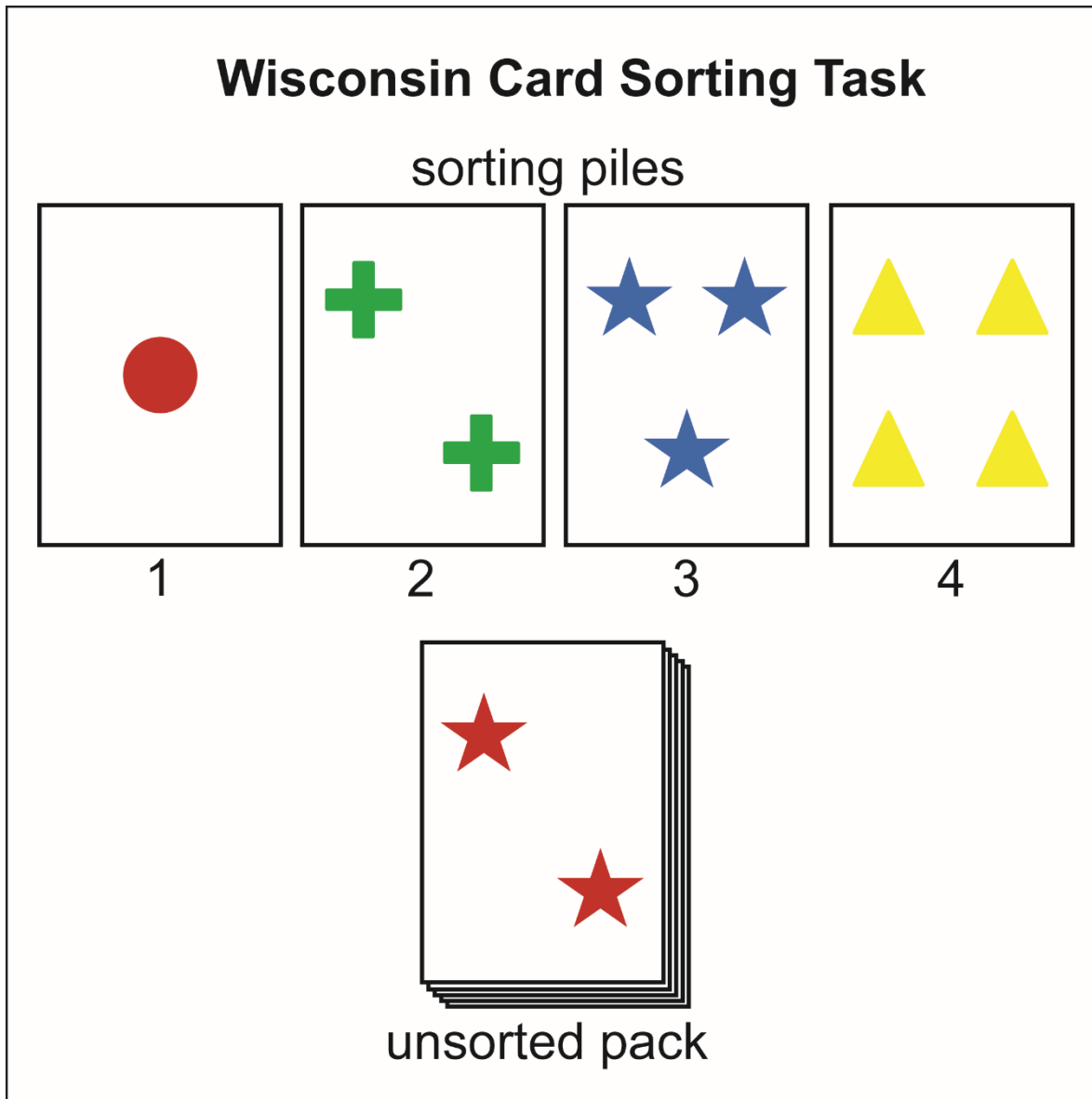
Seminal work by Gelb & Goldstein (1920; in Goldstein & Sheerer, 1941) concluded that war veterans with brain injury suffered from ‘aphasia’ or ‘dementia’ based on their overt, and selective impairment in ‘abstract’ thought, but not ‘concrete’ manipulation. For example, the patients could exercise the ‘concrete’ function of a clock by telling the time, but had difficulty in ‘abstract’ representation, such as setting the clock to a specified hour (*ibid*; Vigotsky & Kasanin, 1934). To formally test for this, the authors designed a ‘colour sorting test’, which presented patients with patches of woollen fabric dyed with several different colours, which also ranged in intensity (*e.g.*, dark green to a lightly saturated green), and instructed patients to sort them based on their colour. This seemingly simple task was met with great difficulty and uncertainty on what constituted a particular colour by the patients, perhaps stemming from impairment in decision-making ability. Modifications to Gelb & Goldstein’s ‘colour sorting test’ by Weigl (1927; translated and republished in Weigl, 1941) replaced fabric swatches with different cardboard shapes varying in colour, along with a change in instructions. Subjects could now sort the shapes however they wished (*e.g.*, based on colour), and upon sorting would then be asked to pick a new strategy and sort them again (*e.g.*, shape), and this continued until the subject could no longer formulate a novel strategy. Brain-damaged patients expressed difficulty in shifting to novel

strategies, and instead perseverated with an existing one. Despite this information, Weigl's task still presented with shortcomings, especially since diverse sorting strategies were admissible owing to many stimulus shapes (e.g., sorting varied quadrilaterals separately) (*ibid: experiment 5*). In order to address this, and in an effort to move towards examining cognitive performance, rather than a simple diagnosis, the Wisconsin Card Sorting Task (WCST) was developed (Berg, 1948).

The WCST, which is still widely used today, presents subjects with a deck of cards, illustrated with a number of coloured shapes, and the subject must learn – through trial and error – the correct strategy for sorting the cards either based on the number of shapes on the card, the colour of the shapes, or the shapes themselves. The subject is requested to proceed through a deck of cards and asked to sort each 'response card' in to one of the four 'stimulus card' piles in front of them, and sorting of a 'response card' is followed by feedback indicating whether their selection was correct or incorrect (figure 1.1). Following attainment of learning criterion (*i.e.*, five consecutive correct responses in Berg, 1948), the experimenter will 'shift' the sorting strategy to a novel one, which provides a measure of cognitive flexibility, along with a quantitative index for number of errors, which previous sorting tasks failed to measure (Grant & Berg, 1948). Healthy control participants exhibit a behavioural cost (*i.e.*, an increase in trials and errors) when the strategy is shifted, however subsequent experiments revealed that this 'cost' was exacerbated in groups with executive functioning impairment, including patients with dorsolateral prefrontal cortex (dlPFC) damage (Milner, 1963), age-related cognitive decline (Ridderinkhof, Span, & Molen, 2002), schizophrenia (Nieuwenstein, Aleman, & de Haan, 2001), ADHD (Romine *et al.*, 2004), along with neurodegenerative diseases such as Alzheimer's disease (Nagahama, Okina, Suzuki, Nabatame, & Matsuda, 2005) and Parkinson's disease (Paolo, Triister, Axelrod, & Koller, 1995; for review see: Brown & Tait, 2016).

Concurrent to the development of the WCST, a separate group of researchers investigated the mechanisms of discrimination learning in experimental animals. These experiments measured the animals' ability to perceive differences between stimulus qualities (*i.e.*, shapes vs lines) by rewarding one stimulus and not another in various apparatuses, including mazes (Lashley, 1929) and a "jumping stand" (Lashley, 1930). The

latter, as the name implied, required rats to jump from a small, unstable elevated platform towards one of two cardboard cut-outs differing in visual pattern, such as shape (e.g., horizontal line, triangle) and ‘brightness’ (e.g., black or white), with one stimulus pairing



*Figure 1.1: The WCST requires subjects to sort a response card from the unsorted pack to one of the four stimulus cards in the sorting piles; for example, the current card can be sorted based on colour (pile 1), number (2), or shape (3); image from Brown & Tait (2016).*



(*e.g.*, black triangle) being correct, whilst the other pairing (*e.g.*, white horizontal line) was incorrect. The correct stimulus could be easily folded, allowing entry into a reward chamber with a larger platform, whilst the incorrect stimulus was immovable; overall, rats learned to discriminate between the visual patterns on the stimuli cards fairly quickly (*ibid*). Jacobson subsequently adapted Lashley's procedure for work with monkeys, in which subjects were trained to retrieve food by displacing a visual stimulus card to gain access to a food well (Jacobson, 1936), which ultimately inspired the Wisconsin General Testing Apparatus (WGTA), and is still used in both monkey and human research to date (Harlow & Bromer, 1938). With this task, Harlow critiqued 'rat psychologists' for not focussing on how we "learn to learn"; an effect which he observed in primates and referred to as "learning set", and can be regarded as a practice effect of sorts, arising from consecutive discrimination learning/reversal learning (Harlow, 1949).

At the same time, Lawrence investigated the effects of 'transferring' stimulus aspects between discrimination learning trials in rats (1949). In his experiment, rats were trained in a 'two-choice' (either stimulus A or B) apparatus with a 'waiting area' placed in front to two goal compartments, which extended linearly with a reward found at each end. The apparatus was modified such that two "stimulus dimensions" were presented in compound, with one dimension predictive of reward ('relevant'), and the other was non-predictive ('irrelevant'). For example, for one group of rats, the brightness of the goal compartment (black vs white paint) may be relevant, whilst the width of the compartment (or the texture of the floor) was irrelevant for obtaining reward. Following training, rats completed a testing phase in a 'T-Maze' with the same stimulus dimensions present, however the relevancy of the stimulus dimension was switched for half of the rats. Lawrence found that rats completed the testing phase with fewer errors if the relevant dimension stimuli were the same as the training phase ("positive transfer"), compared to rats that were tested on the previously irrelevant dimension stimuli ("negative transfer"; *ibid*). This observation that there is a cost of shifting to an irrelevant dimension formed the basis for 'attentional set', by illustrating that distinct qualities of cues learned during discrimination learning can influence subsequent learning in similar situations.

To reconcile Lawrence's observations, Sutherland & Mackintosh (1971) proposed a 'two-stage' theory of discrimination learning, which assumed that animals must learn to attend to a relevant stimulus dimension (*i.e.*, shapes) *and* learn to attach the correct responses to stimuli (*i.e.*, approach triangle; avoid square). The authors posited that stimulus input is fed into a number of 'analysers', which evaluate this input along particular dimensions. These analysers contain different possible outputs, which form an attachment to a given response (*i.e.*, approach or avoid; see figure 1.2). For example, during a task in which the 'shape' dimension is relevant (triangle=correct; rectangle=incorrect), and the 'brightness' is irrelevant, the rat would receive a reward for selecting a black triangle (vs a white square). This will strengthen the 'shapes analyser' since the output of triangle formed an 'approach' response attachment (whilst square formed an avoidance response attachment). Furthermore, during this trial the 'brightness analyser' would also be strengthened due to partial reinforcement (*i.e.*, the output of 'black' would also form an approach response attachment); however in a subsequent trial, in which a white triangle may be presented, this brightness analyser would lose strength, upon selection of the correct stimulus. The authors' anthropomorphised process of strengthening or 'switching in' analysers forms the basis for attentional set-formation; by focussing attentional resources toward a particular stimulus dimension, novel learning within this dimension is enhanced; this process can also be likened to Harlow's process of 'learning to learn' (Harlow, 1949; Sutherland & Mackintosh, 1971).

### **1.3 Attentional set**

The concept of 'set' has been ascribed various definitions across psychology (see: Gibson, 1941), such as 'concepts' or 'attitudes' (Vigotsky & Kasanin, 1934), a 'mental set' (Grant & Berg, 1948), leading up to the current – and still widely used – 'attentional set' (Sutherland & Mackintosh, 1971). In this context, 'set' refers to a predisposition for attentional resources to be preferentially directed towards relevant stimulus qualities resulting in enhancement of novel learning, whilst concurrently inhibiting irrelevant information (Brown & Tait, 2016). Attentional set can then be regarded as a cognitive shorthand or heuristic, in which knowing about contingencies and rules allows the animal

to anticipate for future events, limiting the range of available options, and increasing the speed of processing (Brown & Tait, 2010). Consequently, when faced with an unexpected

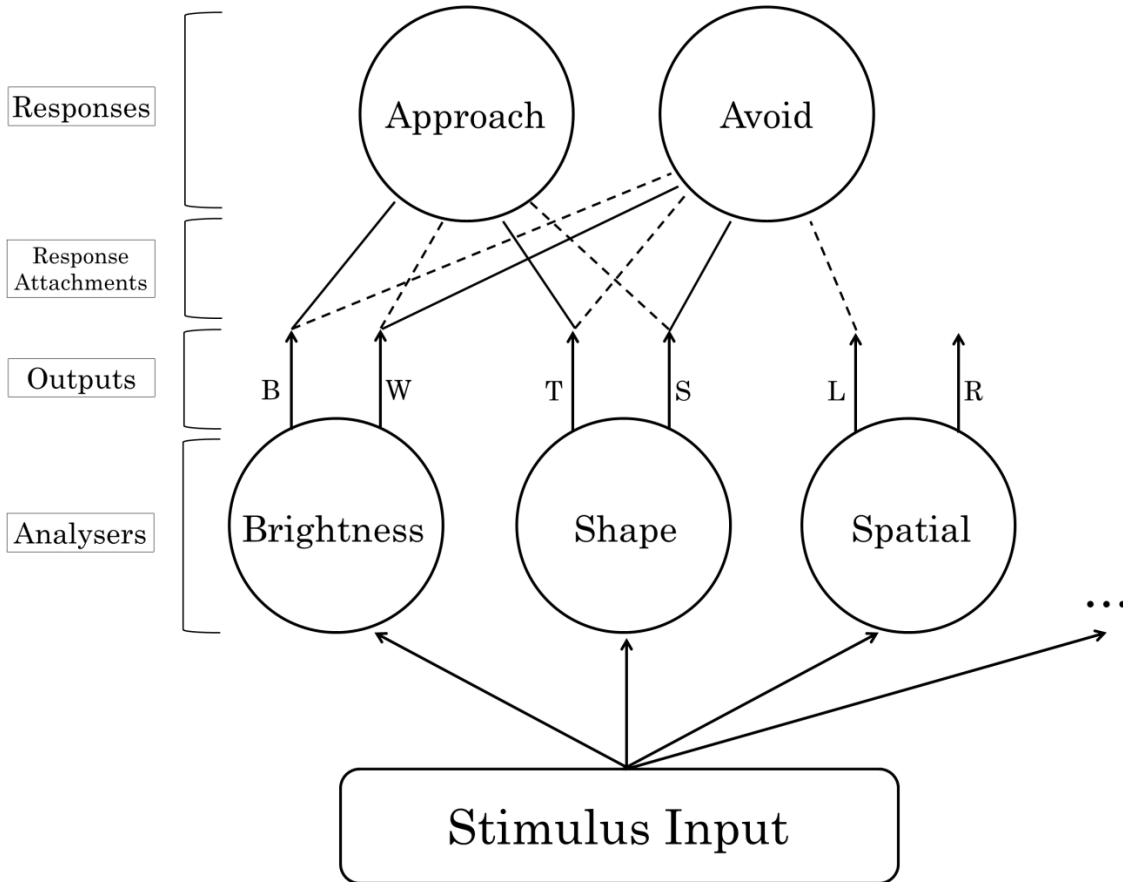


Figure 1.2: Diagram of Sutherland & Mackintosh's two-stage theory of learning; stimulus input is fed through 'analysers', which are strengthened or weakened by the response attachment formed with their outputs; solid lines indicate learned response attachments; hashed lines indicate other possible response attachments; B designates black; W, white; T, triangle; S, square; L, left; R, right; adapted from Sutherland & Mackintosh (1971).

problem, this attentional set must be overridden, and thus remaining cognitively flexible is crucial for the ability to 'shift' attentional set. Measuring the strength of an attentional set can be inferred by examining this 'cost' in learning performance by comparing the number of trials to acquire a discrimination within the same dimension (*i.e.*, intradimensional; ID) with the number of trials when learning requires a shift in attention (*i.e.*, extradimensional; ED). As such, the shifting required for an ED discrimination is not inherently more difficult

than acquiring an ID discrimination; the increased learning requirements arise as a result of a predisposition to selectively attend to one stimulus dimension over another. This consideration is referred to as the “Einstellung effect” (see Luchins, 1942), which posits that novel learning is retarded by the erroneous application of a previous rule or principle. It thus follows that in the absence of an attentional set, there would be no cost of learning an ED acquisition; however, in the absence of set, there would now be an existent deficit during ID learning, when an attentional bias would have facilitated novel learning.

A measure of set-shifting performance presents as a valuable index of several cognitive processes for clinical and experimental neuropsychology, and therefore various tasks have been developed in both humans and animals. These attentional set-shifting tasks employ a ‘total change’ design (Slamecka, 1968), which presents a series of discriminations, including stages in which completely novel exemplars are introduced, along with reversal learning stages. These total change design tasks can be contrasted to ‘rule change’ set-shifting tasks’ (otherwise known as ‘rule-shifting tasks’) (Settlage, Butler, & Odoi, 1956), such as the WCST, which maintain the same stimuli throughout the task. This thesis will exclusively focus on a rodent attentional set-shifting task of total change (Birrell & Brown, 2000), which owes its conception to the Cambridge Neuropsychological Automated Test Battery (CANTAB) (Roberts, Robbins, & Everitt, 1988; Sahakian & Owen, 1992).

The CANTAB ID/ED task – via a touchscreen interface – presents subjects with a series of discriminations based on visual stimuli of shapes with superimposed lines representing two perceptual dimensions (figure 1.3). Subjects begin by learning a simple discrimination (SD) with only one presented dimension, and once criterion performance is achieved, a second – irrelevant – dimension is added in compound to the first. Following a compound reversal stage, novel exemplars are introduced at the ID, which maintains the relevancy of dimension from the previous stages (*i.e.*, shapes). At the ED stage, novel exemplars are again introduced, but here the previously relevant dimension is now rendered irrelevant, and the previously irrelevant dimension is now relevant. Given its interface, the task could be administered to humans (Owen, Roberts, Polkey, Sahakian, & Robbins, 1991), and non-human primates (Dias, Robbins, & Roberts, 1996b; Roberts *et al.*, 1988) alike. Research in humans has expanded our understanding of impairments in behavioural

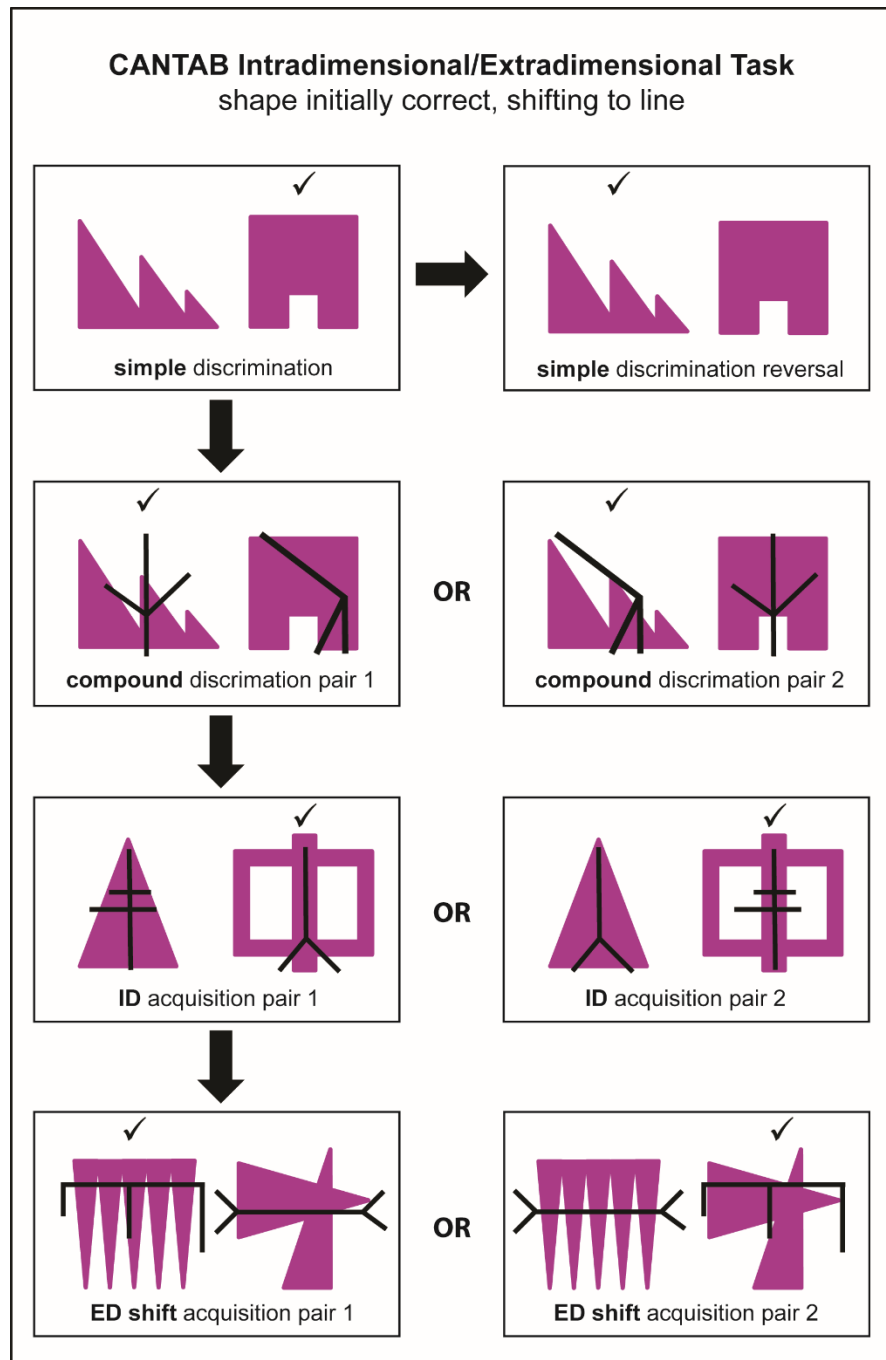


Figure 1.3: Sample stimuli and test procedure from the CANTAB, which is a two-choice visual discrimination task. Subjects initially learn that ‘shape’ (and not ‘line’) is relevant for reward, even after the presentation of novel stimuli at the ID. Relevancy changes to line at the ED and subjects must shift their attention to a previously irrelevant dimension image from Brown & Tait (2016).

flexibility amongst brain-damaged patients and those suffering from mental illness (Lawrence, Sahakian, & Robbins, 1998; Owen *et al.*, 1991; 1993). Excitotoxic lesion studies in monkeys have provided invaluable findings regarding the functional architecture of the PFC (Dias, Robbins, & Roberts, 1996a; Dias *et al.*, 1996b; Roberts, Robbins, Everitt, & Muir, 1992); however the monkey task reportedly takes several weeks to complete (9-11 weeks in Roberts *et al.*, 1992), which complicates the dosing schedule for pharmacological studies, along with increased odds of neuroplasticity throughout the post-surgery test period. To account for this, the CANTAB ID/ED task was adapted for use in rats.

It is well-documented that rats are not particularly visual animals, and possess poor visual acuity for detail (Birch & Jacobs, 1979) and being nocturnal, rats typically flee from brighter environments. Instead, rats rely on their highly attuned sense of smell and touch as primary investigative senses. Whilst previous discrimination learning tasks have employed visual stimuli (Lawrence, 1949; Mackintosh & Little, 1969), training and testing usually spanned hundreds of trials (Bussey, Muir, Everitt, & Robbins, 1997) for rats to attain criterion learning, providing little refinement from the CANTAB; therefore designing a species-appropriate task, which targets the rats' natural tendencies was paramount for the rodent ID/ED task. Work by Wood, Dudchenko, & Eichenbaum (1999) found that rats can be trained to dig in small bowls filled with sawdust to obtain a food reward, which suited their natural digging and foraging behaviour. In a follow-up experiment, the authors added various odours to bowls of sand, some of which were predictive of reward in an effort to investigate non-spatial memory in rats (Dudchenko, Wood, & Eichenbaum, 2000). Birrell & Brown (2000) expanded on the fundamental principle of this task, by introducing various digging media, and textured covers for the digging bowls as stimulus dimensions, which could be used alongside odour cues as another dimension.

The bowl-digging paradigm introduced in Birrell & Brown (2000) matched the primate CANTAB task in that novel stimuli were introduced at the ID and ED stages, respectively (figure 1.4). More importantly, data from control rats matched the established patterns for learning – namely a cost of shifting set from the ID stage to the ED stage – previously evidenced in humans (Owen *et al.*, 1991) and non-human primates (Dias *et al.*, 1996b). Testing in rats could also be accomplished in a single session, presenting as a

technical refinement to examining set-shifting performance in animals. Furthermore, lesions to the rat medial PFC (mPFC), which is the rodent analogue of the primate lateral PFC, led to comparable behavioural deficits – namely impaired set-shifting performance marked by increased trials at the ED stage, whilst lesions of the rat orbital PFC (OFC; structurally analogous in the primate) matched the impaired reversal learning performance seen in primates (Birrell & Brown, 2000; Brown & Bowman, 2002; Dias, Robbins, & Roberts, 1997; McAlonan & Brown, 2003).

Research by Dias *et al.* (1996a) posited that lesioning two different regions of the PFC (*e.g.*, lateral vs orbital) produced a ‘double-dissociation’; they evidence that a lesion of the lateral PFC does not disrupt reversal learning, and that the OFC does not likely play a role in attentional set. More broadly, this implied that the mechanisms for reversal learning were different to those involved in attentional set. It is important to note that the OFC-lesioned monkeys in Dias *et al.* (1996a) took twice as many trials to acquire the ID compared to control and lateral-PFC lesioned animals, yet the data regarding the cost of set-shifting for this group was not reported. The OFC-mediated reversal learning impairment was subsequently replicated in rats (McAlonan & Brown, 2003), and owing to a revised task design in which reversal stages were placed before the ED stage (the reversal stages in Dias were presented after the ED), it was found that lesioned rats failed to present with a ‘positive cost’ of shifting set – in which the ED is greater than the ID – which led McAlonan & Brown to speculate whether an attentional set had even been formed.

The failure to form an attentional set presents a novel behavioural deficit compared to that of a shifting impairment, and can be inferred as a likely cause of the lack of an ID/ED difference, stemming from the rationale that if set was not formed, then it does not require shifting. To aid in the inference of this behavioural deficit, the rodent attentional set-shifting task (ASST) can be modified by removing the reversal stages, and replacing them with multiple ID stages to promote set-formation (Bissonette *et al.*, 2008). Chase *et al.* (2012) found that four consecutive ID stages (the 4-ID task) were sufficient to restore the cost of set-shifting in OFC-lesioned rats, suggesting that OFC-mediated deficits in reversal learning and set-formation were due to a single cognitive deficit, which also challenged the established notion of a ‘double-dissociation’.

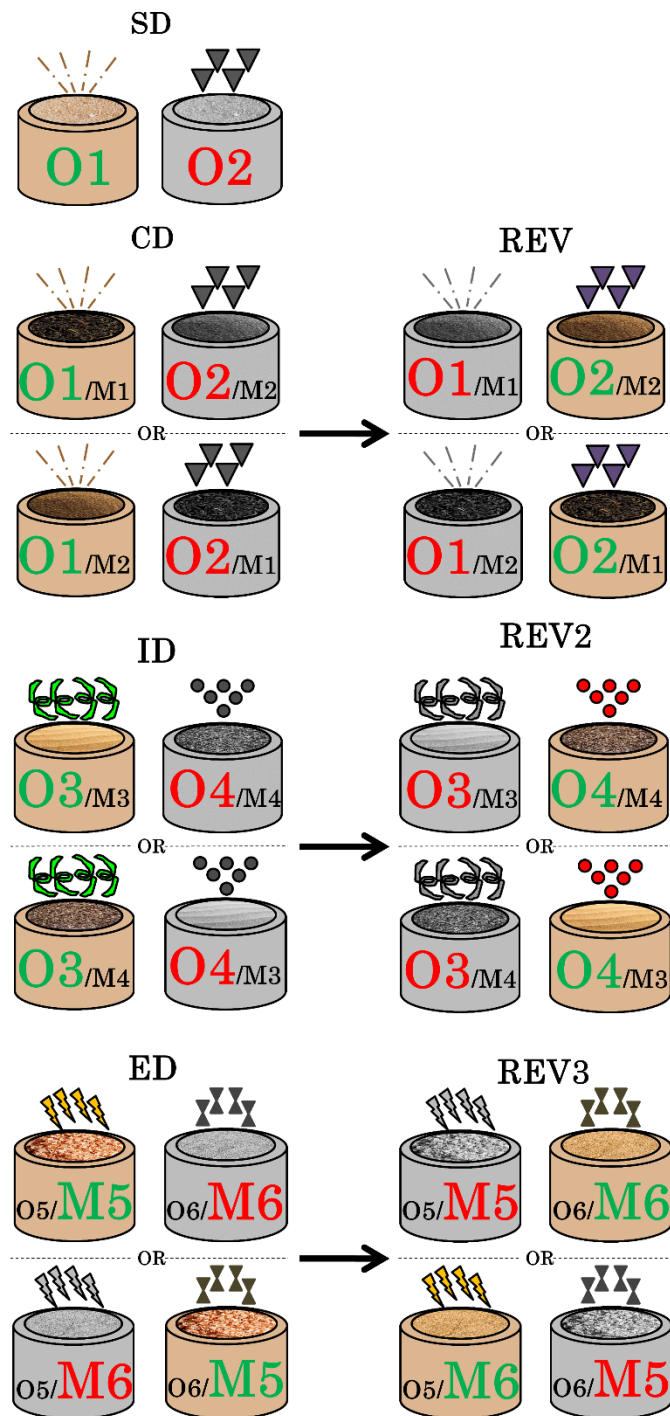


Figure 1.4: Sample stimuli and typical task procedure of the rodent attentional set-shifting task. Rats initially learn that the odour dimension is relevant (with O1 being correct), which is reinforced by novel exemplars at the ID stage (O3 is now correct); the dimension shifts relevancy to medium at the ED stage (now M5 is correct) with the presentation of novel exemplars.



Further research suggested that the impairment in attentional set-formation may involve several neural substrates. It was found that rats with quinolinic acid (QA) lesions of the dorsomedial striatum (DMS) do not present evidence for attentional set-formation, even on the 4-ID task (Lindgren, Wickens, Tait, Brown, & Dunnett, 2013), suggesting that the DMS-mediated set-formation impairment manifests differently than the OFC-mediated impairment. Furthermore, lesions to the non-cholinergic neurons of the rat basal forebrain (Tait & Brown, 2008) may also impair set-formation, as no ID/ED difference was observed, along with replicating the previously observed impairment in reversal learning in monkeys (Roberts *et al.*, 1992); however further research specifically investigating set-formation is needed.

Recent experimental evidence from our lab has suggested that the subthalamic nucleus (STN) and possibly the zona incerta (ZI) may also play a role in the formation of attentional set (Tait, Phillips, Blackwell, & Brown, 2016). The STN and the ZI are anatomically adjacent regions of the BG and are located ventral to the thalamus. Of these two neural substrates, the STN garners considerably more research interest than the ZI. The STN is a key input structure in the BG, responsible for regulation of many components of the BG itself, along with receiving projections from the PFC (Baunez & Lardeux, 2011; Nambu, Tokuno, & Takada, 2002), whereas the ZI, which lies dorsal to the STN and is comprised of a dorsal (ZID) and ventral (ZIV) component, actually remains among some of the least-studied regions in the brain (Urbain & Desche, 2007).

## **1.4 The Basal Ganglia**

BG functioning has largely been researched in relation to the control of movement, stemming from the fact that BG dysregulation has been identified in various movement disorders, such as Parkinson's disease (DeLong, 1990; Obeso *et al.*, 2008). The BG are a group of subcortical nuclei which form a complex network of connectivity, and also project to other regions in the subcortex, along with the neocortex. Classically, the BG were regarded as a series of 'connective loops', and were thought to be a closed 'relay' system which received information from the cerebral cortex, and then "funneled" this information through a series of closed and parallel loops through the striatum, pallidum and substantia

nigra (SN), in order to provide feedback to the cortex via the ventrolateral thalamus (Alexander, DeLong, & Strick, 1986). Over the next few years, this information was formalised into the first coherent model of BG functioning (figure 1.5a), which included two circuits of motor control – the ‘direct’ and ‘indirect’ pathways (Albin, Young, & Penney, 1989; DeLong, 1990; Penney & Young, 1986). Both pathways originate from the striatum, with the ‘direct’ pathway resulting in an increase in thalamo-cortical activity, and the ‘indirect’ pathway, a decrease in thalamo-cortical activity. This created a “rate model” of BG functioning, in which the ‘direct’ pathway facilitated movement, and the ‘indirect’ pathway inhibited/stopped movement. In the ‘direct’ pathway, medium spiny neurons (MSNs) of the striatum express D<sub>1</sub>-type dopamine receptors, which exert tonic, inhibitory control over the SN-reticulata (SNr) and internal globus pallidus (GPi), which are now inhibited, and therefore their projection to the thalamus results in increased thalamo-cortical activity (Albin *et al.*, 1989). In the ‘indirect’ pathway, MSNs expressing D<sub>2</sub>-type receptors from the striatum inhibit the external globus pallidus (GPe), which attenuates the GPe’s inhibitory effect on the STN, which in turn stimulates the SNr and GPi, resulting in decreased thalamo-cortical activity (*ibid*). The modulation of striatal dopamine is crucial for movement, and as expected dopaminergic depletion – the key feature of the Parkinsonian state – renders the STN ‘hyperactive’ and consequently enhances activation of the ‘indirect’ pathway (Gerfen *et al.*, 1990; Obeso & Lanciego, 2011). In turn, silencing the STN via deep-brain stimulation (DBS) has become a popular therapeutic option for amelioration of motor symptoms of Parkinson’s disease (Benabid *et al.*, 1994).

## **1.5 Connections of the STN and ZI**

Advancement in tracing technology has provided anatomical evidence that the STN receives direct input from several key areas of the brain (figure 1.5b). The identification of a third – ‘hyperdirect’ – pathway of the BG, which projects directly from the cortex to the STN, elevated the role of the STN from an intermediary or ‘control’ structure between the GPe and GPi, to a key input structure in the BG (Nambu *et al.*, 2000, 2002). This hyperdirect pathway originates in motor-related cortical areas (including the primary motor cortex, supplementary motor area, and the dorsal and ventral premotor cortices; see Nambu,

Takada, Inase, & Tokuno, 1996; 1997), yet there was additionally evidence from tracing studies that showed that frontal ‘cognitive’ areas, such as the mPFC and OFC, directly project to the STN as well (Janssen, Visser-Vandewalle, & Temel, 2010). It is suggested that the fronto-subthalamo pathway operates in competition with the ‘direct’ pathway (Leblois, Boraud, Meissner, Bergman, & Hansel, 2006), and that this pathway is fast-acting, compared to the slower striatal pathways (Isoda & Hikosaka, 2008; Nambu, 2004), which may work to suppress activation of the ‘direct’ pathway (Aron & Poldrack, 2006a). This connection, coupled with the finding that the STN also has a direct output projection to the ventral thalamus (Rico *et al.*, 2010) – therefore bypassing the pallidum – likely suggests that the STN is crucial in the rapid control and selection of responses.

The STN may also play a modulatory role in inter-BG circuitry, and the identification of an STN-GPe-GPi ‘microcircuit’ also ascribed a new role to the GPe for controlling BG output activity, rather than merely being a ‘go-through’ station in an ‘indirect’ pathway (Obeso, Rodriguez-oroz, Blesa, & Guridi, 2006). The reciprocal connection between the STN and the GPe normally exhibits weakly correlated, irregular activity (Urbain *et al.*, 2000); however, following dopamine depletion in Parkinson’s disease, this reciprocal connection exhibits highly correlated, rhythmic bursting activity (Bergman, Wichmann, Karmon & De Long, 1994), which likely contributes to the increased downstream inhibition of the thalamocortical neurons, which may eventually express as akinesia and rigidity.

In light of these new connections and updated roles for functioning, the view that the BG operates as an ‘on-off’ pathway for action selection is outdated; current modelling suggests that the BG operates via a series of parallel, largely segregated re-entry loops or channels (see Redgrave, Vautrelle, & Reynolds, 2011), which receive input from functionally segregated areas of the cerebral cortex (*i.e.*, limbic, associative, sensory and motor; for review see McGeorge & Faull, 1989) in concert with the dense, and intimately interconnected inter-BG modulatory networks (*i.e.*, the STN-GPe-GPi microcircuit), which contribute to the shaping of output behaviour.

Research regarding the functioning of the ZI – literally, the ‘uncertain zone’ – is sparse, yet evidence exists that the ZI has distinct cytoarchitectonic subdivisions, and that

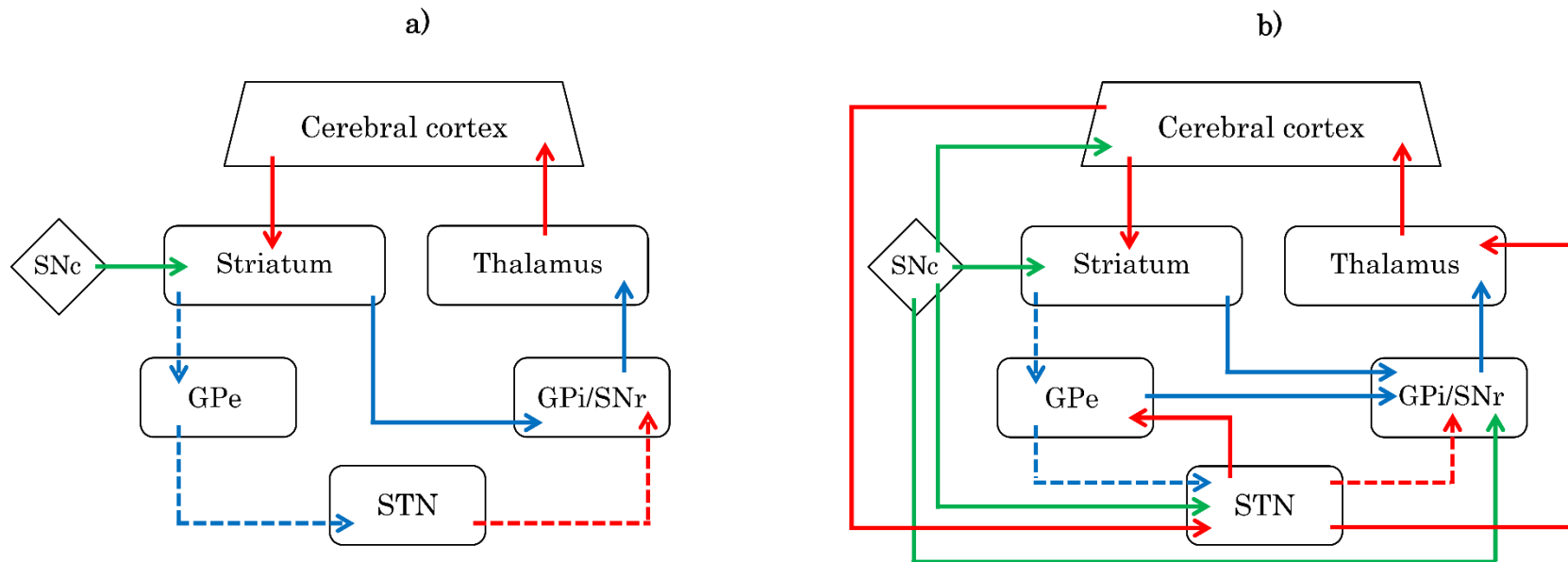


Figure 1.5: a) An illustration of the classic Albin-DeLong model of the Basal Ganglia; red lines indicates excitatory projections, blue lines inhibitory, and green lines dopaminergic. Solid arrows indicate the 'direct' pathway, and hashed lines the 'indirect' pathway, in which the STN was seen as a relay centre; b) A revised model of Basal Ganglia connections based on recent findings, illustrating a revised, and increasingly complex, role for STN functioning (simplified from Obeso and Lanciego, 2011).

each subdivision gives rise to a distinct pattern of efferent projections (for review see Romanowski, Mitchell & Crossman, 1985; also Watanabe & Kawana, 1982). Early research has demonstrated that the rat ZI receives afferent inputs from the thalamus (Swanson, Cowan & Jones, 1974), hypothalamus (Krieger, Conrad & Pfaff, 1979), brain stem (Bowsher, 1975) and the cerebellum (Faull & Carman, 1978). More recent research has determined that projections originating in the cerebral cortex terminate in the ZI, and that the ZI and the STN may work together to process this cortical information, as it was found that cortical representations in these regions have highly overlapped borders, primarily from the layer V neurons of the cortex, but also from areas implicated in higher cognitive functioning, such as the OFC and mPFC (Kita, Osten, & Kita, 2014). Recent research has also evidenced that the ZI projections to cortical layer I of the neonatal rodent brain are crucial during development and maturation, as disrupting this pathway during the first postnatal week increases the likelihood of epileptiform activity in the adult brain (Chen & Kriegstein, 2015).

The ZI also projects to – in some cases reciprocally – and exhibits predominantly inhibitory influence to several key areas of the brain, including the cortex, BG, brainstem, thalamus and spinal cord (for review see Mitrofanis, 2005; Power, Kolmac & Mitrofanis, 1999). The inhibitory projection to the dorsal thalamus (Power, Kolmac, & Mitrofanis, 1999), exerts a powerful influence on the firing properties of thalamic neurons (Bartho *et al.*, 2002). These projections mostly originate in the ZIV – which lies adjacent to the dorsal STN – and plays a role in vibrissal sensorimotor functioning (Shaw, Liao, Chen, Huang, & Lin, 2013; Urbain & Desche, 2007), and furthermore, this thalamic projection also extends into the whisker representation regions of the primary somatosensory cortex (Nicoletis, Chapin, & Lin, 1995), ultimately forming a reciprocal connection with the ZI.

## **1.6 Functional division of the STN and ZI**

Despite the evidence that the STN and ZI are largely involved in motor control, a functional division within these two regions has been presented. This division of the STN has been identified in both non-human primates (Joel & Weiner, 1997) – and in more a simplified subdivision of cytoarchitecture – in rodents (Groenewegen & Berendse, 1990).

Both the primate and rodent STN receives BG input via the GPe, yet the STN also receives direct afferent input from the cortex, which may project differentially within the nucleus itself, and by species.

In the primate, three functional divisions of the STN have been circumscribed: a dorsolateral ‘motor’ part, a medial ‘limbic’ part, and a ventrolateral ‘associative’ part (Parent & Hazrati, 1995). The dorsolateral section projects to sensorimotor territories in the putamen and GPe, the medial section projects to limbic regions of the ventral GPi, whilst the ventrolateral associative (cognitive) regions project to the GPi/SNr and the caudate nucleus (Joel & Weiner, 1997; Parent & Hazrati, 1995). Advancement in anterograde tracing technology has revealed a considerable convergence of projections from functionally diverse cortical areas into the primate STN, creating potentially important interfaces between terminal fields, which appear to overlap rather than be anatomically distinct (Haynes & Haber, 2013). For example, limbic projections from the cortex to the medial STN also extend into the adjacent lateral hypothalamus (LH), and that the processing of limbic information is performed by both regions, suggesting that the lateral LH might be considered the limbic cone of the STN (*ibid*; Berthoud & Munzberg, 2011).

In the rat, the simplified cytoarchitecture compared to the primate brain has given rise to two constituent subregions; the medial and lateral regions, with little evidence for a dorsal/ventral subdivision (Berendse & Groenewegen, 1991a; Groenewegen & Berendse, 1990; Heimer, Zahm, & Alheid, 1995). The lateral section comprises two-thirds of the nucleus, receiving projections from the motor and pre-motor cortices, whilst the medial third of the STN receives input from the anterior cingulate, prelimbic, agranular insular cortices, and orbitofrontal cortices, along with the ventral pallidum (Berendse & Groenewegen, 1991a; for review see Janssen, Visser-Vandewalle, & Temel, 2010). Electrophysiological studies compliment these anatomical tracing results by suggesting there might be a functional division of the STN as well, and similar to the primate STN, the subdivisions of the rodent STN are not entirely segregated from each other (Janssen *et al.*, 2010). STN dendrites can extend nearly the entire length of the nucleus (Heimer *et al.*, 1995), and the limbic projections from the cortex also extend beyond the medial STN, into the adjacent lateral LH (Berendse & Groenewegen, 1991). Experiments investigating the

STN's contribution to cognitive functioning, including the experiments in the current thesis, would benefit by targeting the frontal connections of the medial STN.

Research evidencing a functional division of the ZI is sparse, yet anatomical tracing studies have shown that the ZI receives input from the cortex, BG, brainstem, and spinal cord, leading to partially overlapping regions which contribute differential effects on behaviour, including controlling visceral activity, influencing arousal, orienting visual attention, and maintaining posture and locomotion (Lin, Nicolelis, Schneider, & Chapin, 1990; Mitrofanis, 2005). For example, lesioning or stimulating the rostral ZI (and no other region) will decrease food/water intake, changing ingestive activity (Tonelli & Chiaraviglio, 1995); however the same procedure when applied to the caudal ZI induces changes in posture and locomotion (Edwards & Isaacs, 1991).

Most of the rostral ZI and the ZID receive limbic projections from the cingulate, and the majority of the ZIV and caudal ZI receive projections from the somatosensory cortex (*ibid*). In addition to a rostral-caudal and dorsal-ventral regional division of functioning, the medial third of the ZI contain projections from the dorsomedial frontal and primary motor cortices; projections which also extend into the adjacent LH (*ibid*; Lin *et al.*, 1990). In addition to the identified subdivisions, it is worth noting that this 'subthalamic region', including the STN, ZI, and LHA, with converging and overlapping cortical projections from the mPFC and OFC, likely works in concert to some degree to shape output behaviour (Kita *et al.*, 2014).

## **1.7 Neurochemistry of the STN and ZI**

### **1.7.1 Glutamate**

Considerable research has shown that the dominant postsynaptic receptor in the STN is the glutamate receptor (for review see Clarke & Bolam, 1998). Pioneering electrophysiology research by Robledo & Féger (1990) demonstrated that the STN exerts an excitatory effect on its efferent structures, and that furthermore, chemical blocking of STN neuronal activity with muscimol [a  $\gamma$ -aminobutyric acid receptor-type A (GABA<sub>A</sub>) agonist] produced reductions in neuronal activity in the GP, EP and SNr. Robledo & Féger (1990) also noted that the STN uses glutamate for neurotransmission to the SNr, which

presented new evidence for glutamatergic transmission in subthalamic projections. Subsequent neurophysiological tracing research spearheaded by Atsushi Nambu spanning nearly a decade (1996; 1997; 2000; 2002) built on the seminal findings posited by Hartmann-von Monakow et al. (1978), which originally identified that the STN receives direct excitatory cortical projections from wide-spanning areas of the frontal cortex, including the supplementary motor area (Nambu *et al.*, 1996) and premotor area (Nambu *et al.*, 1997), along with glutamatergic projections from the thalamus and brainstem (Joel & Wiener, 1997; Mathai & Smith, 2011). The ‘hyperdirect’ pathway is the chief excitatory (glutamatergic) input for the STN, and work by Nambu *et al.* (2000) found that stimulation of the primary motor and somatosensory cortices via implanted electrodes in monkeys resulted in a pattern of early, short-latency excitation, followed by a late excitation in the STN, GPe and GPi, in which the early excitation of the STN preceded that of the early excitation for the GPe/GPi. Similar to Robledo & Féger (1990), STN-muscimol treatment in Nambu *et al.* (2000) abolished both the early and late excitation events in the measured GP neurons following cortical stimulation. Building on Robledo & Féger (1990), Nambu (2000) found that injections of *N*-methyl-*D*-aspartate (NMDA) receptor antagonists, and *not*  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptor antagonists into the STN attenuated the early and late excitations found in the GP, which suggests that the ‘hyperdirect’ pathway (cortico-subthalamic) is largely mediated by NMDA receptors. These fast-acting ionotropic receptors (compared to slower-acting metabotropic receptors) convey motor-related cortical information to the GP via the STN, with shorter conduction time than the effects conveyed through the striatum (Nambu *et al.*, 2000).

Of the NMDA receptor, the NR<sub>1</sub>-type receptor is the most prevalent, and despite NMDA being the most prominent ionotropic glutamate receptor type in the STN, and despite the findings in Nambu *et al.* (2000), there also exists evidence that AMPA may play a role in modulating STN activity during a state of nigrostriatal dopamine denervation (*i.e.*, in Parkinson’s disease), in which systemic administration of AMPA antagonists [in conjunction with Levedopa (L-DOPA) – a dopamine agonist and treatment for Parkinson’s disease] have been shown to reverse STN hyperactivity (Vila *et al.*, 1999). This may arise



from the fact that AMPA mediates faster neurotransmission than NMDA (for review see: Ozawa, Kamiya & Tsuzuki, 1998), along with cascades of compensatory neurotransmission within the BG during a dopamine-denervated state (see section 1.7.2; for review see: Galvan, Kuwajima & Smith, 2006). Furthermore, Mouroux & Féger (1993) demonstrated that the expected pattern of STN excitation following electrophysiological stimulation of the parafascicular nucleus of the thalamus was silenced following both NMDA- and AMPA-antagonism, suggesting that both of these ionotropic receptors contributed to STN excitation.

In addition to the ionotropic glutamate receptors of the STN, there exists a population of metabotropic glutamate receptors (mGluRs); these are mostly asymmetrical and colocalised on a single synapse with the ionotropic receptors (Porter, Greene, Higgins, & Greenamyre, 1994). Whilst the ionotropic receptors contribute to fast-acting, excitatory behaviour, the slower-acting mGluRs regulate sustained depolarisation (Ozawa, Kamiya & Tsuzuki, 1998); the colocalised organisation of these two receptor types leads to complex glutamate signalling in the STN (Clarke & Bolam, 1998). There are three distinct families of mGluR (Group I, II, & III) each differing based on their mechanism of action and cellular response outcome; for example, activation of Group I mGluRs – consisting of mGluR<sub>1</sub> and mGluR<sub>5</sub> – leads to a net excitatory response by inducing a mobilisation of intracellular Ca<sup>2+</sup>, along with increasing NMDA (Kuwajima, Hall, Aiba, & Smith, 2004). Conversely, Group II mGluRs, which include mGluR<sub>2</sub> and mGluR<sub>3</sub>, are negatively coupled to adenylyl cyclase, and yield an inhibitory effect on signal transduction (Conn & Pin, 1997; Kuwajima *et al.*, 2004).

*In vitro* electrophysiology studies have determined that Group I (both mGluR<sub>1</sub> and mGluR<sub>5</sub>) receptor types are found in the STN of the rodent (Awad, Hubert, Smith, Levey, & Conn, 2000) and non-human primate (Clarke & Bolam, 1998; Kuwajima *et al.*, 2004; Wang, Ong, Lee, & Huganir, 2000), and that these receptors are mostly localised on postsynaptic dendrites of the STN, and may contribute to an important role in net excitatory drive to several key areas of the BG, including the GPe and GPi, along with the thalamus (Galvan Kuwajima & Smith, 2006; Obeso & Lanciego, 2011). Group II (both mGluR<sub>2</sub> and mGluR<sub>3</sub>) receptor types are also found in the STN, and are presynaptically localised on

STN terminals; the activation of these receptors inhibits excitatory transmission at STN synapses (Bradley *et al.*, 2000). However, both mGluR<sub>2</sub> and mGluR<sub>3</sub> can also be found postsynaptically, and it is postulated that this distribution may aid in regulating output activity of the STN (Clarke & Bolam, 1998; Tamaru, Nomura, Mizuno, & Shigemoto, 2001; Wang *et al.*, 2000).

The usually regular, tonic activity of glutamatergic neurons in the STN (Bevan & Wilson, 1999; Nakanishi, Kita, & Kitai, 1987) is rendered irregular in the Parkinsonian brain, with bursts of high-frequency spikes; this dysregulation appears to be essential for clinical manifestation of the disease (Porter *et al.*, 1994; Tai *et al.*, 2003; Vila *et al.*, 1999). Furthermore, 6-hydroxydopamine (6-OHDA) lesions of the SNc – leading to striatal dopamine depletion – serves as a Parkinsonian disease model, partly by increasing glutamatergic output activity from the STN (for review see Henderson & Dunnett, 1998). During this state of glutamate hyperactivity (*i.e.*, Parkinson's disease), application of an mGluR<sub>5</sub> antagonist (2-methyl-6-(phenylethynyl)-pyridine; MPEP), has been shown to regulate activity, resulting in an attenuation of severe motor and sensorimotor asymmetries (Phillips, Lam, Ackerson, & Maidment, 2006).

Glutamatergic cells are sparsely found throughout the entire rat ZI, with a slightly higher concentration in the medial ZID, making up 40% of the total number of cells in this region, whereas glutamate-immunoreactive cells make up 15% of the cells in the other sectors of the ZI (Kolmac & Mitrofanis, 1999). In a follow-up study, Heise & Mitrofanis (2004) found evidence for glutamatergic projections from the ZI to other parts of the BG, predominantly in both parts of the substantia nigra along with the pedunculopontine tegmental nucleus (only in the *pars dissipata*), with fewer projections to the caudate-putamen, GP, STN and EP. Interestingly, Heise & Mitrofanis (2004) found that very few cells were GABA-positive, which is believed to be the predominant neurotransmitter-type in the ZI (see Nicolelis, Chapin & Lin, 1995; section 1.7.3); however, in a review by Mitrofanis (2005), the author emphasises and details experiments that argue that the ZI is an area with extensive connections to a variety of brain regions, comprised of a heterogeneous collection of cells differing in size and shape. For example, the tracer injection site in Heise & Mitrofanis (2004) was in the medial portion of the ZI, so it is

possible that the cells labelled downstream in the SN and EP do not fully represent the breadth of connections of the ZI, whereas Nicoletis, Chapin & Lin, 1995 used retrograde tracers from the primary somatosensory cortex to map out subdivisions within the ZI. It is clear that glutamate signalling in the ZI influences output behaviour, as infusions of both AMPA or kainic acid agonists (but not NMDA) directly to the medial or rostral ZI induce a marked stimulation of locomotor activity, along with a postural change, and that furthermore, administration of the respective antagonists resulted in an inhibition of locomotor activity (Supko, Uretsky & Wallace, 1991). These excitatory projections place the ZI in a position to induce a modulatory effect on BG and cortical activity, and merit further investigation in an effort to disseminate the subpopulations of neurotransmitters within this area.

### 1.7.2 Dopamine

It has been documented through retrograde tracing and immunohistochemistry studies that the rat STN receives dopaminergic projections from the SNc and the ventral tegmental area (VTA) (Brown & Wolfson, 1978; Brown *et al.*, 1979; Campbell *et al.*, 1985; Hassani, Francois, Yelnik, & Féger, 1997), including sparse arborisations via the striatum (Gauthier, Parent, & Levesque, 1999). This dopaminergic projection has also been identified in the cat (Meibach & Katzman, 1979; Rinvik *et al.*, 1979), and the primate (Rinvik *et al.*, 1979; Galvan *et al.*, 2014) STN; however there is less robust evidence for this in the human brain, with weak, albeit specific binding for D<sub>1</sub>-type dopamine receptors (Augood, Hollingsworth, Standaert, Emson, & Penney, 2000). Indeed several studies have documented that the D<sub>1</sub>, D<sub>2</sub>, D<sub>3</sub>, and D<sub>5</sub> receptor messenger RNAs and binding sites were present in the rodent STN (Flores *et al.*, 1999; Svenningsson & Le Moine, 2002; Baufreton *et al.*, 2003), highlighting a potentially diverse profile of dopamine modulation within the nucleus.

Functionally, earlier *in vivo* research suggested that dopamine largely had an inhibitory effect on STN neurons via D<sub>1</sub> and D<sub>2</sub> dopamine receptors (Campbell *et al.*, 1985; Hassani & Féger, 1999), whilst another group of researchers found that D<sub>1</sub> agonism had a predominantly excitatory effect on STN neurons (Kreiss, Anderson, & Walters, 1996). More recent *in vitro* research has elucidated that activation of D<sub>1</sub>-like receptors (specifically

D<sub>5</sub>) increases burst firing in subthalamic neurons, therefore shaping neuronal activity (Baufreton *et al.*, 2003). There is also evidence that activation of D<sub>2</sub>-like receptors are implicated in excitatory synaptic transmission, with a different mechanism of action compared with D<sub>1</sub>-like receptors (Zhu, Shen & Johnson, 2002; Floran, Floran, Erlij & Aceves, 2004; Galvan *et al.*, 2014). These D<sub>2</sub>-like receptors influence firing either presynaptically or postsynaptically: binding with D<sub>2</sub> and D<sub>3</sub> receptors reduces resting K<sup>+</sup> conductance, and thus promotes firing (presynaptic; Zhu, Shen & Johnson, 2002; Baufreton & Bevan, 2008), whereas selective D<sub>4</sub> agonism decreases GABA release to the STN (see section 1.7.3), thus reducing inhibition of the nucleus (postsynaptic: Shen & Johnson, 2003; Floran, Floran, Erlij & Aceves, 2004). Furthermore, the fact that an inward current induced by dopamine superfusion (*in vitro*) was observed, despite the application of tetrodotoxin (a sodium channel blocker) and ionotropic glutamate receptor antagonists [both AMPA/KA antagonists: (±)-sulpiride and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and NMDA: 2-amino-5-phosphonopentanoic acid (AP5)], suggests that dopamine exerts a direct action on postsynaptic STN neurons (Shen & Johnson, 2003).

The aforementioned studies illustrate that dopamine exerts a direct excitatory effect on STN neurons, and that furthermore it likely works in concert with glutamate to shape the excitatory drive of the STN. This synergy is made particularly salient during a state of dopamine denervation of the nigrostriatal pathway, which is typically seen during Parkinson's disease, resulting in glutamate hyperactivity (Bergman, Wichmann, Karmon & De Long, 1994). Furthermore, increasing dopamine neurotransmission through L-DOPA treatment has a positive effect on regulating the aberrant firing activity of the glutamatergic STN neurons (Hassani & Féger, 1999), and as stated in section 1.7.1, L-DOPA, in conjunction with an AMPA antagonist reverses the hyperactivity of the STN (Vila *et al.*, 1999). More recent research by El Arfani and colleagues (2014) found that rats with 6-OHDA-lesions of the nigrostriatal pathway that received both L-DOPA and MK801 – an NMDA receptor antagonist – experienced enhanced dopamine release, compared to lesioned rats receiving L-DOPA alone. Several studies have also shown that decreasing STN activity via HFS induces a significant increase in extracellular dopamine in the rat striatum (Briet *et al.*, 2001), particularly by increasing D<sub>1</sub> receptor levels, whilst decreasing

D<sub>2</sub> and D<sub>3</sub> receptor levels in the nucleus accumbens (Carcenac *et al.*, 2015). This may partially explain the therapeutic efficacy of STN-HFS to treat motor symptoms of Parkinson's disease (see section 1.7.5).

Functionally, *in vivo* studies have found that dopaminergic inputs to the STN may be crucial in regulating movement, as the application of apomorphine (dopamine agonist) induced an increase in abnormal, nondirected orofacial movements; this behaviour may largely be driven by D<sub>1</sub>-like receptors, since D<sub>1</sub>-like and *not* D<sub>2</sub>-like receptor antagonists blocked the expression of this behaviour (Parry *et al.*, 1994). In the absence of a dopamine agonist, chemical antagonism of the D<sub>1</sub>-like receptors in the STN results in catalepsy in rats (Hauber, 1998). These results support the assertion that dopamine plays a prominent role in the regulation of motor functions, and excitatory drive in the STN.

Within the ZI, a sparse population of dopaminergic neurons have been exclusively identified in the rostral sector, and are found in the medial region (both dorsal and ventral) in both the cat and the rat; these cells comprise roughly 15% of the total number of cells within this region (Wagner, Eaton, Moore & Lookingland, 1995; Cheung, Ballew, Moore, & Lookingland, 1998; Kolmac & Mitrofanis, 1999; Mitrofanis, 2005). These sparse dopaminergic cells have been shown to project to a variety of brain areas, including ipsilateral projections to the adjacent lateral hypothalamus – which contains a large, densely populated group of dopaminergic cells – neighbouring regions such as lateral preoptic area and the limbic structures at the diencephalic-telencephalic juncture, along with more distal regions such as the central nucleus of the amygdala, horizontal diagonal band of Broca, and the paraventricular nucleus (Wagner *et al.*, 1995; Cheung, Ballew, Moore, & Lookingland, 1998; Kolmac & Mitrofanis, 1999).

Research regarding the role of these neurons in behaviour is still limited, with earlier work evidencing that direct injection of a D<sub>1</sub>-receptor agonist to the ZI had a stimulatory control on the release of luteinising hormone and occurrence of ovulation, and selective D<sub>1</sub>-receptor antagonism inhibited ovulation; conversely chemical manipulation of the D<sub>2</sub>-receptor had no effect on sexual cycles (James, Mackenzie, Tuohy-Jones & Wilson, 1987) Subsequent work by Tonelli & Chiaraviglio (1995) demonstrated that administration of a dopamine or a specific D<sub>2</sub>-receptor agonist into the ZI reduced food and water intake,

whilst a D<sub>1</sub>-receptor agonist had no effect, suggesting that D<sub>2</sub> receptors may modulate ingestive behaviour.

### 1.7.3 GABA

The principal inhibitory neurotransmitter, GABA, exerts its effects via two ligand-gated channels (GABA<sub>A</sub> and GABA<sub>C</sub>) and one regulated via G-protein (GABA<sub>B</sub>) (Jones *et al.*, 1998). An important inhibitory input to the STN originates from the GPe, which uses both GABA<sub>A</sub> and GABA<sub>B</sub> to regulate STN output activity, and has been found in both monkeys (Galvan, Charara, Levey, & Smith, 2004) and rats (Bell, Churchill, & Kalivas, 1995). GABAergic boutons from the GPe form symmetric synapses on the STN, which differs from the asymmetric glutamatergic afferents (Smith, Bevan, Shink, & Bolam, 1998). This inhibitory projection from the GPe is critical in shaping STN output activity via the indirect pathway, as it has also been shown that administration of the GABA antagonist bicuculline significantly increases STN activity (Robledo & Féger, 1990). As outlined in the preceding sections, STN activity is largely shaped by glutamate binding, but may also be influenced by dopamine binding; work by Floran *et al.* (2004) found that selective D<sub>4</sub> receptor agonism inhibited GABA release from the GPe to the STN, which would in turn increase STN activity. In addition to the D<sub>4</sub> receptor, Baufreton & Bevan (2008) detailed that dopamine binding at presynaptic D<sub>2</sub>-like receptors attenuates GABAergic transmission (via the GABA<sub>A</sub> receptor). Parkinson's disease is marked by a reduction in GABA and excess glutamate signalling, which contributes to STN hyperactivity, likely resulting from nigrostriatal dopamine denervation (El Arfani *et al.*, 2014).

There is mixed evidence for the existence of GABAergic cells within the STN, and their functional contribution to STN activity. Early work posited that the STN neurons use GABA as an inhibitory transmitter (Nauta & Cuenod, 1982; Rouzair-Dubois, Hammond, Yelnik & Féger, 1984); a consideration which was largely replaced by the predominant excitatory role the STN currently holds, with its robust glutamatergic cell population (for review see Parent & Hazrati, 1995). A separate line of research has maintained that there still exists a GABAergic cell population within the STN, albeit sparse. This population has been identified largely by targeting and labelling – through immunohistochemistry techniques – glutamic acid decarboxylase (GAD): the GABA-synthesising enzyme, which

is a reliable, specific marker for GABA neurons (Kaufman, Houser & Tobin, 1991; Gonzales, Kaufman, Tobin & Chesselet, 1991), along with the use of *in situ* hybridisation histochemistry for GABA transporter 1 (Yasumi *et al.*, 1997). These techniques have been applied to the rat (Oertel & Mugnaini, 1984; Yasumi *et al.*, 1997), monkey (Benson, Isackson, Hendry & Jones, 1991) and human (Nisbet *et al.*, 1996) STN, which revealed few positively labelled cells within the nucleus suggesting that the existence of GABAergic cells in the STN may be minimal. Despite the advancement of these studies, the neuronal nature of these GABAergic cells has not been determined, nor is there a description of the morphological features. In a more recent study, Lévesque & Parent (2005) performed GAD immunohistochemistry on *post mortem* human brain tissue and revealed that these GABAergic cells comprise 7.5% of the total neuronal population of the STN, and are more abundant in the caudal-ventral-medial section of the nucleus, which is largely implicated in limbic and associative functioning.

GABAergic cells are the most populous neurotransmitter founding in the ZI, and are most densely located in the ZIV, but can be found throughout the entire nucleus (Nicolelis *et al.*, 1995; Mitrofanis, 2005; Park, Hoffman, & Keller, 2014). Furthermore, the projections originating in the ZI to the cortex (incertocortical projection) and the dorsal thalamus (incertothalamic projection) are also GABAergic, and in the case of the thalamus, the ZI provides a significant inhibitory influence via the GABA<sub>A</sub> and GABA<sub>B</sub> receptor on the firing properties of the reticular thalamic nucleus – a region implicated in many higher-order functions (Bartho *et al.*, 2002; Nicolelis *et al.*, 1995; Park, Hoffman & Keller, 2014). In addition to the incertocortical and incertothalamic projections, the ZI also exerts GABAergic projections to the superior colliculus (Kim, Gregory & Hall, 1992), which contributes to orienting behaviour, along with inhibitory projections to the brainstem (Nicolelis, Chapin & Lin, 1992). Behaviourally, muscimol (GABA agonist) injected into the rat ZI produced head tilting ipsilateral to the injection site, whilst administration of bicuculline (GABA antagonist) produced head tilting to the contralateral side (Murer & Pazo, 1993). These findings, particularly those documenting the GABAergic projections of the ZI, suggest that the ZI is a major somatosensory relay in the ventral thalamus, carrying inhibitory signals to neocortical and subcortical regions. Consistent with the distinct

cytoarchitectonic subdivisions of the ZI, the predominant distribution of GABAergic cells in the ZIV overlap with the glutamatergic cells in the ZID, and it has been speculated whether this inhibitory ventral region and excitatory dorsal region may divide ZI function (Mitrofanis, 2005).

#### 1.7.4 Serotonin

Early tracing studies found a distribution of serotonin (5-hydroxytryptamine; 5-HT)-immunoreactive nerve fibres in the monkey and rat STN, mostly in the ventral and medial regions of the nucleus, and were more likely found in the caudal sections in the rat (Mori, Takino, Yamada, & Sano, 1985). Recent research has identified that these projections originate in the dorsal raphe nuclei (DRN) and that there are several types of 5-HT receptor in the STN, including evidence of 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>2C</sub> and 5-HT<sub>4</sub> receptors, although only a sparse number of axon terminals were observed in the STN (for review see Reznitsky, Plenge, & Hay-schmidt, 2016; see also Stanford, Kantaria, Chahal, Loucif, & Wilson, 2005).

The effects of 5-HT in the STN can result in two distinct effects on the same neuron: excitation is mediated by the 5-HT<sub>2C</sub> and 5-HT<sub>4</sub> receptors, whilst inhibition is mediated by the 5-HT<sub>1A/B</sub> receptor (Stanford, Kantaria, Chahal, Loucif, & Wilson, 2005). Furthermore, application of exogenous 5-HT or a non-specific 5-HT<sub>2</sub> agonist increases STN neuron firing rate, without changing STN firing pattern, and that these excitations were reduced by 5-HT<sub>2C</sub> and 5-HT<sub>4</sub> receptor antagonists (Stanford, Kantaria, Chahal, Loucif, & Wilson, 2005; Xiang, Wang, & Kitai, 2005). Application of 5-HT elicits a biphasic response on STN neurons – an initial excitation event, followed by inhibition – which were maintained even after application of picrotoxin (a GABA<sub>A</sub> antagonist) and CNQX (AMPA/KA antagonist); however application of a 5-HT<sub>1A</sub> antagonist blocked this inhibitory event, which suggested that although 5-HT receptors are sparse in the STN, 5-HT-mediated excitation and inhibition events are separate entities that arise from direct postsynaptic receptor mediated effects (Stanford, Kantaria, Chahal, Loucif, & Wilson, 2005). It has also been shown that activation of the 5-HT<sub>1B</sub> receptor inhibits neurotransmission in the STN, and that at lower concentrations of 5-HT (10µM) reduces glutamate-mediated excitatory postsynaptic currents by 35%, yet a higher concentration



(100 $\mu$ M) of 5-HT was required to inhibit the GABA-mediated inhibitory postsynaptic currents to a comparable extent (Shen & Johnson, 2008). These findings suggest that 5-HT, similar to dopamine, may exercise a regulatory role in controlling STN output activity.

In the rat ZI, serotonin-immunoreactive cells are sparsely found throughout all regions, with no particular area of concentration, making up around 2% of the total cell population in the dorsal, ventral and caudal sectors and around 5% of the total cell population in the rostral sector (Kolmac & Mitrofanis, 1999). In the ZI, 5-HT plays a role in neuroendocrine function (see Kordon *et al.*, 1980), and it is thought that the serotonergic projections from the medial ZI may modulate prolactin and gonadotrophin secretion (Bosler, Joh, & Beaudet, 1984). Further, and more recent, research found additional evidence that 5-HT in the ZI is implicated in neuroendocrine function: application of 5-HT directly to the ZI inhibits the secretion of lutenising hormone, which in the ZI is mediated by 5-HT<sub>7</sub> receptor activation (Siddiqui, Abu-amara, Aldairy, Hagan, & Wilson, 2004).

### **1.7.5 Neurochemical pathways and HFS**

Seminal work by Benazzouz and colleagues (1993) found that unilateral STN-HFS, applied in monkeys that were rendered hemiparkinsonian with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; dopamine depleting toxin) lesions, alleviated the observed muscular rigidity in the contralateral forelimb (for review see Gubellini, Salin, Kerkerian-Le Goff & Baunez, 2009; but also Baunez & Gubellini, 2010). This work ultimately inspired Benabid *et al.* (1994) to apply HFS to Parkinson's patients, and since then silencing the STN via HFS has become a popular therapeutic option for efficiently alleviating the cardinal symptoms of Parkinson's disease, including akinesia, rigidity and tremors (Benabid *et al.*, 1994; Limousin *et al.*, 1995). There is also more recent evidence suggesting that STN-HFS may also be useful in reducing symptoms of severe forms of obsessive-compulsive disorder (OCD), lending positive therapeutic outcome by targeting the medial STN (Mallet *et al.*, 2008; but see Pelloux & Baunez, 2013). For treatment of OCD symptoms, therapeutic efficacy is benefitted by targeted stimulation of the rostral and medial STN, which is known to receive cortical information from the OFC (see Karachi *et al.*, 2005), and provides further support that impulsive/compulsive behaviour may be

mediated by a network of key brain areas such as the OFC, striatum and the STN (see section 1.8; but also Eagle & Baunez, 2010; Baunez & Lardeux, 2011).

As outlined above, the most compelling evidence for the efficacy of this neurosurgical treatment in Parkinson's disease is the ability for HFS to silence hyperactive glutamate signalling in the STN, which manifests as irregular bursts of high-frequency spikes (Porter *et al.*, 1994; Henderson & Dunnett, 1998; Tai *et al.*, 2003; Vila *et al.*, 1999). It is thought that dopamine depletion alters the activity of the MSNs in the nigro-striatal projection, which in turn, enhances the activation of the 'indirect pathway' (and therefore the glutamatergic STN), ultimately overactivating inhibitory output neurons in the GPi and SNr (Gerfen *et al.*, 1990; Crossman, 1990; Obeso & Lanciego, 2011).

The direct mechanism of action for STN-HFS is still not well understood (see Carcenac *et al.*, 2015); however there is strong evidence that STN-HFS increases striatal dopamine release and metabolism, which consequently attenuates glutamate hyperactivity (Bruet *et al.*, 2001; Messiner *et al.*, 2003; Bergmann *et al.*, 2004; Lacombe *et al.*, 2007; Carcenac *et al.*, 2015). Specifically, STN-HFS causes a significant increase in extracellular D<sub>1</sub> dopamine receptor levels in the rat striatum, which may contribute to the therapeutic effects on motor symptoms (Carcenac *et al.*, 2015). Furthermore, STN-HFS is an effective adjunct to L-DOPA treatment, reducing the required L-DOPA medication by up to 50% amongst Parkinson's patients on chronic STN-HFS (Benabid *et al.*, 1998; Moro *et al.*, 1999). This noted reduction in medication in rodent models of Parkinson's disease is likely due to the fact that STN-HFS coupled with L-DOPA administration results in greater extracellular dopamine release than when rats received either treatment alone (Lacombe *et al.*, 2007). Given that activation of D<sub>1</sub>-like receptors (specifically D<sub>5</sub>) increases burst firing in subthalamic neurons, therefore shaping neuronal activity (see Baufreton *et al.*, 2003), it is speculated that STN-HFS stabilises dopamine levels in the striatum, resulting in a normalisation of STN function (Lacombe *et al.*, 2007).

Despite its clinical efficacy, STN-HFS may result in cognitive and psychiatric impairments, and Parkinson's patients who have been treated by STN-HFS have reported increased apathy, depression, mania, impulse control disorders, and executive functioning (Saint-Cyr *et al.*, 2000; Houeto *et al.*, 2002; Romito *et al.*, 2002; Funkiewiez *et al.*, 2004;

Drapier *et al.*, 2006; Ulla *et al.*, 2006; Frank, 2006; Tan, Hartung, Sharp, & Temel, 2011). It is worth noting that STN-HFS only partially mimics STN inactivation as compared to STN lesioning in rats (see Baunez *et al.*, 2007; but also Gubellini *et al.*, 2009), and that the noted deficits in cognitive performance (see section 1.8) are not as pervasive. Furthermore, STN-HFS is reportedly less effective at treating the cognitive impairments associated with unilateral nigro-striatal dopamine denervation, such as increased trial omissions in a choice reaction time task (for task information see section 1.8), which likely stems from the impairment in initiating contralateral motion acts (see Carli, Evenden & Robbins, 1983), and it therefore generally concluded that the therapeutic effects of STN-HFS are well-suited to treating motor symptoms, but are less effective at treating impaired cognitive functions (see Darbaky, Forni, Amalric & Baunez, 2003). To account for this, recent work by Carcenac *et al.*, (2015) found a significant decrease in D<sub>2</sub> and D<sub>3</sub> receptor levels in the rat nucleus accumbens following STN-HFS – regardless of L-DOPA treatment – and it is speculated that this may partially account for neuropsychiatric and cognitive side effects of STN-HFS. Furthermore, Dalley *et al.* (2007) demonstrated with positron emission tomography that highly impulsive rats also exhibit a reduction in D<sub>2</sub> and D<sub>3</sub> receptor availability in the nucleus accumbens.

In addition to introducing cognitive side-effects, STN-HFS may alter mood and emotional processing, including contributing to depressive symptoms (Funkiewiez *et al.*, 2004; Takeshita *et al.*, 2005; Appleby, Duggan, Regenber, & Rabins, 2007; Tan, Hartung, Sharp, & Temel, 2011). It is worth noting that Parkinson's patients already have increased incidence of developing depression (30-40% of patients; for review see Cummings, 1992), which is partially due to pathological loss of axons and cell bodies of 5-HT neurons in the DRN (Tan *et al.*, 2011). It has been observed that STN-HFS significantly inhibits normal 5-HT neurotransmission in the DRN (inhibiting 40-50% of the firing rate), and whilst the actual pathway originating from the STN to the DRN that is driving this effect has yet to be formally identified, it likely operates via the SNr, mPFC or ventral pallidum (Temel *et al.*, 2007; Tan *et al.*, 2011). Behaviourally, STN-HFS has been shown to acutely induce depressive symptoms which reversed immediately after cessation of the stimulation (Tommasi *et al.*, 2008). These findings suggest that STN-HFS, whilst largely treating

motor symptoms of Parkinson's disease by regulating STN hyperactivity, may introduce a plethora of side effects, along with increasing the likelihood for mood disorders.

In addition to STN-HFS surgery, the caudal ZI (cZI) has also become a target for HFS surgery to treat pervasive motor symptoms of Parkinson's disease (Plaha, Ben-Shlomo, Patel, & Gill, 2006; Guehl *et al.*, 2008; Blomstedt *et al.*, 2012; Watson, Lind, & Thomas, 2014), along with essential tremor (Plaha, Khan, & Gill, 2008; Plaha *et al.*, 2011). The cZI is located posterior and dorsal to the STN and Plaha and colleagues (2006) have reported that cZI-HFS therapy may result in better clinical improvement than STN-HFS therapy for improving contralateral Parkinsonism, yielding greater improvement in tremor activity, bradykinesia, and rigidity. It appears that cZI-HFS is particularly effective at severe forms of Parkinson's disease, resulting in a dramatic improvement in rigidity, akinesia or bradykinesia, and tremors in clinical trials of bilateral cZI-HFS (Plaha *et al.*, 2006; Guehl *et al.*, 2008; Plaha, Khan, & Gill, 2008; Plaha *et al.*, 2011); it appears that unilateral cZI-HFS is effective at treating severe Parkinsonian tremor, but reportedly less effective at treating rigidity and bradykinesia compared to bilateral cZI-HFS (see Blomstedt *et al.*, 2012). Similar to STN-HFS, cZI-HFS therapy decreases the required L-DOPA dosage in patients by up to 50%, suggesting that this area may also play a role in the mediation of Parkinson's symptoms (Plaha *et al.*, 2006; Guehl *et al.*, 2008).

These findings, and the very fact that the ZI is an effective target for HFS are quite remarkable, particularly since the predominant neurotransmitter throughout the ZI is GABA (see section 1.7.3), and that furthermore, rodent STN-HFS studies which report electrode placement/lesions in the ZI observe a different – and generally less efficient – behavioural profile (for review see Gubellini *et al.*, 2009). The exact reason why cZI therapy is effective is still unclear, with some researchers offering interpretations such as the proximity of the cZI to the ventral posterior lateral thalamic nuclei and pallidothalamic fibre tracts, in which the latter has been suggested as a target for HFS (see Gallay, Jeanmonod, Liu, & Morel, 2008; Watson, Lind, & Thomas, 2014). Plaha, Khan, & Gill, (2008) argue that inactivating the GABAergic projections from the ZI to the output nuclei of the BG, including the thalamus serves to normalise aberrant tremor oscillations – during the Parkinsonian state, enhanced activation of the 'indirect pathway' (and STN)

overactivates inhibitory output neurons in the GPi and SNr, which overly inhibits the thalamo-cortical output pathway (Gerfen *et al.*, 1990; Crossman, 1990; Obeso & Lanciego, 2011). This consideration has been subject to scrutiny however, largely due to the fact that GABAergic immunoreactive cells only comprise 10% of the total cell population in the caudal region of the ZI (Kolmac & Mitrofanis, 1999), and it is speculated by other researchers that this relative paucity of GABAergic cells compared to other regions of the ZI may contribute to its clinical efficacy (Watson, Lind, & Thomas, 2014). As stated previously, the ZI has distinct cytoarchitectonic subdivisions, and that each subdivision gives rise to a distinct pattern of efferent projections (see Romanowski, Mitchell & Crossman, 1985). Consequently, a distinct population of calbindin neurons (specifically D28k) have been identified in the cZI, which comprise 25% of the total cells in the caudal region, compared to 5% of the total cells found in rostral, dorsal, dorsolateral and ventral regions (Kolmac & Mitrofanis, 1999), and it is speculated that the inactivation of these calcium-binding proteins aids in the therapeutic effects of cZI-HFS (Watson, Lind, & Thomas, 2014). Additionally, the caudal pole of the ZI has a distinct patch of acetylcholinesterase activity – the enzyme which breaks down acetylcholine – and it is also thought that HFS-inactivation may mimic an acetylcholinesterase inhibitor (Watson, Lind, & Thomas, 2014), which has been used to predominantly treat cognitive symptoms of Parkinson’s disease, but has been also shown to increase the rate of tremor amongst patients (for review see Pagano *et al.*, 2015). In summary, the direct mechanism of action for cZI-HFS is not well established, and more research regarding the contributory neurochemical pathways implicated in this therapy, and ultimately regarding the ZI in general, is essential.

## **1.8 The STN and cognition**

To date, the majority of research regarding the function of the STN has been largely devoted to investigating the STN’s involvement in motor functioning. A simplified interrogation of PubMed’s database illustrated this disparity, in which a search of cognitive research of the STN (subthalamic nucleus [Title] AND cognition [Title/abstract]) yielded 41 results, whilst motor research (subthalamic nucleus [Title] AND motor [Title/abstract])

generated an overwhelming 794 results. An identical search regarding the ZI found 34 articles regarding motor performance, and none pertaining to cognition, further illustrating that this region is under-researched.

Consequently, experimental research with non-human animals regarding the role of the STN originates from a motor research perspective, by examining the effects of lesioning the STN in combination with striatal dopamine (DA) depletion (Baunez *et al.* 1995; Baunez & Robbins, 1999; Phillips & Brown, 1999; El Massioui *et al.*, 2007) or after systemic application of dopamine D1/D2 antagonists (Baunez & Robbins, 1997), to better understand the cognitive deficits that arise in Parkinson's disease, and similarly those which arise from HFS therapy. This preclinical research – predominantly in rats – has suggested that chemical lesioning the STN may result in a variety of cognitive impairments, including deficits in memory, decision-making/impulse control, and visuo-spatial attention (for reviews see: Gubellini *et al.*, 2009; Baunez & Gubellini, 2010; Baunez & Lardeux, 2011). The majority of the lesion-based experiments in rats that have investigated the role of the STN in cognition have typically employed an operant chamber, with either a nose-poke aperture for determining response selection, or a more conventional lever-pressing/-releasing assignment, which will be described in further detail below.

### **1.8.1 Visuo-spatial attention**

This research has predominantly utilised cognitive tasks which require high attentional demand, such as the 5-choice serial reaction time task (5CSRTT), and other tasks of visuo-spatial attention (see Carli, Robbins, Evenden, & Everitt, 1983). These tasks typically train rats to respond to brief flashes of light presented randomly in one of five spatial locations by requiring the rat to poke their nose into the hole that emitted the light. The 5CSRTT provides measures of discriminative accuracy, reaction time, perseveration (nose-poking in additional choice holes, or panel-pushes to initiate the trial), and premature responding (responses made prior to the presentation of the cue light), all of which are useful in measuring impulsivity behaviour, and indeed, there is evidence that the STN may play a role in several forms of impulsive behaviour, including adopting riskier strategies (see section 1.8.2; for review see Jahanshahi, Obeso, Baunez, Alegre, & Krack, 2015; but also see Evenden, 1999 for review on impulsivity). These tasks and others, which measure

impulsivity/compulsivity (e.g., the Go-No Go task & Stop-signal reaction time task) suggest that lesioning the STN results in impulsive response selection, and that the STN likely enables us to “hold our horses” by providing deliberative potential in decision making, perhaps owing to its ability to suppress thalamocortical output activity (Aron & Poldrack, 2006; Frank, Samanta, Moustafa, & Sherman, 2007; see section 1.8.2). This effect has been replicated in both monkeys and in rats (for review see Jahanshahi, Obeso, Baunez, Alegre, & Krack, 2015).

Early work by Baunez *et al.* (1995) hypothesised that lesioning the dopaminergic neurons of the dorsal striatum would induce motor impairments consistent with Parkinson’s symptoms, and further, that subsequently lesioning the STN would provide the same therapeutic effects as it does in humans and monkeys. It was revealed that 6-OHDA lesions resulted in motor initiation deficits, consistent with Parkinson’s symptoms, on a task in which rats were trained to release a lever after the onset of a visual stimulus within a time limit to obtain reward. These 6-OHDA-lesioned rats exhibited a delayed lever-releasing response. When STN lesion surgery was administered two weeks following 6-OHDA surgery, the observed motor initiation impairment was attenuated, yet rats with these combined lesions exhibited premature responding (lever release before visual stimulus onset). Rats that had only received bilateral ibotenic acid lesions of the STN exhibited decreased accuracy, and similar to the combined lesion group, increased premature responding (*ibid*); furthermore, STN-lesioned rats demonstrated ‘speeding’ behaviour, in which their latency to respond significantly decreased. The authors concluded that STN lesions alleviate the motor deficits in a Parkinson’s model rat, but that they might also induce a cognitive impairment, particularly in attention and response control.

In a follow-up study using a 5CSRTT, Baunez & Robbins (1997) partially replicated the lever-releasing findings in Baunez *et al.* (1995), finding that lesioning the STN impairs discriminative accuracy, along with inducing both premature and perseverative responses; interestingly, the lesioned rats in Baunez & Robbins (1997) exhibited increases in latency to make a response (either correct or incorrect), suggesting a slowing of responding. The authors found that increasing the stimulus duration, and decreasing the inter-trial interval (the time between the panel push that signals starts the

trial and the presentation of the visual stimulus) significantly improves accuracy, whilst also reducing the number of premature responses for lesioned animals. Despite this improvement, performance for lesioned rats was still significantly poorer than control rats, and that manipulating stimulus duration and inter-trial interval did not influence perseverative responding, which was remained significantly impaired amongst lesioned animals.

To reconcile these findings the authors suggested that lesioning the STN does not only impair motor performance, and that lesioned rats may have suffered from impairments in orienting attention and impulse control: perseverative panel pushing at the start of the trial may have interfered with the rats' ability to orient itself correctly to the stimulus cue in preparation for signalling, therefore increasing omissions and latency, along with decreasing accuracy. This interpretation also aligns with observation that increasing the stimulus duration would improve performance by permitting more time for the rat to make a correct response. The fact that lesioned rats exhibited increased premature responses implies that for some of the trials rats were oriented correctly, and the observed increase in these anticipatory errors might be indicative of an increase in impulsive action. This consideration is further supported by the result that decreasing the inter-trial interval from five seconds to two seconds essentially eliminates premature responding, and it is possible that the STN may normally act to inhibit behavioural excitation found following increased drive or motivation (see section 1.8.2). It is worth noting that despite the impulsive action seen in Baunez & Robbins (1997), lesioned rats did not evidence the 'speeding' behaviour (decreased latency data) previously observed in Baunez *et al.* (1995). Conversely, lesioned rats were slower than controls to respond in Baunez & Robbins (1997); this effect however was not restricted to trials in which lesioned rats incurred perseverative responses, which led the authors to postulate whether STN-lesioning induces deficits in decision making as well (see section 1.8.2).

Baunez & Robbins (1997) were also interested in investigating whether deficits induced by STN lesions could be minimised by dopamine receptor blockade, and therefore systemically administered  $\alpha$ -flupenthixol, a dopamine D<sub>1</sub>/D<sub>2</sub> receptor antagonist. The authors found a reduction in premature responses and perseverative panel-pushes for STN-



lesioned rats, but no reduction was observed in perseverative nose-poking responses;  $\alpha$ -flupenthixol did not influence accuracy or latency. This finding suggested that some of the observed deficits were independent of striatal dopamine transmission, particularly the perseverative nose-poke response. Furthermore, the authors speculate that perhaps the improvement in premature responses and the partial reduction of perseverative panel-pushing responses may be due to a dopaminergic-mediated involvement in incentive motivation (see Phillips, Pfauss, & Blaha, 1991).

Follow-up research by Baunez & Robbins (1999) attempted to replicate and expand on the findings presented in Baunez & Robbins (1997); instead of using  $\alpha$ -flupenthixol, Baunez & Robbins (1999) performed 6-OHDA lesions of the rat striatum following ibotenic acid lesions of the STN. Test data from the 5CSRTT revealed that STN lesioning alone, as expected, produced several behavioural deficits, including decreased discriminative accuracy suggesting an impairment in attention, along with increasing the number of omissions, premature responses, and both nose-poke and panel-pushing perseverative responses. Consistent with Baunez & Robbins (1997), latency data for STN-lesioned rats illustrated a 'slowing' or an increase in latency per trial. Administration of 6-OHDA lesions of the striatum to STN-lesioned animals exerted an ameliorative effect on the number of premature responses committed only, whilst the other impairments seen following STN lesions remained. These results highlight that the majority of the deficits induced by STN lesions may be independent of a dysfunctional nigrostriatal dopamine projection, whereas the deficit in premature responding, might be amenable to dopamine transmission. Furthermore, 6-OHDA lesions by themselves had no significant effect on premature responses, however when 6-OHDA lesions were made following STN lesions there was an ameliorative effect; this observation still suggests that 6-OHDA lesions did have a behavioural effect, which was potentiated by STN lesions.

Subsequent work by Phillips & Brown (1999) attempted to further investigate the work presented in Baunez *et al.* (1995) and Baunez & Robbins (1997;1999), but by using less debilitating unilateral lesions of the STN and of striatal dopamine, which provided the authors the opportunity to dissociate generalised effects (*e.g.*, hyperkinesia) from response-specific initiation effects, by comparing ipsilateral vs contralateral motor performance

biases. They found – as expected – that rats with striatal dopamine depletion exhibited slower reaction times consistent with a motor initiation impairment, but to the ipsilateral side. The authors also replicated the findings of Baunez *et al.* (1995) and Baunez & Robbins (1997;1999) that STN-lesioned rats (and combined lesion rats) exhibit increased premature responding. However, the combined lesion group favoured a contralateral response bias, which demonstrated that the STN lesion does not merely cancel the akinesia following striatal dopamine depletion by addition of a hyperkinetic impairment, but instead lesioning the STN presents a novel impairment in functioning, which disrupts the balance of the motor system, and by extension of the task, cognition.

This impairment in cognition, particularly attentional behaviour, may result from downstream dysregulation of key neural substrates implicated in cognition – such as the OFC and mPFC – which directly project to both the STN and the ZI (Kita *et al.*, 2014). Disconnection lesions of the mPFC-STN (*i.e.*, lesioning the STN and mPFC unilaterally, but in opposite hemispheres) considerably impaired rats' discriminative accuracy and also increased perseverative responding and response latencies in the 5CSRTT; moreover this functional deficit was remarkably similar to the impairment seen after bilateral STN lesions (Chudasama, Baunez, & Robbins, 2003). This connection to the STN suggests that fronto-subthalamic connection may be important in inhibition of responding, and given that there is existent overlap of receptor terminations in the STN and ZI from cortical areas (see Kita *et al.*, 2014), we could expect the ZI to, in part, play a cognitive role as well.

The majority of cognitive researchers have attributed their findings to be a representative measure of the functioning of the STN, however little experimental evidence is available or has been presented regarding the role of the ZI in cognition. The paucity of cognitive research for the ZI may be partially due the fact that the ZI is an under-researched region of the brain, but also that published research regarding the physical demarcation and admissibility of what constitutes the STN has been inconsistent. Being a small region of interest, studies which use lesions to disrupt the STN sometimes incur tissue damage to the neighbouring ZI region. To address this, some authors have retained these subjects as their inclusion does not influence the behaviour of interest, nor statistical outcome (Phillips & Brown, 2000; Phillips & Brown, 1999). Similarly, electrophysiology or HFS studies, which

record from or stimulate the STN cause unavoidable electrode track damage to the ZI, invariably with behavioural consequences (Baunez, Christakou, Chudasama, & Forni, 2007; Darbaky, Forni, Amalric, & Baunez, 2003; Desbonnet *et al.*, 2004), yet the conclusions drawn from these findings attribute any change in functioning to a manipulation of the STN. In contrast, other studies have either avoided the ZI altogether with discrete lesions (Baunez & Robbins, 1997; Baunez *et al.*, 2001), whereas others have discarded rats that present with cell loss in the ZI, but comment that the data obtained from those animals had little impact on behaviour, suggesting that the ZI may have a minimal role in this type of cognitive behaviour (Baunez *et al.*, 1995; Baunez & Robbins, 1999).

Given the evidence that cortical projections to the STN and ZI overlap in these regions (Kita *et al.*, 2014), it is possible that both the STN and ZI work together to regulate activity, thus contributing to the similar behavioural evidence in some studies. Furthermore, this blending of boundaries applies to other regions that share intimate connections with this area, for example, the cortical projections to both the STN and ZI overlap into the lateral LH as well, suggesting that the entire region may work together to modulate behaviour, and functioning which may very well extend beyond response inhibition (Berthoud & Munzberg, 2011; Lin *et al.*, 1990).

### **1.8.2 Decision making and risky behaviours**

As highlighted above, lesioning the STN leads to evidence of impulsive response selection behaviour, which supports the assertion that STN enables us to “hold our horses” by providing deliberative potential in decision making, perhaps by raising the response threshold to allow time for information gathering (Frank, 2006; Jahanshahi *et al.*, 2014). Impulsivity can manifest many forms, such as responding without deliberation (impulsive action), aversion to delayed gratification, the inability to withhold a prepotent response (motor inhibition), along with engaging in more risky decision making (for reviews Evenden, 1999; Dalley, Everitt, & Robbins, 2011; but also Eagle & Baunez, 2010). In the case of decision making, and particularly decisions made under high-conflict conditions (*i.e.*, choosing the best response under two positively associated responses), inhibition of responding is crucial; it is posited that the STN may play a large role in the ‘stopping’ and

‘integration’ of information prior to response selection, which consequently prevents impulsive or premature responding (Frank, 2006; Jahanshahi *et al.*, 2014).

In humans, this behaviour is typically modeled in a gambling task, such as the Iowa Gambling Task (IGT; see Bechara, 2003), which presents real-world contingencies of reward and punishment (*i.e.*, winning and losing money), and creates a ‘decision conflict’ between an immediate large reward, yet at the cost of a delayed, and inevitable, punishment. Subjects are typically presented with four decks of cards with high-paying (decks A & B) or low-paying (decks C & D) options; the punishment is also higher in the high-paying decks and lower in the low-paying decks, and the task is designed such that the high-paying decks cost more in the long run, and are therefore disadvantageous (Bechara, 2003). Individuals who select riskier options in these tasks tend to accept short-term gains *in lieu* of longer-term objectives, and therefore this task has become a useful diagnostic tool in detecting drug addiction, compulsive binge-eating, and pathological gambling (Bechara, 2003; Dalley, Everitt, & Robbins, 2011).

There is also growing evidence that patients with Parkinson’s disease are at increased risk for developing pathological gambling, along with other impulse control disorders such as hypersexuality, overeating and punding (14% of clinical population), and that furthermore, the likelihood of developing these disorders may be predicted by dopamine agonist therapy (Jahanshahi, 2013). This predisposition in Parkinson’s patients undergoing dopamine agonist therapy may in part be due to a reduction in activation in brain areas implicated in impulse control and response inhibition (OFC, rostral cingulate area, amygdala, & GPe), which results in riskier choices, and may be mediated by outcome devaluation, or reward prediction errors; essentially an interference in the ability to learn from losses (van Eimeren *et al.*, 2009; 2010). It has also been found that Parkinson’s patients who are undergoing STN-HFS respond more impulsively, and are consequently impaired in tasks which require conflict resolution, or response selection under conflict (Frank, Samanta, Moustafa, & Sherman, 2007; Zaghloul *et al.*, 2012; Zénon *et al.*, 2016). It is postulated that the OFC, DMS and STN form a network of regions that may be critical in impulsive/compulsive behaviour, and that dysregulating this network results in increased

impulsive action, premature responding and perseveration, and thus contribute to this impairment in decision making (Eagle & Baunez, 2010; Baunez & Lardeux, 2011).

There is evidence that inactivation of the STN with HFS in Parkinson's patients selectively interferes with the ability slow down, or 'apply the brakes', when faced with a decision conflict (Frank *et al.*, 2007). Furthermore, when faced with decision conflict, patients undergoing HFS demonstrate increased spiking activity in the STN, and that as decision conflict increases, so too does the level of the spiking activity (Zaghloul *et al.*, 2012). More recent research by Zénon *et al.* (2016) has revealed that patients who are undergoing HFS therapy – both on and off L-DOPA – exhibit low-frequency neuronal activity in the STN, and instead of signalling conflict, this activity may indicate the encoding of cost-benefit comparisons. Zénon *et al.* (2016) tested patients on an effort-based decision task, providing monetary incentive for effortful activity (*e.g.*, squeezing a handgrip in varying intensity); behaviourally, the probability of engaging in an effortful task increases with reward and decreases with required effort level. L-DOPA+HFS treatment increased the rate of acceptance for efforts associated with lower rewards, and that when off L-DOPA treatment, this effect was weakened. The authors also found synchronised activity in populations of STN neurons that may reflect the subjective value of reward and the subjective cost of effort, which is postulated by Zénon *et al.* to be the net subjective value of a trial during decision making. These findings indicate that the STN plays a critical role in the mediation of action selection during decision making, and that furthermore this may be mediated by the STN encoding the information required to make cost-benefit comparisons, rather than signalling conflict, which may also implicate the STN in motivational behaviour.

In order to investigate how the STN integrates reward information and to what extent such integration correlates with behaviour, Espinosa-Parrilla, Baunez, & Apicella (2015) conducted electrophysiology recordings of the monkey STN during a two-choice target-reaching task, in which selection of a visual cue (target) from a touch-screen led to the delivery of a higher or lower value reward (*e.g.*, juice *vs* water). Two variants of the task were employed: the standard task, and the choice task. The standard task presented monkeys with an instruction cue (*e.g.*, either a green or yellow circle, on either the left or

right side of the screen), which provided advanced information regarding the nature of the upcoming reward (*e.g.*, green = juice, yellow = water), followed by a 1 seconds waiting period before presented with one trigger stimulus (*e.g.*, red circle) in the same spatial location as the instruction cue, and the animals were permitted to make a selection to receive the designated reward. The information gathered from this standard task helped investigate how the type of reward expected may influence neuronal activity during preparation, initiation and execution of the reaching movement. The choice condition task was formally similar to the standard condition, except it provided two instruction cues (one denoting juice, and the other water), and thereby it permitted the animal to choose its reward when presented with the trigger stimulus. Neuronal recordings were assessed over three time periods: the cue-trigger delay period, the movement period and the reward period. The authors found that when no choice was allowed (*i.e.*, during the standard task), the activity of STN neurons was rarely modulated by adjustments of behaviour, although there was evidence that animals responded faster in juice trials, compared to water trials, therefore indicating a discriminative ability for reward-predictive cues. Conversely, when given a choice in selecting actions that lead to reward (*i.e.*, during the choice task), STN activity was sensitive to the reward type, resulting in an increase STN neuronal activity at the outcome phase (when receiving reward), when the less-preferred reward was chosen (Espinosa-Parrilla, Baunez, & Apicella, 2015). These findings also indicate that the STN may encode whether or not a preferred reward has been received when alternative choices are available, which sheds new light on how the STN may mediate decision making processes.

In studies with rats, our ability to infer complex decision making processes are more limited than in humans (*e.g.*, clinical populations), and therefore we must rely on inference from overt behaviours in modified gambling tasks or delay discounting tasks in operant chambers (Winstanley, Baunez, Theobald, & Robbins, 2005; Uslaner & Robinson, 2006; Aleksandrova *et al.*, 2013). The delay discounting task used by Winstanley *et al.* (2005) assessed impulsive choice behaviour, which defines impulsivity as the selection of a small immediate reward over a larger delayed reward, and can be contrasted with impulsive action behaviour (*i.e.*, motoric disinhibition). Winstanley *et al.* (2005) tested rats in an

operant box which required rats to sustain a nose-poke in a central hole in order to commence a trial; subsequently, two levers were presented, in which one lever provided an immediate food reward of one pellet (delay: 0 seconds), whilst the other lever would produce a reward of four pellets with increasing delays between lever-press and reward delivery as testing proceeded (delay: 0, 10, 20, 30, 40 and 60 seconds). The authors found that preoperatively, rats demonstrated the typical delay-dependant choice behaviour: initially preferring a larger reward when delivery is immediate, but eventually shifting to a smaller reward as delay is increased. Postoperatively, STN-lesioned rats chose the larger reward more frequently than sham-operated controls, which suggests that STN-lesioned rats were less impulsive in this delay-discounting task. Furthermore the evidence that STN lesions results in increased impulsive action (Baunez & Robbins, 1997; Baunez *et al.*, 2001) without increasing impulsive choice may suggest that these behaviours may be subject to independent regulatory mechanisms that operate in concert or an ‘impulsive/compulsive network’ (for reviews see Eagle & Baunez, 2010; Baunez & Lardeux, 2011). In a follow-up study by Uslaner & Robinson (2006), the authors replicated the findings of Winstanley *et al.* (2005), and found in a delay-discounting task, STN-lesioned rats selected the lever which led to delayed gratification more frequently than control rats, but only in the longest delay condition, which supports that lesioning the STN may in fact decrease impulsive choice behaviour. It is also possible that the incentive of the larger reward is more difficult to inhibit, particularly following lesioning, and owing to the task sensitivity of the delay-discounting task we are able to obtain different findings than an impulsive action task (*i.e.*, the 5CSRTT). These findings also align with the studies in human Parkinson’s patient and monkeys presented above, in which the STN may play a critical role in outcome evaluation.

In a recent study by Adams *et al.* (2017), the authors investigated the effects of STN-HFS in rats completing the Rat Gambling Task (rGT) – the rodent analogue of the IGT. Similar to the IGT and delay-discounting tasks, the rGT creates a decision conflict in rats by forcing them to decide the most efficient strategy to maximise food reward by training them to poke their nose in one of four holes which differ in reward volume (1, 2, 3, or 4 pellets given per trial) and with corresponding punishments in the form of a ‘time-out’

(5, 10, 30, or 40 seconds), with larger rewards leading to a higher likelihood of punishment. In typical fashion, the most advantageous option maximised reward, yet limited punishment; essentially, smaller per-trial gains but lower time-out penalties. Adams *et al.* (2017) found that STN-HFS did not influence performance for those rats identified as ‘optimal decision makers’ in the baseline assessment, but that HFS significantly improved choice responding for ‘risk-preferring’ rats (‘high risk, high reward’; roughly 25% of rats from baseline). It is worth noting that the behavioural effects of HFS for this population were progressive, in that improvement in decision making was not apparent until the fourth treatment session. This effect has been seen in the past (see Baunez *et al.*, 2007), which has led Adams *et al.* (2017) to postulate that perhaps HFS itself may not reliably induce changes in decision making, and that perhaps repeated stimulation is required to trigger neuroplasticity, which in turn, results in cognitive change. Ultimately, more work is needed, as it has also been demonstrated that HFS of the STN does not truly mimic the inactivation obtained from lesioning (Baunez *et al.*, 2007; Baunez & Lardeux, 2011), but global evidence from both HFS and lesioning, and across humans, monkeys and rats suggests that STN may play a pivotal role in the processes contributing to decision making.

### **1.8.3 Learning and memory**

There are only a few studies which document the involvement of the STN in learning and memory, and it appears that STN lesions do not directly impair the learning process, yet may impair working memory, depending on the task paradigm (Baunez *et al.*, 2001; El Massioui *et al.*, 2007). Research by Baunez and colleagues (2001) compared performance between a simple (SRT) and choice (CRT) reaction time task with the intention to investigate the role of the STN in the response preparatory process. The SRT, which was adapted from a task designed by Brown & Robbins (1991) for examining the effects of striatal dopamine depletion, required the rats to sustain a nose poke in the central hole of a 5-hole array, which prompted a change in brightness in the neighbouring 2 holes (*e.g.*, an increase or decrease in brightness). Half of the rats were trained to ‘go right if bright, and go left if dim’ (and vice versa for the other half), but the rats could only make a response after the onset of a tone stimulus, thus requiring them to sustain their nose-poke. In the SRT condition, the chamber brightness which specified the location of the reward



was returned to an ‘intermediate’ brightness 0.3 seconds before the tone cue, whereas in the CRT condition, the change in brightness which specified the location of the reward was presented simultaneously with the tone cue; essentially, the SRT condition provided information in advance to aid in response preparation. Pre-operative baseline data suggested that reaction times (RTs) were significantly longer in the CRT condition than in the SRT condition, and that furthermore, a longer ‘foreperiod’ (the time before the presentation of the tone stimulus) decreased RTs; these findings suggested that rats benefitted from advanced information, and that rats demonstrated a ‘motor readiness’ effect.

Comparing performance between the SRT and CRT conditions allows one to infer about the response preparatory process, and following lesions to the STN, the rats in Baunez *et al.* (2001) no longer demonstrated a difference between SRT and CRT conditions, indicating that lesioned animals no longer demonstrated the benefit of information presented in advance. The accuracy for lesioned animals in the SRT condition also significantly decreased, along with a significant increase in premature responding in both SRT and CRT conditions. Interestingly, lesions did not impair the ‘motor readiness’ of the rats, as increasing the foreperiod length still yielded an inverse relationship with RT post-operatively, which contrasts the effects seen after dopamine-depleting lesions of the striatum (Brown & Robbins, 1991). This preserved ‘motor readiness’ suggested that lesioned rats were more likely impaired at response selection rather than ‘motor initiation’, especially since there was no difference between groups when it came to movement time (the time between withdrawal of the nose from the central hole to the nose-poke in the response hole); essentially these rats were impaired at the ‘which’ phase of response selection, and not the ‘when’ phase.

The pattern of responding in premature response trials for STN-lesioned animals in Baunez *et al.* (2001) also changed to a win-stay strategy, resulting in rats perseverating to the same side as the previously rewarded response. The nature of this response preparation deficit led the authors to postulate that lesioning the STN may impair the operation of a ‘response buffer’ – a ‘motor’ working memory. This buffer would hold a selected response in readiness until its execution is required, and would need to be ‘cleared’ following execution to permit subsequent responding. It was theorised that following STN lesions, the

inability to clear the buffer following one response would cause conflict with the subsequent response. It is worth noting that theoretically this conflict should only arise if the subsequent response differs from the previous one, and thus the reduced accuracy and increased reaction time should only apply to alternated responses, and whilst the data are not contradictory, the authors did not provide an analysis of this consideration, which would have bolstered the claim for the impairment of a 'response buffer' following STN lesions.

In a subsequent study by El Massioui, Chéruef, Faure, & Conde (2007), the authors investigated whether they could dissociate learning and memory effects by lesioning the STN in rats either before or after completing a lever-pressing task. In one experiment, El Massioui and colleagues lesioned the STN and then required rats to learn a light-tone discrimination task (*i.e.*, if light is emitted, then press the right lever), which upon criterion was reversed (*i.e.*, if tone is emitted, then press the right lever); in a second experiment, animals were trained to complete the same light-tone discrimination as the first experiment, and upon criterion the STN was lesioned. Following surgery in the second experiment, all animals were subject to a 're-learning' phase, which was identical to the original discrimination and rate of acquisition of this discrimination provided a measure of 'long-term memory'; subsequent to the 're-learning' phase, half of the animals were subjected to a reversal, whilst the other half completed a 'working memory' task, which introduced various delays (5, 10 or 20 seconds) between the offset of the cue stimulus and the presentation of the levers. The authors found that STN lesions did not influence acquisition of discrimination learning, further supporting that inactivating this area has a minimal effect on learning a discrimination. When surgery was completed before the behavioural task, El Massioui *et al.* found that STN lesions facilitated reversal learning performance owing to a more rapid inhibition of the previously correct stimulus; essentially reduced perseveration, which is contrary to previous findings (Baunez *et al.*, 1997; 1999; 2001). It is worth noting however that previous work employed a very short stimulus duration period (0.5 seconds), and that when Baunez *et al.* (1997) extended this stimulus duration period they also noted fewer omissions and premature responses.

When rats were lesioned following the acquisition of discrimination learning (second experiment), lesioning the STN did not impair the re-learning phase, and thus long-term memory, or target control of the task parameters, remained intact. However, during the longest delay condition (20 seconds) for the working memory task, the percentage of STN-lesioned rats that reached criterion significantly decreased (only 57% of STN-lesioned rats managed to), owing to an increase in errors, and thereby suggesting that lesioned rats may have an impairment in working memory. Furthermore, reversal learning performance was comparable between lesioned and control groups for the second experiment, with no evidence for a facilitative effect for either group. The authors postulated that the facilitative effect on reversal learning from the first experiment may have resulted from a rapid forgetting of previous response tendencies. The authors argued that the reversal was not facilitated in the second experiment since the original discrimination stage occurred preoperatively – three weeks before the reversal – and therefore weakening the interaction effect between initial and reversal learning owing to temporal delay. This consideration should be interpreted with some caution, largely because the retraining trials which directly preceded the reversal phase adhered to the same conditions as the initial learning, and therefore a facilitative effect on the reversal should have been apparent in the second experiment as well.

An alternative interpretation posited by the authors suggested that the facilitation of the reversal learning phase could have resulted from an increase in selective attention to the relevant stimuli, which would have facilitated the acquisition of the reversal. This enhancement in attentional selectivity during two-choice discrimination learning typically arises from overtraining (see Chapter 7; but also Sutherland & Mackintosh, 1971), and according to Sutherland and Mackintosh's two-stage theory, may be indicative of attentional set-formation. There is no evidence for overtraining in any of the rats in El Massioui *et al.* (2007) (*i.e.*, comparable performance during discrimination learning and during retraining phases, respectively), and furthermore, recently published data in a task better-suited to make an inference about this type of behaviour (*e.g.*, the ASST) suggests that lesioning the STN/ZI-area may in fact impair the formation of attentional set (Tait,

Phillips, Blackwell, & Brown, 2016); a consideration that will be explored in the subsequent section.

#### 1.8.4 Attentional set

In recent reviews by Baunez & Lardeux (2011) and by Jahanshahi and colleagues (2015), only tasks which predominantly measure inhibition and impulse control behaviour (*i.e.*, visuo-spatial attention tasks and their variations) have been employed thus far to evaluate the role of the STN in cognition. Given that the STN itself is directly connected to frontal areas of the brain – along with key output projections within the BG – we would expect it to play a more complex role in executive functioning. Whilst the tasks thus far present a cognitive role for the STN, visuo-spatial reaction time tasks are not sensitive to measuring a breadth of executive demands, such as those evaluated and required during tasks such as the ASST. It has also been shown in human and rodent literature that tasks which exert strict time pressure, or demand rapid response and response inhibition, may exacerbate STN-mediated deficits (Baunez & Robbins, 1997; El Massioui *et al.*, 2007; Pote *et al.*, 2016). Baunez & Robbins (1997) found that increasing the stimulus duration, and decreasing the inter-trial interval significantly improves accuracy, especially for lesioned animals, whilst also reducing the number of premature responses incurred. Similarly, El Massioui *et al.* (2007) found that extending the stimulus duration period to 10 seconds, and arguably removing temporal requirements and excess load on the motor system, may eliminate perseverative behaviour. Pote *et al.* (2016) investigated this speed-accuracy trade-off in Parkinson's patients undergoing STN-HFS therapy and found that STN inactivation was associated with significantly faster reaction times, but with increased error under increasing speed conditions. It is postulated that the STN may raise response thresholds to permit accumulation of information before response selection, and whilst reaction-time tasks, or similar tasks requiring a time-based response are useful in measuring a particular profile of the STN in cognitive functioning, it may not be well-suited to assess the potential executive role of this area.

Past data from our lab, which has been recently published (Tait *et al.*, 2016), extended the work presented by Phillips & Brown (1999), but with bilateral lesions of the STN and dorsomedial striatum (DMS) in the ASST. The authors found that bilateral lesions

of the STN/ZI-area resulted in impaired discrimination learning at the outset of the task, including at the SD, CD, first reversal and ID stages, and furthermore, no difference between the ID and ED stages (figure 1.6). It was speculated that this impairment at early stages may have stemmed from impairment in inhibition, manifesting as ‘impulsive digging’ – an impulsive action behaviour. However, since performance improved as testing progressed, and since the ED was acquired in visibly fewer trials for STN/ZI-lesioned animals than controls, the authors speculated whether an attentional set had been formed, or if these lesioned rats were capable of forming set. Despite no ID/ED difference, it was not possible to reliably determine whether these rats were impaired at forming set, since the elevated ID performance for STN/ZI-lesioned rats could have stemmed from either an impairment in set-formation, or a latent impairment in inhibition found at the earlier stages of testing.

The ibotenic acid lesions of the STN found in Tait *et al.* (2016) were nearly complete and encompassed both the medial and lateral sections of the nucleus, with only the most posterior sections being spared. Previous research has shown that the size of the lesion or the amplitude and frequency of the HFS directly correlates with the magnitude of the motoric inhibitory deficits, and thus perhaps the selective impairment in earlier stages of testing found in Tait *et al.* (2016) could have partially resulted from large lesions encompassing the lateral STN or ZI. This may have resulted in motor disinhibition, which was gradually brought under control as testing progressed (Desbonnet *et al.*, 2004; Wiener, Magaro, & Matell, 2008); however there is no current evidence for the impact of this functional division on tasks measuring cognition, nor is there evidence lesioning the ZI may impair this type of (or any) cognitive behaviour. Since cognition and executive control is of interest, the medial STN was targeted in this thesis.

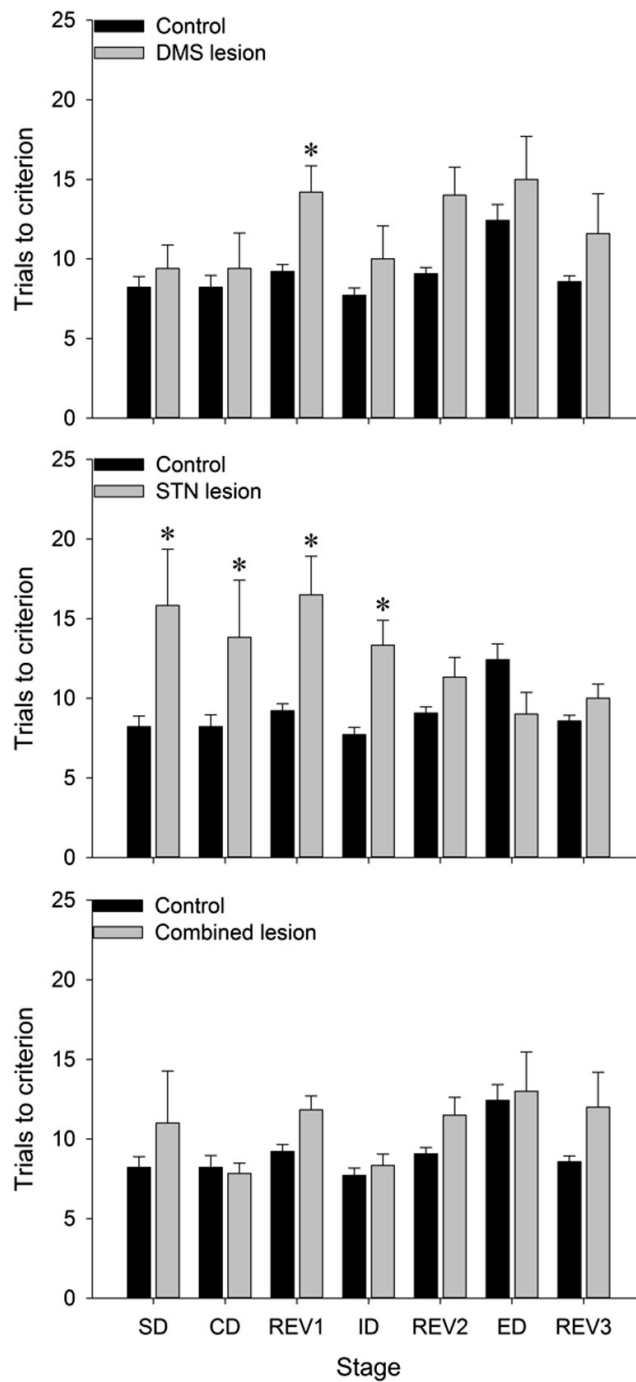


Figure 1.6: Figure from Tait et al., 2016 illustrating the mean TTC (+SEM) for control rats compared to DMS lesioned (top graph), STN lesioned (middle graph) and combined STN+DMS lesioned (bottom graph) rats in each discrimination learning stage; \* $p < 0.05$

## 1.9 Thesis overview

The primary objective of the experiments conducted for this thesis was to further investigate the nature of the cognitive deficits detailed after lesions to the STN. This included manipulating the functioning of the STN, followed by testing behaviour on the rodent ASST to determine whether disrupting the STN induces an impairment in attentional set-formation. This was also in an effort to better understand how learning associations between stimuli contribute to the formation of set.

Chapter 3 will firstly attempt to replicate the findings observed in Tait *et al.* (2016) on the standard 7-stage task, with lesions targeting the medial STN, and the hypothesis that acquisition learning at the earlier stages of the task will not be impaired as a result, along with evaluating the ID-ED comparison to determine the cost of shifting set (if any). A follow-up experiment will use a modified ID/ED task, which has been designed to evaluate several hypotheses which would aid in the inference of set-formation, to further probe this deficit.

The experiments in Chapter 4 introduce another modified task design, which instead of evaluating the cost of shifting set (*i.e.*, ID vs ED), explores the process of set-formation by investigating where attention is being directed to, and the level of distractibility exercised by the irrelevant dimension stimuli. This design is also more succinct than the task variant used in Chapter 3, which was implemented to reduce the time required for testing. This reduction permitted the use of a reduced-stress, palatable ‘jelly’ tablet as a method to deliver orally-bioavailable, cognitive-enhancing drugs (modafinil and ORG49209). The first part of Chapter 4 will demonstrate the efficacy of this ‘jelly’ administration method by examining the bioavailability of modafinil in the brain, along with its effect on behaviour. The second part of the chapter will then test whether rats – either lesioned or control – will benefit from these drugs in the revised task.

In Chapter 5, a refined method of manipulating function of the STN is introduced, which uses a modified viral vector to allow for transient inhibition of a target cell population via application of a synthetic ligand. Being a relatively novel approach, this chapter will be primarily a pilot assessment, determining the best serotype and promoter to

restrict viral transduction to the STN, whilst furthermore measuring changes in c-Fos immunoreactivity levels during an inhibitory state.

Chapter 6 will employ the findings from Chapter 5, and test virally-transduced rats on the same modified ASST from Chapter 3. This task will aid in determining the behavioural consequences of inhibiting the STN on the ASST, along with investigating how rats that are impaired at set-formation might be completing the task.

This is further explored in Chapter 7, where the same group of rats from Chapter 6 are either trained to criterion or receive overtraining at the CD stage, which has been shown to lead to faster reversal acquisition, theoretically owing to a formation of attentional set. The first half of this chapter will attempt to demonstrate this learning effect in the bowl-digging paradigm in unoperated rats, whilst the second part of the chapter will explore the effects of overtraining on virally-transduced rats. This chapter could also shed light on the relationship between associative processes (*i.e.*, reversal learning) and how they are implicated in the formation of attentional set.



# Chapter 2

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## General Methods

The current chapter describes materials and methods commonly used throughout this thesis. This includes information on animal husbandry, general surgical and histological protocol, and the procedure for training and testing on the standard 7-stage attentional set-shifting task, along with procedures for statistical analysis. Variations to these general methods will be described in the respective methods section for that chapter.

## 2.1 Animals

All animals used in the following experiments were Lister hooded rats either obtained from a breeding establishment registered with the UK Home Office or bred in-house (University of St Andrews; *from Charles River stock*). The female rats in Chapter four (experiment I)/Chapter seven (experiment I), and the male rats used in Chapter five were bred in-house, whilst the remaining experiments and experimental chapters utilised male rats from Charles River, UK. All animals were experimentally naïve prior to training for behavioural testing, unless otherwise stated in the respective experimental chapter. Animal welfare and all procedures were in compliance with the Animals Scientific Procedures Act (1986), and conducted under project and personal licenses approved by the UK Home Office and the University of St Andrews Animal Welfare and Ethics committee.

Animal husbandry conditions were nearly identical for all experiments, though minor differences may be reported in each experimental chapter. To provide ‘social enrichment’, rats were group housed when possible, in groups of up to four in large home cages (approximately 50 cm x 30 cm x 25 cm). Following surgery rats were single-housed until their weight stabilised and were then reintegrated into group or pair-housing. In some cases, rats remained single-housed for the duration of the experiment, typically if the rat had difficulty socially reintegrating to group housing or was a potential danger to itself (owing to self-injury from spontaneous seizure activity). These smaller home cages (approximately 40 cm x 23 cm x 19 cm) were also used to pair-house rats in three of the experiments (chapters five, six & seven). In their respective home cages, rats were given “environmental enrichment” in the form of wooden chew bars, shredded cardboard, triangular cardboard “houses” and a plastic tube suspended from the cage lid. Lighting in the colony room was maintained on an artificial twelve hour light/dark cycle (lights on at 07:00), with all behavioural testing conducted during the “lights-on” phase. The colony room and all procedure rooms were kept at  $21^{\circ}\text{C} \pm 2^{\circ}$ , with a relative humidity of  $55\% \pm 10\%$ .

At least one week prior to the start of any experiment, rats were placed on a controlled food diet of 15-20 g of standard laboratory chow (Special Diet Services, Essex, UK) per rat per day; however water was always available *ad libitum*. Food control was employed primarily to encourage motivation for food rewards during

behavioural task work, and to prevent rats from becoming overweight during the experiment. Rats were weighed weekly to ensure that their weight did not decrease suddenly, whilst maintaining a healthy weight gain. Start and end weights for all rats used for the current thesis are summarised below in table 2.1. At the end of the experiment, female rats in experiment I of Chapter 4/7 were terminated using a lethal dose of anaesthetic in accordance with Schedule I of the Animals (Scientific Procedures) Act (1986), whilst rats in all other experimental chapters were transcardially perfused with paraformaldehyde (fixative) under terminal anaesthesia. The procedure for the transcardial perfusion will be described in section 2.5.

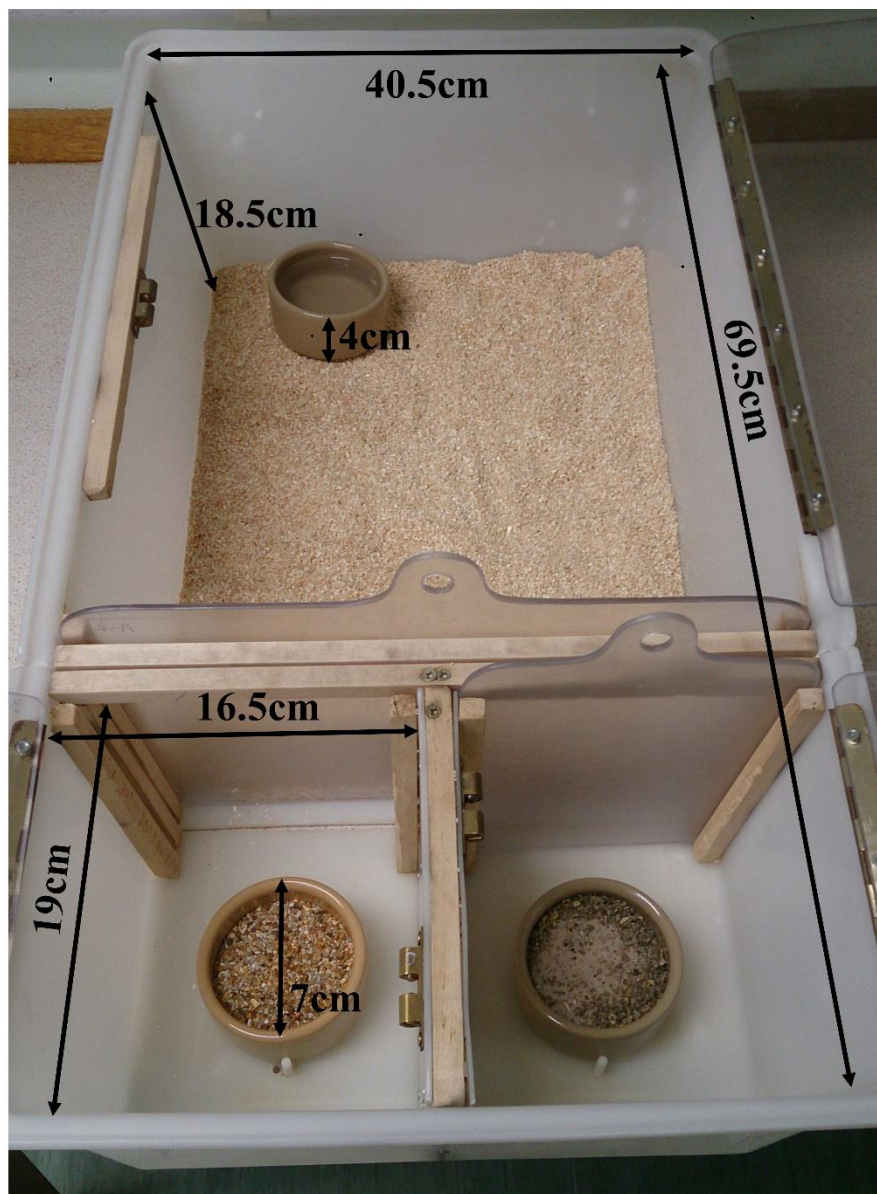
Number of animals	Experimental Chapters	Start weight range	End weight range
20	Chapter 3	358-445g	348-573g
22 (♀)	Chapter 4 - Experiment I Chapter 7 - Experiment I	180-250g	233-328g
20	Chapter 4 - Experiment II	231-286g	405-576g
4	Chapter 5 - Experiment I	369-414g	377-415g
12	Chapter 5 - Experiment II	325-361g	363-425g
22	Chapter 6 Chapter 7 - Experiment II	363-428g	484-575g

*Table 2.1: Start/end weights and sample size of all rats, for all experiments in this thesis.*

## **2.2 Apparatus**

Both training and testing were conducted in the set-shifting apparatus (69.5 cm x 40.5 cm x 18.5 cm). It was constructed from a modified plastic home cage, and divided into a large “waiting area” comprising two-thirds of the box, and the remainder was a

“testing area”, which was further sub-divided into two bowl-digging chambers (figure 2.1). Two acrylic panels were used to occlude entry to the digging chambers – a large panel and a small panel – which could block entry to the entire testing area or to one chamber selectively. The digging bowls were ceramic pet food bowls, which had an internal diameter of 7 cm and a depth of 4 cm. Bowls were filled with digging substrate and odours were subsequently mixed in, until the internal volume was within 0.5 cm of the top of the bowl.



*Figure 2.1: Rat attentional set-shifting apparatus with size dimensions, digging bowls filled with odour and haptic stimuli, small and large acrylic panels, along with a water bowl in the “waiting area”*

## **2.3 Surgery**

### **2.3.1 Neurotoxin**

Chemical lesions to the subthalamic nucleus were accomplished by use of (S)-2-amino-2-(3-hydroxyisoxazol-5-yl) acetic acid or ibotenic acid, a powerful excitotoxin naturally occurring in the *amanita muscaria* mushroom. Ibotenic acid is a conformationally restricted analogue of glutamic acid, and it is evidenced that its application to a target cell population causes protracted pre- and post-synaptic depolarization, which ultimately causes necrosis and cell death (Schwarcz et. al., 1979). Compared to other conventional lesioning methods (*i.e.*, aspiration or kainic acid), IBO is more selective, which minimises extraneous damage to adjacent areas and fibres of passage (Köhler, Schwarcz, & Fuxe, 1979; Jarrard, 1989).

### **2.3.2 Injection materials**

Custom pulled glass micropipettes were used to administer ibotenic acid during all surgical procedures. This was chosen over metal syringes/micro-syringe pumps, which may introduce extraneous toxin damage to adjacent areas, such as the zona incerta and entopeduncular nucleus (EP; Baunez *et. al.*, 1995; Baunez & Robbins, 1997; Phillips & Brown, 1999) during STN lesion surgery. Additionally, metal syringes cause track damage to the ventroposteromedial thalamus, making it difficult to interpret behavioural results (Phillips & Brown, 1999). Thus, the use of a glass micropipette reduces mechanical tissue damage, in addition to a refined toxin administration to the STN, promoting a more localised administration.

Micropipettes were fabricated from borosilicate capillary tubes (0.49mm bore diameter, 1.16-1.19mm outer diameter, 90mm long). Capillary tubes were placed in a vertical pipette puller (Model 720; David Kopf Instruments, Tujunga, CA, USA), which heated the tubes at the centre, whilst pulling the two ends apart. The injection end of the micropipette was then snipped to 30µm under microscope guidance. The micropipettes were marked at 1mm intervals, which denoted an internal volume of 200nl.

### **2.3.3 Surgical procedure**

Induction of anaesthesia was accomplished with 5% isoflurane (Harvard Apparatus, Cambridge, MA, USA) and oxygen (1.5L/min) mixture, in an induction chamber, and maintained between 1-2% throughout surgery via a nosecone fitted on the

incisor bar of the stereotaxic frame. Once anaesthetised, rats were given 0.05ml 5% carprofen (SC: subcutaneous; Carprive, Norbrook Laboratories Ltd., Newry, N. Ireland, UK) to aid in post-operative pain management, and 0.15ml of diazepam (IP: intraperitoneal; Sigma Chemical Company, St Louis, MO, USA) to maintain sedation after recovery from the anaesthesia. Hair was shaved from the scalp to expose the surgical area, and a pre-operative weight reading was taken.

Rats were then secured into a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with atraumatic ear bars, and the skull position was leveled by setting the incisor tooth bar at -3.3mm. A midline incision was made along the scalp, and the skin and fascia tissues were retracted, exposing the skull surface, which was cleaned with sterile saline (sodium chloride 0.9%; Baxter International Inc., Deerfield, IL, USA) prior to drilling. Small burr holes were made at the appropriate stereotaxic coordinates, ventral to the injection site, using a precision dental drill (NSK-Nakanishi International; Kanuma, Tochigi, Japan). Prior to insertion of the micropipette, the dura was lightly scored with a syringe sharp in order for the pipette to enter the cortex without breaking the tip.

Lesions to the STN were made by injection of 200nl of 0.06M ibotenic acid, bilaterally, at the stereotaxic coordinates AP: -3.8mm, ML:  $\pm 2.3$ mm, DV: -7.8mm (Paxinos & Watson, 1998). ML and DV measurements were taken from dura, as the glass micropipette may break by taking measurements from the skull surface. Infusions were given as a bolus injection and the pipettes were left in situ for a further three minutes. Surgery for control rats was identical, except that instead of a toxin, infusions of 200nl of 0.1M phosphate-buffer (0.2M PB: disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate in distilled water) were given. Upon completion, the wounds were cleaned and closed with surgical staples, and the rat was placed in a large recovery cage with a heating pad underneath.

It has been observed that the interaction of ibotenic acid with the STN cell population results in a prolonged and impulsive chewing behaviour whilst recovering from anaesthesia, which may result in self-injury (Baunez & Robbins, 1997). To prevent this, a twine gag was secured in the rat's mouth to provide something to chew on and to limit the risk of self-injury to paws, and the rats were constantly monitored until chewing ceased. Rats were then single-housed for at least one day following

surgery, and returned to group housing following recovery. To account for post-operative pain, rats were given “wet-mash” food, consisting of ground standard laboratory chow mixed with water. Rats that did incur self-injury from “chewing” were given 0.15 mg/kg of meloxicam (Metacam, Boehringer Ingelheil Vetmedica Inc.) mixed into their wet-mash daily to reduce pain, until healed.

## **2.4 The rodent attentional set-shifting task**

### **2.4.1 Training**

The night before training, bowls (one per rat) with six Honey Loops (Kellogg Company, UK) each were filled with sawdust and left in the rats’ home cage to reduce neophobia to the food reward during training. On the day of training, rats were placed in the waiting area of the set-shifting apparatus and two bowls filled with sawdust with a half Honey Loop on the surface were placed in the testing chambers. Upon successful retrieval, the trial was reset and the rat had to retrieve the reward a further five times, yet each time the Honey Loop was baited deeper in the digging bowl (until at the bottom). The rat had to dig for the food reward and a dig was recorded when there was a substantial displacement of digging substrate. The rodent attentional set-shifting task targets the rats’ innate investigative and foraging tendency, thus making it a well-suited measure of goal-directed behaviour.

Training also required the completion of two simple, two-choice discrimination stages, one for medium (two similar smelling digging substrates with no added odour) and one for odour (homecage sawdust with two different added odours). The list of stimuli used for training will appear in the following section in table 2.2. The first four trials of each stage were considered “exploratory trials” and allowed the rat to “self-correct” by retrieving food from the correct bowl following an incorrect dig. An incorrect dig after these exploratory trials, resulted in the small panel being lowered to prevent entry to the correct digging chamber. Upon responding, response latency data was recorded along with whether it encountered only one or both bowls before initiating a digging response. For all discrimination learning stages, rats were given a total of ten minutes to respond before the trial was reset and a “non-dig” response was recorded. As response choice is of interest, “non-digs” were not included in the final computation and statistical analysis. The discrimination learning criterion was set at six consecutive

correct responses (chance  $p=0.0156$ ). Testing typically commenced within two days following training. Whilst there were several variants of the set-shifting task employed in this thesis, the “standard 7-stage task”, as first described in Birrell & Brown (2000) will be described here. Any task variations employed will be detailed in their respective experimental chapter.

#### 2.4.2. Testing on the ‘standard 7-stage task’

A typical testing session on the 7-stage task took between one to three hours to complete, depending on the rats’ satiety level, familiarity with the task, and interest in completing the task. The task itself presented a series of seven discrimination stages, including a combination of novel learning and reversal discriminations. The test started with a **simple discrimination (SD)** stage, which was similar to training and presented a pair of either odour (in home cage sawdust) or medium stimuli. The **compound discrimination (CD)** stage introduced a pair of irrelevant stimuli to the original SD pairing, which served as the irrelevant dimension, and a potential distractor to discrimination learning. The rats then completed a **CD-Reversal (Rev1)** stage, which retained the same stimuli from the CD, but rats now had to learn that the previously correct stimulus was now incorrect, since the correct and incorrect exemplars from the CD were reversed. The **intradimensional acquisition (ID)** stage introduced novel compound odour and medium stimuli, yet the relevant dimension did not change (*i.e.*, if odour stimuli predicted reward in the CD, then the novel odour stimuli in the ID would still predict reward). This was followed by an **intradimensional reversal (Rev2)** stage, in which akin to Rev1, rewarded the previously incorrect stimulus within the same perceptual dimension. The **extradimensional shift acquisition (ED)** stage once again introduced novel odour and medium stimuli, along with a change in the relevancy of dimension (*i.e.*, if odour predicted reward in the ID, then medium would now predict reward in the ED). A difference in trials to criterion between the ED and ID stage is referred to as the “shift cost”, and is an index of the cost of shifting from one dimension to the other during novel learning. The final stage was an **extradimensional reversal (Rev3)** stage, which rewarded the previously incorrect stimulus from the preceding ED stage.

Since there were a large number of possible combinations for odours and digging media, stimuli were arranged into pairs, which were then counterbalanced.



Counterbalancing attempted to control for order of presentation of stimulus pairings, shift type (*e.g.*, medium to odour or vice versa), and the initial correct stimulus within a pairing. The complete list of exemplars for the standard 7-stage task, along with the stimuli used for training are summarised in table 2.2. Additional stimuli used for variations to the 7-stage task will be detailed in their respective experimental chapters, along with modifications to the task design.

	<b>Media</b>		<b>Odours</b>	
Pair I	M1: Coarse tea	M2: Fine tea	O1: Cinnamon	O2: Ginger
Pair II	M3: Sand	M4: Grit	O3: Sage	O4: Paprika
Pair III	M5: Coarse shavings	M6: Fine shavings	O5: Turmeric	O6: Cloves
Training	M9: Polystyrene pieces	M10: Shredded paper	O9: Mint	O10: Oregano

Table 2.2: List of exemplar pairs used in the standard 7-stage task and training.

## **2.5 Histology**

At the end of behavioural testing, rats were terminally anaesthetised with a lethal dose of 0.8ml pentobarbital sodium (ip; JML pentobarbital, Andover, Hampshire, UK), and transcardially perfused with PBS (0.1M PB and sodium chloride in distilled water), followed by 4% paraformaldehyde in 0.1M PB. Brains were removed and refrigerated overnight at 4°C in 20% sucrose solution. The next day, brains were washed three times in distilled water then dried, and placed in wells for fixation in egg yolk. The wells were pre-treated with a spray lubricant (WD-40, San Diego, CA, USA) to aid in removal after the brains set. The brains were covered with egg yolk, then the gaps between the wells were filled with 40% formaldehyde and the yolk was left to set for at least five days. Brains were then sliced into 50µm sections with a freezing microtome (Jung Histoslide, 2000, Reichert-Jung, Cambridge Instruments, GmbH), subdividing tissue collection into eight collected sets, such that one section was collected every 400µm per set. Sections were stored in an anti-freeze solution (sucrose dissolved in 50% 0.2M PB and 40% ethylene glycol in distilled water) at -20°C, until staining for immunohistochemistry. All sections were double-stained for neuronal nuclei (NeuN) and enhanced with cresyl violet to visualise remaining cell bodies, and to map the extent of the lesion.

Staining for NeuN involved first ‘washing’ all sections in 9-hole net wells, which entailed immersion in 0.1M PBS, five times for three minutes each. Sections were then placed on a stirrer for 1 hour in blocking solution (20% normal goat serum, 0.1% triton in 0.1M PBS) and then washed again. Sections were then incubated in mouse anti-NeuN (1:4000; Chemicon International, Temecula, CA, USA) in antibody diluting solution (ADS: 0.1M PBS, 1% normal goat serum, 0.1% triton) and left on a stirrer overnight. The next day, sections were washed and incubated in vector IgG solution (anti-mouse IgG; 5µl/ml; Vector Laboratories Ltd., Peterborough, UK) on a stirrer for one hour. Sections were then washed again, and incubated in reagents A & B (10µl/ml each) of Vectastain ABC complex (Vector Laboratories Ltd., Peterborough, UK) for another hour. Sections were washed again and treated with Sigma Fast 3.3-Diaminobenzidine tablets (DAB: Sigma Chemical Company, St Louis, MO, USA) for approximately 10-15 minutes, until tissue and neuronal nuclei were stained with a dark-brown colour, with minimal background staining. Sections were then washed again and mounted on gelatine-subbed glass slides.

Slides were placed in a slide holder and stored overnight in a formaldehyde gas bath to fix the tissue samples to the gelatine-subbed slides. Sections were immersed in a bath of xylene, which was used as a clearing agent to de-fat tissue. Sections were then re-hydrated with ethanol, followed by 50% ethanol solution (in tap water), and finally tap water. Slides were submerged in cresyl violet solution (cresyl fast violet acetate, dissolved in distilled water and glacial acetic acid, pH adjusted to 3.5 with sodium acetate) for 2 minutes, and then left in a bath of running water for 5 minutes to clean the sections of excess dye. Sections were dehydrated with a 50% ethanol solution, followed by ethanol, and finally xylene, before slides were cover slipped with DPX mountant (BDH Laboratory Supplies, Poole, UK). Lesions to the STN were determined with light microscopy at 10x and 40x magnifications, looking for cell damage either in the form of calcification or lack of neuronal staining. Magnitude of the lesioned area was mapped onto standardised schematics of the rat brain with a stereotaxic atlas (Paxinos & Watson, 1998).

## **2.6 Statistical analysis**

All statistical analyses were computed in IBM SPSS v23 (IBM Corporation, CA, USA) and graphs designed in SigmaPlot v12 (Systat Software Inc., Chicago, USA). Data for trials to criterion, errors to criterion, latency to dig and response order (dig in first or second bowl) were collected. Trials to criterion and errors to criterion typically produces the same pattern in analysis, but trials to criterion yields a larger effect size, and thus increased statistical power (Tait & Brown, 2007; Field, 2009). Data for trials to criterion were analysed with repeated-measures analysis of variance (ANOVA), with specific factors and number of levels detailed in respective experimental chapters. Huynh-Feldt corrections were used when the within-subjects data violated the assumptions of sphericity. Where applicable, Bonferroni-corrected pairwise comparisons were calculated, which is slightly more conservative than Sidak's correction and does result in slight loss of power; however is more robust and better reduces the chances for a type I error (Field, 2009). Significant interactions from the omnibus ANOVA (restricting levels and factors of interest) were re-analysed as restricted analyses with further repeated-measures ANOVA. For the restricted analyses, the error term and degrees of freedom from the omnibus ANOVA were used, and the F-value and thus the p-value (criterion  $p < 0.05$ ) recomputed (Winer, 1971).

# Chapter 3

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## Exploring set-formation with the 11-stage task

### Background:

Recently published work demonstrated that lesions to the STN/ZI-area may impair the formation of attentional set on the 7-stage task, yet further work is needed. This chapter attempts to replicate this set-formation deficit in the 7-stage task, along with evaluate the effects of several manipulations sensitive to the inference of set-formation in the recently designed 11-stage task.

### Methods:

20 rats (12 lesion; 8 control) were tested twice on the 7-stage task, and once on the 11-stage task.

### Results:

Despite histology revealing predominantly unilateral lesion damage, lesioned rats did not demonstrate a cost for shifting set. However control rats also failed to demonstrate a convincing ID-ED difference.

### Conclusions:

It is likely that the amount of intact tissue supported function, leading to the inability to detect a robust behavioural deficit between groups on both the 7-stage and the 11-stage tasks.

### **3.1 Introduction**

In a recently published paper, it was suggested that ibotenic acid lesions to the STN/ZI area may disrupt the formation of attentional set: the expected difference between acquisition at the ED compared to the ID stage of the standard 7-stage test was not seen (Tait, Phillips, Blackwell, & Brown, 2016). Tait *et al.* (2016) also observed an increase in TTC at the early stages (*i.e.*, SD, CD, Rev1 and ID) of the task raising a possibility that there might be a learning impairment masking evidence of an attentional set or perhaps impairing set formation. In subsequent (unpublished) experiments, we have not replicated this increase in TTC at the early stages, although there still appeared to be no ID-ED difference in STN-lesioned rats (Dhawan, Xia, Tait & Brown, *unpublished observations*). Furthermore, testing STN-lesioned rats on multiple ID stages (the 4ID task; adapted from Clarke *et al.*, 2005 and Bissonette *et al.*, 2008) did not result in an ID-ED difference, but there were no impairments at any other stage of testing (Xia, Dhawan, Tait & Brown, *unpublished observations*; Xia, 2014, *University of St Andrews PhD Thesis*).

There are multiple reasons why an attentional set might not form and a lesion of the STN/ZI-area is not the only manipulation that has been associated with set-formation impairments. For example, rats with OFC lesions, with large impairments in the reversal learning stages of the attentional set-shifting task, also fail to exhibit a cost of shifting an attentional set in the standard 7-stage task (Chase, Tait, & Brown, 2012; McAlonan & Brown, 2003). However, when the same OFC-lesioned rats were tested in the 4ID task, omitting reversal stages, they not only formed an attentional set, but had an increased shift cost compared to control rats (Chase *et al.*, 2012). Chase *et al.* (2012) interpreted the increased shift-cost in the 4ID task as another manifestation of the deficit causing a reduced shift-cost in the 7-stage task: namely, slowed set-formation. Although set was slow to form in the 7-stage task, once it had formed after 4IDs, when a shift was required at the ED stage, a new set also formed more slowly. Rats with quinolinic acid lesions of the DMS may also have an impairment in the formation of set: they had no cost of shifting set in either the 7-stage task or in the 4ID task (Lindgren, Wickens, Tait, Brown, & Dunnett, 2013; but see also Tait *et al.*, 2016 who reported ibotenic acid lesions of the DMS resulted in reversal learning impairments but normal set formation). These mixed results suggest different

neural and psychological mechanisms may result in behavioural evidence of impaired set-formation. The aim of this chapter is to explore these possible mechanisms.

A previous attempt to investigate the nature of the set-formation impairment in STN-lesioned rats was, unfortunately, unsuccessful due to a failure of histological confirmation of the lesion damage (Xia, 2014, *unpublished PhD Thesis*). Nevertheless, there were behavioural effects seen in the group receiving ibotenic acid infusions that were not seen in the control group, consistent with a failure to form set in accordance with the hypothesis. In that study, Xia developed an 11-stage task which introduced manipulations to test a number of hypotheses, as outlined below:

1. *Would additional experience result in formation of set?* Although 4 ID stages did not appear to be sufficient for set formation, it is possible that yet further experience might. Therefore, additional stages were included prior to the critical ED stage: in the 7-stage task, there are four stages (2 acquisition (CD and ID) and 2 reversals) and in the 4ID task, there are four acquisition stages prior to the ED. In the 11-stage task, there are 7 stages (5 acquisition and 2 reversals) prior to the ED.
2. *Is there a memory impairment?* If a rat is unable to remember previously rewarded stimuli, it would not be expected to focus attention differentially at the ED stage and there would be no ID/ED difference. There would also be no reversal cost if intervening discriminations caused a rat to forget prior stimuli. Therefore, reversal learning was tested twice, with either one or three novel acquisition stages inserted between the ID and its reversal. If memory for reward-relevant exemplars were disrupted by new stimuli, the reversals would be treated as if they were new learning. More (3 vs 1) intervening stages might also result in an even greater impairment
3. *Is the rat able to differentially attend to the rewarded (and hence not attend to the unrewarded) dimension?* A ‘probe’ stage, in which the exemplars in the relevant (and therefore, attended) dimension were maintained, but the exemplars in the irrelevant (and therefore unattended) dimension were changed. If the rat is attending selectively, the change should not disrupt performance, either because they simply do not notice the

change in the irrelevant dimension or because they ignore it, having learned that the information is irrelevant (Tait & Brown, 2007).

4. *Are the lesioned rats learning the stimuli as configurations, rather than attending to stimulus dimensions?* If the rats are solving the task by treating each of the four bowls as distinct stimuli, each being either baited or not baited, rather than perceiving that reward is predicted by the common exemplar in a perceptual dimension, then acquisition at the ID and ED stages would be equally difficult. However, a bi-conditional stage, where reward is signalled by both stimulus dimensions (*i.e.*, if odour A, then medium B), should be more readily solved by animals who are learning the stimulus configurations compared to those who have had experience in attending to only one of the stimulus dimensions at a time.

The present chapter aims to (i) replicate previous observations that lesions of the STN/ZI impair the formation of attentional set and (ii) extend the observation to demonstrate the nature of set-formation impairments and identify an underlying mechanism.

## **3.2 Methods**

### **3.2.1 Animals**

Twenty male, Lister hooded rats (Charles River, UK) were used in this experiment, with twelve rats in the lesion group, and eight rats in the control group. Rats had a mean weight of 405g (range: 358-445g) at the start of the experiment. At completion of the experiment rats had a mean weight of 515g (range: 348-573g). All rats were experimentally naïve prior to testing and husbandry conditions and housing details are described in the General Methods in Chapter 2.

### **3.2.2 Apparatus**

The attentional set-shifting apparatus is described in the Chapter 2 (General Methods), section 2.2.

### **3.2.3 Surgery**

Ibotenic acid lesion surgery procedures are described in Chapter 2 (General Methods; see section 2.3.3).

### **3.2.4 Behavioural training**

See section 2.4.1 of the General Methods, Chapter 2.

### **3.2.5 Behavioural testing**

Testing, which was carried out with the help of an undergraduate volunteer, was conducted over 14 weeks, starting 2 weeks after surgery. Study I consisted of two test sessions on the 7-stage task, conducted over a period of 9 weeks, with not less than 4 weeks between each test. Study II tested rats on the 11-stage task, completed over a period of 5 weeks.

#### *3.2.5.1 Study I: The 7-stage task*

Rats were tested twice on the 7-stage task, with order of exemplar pairing presentation and shift direction counterbalanced between tests. For a detailed explanation of



the stages and list of stimuli, see section 2.4.2 of the General Methods, Chapter 2. The procedure is summarised in Table 3.1.

<b>Discrimination</b>	<b>Relevant dimension</b>	<b>Discriminanda</b>	<b>Mixed with</b>
Simple Discrimination (SD)	Medium	<i>Coarse tea</i> , not fine tea	<i>Nothing</i>
Compound Discrimination (CD)	Medium	<i>Coarse tea</i> , not fine tea	Cinnamon or ginger
Compound Reversal (Rev1)	Medium	<i>Fine tea</i> , not coarse tea	Cinnamon or ginger
Intradimensional Acquisition (ID)	Medium	<i>Sand</i> , not grit	Sage or paprika
Intradimensional Reversal (Rev2)	Medium	<i>Grit</i> , not sand	Sage or paprika
Extradimensional Shift Acquisition (ED)	Odour	<i>Turmeric</i> , not cloves	Coarse shavings or fine shavings
Extradimensional Reversal (Rev3)	Odour	<i>Cloves</i> , not turmeric	Coarse shavings or fine shavings

*Table 3.1: An example of a test session including typical exemplar order, shift type, and stimuli used in the 7-stage task. Testing was counterbalanced for initial correct exemplars and for shift direction (odour-medium or medium-odour) between tests.*

### *3.2.5.2. Study II: The 11-stage task*

Because the 11-stage task has a large number of novel discrimination stages, a total of eight novel stimulus pairings were required, as shown in table 3.2. Although the media-odour pairings were constant, the order of their use and the correct exemplar within the pairing was counterbalanced within and between groups.

	<b>Media</b>		<b>Odours</b>	
Pair I	M1: Coarse tea	M2: Fine tea	O1: Cinnamon	O2: Ginger
Pair II	M3: Sand	M4: Grit	O3: Sage	O4: Paprika
Pair III	M5: Coarse shavings	M6: Fine shavings	O5: Turmeric	O6: Cloves
Pair IV	M7: Cotton pads	M8: Cigarette filters	O7: Dill	O8: Coriander
Pair V	M11: Coarse cork	M12: Fine cork	O11: Fenugreek	O12: Tarragon
Pair VI	M13: Long wire coat	M14: Short wire coat	M13: Cumin	M14: Marjoram
Pair VII	M15: Beads	M16: Gravel	O15: Thyme	O16: Caraway seeds
Probe	M17: String	M18: String knots	O17: Fennel seeds	O18: Chives

*Table 3.2: List of exemplar pairs in the modified 11-stage task and bi-conditional stage, which added five novel stimulus pairs to the 7-stage task stimuli*

The task started with a compound discrimination (**CD**) stage, which was then succeeded by two intradimensional acquisition stages (**ID1** and **ID2**, respectively). The ID2 stage was followed by a delayed (one intervening stage) ID1 reversal (**ID1R**), which was then succeeded by two further intradimensional acquisition stages (**ID3** and **ID4**, respectively). The ID4 stage was followed by a delayed (three intervening stages) ID2 reversal (**ID2R**), which was then succeeded by the final intradimensional acquisition stage (**ID5**). During the probe stage (**ID5p**), the reward-relevant exemplars from ID5 remained rewarded, and the stimuli from the irrelevant dimension were replaced with novel stimuli. Novel stimuli were reserved for the probe stage, and did not appear at any other stage of testing. The rats were then given an extradimensional (**ED**) shift acquisition stage, followed by a simple discrimination (**SD**) using the reward-relevant stimuli from the preceding ED stage. The purpose of the final SD stage was to validate that change in performance at this point in the task was not due to satiety or fatigue. To avoid satiety, rats were tested the following day on the **bi-conditional** discrimination. In this test, both stimulus dimensions were relevant in cuing the baited bowl. Attending to only one of the two dimensions would make learning impossible: that is to say, because the rule was bi-conditional (of the form “if sand, then sage, but if grit, then paprika”), failing to process the non-discriminable dimension (*e.g.*, whether both bowls contained either sand or grit) would prevent

determination of which odour was baited. The solution is more straightforward if the subject is able to overcome the tendency to process the stimuli as multi-dimensional and rather treats each bowl as an individual configuration that may/may not be baited: for example, sand+sage and grit+paprika are baited, while sand+paprika and grit+sage are not. Rats from the lesion and control groups were matched and paired for counter-balancing, which controlled for order of presentation of stimulus pairings, shift type (*e.g.*, medium to odour or vice versa), and the initial correct stimulus within a pairing.

The bi-conditional task rewarded two stimulus configurations (*e.g.*, O1/M1 and O2/M2) whilst the other two stimulus configurations were incorrect (*e.g.*, O2/M1 and O1/M2). During any given bi-conditional discrimination trial, the bowls differed on only one dimension [*e.g.*, O1/M1 could be paired with either O2/M1 (same medium) or O1/M2 (same odour)]. For a summary of the procedure of the 11-stage task, including the bi-conditional stage, see table 3.3.

### **3.2.6 Repeat testing**

Completion of multiple testing sessions followed a within-subjects protocol, such that all rats completed two tests on the 7-stage task, followed by one test on the 11-stage task. It has been demonstrated that pre-exposure of stimuli is not required for multiple test sessions on the 7-stage task, and that the learning effect (patterns of difference for the shift-cost and evidence of normal reversal learning) are sustained between tests, even after short (*e.g.*, 24-hour) inter-test intervals (Chase *et al.*, 2012; Tait, Marston, Shahid, & Brown, 2009; Wallace, Marston, McQuade, & Gartside, 2014); however, this is not to suggest that performance should be considered identical between test sessions. For this reason, the repeat-test was counterbalanced between and within groups for shift-type, and furthermore all stimuli were associated with reward at some point during each test, either at an acquisition stage or when the acquisition was reversed. As such, it was not deemed necessary to re-expose rats to stimuli between each of the 7-stage tests or prior to testing on the 11-stage task.

### **3.2.7 Data analysis**

Study I was a 2x2x7 mixed factorial design, and TTC data were analysed by repeated-measures ANOVA. There was a within-subjects factor of test (two levels: test 1 and test 2), a within-subjects factor of stage (seven levels) and a between-subjects factor of group (two levels: control or lesion). A restricted analysis with Bonferroni-corrected pairwise comparison was used to analyse the shift-cost between the ID and ED stages. Study II utilised a 2x11 mixed factorial design, with a within-subjects factor of stage (eleven levels) and a between-subjects factor of group (two levels: control or lesion). Restricted analyses investigating factors of interest were conducted to examine the effects of key manipulations, with F-values corrected using error terms from the omnibus ANOVA (Winer, 1971). Trials to criterion data for the bi-conditional discrimination stage were analysed by one-way ANOVA.

### **3.2.8 Histology**

NeuN/Cresyl violet immunohistochemistry procedures are described in Chapter 2 (General Methods; see section 2.5).

<b>Discimination</b>	<b>Relevant dimension</b>	<b>Discriminanda</b>	<b>Mixed with</b>
Compound Discrimination (CD)	Medium	<i>Coarse tea</i> , not fine tea	Cinnamon or ginger
Intradimensional Acquisition I (ID1)	Medium	<i>Sand</i> , not grit	Sage or paprika
Intradimensional Acquisition II (ID2)	Medium	<i>Coarse shavings</i> , not fine shavings	Turmeric or cloves
Intradimensional Reversal I (ID1R)	Medium	<i>Grit</i> , not sand	Sage or paprika
Intradimensional Acquisition III (ID3)	Medium	<i>Cotton pads</i> , not cigarette filters	Dill or coriander
Intradimensional Acquisition IV (ID4)	Medium	<i>Coarse cork</i> , not fine cork	Fenugreek or tarragon
Intradimensional Reversal II (ID2R)	Medium	<i>Fine shavings</i> , not coarse shavings	Turmeric or cloves
Intradimensional Acquisition V (ID5)	Medium	<i>Long wire coat</i> , not short wire coat	Cumin or marjoram
Probe	Medium	<i>Long wire coat</i> , not short wire coat	Fennel seeds or chives
Extradimensional Shift (ED)	Odour	<i>Thyme</i> , not carroway seeds	Beads or gravel
Simple discrimination (SD)	Odour	<i>Thyme</i> , not carroway seeds	<i>Nothing</i>
		Correct combination	Incorrect combination
Bi-conditional (Bi-Con)	N/A	<i>Coarse tea with cinnamon &amp; Fine tea with ginger</i>	Coarse tea with ginger & Fine tea with cinnamon

Table 3.3: An example of the exemplar order, progression of stages, and shift type in a typical test session on the 11-stage task and the bi-condition stage.

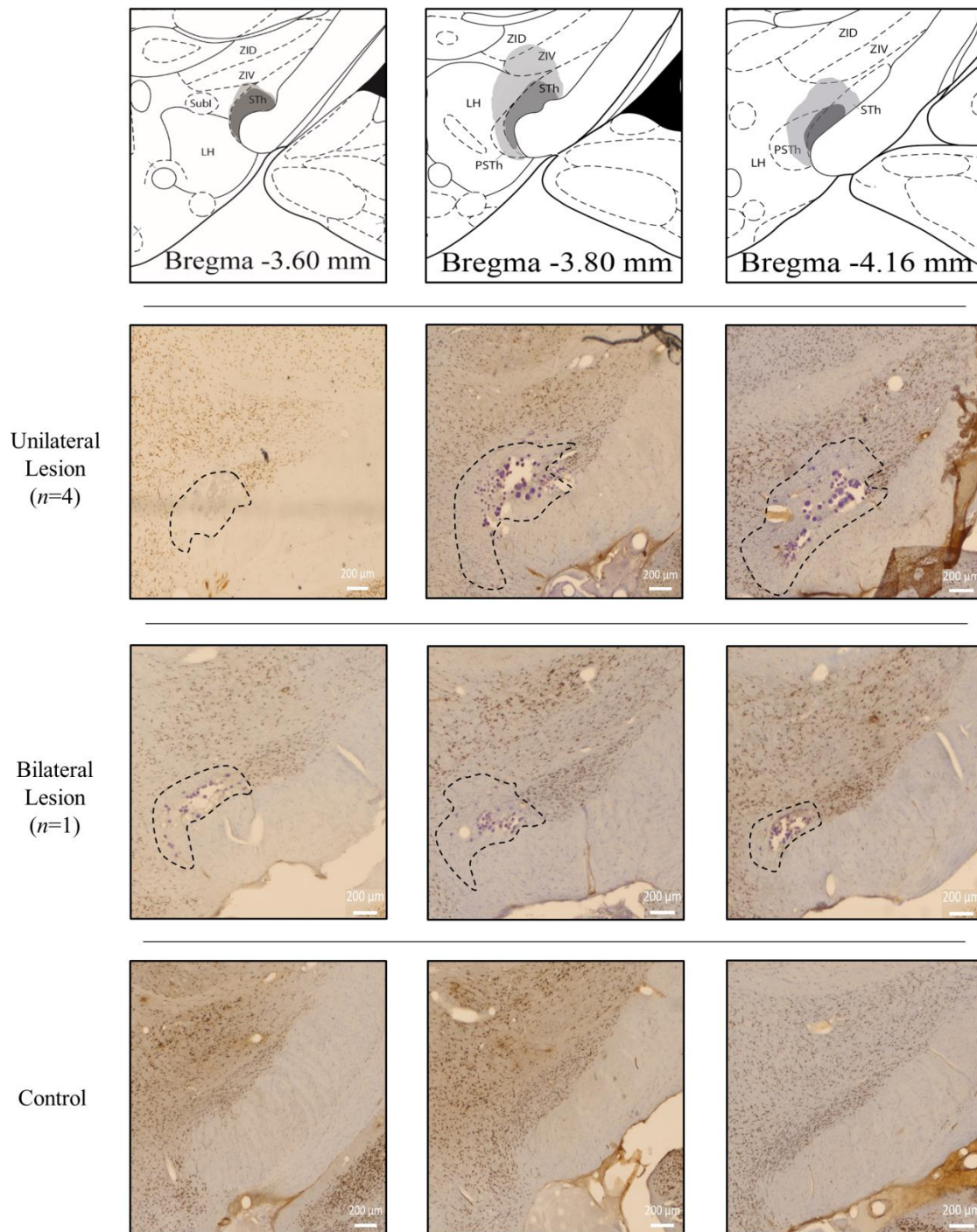
## **3.3 Results**

### **3.3.1 Histology**

One rat from the lesioned group failed to recover post-operatively. One control rat was euthanized between Study I and II, due to the onset of spontaneous seizures. The behaviour for the control rats was not atypical in Study I and therefore his data are included. Consequently, the final group sizes were: lesion:  $n=11$ ; control: Study I,  $n=8$ ; Study II,  $n=7$ .

Lesion extent and cell loss was determined by NeuN/Cresyl Violet double staining. Of the eleven rats in the lesion group, only five rats had discernible lesions of the STN, four of which were only unilateral. Cell-loss in the STN was restricted to the medial portion and extended dorsally into the ventral zona incerta (ZIV) in 3 cases, and included the dorsal zona incerta (ZID) in one case. Figure 3.1 presents schematics of lesion extent, along with photomicrographs of cell-loss found in the bilaterally-lesioned rat, 'typical' cell-loss found amongst unilaterally lesioned rats, and photomicrographs of a control brain.

The remaining six rats from the original 'lesion' group retained a visibly intact STN, despite all eleven rats in this group exhibiting the hallmark 'chewing' behaviour (see Section 2.3.3) during recovery from surgery, along with evidence of calcium deposits in the MGP. Given that these rats did not present with signs of cell-loss in the STN or ZI, these rats were removed prior to statistical analysis reducing the final group size for lesioned rats to five (4 with unilateral lesions; 1 with bilateral lesions).



*Figure 3.1: Schematics and microphotographs of brain sections (NeuN/Cresyl Violet). Top to bottom: schematics illustrate the minimum (dark grey) and maximum (light grey) extent of the lesion; photomicrographs of the ‘typical’ unilateral lesion and the bilaterally-lesioned rat (hashed outline indicates lesion extent); photomicrographs of a control brain. Sth: subthalamic nucleus; ZIV: ventral zona incerta; ZID: dorsal zona incerta; Subl: subincertal nucleus; LH: lateral hypothalamic area; PStH: posterior subthalamic area*

### 3.3.2 Study I: Examining set-formation with the 7-stage task

#### 3.3.2.1 Overall findings

Overall task performance improved between the two testing sessions (confirmed by a main effect of *Test*:  $F_{(1,10)} = 6.16, p < 0.05; \eta^2 = 0.32$ ), and that unexpectedly, the overall performance for all rats on the ID, Rev2, and ED stages improved between test sessions, an effect which may be exacerbated by small, and unequal group sample sizes (*Test by Stage interaction*:  $F_{(5,35,53,46)} = 2.53, p < 0.05; \eta^2 = 0.20$ ; *Huynh-Feldt correction; uncorrected df = 6,60; Bonferroni-corrected pairwise comparison*; see figure 3.2). It was also found that performance did not differ between various stages of the task (main effect of *Stage*:  $F_{(5,18,51,83)} = 1.01, n.s.; \eta^2 = 0.09$ ; *Huynh-Feldt correction*), and that therefore, the expected ID/ED difference was not statistically reliable (Bonferroni-corrected comparison). Furthermore, there were no differences between the control and lesion groups (either bilateral or unilateral), either overall or by Stage or by Test (main effect of *Group*:  $F_{(2,10)} = 2.04, n.s.; \eta^2 = 0.29$ ; *Stage by Group interaction*:  $F_{(10,37,51,83)} = 1.55, n.s.; \eta^2 = 0.24$  *uncorrected df = 12,60; Test by Group*  $F_{(2,10)} = < 1, n.s.; \eta^2 < 0.14$ ).



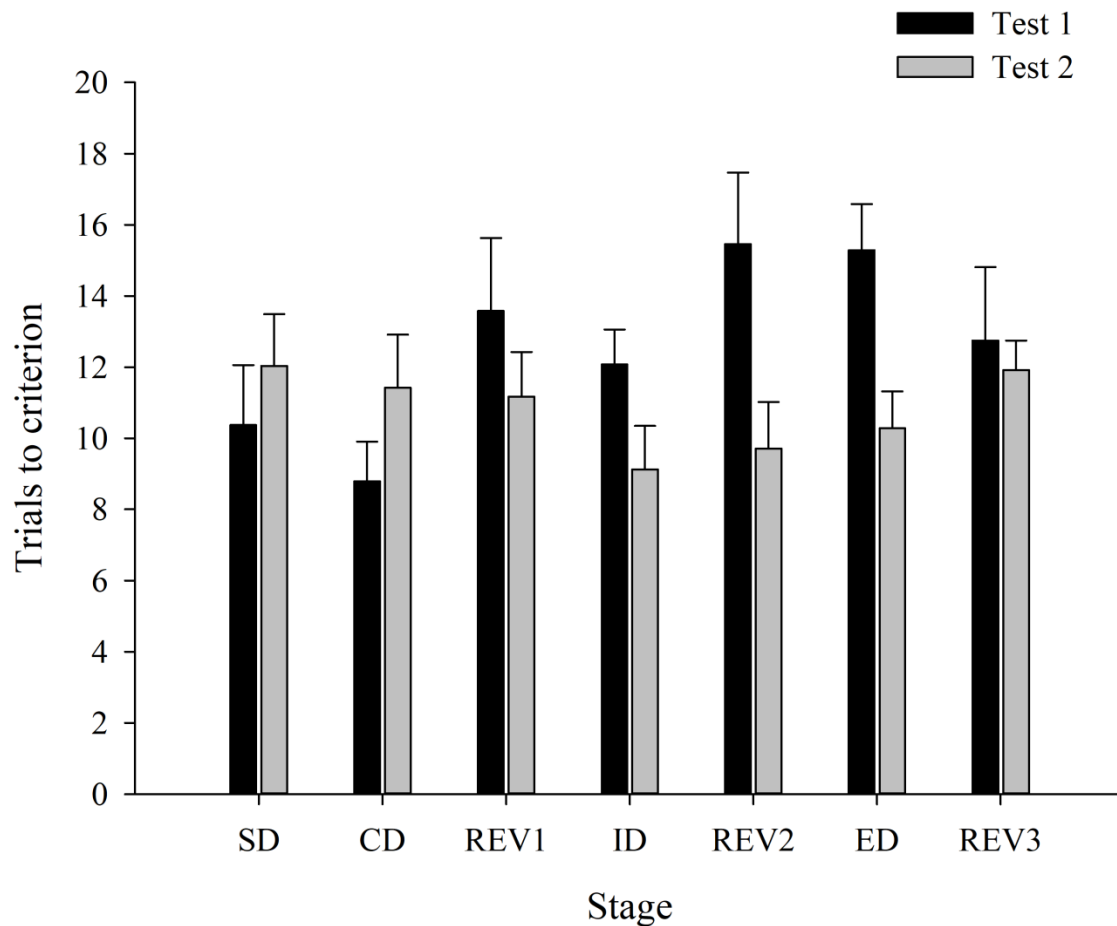


Figure 3.2: Mean TTC performance (+SEM) for all rats separated by test session; TTC performance globally improved between test sessions, particularly at the ID, Rev2, & ED stages.

### 3.3.2.2 Investigating shift cost: ID vs ED stages

Notwithstanding the lack of a significant difference overall between stages, including the ID and the ED stages, or an interaction with lesion groups, nevertheless 7/8 (88%) of the control rats, compared to only 3/5 (60%) of the lesioned rats, had a positive shift cost (see figure 3.3) This observation suggests that there is some evidence for an attentional set in control rats at least. For the lesioned rats, there is not enough evidence to draw a conclusion either way, owing largely to a very small group size: although there was no significant difference between ED and ID, that fact that the difference was also not

significant for the control group makes it unsafe to conclude that the lesion had resulted in a deficit in the formation of set.

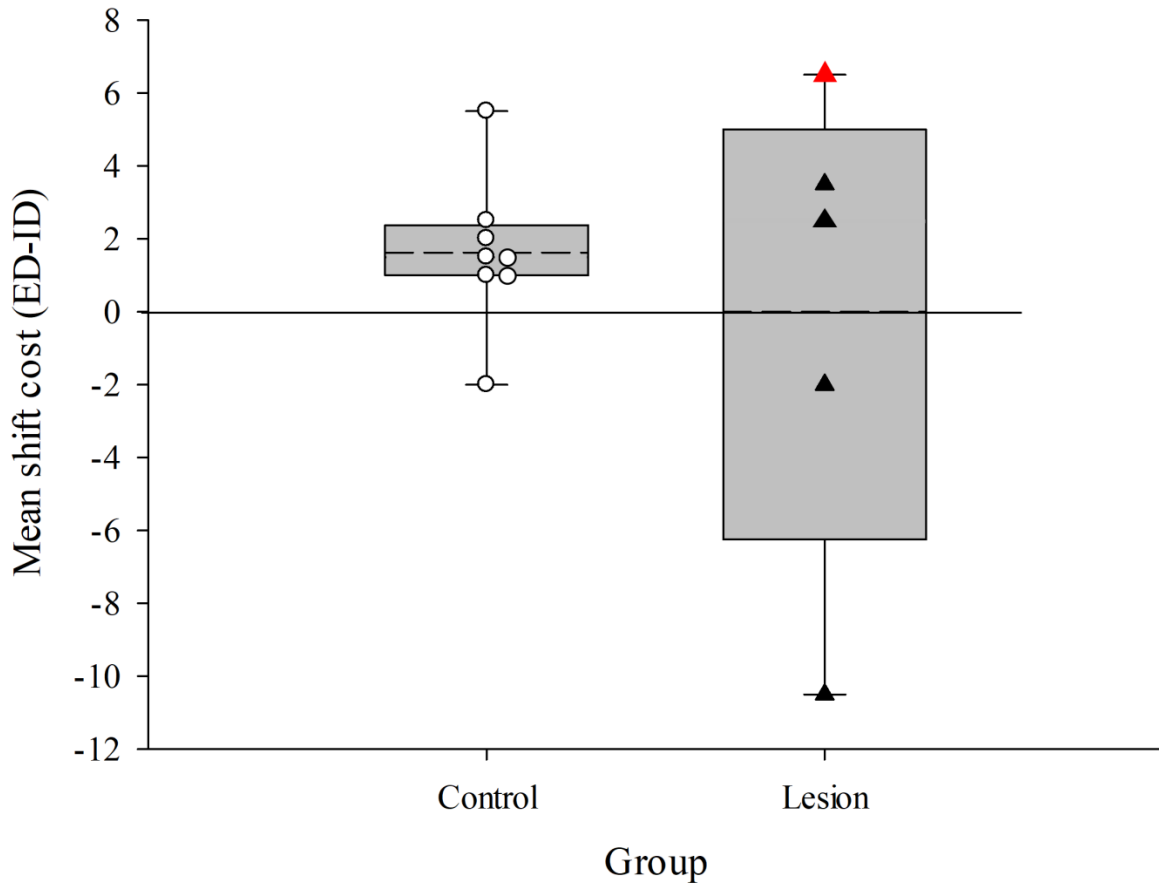


Figure 3.3: Boxplot illustrating the mean shift costs (averaged across the two testing sessions), with hashed lines indicating the mean of the shift cost by group (lesioned rats had a mean cost of zero); superimposed vertical point plot depicting the distribution of the data for each rat, with the red triangle illustrating the shift-cost for the bilaterally-lesioned rat. Shift cost was calculated by computing the difference between the ID and ED stages (ED-ID). Only one control rat failed to exhibit a positive difference between stages.

### 3.3.3 Study II: Investigating set-formation with the 11-stage task

#### 3.3.3.1 Overall findings

As expected, TTC differed as a function of stage (*Stage*:  $F_{(10,90)} = 4.66$ ,  $p < 0.01$ ;  $np^2 = 0.34$ ), but this did not differ as a function of lesion group (*Stage by Group*:  $F_{(20,90)} = 1.27$ , *n.s.*;  $np^2 = 0.22$ ; figure 3.4). Based on this finding, it was parsimonious to conclude

that there was no detectable difference in performance between any of the groups, for any of the manipulations of the 11-stage task, including across multiple ID stages, and at the probe stage. Despite the visually increased TTC at the CD for lesioned rats, this comparison was also non-significant, marked with high variance. Furthermore, 2 of the 3 lesioned rats (including the 1 bilateral rat) that presented with elevated TTC at the CD ( $>17$  TTC) also demonstrated a positive shift cost (*i.e.*, evidence of an attentional set) suggesting that it was not the case that a sub-set of rats were impaired.

There was a significant difference between the groups on the bi-conditional discrimination when the data for lesioned rats was separated into bilateral and unilateral damage (*Group*:  $F_{(2,11)} = 5.36$ ,  $p < 0.05$ ). This effect was largely driven by the data for the lone bilateral-lesioned rat, whose TTC performance was significantly higher than both unilateral lesioned rats and controls. Unfortunately, no conclusions can be drawn from the performance of a single rat, and that when the data for this one rat is combined with the remaining (unilaterally) lesioned animals, the effect of group is lost (*Group*:  $F_{(1,110)} = <1$ , *n.s.*). Irrespective of group, 3/12 (25%) of rats required more TTC in the bi-conditional discrimination compared to any other stage, including the ED, suggesting that for some rats, this was a particularly difficult discrimination to learn. The fact that they did all, eventually, learn the discrimination (the maximum number of TTC required amongst rats was 24) suggested that notwithstanding experience of solving the task by selectively attending to the odour or the medium at different points in the task, they were able to overcome this selective attention.

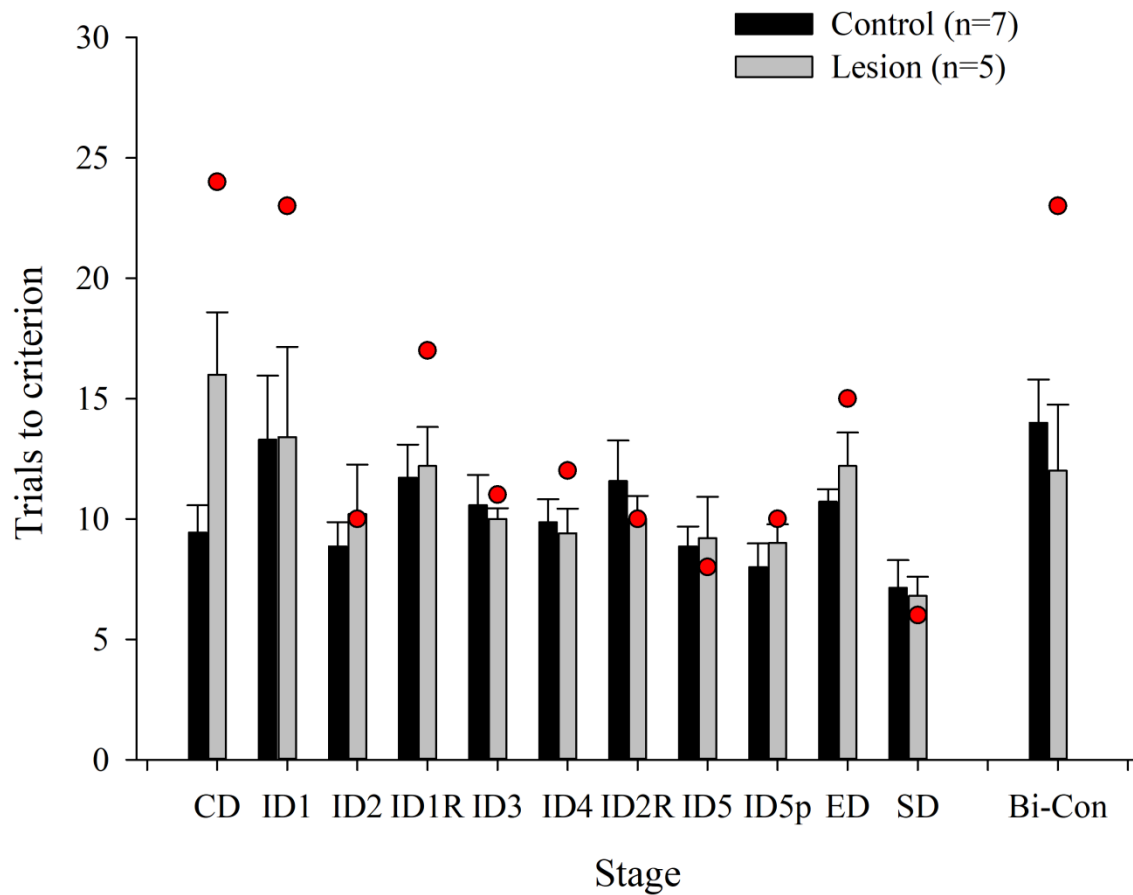
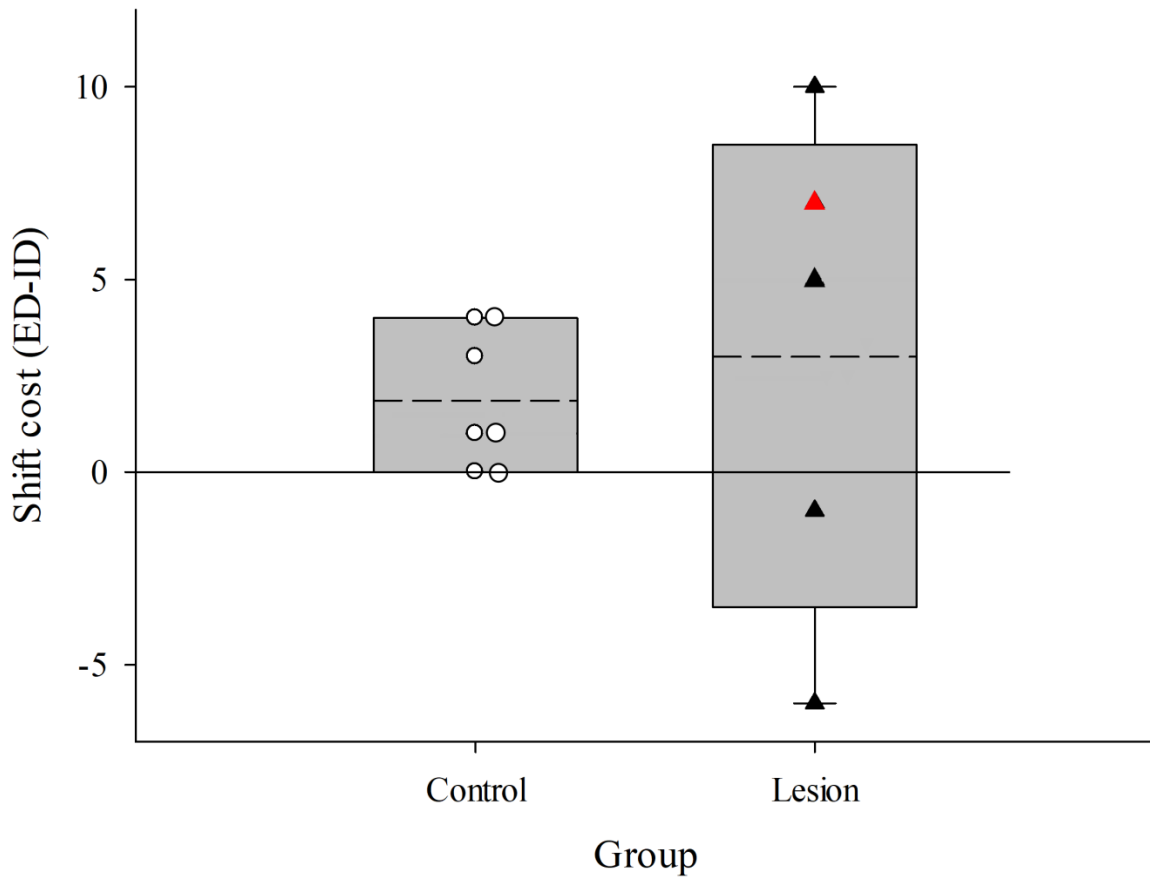


Figure 3.4: Mean TTC performance (+SEM) for lesioned rats and sham-operated control rats on the 11-stage task plus the bi-conditional discrimination; red dots depict the performance for the sole bilaterally lesioned rat.

### 3.3.3.2 Investigating shift cost: ID5 vs ED

Similar to the ID-ED comparison in Study I, despite the lack of a significant difference overall between the ID5 and the ED stage, or an interaction with lesion group, 5/7 (71%) of the control rats had a positive shift cost (the remaining 2 rats acquired the two stages in an equal number of trials) whereas only 3/5 (60%) of the lesioned rats did (see figure 3.5). The conclusion regarding set-shifting in the current study was similar to the one drawn for Study I, namely that the findings present mixed evidence for an attentional set in control rats, and that there is not enough evidence to conclude the formation of set, nor enough evidence to refute its formation, in the rats in the lesion group, largely owing to sample size, and type of lesion.



*Figure 3.5: Boxplot illustrating shift cost, with hashed lines indicating the mean shift cost for rats by group; superimposed vertical point plot depicting the distribution of the data for each rat, with the red triangle illustrating the shift-cost for the bilaterally-lesioned rat; five of the seven control rats evidenced a positive difference in this comparison, whilst the distribution of the data for the lesioned rats illustrates marked variance.*

### **3.4 Discussion**

The first study in this experimental chapter attempted to replicate previous observations that STN/ZI-lesions impair the formation of attentional set. After two sessions of testing on the 7-stage task, lesioned rats did not demonstrate a cost of shifting attentional set. Unfortunately, sham-operated control rats also failed to demonstrate a convincing ID-ED difference and there was no statistical support for group differences in set formation. The most parsimonious conclusion for the first study of this experiment would be that control rats did not present sufficiently robust evidence of set formation and that whilst STN lesions may have disrupted set formation, there is no convincing evidence of absence, owing to a small sample size.

Despite the mixed evidence from the first study, it has been shown that control rats that have failed to demonstrate an ID-ED difference in the 7-stage task benefit from multiple ID stages in a subsequent task – facilitating a significant cost of set-shifting (Lindgren *et al.*, 2013). Therefore, the second study of this experimental chapter presented multiple ID stages, whilst investigating the mechanisms of set-formation using the 11-stage task with the same cohort of rats. The result of the ID5-ED comparison in Study II was similar to the ID-ED comparison from Study I: the study failed to demonstrate robust and statistically-supported evidence of a cost of set-shifting in controls and compromised performance in lesioned rats. Overall, performance for lesioned rats did not differ from control rats, which included performance on the multiple ID stages, reversal stages, the probe, and the bi-conditional discrimination. The lack of clear behavioural effects is most likely due to a lack of consistent lesion damage, in both size/extent and symmetry, which ultimately led to a loss of statistical power owing to a reduced group size (n=5).

#### **3.4.1 Limitations and considerations**

The histology for the ‘lesioned’ group indicated marked asymmetry. Lesions were only discernible at all in five rats, of which only one had clear bilateral damage; the remaining four rats had only unilateral discernible lesions. Another large concern pertained to the consistency in achieving damage to cells in the STN; over half of the rats that received ibotenic acid infusions (n=6) expressed ‘indeterminate’ lesion damage. These rats

were not ‘intact’, in that they had calcium deposits in the MGP (which we have previously reported to accompany subthalamic cell loss; Phillips and Brown, 1999; Xia, Dhawan, Tait & Brown, *unpublished*), along with postoperative chewing. Nevertheless, there was no evidence of cell loss in the STN or ZI of these rats. It is highly likely that the failure to detect a robust behavioural deficit in the lesion group is predominantly due to a small sample size, but also due to the nature of the lesion damage being largely unilateral, and therefore permitting sufficient intact tissue to support function.

#### *3.4.1.1 Unilateral vs bilateral inactivation*

Considerable research from obtained from studies with Parkinson’s patients has detailed a significant difference between unilateral and bilateral approaches to HFS for treatment of motor symptoms. For example, bilateral HFS provides greater improvements in motor functioning than unilateral stimulation (Bastian, Kelly, Revilla, Perlmutter, & Mink, 2003; Goelz *et al.*, 2016; Kumar *et al.*, 1999), but induces cognitive decline not found in unilateral stimulation (Amara *et al.*, 2012), including deterioration in non-verbal recall performance (Williams *et al.*, 2011) along with impaired verbal fluency and trouble inhibiting dominant verbal responses on the Stroop test (York *et al.*, 2008). Furthermore, bilateral HFS – and not unilateral HFS – in Parkinson’s patients, impairs cognition on the “*n*-back task” (see Owen *et al.*, 2005), which tests working memory, encoding and updating (Alberts *et al.*, 2008). Cognitive performance for unilaterally-stimulated PD patients did not differ from patients without stimulation (Alberts *et al.*, 2008), highlighting the importance of bilateral STN inactivation in generating cognitive impairment. This consideration may have contributed to the result found in this chapter; given that of the majority of the lesioned rats presented with unilateral damage (4 of the 5 rats), it is possible that sufficient damage was not incurred in order to evidence a cognitive deficit.

In their study, Alberts *et al.* (2008) postulated that the increased activation in the dorsolateral prefrontal cortex (dlPFC) observed during performance of working memory tasks, such as the *n*-back task (see Jansma, Ramsey, Coppola, & Kahn, 2000; Owen, McMillan, Laird, & Bullmore, 2005) might be disrupted during STN-HFS, owing to the established fronto-subthalamo projections (see: Nambu, Tokuno, & Takada, 2002). It is possible that disruption of information processing and response selection in parts of the

STN which contribute to cognitive impairments perhaps by incorrectly shaping output activity (Alberts *et al.*, 2008). This consideration is also consistent with research in rats, in which the observed cognitive deficits produced by bilateral lesions to the STN could largely stem from the disruption of the STN–medial prefrontal cortex (mPFC) circuit (Chudasama, Baunez, & Robbins, 2003). Disconnection lesions of the mPFC–STN (*i.e.*, lesioning the STN and mPFC unilaterally, but in opposite hemispheres) considerably impaired rats’ discriminative accuracy and also increased perseverative responding and response latencies in the 5CSRTT; moreover this functional deficit was remarkably similar to the impairment seen after bilateral STN lesions (Chudasama *et al.*, 2003). Conversely, unilateral STN lesions induced only a mild deficit, slightly increasing both premature and perseverative responses compared to sham-operated animals, without affecting discriminative accuracy and response latency. Chudasama *et al.* (2003) thus concluded that the cognitive deficits – particularly those of attention and executive function – exhibited by bilateral STN lesioning depend upon the cortico-subthalamic projection, and additionally their research implicitly illustrates the importance of achieving bilateral STN lesions when examining the role of the STN in cognition. The fact that accuracy and latency were unaffected following unilateral lesions also weakens that position that unilateral lesions may induce an attentional impairment, which further suggests that perhaps bilateral lesions are mandatory to obtain cognitive deficits.

Cognitive research, in which lesions to the rat STN are made, predominantly employ bilateral lesions to produce behavioural deficits (See Baunez & Lardeux, 2011; Jahanshahi *et al.*, 2014). Unilateral lesioning has been used in animal studies to dissociate generalised motor effects of striatal dopamine depletion (*e.g.*, hyperkinesia) from response-specific initiation effects, by comparing ipsilateral vs contralateral motor performance biases (Phillips & Brown, 1999; see section 1.8.1), whilst consequently serving as a means to evaluate the quantitative differences between unilateral and bilateral lesioning. Rats with unilateral lesions of the STN in Phillips & Brown (1999) did not exhibit impairments in accuracy, omissions, or perseverative responding in nose-poke visuo-spatial discrimination tasks, as typically expected of rats with bilateral lesions to the STN in similar tasks (Baunez *et al.*, 1995; Baunez & Robbins, 1997; 1999), however they do exhibit increased premature



(anticipatory) responses. The present evidence regarding the effects of bilateral lesions on latency performance in visuo-spatial reaction time tasks is mixed, with evidence that lesioning both ‘speeds up’ (Baunez *et al.*, 1995), and ‘slows down’ (Baunez & Robbins, 1997; 1999) reaction-time performance; in Phillips & Brown (1999), the unilaterally-lesioned animals took longer to respond, however this effect was to the ipsilateral side only. Phillips & Brown (1999) replicated their findings in a subsequent study (Phillips & Brown, 2000), and found that unilateral STN lesions do not change reaction times, nor impair accuracy or error rate in a nose-poke reaction time task, but that unilateral lesions increase the likelihood of premature responses, compared with control surgery. The above evidence – from both clinical populations, and experimental animals – suggests that there is a qualitative and quantitative difference between unilateral stimulation/lesioning of the STN and bilateral. In humans, bilateral HFS for Parkinson’s patients may lead to more reliable improvements in motor dysfunction, but concurrently introduces more severe impairments in cognition. It is worth noting that the therapeutic response to bilateral or unilateral HFS may partially be determined by the asymmetry of the disease (*i.e.*, which hemisphere of the brain is more affected by Parkinson’s disease); in cases where the severity of Parkinsonian degeneration is unilateral, STN-HFS of the contralateral hemisphere may improve therapeutic outcome (Hershey *et al.*, 2008). In rats, unilateral lesions yield a weaker effect on cognition compared with bilateral lesions; bilateral lesions to the STN introduces a variety of cognitive deficits not expressed following unilateral lesions. Therefore, for the present experimental chapter with only one bilaterally lesioned rat, it is most likely that the majority unilaterally-lesioned animals retained sufficient cognitive function to complete the task with minimal impairment.

### **3.4.2 Conclusions**

In summary, predominantly unilateral STN/ZI lesions were insufficient to produce pronounced behavioural deficits on the 7-stage task and the modified 11-stage ID/ED task. There was a suggestion that the unilateral lesions may have induced mild cognitive impairment, but in the absence of robust evidence of set formation in control rats, further research is needed, with a larger sample of bilaterally lesioned rats, before conclusions can be drawn on the explicit contribution of the STN to attentional set-formation.

# Chapter 4

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## Using a palatable jelly tablet to deliver cognitive-enhancing drugs in an ‘early/late probe’ task

### Background:

This chapter presented an alternative behavioural approach; a more succinct alternative to the 11-stage task to infer the formation of attentional set, which provided the opportunity to measure the effects of putative cognitive-enhancing drugs.

### Methods:

The first experiment examines the efficacy of a reduced-stress jelly tablet to deliver modafinil, in which brain concentrations and effects on locomotor activity are measured. The second experiment tested STN-lesioned rats on an ‘early-late’ probe task, with or without cognitive-enhancing drugs. The task inferred set by comparing two probe stages; one before set is likely formed, and one after multiple ID stages.

### Results:

Brain concentrations of modafinil were comparable to oral gavage and modafinil, as expected, sustained locomotor activity, but only for later time-periods. Histology results for experiment II were consistent with Chapter 3; incomplete lesion damage minimised differences between groups, also drugs did not have an effect.

### Conclusions:

Owing to an ongoing inability to visualise lesion damage, which may have attenuated the effects of the drugs, we cannot measure the role of the STN in set-formation from the current experiment. A refinement in how the STN is manipulated is pertinent.

## 4.1 Experiment I

### 4.1.1 Introduction

In Chapter 3, there was no clear evidence for an impairment in attentional set-formation, following STN/ZI lesions. This could have been due to the fact that the lesions were difficult to verify and, where there was clear evidence of cell-loss, the area was small and mostly unilateral. In addition, behavioural evidence for an attentional set (i.e., a robust and statistically reliable ID-ED difference) in the control group was also weak. The purpose of the current experimental chapter was a further attempt to explore the possibility, suggested by previous observations, that lesions of the STN/ZI impair the formation of attentional set. To mitigate some of the issues encountered in Chapter 3, a slightly different approach is taken here. First, we wanted to test an alternative behavioural approach, to explore the possibility that we could detect evidence of an attentional set in a simplified and shorter version of the ASST. Second, it was reasoned that if behaviour is compromised in either lesioned or control rats, drugs which have previously been shown to act as ‘cognitive-enhancers’ might restore performance in one or other group (possibly differentially). It was hoped that, even if the lesions failed, we would still gain information about the usefulness of the modification of the behavioural task and/or the potentially beneficial effects of drugs on either normal, sub-optimal or impaired performance.

The 11-stage task used in Chapter 3 introduced a ‘probe-stage’ after the time at which an attentional set might be expected to have formed, but making an irrelevant change to the exemplars in the supposedly-unattended dimension to challenge the focus of attention of animals. In control animals, we have previously shown (and replicated in Chapter 3) that animals are not distracted by this change. We reasoned that this would only be the case if the probe-stage is presented late (after set-formation) and not early in the session, prior to set-formation. Prior to the formation of set, it is reasoned that rats solve the discriminations by learning about the associations of multiple cues with reward (i.e., odour *and* medium *and* left digging chamber). According to Mackintosh’s learning theory (see General Introduction; Sutherland & Mackintosh, 1971), the cue which is the best predictor of reward will acquire salience, leading to a formation of set. Therefore, we reasoned that rats

that have yet to form set, or rats that – for whatever reason – have failed to form set, would attend to the change in the irrelevant dimension during a probe stage. The probe stage could therefore be used as a measure of ‘pre-set’ and ‘post-set’ learning performance. This manipulation could therefore serve as an alternative to the ID/ED contrast typically used, which is actually a measure of set-shifting ability, rather than a measure of set-formation or even strength of set.

By comparing performance between these two probe stages, along with employing three interim consecutive ID stages to promote set-formation, reduces the total number of stages required for testing. The effects of two putative cognitive-enhancing drugs were also examined, with the hypothesis that one or both might i) ameliorate a set-formation deficit following a lesion; and/or ii) potentially improve overall performance in control rats. Modafinil and ORG49209 were selected as both have been previously shown to have effects on performance of the ASST (Turner, Clark, Dowson, Robbins, & Sahakian, 2004a; Xia, Tait & Brown; Chase, Tait & Brown, *unpublished*).

#### *4.1.1.1 Modafinil*

Modafinil (diphenylmethyl sulfinyl-2-acetamide; marketed as Provigil) is an atypical stimulant drug known for its unique wake-promoting mechanism, limited amphetamine-like side effects, and as an effective treatment for used in the treatment of narcolepsy, shift-work sleep disorder, sleep apnoea/hyponoia and excessive sleepiness (Bastoji & Jouvet, 1988; De Sereville, Boer, Rambert, & Duteil, 1994; Edgar & Seidel, 1997). It has gained particular interest for its unique wake-promoting effects without exerting typical amphetamine-like side-effects such as sleep rebound, along with significantly less abuse liability than amphetamines (Deroche-Gamonet *et al.*, 2002; Edgar & Seidel, 1997; Gold & Balster, 1996; Koob & Bloom, 1988; Leith & Barrett, 1976; Tourev, Sallanon-Moulin, & Jouvet, 1995).

Traditional stimulants (*e.g.*, methylphenidate) exert wake-promoting effects by targeting the reuptake and modulation of catecholamines, particularly dopamine, in the central nervous system, whereas modafinil exerts a different pharmacological profile (Nishino & Mignot, 1997; Scammell *et al.*, 2000). For example, the application of  $\alpha$ -

methyl-*p*-tyrosine ( $\alpha$ MPT; tyrosine hydroxylase enzyme inhibitor) significantly attenuated the effects of amphetamine, whereas the effects of modafinil were only slightly reduced after the same application (Lin *et al.*, 1992). Modafinil is also structurally different to amphetamine, which likely contributes to the observed divergent profile of pharmacological and behavioural effects (for review see Minzenberg & Carter, 2008). Despite this, modafinil still has an affinity for catecholamines, most notable the dopamine transporter (DAT) and noradrenaline (NA); however the neurochemical effect and pattern of brain area activation differs from amphetamines (Mereu, Bonci, Newman, & Tanda, 2013). Figure 4.2.1, which was adapted from Mereu *et al.* (2013), illustrates the complex involvement of the neurotransmitter systems and corresponding target brain areas implicated in modafinil and its application as a cognitive enhancer. In addition to inhibiting dopamine (DA) reuptake, modafinil dose-dependently increases 5-hydroxytryptamine (serotonin; 5HT) in the prefrontal cortex (de Saint Hilaire, Orosco, Rouch, Blanc, & Nicolaidis, 2001) and the central cortex of the amygdala and dorsal raphe nuclei (Ferraro *et al.*, 2002).

Modafinil has also gained interest as a putative cognitive-enhancer in humans – both in clinical populations and in healthy adults – and in experiments with rodents (for review see Minzenberg & Carter, 2008). In a controlled drug study in humans, modafinil enhanced performance for healthy male adults relative to a placebo group on several tests of executive function: improving digit span length, reducing errors in a test of visual pattern recognition memory, and facilitating tasks of spatial planning (Turner *et al.*, 2003). It was speculated that this cognitive enhancement could be due to modafinil’s ability to inhibit pre-potent responses, as additionally – and dose-dependently – modafinil improved stop-signal reaction time (SSRT) latency, whilst reducing the number of errors on “go” trials (trials which require a behavioural response) (Turner *et al.*, 2003). An error during a “go” provides a measurement of anticipatory responding, and consequently suggesting that modafinil may help in reducing impulsive action.

It appears that modafinil’s cognitive-enhancing effect may be subject to individual differences. Müller, Steffenhagen, Regenthal, & Bublak (2004) demonstrated that

modafinil only benefitted working memory performance for lower performing subjects. The authors observed a significant decrease in error rates when the difficulty of the working

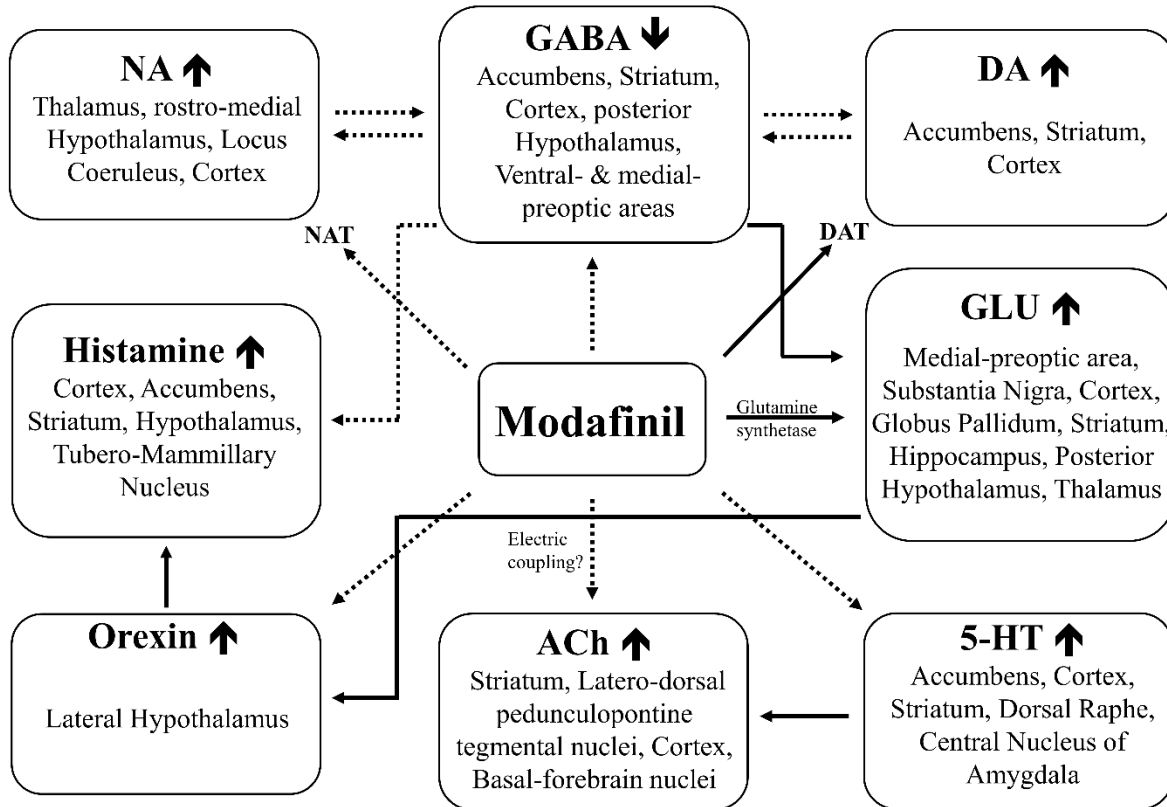


Figure 4.1.1: Brain areas and related neurotransmitter systems that are potentially involved in mediating the therapeutic actions of modafinil as a cognitive enhancer (adapted from Mereu *et al.* 2013). Solid lines indicate direct interactions; dashed lines indicate indirect interactions or those not yet been determined. NA noradrenaline, NAT noradrenaline transporter, GABA gamma-amino-butyric-acid, DA dopamine, DAT dopamine transporter, GLU glutamate, 5HT serotonin, ACh acetylcholine.

memory manipulation increased, and further that participants who were already good at the manipulation task remained good at it, exhibiting no benefit of modafinil administration (Müller *et al.*, 2004). Further research illustrated that modafinil improved visuo-spatial planning and response inhibition, but only amongst ‘lower’ IQ and not ‘higher’ IQ participants (Randall, Shneerson, & File, 2005). It follows that the cognitive-enhancing

effects of modafinil would likely be more readily detected amongst lower levels of performance, where there is more notable room for improvement.

Amongst clinical populations, modafinil has been shown to improve the planning and attentional deficits in adult patients suffering from ADHD, on tasks of digit span, visual pattern memory & spatial planning (Turner, Clark, Dowson, Robbins, & Sahakian, 2004a). In patients with schizophrenia, modafinil improved both forward and backward digit span length compared to placebo, indexing a working memory improvement, and additionally it significantly reduced the typical high levels of attrition seen in the ID/ED task (placebo: 50% attrition; modafinil: 15%), along with reducing the total number of errors at the ED stage (Turner *et al.*, 2004b). Similar to research in healthy volunteers, there was a correlation between the impairment level amongst patients with chronic schizophrenia and modafinil's reported efficacy on restoring cognitive function, in that the most impaired groups derived the greatest benefit from modafinil treatment (Hunter, Ganesan, Wilkinson, & Spence, 2006; Spence, Green, Wilkinson, Hunter, & Hunter, 2005).

In research with rodents, chronic administration of modafinil in mice has been shown to induce faster acquisition of spatial learning for rats in a T-maze, along with improving spatial reversal learning by facilitating a faster target win-shift strategy acquisition compared to vehicle-treated control rats, which suggested that modafinil may be important for the learning process, rather than simply aiding in inhibiting perseverative responses (Beracochea *et al.*, 2002). Morgan, Crowley, Smith, LaRoche, & Dopheide (2007) found that performance on a sustained attention task for modafinil-treated rats, dose-dependently, improved response accuracy, latency and impulse control, without affecting error rate and motor control.

Unpublished observations from our lab have also demonstrated that modafinil may facilitate attentional set-formation in middle-aged rats (Chase, Tait & Brown, *unpublished*). It was found that 30mg/kg (i.p.) of modafinil exacerbated reversal learning deficits in aged rats, but reduced the number of trials required for the subsequent ID stage. Additionally, we have also observed a strong trend that modafinil may aid in ameliorating the set-formation deficit observed after subthalamic lesions, improving novel ID learning along with re-establishing a positive cost of shifting set (Xia, Tait & Brown, *unpublished*). Modafinil

therefore presents as a pertinent cognitive-enhancing drug for the purpose of the current experiment.

#### *4.1.1.2 ORG49209*

ORG49209 (ORG) is a positive allosteric modulator (PAM) of the NMDA receptor with a similar chemical structure to pregnenolone sulphate – an endogenous neurosteroid (Paul & Purdy, 1992). The modulation of NMDA appears to be important in cognitive functioning, and reduced NMDA receptor functioning associated with aging has been implicated in a variety of cognitive deficits, including impaired ED shift performance in rats (Nicolle & Baxter, 2003), and impaired semantic fluency, working memory, set-shifting and spatial planning in aged humans (Zahr, Mayer, Pfefferbaum, & Sullivan, 2008). Additionally, negative modulation of the NMDA receptor with a pharmacological antagonist impairs avoidance and reversal learning along with introducing memory and executive control deficits in mice and rats (Mathis, Vogel, Cagniard, Criscuolo, & Ungerer, 1996; Palencia & Ragozzino, 2006).

It has been shown that NMDA receptor activation is crucial to the induction of long-term potentiation (LTP; for review see Bliss & Gardner-Medwin, 1973), which is a sustained increase in the efficiency of synaptic transmission as a consequence of a cascade of high-frequency stimulation to excitatory pathways, and is a fundamental mechanism of learning (Bliss & Collingridge, 1993). It logically follows that if attenuation of NMDA contributes to a variety of cognitive deficits, then perhaps its amplification should facilitate cognitive enhancement, however it has been demonstrated that increasing levels of extracellular NMDA results in excitotoxicity, and depending on the intensity of the original insult, can result in neuronal cell death (Bonfoco, Krainc, Ankarcrona, Nicotera, & Lipton, 1995; Liu *et al.*, 2007). It appears therefore that NMDA modulation requires a balance, and that either elevated or attenuated levels can result in adverse consequences.

As an alternative, activation of the NMDA receptor via an allosteric binding site has gained interest as an option for positively modulating NMDA function without risking neurotoxicity. Activation of the NMDA receptor at the primary or ‘orthosteric’ binding site is accomplished by the binding of the agonist NMDA molecule, which mimics glutamate –



typically at the NR<sub>1</sub> subunit – which results in channel-opening and signal transduction (Furukawa, Singh, Mancusso, & Gouaux, 2005). Orthosteric ligands bind to an agonist-binding site, which initiates downstream receptor signalling, whereas allosteric ligands are structurally different from orthosteric ligands and bind to distinct sites that are spatially distant from the orthosteric site, for the purpose of modulating orthosteric ligands (De Smet, Christopoulos, & Carmeliet, 2014; May, Leach, Sexton, & Christopoulos, 2007). For example, the endogenous neurosteroid pregnenolone sulphate is a PAM of NMDA, which potentiates NMDA currents, yet does not produce the typical neurotoxic effects of extracellular NMDA similar to those observed from orthosteric modulation (Paul & Purdy, 1992). Furthermore, pregnenolone sulphate has shown promise in ameliorating the cognitive impairments of NMDA receptor antagonism on avoidance learning (Cheney, Uzunov, & Guidotti, 1995; Mathis *et al.*, 1996). Pregnenolone and its sulphated derivative may also present as a promising therapeutic agent for treating the negative symptoms (and potentially cognitive symptoms) of schizophrenia, which may partially stem from the fact that clozapine increases pregnenolone in the rodent hippocampus, which may also contribute to clozapine's superior efficacy as an atypical antipsychotic (Marx *et al.*, 2009).

Pregnenolone sulphate presents with several limitations however, and – like many allosteric modulators – is not entirely selective. In addition to positively modulating NMDA allosterically, pregnenolone sulphate acts as an antagonist for gamma-aminobutyrate-A (GABA<sub>A</sub>), which is an essential inhibitory neurotransmitter for mediating convulsant responses; it is likely that the observed convulsions in experimental animals following pregnenolone sulphate treatment was due to indirect GABA<sub>A</sub> antagonism (Gibbs, Russek, & Farb, 2006; Paul & Purdy, 1992).

In the present experiment, we investigated the potential for cognitive-enhancing effects of ORG49209 (ORG) or (3 $\beta$ )-26,27-dinoregost-5-ene-3,24-diol, which is structurally very similar to the endogenous neurosteroid cerebrosterol. Cerebrosterol – otherwise known as 24(s)-hydroxycholesterol – is abundantly found in the CNS (hence its nomenclature) and is a major metabolite of cholesterol and is partly responsible for regulating the cholesterol activity in the brain (Björkhem, 2007). Cerebrosterol is also an NMDA PAM, and has gained interest for providing potent, direct, and selective positive

allosteric modulation with minimal overlap with other allosteric modulators, whilst not affecting GABA<sub>A</sub>-mediated responses (Paul *et al.*, 2013a). In rats, cerebrosterol also enhances LTP, and reverses the LTP deficits following ketamine treatment to the hippocampus, highlighting potential clinical and cognitive-enhancing uses.

Unpublished findings have suggested that ORG was able to increase an attenuated LTP effect in hippocampal slices, along with being more potent and selective than pregnenolone sulphate (Etherington, 2012, unpublished). Additionally, ORG presents as a choice candidate for the current experiment since the dominant postsynaptic subtype of the STN is glutamatergic (Galvan, Kuwajima, & Smith, 2006), and it has been demonstrated that NMDA receptors contribute to synaptic activity in the STN, which is likely modulated by other glutamate receptors (metabotropic glutamate receptors I & V; see Awad, Hubert, Smith, Levey, & Conn, 2000) providing an orthosteric cascade of activation (Swanger *et al.*, 2015). Behaviourally, our lab has shown that ORG treatment may ameliorate the age-related deficits in reversal learning for middle-aged rats, with evidence supporting a potential improvement in set-shifting performance (Chase, PhD Thesis). This evidence lends promise to the possible ameliorative efficacy for ORG to aid in restoring a potential set-formation-induced deficit in the current experiment.

#### *4.1.1.3 Current experiment*

For the revised set-formation task introduced in this chapter (and any paradigm interested in measuring behaviour), administering a drug and ensuring bioavailability throughout the entire test session is essential. Ideally, we would want peak concentration to occur during the testing of key stages of interest. An important consideration pertains to the route of drug administration, which impacts the pharmacokinetics (*e.g.*, absorption) and influences the timing and magnitude of any behavioural effect; however there are also other practical considerations when selecting a particular route of administration. For example, there may be secondary behavioural effects of giving the drug: an interruption of ongoing behaviour might distract or arouse the animal, such that behaviour after drug administration changes irrespective of any pharmacological effect of the drug (Meijer, Spruijt, van Zutphen, & Baumans, 2006). This is likely to be a particular problem if the route of

administration causes pain or discomfort, as is evident with needle-sticks and gavage. Oral gavage has been reported to induce significant increases in heart rate 2-5 hours post-gavage and increase faecal corticosterone (Bonnichsen, Dragsted, & Hansen, 2005; Walker *et al.*, 2012) – and stress-related arousal will have behavioural consequences.

Drugs can be administered by a variety of routes, for example, intra-cerebrally (via implanted catheters in the brain); intraperitoneally (i.p.; via injection through the abdominal wall); subcutaneously (s.c.; via injection under the skin); transdermally (via a skin-patch or onto a mucous membrane); inhalation; orally (by gavage – insertion directly into the stomach – or through mixing with food or drink). Walker *et al.* (2012) showed that mice which voluntarily consumed a ‘pill’ made from ‘Transgenic Dough Diet’™ (Bioserve, Inc) did not show a stress response. The advantage of the dough diet is that it is readily sourced and the drug can be kneaded into the dough. The disadvantage of using this diet, however, is that it is designed for rodents with chewing, dental or mobility impairments and therefore whilst highly palatable, is also high in protein and fat. This makes it less useful for studies that measure behaviour motivated by food, and in instances where uptake may be affected by food in the gastrointestinal tract.

In the current experiment, we present a refinement of this medium, which used a flavoured and palatable, but calorie-free “jelly” tablet, which can be designed to present an orally bioavailable drug in suspension. In regards to the drugs employed in the current experiment, our lab has previously observed that ORG is particularly difficult to inject, as it requires a high concentration of solvent (>40% hydroxy-propyl- $\beta$ -cyclodextrin; HPBCD) to put it into suspension (Chase, Brown & Tait, *unpublished*). Being an experimental drug, very little information regarding its oral bioavailability has been published, perhaps due to its structural similarity to cerebrosterol, making it difficult to assess levels of concentration in the brain.

Modafinil, on the other hand, is orally bioavailable, and a pharmacokinetic analysis by Waters, Burnham, O’connor, Dawson, & Dias (2005) reported that gavage administration yielded a peak concentration of modafinil between 30-60 minutes post-administration; however the authors did not observe any of the reported cognitive-enhancing effects, and instead found that higher doses (128 mg/kg) of modafinil increased

impulsivity, which is contrary to previous findings (see Morgan, Crowley, Smith, LaRoche, & Dopheide, 2007). It is possible then that the stress-related arousal from gavage may have attenuated the expected behavioural effects of modafinil, suggesting that a reduced-stress method of drug administration should be sought prior to behavioural testing, in order to minimise any secondary effects. Therefore, the first part of this experimental chapter will orally administer modafinil via a palatable jelly and examine:

- i. bioavailability of modafinil, by measuring the time course of brain levels of the drug
- ii. the time course of modafinil's behavioural effect, by measuring the time-course of an expected increase in locomotor activity.

Early studies that investigated the wake-promoting effects of modafinil reported that higher doses of modafinil ( $\geq 40$  mg/kg) elicit an increase in locomotor activity (LMA) in the rat (Simon *et al.* 1996). Subsequent research by Edgar and Seidel (1997) measured this increased LMA with an electroencephalogram (EEG) and determined that the increase was only in proportion to the expected time spent awake during LMA recordings (Simon *et al.* 1996). Additionally, modafinil-treated rats show a normal (*i.e.*, equivalent in magnitude to control vehicle-treated) habituation to the LMA apparatus, with an initial decrease in exploratory behaviour, and then significantly elevated activity at later time-periods (between 40 and 80 minutes post-administration) (Simon *et al.* 1996). We therefore predicted that: oral administration of 30 mg/kg modafinil would induce a similar significant increase in LMA, particularly at later time-periods, compared to vehicle-treated rats following habituation to an actimeter.

The first part of this experiment was made possible by the work of Dr Shuang Xia, who assigned the timing of the sacrifice points, and assisted in the collection of brain tissue. Analysis of the tissue was generously completed by Dr Christoffer Bundgaard on behalf of H. Lundbeck, and special thanks to Miss Ellen Bowman for her innovation in choosing a suitable suspension for the drug. The data for the first part of this experimental chapter has been published in the peer-reviewed journal *Psychopharmacology* (Dhawan *et al.*, 2018).

## 4.1.2 Methods

### 4.1.2.1 Animals

Forty-six (twenty-four male; twenty-two female), naïve Lister hooded rats (bred in-house; University of St Andrews, *from Charles River stock*) were group-housed, but segregated by sex. Male rats (weight range upon sacrifice: 480-630g) were used to establish the pharmacokinetic profile in study I. The female rats were to assess modafinil's effect on LMA in study II, and had a mean weight of 217g (range: 180-250g) at the start of the experiment. At completion of the experiment female rats had a mean weight of 292g (range: 233-328g). Husbandry and housing details are described in the General Methods (Chapter 2; section 2.1). Habituation to jelly-tablets was completed over one week (see habituation procedure below), and behavioural testing of LMA was completed over a period of 3 weeks.

### 4.1.2.2 Apparatus

LMA data was collected by a 7x15 LED infrared actimeter (Motor Monitor, Hamilton-Kinder, Ponway, CA, USA). The number of ambulations per time period was determined by the number of photo beams the rat crossed. A “rearing frame” was also attached to the actimeter, which added an additional measure of “vertical activity” since rearing in rats is indicative of exploratory behaviour and is a useful marker of environmental novelty (Lever, Burton, & O’Keefe, 2006). The software (HK Motor Monitor, Hamilton-Kinder, Ponway, CA, USA) designed for the actimeter, controlled operation of the five actimeters used in this experiment, along with collating raw data into output files.

### 4.1.2.3 Drug preparation and habituation

Modafinil was administered to the rats orally, suspended in a palatable gelatine tablet (jelly) as the vehicle. The jellies were made by heating a water bath to ~70°C, then placing into the water bath a beaker containing 50ml of flavoured, sugar-free, fruit juice concentrate (Robinsons Squash, Britvic PLC, UK), and 50ml of distilled water, and adding 30g gelatine (Dr Oetker, UK). The mixture was stirred until the gelatine was fully dissolved.

Modafinil (Sequoia Research Products Ltd., UK) doses (30mg/kg) for individual rats were weighed out and added to the bottom of 2ml wells in a plastic mould. The gelatine solution was then pipetted into the wells (1.5ml/well), and the mixture carefully stirred with a small pipette tip to suspend the modafinil. Vehicle jellies were made using the same procedure, but without modafinil. The plastic mould was then placed in a fridge (3-5°C) overnight for the jellies to cool and set. Once the jellies were set, they were removed from the moulds and stored in the fridge in airtight containers.

To prevent food neophobia from inhibiting eating, the rats were given jellies without modafinil on several occasions before data collection. Rats were placed individually in a large home-cage and presented with a jelly in a small ceramic pet food bowl and were left until they had fully consumed it. This was repeated once per day until rats had fully consumed the jelly within 5 minutes, which was typically on the third day. We had previously established that there was no evidence that the rats could detect when jellies contained modafinil (*unpublished observations*), therefore it was not necessary to expose them to modafinil prior to testing.

#### *4.1.2.4 The pharmacokinetic profile of orally-administered modafinil*

##### *4.1.2.4.1 Drug administration*

On the day of the experiment, the 24 male rats were single-housed and presented with modafinil-containing jellies. The time at which a rat finished eating the jelly was recorded (typically no more than 5 minutes after it had started eating), and at specific time-points after that (15, 30, 45, 60, 75, 90, 120 and 150 minutes; n=3 per time-point) rats were sacrificed by decapitation. After decapitation, brains were extracted from the skull, the cerebellum was removed, and then the remainder was bisected in the sagittal plane. Each hemisphere was weighed and then rapidly frozen by immersion in isopentane (Sigma-Aldrich, UK) chilled by dry ice. The hemispheres were then wrapped in aluminium foil, individually placed in homogenisation tubes and stored at -80°C.

#### 4.1.2.4.2 Post-mortem bioanalysis

The following bioanalysis was generously completed by Dr Christoffer Bundgaard on behalf of H. Lundbeck in Denmark. Rat brain concentrations of modafinil were determined using Ultra Performance Liquid Chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS). Brain samples were prepared by homogenising the brains 1:3 (v/v) with a mixture of water, 2-propanol and dimethyl sulfoxide (DMSO; 50:30:20 v/v/v). Samples were precipitated with acetonitrile and DMSO (80:20 v/v) containing internal standard. Following centrifugation, 10µl was injected onto the chromatographic system consisting of an Aria TLX2 system (Thermo Fisher Scientific Inc., Massachusetts, USA) connected to a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer. Analytical separation was achieved using a Kinetex C18 column (50x2.1mm, 2.6 µm particles; Phenomenex, California, USA). The mobile phase consisted of 0.01% formic acid in acetonitrile and 0.1% formic acid in water pumped through the column using a 3 minute gradient. Modafinil was detected at a parent > daughter mass to charge ratio (m/z) of 274.01 > 167.00. The retention time was 1.27 minutes. The peak area correlated linearly with the brain concentration of the analyte in the range of 40-4000ng/g brain.

#### 4.1.2.5 *The effects of orally-administered modafinil on locomotion activity*

##### 4.1.2.5.1 Testing procedure

On the day of testing, the 22 female rats were habituated to the infrared actimeter for 60 minutes. Following actimeter habituation, a jelly was presented in a ceramic bowl directly into the actimeter. Habituation commenced four hours after lights-on, and jellies were administered five hours after lights-on (Circadian time 5 (CT-5), where CT0 is lights-on), as it has been observed that sleep loss can impact stimulant drug efficacy (Edgar, Seidel, & Dement, 1991; Roehrs, Zwyghuizen-Doorenbos, Timms, Zorick, & Roth, 1989). CT-5 (in 12 hour light/dark schedule) in particular, yields the least interference from the high variability in wakefulness due to large amounts of sleep (<CT-5) and the normal circadian wakefulness found closer to lights-off (>CT-5) (Edgar & Seidel, 1997). Precisely 10 minutes from the point the rat started eating the jelly (with all jellies consumed within 5 minutes), the actimeter was reset, and testing commenced. This was done to ensure the

sacrifice points from the pharmacokinetic study fell within a measured time period of locomotive activity. The test sessions were 150 minutes, with LMA data compiled to investigate time-periods of interest (0-15, 15-30, 30-45, 45-60, 60-75, 75-90, 90-105, 105-120, 120-135 and 135-150 minutes).

#### 4.1.2.5.2 Counterbalancing

Each rat was tested twice, with 11 rats each receiving modafinil and vehicle jellies in each test, counterbalanced so that each rat received both modafinil and vehicle jellies. There was a minimum of five days between tests to allow for washout of modafinil.

#### 4.1.2.5.3 Data analysis

The 150 minutes of LMA data were compiled into time-periods (0-15, 15-30, 30-45, 45-60, 60-75, 75-90, 90-105, 105-120, 120-135 and 135-150 minutes) and analysed by repeated-measures ANOVA. The dependant variable was the total number of infrared beams crossed within the observed time period. There were two within-subjects factors: dose (two levels – drug and vehicle), and time period (ten levels). A second ANOVA, with two within-subjects factors: test (test 1 vs test 2) and time-period was used to confirm that overall, rats' performance did not change between tests. Huynh-Feldt corrections were applied for sphericity violations.



### 4.1.3 Results

#### 4.1.3.1 *The pharmacokinetic profile of orally-administered modafinil*

Modafinil was detected in brain tissue at all time-points (Figure 4.1.2). A rapid uptake in the brain was observed with average concentrations in the range of 300-400ng/g during the first hour after drug intake, followed by an observed gradual decrease in brain concentrations over time.

#### 4.1.3.2 *The effects of orally administered modafinil on locomotion activity*

There was no effect of running the test twice: as a group, the rats were equally active in both tests (*Test*:  $F_{(1,42)} = 1.42$ , *n.s.*;  $np^2 = 0.03$ ) and the time course of activity was also similar in each test (*Test by Time-period interaction*:  $F_{(4,54, 190.76)} = 1.1$ , *n.s.*; *Huynh-Feldt correction*; *uncorrected degrees of freedom*  $df = 9, 378$ ;  $np^2 = 0.03$ ). Overall, modafinil administration resulted in greater LMA compared to vehicle-treated rats (*Dose*:  $F_{(1, 21)} = 49.3$ ,  $p < 0.05$ ;  $np^2 = 0.70$ ; Figure 4.1.2). Overall activity also decreased as time elapsed (*Time-period*:  $F_{(4,31, 90.49)} = 19.32$ ,  $p < 0.05$ ; *Huynh-Feldt correction*; *uncorrected*  $df = 9, 189$ ;  $np^2 = 0.48$ ), yet modafinil-treated animals remained more active across the time-periods (*Dose by Time-period interaction*:  $F_{(7,11, 149.28)} = 3.66$ ,  $p < 0.05$ ; *Huynh-Feldt correction*; *uncorrected*  $df = 9, 189$ ;  $np^2 = 0.15$ ). Further analysis by pairwise comparison (Bonferroni correction) determined that modafinil-treated rats were significantly more active at nearly all time-periods (15-30 minutes onwards) compared to vehicle-treated rats. As reported in previous studies (Simon, Hémet, & Costentin, 1996), the LMA of control rats decreased rapidly, whilst modafinil-treated rats remained more active at later time-periods.

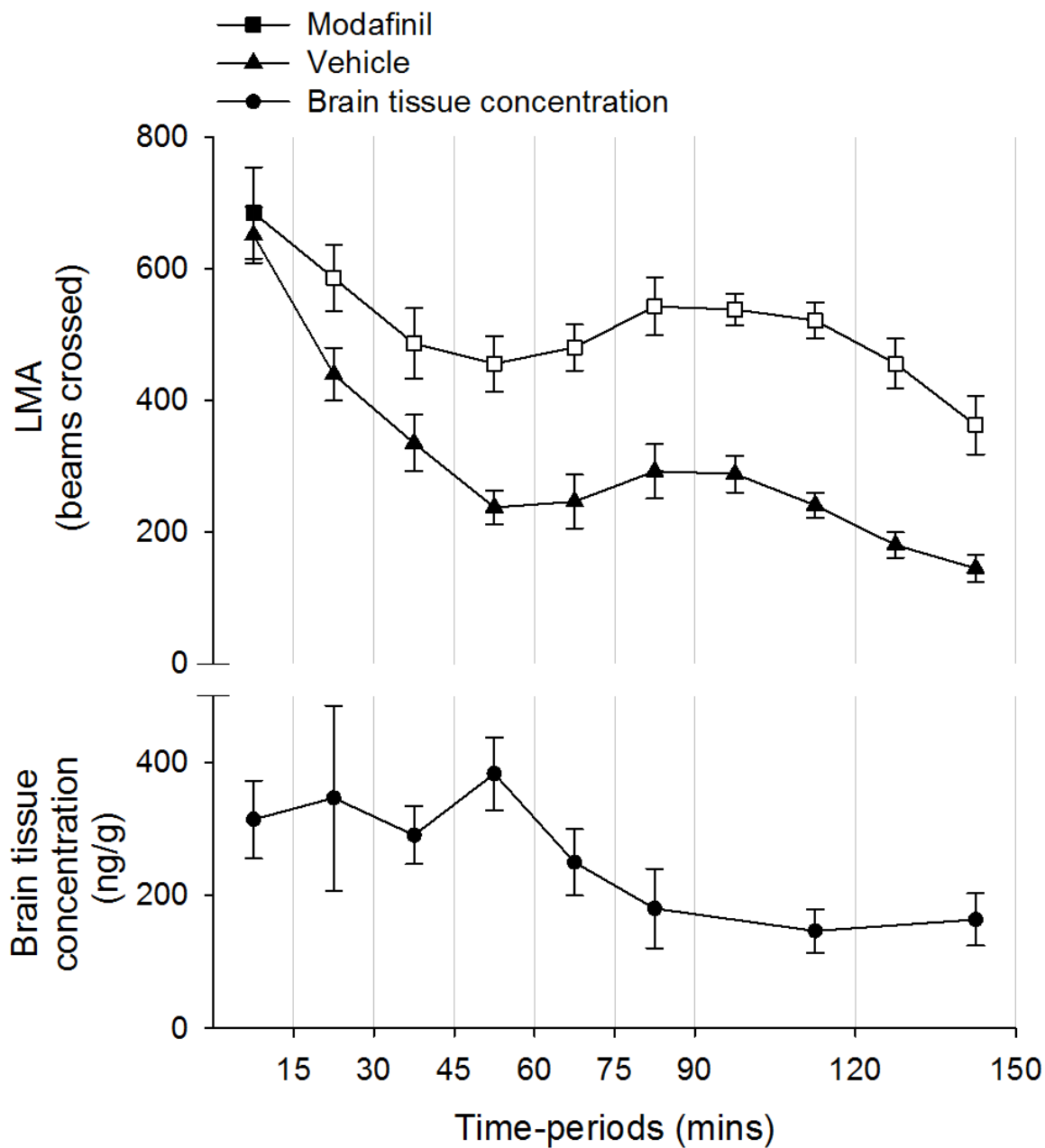


Figure 4.1.2: Top: Mean number of beams crossed ( $\pm$ SEM); modafinil-treated rats remained significantly more active from the 15-30 minute time-period and onward; white boxes  $p < 0.05$ . Bottom: Pharmacokinetic profile of the mean concentration of modafinil in brain tissue  $\pm$  SEM; Modafinil concentration reduces over time, although high variability at the early time-points may mask a true peak at the 60 minutes time-point

#### 4.1.4 Discussion

This study investigated the efficacy of a novel route of administration, in the form of a suspension in a palatable jelly tablet, for thermally stable, bio-orally available drugs. We have shown that modafinil is present in the brain up to at least 150 minutes after consumption of a 30mg/kg modafinil-containing jelly, and that LMA is affected by modafinil on a similar timescale. This range of bioavailability is also comfortably within the projected required test time in a more succinct task variant of the 11-stage task. The results obtained from the pharmacokinetic profile of modafinil concentration in brain tissue show a pattern comparable to previously published data from oral administration of 32mg/kg modafinil via gavage (Waters *et al.*, 2005).

Our data also demonstrate that rats fed modafinil in jelly form show LMA that compares favourably to data from other administration methods, along with aligning with our reported pharmacokinetic profile. For example (Simon *et al.*, 1996), report increased LMA after 40mg/kg modafinil administered via i.p. injection. As in that study, we have shown that following actimeter habituation, compared to control rats, modafinil-treated rats exhibited greater LMA overall and importantly, continuously after the first time-period. This behavioural evidence shows that this jelly method of administration presents as a viable option for delivering orally bioavailable drugs in a revised task investigating set-formation.

##### 4.1.4.1 Conclusions

In conclusion, the current data demonstrate that administration of modafinil in a jelly tablet is an alternative to oral gavage, with the benefit of being less stressful for the animal. The reported pharmacokinetic profile, which aligned with the increase in locomotive behaviour for modafinil-treated rats, presents this as a refinement in drug administration. Reducing the stress-related arousal during the administration of pharmacological agents may be essential to maximise the effects of modafinil on behaviour, which has been addressed through voluntary consumption of the drug.

## **4.2 Experiment II**

### **4.2.1 Introduction**

The introduction to this chapter (4.1.1) outlined that probe stages can present as a useful alternative to an ID/ED comparison in inferring set-formation, allowing for a measurement of both ‘pre-set’ and ‘post-set’ performance. The ID/ED comparison measures learning performance between two stages, whereas alternatively, the probe stage measures the level of distractibility of the irrelevant dimension, and how this ability changes as set is acquired. It is hypothesised here that an ‘early probe’ stage, placed at the outset of the task, should take longer to acquire than a ‘late probe’ stage which occurs after the multiple ID stages, and therefore the opportunity to form set. We hypothesised that:

- i. Data for control rats and lesioned rats should not differ at an early probe stage, in which set is likely not formed, and therefore TTC for both groups should be comparable to the preceding compound learning stage.
- ii. Lesioned rats will find a late probe stage more challenging than control rats, marked by increased TTC. Control rats should be able to ignore or inhibit responding to the irrelevant dimension by this point, and therefore acquire the stage without incurring any errors.

The current experiment also examined whether the application of putative cognitive-enhancing drugs – namely modafinil and ORG – could improve performance in behaviour which is compromised in lesioned rats or control rats. As reported in experiment I, orally bioavailable compounds can be suspended in a palatable jelly tablet as a reduced stress route of administration.

Two undergraduate students completed two sessions of testing on the standard 7-stage task (data not reported here) in the interim period between drug-administered test sessions on the early/late probe task. This also reduced the risk of partial reinforcement, since certain stimuli would never be rewarded, owing to the absence of reversal stages in the early-late probe task.

## **4.2.2 Methods**

### *4.2.2.1 Animals*

Twenty male, Lister hooded rats (Charles River, UK Ltd) were used in this experiment, with twelve rats in the lesion group, and eight rats in the control group. Rats had a mean weight of 268g (range: 231-286g) at the start of the experiment. At completion of the experiment rats had a mean weight of 503g (range: 405-576g). Rats were previously tested as adolescent animals on the 7-stage task as part of another experiment 19 weeks prior to surgery, and 33 weeks prior to the start of behavioural testing on the current experiment.

### *4.2.2.2 Apparatus*

The attentional set-shifting apparatus is described in the Chapter 2 (General Methods), section 2.2.

### *4.2.2.3 Surgery and histology*

Ibotenic acid lesion surgery procedures are described in Chapter 2 (General Methods; see section 2.3.3).

### *4.2.2.4 Experimental design*

A within-subjects design was used so all rats received vehicle, modafinil and ORG49209 jellies over 3 rounds of testing. The order of drug administration was counterbalanced, such that during a given test session one third of the rats received either: vehicle, modafinil, or ORG49209 jellies. Control and lesioned rats were paired and assigned to a drug condition, and order of presentation of stimulus pairings, the initial correct stimulus within a pairing, and ensuring an equal distribution of odour or media dimensions (in the absence of an ED shift) were counterbalanced across test sessions. In the absence of reversal learning stages in the early/late probe task, some stimuli will never be associated with reward, and in an attempt to control for this, an interim 7-stage task was employed to extinguish any conditioned associations with the stimuli.

#### *4.2.2.5 Drug preparation and habituation*

Preparation of vehicle and drug jellies is described in experiment I (see section 4.1.2.3).

#### *4.2.2.6 Behavioural training*

Rats were trained and tested as adolescent animals on the 7-stage task as part of another experiment, and were therefore not trained prior to testing in the current experiment. For training, see section 2.4.1 of the General Methods, Chapter 2.

#### *4.2.2.7 Behavioural testing – The early/late probe task*

Testing was conducted over a period of 10 weeks, commencing 2 weeks after surgery. The three rounds of testing on the probe task were conducted over 2 weeks, 1 week, and 3 weeks, respectively. Prior to testing, rats were placed in the set-shifting apparatus and given a jelly (vehicle, modafinil, or ORG49209) to consume within five minutes. Behavioural testing commenced thirty minutes following consumption of the jelly. The early/late probe, as the name implied, consisted of two probe stages, which replaces only the irrelevant dimension stimuli with novel stimuli. The task also presented multiple ID stages to aid in fostering set-formation. A total of four stimuli pairings were used, plus an additional two stimuli pairings reserved for the two probe stages. The probe stimuli pairings were counterbalanced for order of presentation within each drug group, and for each test session. The full list of stimuli pairing used in the early-late probe task are summarised in table 4.2.1.

The task started with a simple discrimination (**SD**) followed by a compound discrimination (**CD**) stage, in which a novel, yet irrelevant, dimension was added. The fact that this dimension was never predictive of reward, and therefore the CD stage here is also a ‘probe’ stage. However; the probe stage changes irrelevant dimension exemplars, whereas the CD stage introduces a novel dimension, and this introduction might attract attention. Therefore, the subsequent early probe (**CDp**) stage is a better comparison to the late probe stage. This stage retained the reward-relevant exemplars from the preceding CD stage, whilst the stimuli from the irrelevant dimension were changed with novel stimuli. The rats

	<b>Media</b>		<b>Odours</b>	
Pair I	M1: Coarse tea	M2: Fine tea	O1: Cinnamon	O2: Ginger
Pair II	M3: Sand	M4: Grit	O3: Sage	O4: Paprika
Pair III	M5: Coarse shavings	M6: Fine shavings	O5: Turmeric	O6: Cloves
Pair IV	M7: Coarse cork	M8: Fine cork	O7: Fenugreek	O8 Tarragon
Probe I	M11: Cotton pads	M12: Cigarette filters	O11: Dill	O12: Coriander
Probe II	M13: Beads	M14: Gravel	O13: Thyme	O14: Caraway seeds

*Table 4.2.1: The list of stimuli pairings used in the early-late probe task, including the probe stage, which only replaced stimuli in the incorrect dimension.*

then completed three intradimensional acquisition stages (**ID1**, **ID2**, & **ID3**), each rewarding the same perceptual dimension. The late probe (**ID3p**) stage, was formally identical to the early probe stage, and retained the reward-relevant exemplars from the preceding ID3 stage. An example summary of the procedure of the early/late probe task is detailed in table 4.2.2. Testing was carried out with the help of two undergraduate student, as part of their undergraduate final year project work.

#### *4.2.2.8 Data analysis*

The early-late probe experiment was a 2x3x7 mixed factorial design, and data for trials to criterion were analysed by repeated-measures ANOVA. There was a within-subjects factor of drug condition (three levels: vehicle, modafinil, and ORG49209), a within-subject factor of stage (seven levels), and a between-subjects factor of group (two levels: control or lesion). Upon completion of the experiment, data for each drug condition was combined from the three rounds of testing to form a complete set, thus comprising data for all rats in preparation for analysis.

#### *4.2.2.9 Histology*

NeuN/Cresyl violet immunohistochemistry procedures are described in Chapter 2 (General Methods; see section 2.5).

<b>Discrimination</b>	<b>Relevant dimension</b>	<b>Discriminanda</b>	<b>Mixed with</b>
Simple Discrimination (SD)	Medium	<i>Coarse tea</i> , not fine tea	<i>Nothing</i>
Compound Discrimination (CD)	Medium	<i>Coarse tea</i> , not fine tea	Cinnamon or ginger
Early Probe (CDp)	Medium	<i>Coarse tea</i> , not fine tea	Dill or coriander
Intradimensional Acquisition I (ID1)	Medium	<i>Sand</i> , not grit	Sage or paprika
Intradimensional Acquisition II (ID2)	Medium	<i>Coarse shavings</i> , not fine shavings	Turmeric or cloves
Intradimensional Acquisition III (ID3)	Medium	<i>Coarse cork</i> , not fine cork	Fenugreek or tarragon
Late Probe (ID3p)	Medium	<i>Coarse cork</i> , not fine cork	Thyme or caraway seeds

*Table 4.2.2: An example procedure of a typical test session in the early/late probe task. Odour and media as dimensions were counterbalanced, along with initial correct stimulus and order of presentation of probe stimuli.*

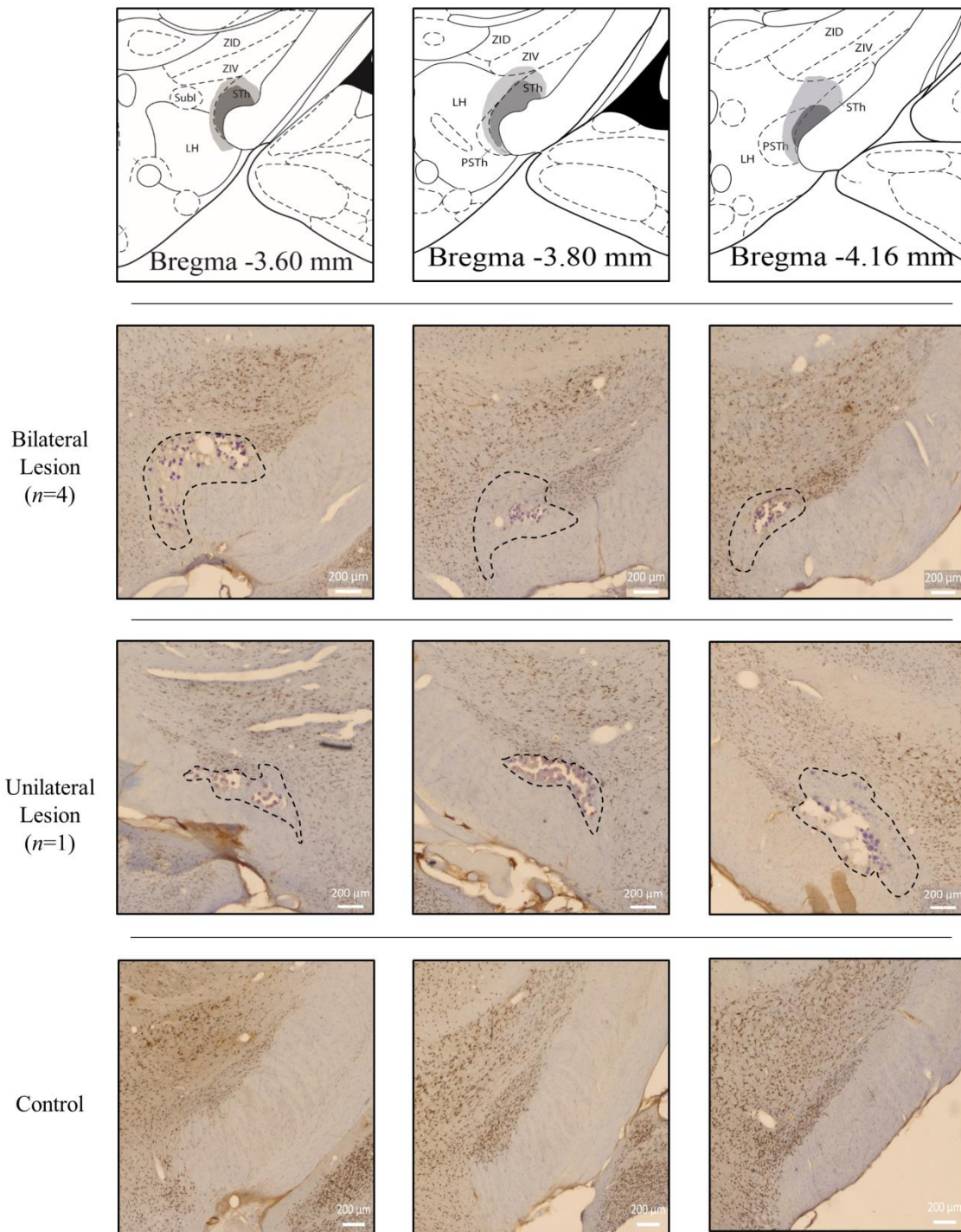


### 4.2.3 Results

#### 4.2.3.1 Histology

Two lesioned rats were excluded from the final analysis: one rat failed to recover post-operatively, and the other was euthanised due to the onset of spontaneous seizures. Consequently, the final group sizes were: lesion  $n=10$ ; control:  $n=8$ .

Lesion extent and cell loss was determined by NeuN/Cresyl Violet double staining, and of the ten rats, only five rats had identifiable lesions to the STN, one of which was unilateral. These rats had STN cell-loss restricted to the medial portion, and the lesion damage for one rat extended medially into PSth and the LHA. Figure 4.2.1 illustrates schematics of lesion extent, along with photomicrographs of the cell-loss found in the unilaterally-lesioned rat, the 'typical' cell-loss found amongst bilaterally lesioned rats, and photomicrographs of a control brain. Given that these rats did not present with signs of cell-loss in the STN or ZI, these rats were removed prior to statistical analysis reducing the final group size for lesioned rats to five (4 with bilateral lesions; 1 with a unilateral lesion). The remaining five rats retained a visibly 'intact' STN with no observable signs of cell-loss, and consistent with Chapter 3, all rats given ibotenic acid infusions presented with evidence of calcification in the MGP, along with 'chewing' behaviour during recovery from surgery.



*Figure 4.2.1: Schematics and example photomicrographs of brain sections (NeuN/Cresyl Violet). Top to bottom: schematics illustrate the minimum (dark grey) and maximum (light grey) extent of the lesion; photomicrographs of the ‘typical’ bilateral lesion and the unilaterally-lesioned rat (hashed outline indicates lesion extent); photomicrographs of a control brain.*

#### 4.2.3.2 Overall analysis

Overall performance on the task improved as testing progressed, with the late probe stage requiring the fewest TTC (*main effect of Stage*:  $F_{(6,60)} = 5.76$ ,  $p < 0.01$ ;  $\eta^2 = 0.37$ ; figure 4.2.2). A simple contrast determined that the late probe was acquired in significantly fewer trials than the early probe ( $F_{(1,10)} = 5.8$ ,  $p < 0.05$ ;  $\eta^2 = 0.37$ ); however, there were no differences between the control and lesion groups, either overall or by stage, including both probe stages (*Group*:  $F_{(2,10)} < 1$ , *n.s.*;  $\eta^2 = 0.04$ ; *Stage by Group interaction*:  $F_{(12,60)} = 1.78$ ,  $p = 0.07$ ;  $\eta^2 = 0.26$ ). Furthermore, there were no differences between Modafinil or ORG administration compared to vehicle, either overall, or by group, or stage (*Drug*:  $F_{(2,20)} = 2.79$ , *n.s.*;  $\eta^2 = 0.22$ ; *Drug by Group interaction*:  $F_{(4,20)} = 1.26$ , *n.s.*;  $\eta^2 < 0.2$ ; *Drug by Stage interaction*:  $F_{(12,120)} = 1.13$ , *n.s.*;  $\eta^2 = 0.1$ ).

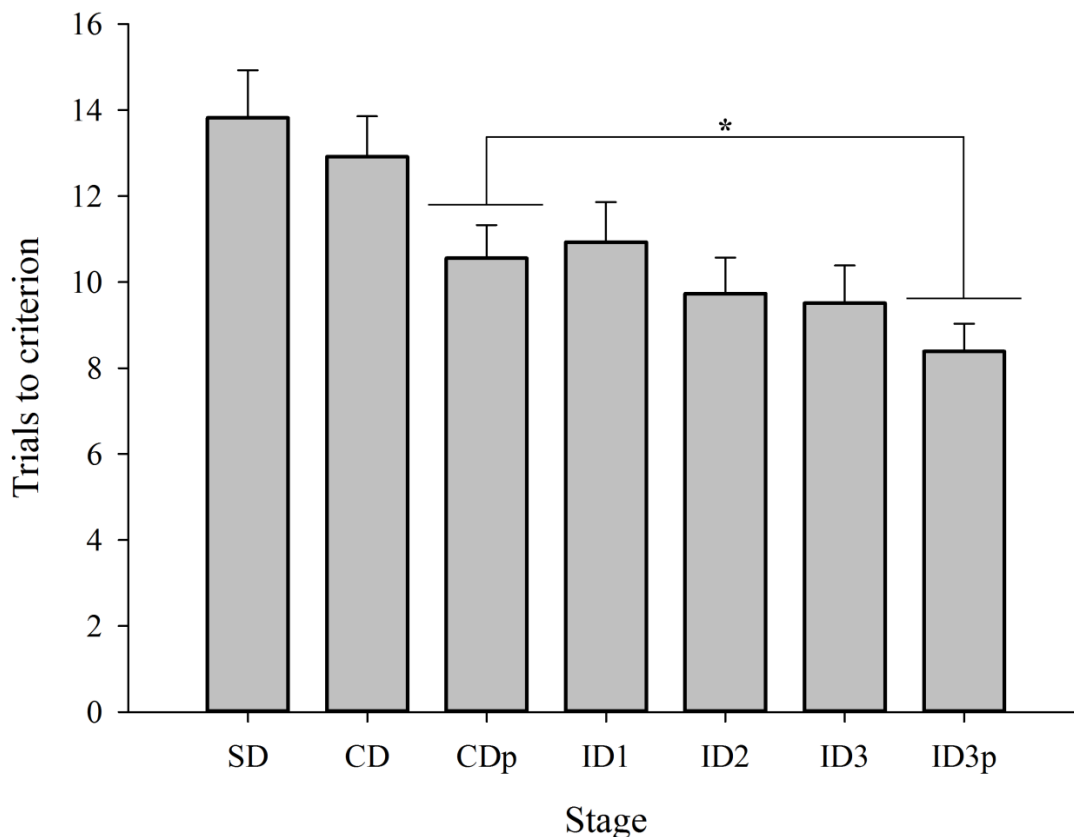


Figure 4.2.2: Mean TTC (+SEM) for all rats across the 7 stages of the early/late probe task, illustrating a stage-wise improvement, and a significant reduction in TTC between the early and late probe stages; data for Drug and Group were combined as neither interacted significantly with stage.

#### 4.2.3.3 Comparison of early and late probe stages

Examination of the raw data (figure 4.2.3) revealed that more than half of the rats (8 out of 13; 62%) exhibited a difference between the early and late probe stage performance (reduction in TTC between CDp and ID3p); however in some cases the difference was very small. Furthermore, we had expected that rats with an attentional set should perform the late-probe stage without any (*i.e.*, in 6 TTC), or with very few, errors, but only a small minority did so. Overall, this was not a convincing demonstration that the rats had formed an attentional set and that the probe stage was no longer distracting.

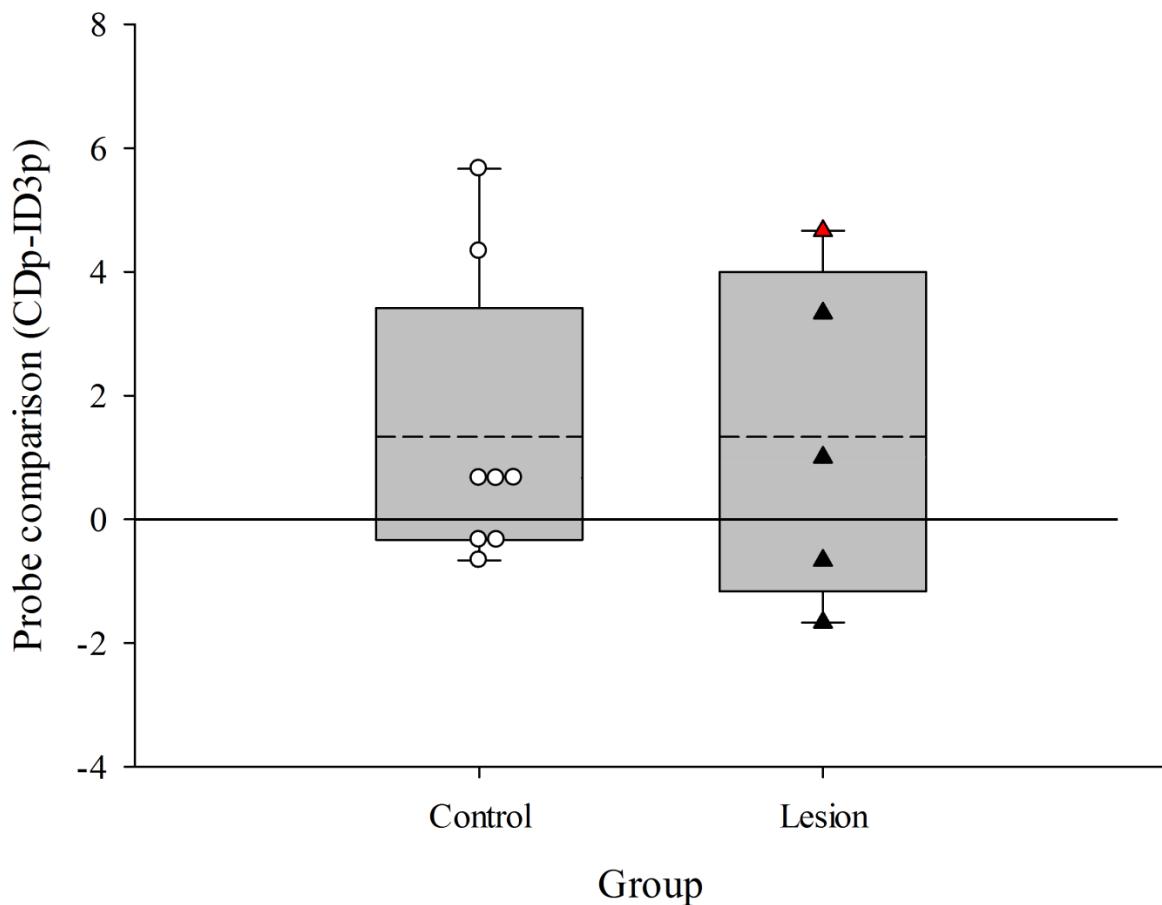


Figure 4.2.3: Boxplot illustrating difference between the probe stages, calculated by subtracting the late probe from the early probe (CDp-ID3p); hashed lines indicate the mean shift cost for rats by group; superimposed vertical point plot depicting the distribution of the data for each rat, with the red triangle illustrating the probe comparison for the unilaterally-lesioned rat; Nearly all of the rats exhibited a positive difference between stages, indicating the late probe was acquired in fewer trials than the early probe.

#### 4.2.4 Discussion

The current experiment introduced a novel manipulation using the bowl-digging paradigm, in which set-formation is inferred not by comparing the acquisition of ID and ED discriminations, but rather by observing the disruptive effect of changing the exemplars in the irrelevant dimension on an already-acquired discrimination. Early in testing, before an attentional set has formed, changing the irrelevant stimuli was expected to distract from performance of the learned discrimination, while later in testing, the change was expected to be unattended and therefore without disruptive effect. The evidence indicated greater disruption of performance of an early probe-stage compared to the later probe-stage. This difference suggests that the formation of attentional set-formation spares ongoing behaviour from the disruptive effects of a change of stimuli in the irrelevant dimension. However there are some limitations of this task, which will be discussed below. The current experiment also examined the effects of lesions of the STN and the potentially ameliorative or even cognitively-enhancing effect of modafinil and ORG49209. However, the lesion manipulation failed and furthermore both drugs were without behavioural effects.

##### *4.2.4.1 Limitations*

In the current experiment, as seen in Chapter 3, once again the lesion manipulation failed to result in consistent observable cell loss. Without verifiable and replicable lesions, it is not possible to draw any conclusion about the contribution of the STN to behaviour. Similar to Chapter 3, the data for the majority of the lesioned group needed to be discarded owing to unobservable cell-loss, despite evidence of calcification in the MGP, along with ‘chewing’ behaviour during recovery from surgery

Although the data are not reported here, there was also a surprising lack of difference between the ID and ED stages of the 7-stage task in the control group. The failure to find an ID/ED difference in the control rats in the 7-stage task, and hence, evidence of set-formation, suggests that the test might have been underpowered but nevertheless makes it difficult to draw any conclusions about lesion effects, even in the small number of rats who had visible damage.

Finally, there were no effects of either modafinil or ORG. It is possible that the early/late probe manipulation is not sufficiently sensitive to any enhancing effects (*i.e.*, if there was little room for improvement). Although Xia, Tait & Brown (*unpublished*) reported that modafinil enhanced learning at an ID3 stage, this was only true for lesioned rats that were impaired at the task. Since it has been documented that modafinil only benefits lower-performing subjects on tasks of increased difficulty (see Müller, Steffenhagen, Regenthal, & Bublak, 2004; Randall, Shneerson, & File, 2005), it is possible that the present manipulation was insufficiently challenging for the rats. This consideration also implicitly supports the conclusion that the lesion damage was insufficient, or that the sample size was too small to evidence a reliable effect. It is likely that the intact tissue for the unilaterally-lesioned rat sustained performance, such that there was no impairment against which to measure ameliorative effects of the drugs, although there was only one unilaterally-lesioned rat. This consideration results in a similar conclusion for the four bilaterally-lesioned rats, in which there may have been an impairment in cognition which may have been amenable to the cognitive-enhancing effects of the drug; however the high variance obtained from small sample size (half of the group had a positive probe comparison, whilst half a negative comparison) makes it unadvisable to draw any conclusions regarding performance influenced by drugs, or regarding the construct validity of the early-late probe task. This likely extends to ORG-treated animals as well, since Chase, Tait & Brown (*unpublished*) reported that performance for ORG-treated aged rats only improved performance in aged-rats at previously impaired reversal stages: novel learning stages and attentional set-shifting were unaffected.

During completion of the current experiment, a paper published by Caliph, Faassen, & Porter (2014) found that whilst ORG is orally bioavailable, its oral bioavailability is low, which may be due to its poor intestinal solubilisation or due to intestinal efflux. It is possible that a dose of 10mg/kg administered orally is too low.

#### *4.2.4.3 The early-late probe task*

The fact that there was a difference between the early and the late probe increases confidence that this is a sensitive measure of the change in attentional focus throughout the

task. The task began, in the same way as the 7-stage ASST, with a simple discrimination in which the bowls only differ on one dimension. The CD stage introduces a reward-*irrelevant* dimension, but the rewarded and unrewarded exemplars of the predictive, relevant dimension are unchanged. It might be expected that, having learned which stimulus is rewarded at the SD stage, rats would complete the CD stage without error, however, only about 35% of control rats do so (Tait, *personal communication*), indicating that, at the CD stage, attention is not solely focussed on the ‘correct’ exemplar. Nevertheless, because irrelevant exemplars are *introduced* at the CD stage, it is not directly comparable to a ‘probe’ stage in which irrelevant exemplars are *changed*. For this reason, it is not surprising that acquisition of the CD stage required significantly more trials than the late probe and did not differ from the early probe.

It is likely that the SD stage is not necessary, and the task could start with a CD stage, followed immediately by the early probe. However, the lack of difference between the CD and early probe is good evidence to suggest that an attentional set has not formed by this point in the test. It might be informative to include an assessment of set-shifting performance following the late-probe stage, not least because this would provide evidence against the suggestion that the rats are simply ‘getting better’ with practice. It would be hypothesised that an ED stage following the ID3p would result in a cost of set-shifting, providing an additional – and robustly replicated – measure of attentional set. It would also be interesting to then to include an additional post-ED probe stage (EDp), as it is speculated that qualities/cues of this newly shifted-to dimension are still being learned, and therefore acquiring an EDp stage may exercise similar learning requirements of a CDp stage. This would also serve to strengthen the probe stage as a useful tool in the inference of set-formation, along with increase our understanding of the mechanisms of attentional set-formation.

#### 4.2.4.4 *The efficacy of a palatable jelly tablet*

The refined delivery method introduced in this experimental chapter presents as a reduced-stress alternative to oral gavage – a forced oral route of administration, which elicits undesirable stress responses and is known to alter an animal’s response to a

xenobiotic (Brown, Dinger, & Levine, 2000; Roberts, Soames, James, Gill, & Wheeldon, 1995). The first study in experiment I reported brain concentrations of modafinil, which are similar to previous observations by Waters *et al.* (2005), and both our data and that of Waters *et al.* demonstrate a rapid decrease in concentration after the 60 minutes time-point. Whilst our data shows a gradual decrease in modafinil concentration as time progresses, our data have high variability in the early time-points, with the greatest concentration actually at 60 minutes – the same as reported in Waters *et al.* Both our study and that of Waters *et al.* sampled three rats per time-point, and given the variability in their data at the highest concentrations reported (60 minutes), we do not think it reasonable to conclude that there is a substantial difference between the pharmacokinetic results. Furthermore, unpublished data from oral gavage using a dose of 64mg/kg show a concentration of 350ng/g modafinil in brain tissue at 60 minutes post administration, although again with high variability (Bundgaard, *unpublished observations*) – comparing favourably to our reported 383ng/g concentration at the same time-point.

Rats fed modafinil in jelly form also exhibit behaviour that compares favourably to data from other administration methods, such as a sustained elevation in LMA (both i.p. and intracerebrally; Simon, Hémet, & Costentin, 1996). Both our data and that of Simon *et al.* illustrate a more rapid decline in LMA in vehicle-treated than modafinil-treated rats, followed by stabilisation during later time-periods. Our observations differ, however, in that after the first time-period, modafinil-treated rats are consistently more active than vehicle-treated – whereas Simon *et al.* report differences only at three time-periods (10-20, 30-40 and 70-80 minutes). That we used more than the twice the number of subjects, and tested each twice, suggests that lower variability in our sample accounts for our more robust effect – rather than, for example, a gender or strain effect.

As previously reported by Edgar & Seidel (1997), EEG recordings support the conclusion that modafinil causes an increase in LMA only in proportion to the expected time spent awake, and therefore “LMA intensity” (a measure of increased LMA per minute of time awake) is not affected by modafinil. In contrast, amphetamine-like stimulants not only increase LMA intensity, but also result in stereotyped behaviours such as “compulsive licking, sniffing, biting, chewing, grooming and head-waving” (Duteil *et al.*, 1990). Even



300mg/kg doses of modafinil (Edgar & Seidel, 1997) do not yield increased LMA intensity in rats, and this is likely this is due to modafinil's minimal involvement in the release of striatal dopamine (Akaoka, Roussel, Lin, Chouvet, & Jouvet, 1991; De Sereville, Boer, Rambert, & Duteil, 1994).

Administering modafinil at CT-5 also aided in maximising stimulant efficacy by controlling the amount of prior sleep the animal received. Edgar, Seidel & Dement (1991) examined the sedative effects of triazolam (benzodiazepine) on CT-18 (12h light/dark schedule = 6 hours post lights-off) rats, CT-18 rats in constant darkness and CT-5 rats. They found that triazolam was most effective at promoting its hypnotic effects if administered during lights-off, high activity periods (CT-18), and actually found a reduction in total sleep if administered during lights-on (CT-5) (Edgar *et al.*, 1991). This highlights the large impact prior sleep presents in pharmacological manipulation of sleep/wakefulness, in relation to circadian phases and a light/dark schedule, respectively. CT-5 was an ideal dosage time to examine the wake-promoting effects of modafinil, as CT-5 is a lights-on, low-activity phase. This presents the opportunity to maximise the pharmacological effects of modafinil as a wake-promoting agent, whilst preventing natural circadian wakefulness from masking measurable effects.

The reported jelly administration method presents as an alternative to both oral gavage, and the 'pill' method described in Walker *et al.* (2012). Rich palatability and a capacity to manufacture a higher volume of pills per batch, makes the 'Transgenic dough diet' a viable alternative to oral gavage, but the high fat and protein content is less desirable for food-motivated experiments, and where there is likely to be slower absorption of a drug because of gastrointestinal contents. Additionally, the dough diet requires the drug to be kneaded in (Walker *et al.*, 2012), and therefore final concentration may be inconsistent as there may be irregular distribution within the dough unless pills are made individually. Some drugs also require a solvent to help dissolve them to aid in uniform distribution, and in some cases a thickening agent was added to the dough mixture to help finalise it for drying. The benefit, therefore, of the individualised jellies is that dosage can be customised to the weight of the rat without having to make a larger batch, thereby reducing wastage and being more cost-effective: the gelatine mixture is pipetted on top of the pre-weighed

drug, which can remain in crystalline form. As with the Walker's pill method, any aversive taste the drug may have should be masked by the palatable flavour of the jelly.

#### *4.2.4.5 Conclusions*

In summary, the results reported in the current experiment suggested that acquisition of the late probe stage required fewer TTC than the early probe, regardless of group or drug administration. Despite this improvement, it is not parsimonious to interpret this as a formation of attentional set since control rats failed to evidence a robust ID/ED difference in the interim 7-stage task. Furthermore, lesion damage from a small sample size makes it difficult to conclude with any certainty how lesions of the STN contribute to behaviour here. This may be supported by the apparent lack of an effect of drug on cognitive-enhancement; modafinil and ORG did not facilitate learning in this experiment, which may have stemmed from an insufficient disruption in behaviour to yield an observable effect of the drugs. A refinement of the methodology in which the subthalamic cell population is modulated is therefore essential for determining the involvement of the STN in attentional set-formation.

# Chapter 5

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## Designer receptors transiently inhibit neuronal functioning in the STN

### Background:

Designer receptors, engineered to be sensitive to a designer drug (clozapine *n*-oxide) can be virally-delivered to transduce a cell population to transiently inhibit neuronal functioning. This chapter investigated the best combination of serotype and promoter to transduce the cells of the STN, and to measure inhibition of neuronal function.

### Methods:

The first experiment measured the efficacy of the CaMKII promoter with either AAV2 or AAV5 serotypes. The second experiment measured labelling of c-Fos in the STN following administration of clozapine *n*-oxide or a vehicle to either unoperated rats or those with designer receptors.

### Results:

AAV5-CaMKII-promoted designer receptors transduced the cells of the STN (in some the ZIV), and that systemic administration of clozapine *n*-oxide significantly reduces c-Fos labelling in the STN.

### Conclusions:

Designer receptors present as a viable alternative to lesion surgery to inhibit the functioning of the STN. It is also confirmed that in the absence of clozapine *n*-oxide, the designer receptors do not significantly influence neuronal functioning.

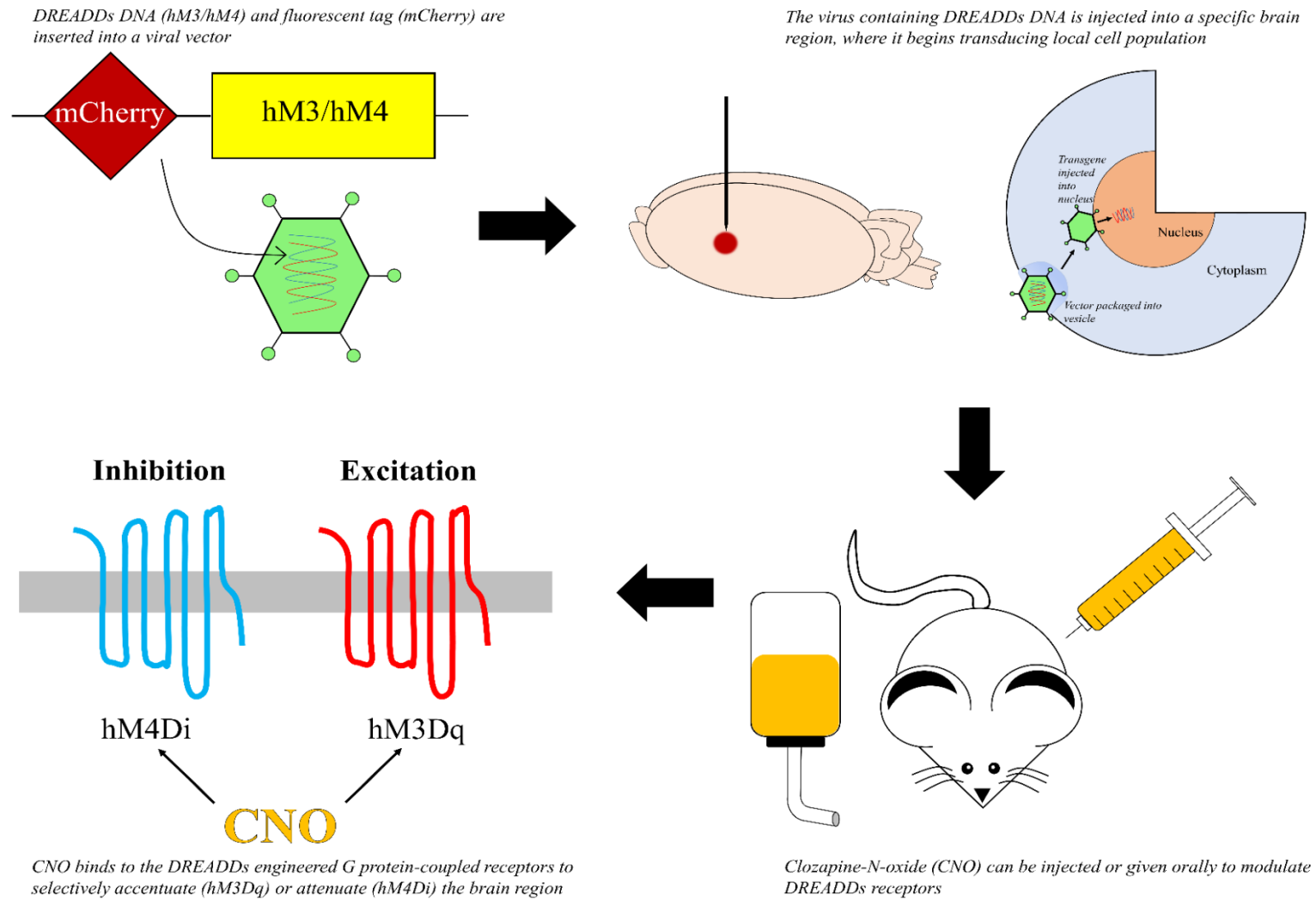
## **5.1 Introduction**

Results from Chapter 3 and 4 (Experiment II) of this thesis were disappointingly consistent, in that the cell damage following ibotenic acid infusions into the medial STN was variable and incomplete. This is in spite of observing reliable behavioural indicators (e.g., immediate post-operative ‘chewing’) that the infusion was located as intended and even observing some behavioural differences in task performance between infused rats and controls, but no differences between infused rats with clear, verifiable lesion damage and infused rats without visible cell-loss. Various measures to improve the consistency of excitotoxin-induced lesions were unsuccessful. Therefore, in the current Chapter, I report the results of transducing the cells within the STN (and ZI) by using pharmacosynthetics or DREADDs (Designer Receptors Exclusively Activated by Designer Drugs). This relatively novel approach to control the elements of signal transduction utilises engineered G protein-coupled receptors (GPCRs) that can be selectively – and non-invasively – modulated by the pharmacologically-inert synthetic ligand clozapine *N*-oxide (CNO) (Urban & Roth, 2015).

Modified human muscarinic acetylcholine receptors are engineered so that they are activated solely by CNO, and are insensitive to their endogenous ligand, acetylcholine (Ferguson & Neumaier, 2012; Rogan & Roth, 2011; Urban & Roth, 2015). Consequently, DREADDs do not themselves, in the absence of CNO, modulate neuronal signalling (Armbruster, Li, Pausch, Herlitze, & Roth, 2007; Farrell & Roth, 2013). This allows for a transient and reversible control of signal transduction: only while CNO is present will the receptors be activated. The receptors are introduced either by packaging into adeno-associated viral (AAV) vectors which are then infused into the brain region of interest, or they can be expressed in a transgenic mouse on a particular population of cells, so that both spatial and temporal control of GPCR signalling cascades can be achieved using DREADDs. DREADDs can be either excitatory (hM3Dq) or inhibitory (hM4Di): depending on the G protein  $\alpha$  subunit, they will either accentuate ( $G_q$ ) or attenuate ( $G_i$ ) cellular activity (figure 5.1).

### **5.1.1 G protein-coupled receptors (GPCRs)**

GPCRs are a type of neurotransmitter membrane receptor with a distinct mechanism of action – each type of GPCR selectively interacts with specific G proteins,



*Figure 5.1: Illustrates the process by which DREADDs-engineered receptors are introduced and modulated in vivo*

and not others, so lending specificity in cellular actions (Feldman, Meyer, & Quenzer, 1997). All G proteins contain a common structure, including three subunits ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) with the  $\alpha$  subunit playing a major role in the outcome cellular process (*i.e.*, excitation or inhibition) and thus determining the nomenclature for that particular G protein ( $G_i$ ,  $G_q$ , etc. with  $i$  and  $q$  denoting the  $\alpha$  subunit) (Feldman *et al.*, 1997).  $G_i$  proteins were one of the first G proteins discovered, and are of particular interest here as they exert an inhibitory effect on adenylyl cyclase, which is an enzyme that promotes intracellular signal transduction via synthesis of the important secondary messenger, cyclic adenosine monophosphate (cAMP), whilst  $G_s$  signalling results in an excitatory effect on cAMP (Feldman *et al.*, 1997; for cAMP review see Robinson, Butcher & Sutherland, 1971). This inhibition of signal transduction – and thus the potential silencing of the subthalamic population – following  $G_i$  DREADDs application is of particular importance for the current experiment. In the case of  $G_i$  DREADDs, the engineered human muscarinic  $M_4$  receptors – now selectively sensitive to CNO – couple to  $G_i$  subunits, which results in hM4Di DREADDs receptors. Following CNO administration these receptors can inhibit forskolin (cAMP agonist)-induced cAMP formation, along with facilitating a G protein inward-rectifying potassium channel (GIRK; see Dascal, 1997) response in the hippocampus; GIRK effectively causes hyperpolarisation and inhibition of firing (Armbruster *et al.*, 2007; Rogan & Roth, 2011).

GPCRs can be found in a wide variety of neurotransmitter receptors, including the muscarinic ACh receptors, all known DA receptors, all known adrenergic receptors, all 5-HT receptors except 5-HT<sub>3</sub>, mGluRs, GABA<sub>B</sub> receptors, along with histamine receptors (Feldman *et al.*, 1997). The dominant postsynaptic projections from STN to the cortex and thalamus are glutamatergic (Galvan, Kuwajima, & Smith, 2006), and activation of Group I mGluRs leads to  $G_q$  signalling, and a net excitatory response, along with increasing NMDA (Kuwajima, Hall, Aiba, & Smith, 2004), whilst Group II mGluRs are negatively coupled to adenylyl cyclase through  $G_i$ , and yield an inhibitory effect on signal transduction (Conn & Pin, 1997; Kuwajima *et al.*, 2004). As presented in the general introduction, *in vitro* electrophysiology studies have determined that both mGluR<sub>1</sub> and mGluR<sub>5</sub> receptor types are found on postsynaptic dendrites of the rodent STN (Awad, Hubert, Smith, Levey, & Conn, 2000), whereas mGluR<sub>2</sub> and mGluR<sub>3</sub> are presynaptically localised on STN terminals (Bradley *et al.*, 2000).

Targeting these mGluRs in the medial STN area with DREADDs-engineered GPCRs should yield neuronal silencing for this population, along with potentially transducing neighbouring populations of GPCRs within the medial ZID. The cortical projections into this region overlap between the medial ZI and the STN, with some suggesting that there is also an overlap in functioning (Kita, Osten, & Kita, 2014). Furthermore, the predominant neurotransmitter of the ZI is GABA (see General Introduction), and the ZI also contains a population of G-protein coupled GABA<sub>B</sub> receptors, increasing the likelihood of transducing cells within this region (Mitrofanis, 2005; Park, Hoffman, & Keller, 2014).

### **5.1.2 Adeno-associated viral vectors**

The AAV itself is a virus responsible for infecting a target cell population for the purpose of delivering modified deoxyribonucleic acid (DNA), and for the current experiment, DNA which is responsible for the replication and proliferation of the DREADDs-engineered receptors (Shevtsova, Malik, Michel, Bahr, & Kugler, 2005). AAV vectors are able to transduce a wide range of dividing and non-dividing cell types, along with providing long-term expression of transgenes – modified genetic material that has been transferred between organisms (Deyle & Russell, 2009). The removal or ‘gutting’ of the endogenous genetic material of the AAV renders it non-infectious and unable to autonomically replicate, and therefore the AAV requires the help of an adenovirus to propagate the desired transgene – packaged as a plasmid (an independently-replicating DNA molecule) – for encoding, and the use of a ‘promoter’ to specify the target-cell population (Berns & Hauswirth, 1979; Muzyczka, 1992; Urban & Roth, 2015). Utilising a cell-type specific promoter, combined with selecting the appropriate ‘serotype’, which controls the ‘spread’ or tropism of the viral application, maximises the likelihood of restricting viral transgene proliferation to a desired target cell population (Rogan & Roth, 2011). For the present experiment, introducing AAVs containing DREADDs-engineered receptors into the rat STN in an effort to achieve localised transduction of the cells within medial area was a challenge; particularly since there are no existing surgical protocols for this novel application to the rat STN, and therefore it was necessary to find an appropriate combination of both serotype and cell-specific promoter.

### 5.1.3 Serotype

The serotype of an AAV provides a method of classification based on the viral tropism exhibited, viral tissue type, and the organisation/frequency of the cell surface viral antigens; early AAV vectors were all based on the AAV2 serotype (Kaplitt *et al.*, 1994; Korecka *et al.*, 2011). The AAV2 serotype is renowned for its relatively conservative tropism compared to other serotypes – such as AAV1 and AAV8 – whilst still exerting a higher transduction percentage of a variety of cell types compared to AAVs with a more widespread distribution (Burger *et al.*, 2004; Paterna, Feldon, Büeler, & Bu, 2004; Vandenberghe *et al.*, 2011; Wang, Wang, Clark, & Sferra, 2003). This conservative tropism makes the AAV2 serotype a prospective candidate for transduction of the cells in the relatively compact medial STN; however a comparison of AAVs with serotypes 1-8 indicated that the novel AAV5 serotype was the most effective overall, by yielding the highest percentage of transduction for dorsal root ganglion neurons, along with a robust green fluorescent protein (GFP) expression (Mason *et al.*, 2010). Paterna *et al.* (2004) conducted a titre-matched comparison of the transduction profiles for AAV5 and AAV2 serotypes in the rat nigrostriatal system, and determined that AAV5 application resulted in a larger GFP-expressing area than AAV2 for neurons in the striatum, along with the dopaminergic neurons in the substantia nigra pars reticulata (SNr) and pars compacta (SNc).

Further evidence suggests that AAV5 and AAV2 are comparable in their efficiency in transducing a variety of neurons in the rat CNS – including the hippocampus, substantia nigra, striatum, globus pallidus and spinal cord – yet AAV5 rats exhibited higher transduction frequencies, which translates into a larger tropism than AAV2 rats (Burger *et al.*, 2004). Given that both AAV2 and AAV5 serotypes transduce cells within the CNS with comparable efficacy, yet the latter induces a larger tropism, both serotypes could serve as suitable candidates for transducing the cells within the medial STN for the current experiment. Successful transduction of these cells can be further controlled by coupling these two serotypes with the correct promoter.

### 5.1.4 Promoter

The promoter is a sequence of genetic code found in the DNA – and in the case of DREADDs, the transgene DNA – and is responsible for binding to the ribonucleic acid polymerase (RNAP) and commencing transcription; essentially the promoter



indicates the starting point for gene expression (which varies depending on the promoter type), and in the case of a viral vector, the promoter drives the translation of the transgene (Losos, Mason, Singer, Raven, & Johnson, 2008). AAV administration of DREADDs, developed by Dr Bryan Roth, is currently limited to four promoters: human elongation factor-1-alpha (Efla); glial-fibrillary acidic protein (GFAP); human synapsin (hSyn); and  $Ca^{2+}$ /calmodulin-dependent protein kinase II alpha (CaMKIIa) (Urban & Roth, 2015).

The GFAP promoter expresses in mainly non-neuronal glial cell types (Urban & Roth, 2015), and Efla is responsible for cell growth, protein synthesis and cytoskeleton organisation (Becker, Kuhse, & Kirsch, 2013; Kim, Uetsuki, Kaziro, Yamaguchi, & Sugano, 1990). Therefore, neither were considered well suited to this particular target population. The hSyn promoter has shown promise in conferring a neuron-specific, long-term transgene expression, lending a pan-neuronal application by transducing the variety of neurons/neurotransmitter systems found in the striatum, thalamus, hippocampus and the SNc, whilst sparing glial cells (Kügler, Kilic, & Bähr, 2003). By transducing all neuronal cell-types, the hSyn promoter is more applicable to the current experiment than GFAP or Efla; however CaMKIIa – which is a  $Ca^{2+}$ -activated enzyme that is highly abundant in the CNS – is central to the regulation of glutamatergic synapses (for review see: Lisman, Schulman, & Cline, 2002).

The major postsynaptic density (PSD) protein found at glutamatergic synapses is CaMKII (Kennedy, Bennett, & Erond, 1983). PSD is a protein that is found on the postsynaptic membrane, and is responsible for regulating synaptic adhesion, organizing neurotransmitter receptor clustering, and modulating receptor sensitivity (Kennedy, 1993). Furthermore, it has been determined that CaMKII phosphorylates the glutamate receptor (GluR), and that PSD CaMKII is critical in strengthening postsynaptic GluR responses (McGlade-McCulloh, Yamamoto, Tan, Brickey, & Soderling, 1993). It is important to note that CaMKII also phosphorylates GABA<sub>A</sub> receptors as well, which are found throughout the rodent ZI, suggesting that CaMKII transcription may regulate GABA<sub>A</sub> receptor activity (Houston, He, & Smart, 2009). Therefore, a CaMKII-promoted DREADDs application was regarded the most likely to result in refined targeting of the STN, compared to other available promoters. Even so, transducing only

the cells within the medial STN, without any activity outside the STN itself, is likely not possible with this technology.

### 5.1.5 Efficacy of DREADDs inhibition

hM4Di DREADDs receptors have demonstrated robust efficacy for – selectively, non-invasively (compared to optogenetics), and transiently – attenuating cellular activity after CNO administration, in a variety of cell types which are not limited to the CNS (Armbruster *et al.*, 2007; Kätzel, Nicholson, Schorge, Walker, & Kullmann, 2014; Ray *et al.*, 2011; Sasaki *et al.*, 2011). Kätzel *et al.* (2014) found that silencing the rat forelimb area of the primary motor cortex – via hM4Di DREADDs – significantly suppressed focal seizures induced by a chemoconvulsant, presenting a novel therapeutic approach for intractable focal epilepsy. Furthermore, *in vitro* transduction of cultured hippocampal neurons with hM4Di induced membrane hyperpolarisation, along with neuronal silencing (Armbruster *et al.*, 2007). The use of hM4Di aided in establishing that serotonergic neurons regulate life-sustaining respiratory and thermoregulatory networks (Ray *et al.*, 2011): after application of hM4Di to the serotonergic neurons of the brainstem, CNO-treated mice showed no increase in respiration as CO<sub>2</sub> concentration increased and exhibited a decrease in core temperature, whilst saline-treated rats increased their respiration in response to rising CO<sub>2</sub> levels, with no noticeable change in thermoregulation.

Targeting the corticostriatal neurons originating from the striatum with hM4Di, followed by chronic CNO administration, resulted in decreased synaptogenesis (the formation of synapses) by day 15 of treatment, which persisted into adulthood at day 28 (Farrell & Roth, 2013; Kozorovitskiy, Saunders, Johnson, Lowell, & Sabatini, 2012). This also demonstrated that DREADDs+CNO can be used to provide chronic neuronal modulation, and that recurrent network activity along this pathway is crucial in synaptogenesis and healthy development. Transient inactivation of neurons via hM4Di in the rat dorsal striatum impaired learning acquisition in a simple operant lever pressing task, which also demonstrated that dysregulation of striatal circuits may contribute to the development of aberrant reward and reinforcement learning (Ferguson & Neumaier, 2012). Being a relatively novel investigative tool, little research has been published regarding the application of DREADDs to the BG for the purpose of investigating cognition.

### 5.1.6 Clozapine N-oxide

DREADDs engineered receptors have been designed to selectively interact only with CNO, which is a pharmacologically inert metabolite of the antipsychotic drug, clozapine (Alexander *et al.*, 2009; Guettier *et al.*, 2009). Developers of DREADDs technology elected to use CNO as a tool for cellular modulation for several key reasons, primarily since CNO's parent compound, clozapine, has a high affinity to M<sub>3</sub> muscarinic receptors, and thus fewer genetic mutations would be required to engineer the receptors (Armbruster *et al.*, 2007). Furthermore, CNO is also highly bioavailable in humans and rodents. It has been shown that in rats, clozapine penetrates the blood-brain barrier faster than CNO, yet concentrations of CNO and clozapine are comparable for time periods >60 minutes (Bender, Holschbach, & Stöcklin, 1994; Chang *et al.*, 1998; Guitton, Abbar, Kinowski, Chabrand, & Bressolle, 1998a). Perhaps most importantly, CNO was selected since it is a pharmacologically inert molecule in rodents, lacking appreciable (<1 µM) affinity for receptors (Armbruster *et al.*, 2007; Weiner *et al.*, 2004); however in humans, CNO undergoes extensive back-metabolism to clozapine (Chang *et al.*, 1998; Guitton, Abbar, Kinowski, Chabrand, & Bressolle, 1998).

CNO modulation of DREADDs receptors illustrates a rapid onset of excitation or inhibition, followed by a prolonged and sustained effect on signal transduction (Guettier *et al.*, 2009; Ray *et al.*, 2011; Rogan & Roth, 2011; Sasaki *et al.*, 2011). In humans the mean elimination half-life of CNO is more than 7 hours (Guitton *et al.*, 1998b), but in mice, plasma levels determine that CNO half-life it is much shorter (~2 hours), yet despite this, the interaction of CNO with DREADDs results in significant behavioural and electrophysiological modulation ranging from 6-10 hours (Alexander *et al.*, 2009; Guettier *et al.*, 2009; Wess, Nakajima, & Jain, 2013). In the aforementioned study, Ray and colleagues (2011) successfully inhibited the serotonergic neurons of the rat brainstem within 10 minutes of CNO application (i.p.), and sustained this inhibition for nearly 12 hours, causing decreased core temperatures, and a blunted response to increased CO<sub>2</sub> concentrations.

There is evidence suggesting that the dosage of CNO required depends on the type of DREADD subtype (G<sub>q</sub> vs G<sub>i</sub>) utilised, in that hM3Dq DREADDs are very effective at depolarising neurons, thus requiring a relatively low dose of CNO (0.03 – 1 mg/kg; Alexander *et al.*, 2009; Boender *et al.*, 2014; Guettier *et al.*, 2009), whereas

hM4Di is reportedly less effective at inhibiting neuronal firing, necessitating the usage of a higher CNO dose (10 mg/kg in rats: Ray *et al.*, 2011; 5 mg/kg in mice: Sasaki *et al.*, 2011; see Farrell & Roth, 2013). Experiments in this thesis should therefore utilise a higher dosage of CNO.

### 5.1.7 Fluorescent proteins

All AAV DREADDs are engineered and tagged with a fluorescent protein (AAV5: mCherry; AAV2: mCitrine; Roth Lab, UNC Vector Core), which can be enhanced with an anti-body and imaged via fluorescence microscopy to determine viral tropism. Both mCherry and mCitrine are fluorophores – chemical compounds that absorb light (excitation: mCherry: 587nm; mCitrine: 516nm) at one wavelength, then emit (emission: mCherry: 610nm; mCitrine: 529nm) it at another – and in the case of mCherry, and mCitrine, of the red and yellow wavelength class, respectively (Losos *et al.*, 2008; Shaner, Steinbach, & Tsien, 2005; Tsien, 1998). Furthermore, mCherry has been praised as the best general-purpose red monomer owing to its superior photostability – a resistance to photobleaching which arises from prolonged excitation – thus increasing long-term viability of the fluorophore (Shaner *et al.*, 2004; 2005).

### 5.1.8 Current experiment

The purpose of the current experiment was to determine the most efficacious combination of serotype to be used with the CaMKIIa promoter to transduce the cells within the STN to provide an inhibitory effect on neuronal transmission. The first study of this experimental chapter will target infusions of both AAV2 and AAV5 serotyped, CaMKIIa-promoted, hM4Di DREADDs into the medial STN, with the same stereotaxic coordinates as employed in Chapter 3 and 4 (Experiment II). Previous experiments have determined that the required volume of the injected virus is similar to that of a neurotoxin in lesion surgery, for example, Kätzel *et al.* (2013) transduced the cells within layer 5 of the forelimb area of the rat right primary motor cortex with 1.5µl of AAV5, whilst Boender *et al.* (2014) utilised 1µl to transduce the dopaminergic neurons that project from the VTA to the nucleus accumbens (Acb). In both experiments, the target brain regions occupied a larger spatial area than the medial STN, and therefore the current experiment should employ a viral volume less than 1µl. Detection of

transduction will be determined by validation of tropism by detecting the tagged fluorescent proteins via fluorescence microscopy.

The second study in this chapter will evaluate the inhibitory effects of DREADDs delivered to the medial STN by measuring and comparing the levels of c-Fos immunoreactivity. The gene c-Fos is expressed within neurons following voltage-gated calcium entry into the cell, which leads to a rapid and transient induction of c-Fos; the transcription of which can be identified by immunohistochemical techniques, and used as a marker for neuronal activity (Bullitt, 1990). A Latin square design was used for this study, which compared c-Fos transcription in unoperated rats with DREADDs-infused animals, both with and without CNO treatment. Since spontaneous c-Fos expression is relatively low in the STN (Sgambato, Abo, Rogard, Besson, & Deniau, 1997; Turner, Gray, Mickiewicz, & Napier, 2008), finding a way to endogenously augment c-Fos transcription in the STN was necessary. A study by Gompf and colleagues (2010) found that rats placed in a novel, environmentally complex setting with other rats spent over 300% more time awake, compared to their routine activity at that time of day. A subsequent study by Qiu, Chen, Huang, & Lu (2014) found that rats exposed to this ‘active-wake’ condition for 2 hours expressed significantly higher counts of c-Fos transcription in several areas of the BG, including the STN. We therefore hypothesise that following an ‘active-wake’ environment, DREADDs+CNO rats will express a reduction in c-Fos transcription owing to inhibition of neural transmission, whereas c-Fos levels for unoperated rats and DREADDs+vehicle rats should be comparable, as it is hypothesised that CNO treatment should not influence c-Fos transcription.

## **5.2 Materials and methods**

### **5.2.1 Animals**

Sixteen male, Lister hooded rats were used in this experimental chapter. Four rats (bred in-house; University of St Andrews, *from Charles River stock*) were used in experiment I, to investigate the most efficacious combination of promoter and serotype. These rats had a mean weight of 387g (range: 369-414g) at the start of the experiment, and a mean weight of 392g (range: 377-415g) upon sacrifice. Twelve rats (Charles River, UK) were used in experiment II, with six rats receiving bilateral, DREADDs infusions targeting the medial STN, whilst the remaining six rats were unoperated. Rats had a mean weight of 346g (range: 325-361g) at the start of the experiment, and a mean weight of 394g (range: 363-425g) upon sacrifice. All rats were experimentally naïve prior to testing in experiment II, which was completed over two days, three weeks post-surgery.

### **5.2.2 Surgery**

The protocol for pharmacosynthetic surgery was nearly identical to ibotenic acid lesion surgery, with some key exceptions. Briefly, rats were anaesthetised with an isoflurane (5% at induction, maintained at 1-2%) and oxygen mixture. Similar to ibotenic acid surgery, rats were given 0.05ml 5% carprofen (s.c.), but were not given any diazepam as there was no administration of an excitotoxin that would require sedation post recovery from the anaesthesia. After drilling burr holes in the skull, the rats were infused with the viral vectors: for study I, two rats were given AAV5-CaMKIIa-hM4D(G<sub>i</sub>)-mCherry (UNC Vector Core, University of North Carolina, USA), whilst another two rats were given AAV2-CaMKIIa-HA-hM4D(G<sub>i</sub>)-IRES-mCitrine (UNC Vector Core, University of North Carolina, USA). For study II, six rats received AAV5-CaMKIIa-hM4D(G<sub>i</sub>)-mCherry only. Infusions were made bilaterally (0.5µl per site) using a Hamilton syringe (Aldrich Chemical Company, Milwaukee, WI, USA) with a 30 gauge round-tipped needle at the stereotaxic coordinates AP: – 3.8mm, ML: ±2.3mm, DV: – 7.8mm (Paxinos & Watson, 1986). Infusion of the viral vector were completed over three minutes, and the needle was left *in situ* for a further three minutes. Rats were returned to pair-housing the day after surgery and were given three weeks to allow for transduction of the viral vector prior to sacrifice.

### 5.2.3 Apparatus

The ‘active-wake’ condition previously described (Gompf *et al.*, 2011; Qiu *et al.*, 2014) employs a novel, environmentally-enriched setting with other (previously unpaired) rats to engage with, in order to increase social and motor activity. Previous experiments employed different apparatuses: either a sleep-recording chamber (Gompf *et al.*) or an open-field environment (Qiu *et al.*); similarly, we designed a large wooden enclosure (66 cm x 66 cm x 40 cm) to serve as an open-field, which could comfortably contain two small home cages without cage lids for study II (see section 2.1 for cage dimensions). The cages were filled with sawdust, and environmental enrichment was presented in the form of cardboard houses, wooden chew-bars, and shredded cardboard.

### 5.2.4 Habituation

Three days before behavioural testing, rats were habituated to i.p. injections by being administered 2ml/kg saline (0.9% NaCl) each day, for two days. This was done to reduce secondary behavioural effects associated with the stress of injection (Meijer, Spruijt, van Zutphen, & Baumans, 2006).

### 5.2.5 Behavioural testing

Rats were assigned to groups in order to complete the open-field manipulation, and were grouped so that no rat was presented alongside its cage-mate, in order to maximise social novelty. Four rats were tested during a given test session: two rats had DREADDs infusions to the medial STN, and two were unoperated; of this, one rat per surgery condition received CNO (10mg/kg in 5mg/ml of 0.9% saline; Sequoia Research Products Ltd., UK), whilst the other received a vehicle injection (0.9% saline). We have previously observed that high concentration doses of CNO (10 mg/ml) causes skin irritations, manifesting as lesions in some rats (*unpublished observations*); therefore a concentration of 5 mg/ml was used in the present study with no observable adverse effects.

Thirty minutes prior to behavioural testing, rats were given an i.p. injection of either CNO ( $n=6$ ) or vehicle ( $n=6$ ) and were placed in a small home cage until the commencement of behavioural testing. Rats were introduced to the open-field thirty minutes following administration of the injection, and were left to explore the

environment and engage with other rats for a duration of two hours. After two hours had elapsed, rats were removed from the open-field and transcardially perfused.

### 5.2.6 Histology

Tissue was collected as described in section 2.5 of the general methods chapter. Briefly, rats were transcardially perfused with PBS and paraformaldehyde and the brains were removed and refrigerated overnight at 4°C in 20% sucrose solution. Brains were then set in egg yolk in a formaldehyde bath to maintain tissue structure during slicing. Sections were sliced into 50µm sections, subdividing tissue collection into eight collected sets, such that one section was collected every 400µm per set. Tissue was stored in an anti-freeze solution at -20°C, until staining for immunohistochemistry.

Fluorescent immunoreactivity was detected by examining the extent of either mCherry or mCitrine fluorescent proteins, and the case of the latter, by targeting the human influenza haemagglutinin (HA) tag that was part of the AAV2-CaMKIIa-**HA**-hm4D(G<sub>i</sub>)-IRES-mCitrine DREADD. Sections were washed with PBS in 9-hole net wells, five times for three minutes each. Sections were then placed on a stirrer for 1 hour in blocking solution (20% normal goat serum, 0.1% triton in 0.1M PBS) and then washed again. Sections were then incubated in the primary antibody, either rabbit anti-mCherry (1:2000; Abcam, Cambridge, UK) or rabbit anti-HA tag (1:2000; ChIP grade, Abcam, Cambridge, UK) in 3ml histology pots, suspended in ADS, and left on a stirrer overnight. The following day sections were washed five times for five minutes each in 9-hole net wells. Sections were then returned to foil-wrapped histology pots and incubated in the secondary antibody, either preadsorbed goat anti-rabbit IgG H&L (1:500; Alexa Fluor 594, Abcam, Cambridge, UK) or goat anti-rabbit IgG H&L 1:500; Alexa Fluor 488, Abcam, Cambridge, UK) in ADS for one hour. Sections were washed five times for five minutes each in 9-hole net wells, and then mounted to glass slides. Vectashield antifade mounting medium with DAPI counterstain (DAPI: 4',6-diamidino-2-phenylindole; Vector Laboratories Ltd., Peterborough, UK) was pipetted on to the slides, which were then coverslipped and the edges sealed with nail polish. DAPI binds to A-T rich sequences of DNA and emits a blue fluorescent stain, aiding in visualisation of nuclear cell bodies (Kapusinski, 1995). Fluorescence microscopy was completed with a confocal microscope (Zeiss, Cambridge, UK) using Novell ZENworks software (Micro Focus, Newbury, UK).



For c-Fos/DAB immunoreactivity, sections were first washed with PBS in 9-hole net wells, five times for three minutes each. Sections were then placed on a stirrer for 1 hour in blocking solution (20% normal goat serum, 0.1% triton in 0.1M PBS) and then washed again. Sections were then placed in histology pots and incubated with 5ml of the primary antibody, biotinylated rabbit anti-c-Fos (1:10000; Calbiochem, San Diego, USA) suspended in antibody diluting solution (ADS: 0.1M PBS, 1% normal goat serum, 0.1% triton) and left on a stirrer overnight. The following day, sections were returned to 9-hole net wells and washed and incubated in vector IgG solution (anti-rabbit IgG; 5µl/ml; Vector Laboratories Ltd., Peterborough, UK) on a stirrer for one hour. Sections were then washed again, and incubated in reagents A & B (10µl/ml each) of Vectastain ABC complex (Vector Laboratories Ltd., Peterborough, UK) for another hour. Sections were washed again and treated with DAB (Sigma Chemical Company, St Louis, MO, USA) for approximately 10-15 minutes, until tissue was stained with a dark-brown colour, with minimal background staining. Sections were then washed again and mounted on gelatine-subbed glass slides. Sections were then de-fatted with xylene before being coverslipped with DPX mountant as described in section 2.5 of the General Methods chapter; however sections were not stained with cresyl violet. c-Fos transcription was determined with light microscopy at 2.5x and 10x magnifications.

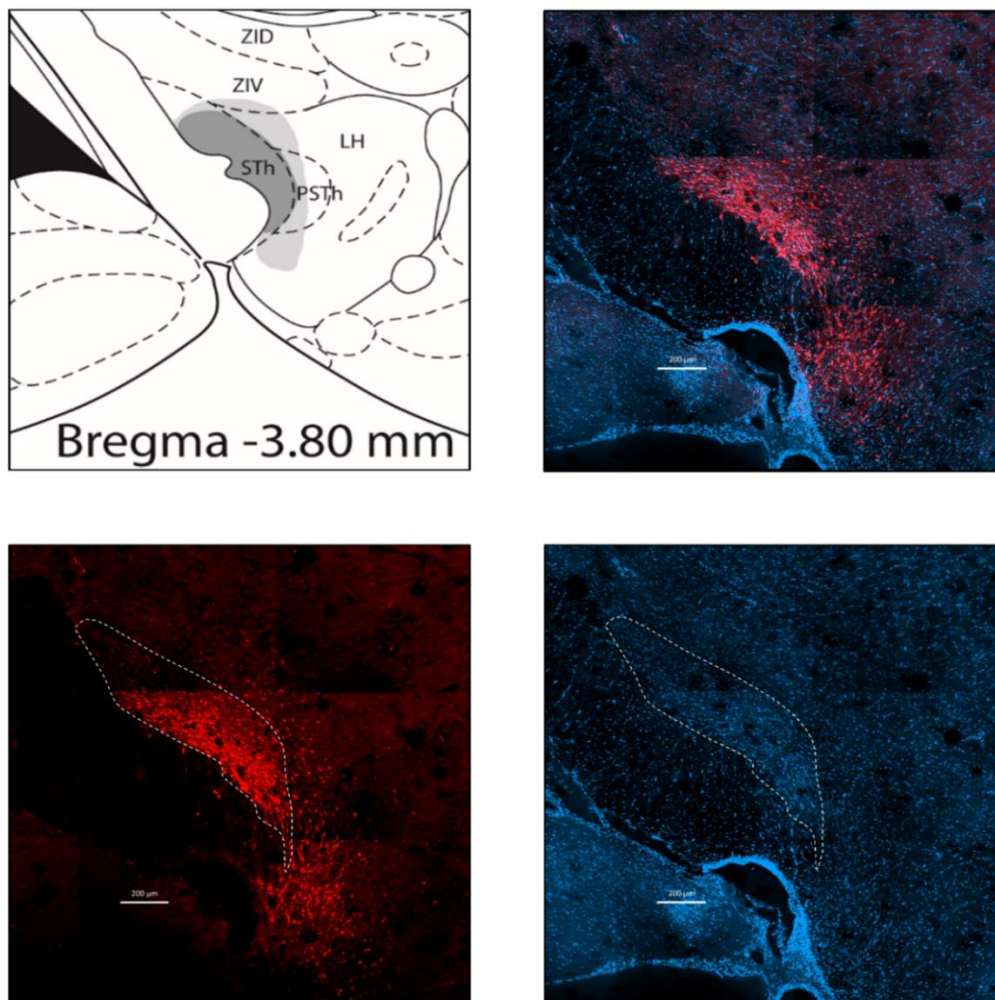
### **5.2.7 Data analysis**

For study II, c-Fos positive cells in four discrete sections of the STN were counted by hand following microscopy, with a transparent grid superimposed over the image to reduce the possibility of 'repeat-counting'. The design followed a 2x2x2x4 mixed factorial design, and c-Fos labelled cells were analysed by repeated-measures ANOVA. There were two within-subjects factors: side (two levels: left hemisphere and right hemisphere), and brain section (four levels), and two between-subjects factors: CNO (two levels: CNO or vehicle), and surgery (two levels: DREADDs transduction or unoperated).

## 5.3 Results

### 5.3.1 Study I

Neither of the two AAV2-mCitrine rats expressed GFP when targeting the HA tag. These sections illustrated minimal background staining with sparse evidence of GFP cells in the STN or ZI. Conversely, mCherry was detected in all rats administered AAV5-mCherry. The AAV5 profile included robust transduction of the medial STN area, whilst sparing the lateral STN; furthermore, viral tropism extended medially into the LH and dorsally into the ZIV (figure 5.1). Given this evidence, AAV5-CaMKIIa-hM4D(G<sub>i</sub>)-mCherry was selected as the best-suited DREADD for transducing the cells within the STN.

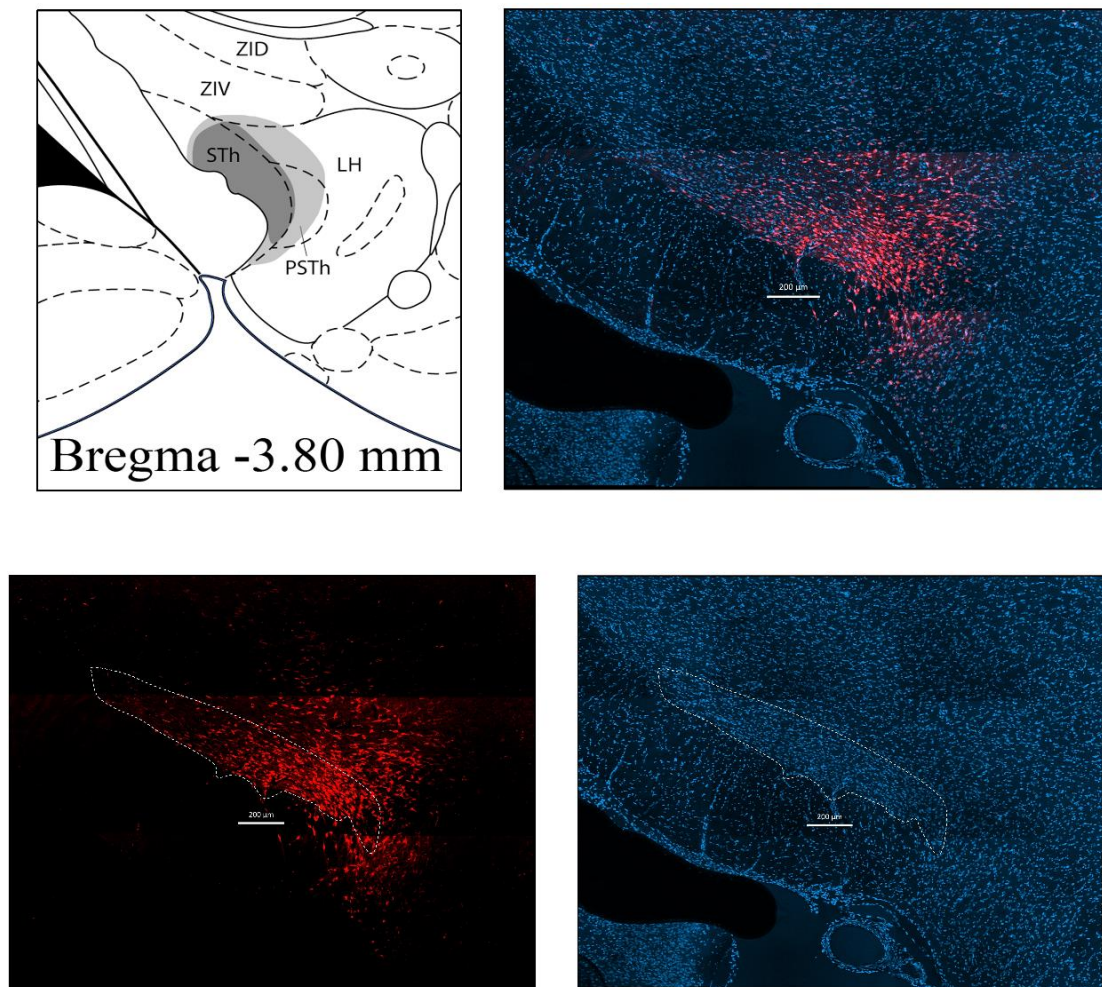


*Figure 5.2: Schematic illustrating the largest (light grey) and smallest (dark grey) area of viral transduction, presented with photomicrographs depicting the mCherry (red) fluorescent protein, with DAPI counterstain of DNA nuclei (blue). White hashed lines indicate the outline of the STN cell population.*

## 5.3.2 Study II

### 5.3.2.1 Viral tropism

Fluorescence microscopy revealed that all six rats which received bilateral DREADDs to the medial STN expressed mCherry in this area. Results indicated a robust transduction of the medial STN, whilst sparing the lateral STN; furthermore, viral tropism extended medially into the LH and dorsally into the ZIV (figure 5.3). However, given that transduction of these adjacent regions was minimal, and mostly near the dorsolateral border of the STN, counting of c-Fos was only conducted in the STN.



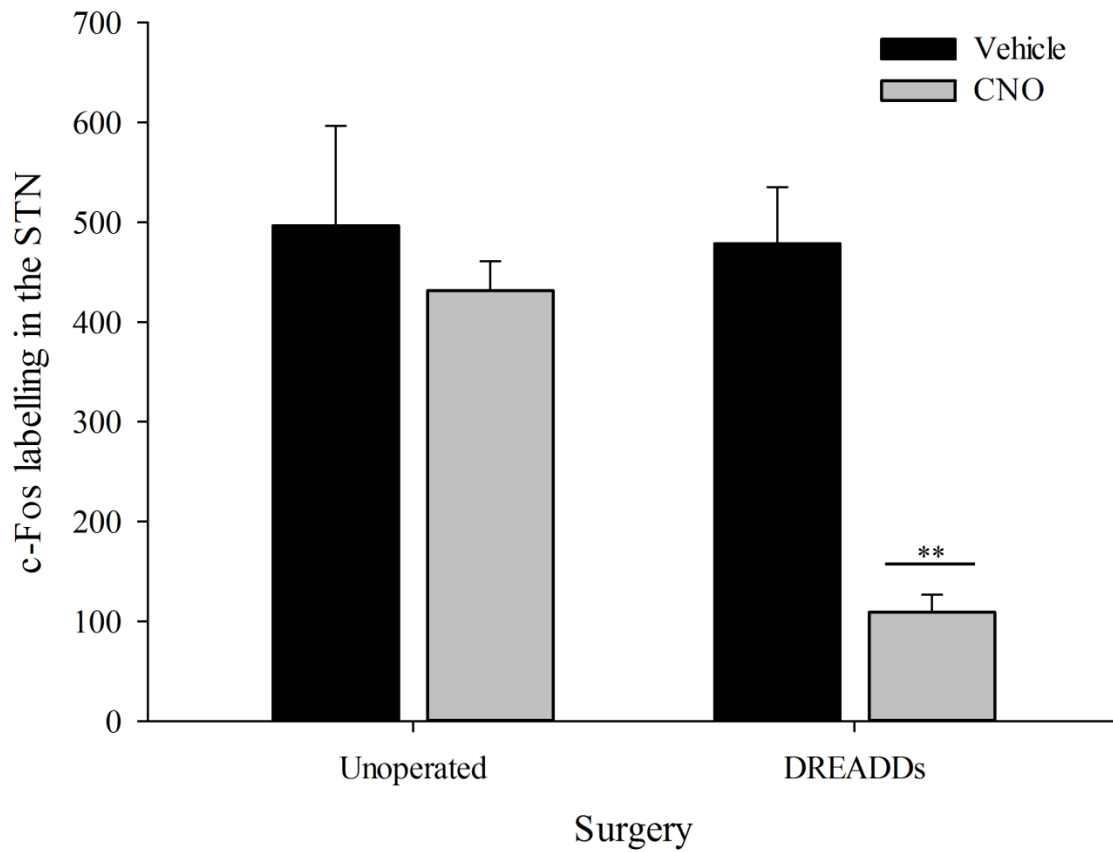
*Figure 5.3: Schematic illustrating the largest (light grey) and smallest (dark grey) area of viral transduction, presented with photomicrographs depicting the mCherry (red) fluorescent protein, with DAPI counterstain of DNA nuclei (blue). White hashed lines indicate the outline of the STN cell population*

### 5.3.2.2 c-Fos

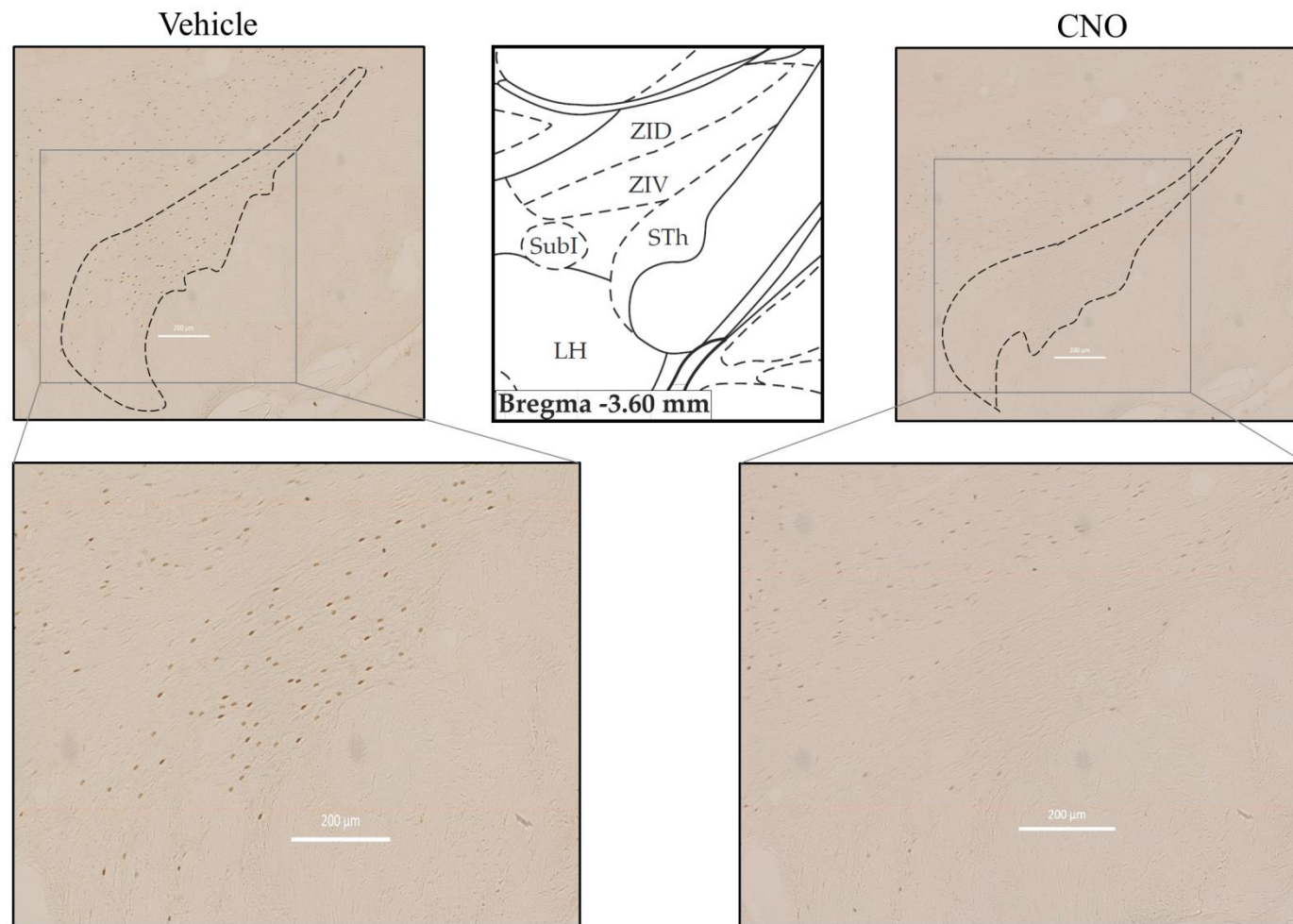
The rodent STN in particular has a very dense population of neurons, compared to the human or monkey brain (Hardman *et al.*, 2002), and given the compact size of this region, taking a total count of c-Fos might incur the possibility of duplicate counting from adjacent tissue without applying a corrective algorithm (see Hedreen, 1998). To account for this potential issue (commonly referred to as ‘lost caps’), I report a total count of c-Fos from four sections (200µm apart) through the STN roughly between Bregma -3.6 mm and -4.3 mm.

A repeated-measures ANOVA revealed that the most caudal STN section presented with reduced c-Fos labelling compared to the remaining three sections, which was expected as the spatial area of the STN varies by section (*main effect of Section*:  $F_{(2.94,23.51)} = 3.47$ ,  $p < 0.05$ ; *Huynh-Feldt Correction*; *Bonferroni-corrected pairwise comparison*; *uncorrected*  $df = 3,24$ ;  $\eta^2 = 0.30$ ). However, this decrease in c-Fos labelling in the most caudal section was consistent for both surgery groups and was observed irrespective of CNO treatment (*Section by Surgery group interaction*:  $F_{(2.93, 23.51)} = <1$ , *n.s.*; *uncorrected*  $df = 3,24$ ;  $\eta^2 = 0.03$ ; *Section by CNO interaction*:  $F_{(2.93, 23.51)} = 1.01$ , *n.s.*; *uncorrected*  $df = 3,24$ ;  $\eta^2 = 0.11$ ). This analysis also determined that c-Fos labelling was comparable in both hemispheres of the brain, and that receiving either DREADDs and/or CNO did not influence this (*main effect of Side*:  $F_{(1,8)} = 1.67$ , *n.s.*;  $\eta^2 = 0.17$ ; *Side by Surgery group interaction*:  $F_{(1,8)} = 2.61$ , *n.s.*;  $\eta^2 = 0.25$ ; *Side by CNO interaction*:  $F_{(1,8)} = 4.67$ ,  $p = 0.063$ ;  $\eta^2 = 0.37$ ).

Although DREADDs-infused rats exhibited less c-Fos labelling overall, and that CNO treatment led to reduced c-Fos labelling as well, a Bonferroni-corrected pairwise comparison revealed that only DREADDs-infused animals that had also received CNO expressed a marked reduction in c-Fos labelling (*main effect of Surgery group*:  $F_{(1,8)} = 8.06$ ,  $p < 0.05$ ;  $\eta^2 = 0.50$ ; *main effect of CNO*:  $F_{(1,8)} = 13.07$ ,  $p < 0.01$ ;  $\eta^2 = 0.62$ ; *Surgery group by CNO interaction*:  $F_{(1,8)} = 6.42$ ,  $p < 0.05$ ;  $\eta^2 = 0.45$ ; figure 5.4: A & B). This result suggested that DREADDs were capable of inhibiting neuronal transmission in the STN, and that in the absence of CNO, they do not exert a significant effect on signal transduction in this area.

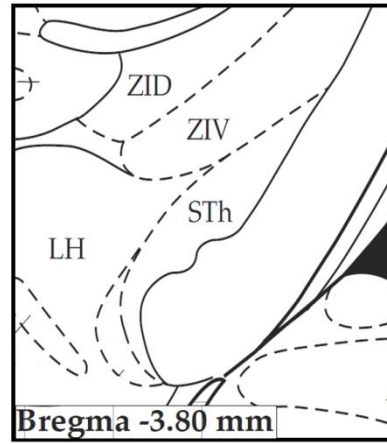
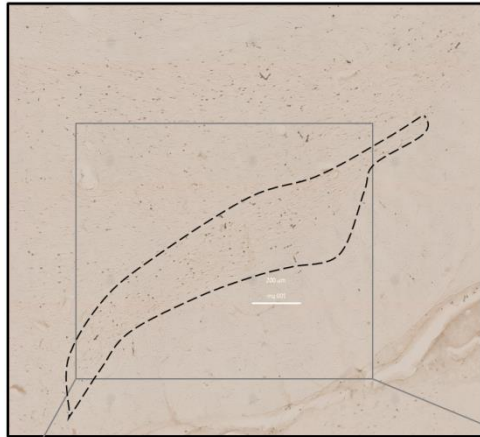


*Figure 5.4a: Mean counts (+SEM) of c-Fos labelling in the STN; counts were taken from four sections of tissue between Bregma -3.6mm to -4.3mm; application of CNO significantly reduced c-Fos expression but only for DREADDs-infused animals.*

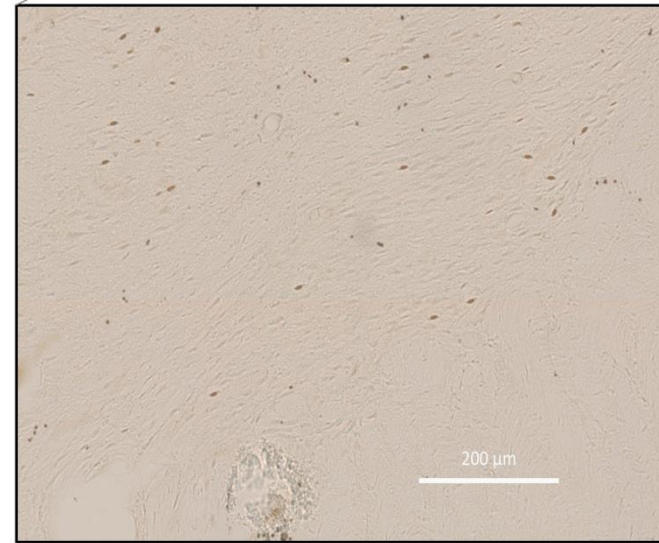
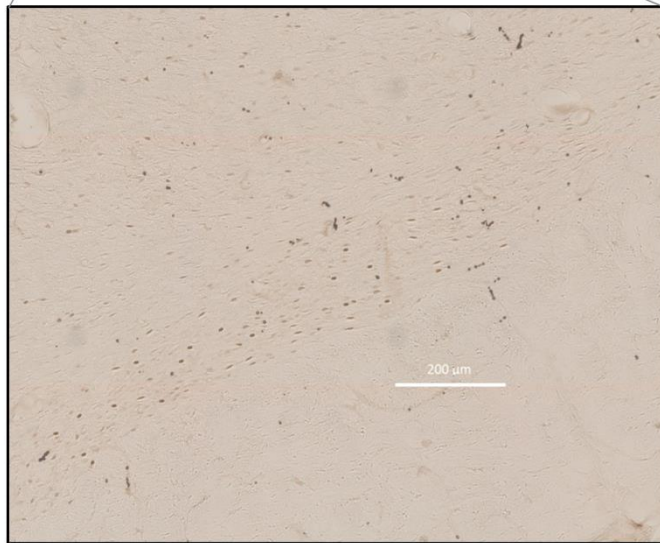
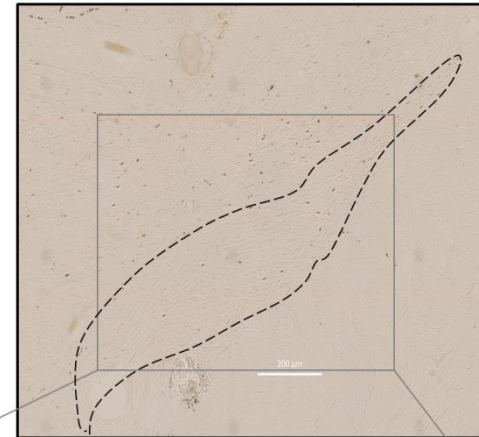


*Figure 3.4b: Photomicrographs and schematics of the STN for DREADDs-infused animals; Staining for c-Fos illustrated a visible reduction in transcription following CNO application, compared to vehicle treatment.*

Vehicle



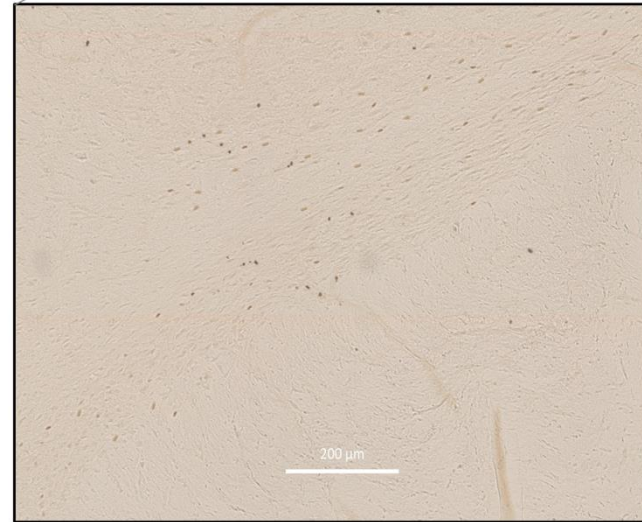
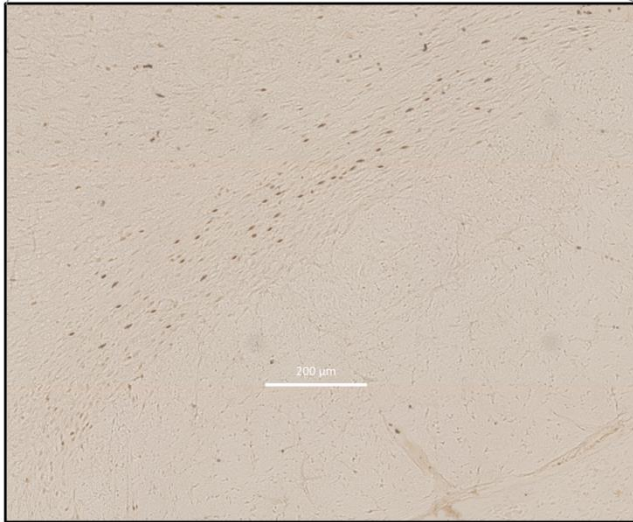
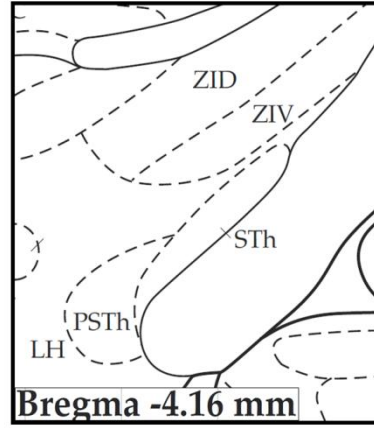
CNO



Vehicle



CNO





## **5.4 Discussion**

The present experiment explored the efficacy of a relatively novel, investigative tool – DREADDs – in providing transient control of signal transduction in the STN. The first study reported that application of an AAV5 serotype, CaMKIIa-promoted, inhibitory-type DREADD resulted in an mCherry expression profile originating from the medial area of the STN. The results of the mCherry immunofluorescence illustrated that transduction of the cells within the STN with AAV5-CaMKIIa-hM4D(G<sub>i</sub>) DREADDs was successful. This was replicated in study II, which further demonstrated that the application of CNO to DREADDs-infused animals provided neuronal silencing, as indicated by significantly decreased c-Fos transcription. This study also validated that in the absence of CNO, the functioning of STN in these DREADDs-infused animals does not differ from unoperated animals. Furthermore, it is reported that CNO by itself, lends no observable impact on signal transduction in the STN.

Although the surgical procedure – and therefore the volume of the virus – was identical, AAV2 subtype rats did not express notable GFP expression. Despite work by Jara et al. (2012) illustrating that AAV2 undergoes retrograde transduction (for corticospinal motor neurons), a titre-matched comparison of serotypes by Burger et al (2004) claim that AAV2 undergoes minimal retrograde transport, and in fact, found that AAV1 and AAV5 do so, more robustly. Therefore, in the current experiment, the inability to visualise GFP for AAV2 may have stemmed from conservative tropism for this serotype, or a less potent viral batch titre; unfortunately, with only two rats per surgery group in study I, it is difficult to ascertain precisely why we failed to visualise an AAV2 transduction profile.

### **5.4.1 Conclusions**

The current experimental chapter presented that AAV infusions of DREADDs to the medial STN results in a transduction profile similar to ibotenic acid lesions, and that systemic application of CNO results in significant decreases in neuronal functioning, consistent with an inhibitory effect. This work reported here also presents as a surgical protocol for subsequent work in this thesis, which will allow for a more precise experimental manipulation of the STN, to further investigate its involvement in attentional set-formation.

# Chapter 6

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## Pharmacosynthetic inhibition of the STN and ZI/LHA-area impairs the formation of attentional set

### Background:

Inhibition of the cellular activity within the STN with designer receptors will allow us to measure the role of this region in set-formation with the 11-stage task.

### Methods:

18 rats were given bilateral DREADDs infusions to the STN; during testing, half of the group received clozapine *n*-oxide, whilst half received a vehicle.

### Results:

CNO-treated rats did not present with evidence for an attentional set; there was neither a set-shifting cost, nor an improvement across multiple ID stages. The probe stage indicated that these rats were still attending to the irrelevant dimension, and the bi-conditional stage suggested this was due to configural learning, which may have ultimately attenuated the reversal costs.

### Conclusions:

The STN is important in attentional selectivity, and that inhibiting this region impairs the ability to parse relevant from irrelevant. Results from the reversal stages suggest more work is needed to explore the relationship between reversal learning and attentional shifting as mediated by the STN.

## **6.1 Introduction**

Chapter 5 illustrated that infusion of AAV5, CaMKIIa-promoted DREADDs to the medial STN led to the expression of G<sub>i</sub>-coupled designer receptors in subthalamic neurons. Viral expression was found primarily in the medial STN, whilst sparing the lateral STN, along with viral expression in the ventral portion of the ZIV and the most lateral portion of the LHA. In a follow-up experiment, systemic administration of CNO resulted in decreased neuronal activity in the STN, as evidenced by reduced c-Fos labelling compared to vehicle-treated rats. Together, these results suggest that AAV5-CaMKIIa-hM4D(Gi) DREADDs present as a refinement to ibotenic acid lesions of the STN to modulate neuronal activity. The current experiment builds on the results from Chapter 5 to investigate the involvement of the STN in the formation of attentional set, using the 11-stage task (see Chapter 3) to compare various hypotheses about the processes that might be compromised when the STN is inhibited.

Briefly, the 11-stage task introduced five ID stages to promote set-formation, of which, data from the last ID stage can be compared with the ED stage to ascertain the cost of shifting attentional set to an unattended stimulus dimension. The task also presented two reversal stages – one with one interim stage between compound learning and reversal, and one with three interim stages – with the hypothesis that if memory for reward-relevant exemplars were disrupted by new stimuli, then reversals (particularly when there is significant interference by the presentation of multiple sets of novel stimuli) would be treated as if they were new learning. Finally, the task also introduced ‘probe’ stage which measured the suppression of attention to the irrelevant dimension, thereby providing additional inference of attentional set. In the probe stage, the rewarded status of the relevant (and, presumably, differentially attended) exemplars from the preceding ID stage remained the same, whilst the stimuli from the irrelevant dimension (which ought to be unattended) were replaced with novel stimuli.

As an adjunct to the 11-stage task, in a bi-conditional discrimination stage, the combination of exemplars from both dimensions indicated the baited bowl (see description in Chapter 3, Section 3.2.5.2). The bi-conditional discrimination, where the baited bowl is

signalled by both stimulus dimensions, should be more readily solved by animals who are learning the stimulus configurations – which may be the result of being unable to form set or may be the cause of it – compared to those who have had experience in attending to only one of the stimulus dimensions at a time.

The 11-stage task is typically completed by rats in fewer than 4 hours, which is comfortably within the time frame of the transient CNO inactivation (10-12 hours; see Chapter 5).

## **6.2 Materials and methods**

### **6.2.1 Animals**

Eighteen male, Lister hooded rats (Charles River, UK) were used in this experiment, with all rats receiving infusions targeted on the medial STN. Rats had a mean weight of 398g (range: 363-428g) at the start of the experiment. At completion of the experiment rats had a mean weight of 525g (range: 484-575g). All rats were experimentally naïve prior to testing and husbandry conditions and housing details are described in the General Methods chapter (Section 2.1). Behavioural testing was completed 3-12 weeks post-surgery. A 7-stage task was completed over 4 weeks, starting at 3 weeks post-surgery, the 11-stage task was completed over 3 weeks, with rats tested at least 10 days after completion of the 7-stage task.

### **6.2.2 Apparatus**

Behavioural testing was completed in the attentional set-shifting apparatus, which is described in section 2.2 of the general methods chapter.

### **6.2.3 Surgery**

The protocol for pharmacosynthetic surgery is described in Chapter 5 (Section 5.2.2).

### **6.2.4 Behavioural training**

The procedure for behavioural training employed in the current experiment is described in section 2.4.1 of the General Methods chapter.

### **6.2.5 Behavioural testing**

#### *6.2.5.1 The 7-stage task*

Rats were first tested on the 7-stage task without CNO to familiarise them with the behavioural requirements of the ID/ED task, so that on subsequent, CNO-administered tests, behavioural data could be collected within the peak availability period for CNO (see: Ray *et al.*, 2011). As stated in section 3.2.6, repeat testing on the attentional set-shifting task results in a similar performance profile (i.e., costs associated with reversal learning and set-

shifting), but the overall time to complete the test reduces, which is advantageous here given the length of the 11-stage task and the transient effect of the CNO.

A detailed explanation of the stages and list of stimuli for the 7-stage task can be found in Section 2.4.2 of the General Methods chapter, and an example summary of the procedure found in Chapter 3 (table 3.1; Section 3.2.5.1). Counterbalancing controlled for the order of presentation of stimulus pairings, shift type (e.g., medium to odour or vice versa), and the initial correct stimulus within a pairing.

#### *6.2.5.2 The 11-stage task*

Three days before behavioural testing, rats were habituated to i.p. injections by being administered 2ml/kg saline (0.9% NaCl) each day, for two days. This was done to reduce secondary behavioural effects associated with the stress of injection (Meijer, Spruijt, van Zutphen, & Baumans, 2006). Thirty minutes prior to behavioural testing, rats were given an i.p. injection of either CNO ( $n=9$ ; 10mg/kg in 5mg/ml of 0.9% saline; Sequoia Research Products Ltd., UK) or a vehicle ( $n=9$ ; 0.9% saline). Following injection, rats were placed in a small home cage (see section 2.1 for cage dimensions) until the commencement of behavioural testing. Testing began thirty minutes following injection. The list of stimuli and procedure of the 11-stage task can be found in Chapter 3 (section 3.2.5.2).

#### **6.2.6 Histology**

Tissue was collected as described in Section 2.5 of the General Methods chapter. Two immunohistochemistry procedures were completed, on two sets of tissue; the first determined the extent of viral tropism in the STN with mCherry/DAB and the second procedure determined the extent of nuclear transduction with colocalised immunofluorescence of neuronal nuclei and mCherry.

For mCherry/DAB immunoreactivity, sections were first washed with PBS in 9-hole net wells, five times for three minutes each. Sections were then placed on a stirrer for 1 hour in blocking solution (20% normal goat serum, 0.1% triton in 0.1M PBS) and then washed again. Sections were then placed in histology pots and incubated with 5ml of the primary antibody, biotinylated rabbit anti-mCherry (1:2000; Abcam, Cambridge, UK) suspended in antibody diluting solution (ADS: 0.1M PBS, 1% normal goat serum, 0.1%

triton) and left on a stirrer overnight. The following day, sections were returned to 9-hole net wells and washed and incubated in vector IgG solution (anti-rabbit IgG; 5µl/ml; Vector Laboratories Ltd., Peterborough, UK) on a stirrer for one hour. Sections were then washed again, and incubated in reagents A & B (10µl/ml each) of Vectastain ABC complex (Vector Laboratories Ltd., Peterborough, UK) for another hour. Sections were washed again and treated with DAB (Sigma Chemical Company, St Louis, MO, USA) for approximately 10-15 minutes, until tissue was stained with a dark-brown colour, with minimal background staining. Sections were then washed again and mounted on gelatine-subbed glass slides. Sections were then de-fatted with xylene before being coverslipped with DPX mountant as described in section 2.6 of the General Methods chapter; however sections were not stained with cresyl violet. mCherry expression was determined with light microscopy at 2.5x and 10x magnifications, examining the extent of transduction, including axonal projections.

For colocalised immunofluorescence, sections were washed with PBS in 9-hole net wells, five times for three minutes each. Sections were then placed on a stirrer for 1 hour in blocking solution (20% normal goat serum, 0.1% triton in 0.1M PBS) and then washed again. Sections were then placed in histology pots and incubated in a 3ml mixture of the primary antibodies: biotinylated rabbit anti-mCherry (1:1000; Abcam, Cambridge, UK) and mouse anti-NeuN (1:1000; Chemicon International, Temecula, CA, USA) suspended in ADS and left on a stirrer overnight. The following day sections were washed five times for five minutes each in 9-hole net wells. Sections were then returned to foil-wrapped histology pots and incubated in a 3ml mixture of the secondary antibodies: preadsorbed goat anti-rabbit IgG H&L (1:250; Alexa Fluor 594, Abcam, Cambridge, UK) and preadsorbed goat anti-mouse IgG H&L (1:250; Alexa Fluor 488, Abcam, Cambridge, UK) in ADS for one hour. Sections were washed five times for five minutes each in 9-hole net wells, and then mounted to glass slides. Vectashield antifade mounting medium (Vector Laboratories Ltd., Peterborough, UK) was pipetted on to the slides, which were then coverslipped and the edges were sealed with nail polish. Fluorescence microscopy was completed with a microscope (Zeiss, Cambridge, UK) using Novell ZENworks software (Micro Focus, Newbury, UK).

### 6.2.7 Data analysis

The procedure for data analysis for the bi-conditional discrimination and 7-stage/11-stage tasks are described in Chapter 3 (Section 3.2.7). To quantify the level of colocalised transduction, photomicrographs were analysed by an irregular shape calculator tool (Dobbs, 2011) and the area of colocalised region (for every section and hemisphere) was calculated and divided by the total area of the respective region of interest (e.g., STN, ZIV, ZID & LHA) to determine a ‘percent transduced’ by area. Data were analysed by repeated-measures ANOVA with a within-subjects factor of Brain Region (four levels: STN, ZIV, ZID, & LHA), a within-subjects factor of Side (two levels: left or right), a within-subjects factor of Section (three levels: -3.6mm, -3.8mm, -4.16mm), and a between-subjects factor of CNO (two levels: CNO and vehicle). In order to correlate behavioural performance with levels of transduction, total observed transduction was computed by summing the transduction percentages across each hemisphere, section and brain region for each animal, which could then be compared with manipulations of interest (e.g., shift-cost, probe, etc.). This correlational analysis was also done for each region to determine in any region-specific effects of transduction influenced behaviour.



## **6.3 Results**

### **6.3.1 Histology**

Preliminary examination of viral tropism with mCherry/DAB staining determined that two rats – one from the CNO and one from the vehicle treatment group – expressed unilateral mCherry expression. The data for these two rats were included in all analyses in this chapter, as excluding the unilateral CNO-treated rat did not influence the statistical outcome; this will be evaluated separately in the subsequent sections. All other rats expressed bilateral mCherry expression in the STN, including the medial region. This mCherry/DAB staining approach revealed the total spread of the virus, including non-cellular transduction (*i.e.*, in axons and dendrites); it was found that the virus was trafficked into the ZIV in all animals ( $n=18$ ), but only a subset of rats exhibited mCherry in the ZID ( $n=8$ ) and LHA ( $n=12$ ; figure 6.1).

Colocalised immunofluorescence (NeuN/mCherry) was used to determine the extent of nuclear transduction of the brain regions identified by the mCherry/DAB staining (figure 6.2). Colocalisation was illustrated by yellow labelling (Green-NeuN + Red-mCherry) in respective brain regions. In order to quantify this labelling, the area of this yellow region (for every section and hemisphere) was calculated by an irregular shape calculator (Dobbs, 2011) and divided by the total area of the respective region of interest to determine a ‘percent transduced’. This was done *in lieu* of cell counting as the strength of the fluorescence signal made it difficult to distinguish the cell perimeter in regions with high cell density (*e.g.*, STN); this was likely owing to the thickness of the tissue in this experiment (50 $\mu$ m); a consideration which will be evaluated in the discussion. Therefore this ‘percent’ measure was an approximation of colocalised spatial area within a region. Despite clear labelling of non-cellular transduction (*i.e.*, fibres of passage) in the colocalised photomicrographs (labelled in red), owing to the thickness of the sections (and therefore the volume of the ‘z-axis’), there is still a possibility that not all of the cells within a particular transduced region were colocalised, as their detection was obfuscated by a superimposed cell. However, since colocalised labelling (yellow) is only visibly present when the cell body (NeuN-green) is labelled, it is argued that the current approach is a

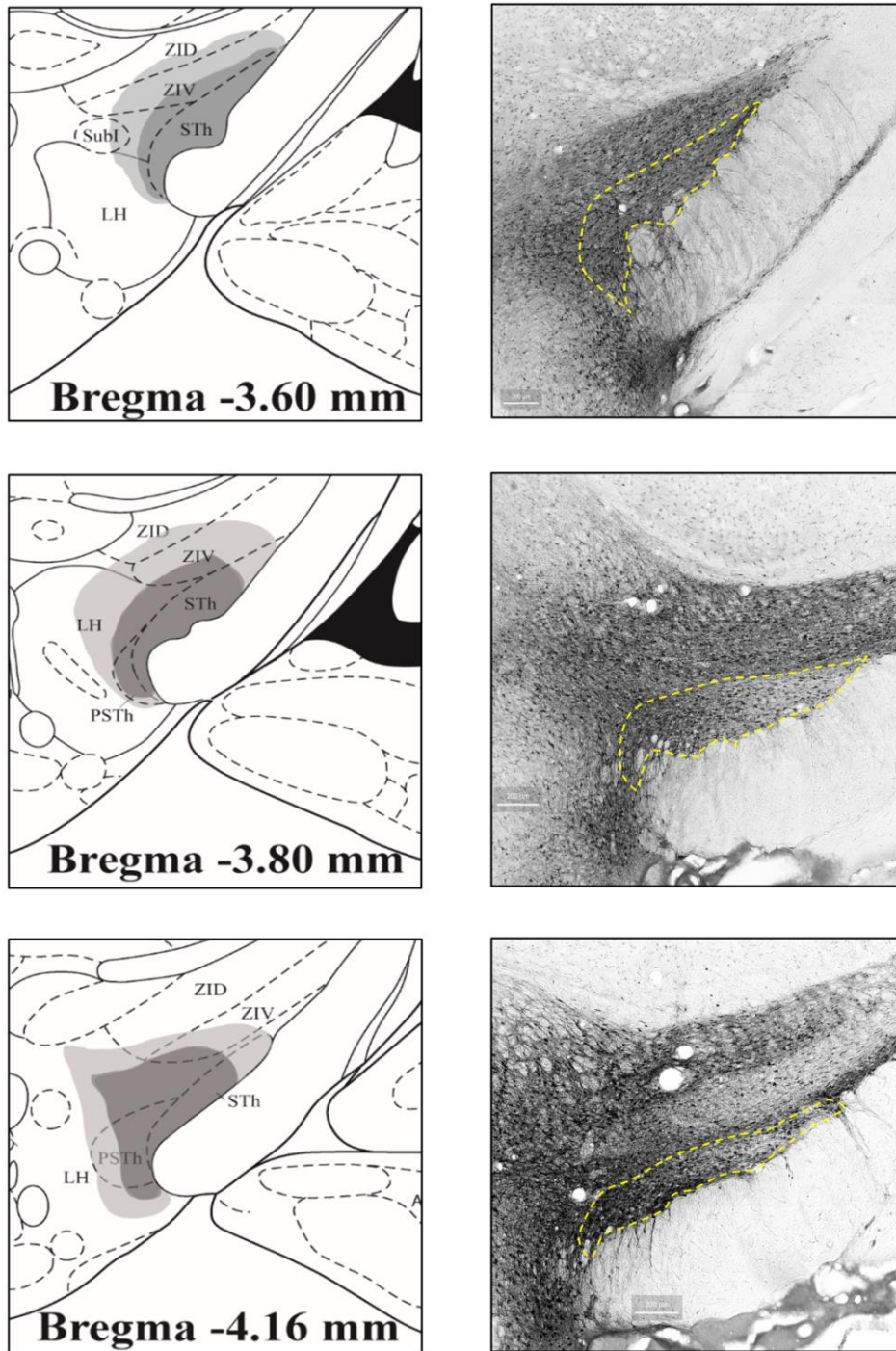
reasonable alternative to cell-counting to provide a comparative measure of colocalised viral transduction.

The analysis of the colocalised transduction suggested that transduction occurred uniformly within the STN, originating from the medial region in all animals ( $n=18$ ; albeit unilaterally for 2 rats) and transducing an average of approximately 40% of the nucleus; in contrast, extraneous transduction to the ZI ( $n=10$ ) and LHA ( $n=10$ ; a different subgroup than the ZI-rats) varied by rat in terms of transduction percentage and frequency of occurrence (Table 6.1). Viral transduction centred on the medial area of the STN, and extended laterally to the lateral STN (range of STN transduction: 25-65% of nucleus) in all animals, whilst for some rats transduction extended medially to the LHA (range: 5-33%), and/or dorsally to the ZIV (range: 5-40%) and ZID ( $n=7$ ; range: 5-36%; figure 6.3). The percentage of colocalised transduction for a given brain region did not significantly differ between sides of the brain (*main effect of Side*:  $F_{(1,16)} = <1$ , *n.s.*;  $np^2 = 0.02$ ), nor did transduction vary rostro-caudally, between sections (*main effect of Section*:  $F_{(1.49,23.8)} = 1.23$ , *n.s.*; *Huynh-Feldt correction*; *uncorrected*  $df = 2, 32$ ;  $np^2 = 0.02$ ); furthermore, transduction patterns were consistent between CNO treatment groups (*main effect of CNO group*:  $F_{(1,16)} = <1$ , *n.s.*;  $np^2 = 0.03$ ).

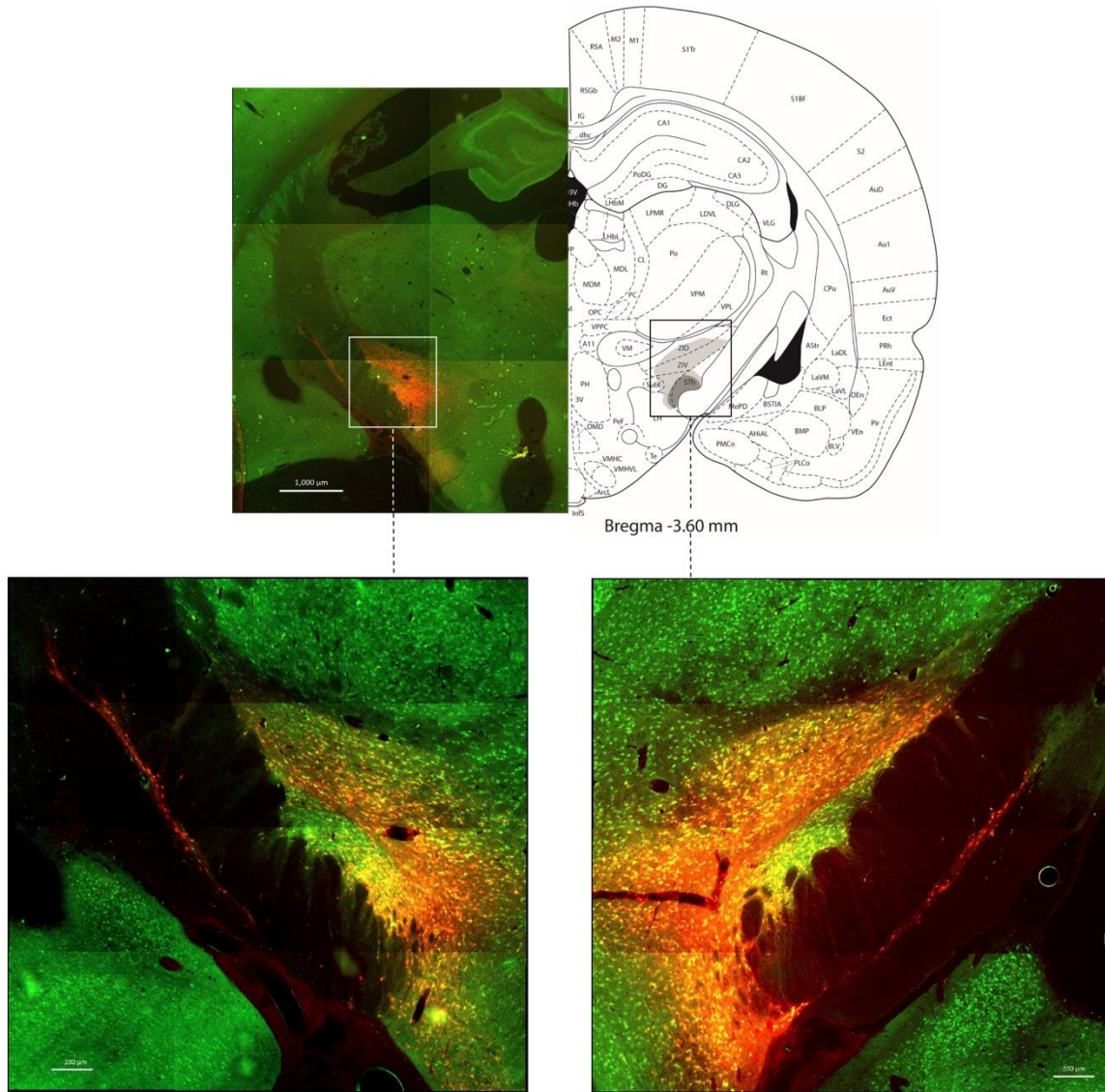
As expected, analysis revealed that percentage of transduction differed between brain regions (*main effect of Region*:  $F_{(2.66,42.48)} = 73.8$ ,  $p < 0.01$ ; *Huynh-Feldt correction*; *uncorrected*  $df = 3, 48$ ;  $np^2 = 0.82$ ), with the STN significantly achieving the greatest percentage of colocalised transduction (M: 40.2% of region transduced); an effect which held for each section of tissue (*Region by Section interaction*:  $F_{(2.2,35.2)} = 4.12$ ,  $p < 0.05$ ; *Huynh-Feldt correction*; *uncorrected*  $df = 6, 96$ ;  $np^2 = 0.21$ ; *Bonferroni-corrected pairwise comparison*). In contrast, extraneous transduction to the neighbouring ZIV (13.3% of nucleus transduced), ZID (8.5%), and LHA (10.7%) was not uniform and varied by rat: 8 of the 18 rats presented with no nuclear transduction of the ZI, whilst a different subset of 8 rats did not present with any nuclear transduction of the LHA, whereas transduction in the STN was present in all rats (with 4 rats presenting with colocalised transduction exclusive to the STN: 1 CNO-treated; 3 vehicle-treated). The amount of transduction of the ZI and LHA also varied considerably between rats, with some rats presenting with high

percentages of bilateral transduction (*e.g.*, 60% of nucleus), others had minimal unilateral transduction (*e.g.*, 10-15% of nucleus), whilst some had no extraneous transduction at all. Subsequent behavioural analyses will evaluate any behavioural effects in light of these differences in transduction, and the details of such analyses will be described in their respective sections.

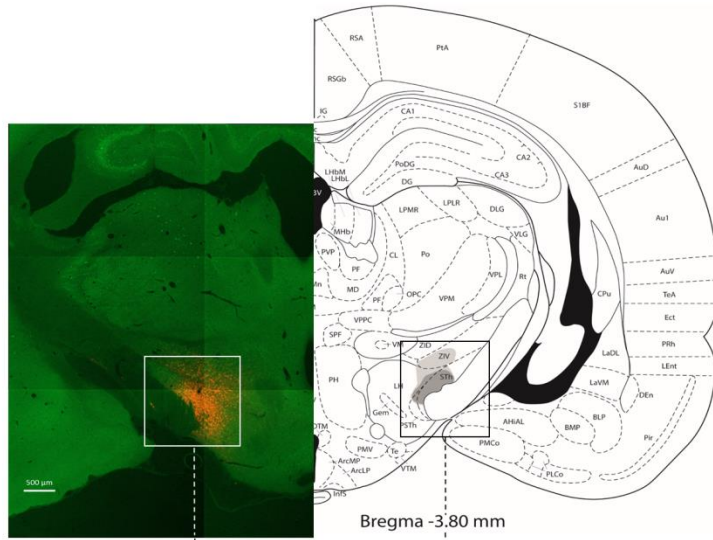
Anatomical/tracing studies have suggested that the medial third (33%) of the STN functionally subdivides the nucleus, with this area being responsible for limbic and associative (cognitive) tasks owing to a direct connection with cortical areas implicated in cognition (*i.e.*, mPFC & lateral OFC; Groenwegen & Berendse, 1990; Heimer *et al.*, 1995; Janssen, Visser-Vandewalle, & Temel, 2010; but see Kita *et al.*, 2014); consequently, this area was the surgical target throughout this thesis. Based on the percentage of STN transduction in this experiment (40%), it is parsimonious to conclude that this area was transduced (to a varying degree) in all animals (apart from the one hemisphere for the two unilaterally-transduced animals).



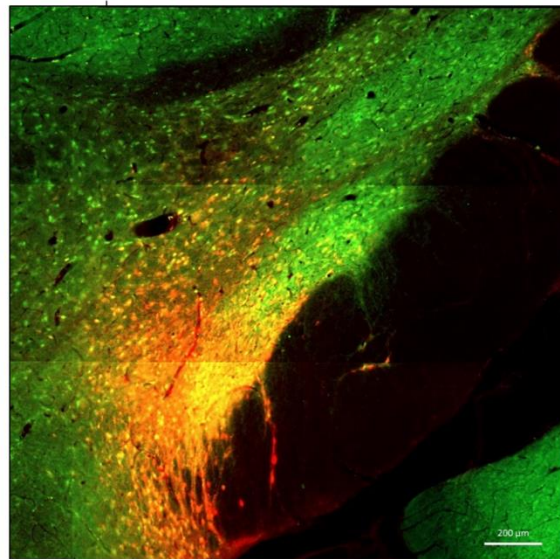
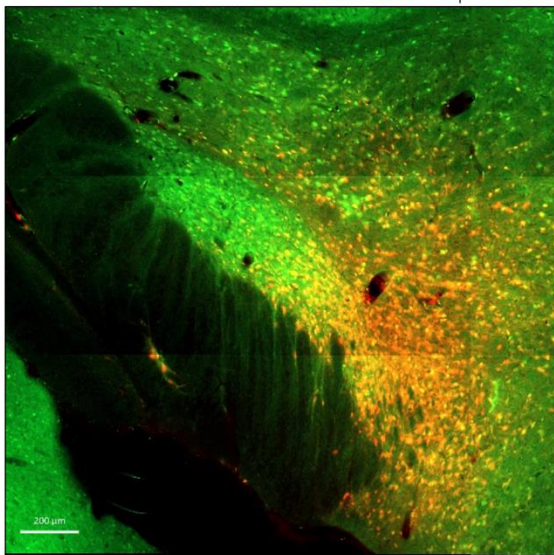
*Figure 6.1: Schematic illustrating the largest (light grey) and smallest (dark grey) area of mCherry viral transduction, presented with photomicrographs of brain sections stained with DAB, which included staining for both neuronal and non-neuronal nuclei; hashed lines indicate the outline of the STN cell population.*

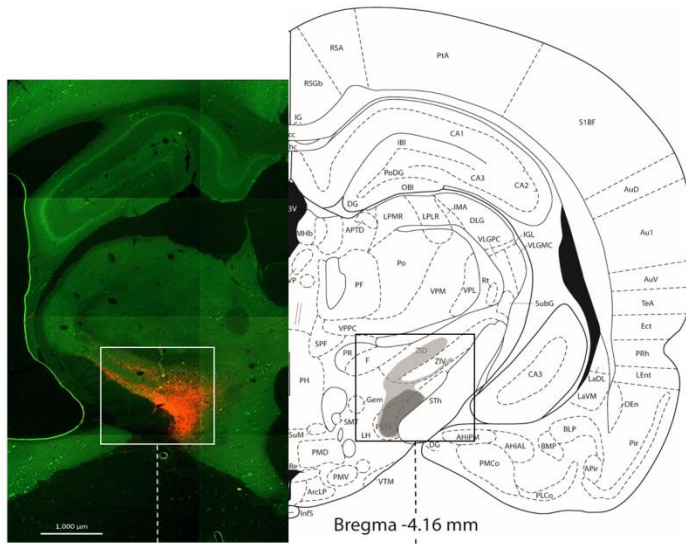


*Figure 6.2: Schematics and example photomicrographs of colocalised sections, stained for NeuN (green) and mCherry (red) with nuclear-viral colocalisation illustrated in yellow. Schematics illustrate the minimum (dark grey) and maximum (light grey) extent of the viral transduction, with photomicrographs of a 'typical' brain from 2.5x and 10x magnification.*

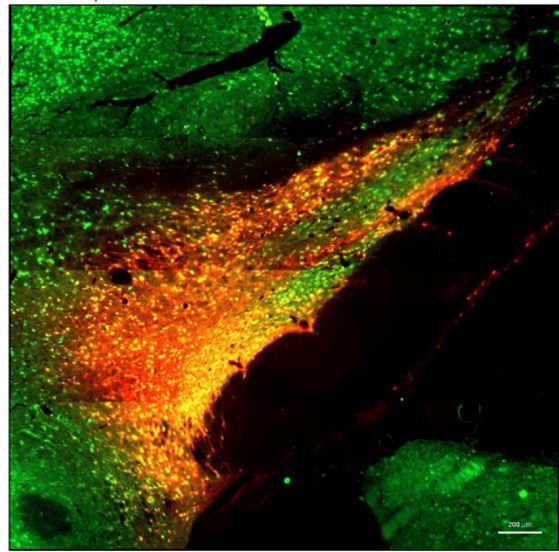
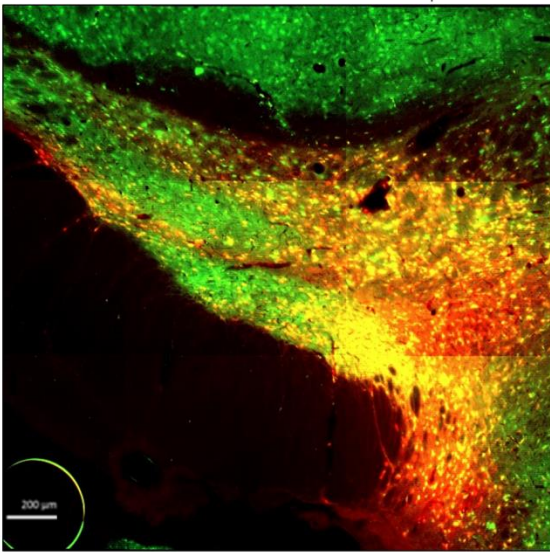


Bregma -3.80 mm





Bregma -4.16 mm



	<b>Bilateral</b>	<b>Unilateral</b>	<b>None</b>
<b>medial STN</b> (n=18)	8/8	1/1	0/0
<b>lateral STN</b> (n=17)	8/7	1/1	0/1
<b>ZIV</b> (n=10)	5/2	2/1	2/6
<b>ZID</b> (n=7)	4/2	1/0	4/7
<b>LHA</b> (n=10)	4/4	1/1	4/4

*Table 6.1: Summary of colocalised transduction results separated by brain region, illustrating the distribution of rats (CNO/vehicle) by extent of transduction (bilateral vs unilateral vs none); colocalised transduction was uniform in the STN and prevalent in every animal, yet the pattern for extraneous transduction to the ZI and LHA varied by rat. Rats with mean transduction percentages exceeding 33% were deemed to have transduction extending into the lateral STN.*



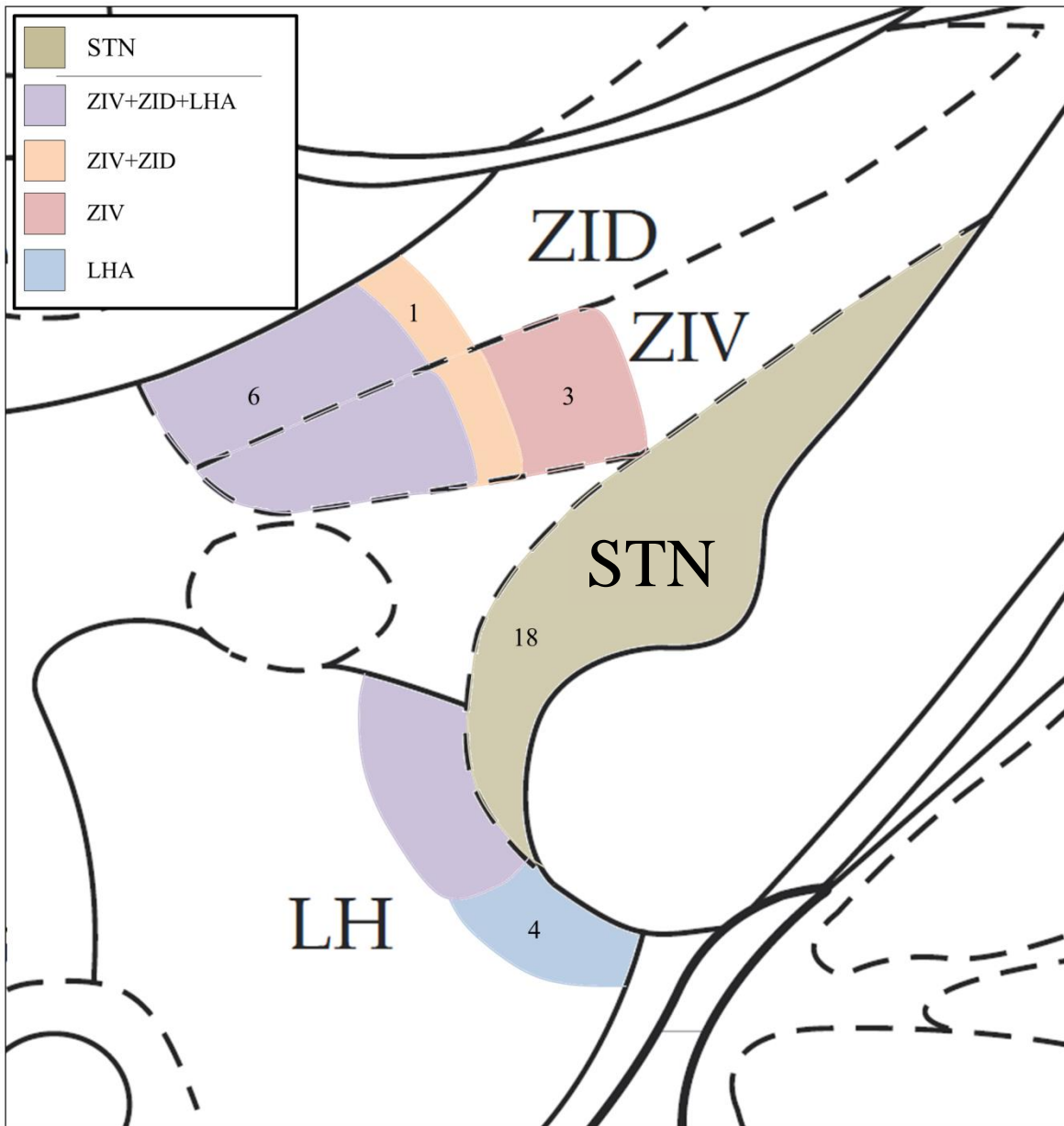


Figure 6.3: A schematic of the STN, ZIV, ZID, & LHA, with coloured shading denoting extent (to one or more extraneous regions) and proportion (by region) of colocalised transduction for all rats; colocalised transduction was found in the STN in all rats ( $n=18$ ; CNO  $n=9$ ), whilst transduction varied in the ZI and LHA: 10 rats had transduction in the ZIV (CNO  $n=7$ ), which extended dorsally to the ZID in 7 animals (CNO  $n=5$ ), and of these animals, 6 (CNO  $n=4$ ) also had transduction of the LHA.

### 6.3.2 Baseline assessment using the standard 7-stage task

The 7-stage task tests novel and reversal learning and therefore performance did, as expected, differ as a function of stage (main effect of stage,  $F_{(6,102)} = 21.4$ ,  $<0.01$ ;  $n\eta^2 = 0.58$ ). The ‘cost’ of shifting set can be expressed as the difference in TTC between the ID and ED stages: a Bonferroni-adjusted pairwise comparison determined that rats required significantly more trials to acquire the ED stage (M: 13.8; SD: 2.44) compared to the ID stage (M: 7.8; SD: 1.82;  $p < 0.01$ ; figure 6.4). This matched the established pattern of behaviour for typical control rats in the attentional set-shifting task (Birrell & Brown, 2000; Tait *et al.*, 2016), along with the demonstrating that in the absence of CNO administration, DREADDs-transduced rats formed an attentional set, as inferred by the significant cost of shifting away from that set.

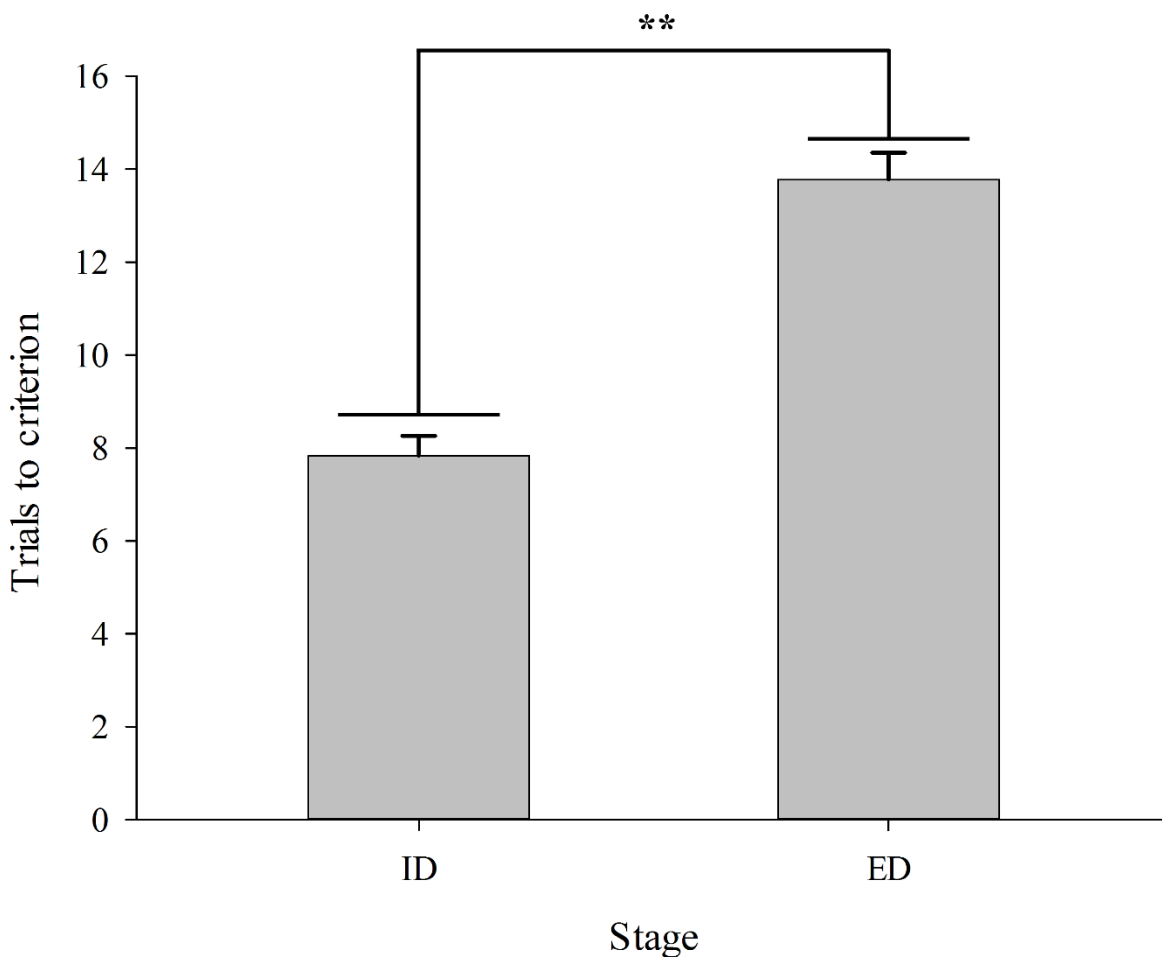


Figure 6.4: Mean trials to criterion (+SEM) demonstrated that rats ( $n=18$ ) exhibited a significant cost of shifting set from an attended stimulus dimension (ID), to an unattended stimulus dimension (ED);  $**p<0.01$ .

### 6.3.3 CNO-treatment impairs set-formation on the 11-stage task

The 11-stage task also includes new learning and reversals and, as expected performance differed as a function of stage (repeated measures ANOVA, main effect of stage:  $F_{(8,8,140.1)} = 21.9$ ,  $p<0.01$ ; *Huynh-Feldt correction*; *uncorrected*  $df = 10,160$ ;  $np^2 = 0.58$ ). More interesting, however, are the stage-specific effects of CNO. It was not expected that CNO would impair simple acquisition (SD), but, as expected, some stages were solved in fewer trials and other stages were required additional trials, compared to control performance (interaction of CNO and Stage:  $F_{(8,8,140.1)} = 18.4$ ,  $p<0.01$ ; *Huynh-Feldt correction*; *uncorrected*  $df = 10,160$ ;  $np^2 = 0.54$ ). Figure 6.5 shows the origin of this

interaction, which was explored in more depth using planned and Bonferroni-corrected pairwise comparisons. As detailed in the histology section, one CNO-treated rat presented with unilateral transduction, but was included in the analysis above since excluding this animal had no effect on the statistical outcome here (interaction of CNO and Stage:  $F_{(7.9,117.9)} = 17.6, p < 0.01$ ; *Huynh-Feldt correction*; *uncorrected*  $df = 10, 150$ ;  $np^2 = 0.52$ ).

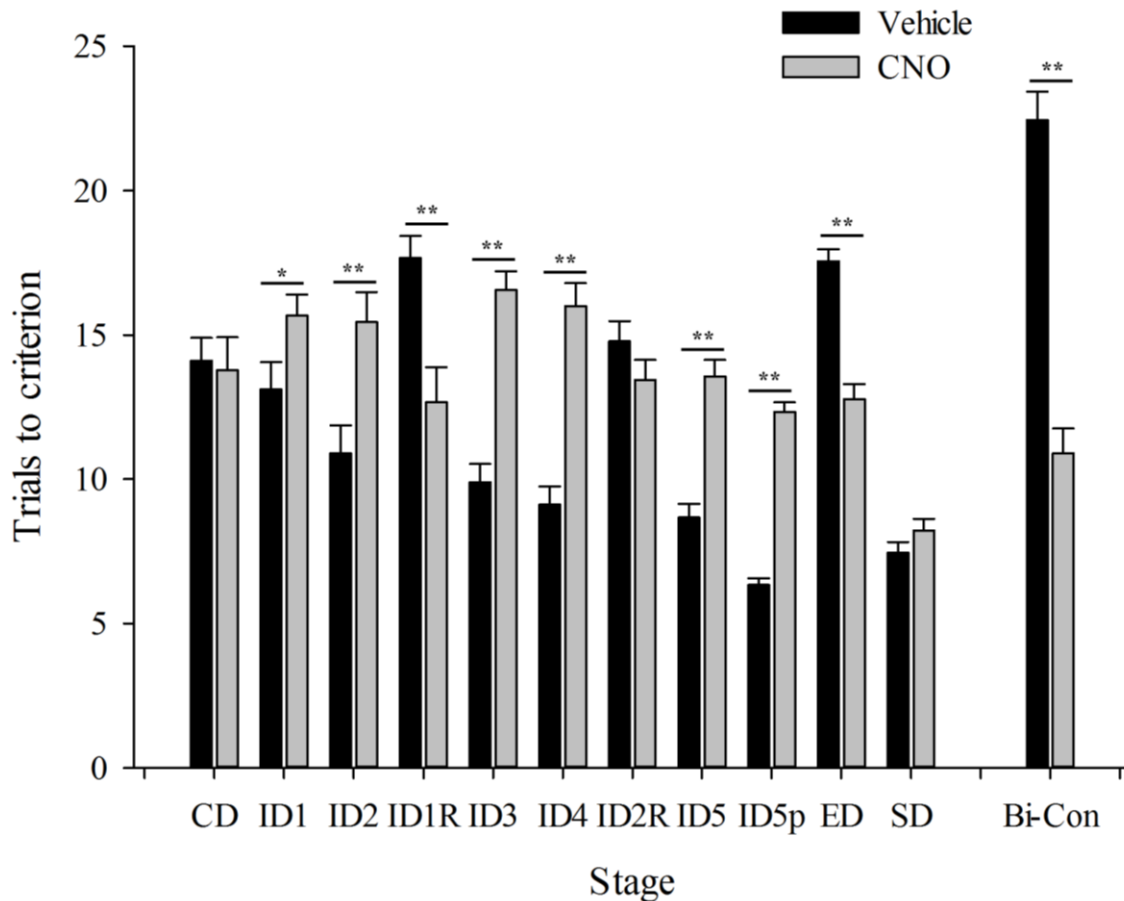


Figure 6.5: Mean trials to criterion (+SEM) illustrating that CNO-treated rats required significantly more trials on all ID learning stages and the probe stage, however required fewer trials on the ID1R and ED stages, along with the adjunct bi-conditional stage.

### 6.3.3.1 Examining shift-cost: ID5 vs ED

To examine the cost of set-shifting, a restricted analysis of the ID5 and ED stages was conducted using a repeated-measures ANOVA, correcting the F-values using the error terms from the omnibus analysis (Winer, 1971). CNO-treated rats did not exhibit a cost of

shifting set between these stages, whilst vehicle-treated rats illustrated the expected ID-ED difference (*Stage by CNO interaction*:  $F_{(1,140.1)} = 39.7, p < 0.01$ ; *uncorrected*  $df = 1,160$ ;  $np^2 = 0.90$ ; figure 6.6). Vehicle-treated rats acquired the ID5 stage with significantly fewer TTC than CNO-treated rats, illustrating the benefit of selective attention to the reward-relevant dimension, whilst conversely, CNO-treated rats completed the ED shift acquisition in fewer trials than vehicle-treated rats (*Bonferroni-adjusted pairwise comparison*; *all*  $ps < 0.01$ ). Due to the decreased TTC at the ED stage, and increased TTC at the ID stage, performance between ID and ED stages did not differ for the CNO-treated rats, suggesting a failure to form an attentional set. Similar to the omnibus analysis, excluding the data for the one unilaterally-transduced CNO-treated rat had no effect on the statistical outcome (*Stage by CNO interaction*:  $F_{(1,117.9)} = 33.7, p < 0.01$ ; *uncorrected*  $df = 1,150$ ;  $np^2 = 0.89$ ). Furthermore, inspection of the ID5-ED comparison suggested that this unilaterally-transduced animal did not form a set, as it required two more TTC for ID5 than ED.

Results from the colocalised transduction analysis revealed that only one CNO-treated rat exhibited viral transduction solely limited to the STN, and therefore any analysis which attempted to draw a conclusion from data with a group size to 1 would be unreliable. Consequently, CNO-treated rats with ‘minimal’ extraneous transduction [*i.e.*, rats with only STN transduction, *plus* only unilateral transduction of only one extraneous brain region (either the ZIV, ZID or LHA): range of transduction for extraneous region = 0-35%] were separated ( $n=4$ ) from CNO-treated rats with ‘moderate’ transduction (*i.e.*, rats with only STN transduction, *plus* bilateral transduction of one or more extraneous brain regions;  $n=5$ ; range of transduction for extraneous region = 0-60%). With this new group assignment, a repeated-measures ANOVA was conducted to determine transduction-specific effects, such as a reduced impairment in shift-cost for minimally-transduced rats compared to moderately-transduced rats; the data for vehicle-treated rats was not reclassified as in the absence of CNO, the DREADDs are not activated and therefore their specific location could be of no relevance. It was found that all of the CNO-treated rats, irrespective of any additional transduction to extraneous areas, did not exhibit a cost of shifting set between the ID5 and ED stages (*Stage by Revised CNO interaction*:  $F_{(2,128.39)} = 10.53, p < 0.01$ ; *Huynh-Feldt correction*; *uncorrected*  $df = 2,150$ ;  $np^2 = 0.95$ ); furthermore acquisition of the ID and

ED stages did not differ between the ‘minimal’ and ‘moderately’ transduced CNO-treated rats, suggesting that set-shifting performance and novel learning acquisition, did not differ between animals with a high amount of extraneous transduction to the ZI and LHA, and animals with minimal extraneous.

An alternative analysis correlated the total amount of observed transduction with shift cost to determine whether rising levels of transduction may correlate with a smaller shift cost (or a ‘negative’ shift cost), or perhaps the inverse, whether less transduction correlates with a reduced impairment in shift-cost. Total observed transduction was computed by summing the transduction percentages across each hemisphere (i.e., L + R), section (i.e., -3.6 + -3.8...) and brain region (i.e., STN + ZIV...) for each animal, and subsequently these total values per animal were correlated against the shift-cost data for each rat. Results of a Pearson correlation indicated no relationship between the total amount of transduction and the magnitude of shift-cost ( $r = 0.07$ ;  $n=9$ ; *n.s.*). Furthermore, there was no relationship between the amount of transduction for each region and the magnitude of shift-cost: differences in transduction within the ZIV ( $r = 0.05$ ;  $n=9$ ; *n.s.*), ZID ( $r = 0.01$ ;  $n=9$ ; *n.s.*), and LHA ( $r = -0.38$ ;  $n=9$ ; *n.s.*) did not significantly correlate with ID5-ED behaviour following CNO treatment. It appears therefore that transduction of extraneous areas near the STN, such as the ZIV, ZID and LHA may play a minimal role in the mediation of the behavioural deficit observed here, which may be largely due to transduction of the STN.

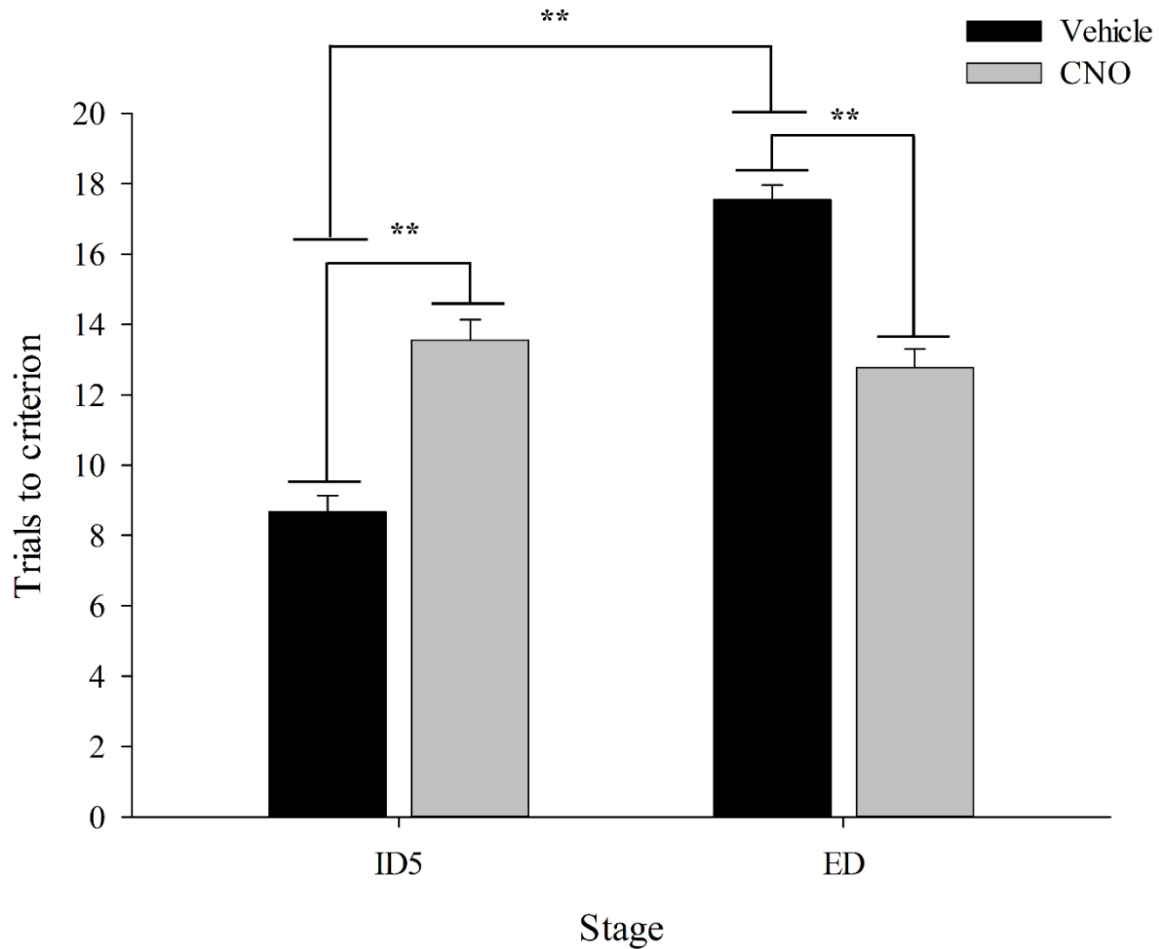


Figure 6.6: Mean trials to criterion (+SEM) for vehicle and CNO-treated rats for the ID5 and ED stages; evidence of set-formation was observed for vehicle-treated rats only, demonstrated by a significant positive cost of shifting attentional set; \*\* $p < 0.01$ .

### 6.3.3.2 Performance across multiple ID stages

To test the hypothesis that CNO-treated rats would not benefit, by forming an attentional set, from repeated experience of novel discriminations, the 11-stage task includes five novel ID discriminations. Restricting the analysis to these five ID stages, it was clear that the CNO-treated rats showed no benefit and required more TTC than vehicle-treated across all of the ID stages, while the vehicle-treated rats showed a gradual decrease in TTC across the five ID stages (*CNO by Stage interaction*:  $F_{(4,140.1)} = 2.65$ ,  $p < 0.05$ ; *uncorrected*  $df = 4,160$ ;  $np^2 = 0.14$ ) Further analysis using Bonferroni-corrected pairwise comparisons determined that vehicle-treated rats showed a significant improvement between ID1 (M: 13.1) and ID5 (M: 8.7,  $p < 0.01$ ), whilst performance for CNO-treated rats

did not differ across the five ID stages ( $p > 0.05$ ; figure 6.7). Also, removing the data for the unilaterally-transduced CNO-treated animal had no effect on the statistical outcome (*CNO by Stage interaction*:  $F_{(3.7,117.9)} = 2.69$ ,  $p < 0.05$ ; *uncorrected*  $df = 4,150$ ;  $np^2 = 0.14$ ); CNO-treated rats exhibited no improvement, and furthermore, the data for the unilateral rat was no different than the remaining bilaterally-transduced group: performance for this rat at ID1 (15 TTC), ID2 (19 TTC), and ID3 (21 TTC) worsened considerably before improving slightly by ID5 (13 TTC).

Similar to the analysis of colocalised transduction presented for the shift-cost above, the same sub-groups of CNO-treated rats (*e.g.*, ‘minimal’ vs ‘moderate’) were employed for the current analysis to determine transduction-specific effects, and whether increased extraneous transduction in the ZI and LHA will worsen performance across the multiple ID stages. Despite subdividing rats, performance between the two groups of CNO-treated rats did not differ (*n.s.*); both ‘minimally-’ and ‘moderately-transduced’ groups of CNO-treated rats still required more TTC than vehicle-treated across all of the ID stages, along with evidencing no significant improvement between any stages, including the ID5 and ID1 stages (*Revised CNO by Stage interaction*:  $F_{(8,128.39)} = 2.26$ ,  $p < 0.05$ ; *uncorrected*  $df = 8,150$ ;  $np^2 = 0.24$ ; *Bonferroni-corrected pairwise comparison*). Results of a Pearson correlation between total transduction percentage and the mean trial difference between ID5 and ID1 (ID5–ID1) revealed no relationship ( $r = 0.43$ ;  $n = 9$ ; *n.s.*), suggesting that increasing the level of transduction does not influence performance between these two stages, or less transduction does not correlate with less impairment. Also, there was no relationship between the amount of transduction for each region and the impairment across the multiple ID stages: differences in transduction within the ZIV ( $r = 0.38$ ;  $n = 9$ ; *n.s.*), ZID ( $r = 0.48$ ;  $n = 9$ ; *n.s.*), and LHA ( $r = 0.15$ ;  $n = 9$ ; *n.s.*) did not significantly correlate with ID behaviour following CNO treatment. Therefore, the conclusion drawn here can be similar to the one regarding the shift-cost: higher levels of colocalised transduction of extraneous areas near the STN, such as the ZIV, ZID and LHA had a minimal impact on performance across multiple ID stages.



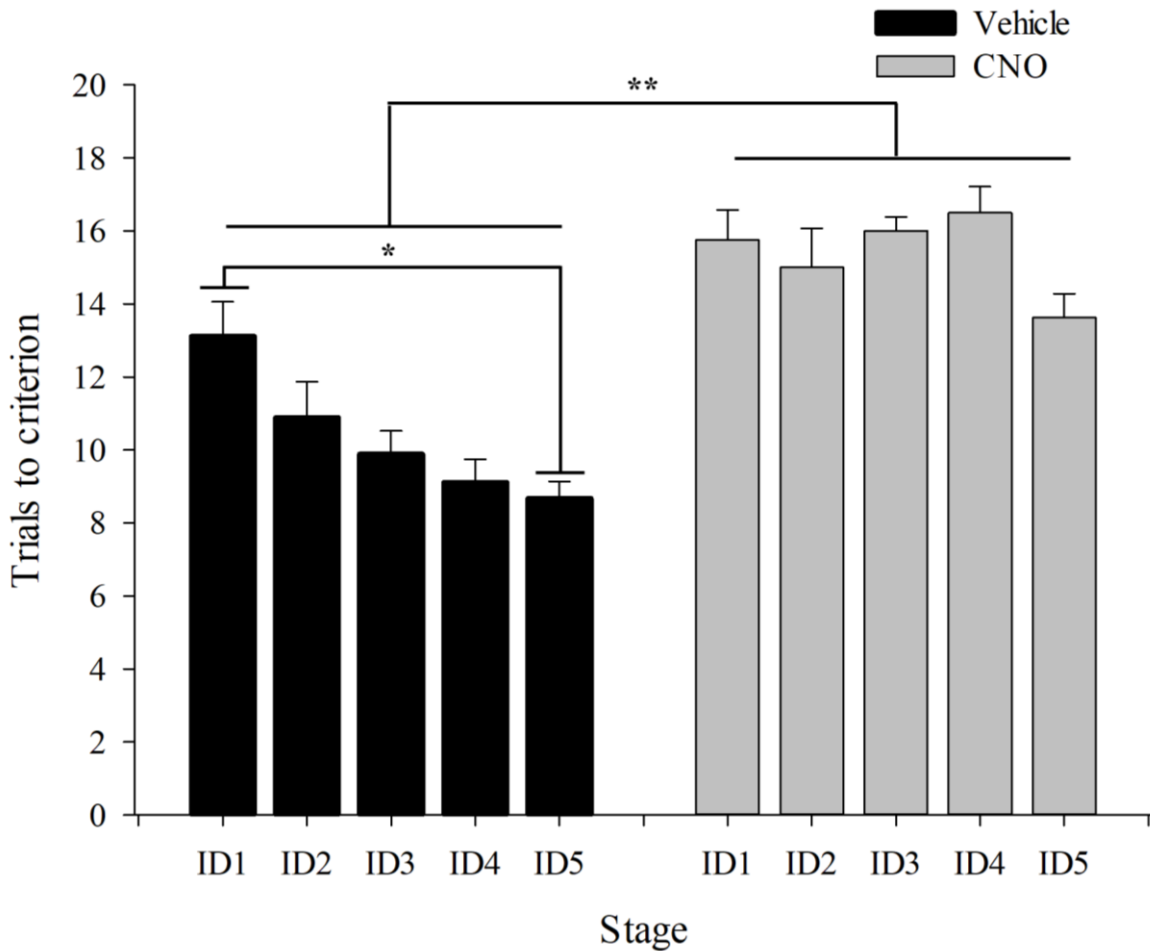


Figure 6.7: Mean trials to criterion (+SEM) illustrating that performance for vehicle-treated rats improved across the five ID stages, aiding in the inference of set-formation; in contrast, CNO-treated rats did not demonstrate an improvement in ID discrimination learning, and required more trials overall; \* $p < 0.05$ ; \*\* $p < 0.01$ .

#### 6.3.3.3 Delayed reversal learning with intermediary stages

A ‘reversal cost’ was clearly evident in vehicle-treated rats, even with multiple intervening stages (figure 6.8). By contrast, there was no obvious evidence of a ‘reversal cost’ in CNO-treated rats: reversal stages appeared to require about the same number of trials as new learning [i.e., there was no significant difference between ID1 and its reversal (ID1R) or between ID2 and its reversal (ID2R); *Stage by CNO interaction*:  $F_{(3,140.1)} = 15.3$ ,  $p < 0.01$ ; *uncorrected*  $df = 3,160$ ;  $\eta^2 = 0.44$ ); Bonferroni-corrected pairwise comparison revealed that vehicle-treated rats required significantly more TTC for both reversal stages

compared to the respective novel learning stages, indicating a ‘reversal-cost’, which was not found for CNO-treated rats]. This effect remained even if the unilaterally-transduced CNO-treated rat was removed from the analysis (*Stage by CNO interaction*:  $F_{(3,117.9)} = 14.2$ ,  $p < 0.01$ ; *uncorrected df* = 3,150;  $np^2 = 0.42$ ; *Bonferroni-corrected pairwise comparison*).

The intervening stages were included to test the hypothesis that a failure to demonstrate a significant reversal-cost following CNO administration could be due to memory impairment: if the presentation of new stimuli essentially disrupts memory of previous stages, all stages would be treated as novel. Although the rats appeared to have no reversal cost, there was nevertheless evidence that the rats did not have a memory impairment: the probability of rats committing an error during the first four ‘exploratory’ trials of the reversal stages was not the same as for new learning. Although CNO-treated rats were less likely to make an error in the initial trials of a reversal than vehicle-treated rats (*main effect of CNO*:  $F_{(1,16)} = 8.25$ ,  $p < 0.05$ ;  $np^2 = 0.33$ ; *Bonferroni-corrected pairwise comparison*; figure 6.9), nevertheless, they were significantly more likely to choose the previously rewarded stimulus, indicating that they clearly did remember the discrimination. Furthermore, there was no difference between the groups in the number of times they rejected the previously incorrect stimulus (*i.e.*, having approached a bowl, they did not dig if the stimulus was the previously-incorrect stimulus, but rather moved away from it; figure 6.10). This was true for both reversal stages (*repeated measures ANOVA*; *no main effect of CNO*:  $F_{(1,16)} < 1$ , *n.s.*;  $np^2 = 0.02$ ; *no interaction of CNO by reversal stage*:  $F_{(1,16)} < 1$ , *n.s.*;  $np^2 < 0.01$ ). Similar to the TTC data, removing the error data for the unilaterally-transduced CNO-treated rat did not change the fact that CNO-treated rats still made fewer errors (*main effect of CNO*:  $F_{(1,15)} = 7.04$ ,  $p < 0.05$ ;  $np^2 = 0.32$ ; *Bonferroni-corrected pairwise comparison*), whilst not differing from control rats in the number of times they rejected the previously incorrect stimulus stages (*repeated measures ANOVA*; *no main effect of CNO*:  $F_{(1,15)} < 1$ , *n.s.*;  $np^2 = 0.02$ ; *no interaction of CNO by reversal stage*:  $F_{(1,15)} < 1$ , *n.s.*;  $np^2 < 0.01$ ).

The evidence that CNO-treated rats remembered previously rewarded stimulus suggested that the observed deficit did not likely stem from a dysfunction in memory, notwithstanding the lack of a reversal-cost following CNO treatment. The attenuation of a

reversal-cost may be the result of less efficient, or less thorough, learning due to an impairment in attentional set which would increase the cognitive load of the discrimination learning.

Similar to the results for the shift-cost and multiple ID stages, reclassifying CNO-treated rats based on their transduction profile (*i.e.*, either ‘minimal’ or ‘moderate’) did not significantly influence statistical outcome: there was still no evidence of a ‘reversal-cost’ in either minimally- or moderately-transduced CNO-treated rats (*Stage by Revised CNO interaction*:  $F_{(5.23,128.39)} = 9.82$ ,  $p < 0.01$ ; *uncorrected*  $df = 6,150$ ;  $np^2 = 0.48$ ; *Bonferroni-corrected pairwise comparison*). Furthermore, both minimally- and moderately-transduced CNO-treated rats were still less likely to make an error in the initial trials of a reversal than vehicle-treated rats (*main effect of CNO*:  $F_{(2,15)} = 2.06$ ,  $p < 0.05$ ;  $np^2 = 0.35$ ; *Bonferroni-corrected pairwise comparison*), and as above, there was no difference between any of the groups in the number of times they rejected the previously incorrect stimulus (*CNO*:  $F_{(2,15)} = <1$ , *n.s.*;  $np^2 = 0.09$ ).

Results of Pearson correlations found that amount of overall transduction percentage by rat did not influence the number of errors incurred during the exploratory trials (*i.e.*, first four trials) of Rev1 ( $r = 0.40$ ;  $n=9$ ; *n.s.*) or Rev2 ( $r = -0.23$ ;  $n=9$ ; *n.s.*), nor were there any region-specific effects of increasing transduction within the ZIV (Rev1:  $r = 0.44$ ; Rev2:  $r = -0.34$ ; *n.s.*), ZID (Rev1:  $r = 0.29$ ; Rev2:  $r = -0.28$ ; *n.s.*), and LHA (Rev1:  $r = 0.34$ ; Rev2:  $r = -0.14$ ; *n.s.*), respectively, on the generation of errors for either stage. Similarly, the mean number of times rats rejected the previously incorrect stimulus for both Rev1 ( $r = -0.23$ ;  $n=9$ ; *n.s.*) and Rev2 ( $r = -0.31$ ;  $n=9$ ; *n.s.*) was also not influenced by changes in overall transduction percentage, nor by changes in transduction within the ZIV (Rev1:  $r = -0.34$ ; Rev2:  $r = -0.25$ ; *n.s.*), ZID (Rev1:  $r = -0.28$ ; Rev2:  $r = -0.29$ ; *n.s.*), or LHA (Rev1:  $r = -0.14$ ; Rev2:  $r = -0.36$ ; *n.s.*). These findings align with the aforementioned analyses from this chapter and suggest that extraneous transduction to ZI and LHA minimally influenced the behavioural outcome during the reversal stages.

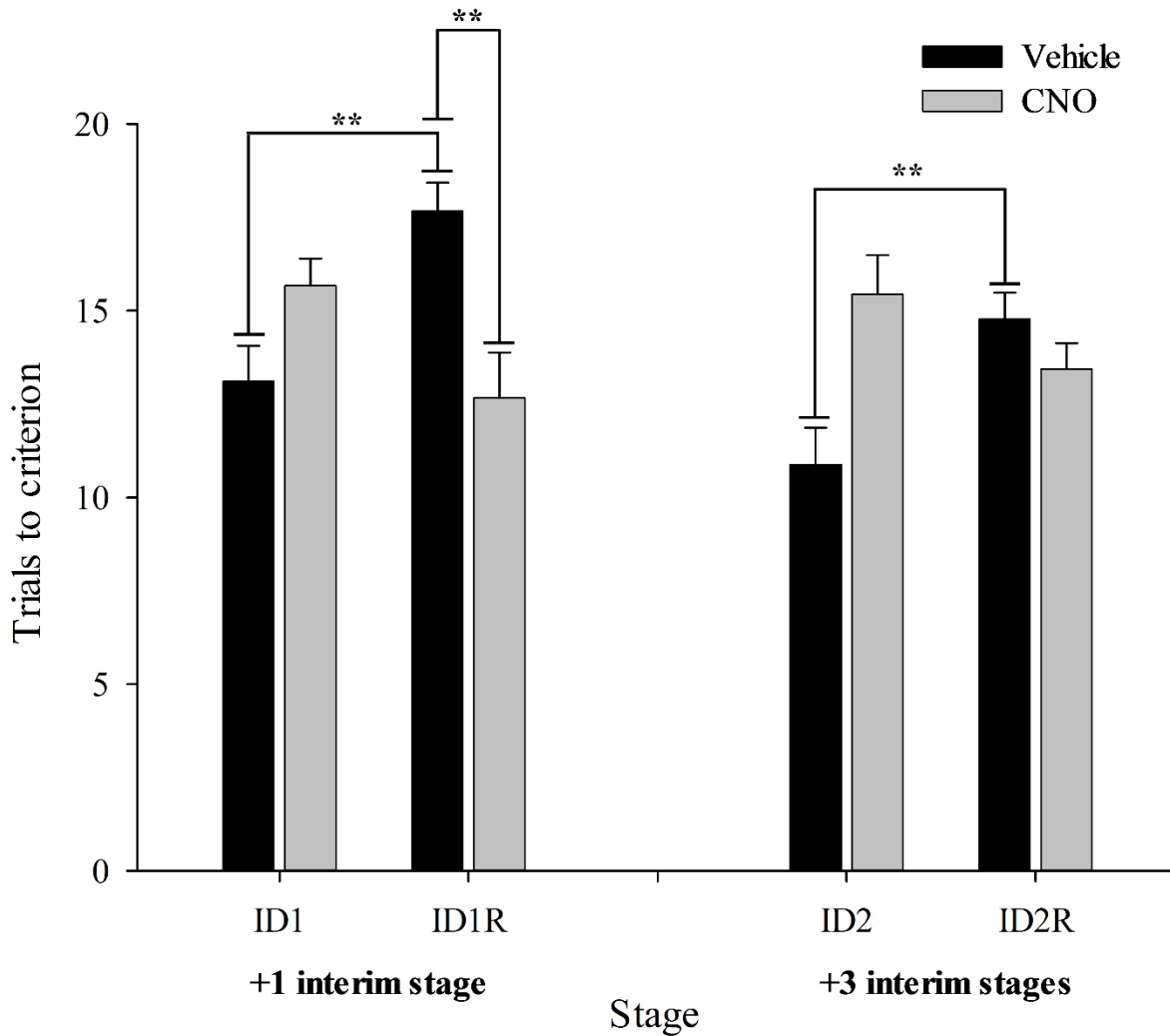


Figure 6.8: Mean trials to criterion (+SEM) illustrating that vehicle-treated rats exhibited a cost for reversing for both reversal stages. In contrast, CNO-treated exhibited no difference between novel and reversal discrimination learning, and reversed faster than vehicle-treated rats in the ID1R stage; \* $p < 0.05$ ; \*\* $p < 0.01$ .

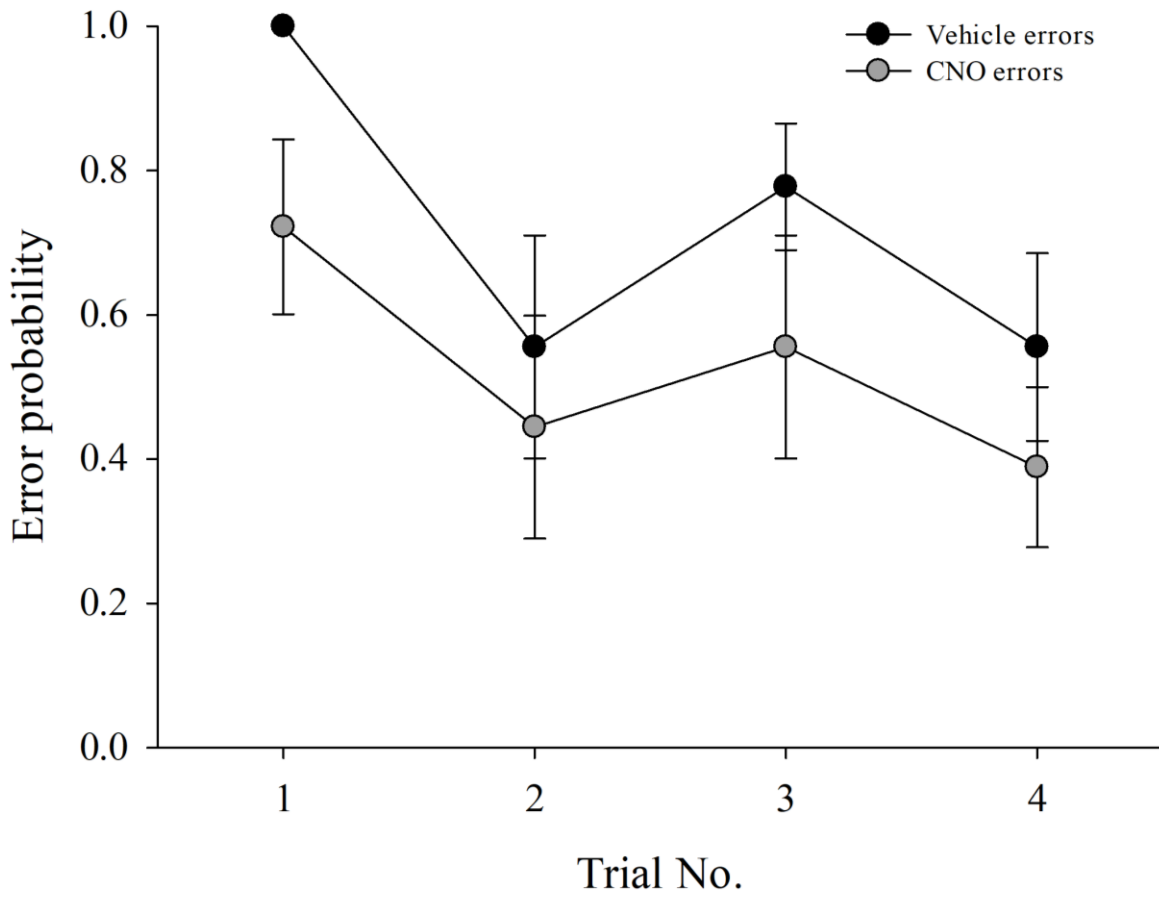


Figure 6.9: Mean  $\pm$ SEM probability of an error for the first four trials of ID1R and ID2R, combined. CNO treatment reduced the probability of an error at the reversal stages.

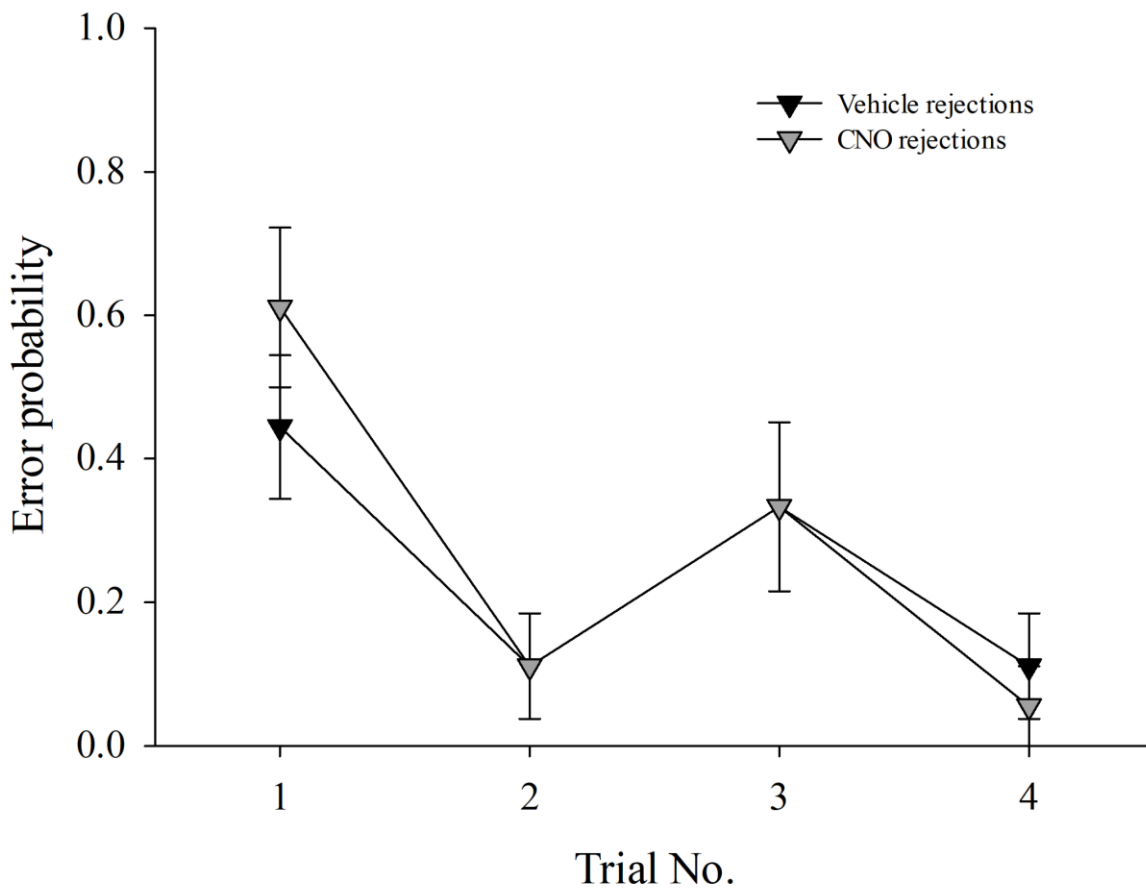


Figure 6.10: Mean  $\pm$ SEM probability of a rejection (i.e., errors where the rewarded bowl was encountered and rejected before the rat dug in the incorrect bowl), for the first four trials of ID1R and ID2R, combined. CNO treatment did not influence the probability of observing this error type.

#### 6.3.3.4 Probe and bi-conditional learning stages

A restricted analysis of the probe stage (figure 6.11) determined that CNO-treated rats required significantly more TTC than vehicle-treated rats (*one-way ANOVA*;  $F_{(1,140.1)} = 30.6$ ,  $p < 0.01$ ; *uncorrected*  $df = 1,160$ ; *Huynh-Feldt correction*). Seven out of nine (78%) vehicle-treated rats obtained criterion in the expected minimum required (six) TTC, whilst the remaining two rats completed the stage with only one mistake. In contrast, it appeared that the change in the irrelevant dimension disrupted performance for CNO-treated rats. At this stage, all rats that were administered CNO made at least two errors before reaching criterion, with 4 out of 9 (44%) rats incurring at least four errors. Consequently, CNO-treated rats (M: 12.3 trials) took twice as many trials to acquire the probe, compared to vehicle-treated rats (M: 6.3 trials). Furthermore, performance for CNO-treated rats on the probe stage did not differ from two of the ID stages, including the preceding ID5 stage (*planned comparison of ID1-ID5p stages*; *Repeated-measures ANOVA Stage by CNO interaction*:  $F_{(4.7,140.1)} = 2.61$ ,  $p < 0.05$ ; *Bonferroni-corrected pairwise comparison*;  $\eta^2 = 0.14$ ).

Excluding the unilaterally-transduced CNO-treated rat from the analysis did not influence the statistical outcome (*one-way ANOVA*;  $F_{(1,117.9)} = 28.5$ ,  $p < 0.01$ ; *uncorrected*  $df = 1,150$ ; *Huynh-Feldt correction*), with the probe data for this unilateral CNO-treated rat (11 TTC; 3 errors) being comparable to the TTC for the remaining bilaterally-transduced CNO-treated animals. Aligned with previous analyses, reclassifying CNO-treated rats as animals with either ‘minimal’ or ‘moderate’ transduction had no effect on the statistical outcome (*one-way ANOVA*;  $F_{(2,128.39)} = 15.7$ ,  $p < 0.01$ ; *uncorrected*  $df = 2,150$ ; *Bonferroni-corrected pairwise comparison*), with no difference in performance between both transduction-groups of CNO-treated rats. Furthermore, changes in transduction percentage did not correlate with behavioural performance at the probe stage ( $r = 0.24$ ;  $n = 9$ ; *n.s.*); higher percentages of transduction did not correlate with increases in impairment. There was also no relationship between the amount of transduction for each region and performance at the probe stage: differences in transduction within the ZIV ( $r = -0.03$ ;  $n = 9$ ; *n.s.*), ZID ( $r = 0.10$ ;  $n = 9$ ; *n.s.*), and LHA ( $r = 0.47$ ;  $n = 9$ ; *n.s.*), respectively, did not significantly correlate with probe TTC following CNO treatment

Analysis of the bi-conditional discrimination (figure 6.11) revealed that CNO-treated rats required significantly fewer TTC than vehicle-treated rats (*one-way ANOVA*;  $F_{(1,17)} = 76.9, p < 0.01$ ). The rapid bi-conditional acquisition by CNO-treated rats compared to vehicle-treated rats supported the hypothesis that CNO-treated rats were attending to both dimensions and likely learning the entire configuration of the stimulus as an approach to solve compound discriminations. Conversely, vehicle-treated rats required considerably more trials, with seven out of nine rats (78%) requiring over twenty TTC. Nevertheless, the vehicle-treated rats did ultimately learn to attend to exemplars in both dimensions to learn the discrimination.

Excluding the data unilaterally-transduced rat from the analysis did not influence the statistical outcome (*one-way ANOVA*;  $F_{(1,15)} = 70.2, p < 0.01$ ); furthermore, the 12 TTC required by this rat for this discrimination was comparable to the remaining 8 bilaterally-transduced CNO-treated rats, of which, 3 also completed this discrimination in 12 TTC. It was also found that reclassifying CNO-treated rats as animals with either ‘minimal’ or ‘moderate’ transduction had no effect on the statistical outcome (*one-way ANOVA*;  $F_{(2,128.39)} = 53.8, p < 0.01$ ; *uncorrected df*=2,150; *Bonferroni-corrected pairwise comparison*), with no difference between the two groups of CNO-treated rats. Furthermore, a change in transduction percentage did not correlate with acquisition of the bi-conditional discrimination ( $r = -0.24; n=9; n.s.$ ); increasing transduction throughout the ZI and LHA does not correlate with a facilitation in acquisition. Similar to the probe, there was also no relationship between the amount of transduction for each region and the acquisition of the bi-conditional discrimination: differences in transduction within the ZIV ( $r = -0.19; n=9; n.s.$ ), ZID ( $r = -0.25; n=9; n.s.$ ), and LHA ( $r = -0.04; n=9; n.s.$ ), respectively, did not significantly correlate with performance following CNO treatment. These findings, coupled with the similar results from this chapter have found that the behavioural data for rats with more robust transduction profiles of the ZI and LHA did not differ from rats with colocalised transduction limited to the STN and minimally to surrounding tissue.



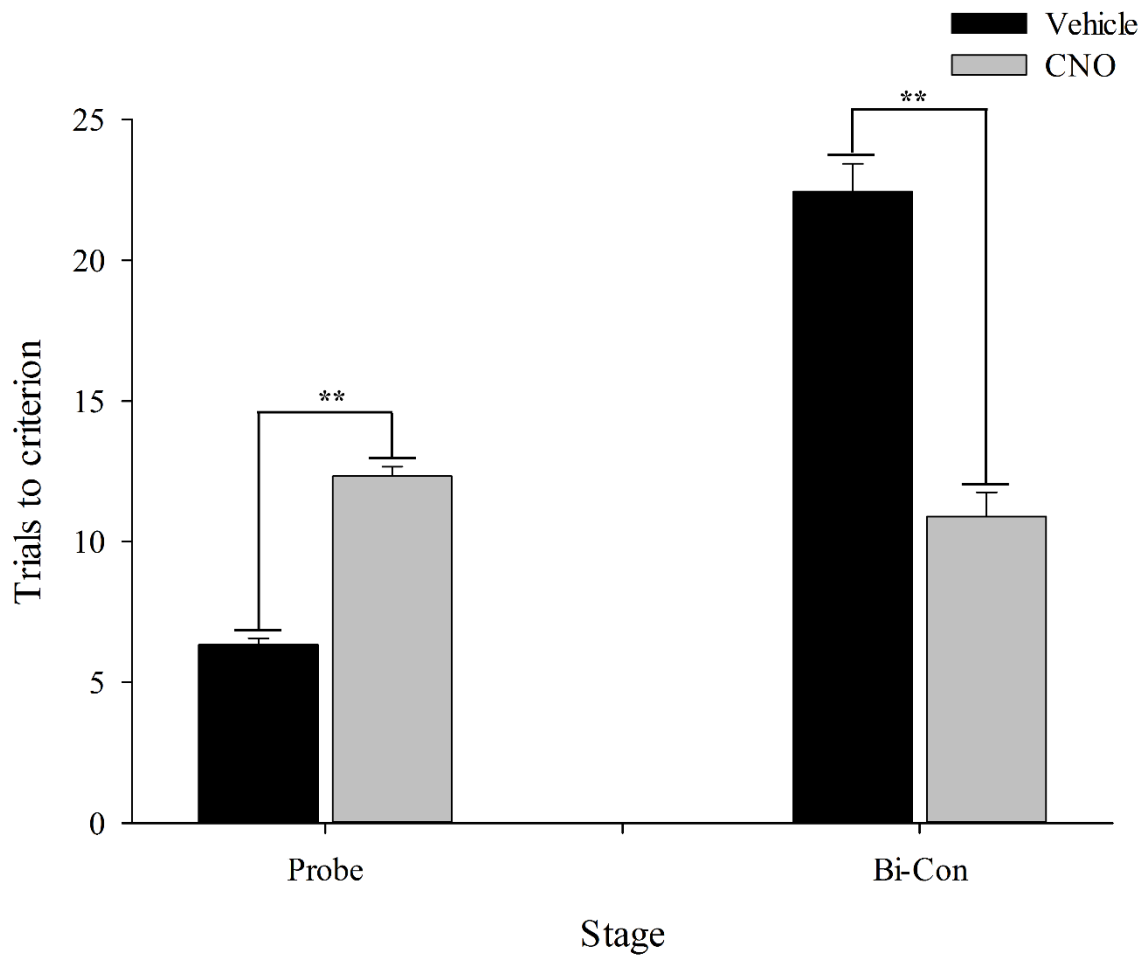


Figure 6.11: Mean trials to criterion (+SEM) for vehicle and CNO-treated rats in the probe and bi-conditional stages. CNO-treated rats were impaired at the probe discrimination, in which only the irrelevant dimension stimuli were changed. Conversely, CNO-treated rats completed the bi-conditional discrimination faster than vehicle-treated rats, in which configural learning rather than an established attentional set would aid in discrimination; \*\* $p < 0.01$ .

## **6.4 Discussion**

The data from the current experiment demonstrated that pharmacosynthetic transduction of the cells within the medial region of the STN and ZI/LHA area with a designer receptor did not disrupt performance at any stage of the 7-stage task in the absence of the designer drug, CNO. Administration of CNO did abolish the expected ID/ED difference in the 11-stage task. The inclusion of additional stages in this task enabled the conclusion that set-formation deficit was not the result of a memory impairment. Neither could ‘slow’ acquisition of set account for the deficit. Rather, the deficit was due to a failure to attend selectively to the dimensions comprising the stimuli resulting in the rat processing the stimuli configurally.

This set-formation impairment was characterised as a disruption of the typical improvement in ID learning performance as a function of experience with ID learning (*i.e.*, no decrease in the number of trials to criterion with successive IDs). The same rats acquired the ED discrimination in fewer trials than vehicle-treated rats, thus not showing the typical ‘shift-cost’, still seen in vehicle-treated rats, when required to shift set between the final ID (ID5) stage and the ED shift stage.

The fact that CNO-treated rats acquired the ED stage in fewer trials than vehicle-treated rats, would at first appear to be an improvement in set-shifting behaviour, and indeed, theoretically, a decrease in trials to solve the ED could stem from an enhanced ability to shift attentional set (Lapiz, Bondi, & Morilak, 2007), yet this claim still requires a positive ID-ED difference, otherwise (*e.g.*, Tunbridge, Bannerman, Sharp, & Harrison, 2004) it is not possible to conclude that an attentional set was present to be shifted away from. Furthermore, if novel ED learning is acquired as quickly as novel ID learning (or faster), this would introduce the likelihood of ‘error shifts’, or the shifting of set when it does not require shifting, which ultimately diminishes the benefit of an attentional set.

### **6.4.1 Memory impairment vs attentional selectivity**

Despite the lack of an apparent reversal cost for CNO-treated rats, and that these rats incur fewer errors during a reversal, which supports the hypothesis for memory impairment, there was nevertheless evidence that these rats did *not* have a memory impairment. CNO-treated rats did not differ from controls in the number of times they

rejected the previously incorrect bowl, and furthermore, these rats were significantly more likely to choose the previously rewarded stimulus, indicating that they remembered the original discrimination.

The attenuation of an observable reversal cost could have then resulted from the larger number of correct/incorrect contingencies that discrimination learning would have demanded, resulting in an increase in cognitive load. This information would have to be retained in memory, whilst learning about novel discriminations presented during the interim stage(s). Theoretically then, increasing the number of interim stages may introduce the possibility of increasing cognitive load to the point which it may attenuate the memory of the original discrimination, yet this was not observed in the current experiment after 3 interim stages (ID2R).

In the current experiment, the hypothesis of attentional dysregulation was investigated by the probe stage, whilst the bi-conditional discrimination stage tested for the potential strategy that CNO-treated rats might have to employ/resort to, in order to complete the task. CNO-treated rats were impaired at a probe stage, which was presented at a point in the test (after the 5<sup>th</sup> ID) when the ID performance of vehicle-treated rats was clearly improved as a result of selective attention to the relevant dimension. Whilst all of the vehicle-treated rats performed with no more than 1 error (7/9 with no errors), none of the CNO-treated rats performed at this level. This indicated that these rats were not attending selectively to the rewarded stimulus dimension: the change in the irrelevant dimension essentially rendered the entire compound stimulus ‘new’, so requiring as many trials to learn as a novel ID stage. This conclusion may at first suggest that CNO-treated rats were simply ‘distracted’ by the stimuli change at the probe stage, yet the data from the bi-conditional discrimination stage illustrated that CNO-treated were, in fact, not distracted by the irrelevant dimension stimuli. Reasoning that, *in lieu* of forming set, CNO-treated rats may be treating the stimuli as novel configurations, the bi-conditional stage was included as a direct test and, indeed, CNO-treated rats solved this discrimination in fewer trials than vehicle-treated rats. This result indicated that CNO-treated rats benefitted when neither dimension predicted reward, such that attending to stimuli holistically was the most adaptive strategy, and therefore was likely employed to solve the task. This also indicated that the STN may play a role in managing the assignment of relevant and irrelevant stimulus information crucial to

attentional set-formation, perhaps by mediating response selection that would be derived from this information, which aligns with its role in action selection (see Frank, 2006; but also Zénon *et al.*, 2016).

In the absence of forming set, employing configural learning may reconcile the noted attenuation of reversal costs; having to retain configurations of stimuli in memory, including those presented during the intervening stages, may have been increased the cognitive load. In light of these findings, the relationship between reversal learning and attentional set merits further investigation, for as it stands associative and attentional shifting are regarded as separate processes, mediated by separate regions of the PFC (Dias, Robbins, & Roberts, 1996). This relationship will be further explored in the subsequent experimental chapter (7).

#### **6.4.2 Evaluating DREADDs technology**

The present experiment utilised a novel, pharmacosynthetic tool to transiently modulate neuronal activity in the target region of the medial STN. Consistent with Chapter 5, we have demonstrated that application of AAV5-CaMKIIa DREADDs infused to the medial area of the STN resulted in expression of G<sub>i</sub>-coupled designer receptors in subthalamic neurons, including transduction of the cells within the medial STN, whilst largely sparing the lateral STN. Studies have suggested that the medial third (33%) of the STN functionally subdivides the nucleus, bestowing a ‘cognitive’ role for the medial region, and a more ‘motor’ role for the lateral (Groenewegen & Berendse, 1990; Heimer *et al.*, 1995); however it is worth noting that the overlap between the cortical projections which are implicated these two behaviours overlap in this nucleus (Janssen, Visser-Vandewalle, & Temel, 2010).

In this chapter, bilateral transduction of the medial STN was achieved in nearly all rats (apart from the unilaterally-transduced animals), however extraneous transduction was incurred to the neighbouring ZIV, ZID and LHA, differentially between rats of both treatment groups. Microscopy revealed that both the mCherry and GFP (NeuN) channels imaged even individually were too ‘noisy’ in areas with high cell density (*e.g.*, medial STN) making it difficult to distinguish the perimeter of the cell in some cases. This was likely due to the thickness of the tissue (and therefore a larger z-axis), which would have increased the amount of signal resulting in overlapping/unclear cell boundaries along the z-axis. This effect remained for imaging at higher

magnification, and was exacerbated by colocalisation. In order to compare the level of transduction quantitatively, spatial area of colocalised transduction was calculated in order to provide an approximate ‘percent transduced’ value. To determine whether this extraneous transduction impacted behaviour when CNO was administered, rats were assigned to either a ‘minimal’ or ‘moderate’ transduction subgroup. Reclassifying nine rats into smaller subgroups increases the risk of Type I error, so to account for this, the mean transduction for each region per rat and total observed transduction per rat was also correlated with a given manipulation of interest. Overall, reclassifying rats into subgroups had no effect on the outcome as rats with ‘minimal’ and ‘moderate’ transduction performed comparably. There was no correlation between the level of transduction of a given region (*e.g.*, ZIV) and any behaviour of interest, and similarly, increasing overall transduction levels to encompass more extraneous regions (or perhaps combinations of regions: *i.e.*, ZI+LHA) does not correlate with levels of impairment for any of the behavioural measures for set-formation in this experiment. These findings are not wholly unexpected as previous work has detailed that including animals with extraneous lesion damage to the ZI in studies with STN-lesioned rats does not influence the behaviour of interest, nor statistical outcome (Baunez *et al.*, 1995; Baunez & Robbins, 1999; Phillips & Brown, 1999; Phillips & Brown, 2000). Furthermore, there is currently no data supporting a robust cognitive role for the ZI and LHA, unlike the STN (see section 1.8). Based on these findings, it was concluded that level of transduction to neighbouring regions, such as the ZI and LHA, had little effect on the behavioural deficits noted in this chapter.

Since level of extraneous transduction to the ZI and LHA had a minimal impact on behaviour, and that data for CNO-treated rats with no transduction of the ZI/LHA did not differ from rats with robust bilateral transduction of these regions, it can be postulated that the deficits observed here likely result from inactivation of the STN. Furthermore, analysis of colocalised transduction revealed that the STN was significantly more transduced than any other region, supporting the notion that inhibition of this area was driving the behavioural outcome. Subsequent work with a larger sample of rats with localised transduction to the STN (and less extraneous transduction) would bolster the assertion that the STN is indeed driving this behaviour; however based on the (lack of a) relationship between rising transduction values and

behavioural deficits reported here, it may be unlikely that further refining transduction will yield a divergent behavioural profile.

Although electrophysiological data were not collected to directly investigate STN inhibition, evidence of decreased c-Fos labelling in DREADDs-infused, CNO-treated animals in Chapter 5, along with compelling behavioural data reported in the current chapter, indexing a robust difference between treatment groups, and colocalised immunofluorescence illustrating nuclear transduction of the STN, provides support that inhibition of the STN was achieved. CNO application in the current experiment yielded significant differences in behaviour at several expected stages of the task, and that furthermore, the magnitude of these effects were more robust than previously observed effects after ibotenic acid lesions of the STN (Xia, Dhawan, Tait & Brown, *unpublished*). The lack of a neurotoxic effect in the current experiment may partially contribute to this difference, along with the specificity of the CaMKIIa-promoted viral vector. CaMKIIa has been identified as a key enzyme in the facilitation of LTP (Lledo *et al.*, 1995; Pettit, Pernman, & Malinow, 1994), which drives learning, and CaMKIIa transcription may be important in consolidation of memory (Lisman, Schulman, & Cline, 2002). The specific targeting of CaMKIIa-positive cells, and the subsequent inhibition of these cells via CNO, may have bolstered the results in this experiment. This assertion may also serve to reconcile the observation that one rat with unilateral transduction of the STN exhibited a comparable behavioural profile to animals with bilateral transduction. This must be interpreted with hesitation as only one rat in the CNO group had unilateral transduction, however based on previous work with STN lesions, we have seen that animals with unilateral lesion damage present with a milder impairment in cognition compared to the more profound impairment in cognition observed in bilaterally-lesioned rats (see section 3.4.1.1). In order to claim that unilateral transduction of the STN is comparable to bilateral, which is *not* being suggested here, a subsequent study with a larger group of unilaterally-transduced animals would be needed, although based on the extant literature, albeit in other cognitive tasks (*e.g.*, visuo-spatial attention, reaction-time, etc.), there is no evidence that unilaterally-inactivated rats should perform comparably to bilaterally-inactivated ones, apart from the data from one rat from this experiment.

The inhibition/excitation of designer receptors with the designer drug CNO undergoes extensive back-metabolism to clozapine in mice and humans (Chang *et al.*, 1998), yet Armbruster *et al.* (2007) demonstrated that CNO lacked appreciable affinity for receptors in rats. Recent research by Maclaren *et al.* (2016) claimed that CNO may not be inert in rats, and found evidence of clozapine back-metabolism in the Long-Evans strain. In mice (Bender, Holschbach, & Stöcklin, 1994) and in schizophrenia patients (Chang *et al.*, 1998), back-conversion of CNO to clozapine results in higher concentrations of clozapine than CNO, yet this was not observed in Maclaren *et al.* At peak concentration (30 min), Maclaren *et al.* reported that plasma levels of clozapine were considerably lower than CNO (0.3µM clozapine compared to 2.2 µM CNO; 5mg/kg CNO).

Maclaren *et al.* (2016) argued that CNO may also induce a behavioural effect, and presented data suggesting that CNO in unoperated rats, dose-dependently, influenced the magnitude of an acoustic startle response, but only for louder tone cues. Interestingly, this effect was only observed for the 1mg/kg dose group, and no effect was found at higher doses (2 and 5mg/kg). In addition, our lab has observed completely different behavioural profiles following inhibitory DREADDs application in the STN, the mPFC (Whyte, Tait & Brown, *unpublished*), and the OFC (Tait & Brown, *unpublished*) after CNO administration. Furthermore, the deficits evidenced in OFC- and mPFC-DREADDs rats are consistent with the observed deficits from lesion studies (Birrell & Brown, 2000; McAlonan & Brown, 2003), further suggesting that CNO offers minimal behavioural impact. The results reported in Chapter 5 also illustrate that CNO administration in unoperated animals does not influence c-Fos labelling in the STN.

To reduce any secondary behavioural effects – particularly those associated with stress – in future experiments with DREADDs, the route of CNO administration can be refined. Since CNO is orally bioavailable (Wess, Nakajima, & Jain, 2013), utilising a palatable jelly (as described in Chapter 4; Whyte, Tait & Brown, *unpublished*) would reduce the stress associated with intraperitoneal administration.

### 6.4.3 Conclusions

In summary, this chapter reports that inhibition of the cells within the STN, ZI and LHA with a designer receptor results in an impairment in attentional set-formation

on the 11-stage task, which may largely be derived from inhibition of the cells of the STN. These data illustrate that CNO-treated rats do not evidence a cost of shifting set, nor the expected improvement across multiple ID stages. Data from the reversal stages indicated that CNO-treated rats clearly remembered the original discrimination, which refuted the hypothesis that these animals had a memory impairment, and instead the attenuated reversal cost likely arose from an increase in cognitive load during discrimination learning. The impaired probe stage performance indicated that CNO-treated rats were still attending to the irrelevant dimension, and the bi-conditional discrimination inferred that this was due to rats responding to the stimuli holistically.

These findings suggest that the STN plays a role in parsing relevant from irrelevant stimulus information. The findings from the reversal stages suggest that associative learning may be intimately linked with set-formation, and therefore more research is needed to examine this relationship. This research would expand our current understanding of the role the STN plays in attentional set, and may elucidate the processes which contribute to set-formation.



# Chapter 7

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## **Examining the relationship between associative and attentional processes with the Overtraining Reversal Effect**

### Background:

Reversal and attentional processes are thought to be doubly dissociated by different regions of the PFC. Evidence from Chapter 6 suggests that these processes may be jointly mediated by the STN. This relationship is explored via the overtraining reversal effect (ORE); a theory which describes that overtraining (OT) a compound discrimination facilitates reversal learning owing to the formation of attentional set.

### Methods:

22 unoperated rats were used to examine the effects of OT in the bowl-digging paradigm in experiment I. In experiment II, the same 18 DREADDs-infused rats from Chapter 6 were used for two test sessions: one with OT, and one without (trained to criterion only).

### Results:

OT facilitated reversal acquisition in experiment I; experiment II revealed that OT exacerbated reversal performance for CNO-treated rats, compared to when these rats were trained to criterion only; this likely resulted from an inability to form attentional set.

### Conclusions:

CNO-treatment prevents the detection of an ORE; OT strengthens the association of the stimulus configuration with reward, which exacerbates reversal learning. Associative and attentional processes do not operate in parallel, and can be mediated by a single brain region.

### 7.1.1 Introduction

As discussed in the General Introduction (see section 1.3; page 28), lesions to the monkey lateral PFC (or rat mPFC) impair ED performance but not reversal learning, whilst lesions of the OFC impair reversal learning but not ED performance (Dias *et al.*, 1996a) (Birrell and Brown, 2000 and McAlonan *et al.*, 2002). This demonstration of a ‘double dissociation’ suggested that reversal learning and ‘attentional’ shifting were independent, operating either in a hierarchy or in parallel (see Dias *et al.*, 1996). However, Chase *et al.* (2012) demonstrated that there was an impairment in attentional set-formation in OFC-lesioned rats and this was why ED performance was not impaired: when additional ID stages were included to encourage set formation, ED performance was impaired. This evidence suggest that reversal learning performance and ED performance are inter-related.

In the case of OFC-lesion deficits, set appeared to be slow to form, but with sufficient experience, it did form. In the case of the STN, the set-formation deficits seem to be far more profound. Although it is not possible to conclude that no amount of additional experience would result in set formation, there is no reason to believe that it would. The implication of this is that the STN-mediated deficit reflects a fundamental impairment in the ability to attend selectively to the stimulus dimensions of multidimensional stimuli. The experiments reported in the current chapter exploit a phenomenon called the ‘overtraining reversal effect’ (ORE) to examine this further.

The ORE was first described by Reid (1953), who trained rats on a simple black-white discrimination and then gave an extra 150 trials of overtraining. Rather than taking longer to reverse the discrimination than rats without overtraining – as might have been expected (intuitively) due to a stronger stimulus-reward association – the opposite effect was found: ‘overtraining’ (OT) resulted in more rapid learning of the reversal than control rats. Sutherland (1959) suggested that OT facilitated reversal learning by increasing the salience of the predictive cues. Mackintosh (1963a) showed that rats with OT of a brightness discrimination reversed more rapidly and spent a smaller proportion of trials responding to irrelevant cues (*i.e.*, responding at chance level) compared to control rats. On the basis of this observation, Sutherland & Mackintosh (1971) suggested that OT had the effect of reducing or eliminating the number of ‘error factors’ (*i.e.*, cues that might be

tested as predictive of reward but which are, in fact, irrelevant), essentially inhibiting the processing of, or down-regulating attention to, irrelevant information. Sutherland and Mackintosh (1971) made 2 predictions about behaviour if the OT resulted in increased attentional focus on the relevant, relative to irrelevant, cues: first, that there would be an increase the number of perseverative responses at the outset of reversal learning; and, second, that there would be a lower probability of responding to irrelevant cues, hence fewer trials of chance responding. Mackintosh's two-stage learning theory, in which the association of stimuli and reward strengthens/weakens attentional 'analysers', explains the ORE as the result of the differential strength of the analysers and, thus, is evidence of attentional set.

Sutherland and Mackintosh (1971) review the literature pertaining to the ORE, and concluded that while there is strong evidence for the ORE, there were also a significant number of studies – at least 35 – that failed to replicate the effect. Mackintosh (1969) demonstrated that the ORE was not generated during an easy spatial discrimination learning task and only for a more difficult visual discrimination when the reward was large. He also noted that 'problem difficulty' was not defined with respect to the absolute number of errors or trials to criterion, but rather the initial probability of responding to the relevant dimension.

In the first experiment reported here, we sought to determine the parameters for observing the ORE in the bowl-digging paradigm as this has not previously been demonstrated. We expected that OT would be an effective manipulation based on the observation by Garner *et al.* (2006), who reported that OT at the ID-reversal increased TTC of the subsequent ED (suggestive of enhanced attentional set), although they did not overtrain an acquisition or demonstrate an ORE. In the second experiment, we explored the effect of inactivation of the STN and the ZI/LHA, using DREADDs, on the ORE. CNO-treated rats do not form attentional set (see Chapter 6), therefore they should also not show an ORE if it results from the same attentional mechanisms. Furthermore, if CNO-treated rats are solving the discriminations 'configurally', then it would also be expected that OT may actually retard reversal performance as a result of a rigid stimulus-response contingency.

## **7.1 Experiment I**

### **7.1.2 Materials and methods**

#### *7.1.2.1 Animals*

Twenty-two experimentally naïve female Lister hooded rats (bred in-house; University of St Andrews, *from Charles River stock*), with a mean starting weight of 217g and ending weight of 292g, were used. Husbandry and housing details are described in the General Methods (see section 2.1).

#### *7.1.2.2 Apparatus and testing*

See section 2.2 of the General Methods chapter.

#### *7.1.2.3 Design*

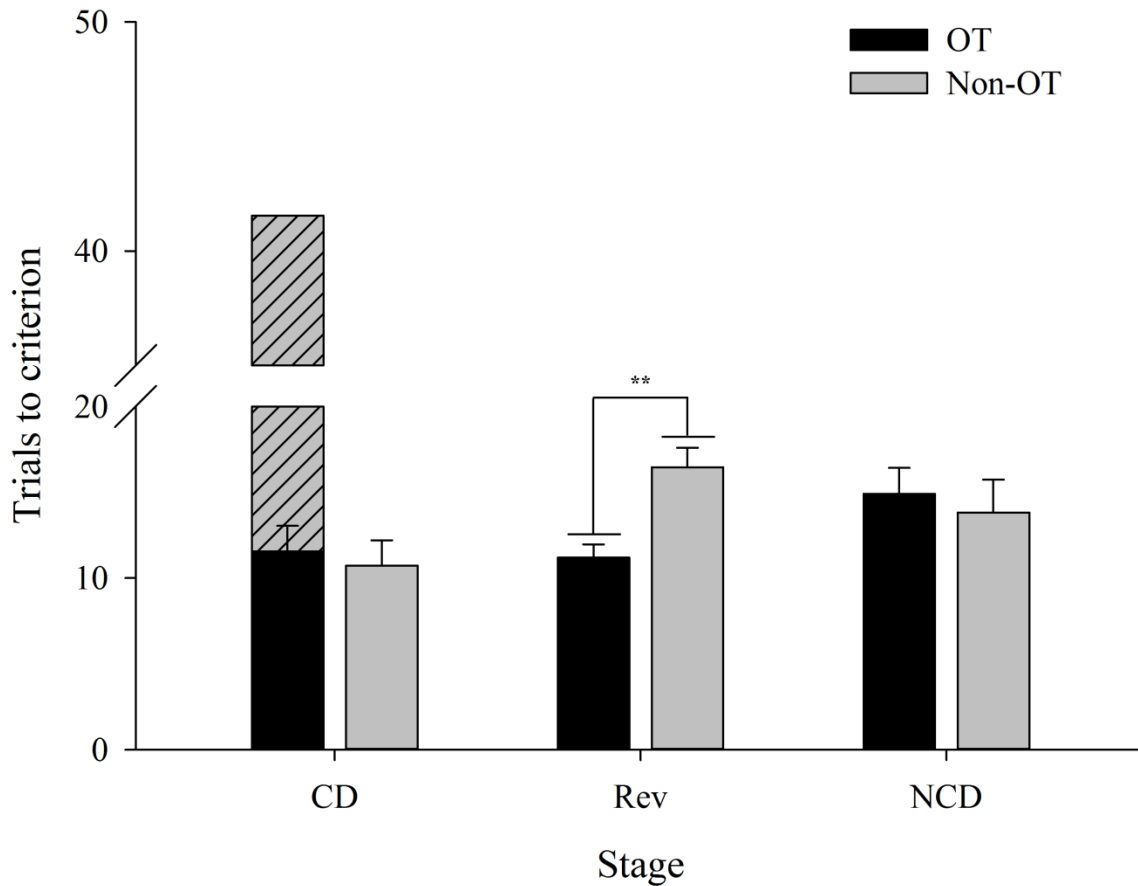
A number of designs were considered and piloted (including adding a third dimension, which we later dropped) before arriving at the design presented here (see table 7.1.1). We used compound odour and media stimuli (with the rewarded dimension counterbalanced) and we omitted the initial SD stage. Thus, the 3 stages were: CD (with (n=11), or without (n=11), 30 additional trials of OT); Rev1; acquisition of a novel compound discrimination (NCD), with the relevant dimension being the previously irrelevant one. The stimuli were the first two stimulus pairings used in the bowl-digging paradigm (see section 2.4.2).

<b>Discimination</b>	<b>Relevant dimension</b>	<b>Discriminanda</b>	<b>Irrelevant</b>
Compound Discrimination (CD)	Medium	<i>Coarse tea</i> , not fine tea	Cinnamon or ginger
Compound Reversal (Rev1)	Medium	<i>Fine tea</i> , not coarse tea	Cinnamon or ginger
Novel Compound Discrimination (NCD)	Odour	<i>Sage</i> , not paprika	Sand or grit

*Table 7.1.1: An example procedure of a typical test session; thirty additional “overtraining” trials were administered at the CD stage for rats in the OT condition.*

### 7.1.3 Results

Rats with OT reached criterion at the reversal stage in significantly fewer trials (M: 11.2) than rats trained to criterion only (M: 16.5; *Stage by OT interaction*:  $F_{(2,40)} = 3.78$ ,  $p < 0.05$ ;  $\eta^2 = 0.16$ ; *figure 7.1.1*). A Bonferroni-corrected pairwise comparison determined OT influenced only TTC at the reversal stage: there was no effect on the novel acquisition.



*Figure 7.1.1: Mean trials to criterion (+SEM) demonstrating that overtraining rats at facilitated a faster reversal learning acquisition, than being trained to criterion only; \*\* $p < 0.01$ .*

#### **7.1.4 Conclusion**

The findings from Experiment 1 demonstrated that 30 OT trials of a compound discrimination resulted in faster learning of its reversal, compared to rats just trained to criterion. This procedure is therefore a useful tool to further investigate the relationship between attention and associability and the role the STN.

## **7.2 Experiment II**

### **7.2.2 Materials and methods**

#### *7.2.2.1 Animals*

The rats used were the same eighteen male, Lister hooded rats (Charles River, UK) (mean starting weight of 398g and a mean end weight of 525g) tested in Chapter 6 (see section 6.2.3 for details of surgery and section 6.2.6 for histology). Rats were assigned to receive CNO (n=9) or vehicle (n=9), according to which they had received in Chapter 6: rats previously given CNO were given vehicle here, and vice-versa.

#### *7.2.2.2 Design*

Experiment 2 used the 7-stage task (see Table 2.2 for exemplar pairings), with the omission of the SD. Each rat completed 2 test sessions, with approximately 10 days between tests, first with overtraining (OT) at just the CD stage and then with learning to criterion only (non-OT). Shift-type, initial correct stimulus and order of presentation were all counterbalanced between groups and test.

#### *7.2.2.3 Data analysis*

The current study was a 2x2x6 mixed factorial design, and trials to criterion (TTC) data were analysed by repeated-measures ANOVA. There was a within-subjects factor of Stage (six levels); a within-subjects factor of Condition (two levels: OT (test 1) and non-OT (test 2) and a between-subjects factor of Treatment group (two levels: CNO or vehicle). Error-type was analysed at the reversal stages, with the number of errors prior to the first correct response classified as ‘perseverative’, and subsequent trials after the first correct response but before the final 6-correct responses, were classified as ‘at chance’ responses.



## 7.2.3 Results

### 7.2.3.1 Histology

As stated for the histology results for Chapter 6 (section 6.3.1), two rats – one from the CNO and one from the vehicle treatment group – expressed unilateral mCherry expression, and that the data for these two rats were included in all analyses in this section, as the inclusion of the data for the unilateral CNO-treated rat here did not influence statistical outcome; as in Chapter 6, this will be evaluated separately in the subsequent behavioural data results. Also, the results of the colocalised transduction analysis are the same as Chapter 6 and are reported in detail in section 6.3.1, with some exceptions owing to the change in in CNO administration for the current experiment. Briefly, 8 of the 18 rats (6 CNO-treated, 2 vehicle-treated) presented with no nuclear transduction of the ZIV or the ZID, whilst a different subset of 8 rats did not present with any nuclear transduction of the LHA (4 CNO-treated, 4 vehicle-treated); conversely, transduction in the STN was present in all rats (with only four rats presenting with exclusive transduction of the STN: 3 CNO-treated; 1 vehicle-treated).

Similar to the behavioural analyses presented for Chapter 6, since any group with an  $n$  of 3 (exclusive STN transduction) would under-power analyses, similar to Chapter 6, CNO-treated animals for this experiment were assigned to a ‘minimally-transduced’, and a ‘moderately-transduced’ group. Rats in the ‘minimally-transduced’ group ( $n=5$ ) had STN transduction, *plus* only minimal unilateral transduction of only one extraneous brain region (either the ZIV or LHA; range of transduction for extraneous region = 0-15%). Conversely, rats in the ‘moderately-transduced’ group ( $n=4$ ) had STN transduction, *plus* bilateral transduction of one or more brain regions (range of transduction for extraneous region = 0-50%). The total amount of observed transduction per rat was also calculated by summing the transduction percentages across each hemisphere, section and brain region for each animal, which could then be compared with manipulations of interest for the current experiment. This correlational analysis was also done for each region to determine in any region-specific effects of transduction influenced behaviour.

### 7.2.3.2 Overtraining impairs reversal learning

Overall, both Condition (OT vs non-OT) and Treatment group (CNO vs vehicle) differentially changed performance at different stages of the task as expected (*Stage by Condition by Treatment group interaction*:  $F_{(3,6,57.6)} = 3.34$ ,  $p < 0.05$ ; *Huynh-Feldt correction*; *uncorrected*  $df = 5,80$ ;  $n\eta^2 = 0.17$ ). Also, removing the data for the unilaterally-transduced CNO-treated rat from the analysis did not change the three-way interaction of Condition, Stage and Treatment group ( $F_{(3,6,54.3)} = 2.98$ ,  $p < 0.05$ ; *Huynh-Feldt correction*; *uncorrected*  $df = 5,75$ ;  $n\eta^2 = 0.17$ ). Therefore, analysis of simple interactions and simple main effects was undertaken.

For the vehicle treatment group, OT of the initial CD discrimination reduced the overall TTC at Rev1, as expected, and also at Rev2 (the reversal following the ID discrimination, which was not overtrained), compared to when they were trained to criterion only ( $p < 0.05$ ), thereby generating evidence of a long-lasting ORE (*Restricted analysis of Reversal stages: Treatment group by Condition by Stage interaction*:  $F_{(2,57.58)} = 3.90$ ,  $p < 0.05$ ; *uncorrected*  $df = 2,80$ ;  $n\eta^2 = 0.45$ ; *Bonferroni-corrected pairwise comparison*; figure 7.2.1). Numerically, the TTC for Rev1 and Rev2 was exactly the same in the OT condition (12.78 for both). There was no effect of OT on Rev3 (the reversal following the ED discrimination).

Responses (errors) prior to the first correct response were deemed perseverative, while the number of trials from the first correct response to the error trial preceding the final 6-correct trials were considered to be ‘at chance’ responding. For rats with more than 1 trial of ‘at chance’ responding, the mean proportion of correct responses to trials was calculated; for vehicle-treated rats, the ratio of correct responses to total ‘at chance’ trials for Rev1 did not differ between OT (Mean responses ‘at chance’: 0.89; proportion: 0.5) and non-OT (Mean responses ‘at chance’: 5.33; proportion: 0.51) conditions. These findings support the contention that the rats were indeed responding at chance.

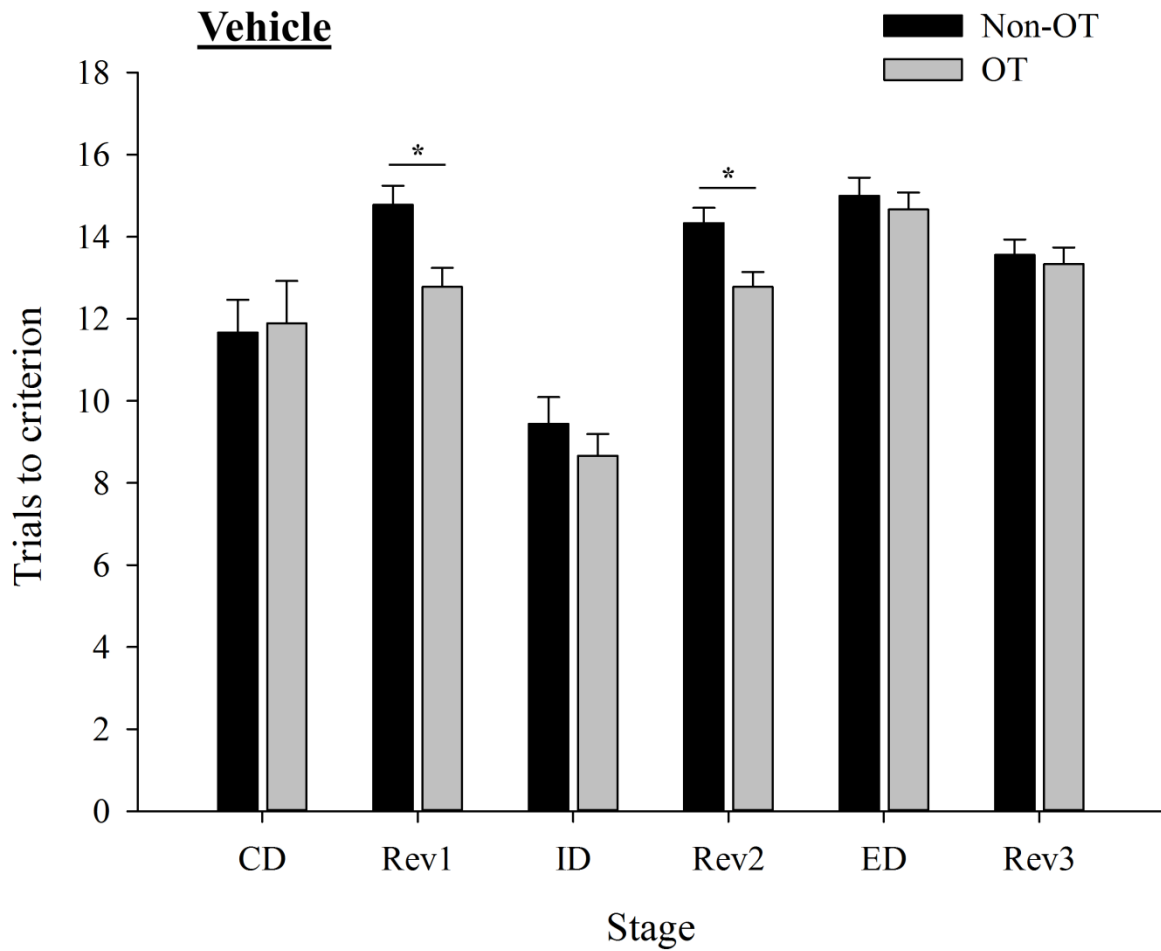
The pattern of errors for the vehicle treatment group changed as a result of OT, with OT significantly increasing the number of perseverative responses for Rev1 (but not Rev2), and also significantly reducing the number of responses made at chance, for both Rev1 and Rev2 compared to rats trained only to criterion (*Condition by Response Type by Treatment*

group by Stage interaction:  $F_{(2,32)} = 7.04$ ,  $p < 0.05$ ; Bonferroni-corrected pairwise comparison;  $np^2 = 0.31$ ; figure 7.2.2). At Rev1, as a result of OT, 5 out of 9 vehicle-treated rats (56%) made only perseverative responses, immediately followed by the final 6-correct responses thus showing no evidence of ‘at chance’ responding. Of these 5 rats, 2 also showed no ‘at chance’ responding for Rev 2.

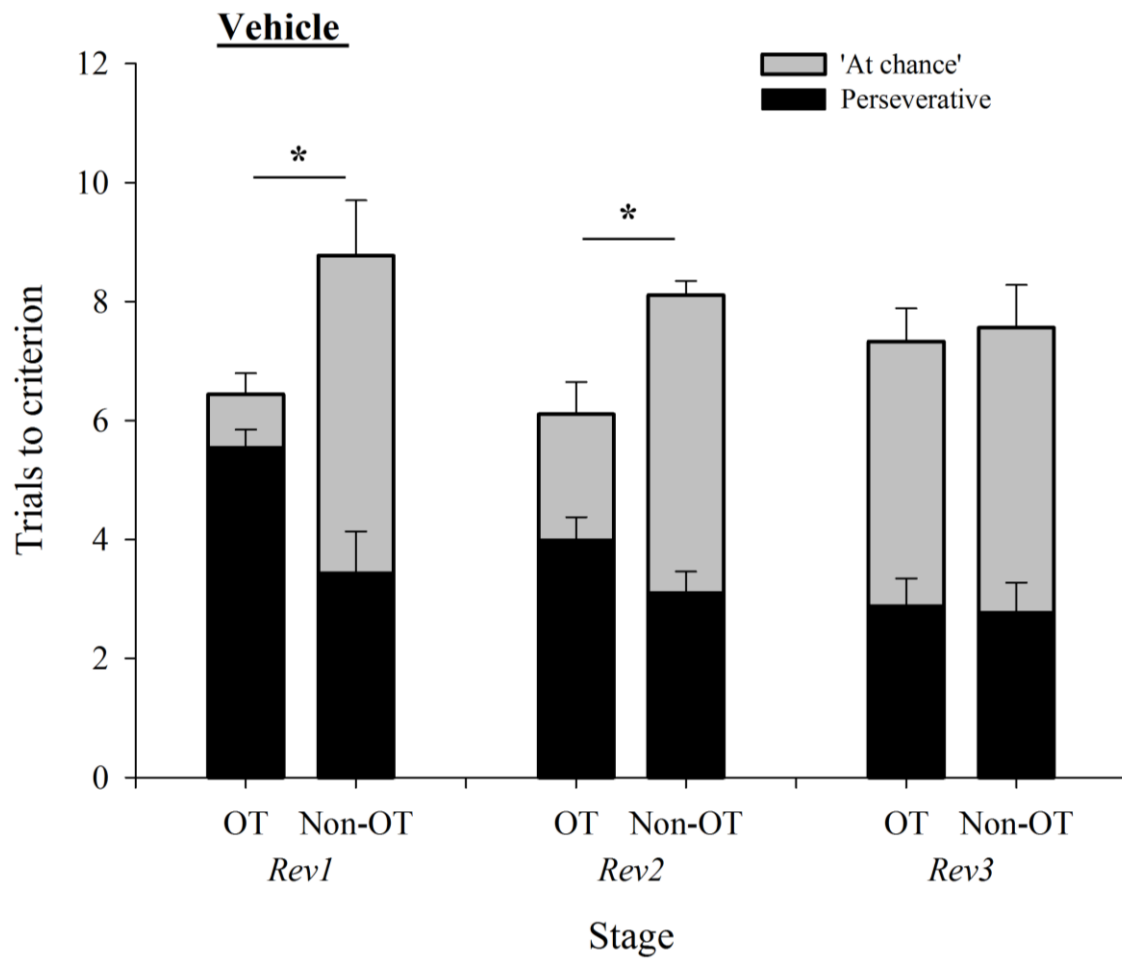
Conversely, in the CNO-treatment group, OT impaired reversal learning performance at Rev1 ( $p < 0.01$ ; figure 7.2.3). The Rev1 mean TTC for the non-OT condition was 14.67, which is very similar to value for the vehicle-treatment group (14.78) (Bonferroni-corrected pairwise comparison, *n.s.*). This pattern indicated that not only did CNO treatment prevent an ORE, but OT actually increased TTC, to a mean of 18.78, at Rev1. Rev2 showed neither benefit nor cost of CD-OT: the mean TTC for OT was 15.78 and for non-OT was 15.22. The increase in TTC as a result of OT was not due to an increase in perseverative errors in the CNO-treatment group, but rather to an increase in the number of responses ‘at chance’ ( $p < 0.05$ ; Bonferroni-corrected pairwise comparison; figure 7.2.4). Removing the data for the unilaterally-transduced animal had no effect on the statistical outcome or conclusion of this section: CNO-treated rats were still impaired at Rev1 when CD-OT was given, compared to being trained to criterion only ( $p < 0.05$ ; Bonferroni-corrected pairwise comparison), and that this impairment was driven by an increase in the number of responses ‘at chance’ ( $p < 0.05$ ; Bonferroni-corrected pairwise comparison). Furthermore, the Rev1 TTC data for this rat for the OT condition (19 TTC) indicates a marked impairment compared to when it was trained to criterion only (14 TTC), suggesting that performance worsened following OT, which is consistent with the remaining bilaterally-transduced animals.

Reclassifying CNO-treated rats as animals with either ‘minimal’ ( $n=5$ ) or ‘moderate’ ( $n=4$ ) transduction had no effect on the statistical outcome for the omnibus ANOVA (Stage by Condition by Revised Treatment group interaction:  $F_{(7,4,55,61)} = 2.15$ ,  $p < 0.05$ ; Huynh-Feldt correction; uncorrected  $df = 10,75$ ;  $np^2 = 0.22$ ), and performance between these two transduction groups did not differ for any stages of the task (Bonferroni-corrected pairwise comparison). Consequently, OT still impaired reversal learning for both CNO-treated transduction groups. Furthermore, a Pearson correlation revealed that increasing levels of

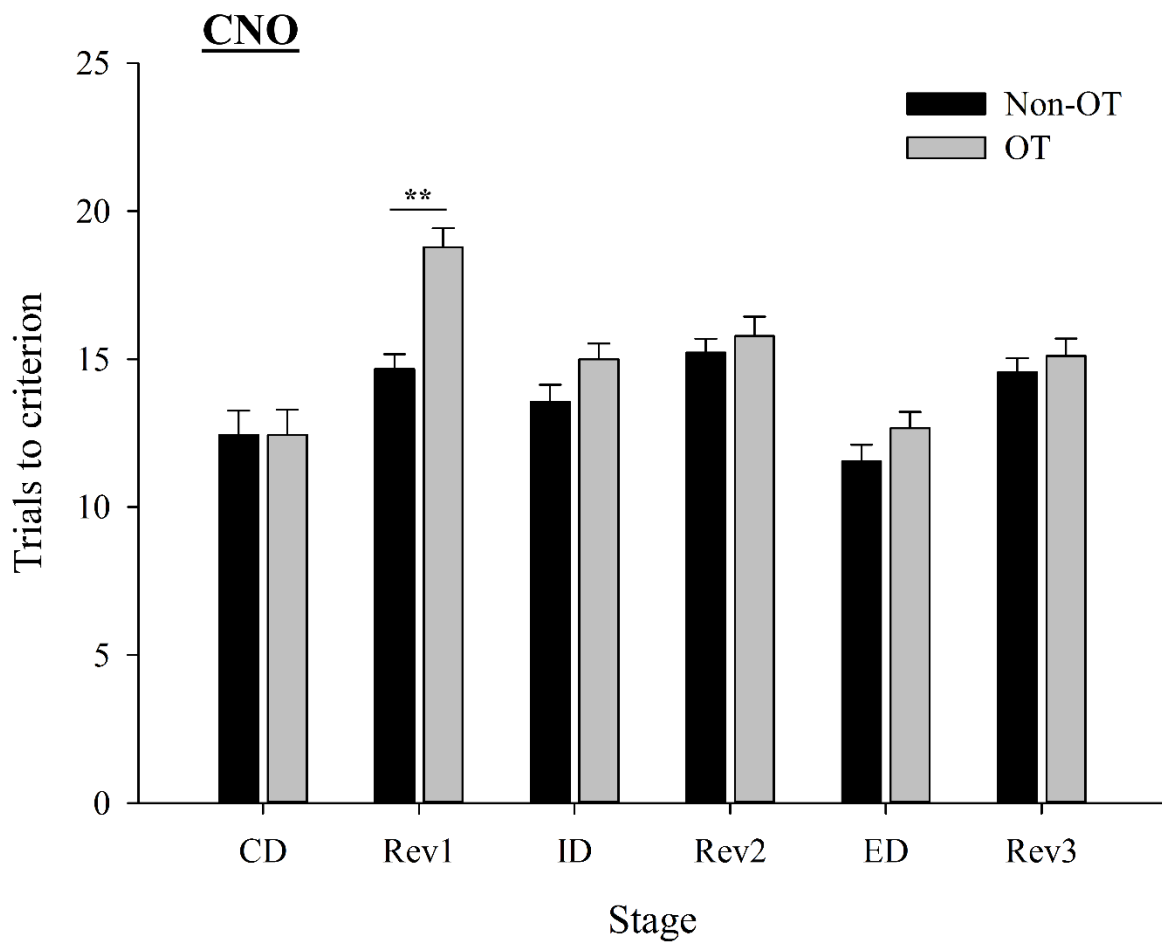
transduction to areas outside the STN (*i.e.*, in the ZI & LHA) did not influence performance for the reversal when OT ( $r = 0.24$ ;  $n=9$ ; *n.s.*); that is, higher levels of transduction are not correlated with exacerbated reversal performance following CNO and OT. Furthermore, there was no relationship between the amount of transduction for each region and the level of impairment at Rev1 when OT: differences in transduction within the ZIV ( $r = -0.04$ ;  $n=9$ ; *n.s.*), ZID ( $r = -0.07$ ;  $n=9$ ; *n.s.*), and LHA ( $r = -0.27$ ;  $n=9$ ; *n.s.*), respectively, did not significantly correlate with Rev1 behaviour following CNO treatment. Overall, reversal data for rats with more robust transduction profiles of the ZI and LHA did not differ from rats with colocalised transduction limited to the STN and minimally to surrounding tissue; this further supports that inactivation of the STN is driving the impairments observed here.



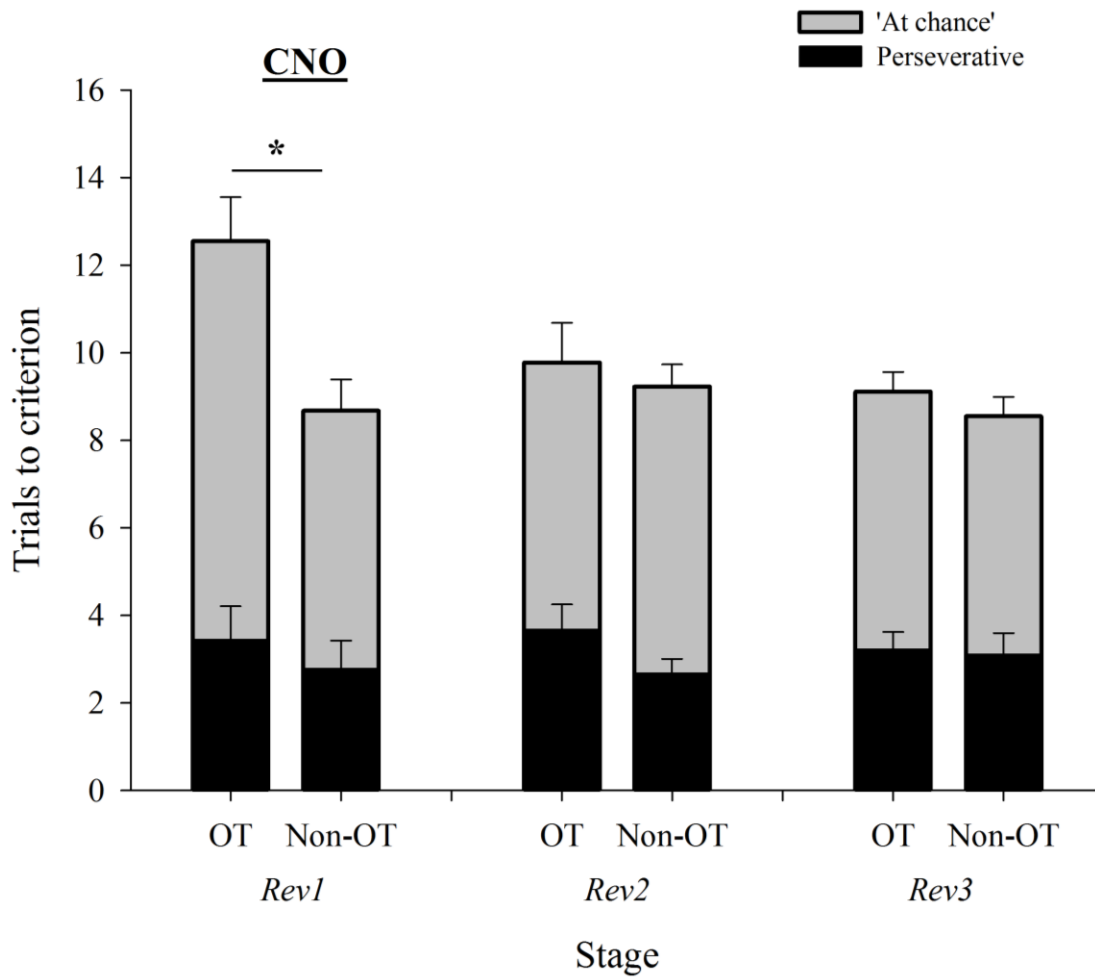
*Figure 7.2.1: Mean trials to criterion (+SEM) for vehicle-treated rats on the modified six stage task, for the OT (+30 post-criterion trials), and non-OT test sessions; OT significantly improved reversal learning performance for Rev1 and Rev2; \* $p < 0.05$ .*



*Figure 7.2.2: Mean number of trials (+SEM) of perseverative and 'at chance' responses for vehicle-treated rats; OT increased the number of perseverative responses (errors) at Rev 1, but decreased the number of 'at chance' responses for Rev1 and Rev2.*



*Figure 7.2.3: Mean trials to criterion (+SEM) for CNO-treated rats on the modified six stage task, for the OT (+30 post-criterion trials), and non-OT test sessions; OT significantly retarded reversal learning performance; \*\* $p < 0.01$ .*



*Figure 7.2.4: Mean number of trials (+SEM) of perseverative and 'at chance' responses for CNO-treated rats; OT increased the number of responses made at chance for Rev 1, but did not influence the number of perseverative responses incurred.*



### 7.2.3.3 CNO-treated rats did not form an attentional set

To investigate the cost of shifting set, a restricted analysis of the ID and ED stages was conducted. There was a very clear main effect of Treatment group, with a large and significant effect of Stage in the vehicle treated group (*Stage by Treatment Group interaction*:  $F_{(1,80)} = 155.20$ ,  $p < 0.01$ ; *Bonferroni-corrected pairwise comparison*;  $np^2 = 0.90$ ) and not in the CNO-treated rats. Condition (OT vs non-OT) had no effect on the size of the shift cost and, furthermore, the CNO-treated rats did not form an attentional set regardless of Condition (*Condition*:  $F_{(1,16)} = <1$ , *n.s.*;  $np^2 = 0.03$ ). Shift-cost data are illustrated for CNO- and vehicle-treated rats below, in figure 7.2.5 for the OT session, and figure 7.2.6 for the non-OT session.

There was a significant difference between the ID and ED stages in the CNO-treatment group, with ID requiring significantly more TTC than ED (*Bonferroni-corrected pairwise comparison*;  $p < 0.05$ ). Conversely, vehicle-treated rats acquired the ID discrimination in significantly fewer trials than the CNO-treated rats (*Bonferroni-adjusted pairwise comparison*,  $p < 0.05$ ), further illustrating the benefit of selective attention to the reward-relevant dimension. Consistent throughout this chapter (and in Chapter 6), removing the unilaterally-transduced rat from the analysis did not influence the statistical outcome of the ID-ED analysis: CNO-treated rats still did not exhibit signs of set-formation regardless of Condition (*Bonferroni-corrected pairwise comparison*), and that the ID required significantly more TTC than ED for this group of rats (*Bonferroni-corrected pairwise comparison*;  $p < 0.05$ ). Furthermore, the raw data for this unilaterally-transduced animal revealed that the ID required more TTC than the ED in both Conditions (OT ED-ID = -2; non-OT ED-ID = -3).

Reclassifying CNO-treated rats as animals with either ‘minimal’ or ‘moderate’ transduction had no effect on the statistical outcome of the ID-ED comparison: vehicle-treated rats still presented with a robust shift-cost, whilst both groups of CNO-treated did not (*Stage by Revised Treatment Group interaction*:  $F_{(2,75)} = 77.94$ ,  $p < 0.01$ ; *Bonferroni-corrected pairwise comparison*;  $np^2 = 0.92$ ). Furthermore, both groups of CNO-treated rats still acquired the ID in significantly more TTC than the ED, whilst demonstrating no between-group differences in performance for either stage (*Bonferroni-corrected pairwise*

*comparison;  $p < 0.05$* ). A Pearson correlation revealed that increasing amount of transduction to areas outside the STN (*i.e.*, in the ZI & LHA) did not influence the magnitude of the shift-cost for CNO-treated rats when OT ( $r = -0.54; n=9; n.s.$ ), nor when trained to criterion only ( $r = -0.22; n=9; n.s.$ ); essentially, higher levels of transduction did not influence a more rapid acquisition of the ED (nor was it exacerbated). Similarly, there was no relationship between the amount of transduction for each region and the magnitude of the ID-ED difference when CNO-treated rats were given OT or non-OT: differences in transduction within the ZIV (OT:  $r = -0.44$ ; non-OT:  $r = -0.12; n.s.$ ), ZID (OT:  $r = -0.46$ ; non-OT:  $r = -0.09; n.s.$ ), and LHA (OT:  $r = -0.31$ ; non-OT:  $r = -0.44; n.s.$ ) did not significantly correlate with shift-cost performance.

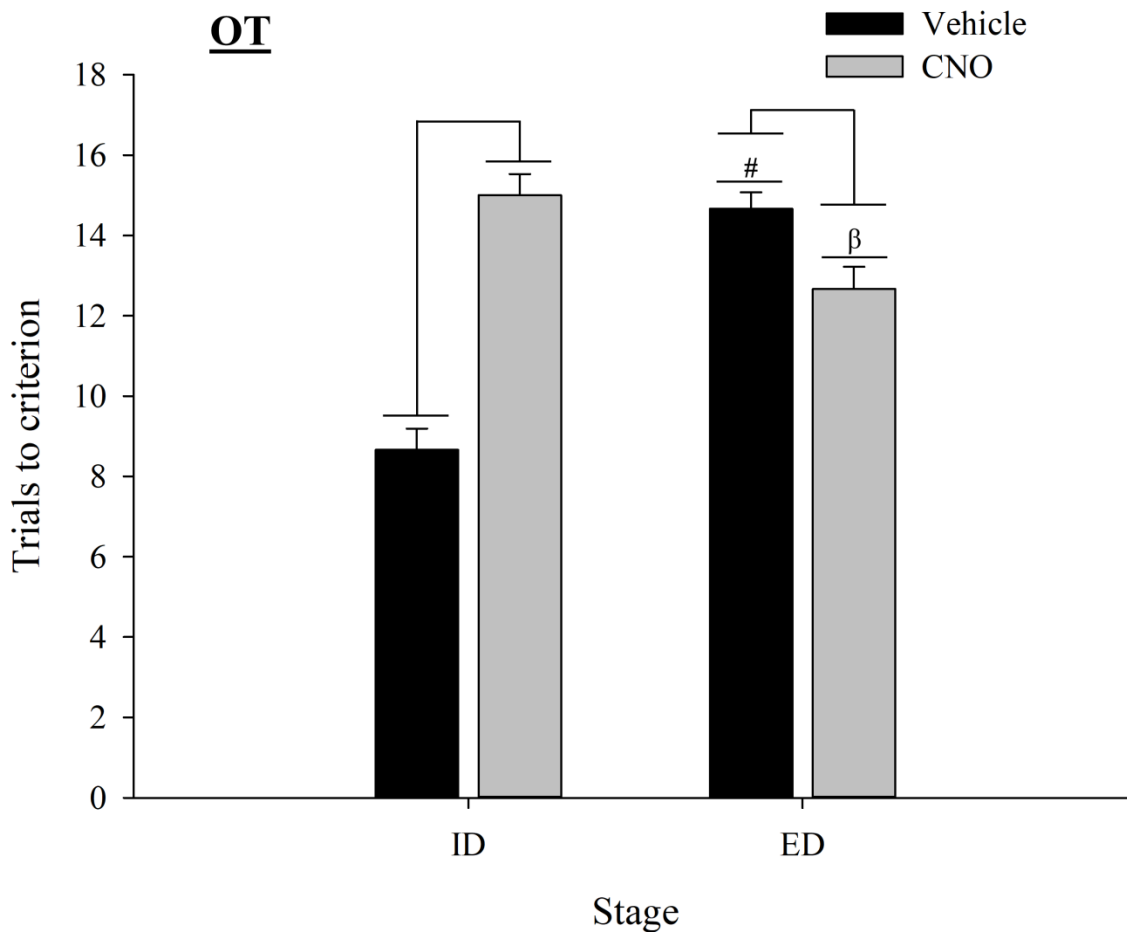
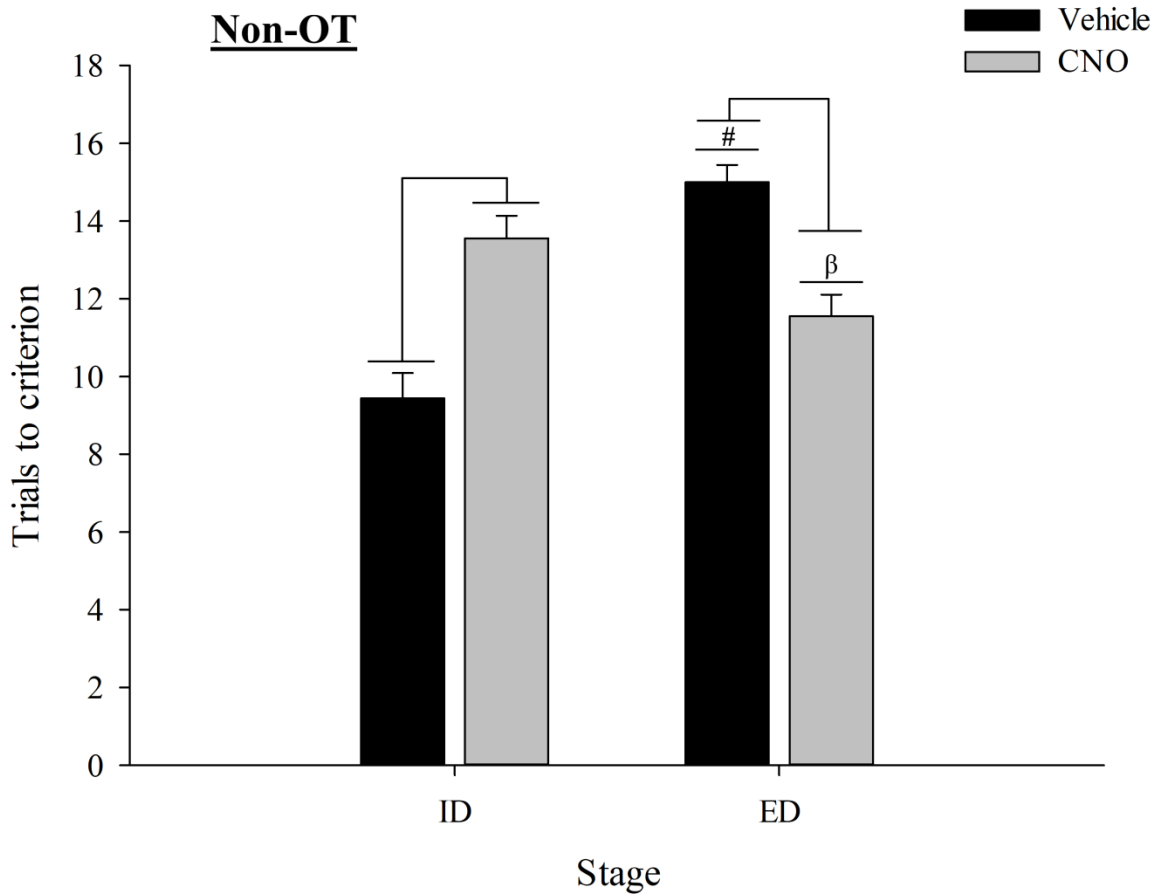


Figure 7.2.5: Mean trials to criterion (+SEM) for vehicle- and CNO-treated rats at the ID and ED stages for the OT test session. ‘#’ indicates that vehicle-treated rats exhibited a significant positive cost of shifting attentional set between the ID and ED stages. ‘β’ indicates that CNO-treated acquired the ED stage in fewer trials than ID. CNO-treated rats were impaired at the ID, but completed the ED in fewer trials than vehicle-treated rats. \*\* $p < 0.01$ .



7.2.6: Mean trials to criterion (+SEM) for vehicle- and CNO-treated rats at the ID and ED stages for the non-OT test session. ‘#’ indicates that vehicle-treated rats exhibited a significant positive cost of shifting attentional set between the ID and ED stages. ‘β’ indicates that CNO-treated acquired the ED stage in fewer trials than ID. CNO-treated rats were impaired at the ID, but completed the ED in fewer trials than vehicle-treated rats. \*\* $p < 0.01$ .

#### 7.2.4 Discussion

The current study found, in two experiments, that rats given 30 OT trials perform a subsequent reversal in fewer trials than if trained only to criterion. When the STN, ZI and LHA were inactivated by administration of CNO in DREADDs-transduced animals, OT resulted in an impairment in reversal learning that was not seen when the same rats were not given OT trials. This suggests that CNO administration – with the consequent inhibition of these areas – prevents an ORE, and provides further support for the hypothesis that rats with inhibition of the STN fail to form set. Furthermore, irrespective of training condition, control rats demonstrated a significant cost of set-shifting (comparison of ID and ED stages), whilst CNO-treated rats did not.

Garner *et al.* (2006) reported that mice given 50 post-criterion trials at Rev2 require significantly more trials at the subsequent ED stage, compared to mice trained only to criterion. In the current experiment, 30 OT trials at the CD had no effect on the size of the shift cost, namely OT did not facilitate ID acquisition, or elevate ED performance with respect to non-OT vehicle-treated rats. Consistent with Sutherland & Mackintosh (1971) however, OT increased the number of perseverative responses in vehicle-treated rats at the first reversal, but also decreased the number of trials spent responding at chance for both the first and second reversal. According to their theory, OT strengthens an ‘analyser’ (*i.e.*, increases the predictiveness of a stimulus dimension), and owing to this, rats are less likely to respond to an irrelevant dimension during a reversal, and consequently spend fewer trials at chance. Consequently, the finding that OT did not increase perseverative responding at Rev2 likely results from the fact that rats were not given OT at the ID stage, however the noted decrease in responses made at chance supports that OT facilitated learning for Rev2 as well.

The previous chapter demonstrated that CNO-treated rats solve the discriminations by learning about the stimuli holistically (see section 6.3.3.4), which may be the result of being unable to form set or may be the cause of it. This approach to discrimination learning likely contributed to the impairment in reversal learning following OT reported here; by responding ‘configurally’ (and being unable to inhibit responding to the irrelevant dimension), OT retarded reversal performance, likely as a result of a rigid stimulus-

response contingency, and the continual strengthening of a configuration with reward. Furthermore, during a reversal stage, these rats will spend more trials responding at chance, learning the ‘new combination’ of stimuli that are now reward-relevant.

The findings in this chapter demonstrated that the ORE results from the same mechanisms as attentional set-formation. This challenges the established ‘double dissociation’ of reversal and attentional processes, by demonstrating that reversal learning and attentional set-formation are not independent processes operating at different levels of response selection. Instead, these findings suggest that reversal and attentional processes are manifestations of a common process of attention and associability, in which response attachments serve to strengthen an attentional set towards a relevant dimension and away from an irrelevant one. These data also expand on the findings of Chase *et al.* (2012), who found that lesions to the OFC not only impaired reversal learning, but also impaired set-formation, which led to the conclusion that both impairments were likely due to a single cognitive deficit.

This experiment used the same cohort of DREADDs-transduced animals as Chapter 6, but with a change in the CNO treatment, such that vehicle-treated rats in Chapter 6 received CNO in this experiment, and vice versa. This arrangement allowed us to test all rats with and without CNO to determine that transiently modifying neuronal signalling in the STN produces detrimental behavioural changes in all animals, and that in the absence of CNO, vehicle-treated rats exhibit typical control-like behaviour. Findings from Chapter 6 also determined that extraneous transduction in the ZI & LHA did not significantly influence behaviour during any of the manipulations of the 11-stage task (see section 6.4.2); similarly here, CNO-treated rats that were subdivided into groups with either ‘minimal’ and ‘moderate’ extraneous transduction did not differ in any measures of behaviour, including the impaired Rev1 performance following OT, along with shift-cost. Furthermore, there was no correlation between the level of transduction of any given region (*e.g.*, ZIV) and behaviour of interest in this experiment, and that CNO-treated rats without extraneous transduction to the ZI/LHA performed similar to rats with robust bilateral transduction of these regions. Similarly, increasing overall transduction levels to encompass more extraneous regions (*i.e.*, globally increasing transduction, or transducing

combinations of regions) does not correlate with levels of impairment for any of the behavioural measures for set-formation in this experiment, suggesting that robust, extraneous transduction to the ZI and LHA had a minimal impact on the behavioural deficits observed here. Previous work has detailed that including animals with extraneous lesion damage to the ZI in studies with STN-lesioned rats does not influence the behaviour of interest, nor statistical outcome (Baunez *et al.*, 1995; Baunez & Robbins, 1999; Phillips & Brown, 1999; Phillips & Brown, 2000). Furthermore, there is currently no data supporting a robust cognitive role for the ZI and LHA, unlike the STN (see section 1.8). The CNO treatment arrangement between Chapter 6 and the current experiment has allowed us to determine that varying levels of extraneous transduction to the ZI and LHA may play a minimal role in the set-formation deficit in this thesis, which may be largely driven by the area which expressed the most robust, and consistent transduction, the STN.

One animal from each CNO dosing group had unilateral transduction of the STN, and as described in Chapter 6, the inclusion of the unilaterally-transduced CNO-treated rat did not influence the outcome of any of the behavioural analyses of the 11-stage task, nor the acquisition of the bi-conditional discrimination. Switching the CNO treatment groups for the current experiment assigned the unilaterally-transduced *vehicle*-treated rat from Chapter 6 to the CNO group here, and similar to Chapter 6, excluding the data for this rat had no effect on the outcome of the CNO-treated group. Again, this must be interpreted with hesitation as only one rat in the CNO group had unilateral transduction, however based on previous work with STN lesions, we have seen that animals with unilateral lesion damage present with a milder impairment in cognition compared to the more profound impairment in cognition observed in bilaterally-lesioned rats (see section 3.4.1.1). Furthermore, the transduction profiles of the two unilaterally-transduced rats are nearly identical, except transduction is found in the opposing hemispheres: colocalised transduction of the STN encompassed between 40-45% of the nucleus, with minimal (10%) transduction of the ZIV.

#### *7.2.4.1 Conclusions*

In summary, the current experiment illustrated that transient inactivation of the STN and ZI/LHA prevents the ORE, leading to impairment in reversal learning, and consequently the ability to form and shift attentional set. The impairment in reversal learning following OT in CNO-treated rats results in more trials in which the rats respond 'at chance'. Conversely, following OT, vehicle-treated rats spend fewer trials responding at chance, but incur more perseverative errors, owing to the formation of attentional set. These findings provide evidence that the associative processes of reversal learning and attentional processes in set-formation are inherently linked as a unified process of attention and associability, rather than independent processes. It was also revealed that extraneous transduction to the ZI and LHA may have a minimal role in this behaviour, which may be mediated by the STN.



# Chapter 8

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## General Discussion

The ability to attend selectively allows us to enhance our focus towards relevant information, whilst filtering out irrelevant information; this process ultimately leads to the formation of an attentional set. The work presented throughout the experimental chapters examined the involvement of the STN in this process. Modifications to the task design with manipulations that aid in inferring set-formation, along with a relatively novel approach to manipulating cellular activity, led to the findings that inhibition of the STN, and perhaps to some degree the ZI & LHA, leads to a disruption in attentional selectivity. It was found that inhibition of this region results in configural learning which may arise as a result of being unable to form set, or may be the cause of it. The findings from Chapter 7 suggest that impairments in reversal learning and attentional selectivity can originate from a single brain region, and these findings align with Mackintosh's two-stage learning theory. The involvement of STN in set-formation raises several theoretical questions, including whether this region participates with others to form a network of brain areas involved in the mediation of set-formation. In this chapter, I will evaluate how the STN may participate in selecting actions which ultimately facilitates the formation of attentional set.

The experiments conducted in this thesis sought to explore the involvement of the STN in processes that are considered to represent ‘executive functions’. The general protocol applied throughout this thesis is identical to the approach taken in neuropsychopharmacology research, which follows several fundamental steps in order to assess whether a brain region (and/or a cognitive-enhancing drug) may influence observable behaviour. These steps include:

1. Select the most suitable animal model to investigate the cognitive function of interest,
2. Choose an appropriate and reliable behavioural task,
3. Manipulate the brain area of interest and/or administer treatment,
4. Look for changes in task performance relative to control animals.

In the current section, the experimental chapters presented throughout this thesis will be evaluated in light of these four steps. To examine set-formation, and how the STN may contribute to the processes that lead to it, the majority of the experiments in this thesis involved modifying an existing behavioural task (*i.e.*, the ASST). At the outset of this thesis, we knew that animals can be trained to respond to one set of stimuli in compound, evidencing an associative relationship between stimulus and reward, and that through ‘sufficient experience’, an attentional set is formed (Lawrence, 1949; Sutherland & Mackintosh, 1971), yet exactly how it arises, or why this occurs is still not well understood. This position underscored that step 2 required careful consideration in order to design manipulations which accurately measure behaviours reflective of set-formation, rather than solely inferring this behaviour from the inability to demonstrate a cost of set-shifting.

## **8.1 Findings**

The first study reported in Chapter 3 attempted to replicate, and expand on recently published findings (Tait et al., 2016), which reported an impairment in attentional set-formation following STN/ZI-area lesions; however this impairment may have resulted from an impairment in discrimination learning at early stages of the task. To reconcile this, subsequent (unpublished) findings with lesions that spared the lateral portion of the STN have found that despite unimpaired early discrimination learning

stages, lesioned rats still do not demonstrate a robust cost of shifting set. Unfortunately, in an effort to replicate this effect, the histological results from Chapter 3 revealed that predominantly unilateral STN lesions were insufficient to produce pronounced behavioural deficits, and that furthermore control rats did not exhibit robust evidence for attentional set-formation either. This asymmetry in lesion damage suggested the minimum conditions for step 3 in the aforementioned protocol (manipulation of the brain area of interest) were not met, which likely led to minimal comparable differences between lesioned and control rats (step 4). This limitation resulted in a similar outcome for the second study in Chapter 3, which presented a modified 11-stage task. This task examined several hypotheses relevant to the inference of set-formation, including: whether additional experience would aid in set-formation (multiple ID stages), examine if the set-formation impairment stemmed from a memory impairment (delayed reversal stages), and assess the degree to which rats were still attending to the irrelevant dimension ('probe' stage). The task also included an adjunct bi-conditional stage, which investigated whether lesioned rats were learning the stimuli as configurations, rather than attending to a stimulus dimension. A mild cognitive deficit induced by mostly unilateral lesions, coupled with a significantly reduced sample size is likely what led to the failure in producing a pronounced behavioural deficit in attentional set-formation.

The research reported in Chapter 4 further attempted to explore the possibility that lesions of the STN impaired the formation of attentional set by utilising an alternative behavioural approach in the form of a succinct revision to the 11-stage task, and investigating whether putative 'cognitive-enhancing' drugs – modafinil and ORG49209 – might restore performance in either lesioned or control animals. To more effectively deliver these drugs, the first experiment in Chapter 4 reported that a reduced-stress jelly tablet was an efficacious method in delivering orally bioavailable drugs, and reported that voluntary consumption of modafinil yielded brain concentrations comparable to that of oral gavage. Consistent with previous findings, this study also evidenced that modafinil increased LMA, but only at later time-points, replicating that modafinil does not increase LMA intensity, and rather exerts an alternative 'wake-promoting' effect. The second experiment in Chapter 4 utilised these jelly-tablets to deliver either ORG or modafinil in the modified 'early-late probe' task, which – as the name implied – utilised two 'probe' stages, in which only the irrelevant dimension

stimuli were changed. Instead of evaluating the cost of shifting set via an ID/ED comparison, the early-late probe task tested the (potentially) disruptive effects of changing the stimuli in the irrelevant dimension, with the hypothesis that an ‘early probe’ stage, presented before an attentional set has formed, would provide a greater distraction than a ‘late probe’ stage, in which an attentional set would aid in inhibiting responding toward an unattended dimension. The results determined that the late probe indeed was acquired in fewer trials than the early probe; however, since control rats did not exhibit an ID/ED difference during an interim 7-stage task, it was not parsimonious to interpret this improvement between the probe stages as evidence for the formation of attentional set. Similar to the results reported for Chapter 3, the majority of lesions in Chapter 4 were undetectable, thereby significantly reducing the sample size, and it was therefore also not possible to draw any conclusion about the contribution of the STN to this behaviour. The intact tissue in the unilaterally-lesioned rat, and the high variance for the four bilaterally-lesioned rats may partially explain the lack of an observable effect for either drug on performance; this tissue likely sustained performance, such that there was no impairment against which to measure ameliorative effects of the drugs. Therefore, owing to an ongoing challenge in manipulating the STN (step 3), we were again unable to ascertain how lesioned rats may express a behavioural deficit compared to control rats (step 4), and also unable to robustly validate the efficacy of the early-late probe task (step 2).

The inability to visualise lesion damage following ibotenic acid infusions to the STN presented in Chapters 3 and 4 were addressed in Chapter 5 by introducing a relatively novel investigative tool – DREADDs. This approach provided increased experimenter control of neuronal functioning by delivering a viral vector which transduced the cells within the STN with an inhibitory designer receptor; this allowed for transient inhibition following administration of a designer drug (CNO). The experiments reported in this chapter firstly demonstrated that an efficacious combination of serotype (AAV5) and promoter (CaMKII) yielded transduction of the cells within medial, and not within the lateral, STN; furthermore transduction also included a relatively small proportion of neighbouring regions (*e.g.*, ZI and LHA). The second experiment in Chapter 5 reported that application of CNO to animals with DREADDs infusions to the medial STN expressed a reduction in c-Fos labelling in the

STN, providing an index of inhibition of cellular activity. This chapter detailed that DREADDs present as a refinement in how the cells of the STN are manipulated (step 3) compared to excitotoxic lesion surgery.

Using the surgical protocol reported in Chapter 5, Chapter 6 found that inhibition of the STN, ZI, & LHA resulted in behavioural deficits consistent with an attentional set-formation impairment on the 11-stage task. This was inferred from several manipulations presented in the task: CNO-treated rats did not demonstrate a cost of shifting attentional set, nor did they exhibit an improvement in learning across multiple ID stages. The error data from the first four trials of the delayed reversal stages allowed us to reject the hypothesis that the subthalamic-mediated set-formation impairment stemmed from impairment in memory. It was evident that CNO-treated rats remembered the stimuli from the original discrimination, illustrating a reversal cost, despite requiring fewer trials to learn the reversal. Furthermore, the probe stage indicated that these rats were distracted by changes in the irrelevant dimension, whereas control rats completed this stage with little detriment on performance. The results reported for the adjunct bi-conditional discrimination suggested that CNO-treated rats were processing the stimuli holistically and that by responding to the configurations of the stimuli, they were able to complete the task with relatively little impairment. This served to reconcile the reversal learning data; having to retain multiple configurations of stimuli in memory (*e.g.*, the reward pairing of four distinct stimuli) increased the cognitive load for discrimination learning, compared to retaining one rule (*e.g.*, ‘the bowl smelling of cinnamon is baited’). Less effective learning about each of the four stimuli might mean that they (or their response costs) are less well retained and, with further interference from the novel stimuli of the intervening stages, fewer trials were required to learn the new stimulus-reward relationships than were required to learn a new rule (*e.g.*, ‘the bowl smelling of ginger is baited’). It was also found that higher levels of extraneous transduction to the ZI and LHA did not serve to exacerbate, nor facilitate behavioural performance, and that irrespective of level of colocalised transduction to these areas, all CNO-treated rats exhibited a similar pattern of behavioural deficits, perhaps owing to consistent transduction of the STN.

To investigate the relationship between the processes of associative learning and set-formation further, Chapter 7 explored the ORE – a paradoxical phenomenon

formalised by Sutherland & Mackintosh (1971) detailing that OT leads to a faster reversal acquisition due to the formation of an attentional set. The first half of the chapter replicated the ORE in the rodent bowl-digging paradigm in unoperated rats. The second experiment in Chapter 7 used the same cohort of DREADDs-transduced rats from Chapter 6, and reported that OT facilitated reversal learning for Rev1 and Rev2 for control rats only; CNO treatment prevented the ORE, resulting in impaired reversal learning performance. This indicated that OT impairs reversal learning when set cannot be formed, and that owing to configural learning, OT continuously strengthening the associative relationship between the correct configurations and reward. These findings also suggested that STN-mediated set-formation impairment is profound, likely resulting in a permanent inability to form set. This also illustrated that reversal learning and set-shifting are not independent ‘types’ of learning, and instead are a manifestation of a common process of attention and associability. Finally, increased extraneous transduction to the ZI and LHA did not impact behavioural performance in this experiment as well, and irrespective of level of colocalised transduction in these areas, all CNO-treated rats exhibited a similar pattern of behavioural deficits; these findings coupled with the identical null results from Chapter 6 suggested that the impairment in attentional set-formation here may be predominantly derived from the inactivation of the STN.

## **8.2 The role of the STN in attentional set-formation**

The experiments summarised above have elevated the role of the STN in cognitive functioning, along with expand our understanding of the processes involved in attentional set-formation. As a recently identified behavioural deficit, our current inference of an attentional set-formation impairment has relied on the lack of a shift-cost, which conversely, is actually a measure of set-shifting behaviour. By using this approach, we have previously observed a lack of a shift-cost in the 7-stage task after ibotenic acid lesions to the OFC (McAlonan & Brown, 2003; Chase, Tait, & Brown, 2012), basal forebrain (Tait & Brown, 2008), and STN/ZI (Tait, Phillips, Blackwell, & Brown, 2016) and in the 4ID task, after quinolinic acid lesions of dorsomedial striatum (Lindgren, Wickens, Tait, Brown, & Dunnett, 2013). The work in this thesis sought to expand on how we measure set-formation by introducing several manipulations

sensitive to its inference, in an effort better understand set-formation as a process. As it stands, we don't fully understand *why* set is formed. In the absence of set, an individual can remain cognitively flexible, being able to rapidly shift behaviour to changing circumstances, and in the current thesis – particularly Chapters 6 and 7 – this was illustrated by decreases in the number of trials required for ED acquisitions. However in the absence of set, all the benefits of selectively attending are lost, such as the enhancement of novel learning when it can be predicted – as illustrated by the reduction in multiple ID stages for vehicle-treated rats in Chapter 6.

The deficit in set-formation presented in this thesis has illustrated this pattern; namely an increase in ID learning, and a decrease in ED with respect to control rats. It was found that in the absence of attending to stimulus dimensions, subjects learned that configurations of stimuli best predicted reward, which allowed them to complete the task with relatively little impairment, compared with control animals. Despite this, the inability to form set and instead treat complex stimuli configurally would beset a series of challenges to daily life. For example, as an occasional consumer of Coca-Cola, I would find it quite easy to pick out a diet Coke when navigating the soft drink aisle at my local newsagent, owing to my experience and knowledge of certain aspects of a Coke bottle as a multidimensional stimulus (*i.e.*, cap colour, bottle shape, manufacturer font/script, etc.). In the past I could readily select a diet Coke owing to the distinct white cap colour, which has recently been changed to red – the same colour as normal Coke – and thus I find myself having to re-check the label to ensure that I have selected the right product; essentially this change has prompted a shift in my attention to other pertinent predictors of 'diet Coke'. If I possessed a dysregulated STN, and an impairment in set-formation that has been compensated by (or resulting from) holistic processing, I would attempt to process all of the multidimensional qualities of a diet Coke bottle, which owing to its relative complexity, and similarity to other soft drinks at the newsagent, may result in me vacillating between drink options with similar qualities (*i.e.*, normal Coke, Dr Pepper, etc.). Furthermore, the recent change in cap colour would also yield disruption in this processing, or delayed learning in acquiring what *now* constitutes a diet Coke bottle.

The above is merely a qualitative example of what may arise from subthalamic dysregulation; however, patients who undergo HFS for treatment of Parkinson's

symptoms exhibit cognitive declines in various processes of executive control, such as selective attention, working memory, speed of mental processing, and susceptibility to interference (Aono et al., 2014; Bronstein et al., 2011; Saint-cyr, Trepanier, Kumar, Lozano, & Lang, 2000; Smeding et al., 2006). Although HFS, unlike lesioning, does not truly mimic an inactivation of the STN (much of the deficits induced by HFS are transient; see: Baunez, Christakou, Chudasama, & Forni, 2007; Hershey et al., 2003), current research with Parkinson's patients has not examined whether this group is impaired at attentional set-formation on the ASST, since the impairments in selective attention and susceptibility to interference noted in this thesis attest to the importance in undertaking this type of research in clinical populations.

A disruption in attentional selectivity was identified to be likely driving the set-formation impairment in this thesis, which is different than the set-formation deficits induced by disrupting other brain regions, such as the OFC. Unlike in OFC-lesioned rats (Chase et al., 2012), additional ID stages did not contribute to evidence of set-formation in subthalamic-inhibited rats. Furthermore, OFC-lesioned rats, and not subthalamic-inhibited rats, are impaired at reversal learning (McAlonan & Brown, 2003; Chase et al., 2012). Thus, in the absence of a reversal deficit (and no information on set-shifting), we must consider that the subthalamic-mediated set-formation impairment arises from different mechanisms than the OFC-mediated one.

It also appears that the subthalamic-mediated deficit does not result from a failure to downregulate attention to redundant information, such as after mPFC lesions (Sharpe & Killcross, 2014), especially since mPFC-lesioned rats demonstrate robust evidence of set-formation, along with significantly impaired ED performance (Birrell & Brown, 2000; Tait, Marston, Shahid, & Brown, 2009). Our lab has also recently shown that rats with DREADDs-inhibition of the mPFC exhibit a distinctly different pattern of behaviour to the DREADDs-inhibited rats in this thesis by demonstrating a shift-cost, despite also presenting evidence that they learn about the irrelevant cues during a probe stage (Whyte, Tait & Brown, *unpublished*). This suggests that whilst mPFC-inhibited rats fail to downregulate attention to irrelevant information, this doesn't disrupt attentional set-formation. In contrast, CNO-treated rats in this thesis failed to demonstrate the ability to parse relevant from irrelevant (implying limited attentional



selectivity), and instead solve the compound discriminations by learning the correct/incorrect stimulus configurations.

Seminal research of the STN's involvement in cognition, spearheaded by Christelle Baunez, reported that lesioning the STN results in premature or impulsive responding on visuo-spatial attention tasks (Baunez & Robbins, 1997; Baunez, Nieoullon, & Amalric, 1995); an effect which has been robustly replicated in similar tasks in both lesioned rats (Baunez & Robbins, 1999; Chudasama, Baunez, & Robbins, 2003; Phillips & Brown, 2000) and following DBS treatment in humans (Aron & Poldrack, 2006). Subsequent research by Baunez and colleagues (2001) investigated the effect of STN lesions on the response preparatory processes in a modified reaction-time task. In one test, rats were presented with information regarding the response location in advance, whilst in another test, the information was not presented. Contrasting performance between these two tests revealed that STN lesions do not impair the 'readiness to respond' to cues in either task (*i.e.*, 'motor readiness'), suggesting that the signs of impulsive responding seen following STN lesions are likely not due to an impairment in the 'when' phase of action preparation. Instead, the authors suggested that lesions abolish the beneficial effects of information presented in advance (*i.e.*, the 'which' phase of response preparation); implying an impairment in response selection.

Thus, the function of the STN, namely the ability to 'hold a response' (see Frank, 2006), is impaired following lesioning, resulting in 'impulsive action' (not being able to inhibit the initiation of a response), which actually may reflect a global impairment in action selection. This consideration may reconcile the results reported in this thesis, in which subthalamic dysregulation here may have introduced a form of 'cognitive disinhibition'; it is speculated that subthalamic-inhibited animals were unable to inhibit responding to the irrelevant dimension, such that they never learned that this dimension was irrelevant (and by extension, which dimension was relevant). This impairment in selecting the correct responses led to an inability to assign relevancy (*i.e.*, switch in analysers), and perhaps as a consequence, these rats treated combinations of the cues as correct/incorrect. Recent electrophysiology research in monkeys by Espinosa-Parrilla, Baunez, & Apicella, (2015) found evidence that STN neurons encode whether or not a preferred reward had been received when a choice between response alternatives was required. Therefore, by inactivating these neurons within the STN the rats may not have

been able to perform ‘outcome evaluation’; updating response attachments with stimuli in light of reward or punishment. The inability to update response attachments would ultimately render a failure to downregulate redundant (irrelevant) information and upregulate relevant information, leading to a failure in set-formation.

This exercising of ‘impulsive action’ can be contrasted from ‘impulsive choice’ responding (for review see Eagle & Baunez, 2010; but also Evenden, 1999), in which the latter entails the selection of actions without deliberation of other possible outcomes or options. ‘Impulsive choice’ behaviour is typically modelled in a delay-discounting task, which presents subjects with the option of a small but immediate reward, or a large but delayed reward. Interestingly, STN-lesioned rats were able to overcome impulsivity to wait for a larger reward (Winstanley, Baunez, Theobald, & Robbins, 2005), and it is theorised that this inhibitory control may be modulated by motivation for the outcome (Uslaner & Robinson, 2006). This consideration may also entertain why we did not observe ‘impulsive digging’ behaviour in this thesis (*i.e.*, no impairment in compound discrimination learning), as the option for the ‘other bowl’ was sufficiently motivating to subthalamic-inhibited rats that they were able to withhold an impulsive digging response. The ability to withhold impulsive/compulsive digging in the incorrect bowl also implied that these rats were able to inhibit a prepotent motor response, and perhaps the ‘impulsive action’ behaviour previously seen in lesioned animals (Baunez *et al.*, 1995; Baunez & Robbins, 1997; 1999) manifests differently in the ASST as a failure to form attentional set. This also supports the previous observations that tasks which do not exert strict time constraints during a given trial (*i.e.*, goal-directed research, increased stimulus duration) find fewer deficits associated with impulse control in STN-lesioned animals (Baunez & Robbins, 1997; El Massioui *et al.*, 2007; Pote *et al.*, 2016).

Although previous work detailed that lesioning the rat STN may induce impairments in working memory, either by impairing the normal operation of a ‘motor working memory buffer’ (Baunez *et al.*, 2001), or by inducing an inclination to rapidly forget previous response associations between stimuli (El Massioui *et al.*, 2007), there was no evidence to suggest that inactivating the STN impairs working memory in this thesis. The anthropomorphised ‘motor working memory buffer’ in Baunez *et al.* (2001) is purported to hold a selected response in readiness until its execution is required; this ability is rendered dysfunctional in STN lesioned rats, resulting in a conflict in response

selection and the observed perseverative behaviour in visuo-spatial attention tasks. Conversely, in another study in which working memory deficits are reported in STN-lesioned rats (El Massioui *et al.*, 2007), the authors did not report increased perseverative behaviour. In El Massioui *et al.* (2007), when a sufficient delay between the offset of a cue and presentation of the response levers are introduced, rats apparently ‘forget’ where to respond, indexing more errors and incomplete trials. By delaying or offsetting reversal stages in Chapter 6, we were able to evaluate working memory performance by requiring rats to retain pertinent information regarding qualities of the stimuli learned during a compound discrimination stage, along with their respective response strengths. As reported in Chapter 6, even after three intervening stages, subthalamic-inhibited rats clearly retained a memory of the stimulus-response attachments by rejecting the previously incorrect bowl as readily as control rats during early trials of the delayed reversal stages. Thus, the working memory deficits previously observed could again result from the task requirements; the ASST permits up to ten minutes per trial to make a response, and therefore the nature of the reported STN-mediated working memory deficit may depend on task parameters and time constraints.

STN-lesioned rats in El Massioui *et al.* (2007) also acquired the reversal in fewer sessions than control animals, and the authors postulated that reversal performance might be enhanced, stemming from an improvement in attentional selectivity following STN lesions. As demonstrated in Chapter 7, facilitation of reversal performance owing to improvements in attentional selectivity during two-choice discrimination learning typically arises from overtraining (see Sutherland & Mackintosh, 1971), and is indicative of attentional set-formation. Despite this interpretation, El Massioui *et al.* (2007) did not report any evidence for overtraining in any of the rats in, such as comparable performance during discrimination learning and during retraining phases, respectively. Furthermore, the interpretation that STN lesions may enhance attentional selectivity is inconsistent with the findings reported in this thesis, which details an impairment in attentional selectivity following inhibition of the STN. El Massioui *et al.* (2007) postulated that this focussed attention would enhance learning to relevant stimuli, but “to the detriment of a general attentional process devoted to all components of the experimental situation”. The detriment being referred to here actually pertains to the cost associated with shifting set, and therefore this interpretation is

problematic in the context of the lever-pressing task used in El Massioui *et al.* (2007), in which there is no task requirement which mimics an ED shift.

### **8.3 Why the STN?**

Nearly seventeen years ago in our lab, whilst investigating whether lesions to the STN after dopamine depletion would ameliorate the reversal deficits seen in the ASST, there was a serendipitous finding: the ‘control’ group of rats that had received only STN lesions unexpectedly exhibited a profile of impairment that was not previously observed following lesions to any other region of the brain. The experiment was presented as a poster at the annual meeting of the Society for Neuroscience but a subsequent manuscript was rejected because the authors could not give the reviewers an adequate explanation of the findings. The manuscript was not published until 2016 and, in the intervening years, a number of studies have been undertaken in an attempt to determine both *how* and *why* this area was involved in attentional set, culminating to the work conducted for this thesis. This work addresses one of these two considerations, yet further investigation is needed to determine *why* this region is involved. Perhaps this question can be better fielded by considering how disruption of the STN affects downstream neural functioning.

It has been stressed throughout this thesis that the STN is not the only region of the brain that may be implicated in attentional set-formation, with evidence suggesting that lesioning the OFC (McAlonan & Brown, 2003; but see Chase *et al.*, 2012) or the DMS (Lindgren *et al.*, 2013) also impairs this behaviour. As previously stated, the OFC-mediated deficit results in a *delayed* ability to form set: with sufficient experience, after multiple ID stages, a set does form. A DMS-mediated deficit, akin to the subthalamic-mediated deficit, has been reported (Lindgren *et al.*) which was more profound, with no evidence of set-formation following at least 4 consecutive ID stages. However, it should also be noted that we have not seen such a deficit (Tait *et al.*, 2016) and have failed to replicate one (Garcia, *unpublished* PhD thesis, 2016). Nevertheless, possibility that both the STN and DMS are involved in attentional set-formation introduces the possibility that dysregulation of input regions of the basal ganglia may cause this lasting set-formation impairment; however further research – perhaps with the 11-stage task – is

needed in this regard to ascertain how similar the DMS-mediated deficit is to the subthalamic-mediated one.

It has been shown that transient inactivation of the dorsal striatum with lidocaine before reversal learning, and not before compound discrimination OT, in a lever-pressing operant task, prevented the ORE (Racht-delatour & El Massioui, 2000), which suggested that inactivating the DMS likely disrupts the ability to rapidly change responding to novel situations (*i.e.*, learning a new rule), likely resulting in perseveration or learned non-reward (see Tait & Brown, 2007). Consequently, lesioning the DMS does not likely induce a deficit in attentional selectivity – as evidenced by an ORE – whereas inactivating the STN does.

Although there is clear evidence that the dorsal striatum and the STN exercise different roles in cognition, a recent review by Tewari, Jog, & Jog (2016) stresses the collaborative nature of these two areas in response selection. They suggest that the striatum and the STN work in concert via the ‘indirect’ pathway for selective response inhibition, with the striatum promoting action execution via the ‘direct’ pathway, and the STN as a rapid response inhibitor via the ‘hyperdirect’ pathway. Tewari *et al* (2016) credit Frank's (2006) neurocomputational model of BG functioning, which illustrates that in spite of different inherent functions, the roles exercised by the DMS and STN allows for an indirect functional contribution to other brain regions, and possibly reciprocal modulation between the STN and striatum themselves. It is therefore possible that DMS inactivation may have incurred a downstream effect on STN regulation via the GPe, or vice versa; that inactivation of the STN may subsequently dysregulate cortico-striatal input, yet further work on this is needed.

It is also well-documented that both the STN and the striatum receive considerable cortical input from regions that have been identified to play a role in executive functioning, including the OFC and the mPFC (Joel & Weiner, 1997; Kita *et al.*, 2014; for reviews see Haber, 2016; Parent & Hazrati, 1995). The OFC in particular, along with the DMS and the STN form a network of regions that may be critical in impulsive/compulsive behaviour, and that dysregulating this network results in increased impulsive action, premature responding and perseveration (Eagle & Baunez, 2010; Baunez & Lardeux, 2011). Furthermore, this dysregulation in response selection

may differentially disrupt set-formation when the normal functioning of these regions is inhibited.

Recent fMRI research by Morris, Baek, & Voon (2017) found higher connectivity between the STN and the medial OFC (mOFC) in healthy volunteers who excelled at tasks of ‘goal directed model-based’ behaviour: slower, deliberative behavioural control that required flexible, goal-directed action with an understanding of task-structure (see Daw, Gershman, Seymour, Dayan, & Dolan, 2011). This goal directed model-based behaviour can be contrasted with a ‘model-free system’, in which fast, reactive habitual learning is employed. This finding detailed the importance of the ‘hyperdirect’ pathway on STN functioning, which aligned with evidence from previous studies (Chudasama et al., 2003; Baunez & Lardeux, 2011; Kita, Osten, & Kita, 2014). Furthermore this involvement of the OFC-STN connection in modulating deliberative response control may suggest that the silencing of these post-synaptic neurons found in the (predominantly medial) STN may have contributed to the observed behavioural deficits reported in this thesis. It is possible that pertinent information regarding the nature of the stimuli was relayed by the OFC to the STN, which was inhibited, and therefore the selection of responses that would have led to the formation of set were consequently not selected; as stated before, this may have resulted from the inability to inhibit responding to the irrelevant dimension. However, *how* the STN encodes this direct cortical (and indirect striatal) information is currently not known, but perhaps it plays a crucial role in outcome evaluation (Espinosa-Parilla, Booneze & Apicella, 2013; 2015).

Throughout this thesis, attributing behavioural results solely to the STN has not been directly possible, since all of the attempts to manipulate medial STN cell population have incurred damage/transduction to the ZI and LHA. Despite restricting lesion damage or viral transduction to the medial and not the lateral portion of the STN, extraneous manipulation of the ZIV (and in some cases the ZID), along with the lateral LH area (LHA) was found in some animals. In an effort to better understand how this varying level of cellular manipulation might affect the significant behavioural effects reported in Chapters 6 & 7, transduction percentages by spatial area were analysed, which suggested that increasing levels of bilateral transduction within the ZIV, ZID and LHA have a minimal impact on the behavioural deficits found in this thesis, and that

furthermore, the behaviour for CNO-treated animals with more overall transduction of the ZI/LHA did not differ from those with conservative or no extraneous transduction. This conclusion strongly supports that the STN was driving the majority of the effect here, but another study in which transduction is limited to the medial STN in all animals (or in a larger sample) would bolster this claim. Additionally, future work in this field would largely benefit from a more systematic analysis of the transduction percentage by counting the colocalised cells. Thickness of the tissue (and therefore a larger z-axis) increased the amount of signal such that mCherry and GFP (NeuN) channels imaged even individually were too 'noisy' in areas with high cell density (*e.g.*, STN), making it difficult to distinguish the perimeter of the cell as it was overlapping/unclear along the z-axis. This effect remained for imaging at higher magnification, and was exacerbated by colocalisation. Whilst the tissue thickness here (50  $\mu\text{m}$ ) allowed for detection of colocalised transduction within a given region, it did not permit for cell counting, and therefore in subsequent research, histological methods should be altered to collect thinner sections (5-15 $\mu\text{m}$ ) to permit accurate counting of colocalised cells.

Despite this methodological shortcoming, there is no evidence that these extraneous regions play a role in executive functions; in previous work investigating the cognitive impact of STN-lesioning, some authors have retained rats with ZI-lesions as their inclusion does not influence the behaviour of interest, nor statistical outcome (Phillips & Brown, 2000; Phillips & Brown, 1999), whilst others have discarded rats that present with cell loss in the ZI, but comment that the data obtained from those animals had little impact on behaviour, suggesting that the ZI may have a minimal role in this type of cognitive behaviour (Baunez *et al.*, 1995; Baunez & Robbins, 1999). Despite attempting to restrict DREADDs transduction to the medial STN with a CaMKII promoter, the resulting nuclear transduction of the ZI possibly arose from the transduction of the glutamatergic cells that are found in the medial ZID, or since CaMKII also phosphorylates GABA<sub>A</sub>, transduction of the GABA-ergic cells of the ZIV (Mitrofanis, 2005). Given the overlapping projections found in this area, particularly cortical projections from the OFC to the STN and ZI (Kita *et al.*, 2014), it is still possible that this region works in concert to modulate output activity, and perhaps inhibition of the STN, along with this select region led to the noted behavioural deficits

reported here. However, more research regarding the overall role of the ZI and LHA is needed, especially to determine if they exclusively hold executive roles.

The function of the LHA has predominantly been implicated in the modulation of attention and sleep/wake cycles, along with food intake/weight management (Sakurai et al., 1998), however recent evidence suggests that lesioning these – principally orexinergic – neurons impairs the acquisition of a conditioned orienting response, which may extend the functional role of this region into associative learning (Wheeler et al., 2015). Recent research has suggested that the overlapping region between the ventrolateral LHA and the medial ZI work together to modulate attention and arousal, yet the ZI (with projections from the frontal eye field), and the LHA (with projections from the mPFC) also operate independently; the ZI may modulate oculomotor control in attention, and the LHA may modulate exploratory behaviour (Chometton et al., 2017). Overall however, there is little evidence to suggest that the ZI or the LHA are implicated in executive functioning, and more research regarding this is needed.

#### **8.4 Reconciling attention and associability**

This thesis, whilst trying to further elucidate a cognitive role for the STN, was also interested in further understanding and exploring how attentional set is formed. Two-stage theories of animal discrimination learning (*i.e.*, Mackintosh's model; see Sutherland & Mackintosh, 1971) offer a theoretical reconciliation for processes of attentional set and associative learning, which are seen as complimentary, such that strengthening an anthropomorphised 'analyser' (*i.e.*, a perceptual dimension) will increase the salience of a cue and its association with reward. Early ASST work with marmoset monkeys revealed evidence for a dissociation of these two processes, and it was found that lesioning different parts of the PFC impairs either reversal learning or set-shifting ability (Dias, Robbins, & Roberts, 1996). These authors contended that this double-dissociation of 'associative' and 'attentional' shifting processes may be hierarchically organised, or conversely may operate independently. In subsequent work, this same double-dissociation has also been found in rats (Birrell & Brown, 2000; McAlonan & Brown, 2003; Young & Shapiro, 2009). Interestingly, whilst serial reversal learning does not reduce the switching deficit following mPFC-muscimol infusions (Rich & Shapiro, 2007), Young & Shapiro found that serial strategy-switching



in OFC-muscimol animals normalised spatial reversal learning, which led the authors to conclude that ‘switching’ behaviour may engage reversal mechanisms, and thus the two processes are likely hierarchically organised.

Despite supporting a double dissociation, along with measuring strategy shifting (and not set-shifting), the evidence presented by Young & Shapiro illustrating a relationship between reversal learning and attentional processing aligns with our findings from this thesis. The results reported in Chapter 7 demonstrated that reversal learning and attentional set are not independent processes, and instead, by inhibiting the STN it was possible to impair reversal learning in animals that are unable to form an attentional set by presenting these rats with OT. This finding illustrated that these two processes could be mediated by a single brain region; a conclusion which also challenged whether attentional and reversal functions were two, doubly-dissociable processes. This finding also provides credence to Chase *et al.*’s (2012) claim that the reversal and set-formation impairments in OFC-lesioned rats may arise from a single cognitive deficit.

The findings in this thesis, including the notion for a hierarchical relationship between associative and attentional processes, supports Mackintosh’s model of discrimination learning (Sutherland & Mackintosh, 1971). Currently, the two competing theories of animal discrimination learning – the Mackintosh model and the Pearce-Hall model (Pearce & Hall, 1980) – both acknowledge the important link between learned associations and the driving of attention salience (*i.e.*, where attention is directed). Pearce’s model asserts that uncertainty, or novelty in cue quality will drive salience, and ultimately attention, whereas Mackintosh’s model posits that the cue which is the best predictor for reward will drive attention. According to Pearce’s theory, discrimination learning is achieved through configural learning, and therefore subjects associate each positively reinforced compound separately with reward. Consequently, Pearce’s model cannot readily account for attentional set-shifting, nor offer any reconciliation for an observation that requires a two-stage theory, such as the ORE.

Recent computational modelling by Esber & Haselgrove (2011) has introduced a ‘hybrid model’ of attention, which in an effort to reconcile Pearce and Mackintosh’s contradicting models, utilises both predictiveness *and* uncertainty to explain how cue salience drives attention. Esber and Haselgrove’s model emphasises a single attentional

process based on ‘total predictiveness’; during a discrimination learning trial, a calculation is made about how well a cue predicts a reward (Mackintosh), and the extent to which each cue is followed by an accurately predicted reward (Pearce-Hall), and the result of this calculation will determine whether there is an increase or decrease in attention.

In this hybrid model, the ‘Mackintosh mechanism’ would be employed when an established predictive cue (*i.e.*, one learned in a pre-training phase) would be presented during a discrimination trial alongside other cues varying in attentional salience. This comparison of the *relative predictiveness* (*i.e.*, the extent to which one cue is a better predictor than another; see Kattner, 2015) of these competing cues is what drives attentional selection in Mackintosh’s model, in which the best predictor will acquire salience. For example, the salience of attention toward sudden ripples in water will increase for a wetland heron if the ripples predict the catching of a fish, and the salience will further increase if it also predictively signals a lurking alligator since adaptively learning about different types of ripples may be the difference between eating and being eaten.

The ‘Pearce-Hall mechanism’ would switch in when two cues do not differ in their *relative* predictiveness but do differ in terms of their *absolute* predictiveness (*i.e.*, the overall probability of being *followed by* a well-predictable outcome). The omission of a reinforcer ( $\Sigma\bar{V}$ ) is a motivationally potent event (*e.g.*, relief if the reinforcer is aversive, frustration if it is appetitive), and drives the acquisition of salience in the Pearce-Hall model; the hybrid model suggests that the expectation of ‘no-reinforcement’ detracts from the expectation of reinforcement ( $\Sigma V$ ) in order to determine the *overall* prediction of reinforcement ( $\Sigma V - \Sigma\bar{V}$ ). For example, the fishing heron will not make a catch at the detection of every ripple, as occasionally the fish will get away. The hybrid model would maintain that the harder it is for the heron to make a catch, the more frustrated it would become as the expectation of reinforcement (*i.e.*, making a catch) decreases, which will subsequently enhance the salience of the ripples to a level greater than the association of the ripple with fish. This will subsequently increase the heron’s attention toward qualities of different types of ripples, and will improve the likelihood of making a catch.

The formalisation of this hybrid model may serve to reconcile existing evidence that separate neural circuits or brain regions can be conceptualised as either a ‘Mackintoshian’ or one that operates in accordance with a ‘Pearce-Hall’ attentional system (*e.g.*, Sharpe & Killcross, 2014). The role of the mPFC in downregulating attention toward poor predictors of reward, whilst up-regulating attention toward good predictors led Sharpe & Killcross to conceptualise this region as one that participates in a Mackintosh attentional system. Conversely, recent research by Esber, Torres-tristani, & Holland (2015) suggest that the central nucleus of the amygdala (CeA) and the DMS are essential for surprise-induced salience enhancements of attention, suggesting that these areas may participate in a Pearce-Hall attentional system.

The findings reported in this thesis would illustrate a condition in which it is possible to dissociate the functioning of the hybrid model, in that, dysregulating the STN selectively disrupts the Mackintosh mechanism, whilst potentially leaving the Pearce-Hall mechanism intact, or perhaps resulting in a state in which the Pearce-Hall mechanism is defaulted to. In this state, the hybrid model may illustrate that a heron would attempt to associate the configuration of a type of ripple (stimulus) in the water with a fish (reward), which may prove costly if the ripple is in fact predictive of an alligator. Pending the heron survives, it will learn to configure a particular type of ripple as appetitive (*i.e.*, the ripple which led to the catching of a fish), and another ripple-type will be configured as aversive (*i.e.*, the ripple that nearly led to being eaten); yet without the Mackintosh mechanism, the heron will not learn about the predictiveness of ripples in general, which can be retained and utilised when faced with the presentation of newer ‘ripple types’. In this thesis, this consideration is best supported by an enhanced bi-conditional acquisition in CNO-treated rats, whilst failing to evidence an attentional set consistent with Mackintosh’s model. It is worth noting that the nature of the subthalamic-mediated set-formation impairment requires further exploration since we still do not know whether learning stimulus configurations results from an inability to form set, or whether learning configurations causes the impairment in the first place. Essentially, is inactivation of the STN disabling the Mackintosh mechanism? Or is it ‘locking in’ the Pearce-Hall mechanism?

## **8.5 Limitations: persisting with ibotenic acid lesions**

The chief limitation noted in this thesis pertained to the failure to produce consistent and observable cell loss in the STN following infusions of ibotenic acid. Without verifiable and replicable lesions, it was not possible to draw any conclusions about the contribution of the STN to behaviour in Chapters 3 and 4. Despite this shortcoming being an issue in previously unpublished work (Xia, Dhawan, Tait & Brown), we persisted with ibotenic acid as a means to lesions the STN in Chapters 3 & 4 since when visible damage was evidenced, it was detectable in, and restricted to the target region. Furthermore, all of the rats which were administered ibotenic acid infusions exhibited the expected ‘chewing’ behaviour following surgery (see section 2.3.3 and Baunez & Robbins, 1997), along with calcium deposits in the MGP, which is usually indicative of accurate lesion placement within the STN, and was not observed in control rats. However, as Chapters 3 & 4 illustrated, post-operative chewing behaviour, and MGP calcium deposits do not always indicate visible lesion damage.

Throughout this thesis, the targeting of the mSTN has employed the same stereotaxic coordinates as those reported in previous studies, with a minor modification of the dorsal-ventral (DV) coordinates (Baunez & Robbins, 1997; Chudasama et al., 2003; Phillips & Brown, 2000; Tait et al., 2016). Previous research took DV coordinates from bregma with minor variations (Phillips: -8.5mm; Baunez: -8.35mm) yet report comparable lesion damage, whilst for this thesis, coordinates were taken from dura, rather than bregma, as a glass micropipette (for chapters 3 & 4) was used to deliver the toxin (which may have broken if contacted with the skull surface). Xia, Dhawan, Tait and Brown (*unpublished*), along with the rats that presented with discernible lesion damage in this thesis, have demonstrated that our lesion coordinates are indeed correct for generating cell loss localised to the medial portion of the STN (and minimally to adjacent areas near the STN), yet this visible damage was only evident in roughly half of the total group which received ibotenic acid infusions. In order to restrict the lesion size to the medial STN, we reduced the toxin volume from the widely published 0.4-0.5µl to 0.2µl, whilst maintaining the same concentration of the toxin (0.06M). It is possible, however unlikely, that lesioned rats without discernible damage retained a perfectly ‘intact’ STN or ZI, or that the toxin was incorrectly injected ventrally into the internal capsule, since all rats presented with calcium deposits in the MGP, and chewing

behaviour during recovery. In an effort to detect lesions in this group, Xia, Tait & Brown (*unpublished*) utilised a variety of histological staining techniques, including tyrosine hydroxylase, parvalbumin, and glial fibrillary acidic protein, yet no observable damage in the STN was detected.

Previous studies that have reported robust STN lesion damage have utilised either a barbiturate anaesthetic, such as pentobarbital sodium (Phillips & Brown, 2000; Tait et al., 2016), or a combination of xylazine and ketamine (Baunez et al., 1995). Whilst both approaches provide prolonged sedation compared to an inhalant anaesthetic (such as isoflurane used in this thesis), they are not currently available for use for surgery on animals. Prolonged sedation allows for the excitotoxin to exert its effect without having to use diazepam as an adjunct; past observations in our lab suggest that administering diazepam before toxin infusion, rather than before surgery may minimise subthalamic lesion extent (Xia, Tait & Brown, *unpublished*). Furthermore, it has also been demonstrated that benzodiazepine treatment before administration of glutamatergic excitotoxins such as ibotenic acid (Perez-Rico & Gomez-Ramos, 1984) kainic acid (Ben-Ari et al., 1978) and quinolinic acid (Lapin, Prakhie, & Kiseleva, 1986) attenuates acid damage; it has been argued that benzodiazepines do not act as receptor antagonists for kainic, ibotenic and quinolinic acids, but instead may facilitate GABA inhibition (Perez-Rico & Gomez-Ramos, 1984). It is possible that given the small volume of excitotoxin infused, that the administration of diazepam prior to the injection of ibotenic acid may have attenuated the some of the deleterious effects. It is not being suggested here that the addition of diazepam is nullifying the effect of ibotenic acid, especially since administration of diazepam both before (Chase et al., 2012) and after (Lindgren et al., 2013) excitotoxic surgery still induces measurable cell loss, but perhaps it played a part in reducing the spread of the lesion, and contributed to preventing lesion detection.

Following the histology results from Chapter 4, which were consistent with Chapter 3 and previous findings, it was determined that the ibotenic acid surgery protocol did not satisfy expectations for manipulating the STN, and therefore alternative options were sought, which ultimately led to a relatively novel option – DREADDs. These designer receptors were transiently controlled by the designer drug CNO, and also reconciled the shortcoming of ibotenic acid infusions, namely, the visualisation of the surgical manipulation. DREADDs infusions were also made to the same stereotaxic

coordinates as the ibotenic acid infusions, validating that these coordinates were indeed accurate, whilst concurrently presenting as a refinement which incurred less harm to the animal (*i.e.*, no cellular necrosis, and therefore no chewing).

## **8.6 Conclusions**

The work presented in this thesis has highlighted the importance of the subthalamic area in mediating the processes which lead to the formation of attentional set. By inhibiting cells within this region, including the cells of the medial STN, we found evidence that this area mediates attentional selectivity, and perhaps arises as a failure to inhibit responding to an irrelevant stimulus dimension. Additionally, this thesis elucidated that reversal and attentional process are not independent functions, but rather exist in a hierarchy and stem from a common manifestation of attention and associability. It is possible that the STN is essential in enabling and maintaining a ‘Mackintosh-ian attentional system’, which consequently permits the formation of an attentional set, and in the absence of this system, we may resort to a Pearce-Hall attentional system. With this system engaged, it is easier to acquire an ED stage since there is no attentional set to shift away from, corroborating the findings that inhibiting the STN may facilitate cognitive flexibility (see neurocomputational model by Frank, 2006). Given that the nature of the set-formation deficit here is profound (*i.e.*, showing little improvement in learning after multiple ID stages), future experiments can attempt to ameliorate these deficits through the application of cognitive-enhancing drugs, such as modafinil.

The overall findings here ascribe many ‘frontal’ like functions to a subcortical region, and that several of the processes examined by the ASST exemplify what is known as ‘executive control’. The maintenance of these executive functions has classically been the ascribed role of the PFC, yet a newer school of thought has postulated that the notion of a ‘central executive’ may be outdated, and that regions such as the basal ganglia, hippocampus, and the posterior cortex work in concert with the PFC to create an executive network (Hazy, Frank, & Reilly, 2006). Having a central executive also raises several problems, such as: how does the PFC ‘know’ which action or response profile to select? Essentially, what is controlling the controller? Furthermore, how does learning and experience guide the actions taken by the PFC?

Without answering these questions, the current concept of the central executive can be likened to that of a homunculus (small man) living inside the PFC and dictating action in response to stimuli, which, given the important role of subcortical regions – like the STN – in this process, may render a central executive outdated (Hazy, Frank, & Reilly, 2007). It is clear from the work in this thesis that the STN plays a critical role in several cognitive processes that would substantially aid us in daily life. The control exerted by the STN facilitates a range of tasks we take for granted, from buying a diet Coke at your local newsagent, to planning and executing your next challenge in Scotland: whether it's embarking on a holiday to Ballachulish or writing a PhD thesis in St Andrews.

# References

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- Akaoka, H., Roussel, B., Lin, J. S., Chouvet, G., & Jouvet, M. (1991). Effect of modafinil and amphetamine on the rat catecholaminergic neuron activity. *Neuroscience Letters*, 123(1), 20–22.
- Alberts, J. L., Voelcker-Rehage, C., Hallahan, K., Vitek, M., Bamzai, R., & Vitek, J. L. (2008). Bilateral subthalamic stimulation impairs cognitive - Motor performance in Parkinson's disease patients. *Brain*, 131(12), 3348–3360.
- Albin, R. L., Young, A. B., & Penney, J. B. (1989). The functional anatomy of basal ganglia disorders. *Trends in Neurosciences*, 12(10), 366–375.
- Aleksandrova, L. R., Creed, M. C., Fletcher, P. J., Lobo, D. S., Hamani, C., & Nobrega, J. N. (2013). Deep brain stimulation of the subthalamic nucleus increases premature responding in a rat gambling task. *Behavioural brain research*, 245, 76–82.
- Alexander, G. E., DeLong, M. R., & Strick, P. L. (1986). Parallel organization of functionally segregated circuits linking basal ganglia and cortex. *Annual Review of Neuroscience*, 9, 357–381.
- Alexander, G. M., Rogan, S. C., Abbas, A. I., Armbruster, B. N., Pei, Y., Allen, J. a, ... Roth, B. L. (2009). Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors. *Neuron*, 63(1), 27–39.
- Amara, A. W., Standaert, D. G., Guthrie, S., Cutter, G., Watts, R. L., & Walker, H. C. (2012). Unilateral subthalamic nucleus deep brain stimulation improves sleep quality in Parkinson's disease. *Parkinsonism & Related Disorders*, 18(1), 63–8.
- Aono, M., Iga, J., Ueno, S., Agawa, M., Tsuda, T., & Ohmori, T. (2014). Neuropsychological and psychiatric assessments following bilateral deep brain stimulation of the subthalamic nucleus in Japanese patients with Parkinson's disease. *Journal of Clinical Neuroscience*, 21(9), 1595–1598.
- Appleby, B. S., Duggan, P. S., Regenberg, A., & Rabins, P. V. (2007). Psychiatric and neuropsychiatric adverse events associated with deep brain stimulation: A meta-analysis of ten years' experience. *Movement disorders*, 22(12), 1722–1728.
- Armbruster, B. N., Li, X., Pausch, M. H., Herlitze, S., & Roth, B. L. (2007). Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proceedings of the National Academy of Sciences of the United States of America*, 104(12), 5163–8.
- Aron, A. R., & Poldrack, R. A. (2006). Cortical and Subcortical Contributions to Stop Signal Response Inhibition: Role of the Subthalamic Nucleus. *The Journal of Neuroscience*, 26(9), 2424–2433.
- Atkinson, R. C., & Shiffrin, R. M. (1968). Human memory: A proposed system and its control processes. In K. Spence & J. Spence (Eds.), *The psychology of learning and motivation* (2nd ed., pp. 89–195). New York: Academic Press.
- Atkinson, R. C., & Shiffrin, R. M. (1971). The control processes of short-term memory. *Technical Reports*, 173.
- Augood, S. J., Hollingsworth, Z. R., Standaert, D. G., Emson, P. C., & Penney, J. B. (2000). Localization of Dopaminergic Markers in the Human Subthalamic Nucleus. *The Journal of Comparative Neurology*, 421(January), 247–255.
- Awad, H., Hubert, G. W., Smith, Y., Levey, A. I., & Conn, P. J. (2000). Activation of metabotropic glutamate receptor 5 has direct excitatory effects and potentiates NMDA receptor currents in neurons of the subthalamic nucleus. *The Journal of Neuroscience*, 20(21), 7871–7879.



- Baddeley, A. (1996). Exploring the central executive. *The Quarterly Journal of Experimental Psychology*, 49A(1), 5–28.
- Baddeley, A. D. (1992). Working Memory. *Science*, 255, 556–559.
- Baddeley, A. D., & Hitch, G. (1974). Working memory. In G. Bower (Ed.), *The psychology of learning and motivation* (8th ed., pp. 47–89). New York: Academic Press.
- Bartho, P., Freund, T., & Acsady, L. (2002). Selective GABAergic innervation of thalamic nuclei from zona incerta. *European Journal of Neuroscience*, 16, 999–1014.
- Bastian, A. J., Kelly, V. E., Revilla, F. J., Perlmutter, J. S., & Mink, J. W. (2003). Different Effects of Unilateral Versus Bilateral Subthalamic Nucleus Stimulation on Walking and Reaching in Parkinson ' s Disease, 18(9), 1000–1007.
- Bastoji, H., & Jouvet, M. (1988). Successful treatment of idiopathic hypersomnia and narcolepsy with modafinil. *Progress in Neuropsychopharmacology and Biological Psychiatry*, 12(5), 695–700.
- Baufreton, J., & Bevan, M. D. (2008). D2-like dopamine receptor-mediated modulation of activity-dependent plasticity at GABAergic synapses in the subthalamic nucleus. *J Physiol*, 586(8), 2121–2142.
- Baufreton, J., Garret, M., Rivera, A., de la Calle, A., Gonon, F., Dufy, B., ... & Taupignon, A. (2003). D5 (not D1) dopamine receptors potentiate burst-firing in neurons of the subthalamic nucleus by modulating an L-type calcium conductance. *Journal of Neuroscience*, 23(3), 816–825.
- Baunez, C., Christakou, A., Chudasama, Y., Forni, C., & Robbins, T. W. (2007). Bilateral high-frequency stimulation of the subthalamic nucleus on attentional performance: transient deleterious effects and enhanced motivation in both intact and parkinsonian rats. *European Journal of Neuroscience*, 25(4), 1187–1194.
- Baunez, C., Humby, T., Eagle, D. M., Ryan, L. J., Dunnett, S. B., & Robbins, T. W. (2001). Effects of STN lesions on simple vs choice reaction time tasks in the rat: Preserved motor readiness, but impaired response selection. *European Journal of Neuroscience*, 13(8), 1609–1616.
- Baunez, C., & Lardeux, S. (2011). Frontal cortex-like functions of the subthalamic nucleus. *Frontiers in Systems Neuroscience*, 5(October), 83.
- Baunez, C., & Robbins, T. W. (1999). Effects Of Dopamine Depletion Of The Dorsal Striatum And Further Interaction With Subthalamic Nucleus Lesions In An Attentional Task In The Rat, 92(4), 1343–1356.
- Baunez, C., Nieoullon, A., & Amalric, M. (1995). In a rat model of parkinsonism, lesions of the subthalamic nucleus reverse increases of reaction time but induce a dramatic premature responding deficit. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 15(10), 6531–6541.
- Baunez, C., & Robbins, T. W. (1997). Bilateral lesions of the subthalamic nucleus induce multiple deficits in an attentional task in rats. *European Journal of Neuroscience*, 9(10), 2086–2099.
- Baunez, C., & Gubellini, P. (2010). Effects of GPi and STN inactivation on physiological, motor, cognitive and motivational processes in animal models of Parkinson's disease. In *Progress in brain research* (Vol. 183, pp. 235–258). Elsevier.
- Bechara, A. (2003). Risky business: emotion, decision-making, and addiction. *Journal of gambling studies*, 19(1), 23–51.
- Becker, M., Kuhse, J., & Kirsch, J. (2013). Effects of two elongation factor 1A isoforms on the formation of gephyrin clusters at inhibitory synapses in hippocampal neurons. *Histochemistry and Cell Biology*, 140(6), 603–609.
- Bell, K. E. N., Churchill, L., & Kalivas, P. W. (1995). GABAergic Projection From the Ventral Pallidum and Globus Pallidus to the Subthalamic Nucleus. *Synapse*, 20, 10–18.

- Benabid, A. L., Pollak, P., Gross, C., Hoffmann, D., Benazzouz, A., Gao, D. M., ... Perret, J. (1994). Acute and long-term effects of subthalamic nucleus stimulation in Parkinson's disease. *Stereotact Funct Neurosurg*, *62*, 76–84.
- Benabid, A. L., Benazzouz, A., Hoffmann, D., Limousin, P., Krack, P., & Pollak, P. (1998). Long-term electrical inhibition of deep brain targets in movement disorders. *Movement Disorders*, *13*(S3), 119-125.
- Ben-Ari, Y., Lagowska, Y., Le Gal La Salle, G., Tremblay, E., Ottersen, O. P., & Naquet, R. (1978). Diazepam pretreatment reduces distant hippocampal damage induced by intramygdaloid injections of kainic acid. *European Journal of Pharmacology*, *52*, 419–420.
- Bender, D., Holschbach, M., & Stöcklin, G. (1994). Synthesis of n.c.a. carbon-11 labelled clozapine and its major metabolite clozapine-N-oxide and comparison of their biodistribution in mice. *Nuclear Medicine and Biology*, *21*(7), 921–925.
- Benazzouz, A., Gross, C., Féger, J., Boraud, T., & Bioulac, B. (1993). Reversal of rigidity and improvement in motor performance by subthalamic high-frequency stimulation in MPTP-treated monkeys. *European Journal of Neuroscience*, *5*(4), 382-389.
- Benson, D. L., Isackson, P. J., Hendry, S. H., & Jones, E. G. (1991). Differential gene expression for glutamic acid decarboxylase and type II calcium-calmodulin-dependent protein kinase in basal ganglia, thalamus, and hypothalamus of the monkey. *Journal of Neuroscience*, *11*(6), 1540-1564.
- Beracochea, D., Celerier, A., Borde, N., Valleau, M., Peres, M., & Pierard, C. (2002). Improvement of learning processes following chronic systemic administration of modafinil in mice. *Pharmacology Biochemistry and Behavior*, *73*(3), 723–728.
- Berendse, H. W., & Groenewegen, H. J. (1991). The connections of the medial part of the subthalamic nucleus in the rat: Evidence for a parallel organization. In G. Bernardi, M. B. Carpenter, G. Di Chiara, M. Morelli, & P. Stanzione (Eds.), *The Basal Ganglia III* (pp. 89–98). New York: Springer.
- Berg, E. A. (University of W. (1948). A simple objective technique for measuring flexibility in thinking. , *Journal of General Psychology*, *39* (1948) p.15. *The Journal of General Psychology*, *39*, 15–22.
- Bergman, H., Wichmann, T., & Karmon, B. (1994). The Primate Subthalamic Nucleus . II . Neuronal Activity in the MPTP Model of Parkinsonism. *Journal of Neurophysiology*, *72*(2), 507–520.
- Bergmann, O., Winter, C., Meissner, W., Harnack, D., Kupsch, A., Morgenstern, R., & Reum, T. (2004). Subthalamic high frequency stimulation induced rotations are differentially mediated by D1 and D2 receptors. *Neuropharmacology*, *46*(7), 974-983.
- Berns, K., & Hauswirth, W. (1979). Adeno-associated viruses. *Advances in Virus Research*, *25*, 407–449.
- Berthoud, H., & Munzberg, H. (2011). The lateral hypothalamus as integrator of metabolic and environmental needs: from electrical self-stimulation to opto-genetics. *Physiol Behav*, *104*(1), 29–39.
- Bevan, M. D., & Wilson, C. J. (1999). Mechanisms underlying spontaneous oscillation and rhythmic firing in rat subthalamic neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *19*(17), 7617–7628.
- Birch, D., & Jacobs, G. H. (1979). Spatial contrast sensitivity in albino and pigmented rats. *Vision Research*, *19*(8), 933–937.
- Birrell, J. M., & Brown, V. J. (2000). Medial frontal cortex mediates perceptual attentional set shifting in the rat. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *20*(11), 4320–4.
- Bissonette, G. B., Martins, G. J., Franz, T. M., Harper, E. S., Schoenbaum, G., & Powell, E. M. (2008). Double dissociation of the effects of medial and orbital prefrontal cortical

- lesions on attentional and affective shifts in mice. *Journal of Neuroscience*, 28(44).
- Björkhem, I. (2007). Rediscovery of cerebrosterol. *Lipids*, 42(1), 5–14.
- Boender, A. J., De Jong, J. W., Boekhoudt, L., Luijendijk, M. C. M., Van Der Plasse, G., & Adan, R. A. H. (2014). Combined use of the canine adenovirus-2 and DREADD technology to activate specific neural pathways in vivo. *PLoS ONE*, 9(4), 1–6.
- Bliss, T. V., & Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, 361(6407), 31–39.
- Bliss, T. V., & Gardner-Medwin, A. R. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the unanaesthetized rabbit following stimulation of the perforant path. *The Journal of Physiology*, 232(2), 357–374.
- Blomstedt, P., Fyttagoridis, A., Åström, M., Linder, J., Forsgren, L., & Hariz, M. I. (2012). Unilateral caudal zona incerta deep brain stimulation for Parkinsonian tremor. *Parkinsonism & related disorders*, 18(10), 1062–1066.
- Bonfoco, E., Krainc, D., Ankarcona, M., Nicotera, P., & Lipton, S. A. (1995). Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proceedings of the National Academy of Sciences of the United States of America*, 92(16), 7162–6.
- Bonnichsen, M., Dragsted, N., & Hansen, A. K. (2005). The welfare impact of gavaging laboratory rats. *Animal Welfare*, 14(3), 223–227.
- Bosler, O., Joh, T. H., & Beaudet, A. (1984). Ultrastructural relationships between serotonin and dopamine neurons in the rat arcuate nucleus and medial zona incerta: a combined radioautographic and immunocytochemical study. *Neuroscience letters*, 48(3), 279–285.
- Bowsher, D. (1975). Diencephalic projections from the midbrain reticular formation. *Brain research*, 95(2-3), 211–220.
- Bradley, S. R., Marino, M. J., Wittmann, M., Rouse, S. T., Awad, H., Levey, A. I., & Conn, P. J. (2000). Activation of group II metabotropic glutamate receptors inhibits synaptic excitation of the substantia nigra pars reticulata. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 20(9), 3085–3094.
- Bronstein, J. M., Tagliati, M., Alterman, R. L., Lozano, A. M., Volkmann, J., Stefani, A., ... DeLong, M. R. (2011). Deep Brain Stimulation for Parkinson Disease. *Archives of Neurology*, 68(2), 165.
- Brown, A. P., Dinger, N., & Levine, B. S. (2000). Stress produced by gavage administration in the rat. *Contemporary Topics in Laboratory Animal Science / American Association for Laboratory Animal Science*, 39(1), 17–21.
- Brown, L. L., Markman, M. H., Wolfson, L. I., Dvorkin, B., Warner, C., & Katzman, R. (1979). A direct role of dopamine in the rat subthalamic nucleus and an adjacent intrapeduncular area. *Science*, 206(4425), 1416–1418.
- Brown, L. L., & Wolfson, L. I. (1978). Apomorphine increases glucose utilization in the substantia nigra, subthalamic nucleus and corpus striatum of rat. *Brain research*, 140(1), 188–193.
- Brown, V. J., & Bowman, E. (2002). Rodent models of prefrontal cortical function. *Trends in Neurosciences*, 25(7), 340–343.
- Brown, V. J., & Robbins, T. W. (1991). Simple and choice reaction time performance following unilateral striatal dopamine depletion in the rat: impaired motor readiness but preserved response preparation. *Brain*, 114(1), 513–525.
- Brown, V. J., & Tait, D. S. (2015). Behavioral flexibility: Attentional shifting, rule switching and response reversal. In L. Price & I. P. Stolerman (Eds.), *Encyclopedia of Psychopharmacology* (pp. 1–5). Berlin, Heidelberg: Springer.
- Brown, V. J., & Tait, D. S. (2016). Attentional set-shifting across species. In *Current Topics Behavioural Neuroscience* (pp. 289–320).

- Brown, V. J., & Tait, D. S. (2010). Behavioral flexibility: Attentional shifting, rule switching and response reversal. In L. Price & I. P. Stolorman (Eds.), *Encyclopedia of Psychopharmacology* (pp. 1–5). Berlin, Heidelberg: Springer.
- Bruet, N., Windels, F., Bertrand, A., Feuerstein, C., Poupard, A., & Savasta, M. (2001). High Frequency Stimulation of the Subthalamic Nucleus Increases the Extracellular Contents of Striatal Dopamine in Normal and Partially Dopaminergic Denervated Rats. *Journal of Neuropathology and Experimental Neurology*, *60*(1), 15–24.
- Bullitt, E. (1990). Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. *The Journal of Comparative Neurology*, *296*(4), 517–530.
- Burger, C., Gorbatyuk, O. S., Velardo, M. J., Peden, C. S., Williams, P., Zolotukhin, S., ... Muzyczka, N. (2004). Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Molecular Therapy*, *10*(2), 302–317.
- Bush, G., Valera, E. M., & Seidman, L. J. (2005). Hyperactivity Disorder : A Review and Suggested Future Directions. *Biological Psychiatry*, *57*, 1273–1284.
- Bussey, T. J., Muir, J. L., Everitt, B. J., & Robbins, T. W. (1997). Triple dissociation of anterior cingulate, posterior cingulate, and medial frontal cortices on visual discrimination tasks using a touchscreen testing procedure for the rat. *Behavioral Neuroscience*, *111*(5), 920–936.
- Caliph, S. M., Faassen, F. W., & Porter, C. J. H. (2014). The influence of intestinal lymphatic transport on the systemic exposure and brain deposition of a novel highly lipophilic compound with structural similarity to cholesterol. *Journal of Pharmacy and Pharmacology*, *66*(10), 1377–1387.
- Campbell, G. A., Eckardt, M. J., & Weight, F. F. (1985). Dopaminergic mechanisms in subthalamic nucleus of rat: analysis using horseradish peroxidase and microiontophoresis. *Brain research*, *333*(2), 261–270.
- Carcenac, C., Favier, M., Vachez, Y., Lacombe, E., Savasta, M., & Boulet, S. (2015). Subthalamic Deep Brain Stimulation Differently Alters Striatal Dopaminergic Receptor Levels in Rats. *Movement Disorder*, *30*(13), 1739–1749.
- Carli, M., Robbins, T. W., Evenden, J. L., & Everitt, B. J. (1983). Effects of lesions to ascending noradrenergic neurones on performance of a 5-choice serial reaction task in rats; Implications for theories of dorsal noradrenergic bundle function based on selective attention and arousal, *9*, 361–380.
- Chakirova, G., Welch, K. A., Moorhead, T. W. J., Stanfield, A. C., Hall, J., Skehel, P., ... McIntosh, A. M. (2010). Orbitofrontal morphology in people at high risk of developing schizophrenia. *European Psychiatry*, *25*(6), 366–372.
- Chan, R. C. K., Shum, D., Touloupoulou, T., & Chen, E. Y. H. (2008). Assessment of executive functions : Review of instruments and identification of critical issues, *23*, 201–216.
- Chang, W. H., Lin, S. K., Lane, H. Y., Wei, F. C., Hu, W. H., Lam, Y. W. F., & Jann, M. W. (1998). Reversible metabolism of clozapine and clozapine N-oxide in schizophrenic patients. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *22*(5), 723–739.
- Chase, E. A., Tait, D. S., & Brown, V. J. (2012). Lesions of the orbital prefrontal cortex impair the formation of attentional set in rats. *The European Journal of Neuroscience*, *36*(3), 2368–75.
- Chen, J., & Kriegstein, A. R. (2015). A GABAergic projection from the zona incerta to cortex promotes cortical neuron development. *Science*, *350*(6260), 554–558.
- Cheney, D. L., Uzunov, D., & Guidotti, A. (1995). Pregnenolone sulfate antagonizes dizocilpine amnesia: role for allopregnanolone. *Neuroreport*, *6*, 1697–1700.

- Cheung, S., Ballew, J. R., Moore, K. E., & Lookingland, K. J. (1998). Contribution of dopamine neurons in the medial zona incerta to the innervation of the central nucleus of the amygdala, horizontal diagonal band of Broca and hypothalamic paraventricular nucleus. *Brain Research*, 808, 174–181.
- Chometton, S., Charrière, K., Bayer, L., Houdayer, C., Franchi, G., Poncet, F., ... Risold, P. (2017). The rostromedial zona incerta is involved in attentional processes while adjacent LHA responds to arousal: c-Fos and anatomical evidence. *Brain Structure and Function*, 222(6), 2507–2525.
- Chudasama, Y., Baunez, C., & Robbins, T. W. (2003). Functional Disconnection of the Medial Prefrontal Cortex and Subthalamic Nucleus in Attentional Performance: Evidence for Corticosubthalamic Interaction, 23(13), 5477–5485.
- Clarke, H. F., Walker, S. C., Crofts, H. S., Dalley, J. W., Robbins, T. W., & Roberts, a C. (2005). Prefrontal serotonin depletion affects reversal learning but not attentional set shifting. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 25(2), 532–8.
- Clarke, N. P., & Bolam, J. P. (1998). Distribution of glutamate receptor subunits at neurochemically characterized synapses in the entopeduncular nucleus and subthalamic nucleus of the rat. *Journal of Comparative Neurology*, 397(3), 403–420.
- Conn, P. J., & Pin, J.-P. (1997). Pharmacology and Functions of Metabotropic Glutamate Receptors. *Annual Review of Pharmacology and Toxicology*, 37(1), 205–237.
- Crossman, A. R. (1990). A hypothesis on the pathophysiological mechanisms that underlie levodopa-or dopamine agonist-induced dyskinesia in Parkinson's disease: implications for future strategies in treatment. *Movement Disorders*, 5(2), 100-108.
- Cummings, J. L. (1992). Depression and Parkinson's disease: a review. *The American journal of psychiatry*, 149(4), 443.
- Dalley, J. W., Fryer, T. D., Brichard, L., Robinson, E. S., Theobald, D. E., Lääne, K., ... & Abakumova, I. (2007). Nucleus accumbens D2/3 receptors predict trait impulsivity and cocaine reinforcement. *science*, 315(5816), 1267-1270.
- Dalley, J. W., Everitt, B. J., & Robbins, T. W. (2011). Impulsivity, compulsivity, and top-down cognitive control. *Neuron*, 69(4), 680-694.
- Darbaky, Y., Forni, C., Amalric, M., & Baunez, C. (2003). High frequency stimulation of the subthalamic nucleus has beneficial antiparkinsonian effects on motor functions in rats, but less efficiency in a choice reaction time task, 18.
- Dascal, N. (1997). Signalling via the G protein-activated K<sup>+</sup> channels. *Cellular Signalling*, 9(8), 551–573.
- Daw, N. D., Gershman, S. J., Seymour, B., Dayan, P., & Dolan, R. J. (2011). Model-Based Influences on Humans' Choices and Striatal Prediction Errors. *Neuron*, 69(6), 1204–1215.
- DeLong, M. (1990). Primate models of movement disorders of basal ganglia origin. *Trends in Neurosciences*, 13, 281–285.
- DeLong, M. R. (1990). Primate models of movement disorders of basal ganglia origin. *Trends in Neurosciences*, 13(7), 281–285.
- De Saint Hilaire, Z., Orosco, M., Rouch, C., Blanc, G., & Nicolaidis, S. (2001). Variations in extracellular monoamines in the prefrontal cortex and medial hypothalamus after modafinil administration: a microdialysis study in rats. *Neuroreport*, 12(16), 3533–7.
- Desbonnet, L., Temel, Y., Visser-vandewalle, V., Blokland, A., Hornikx, V., & Steinbusch, H. W. M. (2004). Premature responding following bilateral stimulation of the rat subthalamic nucleus is amplitude and frequency dependent. *Brain Research*, 1008, 198–204.
- De Sereville, J. E., Boer, C., Rambert, F. A., & Duteil, J. (1994). Lack of pre-synaptic

- dopaminergic involvement in modafinil activity in anaesthetized mice: In vivo voltammetry studies. *Neuropharmacology*, 33(6), 755–761.
- De Smet, F., Christopoulos, A., & Carmeliet, P. (2014). Allosteric targeting of receptor tyrosine kinases. *Nature Biotechnology*, 32(11), 1113–20.
- Deroche-Gamonet, V., Darnaudéry, M., Bruins-Slot, L., Piat, F., Le Moal, M., & Piazza, P. (2002). Study of the addictive potential of modafinil in naive and cocaine-experienced rats. *Psychopharmacology*, 161(4), 387–395.
- Deyle, D. R., & Russell, D. W. (2009). Adeno-associated virus vector integration. *Current Opinion in Molecular Therapeutics*, 11(4), 442–7.
- Dhawan, S.S., Xia, S., Tait, D.S., Bundgaard, C., Bowman, E., & Brown, V.J. (2018). Oral dosing of rodents using a palatable tablet. *Psychopharmacology*. *In Press*.
- Diamond, A. (2013). Executive functions. *Annual Review of Psychology*, 64, 135–168.
- Dias, R., Robbins, T. W., & Roberts, A. C. (1996a). Dissociation in prefrontal cortex of affective and attentional shifts. *Nature*.
- Dias, R., Robbins, T. W., & Roberts, A. C. (1996b). Primate Analogue of the Wisconsin Card Sorting Test : Effects of Excitotoxic Lesions of the Prefrontal Cortex in the Marmoset " 110(5), 872–886.
- Dias, R., Robbins, T. W., & Roberts, A. C. (1997). Dissociable forms of inhibitory control within prefrontal cortex with an analog of the Wisconsin Card Sort Test: Restriction to novel situations and independence. *The Journal of Neuroscience*, 17(23), 9285–9297.
- Donix, M., Small, G. W., & Bookheimer, S. Y. (2012). Family History and APOE-4 Genetic Risk in Alzheimer’s Disease. *Neuropsychology Review*, 22, 298–309.
- Drapier, D., Drapier, S., Sauleau, P., Haegelen, C., Raoul, S., Biseul, I., Peron, J., Lallement, F., Rivier, J.M., Reymann, J.M., Edan, G., Verin, M., & Millet, B. (2006). Does subthalamic nucleus stimulation induce apathy in Parkinson’s disease?. *Journal of neurology*, 253(8), 1083.
- Dudchenko, P. A., Wood, E. R., & Eichenbaum, H. (2000). Neurotoxic hippocampal lesions have no effect on odor span and little effect on odor recognition memory but produce significant impairments on spatial span, recognition, and alternation. *The Journal of Neuroscience*, 20(8), 2964–2977.
- Duncan, J. (2001). An adaptive coding model of neural function in prefrontal cortex. *Nature Reviews Neuroscience*, 2, 820–829.
- Duteil, J., Rambert, F. a, Pessonnier, J., Hermant, J. F., Gombert, R., & Assous, E. (1990). Central alpha 1-adrenergic stimulation in relation to the behaviour stimulating effect of modafinil; studies with experimental animals. *European Journal of Pharmacology*, 180(1), 49–58.
- Eagle, D. M., & Baunez, C. (2010). Is there an inhibitory-response-control system in the rat ? Evidence from anatomical and pharmacological studies of behavioral inhibition. *Neuroscience and Biobehavioral Reviews*, 34, 50–72.
- Edgar, D. M., & Seidel, W. F. (1997). Modafinil induces wakefulness without intensifying motor activity or subsequent rebound hypersomnolence in the rat. *The Journal of Pharmacology and Experimental Therapeutics*, 283(2), 757–769.
- Edgar, D. M., Seidel, W. F., & Dement, W. C. (1991). Triazolam-induced sleep in the rat: influence of prior sleep, circadian time, and light/dark cycles. *Psychopharmacology*, 105(3), 374–80.
- Edwards, D., & Isaacs, S. (1991). The zona incerta lesions: effects on copulation, partner preference and other socio-sexual behaviours. *Behavioural Brain Research*, 44, 145–150.
- El Arfani, A., Bentea, E., Aourz, N., Ampe, B., De Deurwaerdère, P., Van Eeckhaut, A., ... Michotte, Y. (2014). NMDA receptor antagonism potentiates the l-DOPA-induced extracellular dopamine release in the subthalamic nucleus of hemi-parkinson rats.

- Neuropharmacology*, 85, 198–205.
- El Massioui, N., Chéruel, F., Faure, A., & Conde, F. (2007). Learning and memory dissociation in rats with lesions to the subthalamic nucleus or to the dorsal striatum. *Neuroscience*, 147(4), 906–918.
- Esber, G. R., & Haselgrove, M. (2011). Reconciling the influence of predictiveness and uncertainty on stimulus salience: a model of attention in associative learning. *Proceedings of the Royal Society B*, 278(1718), 1–9.
- Esber, G. R., Torres-tristani, K., & Holland, P. C. (2015). Amygdalo-striatal interaction in the enhancement of stimulus salience in associative learning. *Behavioral Neuroscience*, 129(2), 87–95.
- Eslinger, P., & Damasio, A. (1985). Severe disturbance of higher cognition after bilateral frontal lobe ablation : Patient EVR. *Neurology*, 35, 1731–1741.
- Espinosa-Parrilla, J. F., Baunez, C., & Apicella, P. (2015). Modulation of neuronal activity by reward identity in the monkey subthalamic nucleus. *European Journal of Neuroscience*, 42(1), 1705-1717.
- Evenden, J. L. (1999). Varieties of impulsivity. *Psychopharmacology*, 146(4), 348-361.
- Farrell, M. S., & Roth, B. L. (2013). Pharmacosynthetics: Reimagining the pharmacogenetic approach Martilias. *Brain Re*, 1511, 6–20.
- Faull, R. L. M., & Carman, J. B. (1978). The cerebellofugal projections in the brachium conjunctivum of the rat. I. The contralateral ascending pathway. *Journal of Comparative Neurology*, 178(3), 495-517.
- Feldman, R., Meyer, J., & Quenzer, L. (1997). *Principles of Neuropsychopharmacology*. Sunderland, MA: Sinauer Associates Inc.
- Ferguson, S. M., & Neumaier, J. F. (2012). Grateful DREADDs: engineered receptors reveal how neural circuits regulate behavior. *Neuropsychopharmacology*, 37(1), 296–297.
- Ferraro, L., Fuxe, K., Tanganelli, S., Tomasini, M. C., Rambert, F. A., & Antonelli, T. (2002). Differential enhancement of dialysate serotonin levels in distinct brain regions of the awake rat by modafinil: Possible relevance for wakefulness and depression. *Journal of Neuroscience Research*, 68(1), 107–112.
- Field, A. (2009). *Discovering statistics using SPSS* (Third). London: Sage Publications Ltd.
- Floran, B., Floran, L., Erlij, D., & Aceves, J. (2004). Dopamine D4 receptors inhibit depolarization-induced [3 H] GABA release in the rat subthalamic nucleus. *European journal of pharmacology*, 498(1), 97-102.3-
- Flores, G., Liang, J. J., Sierra, A., Martinez-Fong, D., Quirion, R., Aceves, J., & Srivastava, L. K. (1999). Expression of dopamine receptors in the subthalamic nucleus of the rat: characterization using reverse transcriptase–polymerase chain reaction and autoradiography. *Neuroscience*, 91(2), 549-556.
- Frank, M. J. (2006). Hold your horses : A dynamic computational role for the subthalamic nucleus in decision making. *Neural Networks*, 19, 1120–1136.
- Frank, M. J., Samanta, J., Moustafa, A. A., & Sherman, S. J. (2007). Hold your horses: Impulsivity, deep brain stimulation, and medication in Parkinsonism. *Science*, 318(November), 1309–1312.
- Funkiewiez, A., Ardouin, C., Caputo, E., Krack, P., Fraix, V., Klinger, H., ... & Pollak, P. (2004). Long term effects of bilateral subthalamic nucleus stimulation on cognitive function, mood, and behaviour in Parkinson's disease. *Journal of Neurology, Neurosurgery & Psychiatry*, 75(6), 834-839.
- Furukawa, H., Singh, S. K., Mancusso, R., & Gouaux, E. (2005). Subunit arrangement and function in NMDA receptors. *Nature*, 438(7065), 185–192.
- Fuster, J. (2015). *The Prefrontal Cortex* (5th ed.). Los Angeles: Academic Press Inc.
- Gallay, M. N., Jeanmonod, D., Liu, J., & Morel, A. (2008). Human pallidothalamic and

- cerebellothalamic tracts: anatomical basis for functional stereotactic neurosurgery. *Brain Structure and Function*, 212(6), 443-463.
- Galvan, A., Kuwajima, M., & Smith, Y. (2006). Glutamate and GABA receptors and transporters in the basal ganglia: What does their subsynaptic localization reveal about their function? *Neuroscience*, 143(2), 351-375.
- Galvan, A., Charara, A., Levey, A. I., & Smith, Y. (2004). Differential subcellular and subsynaptic distribution of GABAA and GABAB receptors in the monkey subthalamic nucleus. *Neuroscience*, 127, 709-721.
- Galvan, A., Hu, X., Rommelfanger, K. S., Pare, J., Khan, Z. U., Smith, Y., & Wichmann, T. (2014). Localization and function of dopamine receptors in the subthalamic nucleus of normal and parkinsonian monkeys. *J Neurophysiol*, 112, 467-479.
- Garner, J. P., Thogerson, C. M., Würbel, H., Murray, J. D., & Mench, J. A. (2006). Animal neuropsychology: Validation of the Intra-Dimensional Extra-Dimensional set shifting task for mice. *Behavioural Brain Research*, 173(1), 53-61.
- Gauthier, J., Parent, M., & Levesque, M. (1999). The axonal arborization of single nigrostriatal neurons in rats. *Brain Research*, 834, 228-232.
- Gerfen, C. R., Engber, T. M., Mahan, L. C., Susel, Z. V. I., Chase, T. N., Monsma, F. J., & Sibley, D. R. (1990). D1 and D2 Dopamine Receptor-Regulated Gene Expression of Striatonigral and Striatopallidal Neurons dopamine. *Science*, 250(December), 1429-1432.
- Gibbs, T. T., Russek, S. J., & Farb, D. H. (2006). Sulfated steroids as endogenous neuromodulators. *Pharmacology Biochemistry and Behavior*, 84(4), 555-567.
- Gibson, J. (1941). A critical review of the concept of set in contemporary experimental psychology. *Psychological Bulletin*, 38(9), 781-817.
- Goelz, L. C., David, F. J., Sweeney, J. A., Vaillancourt, D. E., Poizner, H., Metman, L. V., & Corcos, D. M. (2016). The effects of unilateral versus bilateral subthalamic nucleus deep brain stimulation on prosaccades and antisaccades in Parkinson's disease. *Experimental Brain Research*, 1-12.
- Gold, L., & Balster, R. (1996). Evaluation of the cocaine-like discriminative stimulus effects and reinforcing effects of modafinil. *Psychopharmacology*, 126(4), 286-292.
- Goldstein, K., & Scheerer, M. (1941). Abstract and concrete behavior: An experimental study with special tests. In J. Dashiell (Ed.), *Psychological Monographs* (Vol. 53). Ann Arbor, MI: Edwards Brothers, Inc.
- Gompf, H. S., Mathai, C., Fuller, P. M., Wood, D. A., Pedersen, N. P., Saper, C. B., & Lu, J. (2011). Locus coeruleus (LC) and anterior cingulate cortex sustain wakefulness in a novel environment. *Journal of Neuroscience*, 30(43), 14543-14551.
- Gonzales, C., Kaufman, D. L., Tobin, A. J., & Chesselet, M. F. (1991). Distribution of glutamic acid decarboxylase (Mr 67 000) in the basal ganglia of the rat: an immunohistochemical study with a selective cDNA-generated polyclonal antibody. *Journal of neurocytology*, 20(12), 953-961.
- Grant, B. Y. D. A., & Berg, E. A. (1948). A behavioural analysis of degree of reinforcement and ease of shifting to new responses in a Weigl-type card-sorting problem. *Journal of Experimental Psychology*, 38, 404-411.
- Groenewegen, H. J., & Berendse, H. W. (1990). Connections of the subthalamic nucleus with ventral striatopallidal parts of the basal ganglia in the rat. *The Journal of Comparative Neurology*, 294(4), 607-22.
- Gubellini, P., Salin, P., Kerkerian-Le Goff, L., & Baunez, C. (2009). Deep brain stimulation in neurological diseases and experimental models: from molecule to complex behavior. *Progress in neurobiology*, 89(1), 79-123.
- Guehl, D., Vital, A., Cuny, E., Spampinato, U., Rougier, A., Bioulac, B., & Burbaud, P.



- (2008). Postmortem proof of effectiveness of zona incerta stimulation in Parkinson's disease. *Neurology*, *70*, 1489–1490.
- Guettier, J.-M., Gautam, D., Scarselli, M., Ruiz de Azua, I., Li, J. H., Rosemond, E., ... Wess, J. (2009). A chemical-genetic approach to study G protein regulation of beta cell function in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(45), 19197–202.
- Guitton, C., Abbar, M., Kinowski, J. M., Chabrand, P., & Bressolle, F. (1998a). Multiple-dose pharmacokinetics of clozapine in patients with chronic schizophrenia. *Journal of Clinical Psychopharmacology*, *18*(6), 470–6.
- Guitton, C., Abbar, M., Kinowski, J. M., Chabrand, P., & Bressolle, F. (1998b). Multiple-dose pharmacokinetics of clozapine in patients with chronic schizophrenia. *Journal of Clinical Psychopharmacology*, *18*(6), 470–6.
- Haber, S. N. (2016). Corticostriatal circuitry. *Dialogues in Clinical Neuroscience*, *18*(1), 7–21.
- Hardman, C. D., Henderson, J. M., Finkelstein, D. I., Horne, M. K., Paxinos, G., & Halliday, G. M. (2002). Comparison of the Basal Ganglia in Rats, Marmosets, Macaques, Baboons, and Humans: Volume and Neuronal Number for the Output, Internal Relay, and Striatal Modulating Nuclei. *The Journal of Comparative Neurology*, *445*, 238–255.
- Harlow, H., & Bromer, J. (1938). A test apparatus for monkeys. *The Psychological Record*, *19*, 434–436.
- Harlow, H. F. (1949a). The formation of learning sets. *Psychological Review*, *56*(1), 51–65.
- Harlow, H. F. (1949b). The formation of learning sets. *Psychological Review*, *56*(1), 51–65.
- Hartmann-von Monakow, K., Akert, K., & Künzle, H. (1978). Projections of the precentral motor cortex and other cortical areas of the frontal lobe to the subthalamic nucleus in the monkey. *Experimental brain research*, *33*(3-4), 395-403.
- Hassani, O. K., & Feger, J. (1999). Effects of intrasubthalamic injection of dopamine receptor agonists on subthalamic neurons in normal and 6-hydroxydopamine-lesioned rats: an electrophysiological and c-Fos study. *Neuroscience*, *92*(2), 533-543.
- Hassani, O., Francois, C., Yelnik, J., & Feger, J. (1997). Evidence for a dopaminergic innervation of the subthalamic nucleus in the rat. *Brain Research*, *749*, 88–94.
- Hauber, W. (1998). Blockade of subthalamic dopamine D1 receptors elicits akinesia in rats. *NeuroReport*, *9*(18), 4115–4118.
- Haynes, W. I. A., & Haber, S. N. (2013). The organization of prefrontal-subthalamic inputs in primates provides an anatomical substrate for both functional specificity and integration: implications for Basal Ganglia models and deep brain stimulation. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *33*(11), 4804–14.
- Hazy, T. E., Frank, M. J., & Reilly, R. C. O. (2006). Banishing The Homunculus : Making Working Memory Work. *Neuroscience*, *139*, 105–118.
- Hazy, T. E., Frank, M. J., & Reilly, R. C. O. (2007). Towards an executive without a homunculus : computational models of the prefrontal cortex/basal ganglia system. *Philosophical Transactions of the Royal Society B*, *362*(April), 1601–1613.
- Hedreen, J. C. (1998). Lost Caps in Histological Counting Methods. *The Anatomical Record*, *250*, 366–372.
- Heimer, L., Zahm, D. S., & Alheid, G. F. (1995). Basal Ganglia. In G. Paxinos (Ed.), *The Rat Nervous System* (Second, pp. 579–614). San Diego: Academic Press.
- Heise, C. E., & Mitrofanis, J. (2004). Evidence for a glutamatergic projection from the zona incerta to the basal ganglia of rats. *Journal of Comparative Neurology*, *468*(4), 482-495.
- Henderson, J. M., & Dunnett, S. B. (1998). Targeting the subthalamic nucleus in the treatment of Parkinson's disease. *Brain Research Bulletin*, *46*(6), 467–474.

- Hershey, T., Revilla, F. J., Wernle, A. R., McGee-Minnich, L., Antenor, J. V., Videen, T. O., ... Perlmutter, J. S. (2003). Cortical and subcortical blood flow effects of subthalamic nucleus. *Neurology*, *61*, 816–821.
- Hershey, T., Wu, J., Weaver, P. M., Perantie, D. C., Karimi, M., Tabbal, S. D., & Perlmutter, J. S. (2008). Unilateral vs. bilateral STN DBS effects on working memory and motor function in Parkinson disease. *Experimental neurology*, *210*(2), 402–408.
- Houeto, J. L., Mesnage, V., Mallet, L., Pillon, B., Gargiulo, M., Du Moncel, S. T., Bonnet, A.M., Pidoux, B., Dormont, D., Cornu, P., & Agid, Y. (2002). Behavioural disorders, Parkinson's disease and subthalamic stimulation. *Journal of Neurology, Neurosurgery & Psychiatry*, *72*(6), 701–707.
- Houston, C. M., He, Q., & Smart, T. G. (2009). CaMKII phosphorylation of the GABA A receptor : Receptor subtype- and synapse-specific modulation. *J Physiol*, *10*, 2115–2125.
- Hunter, M. D., Ganesan, V., Wilkinson, I. D., & Spence, S. A. (2006). Impact of modafinil on prefrontal executive function in schizophrenia. *American Journal of Psychiatry*, *163*(12), 2184–2186.
- Isoda, M., & Hikosaka, O. (2008). Role for Subthalamic Nucleus Neurons in Switching from Automatic to Controlled Eye Movement. *The Journal of Neuroscience*, *28*(28), 7209–7218.
- Jacobson, C. (1936). Studies of cerebral function in primates. *Comparative Psychology Monographs*, *13*, 1–60.
- Jahanshahi, M., Obeso, I., Baunez, C., Alegre, M., & Krack, P. (2015). Parkinson's Disease , the Subthalamic Nucleus , Inhibition , and Impulsivity. *Movement Disorder*, *30*(2), 128–140.
- Jahanshahi, M. (2013). Risky choices link the subthalamic nucleus with pathological gambling in Parkinson's disease. *Movement Disorders*, *28*(12), 1617–1619.
- James, M. D., MacKenzie, F. J., Tuohy-Jones, P. A., & Wilson, C. A. (1987). Dopaminergic neurones in the zona incerta exert a stimulatory control on gonadotrophin release via D1 dopamine receptors. *Neuroendocrinology*, *45*(5), 348–355.
- Janssen, M., Visser-Vandewalle, V., & Temel, Y. (2010). Cortico-Subthalamic Projections In The Rat. *Journal of Experimental and Clinical Medicine*, *27*, 04–12.
- Jansma, J. M., Ramsey, N. F., Coppola, R., & Kahn, R. S. (2000). Specific versus nonspecific brain activity in a parametric N-back task. *NeuroImage*, *12*(6), 688–697.
- Jara, J.H., Villa, S.R., Khan, N.A., Bohn, M.C., Ozdinler, P.H. (2012). AAV2 mediated retrograde transduction of corticospinal motor neurons reveals initial and selective apical dendrite degeneration in ALS. *Neurobiology of Disease*, *47*(2), 174–183.
- Jarrard, L. (1989). On the use of ibotenic acid to lesion selectively different components of the hippocampal formation. *J Neurosci Methods*, *29*(3), 251–259.
- Joel, D., & Weiner, I. (1997). The connections of the primate subthalamic nucleus : indirect pathways and the open-interconnected scheme of basal ganglia-thalamocortical circuitry. *Brain Research*, *23*, 62–78.
- Jones, K. A., Borowsky, B., Tamm, J. A., Craig, D. A., Durkin, M. M., Dai, M., ... Gerald, C. (1998). GABAB receptors function as a heteromeric assembly of the subunits GABABR1 and GABABR2. *Nature*, *396*(December), 674–679.
- Kaplitt, M., Leone, P., Samulski, R., Xiao, X., Pfaff, D., O'Malley, K., & Doring, M. (1994). Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nature Genetics*, *8*(4), 148–154.
- Karachi, C., Yelnik, J., Tandé, D., Tremblay, L., Hirsch, E. C., & François, C. (2005). The pallidosubthalamic projection: an anatomical substrate for nonmotor functions of the subthalamic nucleus in primates. *Movement disorders*, *20*(2), 172–180.
- Kattner, F. (2015). Transfer of absolute and relative predictiveness in human contingency

- learning. *Learning & Behavior*, 43(1), 32–43.
- Kätzel, D., Nicholson, E., Schorge, S., Walker, M. C., & Kullmann, D. M. (2014). Chemical-genetic attenuation of focal neocortical seizures. *Nature Communications*, 5(May), 3847.
- Kaufman, D. L., Houser, C. R., & Tobin, A. J. (1991). Two forms of the  $\gamma$ -aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. *Journal of neurochemistry*, 56(2), 720-723.
- Kennedy, M. B. (1993). The postsynaptic density. *Current Opinion in Neurobiology*, 3(5), 732–737.
- Kennedy, M. B., Bennett, M. K., & Erondy, N. E. (1983). Biochemical and immunochemical evidence that the “major postsynaptic density protein” is a subunit of a calmodulin-dependent protein kinase. *Proceedings of the National Academy of Sciences of the United States of America*, 80(23), 7357–7361.
- Kim, D. W., Uetsuki, T., Kaziro, Y., Yamaguchi, N., & Sugano, S. (1990). Use of the human elongation factor 1 alpha promoter as a versatile and efficient expression system. *Gene*, 91(2), 217–23.
- Kim, U., Gregory, E., & Hall, W. C. (1992). Pathway from the zona incerta to the superior colliculus in the rat. *Journal of Comparative Neurology*, 321(4), 555-575.
- Kita, T., Osten, P., & Kita, H. (2014). Rat subthalamic nucleus and zona incerta share extensively overlapped representations of cortical functional territories. *J Comp Neurol*, 522(18), 4043–4056.
- Kohler, C., Schwarcz, R., & Fuxe, K. (1979). Intrahippocampal injections of ibotenic acid provide histological evidence for a neurotoxic mechanism different from kainic acid. *Neuroscience Letters*, 15(2), 223–228.
- Kolmac, C., & Mitrofanis, J. (1999). Distribution of various neurochemicals within the zona incerta: an immunocytochemical and histochemical study. *Anatomy and embryology*, 199(3), 265-280.
- Koob, G. F., & Bloom, F. E. (1988). Cellular and Molecular Mechanisms of Drug Dependence. *Science*, 242(4879), 715–723.
- Kordon, C., Enjalbert, A., H~ry, M., Joseph-Bravo, P.I., Rotsztejn, W. and Ruberg, M., Role of neurotransmitters in the control of adenohipophyseal secretion. In P.J. Morgane and J. Panksepp (Eds.), *Handbook of the Hypothalamus*, Vol. 2, Dekker, New York, 1980, pp 253-306
- Korecka, J., Schouten, M., Eggers, R., Ulusoy, A., Bossers, K., & Verhaagen, J. (2011). Comparison of AAV serotypes for gene delivery to dopaminergic neurons in the Substantia Nigra. *Viral Gene Therapy*, 2, 205–224.
- Kozorovitskiy, Y., Saunders, A., Johnson, C. a., Lowell, B. B., & Sabatini, B. L. (2012). Recurrent network activity drives striatal synaptogenesis. *Nature*, 485(7400), 646–650.
- Kreiss, D. S., Anderson, L., & Walters, J. (1996). Apomorphine and dopamine D1 receptor agonists increase the firing rates of subthalamic nucleus neurons. *Neuroscience*, 72(3), 863–876.
- Krieger, M. S., Conrad, L. C., & Pfaff, D. W. (1979). An autoradiographic study of the efferent connections of the ventromedial nucleus of the hypothalamus. *Journal of Comparative Neurology*, 183(4), 785-815.
- Kügler, S., Kilic, E., & Bähr, M. (2003). Human synapsin 1 gene promoter confers highly neuron-specific long-term transgene expression from an adenoviral vector in the adult rat brain depending on the transduced area. *Gene Therapy*, 10(4), 337–347.
- Kumar, R., Lozano, A. M., Sime, E., Halket, E., & Lang, A. E. (1999). Comparative effects of unilateral and bilateral subthalamic nucleus deep brain stimulation. *Neurology*, 53(3), 561–566.
- Kuwajima, M., Hall, R. A., Aiba, A., & Smith, Y. (2004). Subcellular and subsynaptic

- localization of group I metabotropic glutamate receptors in the monkey subthalamic nucleus. *Journal of Comparative Neurology*, 474(4), 589–602.
- Lacombe, E., Carcenac, C., Boulet, S., Feuerstein, C., Bertrand, A., Poupard, A., & Savasta, M. (2007). High-frequency stimulation of the subthalamic nucleus prolongs the increase in striatal dopamine induced by acute 1-3, 4-dihydroxyphenylalanine in dopaminergic denervated rats. *European Journal of Neuroscience*, 26(6), 1670–1680.
- Lapin, I. P., Prakhie, I. B., & Kiseleva, I. P. (1986). Antagonism of seizures induced by the administration of the endogenous convulsant quinolinic acid into rat brain ventricles. *J Neural Transm.*, 65(3–4), 177–185.
- Lapiz, S., Bondi, C. O., & Morilak, D. A. (2007). Chronic Treatment with Desipramine Improves Cognitive Performance of Rats in an Attentional Set-Shifting Test. *Neuropsychopharmacology*, 32, 1000–1010.
- Lashley, K. (1929). *Brain Mechanisms and Intelligence* (1963 ed.). Dover, New York.
- Lashley, K. S. (1930). The Mechanism of Vision: I. A Method for Rapid Analysis of Pattern-Vision in the Rat. *The Pedagogical Seminary and Journal of Genetic Psychology*, 37(4), 453–460.
- Lawrence, A. D., Sahakian, B. J., & Robbins, T. W. (1998). Cognitive functions and corticostriatal circuits: Insights from Huntington's disease. *Trends in Cognitive Sciences*, 2(10), 379–388.
- Lawrence, D. H. (1949). Acquired distinctiveness of cues: I. Transfer between discriminations on the basis of familiarity with the stimulus. *Journal of Experimental Psychology*, 39(6), 770–784.
- Leblois, A., Boraud, T., Meissner, W., Bergman, H., & Hansel, D. (2006). Competition between Feedback Loops Underlies Normal and Pathological Dynamics in the Basal Ganglia. *The Journal of Neuroscience*, 26(13), 3567–3583.
- Lévesque, J. C., & Parent, A. (2005). GABAergic interneurons in human subthalamic nucleus. *Movement Disorders*, 20(5), 574–584
- Lisman, J., Schulman, H., & Cline, H. (2002). The Molecular Basis Of Camkii Function In Synaptic And Behavioural Memory. *Nature Reviews Neuroscience*, 3(3), 175–190.
- Lin, C., Nicolelis, M. A. L., Schneider, J. A. Y. S., & Chapin, J. K. (1990). A Major Direct GABAergic Pathway from Zona Incerta to Neocortex. *Science*, 248(4962), 1553–1556.
- Lindgren, H. S., Wickens, R., Tait, D. S., Brown, V. J., & Dunnett, S. B. (2013). Lesions of the dorsomedial striatum impair formation of attentional set in rats. *Neuropharmacology*, 71, 148–53.
- Leith, N. J., & Barrett, R. J. (1976). Amphetamine and the reward system: Evidence for tolerance and post-drug depression. *Psychopharmacologia*, 46(1), 19–25.
- Lever, C., Burton, S., & O'Keefe, J. (2006). Rearing on hind legs, environmental novelty, and the hippocampal formation. *Rev Neurosci*, 17(1–2), 111–133.
- Lin, J. S., Roussel, B., Akaoka, H., Fort, P., Debilly, G., & Jouvet, M. (1992). Role of catecholamines in the modafinil and amphetamine induced wakefulness, a comparative pharmacological study in the cat. *Brain Research*, 591(2), 319–326.
- Liu, Y., Wong, T. P., Aarts, M., Rooyackers, A., Liu, L., Lai, T. W., ... Wang, Y. T. (2007). Neurobiology of Disease NMDA Receptor Subunits Have Differential Roles in Mediating Excitotoxic Neuronal Death Both In Vitro and In Vivo. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 27(11), 2846–2857.
- Lledo, P. M., Hjelmstad, G. O., Mukherji, S., Soderling, T. R., Malenka, R. C., & Nicoll, R. A. (1995). Calcium/calmodulin-dependent kinase II and long-term potentiation enhance synaptic transmission by the same mechanism. *Proceedings of the National Academy of Sciences of the United States of America*, 92(24), 11175–9.
- Losos, J., Mason, K., Singer, S., Raven, P., & Johnson, G. (2008). *Biology* (Eighth). New

- York: McGraw-Hill.
- Luchins, A. S. (1942). Mechanization in problem solving: The effect of Einstellung. *Psychological Monographs*, *54*(6), i-95.
- Mackintosh, N. J. (1963). The effect of irrelevant cues on reversal learning in the rat. *British Journal of Psychology*, *54*(2), 127–134.
- Mackintosh, N. J. (1969). Further analysis of the overtraining reversal effect. *Journal of Comparative and Physiological Psychology Monograph*, *67*(2), 1–18.
- Mackintosh, N. J., & Little, L. (1969). Selective attention and response strategies as factors in serial reversal learning. *Canadian Journal of Psychology/Revue Canadienne de Psychologie*, *23*(5), 335–346.
- Maclaren, D., Browne, R. W., Shaw, J. K., Radhakrishnan, S. K., Khare, P., España, R. A., & Clark, S. D. (2016). Clozapine-n-oxide administration produces behavioral effects in Long-Evans rats - implications for designing DREADD experiments CNO converts into bioactive compounds in rats. *Integrative Systems*, *10*(October), 219–16.
- Mallet, L., Polosan, M., Jaafari, N., Baup, N., Welter, M. L., Fontaine, D., ... & Raoul, S. (2008). Subthalamic nucleus stimulation in severe obsessive–compulsive disorder. *New England Journal of Medicine*, *359*(20), 2121-2134.
- Mann, J. J., Malone, K. M., Diehl, D. J., Perel, J., Nichols, T. E., & Mintun, M. A. (1996). Positron Emission Tomographic Imaging of Serotonin Activation Effects on Prefrontal Cortex in Healthy Volunteers. *Journal of Cerebral Blood Flow and Metabolism*, *16*, 418–426.
- Marx, C. E., Keefe, R. S. E., Buchanan, R. W., Hamer, R. M., Kilts, D., Bradford, D. W., ... Shampine, L. J. (2009). Targeting Cognitive and Negative Symptoms in Schizophrenia, *34*(8), 1885–1903.
- Mason, M. R. J., Ehler, E. M. E., Eggers, R., Pool, C. W., Hermening, S., Huseinovic, A., ... Verhaagen, J. (2010). Comparison of AAV serotypes for gene delivery to dorsal root ganglion neurons. *Molecular Therapy: The Journal of the American Society of Gene Therapy*, *18*(4), 715–24.
- Mathis, C., Vogel, E., Cagniard, B., Criscuolo, F., & Ungerer, A. (1996). The neurosteroid pregnenolone sulfate blocks deficits induced by a competitive NMDA antagonist in active avoidance and lever-press learning tasks in mice. *Neuropharmacology*, *35*(8), 1057–1064.
- May, L. T., Leach, K., Sexton, P. M., & Christopoulos, A. (2007). Allosteric modulation of G protein-coupled receptors. *Ann Rev Pharmacol Toxicol*, *47*, 1–51.
- McAlonan, K., & Brown, V. J. (2003). Orbital prefrontal cortex mediates reversal learning and not attentional set shifting in the rat. *Behavioural Brain Research*, *146*(1–2), 97–103.
- McGeorge, A. J., & Faull, R. L. M. (1989). The organization of the projection from the cerebral cortex to the striatum in the rat. *Neuroscience*, *29*(3), 503–537.
- McGlade-McCulloh, E., Yamamoto, H., Tan, S., Brickey, D., & Soderling, T. R. (1993). Phosphorylation and regulation of glutamate receptors by calicum/calmodulin-dependent protein kinase II. *Nature*, *363*, 640–642.
- Meibach, R. C., & Katzman, R. (1979). Catecholaminergic innervation of the subthalamic nucleus: evidence for a rostral continuation of the A9 (substantia nigra) dopaminergic cell group. *Brain research*, *173*(2), 364-368.
- Meijer, M. K., Spruijt, B. M., van Zutphen, L. F., & Baumans, V. (2006). Effect of restraint and injection methods on heart rate and body temperature in mice. TL - 40. *Laboratory Animals*, *40* VN-r(4), 382–391.
- Mereu, M., Bonci, A., Newman, A. H., & Tanda, G. (2013). The neurobiology of modafinil as an enhancer of cognitive performance and a potential treatment for substance use disorders. *Psychopharmacology*, *144*(3), 415–434.

- Meissner, W., Harnack, D., Reese, R., Paul, G., Reum, T., Ansorge, M., Kusserow, H., Winter, C., Mogenstern, R., & Kupsch, A. (2003). High-frequency stimulation of the subthalamic nucleus enhances striatal dopamine release and metabolism in rats. *Journal of neurochemistry*, *85*(3), 601-609.
- Meyer-lindenberg, A. S., Olsen, R. K., Kohn, P. D., Brown, T., Egan, M. F., Weinberger, D. R., & Berman, K. F. (2005). Regionally Specific Disturbance of Dorsolateral Prefrontal–Hippocampal Functional Connectivity in Schizophrenia. *Arch Gen Psychiatry*, *62*, 379–386.
- Miller, E., & Cohen, J. (2001). An integrative theory of prefrontal cortex function. *Annual Review of Neuroscience*, *24*, 167–202.
- Milner, B. (1963). Effects of different brain lesions on card sorting: The role of the frontal lobes. *Arch Neurol*, *9*, 90–100.
- Minzenberg, M. J., & Carter, C. S. (2008). Modafinil: A Review of Neurochemical Actions and Effects on Cognition. *Neuropsychopharmacology*, *33*(7), 1477–1502.
- Mitrofanis, J. (2005). Some certainty for the “zone of uncertainty”? Exploring the function of the zona incerta. *Neuroscience*, *130*, 1–15.
- Mori, M. S., Takino, T., Yamada, H., & Sano, Y. (1985). Immunohistochemical demonstration of serotonin nerve fibers in the subthalamic nucleus of the rat, cat and monkey. *Neuroscience Letters*, *62*, 305–309.
- Morris, L. S., Baek, K., & Voon, V. (2017). Distinct cortico-striatal connections with subthalamic nucleus underlie facets of compulsivity. *Cortex*, *88*, 143–150.
- Morgan, R. E., Crowley, J. M., Smith, R. H., LaRoche, R. B., & Dopheide, M. M. (2007). Modafinil improves attention, inhibitory control, and reaction time in healthy, middle-aged rats. *Pharmacology, Biochemistry, and Behavior*, *86*(3), 531–41.
- Moro, E., Scerrati, M., Romito, L. M. A., Roselli, R., Tonali, P., & Albanese, A. (1999). Chronic subthalamic nucleus stimulation reduces medication requirements in Parkinson’s disease. *Neurology*, *53*(1), 85-85.
- Mouroux, M., & Feger, J. (1993). Evidence that the parafascicular projection to the subthalamic nucleus is glutamatergic. *Neuroreport*, *4*(6), 613-615.
- Murer, M. G., & Pazo, J. H. (1993). Circling behaviour induced by activation of GABAA receptors in the subthalamic nucleus. *Neuroreport*, *4*(11), 1219-1222.
- Müller, U., Steffenhagen, N., Regenthal, R., & Bublak, P. (2004). Effects of modafinil on working memory processes in humans. *Psychopharmacology*, *177*(1–2), 161–169.
- Muzyczka, N. (1992). Use of Adeno-Associated Virus as a General Transduction Vector for Mammalian Cells. In N. Muzyczka (Ed.), *Viral Expression Vectors* (Vol. 158, pp. 97–129). New York: Springer Berlin Heidelberg.
- Nagahama, Y., Okina, T., Suzuki, N., Nabatame, H., & Matsuda, M. (2005). The cerebral correlates of different types of perseveration in the Wisconsin Card Sorting Test. *J Neurol Neurosurg Psychiatry*, *76*, 169–175.
- Nakanishi, H., Kita, H., & Kitai, S. T. (1987). Intracellular study of rat substantia nigra pars reticulata neurons in an in vitro slice preparation: electrical membrane properties and response characteristics to subthalamic stimulation. *Brain Research*, *437*, 45–55.
- Nambu, A. (2004). A new dynamic model of the cortico-basal ganglia loop. *Progress in Brain Research*, *143*, 461–466.
- Nambu, A., Takada, M., Inase, M., & Tokuno, H. (1996). Dual Somatotopical Representations in the Primate Subthalamic Nucleus: Evidence for Ordered but Reversed Body-Map Transformations from the Primary Motor Cortex and the Supplementary Motor Area. *The Journal of Neuroscience*, *16*(8), 2671–2683.
- Nambu, A., Tokuno, H., Hamada, I., Kita, H., Imanishi, M., Akazawa, T., ... Hasegawa, N. (2000). Excitatory Cortical Inputs to Pallidal Neurons Via the Subthalamic Nucleus in

- the Monkey. *Journal of Neurophysiology*, 84, 289–300.
- Nambu, A., Tokuno, H., Inase, M., & Takada, M. (1997). Corticosubthalamic input zones from forelimb representations of the dorsal and ventral divisions of the premotor cortex in the macaque monkey: comparison with the input zones from the primary motor cortex and the supplementary motor area. *Neuroscience Letters*, 239, 13–16.
- Nambu, A., Tokuno, H., & Takada, M. (2002). Functional significance of the cortico-subthalamo-pallidal “hyperdirect” pathway. *Neuroscience Research*, 43(2), 111–117.
- Nauta, H. J. W., & Cuenod, M. (1982). Perikaryal cell labeling in the subthalamic nucleus following the injection of 3H- $\gamma$ -aminobutyric acid into the pallidal complex: an autoradiographic study in cat. *Neuroscience*, 7(11), 2725–2734.
- Nicolelis, M. A., Chapin, J. K., & Lin, R. C. (1992). Somatotopic maps within the zona incerta relay parallel GABAergic somatosensory pathways to the neocortex, superior colliculus, and brainstem. *Brain research*, 577(1), 134–141.
- Nicolelis, M., Chapin, J., & Lin, R. (1995). Development of direct GABAergic projections from the Zona Incerta to the somatosensory cortex of the rat. *Neuroscience*, 65(2), 609–631.
- Nieuwenstein, M. R., Aleman, A., & de Haan, E. H. (2001). Relationship between symptom dimensions and neurocognitive functioning in schizophrenia: A meta-analysis of WCST and CPT studies. *Journal of Psychiatric Research*, 35, 119–125.
- Nisbet, A. P., Eve, D. J., Kingsbury, A. E., Daniel, S. E., Marsden, C. D., Lees, A. J., & Foster, O. J. F. (1996). Glutamate decarboxylase-67 messenger RNA expression in normal human basal ganglia and in Parkinson's disease. *Neuroscience*, 75(2), 389–406.
- Nishino, S., & Mignot, E. (1997). Pharmacological aspects of human and canine narcolepsy. *Progress in Neurobiology*, 52(1), 27–78.
- Obeso, J. A., & Lanciego, J. L. (2011). Past, present, and future of the pathophysiological model of the Basal Ganglia. *Frontiers in Neuroanatomy*, 5, 39.
- Obeso, J. A., Rodriguez-oroz, M. C., Blesa, F. J., & Guridi, J. (2006). The globus pallidus pars externa and Parkinson's disease. Ready for prime time? *Experimental Neurology*, 202, 1–7.
- Obeso, J. A., Rodriguez, M. C., Benitez-temino, B., Blesa, F. J., Guridi, J., Marin, C., & Rodriguez, M. (2008). Functional organization of the basal ganglia: Therapeutic implications for Parkinson's disease. *Movement Disorders*, 23, 548–559.
- Oertel, W. H., & Mugnaini, E. (1984). Immunocytochemical studies of GABAergic neurons in rat basal ganglia and their relations to other neuronal systems. *Neuroscience letters*, 47(3), 233–238.
- Owen, A. M., McMillan, K. M., Laird, A. R., & Bullmore, E. (2005). N-back working memory paradigm: A meta-analysis of normative functional neuroimaging studies. *Human Brain Mapping*, 25(1), 46–59.
- Owen, A. M., Roberts, A. C., Hodges, J. R., Summers, B. A., Polkey, C. E., & Robbins, T. W. (1993). Contrasting mechanisms of impaired attentional set-shifting in patients with frontal lobe damage or Parkinson's disease. *Brain*, 116, 1159–1175.
- Owen, AM., Roberts, AC., Polkey, CE., Sahakian, B. J., & Robbins, T. W. (1991). Extra-dimensional versus intra-dimensional set shifting performance following frontal lobe excisions, temporal lobe excisions or amygdalo-hippocampectomy in man. *Neuropsychologia*, 29(10), 993–1006.
- Ozawa, S., Kamiya, H., & Tsuzuki, K. (1998). Glutamate receptors in the mammalian central nervous system. *Progress in neurobiology*, 54(5), 581–618.
- Pagano, G., Rengo, G., Pasqualetti, G., Femminella, G. D., Monzani, F., Ferrara, N., & Tagliati, M. (2014). Cholinesterase inhibitors for Parkinson's disease: a systematic review and meta-analysis. *J Neurol Neurosurg Psychiatry*, jnnp-2014.

- Palencia, C. A., & Ragozzino, M. E. (2006). The effect of N-methyl-D-aspartate receptor blockade on acetylcholine efflux in the dorsomedial striatum during response reversal learning. *Neuroscience*, *143*(3), 671–678.
- Paolo, A. M., Triister, A. I., Axelrod, B. N., & Koller, W. C. (1995). Construct validity of the WCST in normal elderly and persons with Parkinson's disease. *Archives of Clinical Neuropsychology*, *10*(5), 463–473.
- Parent, A., & Hazrati, L. (1995). Functional anatomy of the basal ganglia . II. The place of subthalamic nucleus and external pallidum in basal ganglia circuitry. *Brain Research Reviews*, *20*, 128–154.
- Park, A., Hoffman, K., & Keller, A. (2014). Roles of GABA A and GABA B receptors in regulating thalamic activity by the zona incerta : A computational study. *J Neurophysiol*, *112*, 2580–2596.
- Paterna, J., Feldon, J., Büeler, H., & Bu, H. (2004). Transduction Profiles of Recombinant Adeno-Associated Virus Vectors Derived from Serotypes 2 and 5 in the Nigrostriatal System of Rats Transduction Profiles of Recombinant Adeno-Associated Virus Vectors Derived from Serotypes 2 and 5 in the Nigrostriatal. *Journal of Virology*, *78*(13), 6808–6817.
- Paul, S. M., Doherty, J. J., Robichaud, A. J., Belfort, G. M., Chow, B. Y., Hammond, R. S., ... Zorumski, C. F. (2013). The major brain cholesterol metabolite 24(s)-hydroxycholesterol is a potent allosteric modulator of N-methyl-d-aspartate receptors. *J Neurosci*, *33*(44), 17290–17300.
- Paul, S. M., & Purdy, R. H. (1992). Neuroactive steroids. *The FASEB Journal*, *6*, 2311–2322.
- Paxinos, G., & Watson, C. (1998). *The rat brain in stereotaxic coordinates*. San Diego: Academic Press.
- Pearce, J. M., & Hall, G. (1980). A Model for Pavlovian Learning: Variations in the Effectiveness of Conditioned But Not of Unconditioned Stimuli. *Psychological Review*, *87*(6), 532–552.
- Pelloux, Y., & Baunez, C. (2013). Deep brain stimulation for addiction: why the subthalamic nucleus should be favored. *Current opinion in neurobiology*, *23*(4), 713-720.
- Penney, J. B., & Young, A. B. (1986). Striatal inhomogeneities and basal ganglia function. *Movement Disorders*, *1*(1), 3–15.
- Perez-Rico, C., & Gomez-Ramos, P. (1984). Histological study of ibotenic acid-induced modifications of rat retina and their attenuation by diazepam. *Cell and Tissue Research*, *238*(1), 81–85.
- Pettit, D., Pernman, S., & Malinow, R. (1994). Potentiated transmission and prevention of further LTP by increased CaMKII activity in postsynaptic hippocampal slice neurons. *Science*, *266*, 1881–1885.
- Phillips, A. G., Pfaus, J. G., & Blaha, C. D. (1991). Dopamine and motivated behavior: insights provided by in vivo analyses. *The mesolimbic dopamine system: From motivation to action*, 199-224.
- Phillips, J. M., & Brown, V. J. (1999). Reaction time performance following unilateral striatal dopamine depletion and lesions of the subthalamic nucleus in the rat. *Eur.J.Neurosci.*, *11*(3), 1003–1010.
- Phillips, J. M., & Brown, V. J. (2000). Anticipatory errors after unilateral lesions of the subthalamic nucleus in the rat: evidence for a failure of response inhibition. *Behavioral Neuroscience*, *114*(1), 150–157.
- Phillips, J. M., Lam, H. A., Ackerson, L. C., & Maidment, N. T. (2006). Blockade of mGluR 5 glutamate receptors in the subthalamic nucleus ameliorates motor asymmetry in an animal model of Parkinson ' s disease. *European Journal of Neuroscience*, *23*, 151–160.
- Plaha, P., Patel, N. K., & Gill, S. S. (2006). Stimulation of the caudal zona incerta is superior



- to stimulation of the subthalamic nucleus in improving contralateral parkinsonism. *Brain*, 129, 1732–1747.
- Plaha, P., Khan, S., & Gill, S. S. (2008). Bilateral stimulation of the caudal zona incerta nucleus for tremor control. *Journal of neurology, neurosurgery & psychiatry*, 79(5), 504-513.
- Plaha, P., Javed, S., Agombar, D., O'Farrell, G., Khan, S., Whone, A., & Gill, S. (2011). Bilateral caudal zona incerta nucleus stimulation for essential tremor: outcome and quality of life. *Journal of Neurology, Neurosurgery & Psychiatry*, jnnp-2010.
- Porter, H. P., Greene, G., Higgins, D., & Greenamyre, J. (1994). Polysynaptic Regulation of Glutamate Receptors and Mitochondrial Enzyme Activities in the Basal Ganglia of Rats with Unilateral Dopamine Depletion. *The Journal of Neuroscience*, 14(11), 7192–7199.
- Pote, I., Torkamani, M., Kefalopoulou, Z. M., Zrinzo, L., Limousin-Dowsey, P., Foltyniec, T., ... & Jahanshahi, M. (2016). Subthalamic nucleus deep brain stimulation induces impulsive action when patients with Parkinson's disease act under speed pressure. *Experimental brain research*, 234(7), 1837-1848.
- Power, B. D., Kolmac, C. I., & Mitrofanis, J. (1999). Evidence for a Large Projection From the Zona Incerta to the Dorsal Thalamus. *The Journal of Comparative Neurology*, 404, 554–565.
- Qiu, M., Chen, M. C., Huang, Z., & Lu, J. (2014). Neuronal activity (c-Fos) delineating interactions of the cerebral cortex and basal ganglia. *Frontiers in Neuroanatomy*, 8(13), 1–10.
- Racht-delatour, B. V. G., & El Massioui, N. (2000). Alleviation of overtraining reversal effect by transient inactivation of the dorsal striatum, 12(L), 3343–3350.
- Randall, D. C., Shneerson, J. M., & File, S. E. (2005). Cognitive effects of modafinil in student volunteers may depend on IQ. *Pharmacology Biochemistry and Behavior*, 82(1), 133–139.
- Ray, R. S., Corcoran, A. E., Brust, R. D., Kim, J. C., Richerson, G. B., Nattie, E., & Dymecki, S. M. (2011). Impaired respiratory and body temperature control upon acute serotonergic neuron inhibition. *Science*, 333, 637–642.
- Redgrave, P., Vautrelle, N., & Reynolds, J. N. J. (2011). Functional properties of the basal ganglia's re-entrant loop architecture: Selection and reinforcement. *Neuroscience*, 198, 138–151.
- Reid, L. S. (1953). The development of noncontinuity behavior through continuity learning. *Journal of Experimental Psychology*, 46(2), 107–112.
- Reznitsky, M., Plenge, P., & Hay-schmidt, A. (2016). Serotonergic projections from the raphe nuclei to the subthalamic nucleus; A retrograde- and anterograde neuronal tracing study. *Neuroscience Letters*, 612, 172–177.
- Rich, E. L., & Shapiro, M. L. (2007). Prelimbic / Infralimbic Inactivation Impairs Memory for Multiple Task Switches , But Not Flexible Selection of Familiar Tasks. *The Journal of Neuroscience*, 27(17), 4747–4755.
- Rico, A. J., Barroso-chinea, P., Conte-perales, L., Roda, E., Gómez-bautista, V., Gendive, M., ... Lanciego, J. L. (2010). Neurobiology of Disease A direct projection from the subthalamic nucleus to the ventral thalamus in monkeys. *Neurobiology of Disease*, 39(3), 381–392.
- Ridderinkhof, K. R., Span, M. M., & Molen, M. W. Van Der. (2002). Perseverative behavior and adaptive control in older adults: Performance monitoring, rule induction, and set shifting. *Brain and Cognition*, 49, 382–401.
- Rinvik, E., Grofova, I., Hammond, C., Feger, J., & Deniau, J. M. (1979). A study of the afferent connections to the subthalamic nucleus in the monkey and the cat using the HRP technique. *Advances in neurology*, 24, 53-70.

- Roberts, A. C., Robbins, T. W., & Everitt, B. J. (1988). The Effects of Intradimensional and Extradimensional Shifts on Visual Discrimination Learning in Humans and Non-human Primates, (789284501).
- Roberts, A. C., Robbins, T. W., Everitt, B. J., & Muir, J. L. (1992). A specific form of cognitive rigidity following excitotoxic lesions of the basal forebrain in marmosets. *Neuroscience*, *47*(2), 251–264.
- Roberts, R. A., Soames, A. R., James, N. H., Gill, J. H., & Wheeldon, E. B. (1995). Dosing-induced stress causes hepatocyte apoptosis in rats primed by the rodent nongenotoxic hepatocarcinogen cyproterone acetate. *Toxicology*, *135*, 192–199.
- Robinson, G., Butcher, R., & Sutherland, E. (1971). *Cyclic AMP*. Academic Press.
- Robledo, P., & Féger, J. (1990). Excitatory influence of rat subthalamic nucleus to substantia nigra pars reticulata and the pallidal complex: electrophysiological data. *Brain research*, *518*(1-2), 47-54.
- Roehrs, T., Zwyghuizen-Doorenbos, a, Timms, V., Zorick, F., & Roth, T. (1989). Sleep extension, enhanced alertness and the sedating effects of ethanol. *Pharmacology, Biochemistry, and Behavior*, *34*(2), 321–4.
- Rohrer, J. D., & Warren, J. D. (2011). Phenotypic signatures of genetic frontotemporal dementia. *Current Opinion in Neurology*, *24*, 542–549.
- Rogan, S., & Roth, B. (2011). Remote control of neuronal signaling. *Pharmacological Reviews*, *63*(2), 291–315.
- Romanowski, C. A., Mitchell, I. J., & Crossman, A. R. (1985). The organisation of the efferent projections of the zona incerta. *Journal of anatomy*, *143*, 75.
- Romine, C. B., Lee, D., Wolfe, M. E., Homack, S., George, C., & Riccio, C. A. (2004). Wisconsin Card Sorting Test with children: A meta-analytic study of sensitivity and specificity. *Archives of Clinical Neuropsychology*, *19*, 1027–1041.
- Romito, L. M., Raja, M., Daniele, A., Contarino, M. F., Bentivoglio, A. R., Barbier, A., Scerrati, M., & Albanese, A. (2002). Transient mania with hypersexuality after surgery for high frequency stimulation of the subthalamic nucleus in Parkinson's disease. *Movement Disorders*, *17*(6), 1371-1374.
- Ross, C. A., & Margolis, R. L. (2001). Huntington's disease. *Clinical Neuroscience Research*, *1*, 142–152.
- Rouzaire-Dubois, B., Hammond, C., Yelnik, J., & Féger, J. (1984). Anatomy and neurophysiology of the subthalamic efferent neurons. In *The Basal Ganglia* (pp. 205-234). Springer, Boston, MA.
- Sahakian, B. J., & Owen, A. M. (1992). Computerized assessment in neuropsychiatry using CANTAB: discussion paper. *Journal of the Royal Society of Medicine*, *85*(July), 399–402.
- Saint-cyr, J. A., Trepanier, L. L., Kumar, R., Lozano, A. M., & Lang, A. E. (2000). Neuropsychological consequences of chronic bilateral stimulation of the subthalamic nucleus in Parkinson's disease. *Brain*, *123*, 2091–2108.
- Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R. M., Tanaka, H., ... Yanagisawa, M. (1998). Orexins and Orexin Receptors : A Family of Hypothalamic Neuropeptides and G Protein-Coupled Receptors that Regulate Feeding Behavior. *Cell*, *92*, 573–585.
- Sasaki, K., Suzuki, M., Mieda, M., Tsujino, N., Roth, B., & Sakurai, T. (2011). Pharmacogenetic modulation of orexin neurons alters sleep/wakefulness states in mice. *PLoS ONE*, *6*(5).
- Settlage, P., Butler, R., & Odoi, H. (1956). Perseverative interference in monkeys following bilateral removal of the prefrontal areas. *J Gen Psych*, *54*, 255–262.
- Scammell, T. E., Estabrooke, I. V, McCarthy, M. T., Chemelli, R. M., Yanagisawa, M.,

- Miller, M. S., & Saper, C. B. (2000). Hypothalamic arousal regions are activated during modafinil-induced wakefulness. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *20*(22), 8620–8628.
- Schwarcz, R., Fuxe, K., Jonsson, G., Goldstein, M., & Terenius, L. (1979). Brain Original Articles Ibotenic Acid-induced Neuronal Degeneration: A Morphological and Neurochemical Study. *Experimental Brain Research*, *216*, 199–216.
- Sgambato, V., Abo, V., Rogard, M., Besson, M. J., & Deniau, J. M. (1997). Effect of electrical stimulation of the cerebral cortex on the expression of the fos protein in the basal ganglia. *Neuroscience*, *81*(1), 93–112.
- Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N. G., Palmer, A. E., & Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nature Biotechnology*, *22*(12), 1567–72.
- Shaner, N., Steinbach, P., & Tsien, R. (2005). A guide to choosing fluorescent proteins. *Nature Methods*, *2*(12), 905–909.
- Sharpe, M. J., & Killcross, S. (2014). The prelimbic cortex contributes to the down-regulation of attention toward redundant cues. *Cerebral Cortex*, *24*(4), 1066–1074.
- Shaw, F., Liao, Y., Chen, R., Huang, Y., & Lin, R. C. S. (2013). The zona incerta modulates spontaneous spike-wave discharges in the rat. *J Neurophysiol*, *109*, 2505–2516.
- Shen, K. Z., & Johnson, S. W. (2003). Presynaptic inhibition of synaptic transmission by adenosine in rat subthalamic nucleus in vitro. *Neuroscience*, *116*(1), 99–106.
- Shen, K., & Johnson, S. (2008). 5-Hydroxytryptamine inhibits synaptic transmission in rat subthalamic nucleus neurons in vitro. *Neuroscience*, *151*(4), 1029–1033.
- Shevtsova, Z., Malik, J. M., Michel, U., Bahr, M., & Kugler, S. (2005). Promoters and serotypes: targeting of adeno-associated virus vectors for gene transfer in the rat central nervous system in vitro and in vivo. *Exp Physiol*, *90*(1), 53–59.
- Siddiqui, A., Abu-amara, M., Aldairy, C., Hagan, J. J., & Wilson, C. (2004). 5-HT<sub>7</sub> receptor subtype as a mediator of the serotonergic regulation of luteinizing hormone release in the zona incerta. *European Journal of Pharmacology*, *491*, 77–84.
- Simon, P., Hémet, C., & Costentin, J. (1996). Analysis of stimulant locomotor effects of modafinil in various strains of mice and rats. *Fundamental & Clinical Pharmacology*, *10*(5), 431–5.
- Slamecka, N. (1968). A methodological analysis of shift paradigms in human discrimination learning. *Psychological Bulletin*, *69*(6), 423–438.
- Smeding, H. M. M., Speelman, J. D., Schuurman, P. R., Nijssen, P., Laar, T. Van, & Schmand, B. (2006). Neuropsychological effects of bilateral STN stimulation in Parkinson disease A controlled study. *Neurology*, *66*, 1830–1836.
- Smith, Y., Bevan, M. D., Shink, E., & Bolam, J. P. (1998). Microcircuitry of the direct and indirect pathways of the Basal Ganglia. *Neuroscience*, *86*(2), 353–387.
- Spence, S. A., Green, R. D., Wilkinson, I. D., Hunter, M. D., & Hunter, M. D. (2005). Modafinil modulates anterior cingulate function in chronic schizophrenia Modafinil modulates anterior cingulate function in chronic schizophrenia. *British Journal of Psychiatry*, *187*, 55–61.
- Stanford, I. M., Kantaria, M. A., Chahal, H. S., Loucif, K. C., & Wilson, C. L. (2005). 5-Hydroxytryptamine induced excitation and inhibition in the subthalamic nucleus: Action at 5-HT<sub>2C</sub>, 5-HT<sub>4</sub> and 5-HT<sub>1A</sub> receptors. *Neuropharmacology*, *49*, 1228–1234.
- Stanley, M., Virgilio, J., & Gershon, S. (1982). Tritiated Imipramine Binding Sites are Decreased in the Frontal Cortex of Suicides. *Science*, *216*(4552), 1337–1339.
- Supko, D. E., Uretsky, N. J., & Wallace, L. J. (1991). Activation of AMPA/kainic acid glutamate receptors in the zona incerta stimulates locomotor activity. *Brain*

- research*, 564(1), 159-163.
- Sutherland, N. S. (1959). Stimulus analyzing mechanisms. In *Proceedings of a symposium on the mechanization of thought processes* (pp. 575–609). London: HM Stationary Office.
- Sutherland, N. S., & Mackintosh, N. J. (1971). *Mechanisms of animal discrimination learning*. New York: Academic Press Inc.
- Svenningsson, P., & Le Moine, C. (2002). Dopamine D1/5 receptor stimulation induces c-fos expression in the subthalamic nucleus: possible involvement of local D5 receptors. *European Journal of Neuroscience*, 15(1), 133-142.
- Swanger, S. A., Vance, K. M., Pare, J.-F., Sotty, F., Fog, K., Smith, Y., & Traynelis, S. F. (2015). NMDA Receptors Containing the GluN2D Subunit Control Neuronal Function in the Subthalamic Nucleus. *Journal of Neuroscience*, 35(48), 15971–15983.
- Swanson, L. W., Cowan, W. M., & Jones, E. G. (1974). An autoradiographic study of the efferent connections of the ventral lateral geniculate nucleus in the albino rat and the cat. *Journal of Comparative Neurology*, 156(2), 143-163.
- Tai, C., Boraud, T., Bezdard, E., Bioulac, B., Gross, C., & Benazzouz, A. (2003). Electrophysiological and metabolic evidence that high-frequency stimulation of the subthalamic nucleus bridges neuronal activity in the subthalamic nucleus and the substantia nigra reticulata. *FASEB J*, 17(13), 1820–1830.
- Tait, D. S., & Brown, V. J. (2008). Lesions of the basal forebrain impair reversal learning but not shifting of attentional set in rats. *Behavioural Brain Research*, 187(1), 100–8.
- Tait, D. S., & Brown, V. J. (2007). Difficulty overcoming learned non-reward during reversal learning in rats with ibotenic acid lesions of orbital prefrontal cortex. *Annals of the New York Academy of Sciences*, 1121, 407–20.
- Tait, D. S., Marston, H. M., Shahid, M., & Brown, V. J. (2009). Asenapine restores cognitive flexibility in rats with medial prefrontal cortex lesions. *Psychopharmacology*, 202(1–3), 295–306.
- Tait, D. S., Phillips, J. M., Blackwell, A. D., & Brown, V. J. (2016). Effects of lesions of the subthalamic nucleus/zona incerta area and dorsomedial striatum on attentional set-shifting in the rat. *Neuroscience*, 16.
- Takayanagi, Y., Takahashi, T., Orikabe, L., Masuda, N., Yamasue, H., Kasai, K., ... Suzuki, M. (2010). Volume reduction and altered sulco-gyral pattern of the orbitofrontal cortex in first-episode schizophrenia. *Schizophrenia Research*, 121, 55–65.
- Takeshita, S., Kurisu, K., Trop, L., Arita, K., Akimitsu, T., & Verhoeff, N. P. L. (2005). Effect of subthalamic stimulation on mood state in Parkinson's disease: evaluation of previous facts and problems. *Neurosurgical review*, 28(3), 179-186.
- Tamaru, Y., Nomura, S., Mizuno, N., & Shigemoto, R. (2001). Distribution of metabotropic glutamate receptor mGluR3 in the mouse CNS: Differential location relative to pre- and postsynaptic sites. *Neuroscience*, 106(3), 481–503.
- Tan, S. K. H., Hartung, H., Sharp, T., & Temel, Y. (2011). Serotonin-dependent depression in Parkinson's disease: A role for the subthalamic nucleus? *Neuropharmacology*, 61(3), 387–399.
- Taylor, A., Saint-Cyr, J., & Lang, A. (1986). Frontal lobe dysfunction in Parkinson's disease. *Brain*, 109, 845–883.
- Temel, Y., Boothman, L. J., Blokland, A., Magill, P. J., Steinbusch, H. W., Visser-Vandewalle, V., & Sharp, T. (2007). Inhibition of 5-HT neuron activity and induction of depressive-like behavior by high-frequency stimulation of the subthalamic nucleus. *Proceedings of the National Academy of Sciences*, 104(43), 17087-17092.
- Tewari, A., Jog, R., & Jog, M. S. (2016). The Striatum and Subthalamic Nucleus as Independent and Collaborative Structures in Motor Control. *Frontiers in Systems Neuroscience*, 10(17), 1–13.

- Tonelli, L., & Chiaraviglio, E. (1995). Dopaminergic Neurons in the Zona Incerta Modulates Ingestive Behavior in Rats. *Physiology and Behavior*, 58(4), 725–729.
- Tommasi, G., Lanotte, M., Albert, U., Zibetti, M., Castelli, L., Maina, G., & Lopiano, L. (2008). Transient acute depressive state induced by subthalamic region stimulation. *Journal of the neurological sciences*, 273(1), 135–138.
- Tourev, M., Sallanon-Moulin, M., & Jouvet, M. (1995). Awakening properties of modafinil without paradoxical sleep rebound: Comparative study with amphetamine in the rat. *Neuroscience Letters*, 189(1), 43–46.
- Tsien, R. Y. (1998). The Green Fluorescent Protein. *Annual Review of Biochemistry*, 67, 509–544.
- Tunbridge, E. M., Bannerman, D. M., Sharp, T., & Harrison, P. J. (2004). Catechol-O-Methyltransferase Inhibition Improves Set-Shifting Performance and Elevates Stimulated Dopamine Release in the Rat Prefrontal Cortex. *The Journal of Neuroscience*, 24(23), 5331–5335.
- Turner, M. S., Gray, T. S., Mickiewicz, A. L., & Napier, A. T. C. (2008). Fos expression following activation of the ventral pallidum in normal rats and in a model of Parkinson's Disease: implications for limbic system and basal ganglia interactions. *Brain Structure and Function*, 213, 197–213.
- Turner, D. C., Clark, L., Dowson, J., Robbins, T. W., & Sahakian, B. J. (2004). Modafinil improves cognition and response inhibition in adult attention-deficit/hyperactivity disorder. *Biological Psychiatry*, 55(10), 1031–1040.
- Turner, D. C., Clark, L., Pomarol-Clotet, E., McKenna, P., Robbins, T. W., & Sahakian, B. J. (2004). Modafinil improves cognition and attentional set shifting in patients with chronic schizophrenia. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, 29(7), 1363–1373.
- Turner, D. C., Robbins, T. W., Clark, L., Aron, A. R., Dowson, J., & Sahakian, B. J. (2003). Cognitive enhancing effects of modafinil in healthy volunteers. *Psychopharmacology (Berl)*, 165(3), 260–269.
- Ulla, M., Thobois, S., Lemaire, J. J., Schmitt, A., Derost, P., Broussolle, E., Llorca, P.M., & Durif, F. (2006). Manic behaviour induced by deep-brain stimulation in Parkinson's disease: evidence of substantia nigra implication?. *Journal of Neurology, Neurosurgery & Psychiatry*, 77(12), 1363–1366.
- Uslaner, J. M., & Robinson, T. E. (2006). Subthalamic nucleus lesions increase impulsive action and decrease impulsive choice - mediation by enhanced incentive motivation? *European Journal of Neuroscience*, 24, 2345–2354.
- Urbain, N., & Desche, M. (2007). Motor Cortex Gates Vibrissal Responses in a Thalamocortical Projection Pathway. *Neuron*, 56, 714–725.
- Urbain, N., Gervasoni, D., Souliere, F., Lobo, L., Rentero, N., Windels, F., ... G, C. (2000). Unrelated course of subthalamic nucleus and globus pallidus neuronal activities across vigilance states in the rat. *European Journal of Neuroscience*, 12, 3361–3374.
- Urban, D. J., & Roth, B. L. (2015). DREADDs (Designer Receptors Exclusively Activated by Designer Drugs): Chemogenetic Tools with Therapeutic Utility. *Annual Review of Pharmacology and Toxicology*, 55(1), 399–417.
- Vandenberghe, L. H., Bell, P., Maguire, A. M., Cearley, C. N., Xiao, R., Calcedo, R., ... Bennett, J. (2011). Dosage thresholds for AAV2 and AAV8 photoreceptor gene therapy in monkey. *Science Translational Medicine*, 3(88).
- Van Eimeren, T., Ballanger, B., Pellecchia, G., Miyasaki, J. M., Lang, A. E., & Strafella, A. P. (2009). Dopamine agonists diminish value sensitivity of the orbitofrontal cortex: a trigger for pathological gambling in Parkinson's disease?. *Neuropsychopharmacology*, 34(13), 2758.

- Van Eimeren, T., Pellecchia, G., Cilia, R., Ballanger, B., Steeves, T. D., Houle, S., ... & Strafella, A. P. (2010). Drug-induced deactivation of inhibitory networks predicts pathological gambling in PD. *Neurology*, *75*(19), 1711-1716.
- Vigotsky, L., & Kasanin, J. (1934). Thought in schizophrenia. *Arch Neurol Psychiatry*, *31*(5), 1063–1077.
- Vila, M., Ruberg, M., Jimenez, A., Raisman-vozari, R., Agid, Y., Tolosa, E., ... Inserm, U. (1999). Systemic Administration of NMDA and AMPA Receptor Antagonists Reverses the Neurochemical Changes Induced by Nigrostriatal Denervation in Basal Ganglia, 344–352.
- Volkow, N. D., & Fowler, J. S. (2000). Addiction , a disease of compulsion and drive : Involvement of the orbitofrontal cortex. *Cerebral Cortex*, *10*, 318–325.
- Wagner, C. K., Eaton, M. J., Moore, K. E., & Lookingland, K. J. (1995). Efferent projections from the region of the medial zona incerta containing A13 dopaminergic neurons: a PHA-L anterograde tract-tracing study in the rat. *Brain research*, *677*(2), 229-237.
- Walker, M. K., Boberg, J. R., Walsh, M. T., Wolf, V., Trujillo, A., Duke, M. S., ... Felton, L. A. (2012). A less stressful alternative to oral gavage for pharmacological and toxicological studies in mice. *Toxicology and Applied Pharmacology*, *260*(1), 65–69.
- Wallace, J., Marston, H. M., McQuade, R., & Gartside, S. E. (2014). Evidence that the attentional set shifting test in rats can be applied in repeated testing paradigms. *Journal of Psychopharmacology (Oxford, England)*, *28*(7), 691–696.
- Wang, X. S., Ong, W. Y., Lee, H. K., & Haganir, R. L. (2000). A light and electron microscopic study of glutamate receptors in the monkey subthalamic nucleus. *J Neurocytol*, *29*(10), 743–54.
- Wang, C., Wang, C.-M., Clark, K. R., & Sferra, T. J. (2003). Recombinant AAV serotype 1 transduction efficiency and tropism in the murine brain. *Gene Therapy*, *10*(17), 1528–34.
- Warrington, E. K., & Shallice, T. (1969). The selective impairment of auditory verbal short-term memory, 885–896.
- Watanabe, K., & Kawana, E. (1982). The cells of origin of the incertofugal projections to the tectum, thalamus, tegmentum and spinal cord in the rat: a study using the autoradiographic and horseradish peroxidase methods. *Neuroscience*, *7*(10), 2389-2406.
- Waters, K. A., Burnham, K. E., O’connor, D., Dawson, G. R., & Dias, R. (2005). Assessment of modafinil on attentional processes in a five-choice serial reaction time test in the rat. *Journal of Psychopharmacology (Oxford, England)*, *19*, 149–158.
- Watson, C., Lind, C. R. P., & Thomas, M. G. (2014). The anatomy of the caudal zona incerta in rodents and primates. *Journal of Anatomy*, *224*, 95–107.
- Weigl, E. (1941). On the psychology of so-called processes of abstraction. *Journal of Abnormal and Social Psychology*, *36*, 3–33.
- Weiner, D. M., Meltzer, H. Y., Veinbergs, I., Donohue, E. M., Spalding, T. A., Smith, T. T., ... Brann, M. R. (2004). The role of M1 muscarinic receptor agonism of N-desmethylozapine in the unique clinical effects of clozapine. *Psychopharmacology*, *177*(1–2), 207–216.
- Wess, J., Nakajima, K., & Jain, S. (2013). Novel designer receptors to probe GPCR signaling and physiology. *Trends in Pharmacological Sciences*, *34*(7), 385–392.
- Wheeler, D. S., Wan, S., Miller, A., Angeli, N., Adileh, B., Hu, W., & Holland, P. C. (2015). Role of lateral hypothalamus in two aspects of attention in associative learning. *European Journal of Neuroscience*, *40*(2), 2359–2377.
- Wiener, M., Magaro, C. M., & Matell, M. S. (2008). Accurate timing but increased impulsivity following excitotoxic lesions of the subthalamic nucleus. *Neuroscience Letters*, *440*(2), 176–180.
- Williams, A. E., Arzola, G. M., Strutt, A. M., Simpson, R., Jankovic, J., & York, M. K.

- (2011). Cognitive outcome and reliable change indices two years following bilateral subthalamic nucleus deep brain stimulation. *Parkinsonism and Related Disorders*, 17(5), 321–327.
- Winer, B. J. (1971). *Statistical principles in experimental design*. New York: McGraw-Hill.
- Winstanley, C. A., Baunez, C., Theobald, D. E. H., & Robbins, T. W. (2005). Lesions to the subthalamic nucleus decrease impulsive choice but impair autoshaping in rats: the importance of the basal ganglia in Pavlovian conditioning and impulse control. *European Journal of Neuroscience*, 21, 3107–3116.
- Wood, E. R., Dudchenko, P. a, & Eichenbaum, H. (1999). The global record of memory in hippocampal neuronal activity. *Nature*, 397(6720), 613–6.
- Wright, I., Rabe-Hesketh, S., Woodruff, P., David, A., Murray, R. M., & Bullmore, E. T. (2000). Meta-Analysis of Regional Brain Volumes in Schizophrenia. *American Journal of Psychiatry*, 157(January), 16–25.
- Xiang, Z., Wang, L., & Kitai, S. T. (2005). Modulation of Spontaneous Firing in Rat Subthalamic Neurons by 5-HT Receptor Subtypes, 1145–1157.
- Yasumi, M., Sato, K., Shimada, S., Nishimura, M., & Tohyama, M. (1997). Regional distribution of GABA transporter 1 (GAT1) mRNA in the rat brain: comparison with glutamic acid decarboxylase67 (GAD67) mRNA localization. *Molecular brain research*, 44(2), 205-218.
- York, M. K., Dulay, M., Macias, a, Levin, H. S., Grossman, R., Simpson, R., & Jankovic, J. (2008). Cognitive declines following bilateral subthalamic nucleus deep brain stimulation for the treatment of Parkinson’s disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, 79(7), 789–795.
- Young, J., & Shapiro, M. (2009). Double Dissociation and Hierarchical Organization of Strategy Switches and Reversals in the Rat PFC. *Behavioral Neuroscience*, 123(5), 1–17.
- Zaghloul, K. A., Weidemann, C. T., Lega, B. C., Jaggi, J. L., Baltuch, G. H., & Kahana, M. J. (2012). Neuronal activity in the human subthalamic nucleus encodes decision conflict during action selection. *Journal of Neuroscience*, 32(7), 2453-2460.
- Zahr, N. M., Mayer, D., Pfefferbaum, A., & Sullivan, E. V. (2008). Low striatal glutamate levels underlie cognitive decline in the elderly: Evidence from in vivo molecular spectroscopy. *Cerebral Cortex*, 18(10), 2241–2250.
- Zénon, A., Duclos, Y., Carron, R., Witjas, T., Baunez, C., Régis, J., ... & Eusebio, A. (2016). The human subthalamic nucleus encodes the subjective value of reward and the cost of effort during decision-making. *Brain*, 139(6), 1830-1843.
- Zhu, Z. T., Shen, K. Z., & Johnson, S. W. (2002). Pharmacological identification of inward current evoked by dopamine in rat subthalamic neurons in vitro. *Neuropharmacology*, 42(6), 772-781.