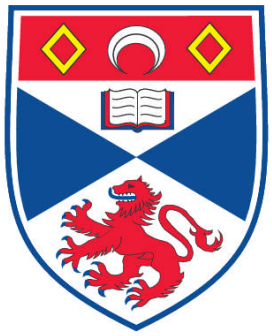
LINKING ACTIONS TO OUTCOMES: THE ROLE OF THE POSTERIOR PEDUNCULOPONTINE TEGMENTAL NUCLEUS IN INSTRUMENTAL LEARNING

Duncan A.A. MacLaren

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



2012

Linking actions to outcomes: the role of the posterior pedunculopontine tegmental nucleus in instrumental learning

Duncan AA MacLaren

A thesis submitted for the degree of PhD at the University of St Andrews

March 2012

1. Candidate's declarations:

I Duncan AA MacLaren hereby certify that this thesis, which is approximately 55,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in September 2007 and as a candidate for the degree of PhD in September 2007; the higher study for which this is a record was carried out in the University of St Andrews between 2007 and 2012.

Date signature of candidate ……..

2. Supervisor's declaration:

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of PhD in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date signature of supervisor ……..

3. Permission for electronic publication:

In submitting this thesis to the University of St Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and the abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker, that my thesis will be electronically accessible for personal or research use unless exempt by award of an embargo as requested below, and that the library has the right to migrate my thesis into new electronic forms as required to ensure continued access to the thesis. I have obtained any third-party copyright permissions that may be required in order to allow such access and migration, or have requested the appropriate embargo below.

The following is an agreed request by candidate and supervisor regarding the electronic publication of this thesis:

(i) Access to printed copy and electronic publication of thesis through the University of St Andrews.

Date signature of candidate signature of supervisor

Acknowledgements

I wish to thank Professor Philip Winn, who, despite being pretty near the top of two universities and (surprisingly) often not in the same country, managed to seemingly effortlessly achieve the pivotal balance between letting me conduct my work independently and providing supervision and advice whenever (and wherever) required. It was Prof Winn's enthusiasm while teaching during my undergraduate degree which caused me to consider doing research in a behavioural neuroscience laboratory. I would also like to thank Dr Eric Bowman for his advice over the years and for remaining my primary contact in St Andrews after I moved to Strathclyde.

My greatest thanks and acknowledgements go to Nadine Gut, David Wilson, Mary Latimer and Morag Farquhar. During the years we worked together I gained crucial insight from them in how to conduct and think about research. The completion of the work presented here is in no small part attributable to the assistance, advice and support they gave me. Moreover, we also had a lot of fun, those years remain some of my most enjoyable at St Andrews and are memories I hope I never loose.

Thanks to Claire Rostron, Stewart Clark, Marilyn Cyr, Mariza Bortolanza, Nicholas Scott, Magda Schlesiger, and, especially my family, who have all contributed over the years or offered moral support.

I would also like to acknowledge the help and support of the staff at the animal facilities at the University of St Andrews and the University of Strathclyde.

Finally, I am very grateful for the generous financial support from the Wellcome Trust and the School of Psychology, University of St Andrews, which was essential for conducting this work and enabling me to present it at conferences (and in doing so giving me the opportunity to enjoy seeing some of the world).

Abstract

Located in the mesopontine tegmentum, the pedunculopontine tegmental nucleus (PPTg) is comprised principally of glutamatergic, cholinergic and GABAergic neurons. In addition to being fully integrated into basal ganglia, PPTg projects to thalamus and motor output sites in the brainstem. Previous studies have shown a range of behavioural changes after PPTg manipulation. Prominent amongst these is an apparent deficit in the ability to learn the consequences of actions. PPTg is divisible into a posterior component (pPPTg) in receipt of rapid polymodal sensory input and projecting into VTA/SNc dopamine neurons and an anterior component (aPPTg) in receipt of basal ganglia outflow and projecting into SNc and lower brainstem structures. The research described here assesses the role of the pPPTg in instrumental learning. Using a contingency degradation paradigm, it was shown that inactivation of the pPPTg (by muscimol microinfusion) specifically blocked the updating of associations between actions and outcomes, without the affecting the ability to re-execute previously learned instrumental actions. Selective bilateral destruction of pPPTg cholinergic neurons (with the fusion toxin diphtheria toxin – urotensin II [Dtx-UII]) resulted in >90% loss of pPPTg cholinergic neurons. These lesions produced no detectable changes on any measured aspect of an instrumental learning task consisting of various fixed and variable ratio schedules of reinforcement and extinction. Subsequent experiments found that the same selective cholinergic pPPTg lesions also produced no changes in the locomotor response to nicotine or rate of nicotine sensitisation. These results are the first to demonstrate a brainstem role in action-outcome learning. Results support the view that PPTg performs a 'first pass' analysis on incoming sensory data and interfaces salient aspects of this with appropriate basal ganglia and brainstem circuitry, with glutamatergic pPPTg projections sending an essential signal and cholinergic projections performing as part of a wider modulatory system.

Table of Contents

List of tables and figures

Chapter 1 – figures

Chapter 3 - figures

Chapter 3 - tables

Chapter 4 - figures

Chapter 4 - tables

Chapter 5 - figures

Chapter 5 - tables

Chapter 6 - figures

Chapter 7 - figures

Chapter 7 - tables

List of abbreviations

6-OHDA - 6-hydroxydopamine

ACh - acetylcholine

AChR - acetylcholine receptor

ADS - antibody diluting solution

AF64A - ethylcholine mustard aziridinium ion

ANOVA - analysis of variance

A-O - action-outcome

AP - anterior-posterior

aPPTg - anterior pedunculopontine tegmental nucleus

ARAS - ascending reticular activating system

BG - basal ganglia

BNST - the bed nucleus of the stria terminalis

Cd - caudate

ChAT - choline acetyltransferase

CL – centrolateral nucleus (of the thalamus)

CNS - central nervous system

CPP - conditioned place preference

CPu - caudate-putamen

CR - conditioned response

CS - conditioned stimuli

DA - dopamine

DAB - 3,3-diaminobenzidine

DLS - dorsolateral striatum

DMS - dorsalmedial striatum

Dtx-UII - diphtheria toxin - urotensin II

DV - dorsoventral

EEG - electroencephalography

fMRI - functional magnetic resonance imaging

FR - fixed ratio

GABA - gamma-amino butyric acid

GLW - glycine leucine tryptophan

GPe - globus pallidus external segment

Gpi - globus pallidus internal segment

id - internal diameter

IAL - intra aural line

IC - inferior colliculus

ICSS - intracranial self-stimulation

ITI - inter-trial interval

IVSA - intravenous self-administration

LGN - lateral geniculate nucleus

LC - locus coeruleus

LDTg - laterodorsal tegmental nucleus

LED - light emitting diode

LH - lateral habenula

mAChR - muscarinic acetylcholine receptor

MFB - medial forebrain bundle

mLDTg - medial laterodorsal tegmental nucleus

mRF - medial reticular formation

mRNA - messenger ribonucleic acid

NAcc - nucleus accumbens

nAChR - nicotinic acetylcholine receptor

NACWO - named animal care and welfare officer

NADPHd - nicotinamide adenine dinucleotide phosphate

NbM - nucleus basalis magnocellularis

NeuN - neuronal nuclei

NMDA - N-Methyl-D-aspartic acid

NOS - nitric oxide synthase

od - outer diameter

PB - phosphate buffer

PBS - phosphate buffered saline

PD - Parkinson's disease

Pf - parafascicular nuclues (of the thalamus)

PIT - Pavlovian to instrumental transfer

PnC - pontine reticular nucleus

PnO - pontine reticular nucleus, oral part

PPI - pre-pulse inhibition

pPPTg - posterior pedunculopontine tegmental nucleus

PPTg - pedunculopontine tegmental nucleus

PR - progressive ratio

PRP - post-reinforcement pause

Put - putamen

RCL - reward collection latency

REM - rapid eye movement

RGC - retinal ganglion cells

RMTg - rostromedial tegmental nucleus

RPE - reward prediction error

RPO - reticularis pontis oralis nucleus;

RR - random ratio

Rt - reticular thalamic nucleus

RT - random time

SEM - standard error of the mean

SC - superior colliculus

SN – substantia nigra

SNc - substantia nigra pars compacta

SNr - substantia nigra pars reticulata

S-O - stimulus-outcome

S-R - stimulus-response

STN - subthalamic nucleus

SW - slow wave

TH - tyrosine hydroxylase

UII - urotensin-II

UR - unconditioned response

VR - variable ratio

VTA - ventral tegmental area

WS - widespread

Chapter 1: General introduction

General overview

The pedunculopontine tegmental nucleus (PPTg) is an evolutionary old structure in the upper brainstem. Evidence of the existence of PPTg and its closest interconnected structures is apparent in the oldest group of vertebrates (cyclostomes) which existed some 560 million years ago (Stephenson-Jones et al., 2011). Remaining well preserved through evolution, all 'modern' species studied (including human) have a PPTg with a remarkably similar structure and pattern of connections (Brantley and Bass, 1988; Medina and Reiner, 1994; Manaye et al., 1999; Wang and Morales, 2009 For reviews see: Winn, 2008; Martinez-Gonzalez et al., 2011). Early description of the PPTg appeared in the first half of the 20th century and prompted theories regarding the function of this 'old' area of brain which were largely focused on the basic mammalian functions of sleep (for review see: Steriade and McCarley, 1990) and locomotion (eg: Shik et al., 1966). In recent decades the ways in which the possible functions of brain regions are considered has changed considerably, moving from an almost phrenological approach where one brain region or system is seen as subserving a small range of functions, to approaches where the brain is considered as a highly distributed yet highly organised set of interconnected systems (for an example of early criticism of the phrenological approach see: Koob, 1982). Theories regarding the function of the PPTg have advanced in a similar manner. While still involved in, but critically not required for sleep (Deurveilher and Hennevin, 2001) and locomotion (for review see: Gut and Winn, 2011), the PPTg is now viewed as only one of many structures contributing to these complex and still not well understood behaviours. Moreover, the PPTg is no longer considered as a homogenous entity, but a heterogeneous structure containing distinct anatomical and neurochemically identifiable sub-regions that contribute to different functions (Winn, 2006; Wang and Morales, 2009;

Martinez-Gonzalez et al., 2011). Contemporary theories of PPTg function focus on involvement in learning (eg: Alderson et al., 2004), action selection (eg: Rostron et al., 2008), attention (eg: Inglis et al., 2001), sensory processing (eg: Diederich and Koch, 2005) and behavioural state control (eg: Mena-Segovia et al., 2008b). While seemingly advanced cognitive or high order processes, these are still 'old' functions of the brain: indeed the problem of action selection (selecting and executing one out of many possible actions in a given situation) is one of the most fundamental and essential problems faced by any mammalian brain (or any other for that matter). The work conducted and presented in this thesis addresses two questions directly raised by contemporary research into PPTg function: (1) what is the involvement of the posterior PPTg (pPPTg) in learning the consequences of actions? Previous research strongly suggests that the PPTg is critically involved in learning of new associations between actions and outcomes; however this has not been conclusively assessed and previous results are open to other interpretations; and (2) What is the involvement of cholinergic pPPTg systems in learning? Considerable emphasis has been placed on the functions of the cholinergic subpopulation of PPTg neurons. However, until recently there has been no method to reliably selectively manipulate this neuronal type. Using a novel fusion toxin (Dtx-UII) which can selectively destroy cholinergic PPTg neurons, the role of this neuronal subpopulation in learning is assessed. The results of these behavioural studies are interpreted in terms of considering pedunculopontine as being in a position of influence in both basal ganglia and brainstem systems. The experimental results support the view that PPTg rapidly detects and extracts salient aspects of incoming sensory information and interfaces these with the appropriate basal ganglia or brainstem systems. The role of cholinergic and non-cholinergic mesopontine tegmental projections are discussed with reference to reinforcement learning and reinforcement enhancement.

PPTg - location and structure

The PPTg is situated in the mesopontine tegmentum of the upper brainstem, beneath the level of the cuneiform nucleus, immediately in front of the parabrachial nuclei and stretching forward to the most posterior part of substantia nigra (Paxinos and Watson, 2005; Winn, 2006). The PPTg is medially adjacent to the laterodorsal tegmental nucleus (LDTg) (discussed in chapter 6) where the posterior PPTg is joined to the LDTg by sparse neurons stretching under the fibres of the superior cerebellar peduncle. Taking a very similar form in every species studied (for example rat (Honda and Semba, 1995), human (Manaye et al., 1999) and monkey (Lavoie and Parent, 1994b), for review see: Winn, 2008)) it appears to have remained well preserved across evolution. The PPTg consists of a interdigitated collection of three main neuronal populations: cholinergic (acetylcholine releasing), glutamatergic (glutamate releasing) and GABAergic (γ-aminobutyric acid releasing) (Mesulam et al., 1983; Lavoie and Parent, 1994b; Charara et al., 1996; Wang and Morales, 2009). While these three neuronal types exist in all areas of the PPTg, the distribution of each is not homogenous throughout the structure. In the posterior part glutamatergic neurons are the largest neuronal population (50%), followed by cholinergic (31%) and then GABAergic (19%) (Wang and Morales, 2009). In the anterior section GABAergic neurons are the most numerous (40% of aPPTg), followed by glutamatergic (37%) then cholinergic (23%) (Wang and Morales, 2009). Over 95% of cholinergic neurons also express nitric oxide as a neurotransmitter (Vincent and Kimura, 1992) which in turn is virtually absent in non-cholinergic PPTg neurons. In contrast, populations of non-cholinergic PPTg neurons express the calcium binding proteins calbindin and calretinin which only have very low levels of expression in cholinergic PPTg neurons (Dun et al., 1995; Fortin and Parent, 1999; Martinez-Gonzalez et al., 2012). Various neuropeptides are also present in PPTg neurons, including substance P (Vincent et al., 1986), atrial natriuretic peptide (Ryan and Gundlach, 1995) and GLW-amide-like peptides (Hamaguchi-Hamada et al.,

2009) with no known pattern of location or co-expression with neurotransmitters. PPTg neurons have been identified as having receptors for: acetylcholine (ACh) (Azam et al., 2003), glutamate (Inglis and Semba, 1996), GABA (Saitoh et al., 2003), glycine (Fort et al., 1993), noradrenaline (Williams and Reiner, 1993) and urotensin II (Clark et al., 2001). There is no clearly defined pattern of differential expression of receptors across neuronal types, the (so far) only known exception to this being the urotensin II receptor, which is exclusively found on cholinergic neurons (Clark et al., 2001). There is some debate over the possibility of single PPTg neurons co-expressing combinations of acetylcholine, glutamate and γ-aminobutyric acid (GABA). Older studies claim that up to 50% of cat PPTg cholinergic neurons are able to co-express GABA (Jia et al., 2003) and there is evidence that co-expression may occur in the monkey brain (Lavoie and Parent, 1994a, b; Charara et al., 1996). However, these studies use immunohistochemistry or histochemistry as a marker of transmitter expression. While very reliable immunohistochemical markers of cholinergic PPTg neurons exist, immunohistochemically marking PPTg glutamate or γ-aminobutyric acid expressing neurons is notoriously difficult. Due to the rapid rate of synthesis and release of transmitter, dependant on the antibody used, immunohistochemical markers are highly susceptible to both false positive and false negative results (see chapter 10 of: Porter (2007) for full discussion, also see: Wang and Morales, 2009). More recent work using in-situ hybridization (which does have reliable markers of the three main PPTg neuronal types) found that only 0.7% of rat cholinergic PPTg neurons were able to co-express GABA and 1.2% glutamate (Wang and Morales, 2009). Further investigation into the possibility that neurons only co-express under certain conditions will hopefully resolve these issues, but the interim conclusion is that co-expression, if it does occur, is at a very low rate. While small numbers of other types of neuron have been found in some species, for example tyrosine hydroxylase (therefore either dopaminergic or noraderenergic) in guinea pig (Leonard et al., 1995), this has not been assessed in all species

(Winn, 2006) and even when found comprises only a very small proportion of the entire structure. As a result the experimental work on the PPTg focuses almost exclusively on the cholinergic, GABAergic and glutamatergic neurons.

PPTg - connections

The PPTg is highly interconnected with other brain regions. Connections can largely be grouped into the following categories: (1) to other structures in the mesopontine tegmentum, midbrain, brainstem and spinal cord; (2) to basal ganglia structures and the VTA; (3) to thalamus and (4) to prefrontal and cortical areas.

(1) PPTg sends a mixture of cholinergic and non-cholinergic projections to the pontine reticular formation (Semba et al., 1990; Takakusaki et al., 1996; Rolland et al., 2011), areas of the medulla (medioventral medulla (Skinner et al., 1990b), medulla oblongata (Nakamura et al., 1989) and rostral ventro-lateral medulla (Yasui et al., 1990)) and the motor trigeminal (Fay and Norgren, 1997c, b, a). Projections also target the spinal cord itself (Skinner et al., 1990a) but these appear to be predominantly non-cholinergic (for review see: Martinez-Gonzalez et al., 2011). PPTg sends cholinergic (and an un-quantified smaller quantity of non-cholinergic) innervation to the inferior (Motts and Schofield, 2009) and superior (Hall et al., 1989) colliculus. Interestingly, cholinergic projections to the inferior colliculus (IC) arise predominantly from the pPPTg, whereas non-cholinergic projections arise from the area around the aPPTg (Beninato and Spencer, 1986). Projections from the superior colliculus (SC) to the PPTg and surrounding area have also been documented (Redgrave et al., 1987). The direction of information flow is unclear here (and may be bidirectional): it has been hypothesised that SC outflow to PPTg forms a functional part of pre-pulse inhibition pathways

(Bosch and Schmid, 2008) (PPI is discussed in more detail in subsequent sections; pp18-19). However, in a clever double-tracer triple-stain analysis, Schofield and colleagues revealed that neurons in auditory cortex project directly onto the same PPTg cholinergic neurons which then innervate the IC, showing that the PPTg is also an auditory input to IC (Schofield, 2010). PPTg (particularly aPPTg) is in receipt of output from and sends input to the rostromedial tegmental nucleus (RMTg) (Jhou et al., 2009b). Finally, PPTg is in reciprocal connection with contralateral PPTg and unilateral and contralateral LDTg, bed nucleus of the stria terminalis (BNST), central grey and receives innervation from the locus coeruleus (LC) (Semba and Fibiger, 1992).

(2) The degree and nature of interconnection between the PPTg and basal ganglia (BG) has led to the proposition that it should be considered part of the BG family (Mena-Segovia et al., 2004a). The PPTg is directly reciprocally connected with the STN, GPe, SNr and projects to both the SNc and VTA; through indirect connections, PPTg can influence the activity of GPe (via STN) and, importantly, all areas of the striatum (via SNc, VTA and the STN - GPe pathways) (Moriizumi and Hattori, 1992; Semba and Fibiger, 1992; Lavoie and Parent, 1994b, a). PPTg receives direct connections from SNr (predominantly GABAergic) and GPi which in turn can direct striatal outflow towards PPTg (see fig 1.1 for connection diagram) (Moriizumi and Hattori, 1992; Semba and Fibiger, 1992; Winn et al., 2005; Rolland et al., 2011 ; for review see: Mena-Segovia et al., 2004a). The projections to the SNc and VTA form a topographical gradient where SNc is principally targeted by the aPPTg and the VTA by pPPTg and neighbouring LDTg (Oakman et al., 1995b); For review see: Maskos, 2008). Arising from both cholinergic and glutamatergic PPTg neurons (Parent et al., 1999), recent evidence suggests these projections may be predominantly glutamatergic (Wang et al., 2010). Likewise,

connections to the STN have been shown to be principally non-cholinergic (90%) in nature and arising mainly from rostrodorsomedial and caudoventral areas of PPTg (Kita and Kita, 2011). What is important to consider is the degree of functional interdependence between BG structures and PPTg: PPTg is not simply a relay station passing information into and receiving output from BG. Rather, its feed forward and feedback circuits place it in a position to influence activity throughout the BG network (Mena-Segovia et al., 2004a). Moreover, different regions of PPTg are positioned to have differential effects on BG and striatum sub-components, with aPPTg influencing SNc and subsequent dorsal striatal processing whereas pPPTg output is also directed to the VTA and the ventral striatal stream; see fig 1.1 and further sections (pp40-46) for full discussion of the functions of the BG.

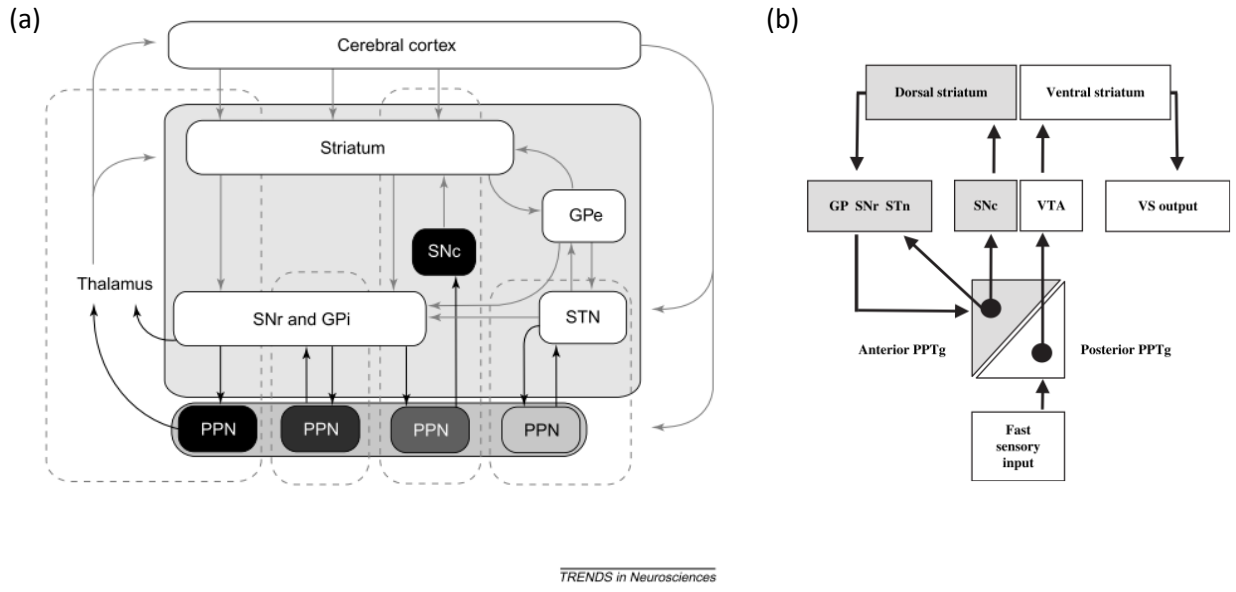


Figure 1.1: Schematic of the connections between PPTg and BG: (a) shows interconnections between PPTg and other basal ganglia nuclei (b) shows the preferential connection and subsequent projection pathways of anterior and posterior PPTg. (a) redrawn from: Mena-Segovia et al., 2004 and (b) from: Winn, (2008).

(3) Virtually all of the thalamus receives cholinergic innervation from the PPTg, LDTg and nearby parabigeminal nucleus, with some nuclei receiving over half of their cholinergic innervation from the PPTg (the centrolateral nucleus receives 78% of its cholinergic innervation from PPTg and ventrolateral nucleus 62%) (Hallanger et al., 1987; Hallanger and Wainer, 1988; Heckers et al., 1992; Kolmac and Mitrofanis, 1998; Capozzo et al., 2003; Holmstrand and Sesack, 2011). It has been hypothesised (and seems logical) that these extensive brainstem-thalamic projections form an organised topographical system rather than *en masse* non-specific innervation. However, the exact nature of such a system remains undefined – the studies referenced above have not uncovered a systematic underlying pattern in the projections. In functional terms, unilateral stimulation of PPTg ipsilaterally activated thalamic centrolateral and ventrolateral nuclei and caused bilateral activation of the reticular nucleus (Ainge et al., 2004). In contrast to this, lesions of the PPTg had no effect on Ach levels in reticular nucleus, but caused a decrease of Ach in anteroventral nucleus and time dependant increase in mediodorsal nucleus (Jenkins et al., 2002). These results have led to the conclusion that PPTg-thalamic projections form a "dynamic balance" (Jenkins et al., 2002) of influence in thalamic activity rather than simple innervation. It should not be forgotten that while much emphasis is on the cholinergic projections to thalamus, there is also a considerable non-cholinergic component to the projections (Ye et al., 2010). Despite the uncertainty of the projection pattern, it is clear that PPTg sends substantial cholinergic innervation to the thalamus, which in turn can influence thalamic output to the striatum and neocortex and control the level of thalamic burst firing.

(4) Much of the literature on the PPTg and cortical innervation is concerned with the relationship PPTg cholinergic neurons have with cortical EEG activity and their possible role in changing between behavioural states (waking state, slow wave sleep states and REM sleep state). Cholinergic PPTg neurons form part of the ascending reticular activating system (ARAS) (Moruzzi and Magoun, 1949; Steven R, 2000; Mena-Segovia et al., 2008b) which is believed to control levels of cortical activation and the shift between behavioural states (for discussion of the history of and one current view of ARAS see: Fuller et al., 2007). Cholinergic PPTg neurons do indeed change their firing properties depending on behavioural state (Mena-Segovia et al., 2008b) as do sub-populations of non-cholinergic PPTg neurons (Ros et al., 2010). However, the relationship is not as simple as cholinergic activity driving cortical activity during wakefulness and REM sleep, then being at a low level during slow wave sleep (as might be predicted by a standard ARAS model). Instead, during periods of experimentally induced slow wave sleep, PPTg neurons showed dynamic firing patterns: 80% of cholinergic neurons fired at around 1 Hz during the 'active' component of slow wave sleep (the upstate of cortical slow oscillations) with the remaining 20% showing the opposite pattern - firing only during the 'inactive' down state and at a significantly higher (30 Hz) firing rate. Putative glutamatergic neurons fired reliably during the transition from upstate to downstate (Mena-Segovia and Bolam, 2011). A similar time locked firing pattern at the transition from down to up state during the SW phase of naturally sleeping rats has been observed in the LC (Eschenko et al., 2011). These systematic firing patterns during the SW state have been hypothesised to have a role in facilitating or gating communication between different neuronal systems during now recognised 'waking like microstates' present in the SW state (Eschenko et al., 2011; Mena-Segovia and Bolam, 2011). Excitotoxic lesions of the PPTg do not alter

spontaneously generated REM sleep (Deurveilher and Hennevin, 2001) but interestingly do change the response to rebound sleep after periods of sleep deprivation. Together, these results show that PPTg activity is related to cortical EEG activity in a manner dependant on behavioural state (and sub-components of behavioural state), but that PPTg itself is neither critical for the shift between behavioural states nor maintenance of normal cortical EEG. Instead, PPTg appears to be one of many components of an extended system active during both the change in behavioural state and the processes which occur within individual states. The exact nature of this involvement remains to be defined.

Reports of direct connections between PPTg and cortical areas exist but are sparse: PPTg projects to medial and sulcal frontal cortical areas (Saper and Loewy, 1982) and sends cholinergic and non-cholinergic innervation to the nucleus basalis magnocellularis (NbM) (which in turn sends considerable cholinergic innervation to cortex) (Semba et al., 1988; Losier and Semba, 1993). There is evidence from anesthetized rats that the main functional component of this projection may be non-cholinergic (Rasmusson et al., 1994). Both auditory (Rolland et al., 2011) and motor (Matsumura et al., 2000) cortex project to PPTg. Non-cholinergic projections from pPPTg target visual cortex in the cat (Higo et al., 1996) but there is only circumstantial evidence that visual cortex projects to the PPTg (for discussion of the relatively old tracing studies this conclusion is based on, see: Higo et al., 1996). Reciprocal connections exist between PPTg and lateral hypothalamus (LH) (Semba and Fibiger, 1992) and extended amygdala circuitry projects to PPTg (Zahm et al., 2001).

In the pattern of connections reviewed above, there is no systematic difference between cholinergic and non-cholinergic projections. Indeed, very few connections seem to be entirely composed of, or entirely devoid of, a cholinergic component. However, one striking difference between cholinergic and non-cholinergic projections has recently been revealed by the excellent juxtacellular labelling and tracing studies of Mena-Segovia and colleagues. This technique enables the visual reconstruction of dendritic and axonal pathways of individual neurons. Cholinergic axonal projections were found to split into 4 - 6 collaterals, some of which diverged down lower into the brainstem while the remainder travelled upwards to basal ganglia, thalamic and/or other areas. Individual cholinergic neurons, therefore, formed projections to multiple target structures. In sharp contrast to this, non-cholinergic neurons were found to have far simpler projection patterns, typically consisting of one or two branches travelling in the same direction - none of these neurons formed nearly as complex a projection pattern as the cholinergic neurons (Mena-Segovia et al., 2008b). See figure 1.2). While only a small sample of neurons have been identified by this method (10 cholinergic and 6 non-cholinergic), and it has been known for some time that PPTg neurons send collateral projections (Semba et al., 1990; Losier and Semba, 1993; Motts and Schofield, 2009), this may give insight into a fundamental difference between cholinergic and non-cholinergic projections.

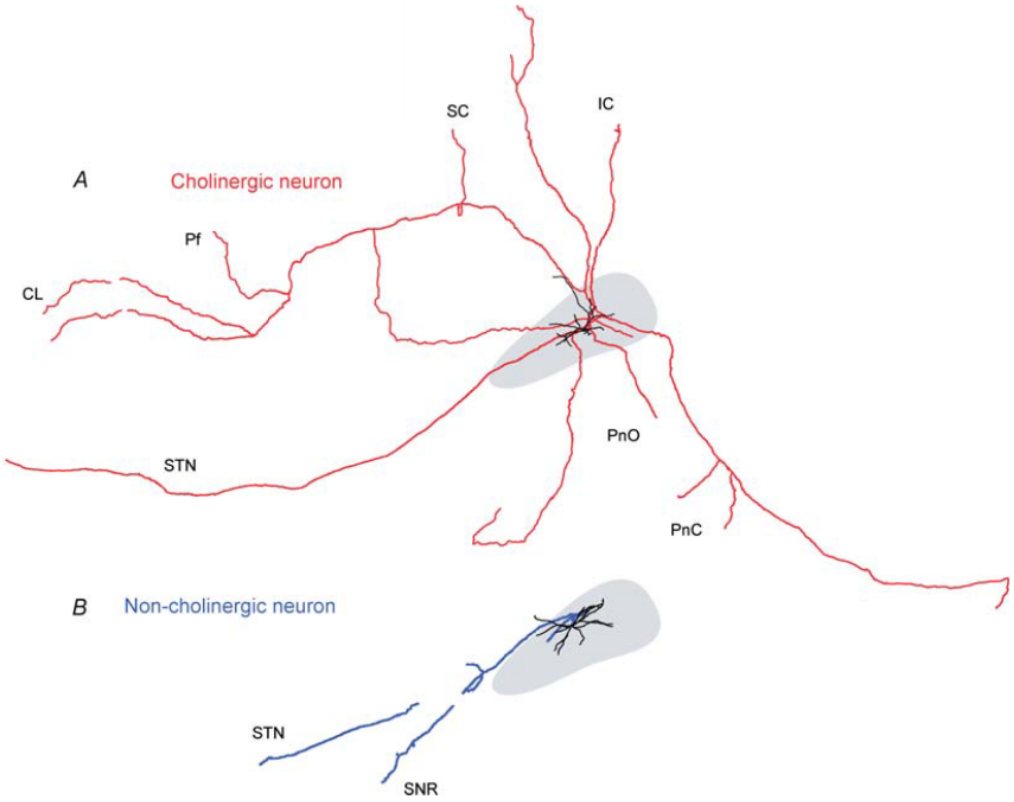


Figure 1.2: Illustration of the connectivity patterns of single cholinergic (red) and non-cholinergic (blue) PPTg neurons. Figure shows reconstructions of the dendrites in black and axons in colour and indicates the structures targeted by each neuron. Image reproduced from Mena-Segovia et al., (2008b).

PPTg - nomenclature

While nomenclature may seem a trivial point, a recent neurosurgeons report debating whether the terms pedunculopontine nucleus and peripeduncular nucleus (a nearby but unrelated structure) had been erroneously confused (Zrinzo and Zrinzo, 2008) highlights the importance of precision in terminology. There is no standardised name for the PPTg. This laboratory has always used the name *pedunculopontine tegmental nucleus* (abbreviated to PPTg) which is one of the most common names given for it in the literature. It is also the case that until recent editions it was the name given in the popular brain atlases of Paxinos and colleagues (eg human (Paxinos and Huang, 1995), rat (Paxinos and Watson, 2005) and mouse (Paxinos and Franklin, 2003)). However, in the 6th edition of *The Rat Brain* (Paxinos and

Watson, 2007) the structure became *pedunculopontine tegmental* (no 'nucleus') abbreviated to PTg (a rather surprising change given that PTg is not found in the literature prior to this and nearly all other names used include the term nucleus). Another routinely used term is *pedunculopontine nucleus* (abbreviated to PPN) and within the human literature there is a tendency to use this over PPTg. Other variations of the same abbreviations (for example: PPT; PPTG; PPTN; PptgN) as well as NTPP (nucleus tegmenti pedunculopontinus) and TPP (tegmental pedunculopontine nucleus) also appear with differing degrees of frequency. The important point here is that names for the PPTg are interchangeable and refer to the same structure. In other brain regions variations of similar terms represent differences in underlying anatomy. For example, primates have a separable caudate (Cd) and putamen (Put) whereas in the rat these two structures appear as one conjoined complex which is accordingly termed caudate-putamen (CPu). The different terms used for the whole PPTg are not reflective or indicative of any anatomical or species differences. To describe sub-regions within PPTg, again there is no standardised terminology. The cholinergic PPTg neurons are denoted as Ch5 in the classification of Mesulam et al. (Mesulam et al., 1983) and this terminology is still evident in the literature (with cholinergic neurons of the neighbouring LDTg being Ch6). However, perhaps partly due to the increased discussion about non-cholinergic PPTg neurons, it is becoming more common to simply name neurons by their transmitter type. To refer to the distinction between anterior and posterior PPTg components, the most frequently used terms are anterior (aPPTg) and posterior (pPPTg) or caudal and rostral PPTg. Based on the distribution of the cholinergic neurons the terms pars dissipates (PPTg-pd) and pars compacta (PPTg-pc) have also been used to describe the anterior and posterior PPTg. However, again in combination with the increasing discussion of non-cholinergic PPTg components (which do not have the same distribution) this terminology is now less frequently used.

Functions of the PPTg

Methods of assessing PPTg function

Extensive experimental work investigating the functions of the PPTg has been performed on a wide range of animal species and humans. Rodents and non-human primates are the subjects most frequently used in experimental laboratories. Due to the conserved nature of the PPTg, studies in animals carry validity across species meaning evolutionarily 'lower' species can be used without compromising the integrity of the experimental results. The rat is thus an excellent choice of experimental subject because it is large enough for precise experimental manipulation and behavioural assessment while still being small enough for easy housing and care. Non-human primate species are more preferable for some behavioural experiments, for example electrophysiological studies involving complex visual tasks which could not be performed by rats. However, they are less suited for use in lesion studies: the need for precisely sized lesions and group sizes large enough for rigorous statistical analysis makes it an unpractical choice; moreover, it raises ethical concerns about the number of subjects required. Studies in human (generally post-mortem anatomical, fMRI and more recently using deep brain stimulation electrodes implanted for Parkinson's Disease treatment) comprise only a small proportion of the PPTg literature. Located deep in the brainstem and with no clear anatomical boundaries, precisely locating the PPTg with fMRI or other imaging techniques is challenging. Nonetheless, as advances in both imaging techniques and brain atlases continue, it can be expected that there will be improvement in the accuracy of imaging in the future.

The most common laboratory methods of studying the functions of the PPTg are: (1) lesion studies (either permanent or transient lesions). Fibre sparing lesions of the PPTg (or restricted to aPPTg and pPPTg (eg: Wilson et al., 2009a) can be made with various excitotoxins

including ibotenic acid, kainic acid, quinolinic acid and NMDA, with the most frequently used being ibotenic acid. Excitotoxic lesions can be considered as 'non-selective' in that while they spare fibres of passage, these agents have no specificity for one neuronal type within PPTg (while there is some evidence that, for example, quinolinate may be able to preferentially damage cholinergic neurons, it remains the case that some level of damage to all neuronal population is expected after excitotoxic lesion (Rugg et al., 1992)). In terms of lesions selective for one neuronal population, reports that the ethylcholine mustard aziridinium ion (AF64A) selectively destroyed PPTg cholinergic neurons looked like a promising emerging lesion technique (Sandberg et al., 1985; Lanca et al., 2000b). However, authors have reported (and experience in this laboratory has confirmed) that it actually produces non-selective and even physical damage at the site of infusion (Rodriguez et al., 1998). Recently, a novel fusion toxin Dtx-UII has been developed which is highly selective for PPTg cholinergic neurons (Clark et al., 2007). This toxin is diphtheria toxin in which the natural targeting domain has been changed to urotensin II. Taking advantage of the discovery that in the mesopontine tegmentum only the cholinergic neurons express urotensin II receptors (Clark et al., 2001), it selectively binds to, then (after internalisation and inducing protein synthesis inhibition) destroys cholinergic neurons. Older studies often used electrolytic lesions which in addition to being non-selective for neuronal type also damage fibres of passage; however their usage has generally faded. Temporary inactivation of the PPTg can be achieved by direct microinfusion of the GABA agonist muscimol (eg: Corrigall et al., 2001) or local anaesthetics such as lidocaine (eg: Conde et al., 1998). These are believed to be non-selective for neuronal population and lidocaine appears to also inactivate fibres of passage (see chapter 3, pp68-69 for full discussion of inactivation techniques). (2) histological based analysis. A wealth of knowledge has been gained from anatomical assessment of the structure (eg: Wang and Morales, 2009) and tracing studies assessing the connections (eg: Semba and Fibiger, 1992) of the PPTg. Tracing

studies are especially powerful when combined with markers selective for the neuronal population from which the projection arises or targets (eg: Kita and Kita, 2011). It is also possible to measure changes in recent neuronal activity by the histological assessment of post–mortem tissue. Detection of evidence of immediate early gene expression (eg *c-fos*) is a reliable measure of recent neuronal activation (this approach is also discussed in chapter 6, pp171-174). While the technique is somewhat limited (for example having to be performed in post-mortem tissue means it is only suitable for some experimental designs) it has the powerful capability of not only indicating neuronal activity, but through double staining, being able to indicate activity levels in different neuronal populations (eg: Lanca et al., 2000a). (3) Electrophysiological recording from single neurons. The activity of single and multiple PPTg neurons can be recorded in anesthetized (eg: Mena-Segovia et al., 2008b) and awake behaving animals performing cognitive tasks (eg: Norton et al., 2011). This is a powerful technique that enables the assessment of neuronal responses to environmental changes and/or performance during behavioural tasks. While there is no current conclusive way to identify easily from which kind of neuron (cholinergic, glutamatergic or GABAergic) is being recorded from during the recording session, it is hoped that as knowledge of the different neuronal populations develops it will become easier to identify them by their neurophysiological characteristics. Indeed, there has already been some progress in this regard (Zhang et al., 2008). (4) Direct stimulation. The PPTg can be stimulated pharmacologically (for example with the Ach agonist carbachol (eg: Kinney et al., 1998) or the glutamate uptake inhibitor l-trans-pyrrolidine-2,4-dicarboxylic acid (Ainge et al., 2004)) and with current delivered from an electrode (eg: Lokwan et al., 1999). Pharmacological stimulation offers a means of restricting the effects to those of one receptor type, however electrical stimulation offers considerably more flexibility in terms of precise timing and duration of the stimulation.

PPTg function

PPTg and basic behaviours

Rats bearing fibre sparing bilateral excitotoxic PPTg lesions show no changes in basic behaviours: consumption of homecage food and drinking water is unaffected by lesion (Inglis et al., 1994), as are rates of spontaneous generated locomotion (Inglis et al., 1994; Olmstead and Franklin, 1994; Wilson et al., 2009a) and (as discussed earlier) natural sleep (Deurveilher and Hennevin, 2001). Likewise, in an open field, lesions of the PPTg did not change locomotor or exploratory behaviour (spontaneous horizontal motor activity, rearing and centre activity) in either the first test or after repeated exposure to the testing environment (Steiniger and Kretschmer, 2004). There was some confusion over the relationship between PPTg and anxiety: studies using electrolytic and large NMDA lesions of the PPTg reported increases in anxiety-like behaviour (elevated plus maze and social interaction test) (Podhorna and Franklin, 1999; Podhorna and Franklin, 2000) and even the restoration of normal behaviour after treatment with the anxiolytic drug diazepam (Leri and Franklin, 1998). However, this was not consistent with other reports of electrolytic PPTg lesions producing no change in anxiety-like behaviour on the elevated plus maze (Homs-Ormo et al., 2003). To specifically investigate this discrepancy, Susannah Walker and colleagues investigated the effects of ibotenic acid PPTg lesions (which destroyed the PPTg but did not consistently extend into other structures), NMDA lesions of the PPTg (which frequently encroached on the cuneiform nucleus, which is immediately ventral to the PPTg, known to be involved in defensive and anxiety related behaviours and furthermore is activated by exposure to the elevated plus maze (Silveira et al., 1993)) and ibotenic acid lesions of cuneiform nucleus itself. The results were clear: lesions restricted to the PPTg produced no indication of increases in anxiety related behaviour, whereas both lesions of the cuneiform, and PPTg lesions which extended into the cuneiform,

did produce an increase in anxiety related behaviours (Walker and Winn, 2007). PPTg lesioned rats also show normal taste perception and contrast effects (response to changes in value of freely offered rewards) (Olmstead et al., 1999).

What these results show is that bilateral lesions of the PPTg do not affect normal homecage behaviour or cause an overt behavioural change; this is supported by the anecdotal evidence that, solely through observation of rats in their cages, it is not possible reliably to distinguish between PPTg lesioned and sham lesioned control rats (or rats that have had no surgery at all).

PPTg function – simple and complex behavioural tasks

Despite their seemingly normal homecage behaviour, PPTg lesioned rats show a clear and enduring deficit in behavioural tasks designed to assess cognitive functioning and responses to environmental stimuli. The specific deficits observed will be described here and then (starting on p28) interpreted with reference to the broader literature.

PPTg and startle response

Loud and unexpected sounds induce a characteristic startle response known as the acoustic startle response (in the rat this is a freezing and twitch of the facial, neck and limb muscles). If this startle inducing sound is preceded by a quieter pulse of sound or other stimuli (of insufficient intensity to induce a startle response) this quieter pulse will reduce or abolish the normal startle response to a high intensity sound delivered shortly (20 – ~1000 ms) afterwards (for review see: Koch and Schnitzler, 1997; Fendt et al., 2001). This phenomenon, known as pre-pulse inhibition (PPI) of the startle response is considered to be an indicator of normal sensorimotor gating and thought not to be the result of conditioning or learning (Wu et

al., 1984). PPI has attracted much interest in recent years due to being abnormal in both schizophrenic patients (Turetsky et al., 2007) and many animal models of schizophrenia (Marcotte et al., 2001). Bilateral electrolytic (Swerdlow and Geyer, 1993a), excitotoxic (Koch et al., 1993) and muscimol induced transient lesions (Diederich and Koch, 2005) of the PPTg reduced PPI of the acoustic startle reflex. This is indicative of impaired sensorimotor gating and is believed to the result of loss of cholinergic PPTg innervation of the caudal pontine reticular nucleus (PnC), a critical component of the primary startle pathway (Koch et al., 1993; Bosch and Schmid, 2008). It should be noted that of the three lesion techniques used in the studies described, none of them have selectivity for cholinergic PPTg neurons and so the interpretation of results with regard to cholinergic PPTg neurons cannot be conclusive. However, there is *in vitro* evidence that the functional link between PPTg and PnC is cholinergic (Bosch and Schmid, 2008) and further support for this being a cholinergic mediated behaviour comes from finding showing that PPI was transiently improved in schizophrenic patients after nicotine administration (Hong et al., 2008). This is an area where Dtx-UII selective cholinergic lesions show promise for significantly advancing understanding of the functions of the different PPTg neuronal subpopulations and related circuitry.

PPTg and the radial maze

On the 8 arm radial maze PPTg lesioned rats performed both the random foraging (all arms open and 4 baited with food) and the delayed spatial win shift task (comprised of an initial exposure to the maze with 4 baited arms open, then, after a brief delay, re-exposure to the maze with all arms open but only the previously closed ones baited) significantly worse than sham controls. Indeed, the performance of PPTg lesioned rats remained at around chance level (Keating and Winn, 2002). A subsequent experiment used high value and low value rewards as bait on the maze. While the performance of PPTg lesioned rats in the high value

reward group was better than performance in the low value reward group, it still failed to reach the level of controls (Taylor et al., 2004). This improved performance with high value rewards confirms lesioned rats were motivated to perform the task. That they were still unable to solve it without frequent errors suggests that the defects were in the integration of information (spatial information, memory of visited arms, sequencing of which arm to visit), learning which arms are baited in each individual trial (baited locations changed daily) or in actually selecting which arm to enter.

Learning and performance of operant tasks

During simple low fixed and low variable ratio schedules of reinforcement (that is, when pressing on one lever reliably leads to reward delivery in a predicable manner, typically after 1-5 presses) disruption in lever pressing has been found after PPTg lesion for the following rewards: amphetamine (Alderson et al., 2004), heroin (Olmstead et al., 1998), nicotine (Lanca et al., 2000b; Corrigall et al., 2001; Alderson et al., 2006), ethanol (Samson and Chappell, 2001) and food pellets (Alderson et al., 2004; Diederich and Koch, 2005; Wilson et al., 2009a). While these studies utilized various different testing and training protocols and offered many explanations of the data, a common theme in the results appears to be that PPTg lesioned rats showed abnormal behaviour when adapting to new testing conditions in a manner that can be interpreted as a learning impairment. If the rats have learned that lever pressing is rewarded prior to surgery then they will continue to lever press at normal rates after PPTg lesion (Olmstead et al., 1998; Samson and Chappell, 2001; Alderson et al., 2004). However, if they have no experience of operant tasks prior to surgery (Olmstead et al., 1998; Alderson et al., 2004; Wilson et al., 2009a) or the task changes at some point after surgery (Olmstead et al., 1998; Alderson et al., 2004; Diederich and Koch, 2005; Wilson et al., 2009a) then lesioned rats fail to acquire normal rates of lever pressing. Alderson and colleagues

succinctly show both effects in one experiment: they had two groups of rats, a pre-trained group (who learned that lever pressing led to food reward prior to surgery) and a naïve group (no pre-training). The rats who had learned that lever pressing was rewarded on a fixed ratio (FR) 1 then FR2 schedule of reinforcement prior to lesion had normal rates of lever pressing for both food (1 post surgery session) then amphetamine (8 sessions) reward after surgery. However, when the reinforcement schedule was subsequently changed to progressive ratio (PR) 5 (where the number of correct lever presses required for reward delivery increases by 5 after each reward) both the pre-trained and naïve lesion groups had significantly lower rates of lever pressing than shams (Alderson et al., 2004). These strongly suggest that the underlying deficit in PPTg lesioned rats was an inability to form or update an association between action (lever press) and outcome (reward). There are two exceptions to this learning impairment hypothesis: (1) Samson and colleagues (2001) reported that in rats trained to lever press for ethanol reward, subsequent muscimol induced inactivation of the PPTg caused reduced rates of lever pressing. However, an examination of the results reveals that of the three doses of muscimol used, only the highest (0.15 μg muscimol per hemisphere) caused a reduction in lever pressing. This highest dose corresponds to a volume of muscimol that is three times that of the standard one used in this laboratory (0.05 μg; see chapter 3 p68). Moreover, it is higher than a dose which our unpublished observations have shown causes signs of disorientation and motor impairment (Wilson and MacLaren; unpublished pilot study, (2009)). It is therefore possible (and acknowledged by the authors themselves) that this high dose may have spread to other nearby structures and contributed to the behavioural changes observed (Samson and Chappell, 2001). Of their remaining two doses, one is the same as our standard dose and one is lower, in the ethanol experiment neither of these produced changes in the performance of the previously learned lever pressing task. (2) It is reported that in trained rats lesion and inactivation of the PPTg reduces rates of lever pressing for nicotine (but not cocaine in an

identical paradigm) under an FR5 schedule of reinforcement (Lanca et al., 2000b; Corrigall et al., 2001). It has also been reported that, in rats trained prior to surgery to lever press for food reward, responding for nicotine is *enhanced* after pPPTg lesion and unaffected by aPPTg lesion (Alderson et al., 2006). Initially these results seem hard to reconcile. One explanation could be in the anatomical differences between lesion location: the response enhancing lesions were restricted to the pPPTg (which has strong projections to the VTA, a structure of considerable interest in relation to the reinforcing properties of nicotine) and were not seen after aPPTg lesions (which does not project to VTA but instead targets the medially adjacent SNc) whereas the response reducing lesions and inactivations were targeting the whole PPTg. However, without further support this does not seem a satisfactory explanation for such contrasting effects. There were also differences in training and testing protocols: the Alderson et al. study had a programmed conditioned stimuli (CS) concurrent with nicotine delivery and this alone appears to have been sufficient to drive lever pressing (when switched to saline rather than nicotine all groups maintained higher levels of pressing on the active than inactive lever) whereas there was no programmed CS in studies which found reduced responding to nicotine. Given that nicotine in the VTA has been shown to enhance the rewarding effects of conditioned stimuli without being a primary reinforcer by itself (Farquhar et al., 2011), it is possible the different effects are a product of the presence or absence of a CS in combination with disrupted signalling in the VTA caused by loss of innervation from the PPTg. This is clearly a tentative hypothesis that requires further investigation. However, regardless of the explanation, these diverging effects are highly specific to nicotine and are not evident in lever pressing for other reinforces (explicitly shown by Corrigall et al. (2001) who, using identical paradigms, showed that in trained rats, rates of lever pressing for cocaine were not affected by PPTg inactivation, but rates of lever pressing for nicotine were).

In other studies using progressive ratio schedules of reinforcement (PR), it has been shown that PPTg lesioned rats have reduced breaking points (the ratio at which the rat is deemed to have stopped lever pressing) and reduced rates of lever pressing as the ratio of the schedule increases (Olmstead et al., 1998; Alderson et al., 2002; Alderson et al., 2004; Diederich and Koch, 2005). While a lower breaking point is often used as an indication of reduced motivation, several factors suggest that this is not the case in PPTg lesioned rats: (1) as schedule demands increased, lesioned rats made significantly more approaches to the food hopper (if they were less motivated no increase in would be predicted) (Alderson et al., 2002); (2) as the progressive ratio increased, lesioned rats developed significantly increased rates of pressing on the inactive lever, a behaviour they did not show at low ratios on the PR schedule (again, reduced motivation would not explain this increase in lever pressing) (Alderson et al., 2002); (3) lesioned rats had the same latency to collect reward as controls, suggesting that once earned, both groups had equal levels of motivation to collect the reward (Alderson et al., 2002); (4) a closer examination of the pressing rates on the active lever revealed that while lesioned rats had reduced rates of pressing during active parts of the task (leading to the lower breaking point) they also pressed more during inactive parts of the task (where pressing had no consequence) leading to the overall rate of lever pressing during the testing session not being significantly different to shams (Diederich and Koch, 2005). Combining these factors, rather than being a reduction in motivation (which would not explain the increased pressing on the inactive lever, increased approaches to the hopper and high levels of pressing during inactive parts of the tasks) a more fitting explanation is that PPTg lesioned rats were unable to appropriately adjust behaviour in response to the ever changing task demands of the PR schedule. This could either be through a failure to learn the ways in which the task was changing, or (perhaps and) a failure to select the appropriate action in response to the ever increasing nature of the reinforcement schedule.

The results of these studies show a clear pattern – if the rats are trained to lever press before PPTg lesion then they can subsequently re-perform the same task at the same rate as control rats. However, if there is no pre-training, or when task demands change, PPTg lesion reliably causes persistent impairment in task performance. This is highly suggestive of a deficit in the forming and updating of associations between actions (lever pressing) and outcomes (reward delivery) but not in performance of previously learned action-outcome associations.

Behavioural changes after PPTg lesion also appear in operant tasks designed to measure attention. In a task where rats were required to attend to and then rapidly respond to a light presented at unpredictable time intervals, PPTg lesioned showed increased numbers of omissions (trials where they failed to respond to the light cue) and increased rates of responding during the dark period (where responding had no consequence) (Kozak et al., 2005). Increasing the time period in which rats could respond to the light significantly improved the performance of lesioned rats. One standard interpretation of this would be that improved performance in sessions with a longer response time window is a reflection of impaired attentional processing – the rats were given longer to notice the light and this compensated for impaired attention. However, in a repetition of this experiment, observation of the rats during the task showed that lesioned rats had an increase in unconditioned behaviour (orientating to the houselight, rearing) in response to the light (activity which was not increased during the dark phase of the task) (Rostron et al., 2008). The deficit therefore was not simply a failure to respond to the light in a timely manner, but a failure to execute the *correct* response in a timely manner. The increase in time during which rats could respond appears to have increased correct responses by giving lesioned rats more time to execute various behaviours, including the correct one. Similar results were seen in a task where rats were required to hold a lever down until a stimulus (presented at unpredictable time intervals) was presented. Bilateral excitotoxic lesions of the PPTg significantly reduced the number of

correct responses and, when correct responses were made, increased the time taken to make them (Florio et al., 1999). The authors note that PPTg lesions caused "*a sharp increase of unconditioned responses to the instruction starting each trial"* which is strikingly similar to the reports from Rostron and colleagues of increases in incorrect behaviours in response to the light cue and supports the view that in both experiments rats were noticing and responding to the stimuli, but failing to successfully engage in a correct response. It is still possible that there was some degree of attentional deficit concurrent with this action selection problem and the data from these experiments cannot separate out these two factors. In the 5 choice serial reaction time task (which requires the continual detection of and response to briefly presented visual stimuli over 5 spatial locations) PPTg lesions decreased accuracy, increased rats of omission (failure to respond to a light) and while lesions also increased response latency (the time to make a correct response) they did not affect magazine latency (time to collect pellet following correct response) (Inglis et al., 2001). Supporting the view that these rats had an attentional impairment was the finding that increasing the attentional component of the task (by decreasing stimulus brightness and introducing random white noise busts) decreased performance of lesioned rats over and above the decrease caused in sham controls. However, while being a better assessment of attention than increasing the response window, this also does not rule out the possibility of an action selection deficit. For example, depending on the cause of deficit, the unexpected noise bursts could be a source of distraction or trigger further interference in an action selection mechanism reducing to an even greater degree the ability to execute the correct response. The PPTg is well placed to have involvement in both attention and action selection processes (see subsequent sections, pp38-46) and it is hoped further research will help disambiguate the results of these studies.

PPTg and sucrose solution consumption

The effect of PPTg lesions on the consumption of sucrose solution warrants its own sub-section for two reasons: firstly, the results do not clearly fit into any other sub-section, and secondly, despite initially perhaps seeming simply an odd curiosity, it is a strong and easily reproducible effect that deserves proper consideration. When offered high concentration sucrose solution (12% or above), either in the homecage or a testing environment, PPTg lesioned rats will consume significantly more than sham controls (Olmstead et al., 1999; Alderson et al., 2001; Ainge et al., 2006). This effect of overconsumption is not observed for low concentration (4%), normal drinking water or standard lab chow. Indeed, lesioned rats which over-consume sucrose solution subsequently reduce the amount of lab chow they consume (presumably to compensate for the increased energy intake from the sucrose) (Keating et al., 2002). The simplest interpretation would be that PPTg lesioned rats like, or want, sucrose solution more than controls. This has been explicitly tested: when assessed in a conditioned place preference (CPP) paradigm, while PPTg lesions caused significant overconsumption of 12% and 20% (but not 4%) sucrose solution, the strength of the place preference effect for high concentration sucrose was unaffected by the lesion (Alderson et al., 2001; Keating et al., 2002). When using runway transversal speed to measure motivation, PPTg lesioned rats again consumed more 20% (but not 4%) sucrose than controls and while they also had reduced run speeds for the higher concentration, this reduction was somewhat lower and slower to develop than in controls (Ainge et al., 2006). If PPTg lesioned rats caused an increase in the liking or wanting of high concentration sucrose solution it would be expected that a stronger CPP and sharply decreased runway times for the higher concentration would be seen. Together these results show that PPTg lesions only affect sucrose consumption during actual consummatory behaviour, while behaviours connected to the acquisition of sucrose solution are unaffected by lesion. An interesting development was the discovery that in

addition to over-consuming sucrose solution, PPTg lesioned rats consumed significantly more quinine solution that sham controls (Walker and Winn, 2007). Quinine produces aversive reactions in rats and is often used to devalue liquid rewards (Berridge and Robinson, 1998); rats normally only consume very low amounts of it. One explanation proposed is that once engaged in strongly selected behaviours, PPTg lesioned rats find it harder to disengage this behaviour and so perserverate in it (Ainge et al., 2006; Walker and Winn, 2007). This could be a result of disrupted input to the STN (Ainge et al., 2006) which has been proposed to send a "breaking signal" which causes disengagement of the currently selected behaviour (see: Gillies and Willshaw, 1998). The hypothesis here would be that once engaged in drinking of highly pleasant (sucrose) or aversive (quinine) solution, PPTg lesioned rats are late in generating the break signal, but in normal conditions (drinking water) they perform as normal. A recent advance has been the observation that pPPTg lesions, and not aPPTg lesions, cause over-consumption of 20% sucrose (Wilson et al., 2009a). However, as projections to the STN arise from the entire PPTg (but are greatest in central areas and are predominantly non-cholinergic) (Kita and Kita, 2011) this recent finding does not explicitly support or refute the STN-breaking point hypothesis.

Summary

To summarise, several patterns of behavioural change emerge after PPTg lesion: (1) the motor skills to perform operant and maze tasks and willingness to work for reward is intact; (2) while performance of previously learned simple lever pressing tasks is unaffected by PPTg lesion, there is clear impairment in changing behaviour in response to changes in the task demands, the most likely explanation of this being a learning impairment. A learning impairment also explains the poor performance of lesioned rats on the radial maze; (3) in complex tasks requiring a timely response to specific unpredictable cues, there is a deficit in

executing the correct response at the correct time. This deficit (in part at least) appears to be due to impaired or altered attentional processing. However, concurrent with this: (4) lesioned rats appear to suffer from behavioural disorganisation or deficits of response selection. They respond during unreinforced task periods and omit responses during reinforced periods. Moreover, despite selectively increasing activity in response to appropriate cues, this increased activity is often in the wrong behaviours. This is indicative of impairment in action selection processes, an explanation which also may explain some of the deficits observed on the radial maze.

The extent to which these factors contribute to one another is unclear. While it seems reasonable to assume that there is some overlap of deficits it is also the case that careful assessment of behaviour can, to a large extent, tease these apart. For example, learning to press one active lever on a low schedule of reinforcement has a very low attentional load but a high learning component, whereas maintaining performance during subtle changes in stimulus brightness has little requirement to learn but dramatically increases attentional demands.

Interpreting the deficits observed

The role of the PPTg in associative learning

Associative learning (the process of learning associations between combinations of stimuli, actions and events in the environment) can be split into two main sub-types: instrumental conditioning (when the agent forms associations between stimuli and actions performed) and Pavlovian conditioning (where the agent passively forms associations without causing any change in the environment). Instrumental learning can then be further split into action-outcome (A-O) (learning that actions produce specific outcomes) and stimulus-response (S-R) (forming a habitual response to a particular stimuli). Pavlovian conditioning, also known

as stimulus-outcome (S-O) learning, can in turn be further divided into preparatory and consummatory responses (for review see: Yin et al., 2008). It is believed that, during instrumental learning, all of the above learning types develop and have varying degrees of contribution to behavioural control. For example, if a rat is placed in an operant box with no experience of lever pressing, it will, through exploration and formation of associations between actions and outcomes, learn that pressing a lever leads to a pellet being delivered simultaneously with the illumination of a bright light. However, concurrent to the development of these A-O associations, S-O associations will also be formed: the bright light will lead to preparatory S-O responses and, over time, even the slight of the lever itself may do so. In addition the lever may also cause the formation of S-R associations such that the sight of the lever will then trigger the habitual response of lever pressing. Under certain conditions (including repeated performance of the same action, interval schedules of reinforcement and particular drugs) instrumental behavioural control becomes increasingly driven by S-R associations rather than A-O (Hilario and Costa, 2008; DeRusso et al., 2010). Moreover, in addition to this, S-O associations form between the stimuli related to acquiring the outcome and the outcome itself, casing automatic preparatory and consummatory responses to these stimuli which are believed to facilitate instrumental performance for the associated outcome. This process is known as "Pavlovian to instrumental transfer" (PIT) and specifically refers to the capacity of a Pavlovian stimulus that predicts reward *to elicit or increase instrumental responses for the same (or a similar) reward* (Holmes et al., 2010) and is believed to be part of the process that triggers cravings upon perceiving stimuli associated with particular outcomes (Holmes et al., 2010; Belin and Everitt, 2011; Pielock et al., 2011). Combing these effects, behaviour which was initially goal directed, can, under certain circumstances, become driven by responses to the stimuli related to the action rather than the perceived outcome of the action: that is, the behaviour becomes a habit (Everitt and Robbins, 2005). These are natural

processes which lead to efficient behavioural control: for example the shift from A-O to S-R performance enables the low cognitive demand automatic execution of actions in situations where they are frequently performed (and therefore likely to need re-performed often) or the rapid execution of habitual responses in emergency situations (Hilario and Costa, 2008; Redgrave et al., 2010b; Schwabe and Wolf, 2011). However, maladaptive learning can have severe consequences. One notable maladaptive learning pattern is addiction, a core feature of which is believed to be the transfer of behavioural control from action-outcome to stimulus-response performance (see: Everitt and Robbins, 2005) which is enhanced by the strong PIT effects where drug related stimuli trigger cravings and the desire to work (put energy/resources into) acquiring drugs. These stimulus driven processes go some way in explaining the phenomena where drug addicts report that they "want" drugs despite knowing that they no longer "like" them: as their behaviour has become governed by S-R (enhanced and perhaps even triggered by S-O) processes rather than A-O, not liking the drug is in itself insufficient to strongly influence drug seeking behaviour (Berridge, 2007). Disambiguating the different brain processes contributing to learning is therefore of practical as well as theoretical importance. Considerable knowledge has already been gained in understanding the neuronal basis of these different components of learning. Dopaminergic systems of the midbrain, striatum and prefrontal cortex are fundamentally implicated in all of them. More detailed and systematic analysis has revealed that action-outcome learning is crucially dependent on a wide cortical and subcortical system centered on the posterior dorsal medial striatum (pDMS) (Yin et al., 2005a; Yin et al., 2005b; Lex and Hauber, 2010a). In contrast to this the dorsal lateral striatum subserves automatic habit driven performance (Featherstone and McDonald, 2004; Yin et al., 2004; Featherstone and McDonald, 2005). S-O associations are dependant in the integrity of the nucleus accumbens (NAcc) (Parkinson et al., 2000; Dalley et al., 2005) and normal instrumental learning (albeit with a reduced level of overall activity) can occur after

NAcc lesion (Corbit et al., 2001; de Borchgrave et al., 2002; Lex and Hauber, 2010b). PIT also appears dependant on an intact NAcc (Corbit et al., 2001; Wiltgen et al., 2007). Based on these and similar findings, Yin and colleagues (2008) have recently proposed the four-part subdivision of the striatum shown in fig 1.3. Two important points to make here are: (1) while the NAcc is deemed to be involved in Pavlovian conditioning and PIT rather than instrumental learning, both of these strongly contribute to and enhance instrumental learning (Lovibond, 1983; Lex et al., 2011) so it is not possibly to easily separate them; (2) These are far from the only structures involved in these behaviours, indeed they form a central part of an extended cortico-striatal, cortico-thalamic and subcortical system, the full extent and function of which is discussed in subsequent sections (pp40-46).

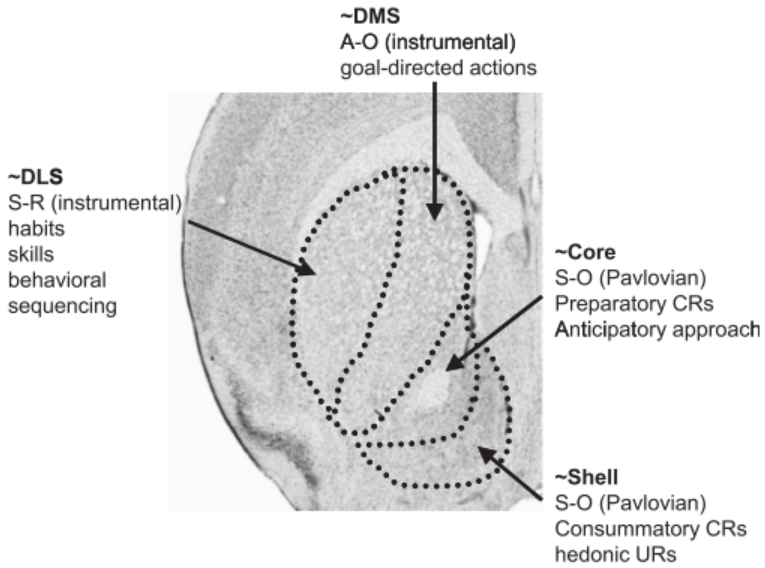


Figure 1.3: Illustration of the major functional domains of the stratum as proposed by Yin et al (2008). Image reproduced from Yin et al. (2008).

The reward prediction error signal

A series of ground-breaking discoveries showed that the activity of midbrain dopamine (DA) neurons in the SNc and VTA reliably develop a characteristic response to reward and reward-predicting stimuli (Ljungberg et al., 1992; Mirenowicz and Schultz, 1994; Mirenowicz and Schultz, 1996 ; for early review see: Schultz, 1998). After unexpected reward delivery, midbrain DA neurons exhibit a short latency short burst of phasic activity (~70 ms after stimuli presentation and lasting 100 – 200 ms) and then return to baseline tonic firing rates. If the same reward is delivered in the same circumstance, phasic activity develops in response to stimuli that reliably precede the reward (for example a tone) and the response to the reward itself diminishes. Once this is established, if the reward preceding stimulus is not followed by the expected reward delivery, a reduction in tonic firing occurs at the time of reward delivery (see figure 1.4 for illustration) (Schultz, 1999). This has been described as a 'reward prediction error' (RPE) signal: reward predicting stimuli elicit firing of the RPE signal, and if the reward is subsequently presented then tonic firing remains at the normal constant level, indicating the situation is as predicted. But if the predicted reward is not delivered then the reduction in tonic firing signals unexpected absence of predicted reward – the situation is worse than predicted – and if a larger than expected, or an entirely unexpected reward is delivered, then there is a burst increase in firing because the situation is better than predicted. It is hypothesised that this signal propagates to the striatum and other BG and cortical systems where it is used to drive reinforcement learning (Schultz, 2010). It has even been interpreted as fitting the requirements of formal learning theories that predate the discovery of this neuronal response (Rescorla and Wagner, 1972 ; for review see: Schultz, 2010). While still regarded by many as a reward prediction error signal which cortico-striatal systems depend on for reward based learning, this view is not unanimously accepted and there are other interpretations.

The midbrain DA reward prediction error / phasic DA signal:

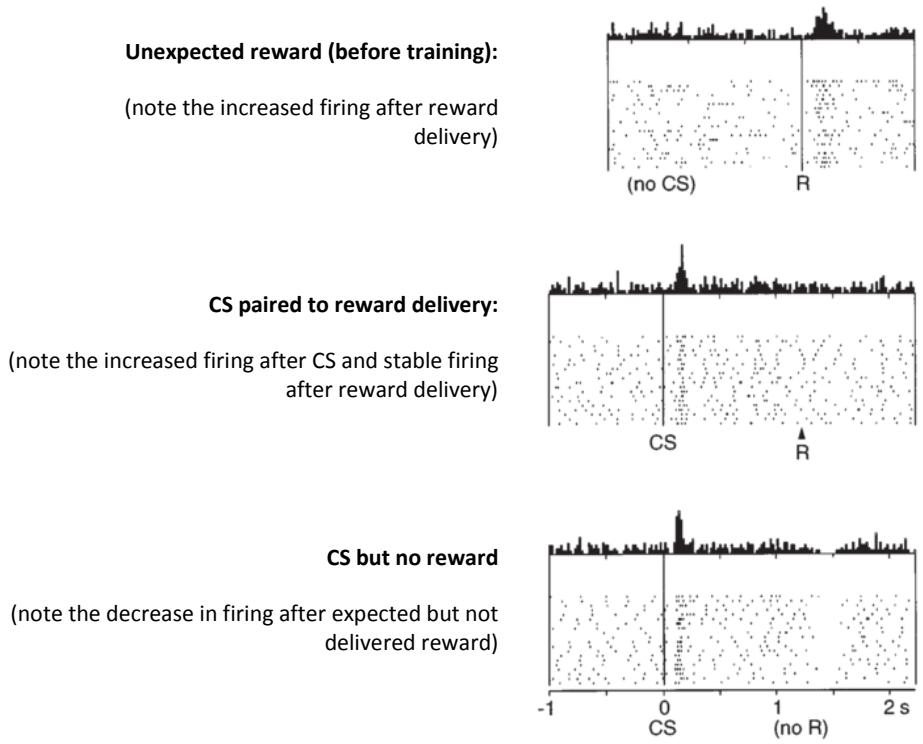


Figure 1.4: Illustration of the firing pattern of midbrain DA neurons in response to unpredicted reward (top), predicted reward (middle) and absence of predicted reward (bottom). Each line of dots shows one trial. Image adapted from: Schultz, W. (1999).

Midbrain DA responses – a role in the determination of agency?

A second theory of the function of this phasic DA signal draws on aspects of the phasic signal which are not well explained by the RPE hypothesis but instead are entirely consistent with the idea that the short latency DA signal plays a crucial role in the discovery of agency in unpredicted events (including rewards) (Redgrave and Gurney, 2006; Redgrave et al., 2008; Redgrave et al., 2010a). That is, determining if the agent (oneself) caused the unexpected event, and if so, discovering which specific actions caused it. This is a major problem faced by organisms. While the lab rat in the operant box (or primate in primate chair) may have a very

limited environment and range of actions that could lead to reward delivery or environmental change, the natural world is constantly changing and one can be performing many simultaneous actions that might be influencing the environment. Efficiently discovering which action leads to something new happening, or a better-than-expected or worse-than-expected occurrence, and which events happen independently of ones actions, is an essential adaptive process.

This argument is supported by the claim that the short latency DA signal (70 - ~100 ms after stimulus presentation) occurs at too short a latency for accurate reward prediction error calculation due to insufficient information being present at this latency. For example, in visually guided tasks (which are frequently used in electrophysiological recording experiments), the main cortical and striatal areas which assemble a detailed visual representation and subserve object identification do not complete this until after the midbrain DA systems have responded (the temporal cortex and temporal-occipital cortex identify objects at 70 - 90 ms and 80 – 100 ms respectively (Rousselet et al., 2004); the amygdala at a mean of 120 ms (Sugase-Miyamoto and Richmond, 2005) and within BG itself the caudate nucleus does not response until 100 - 200 ms after stimulus presentation (Hikosaka et al., 1989) (reviewed in: Redgrave et al., 2008)). This highlights the critical point that the short latency DA response must be dependent on a very short latency input and that in general highly processed cortical inputs would arrive at midbrain DA too late to be useful. Based on this it is argued that the DA response must be using very short latency midbrain and brainstem inputs, of which one prominent source of visual input is the SC (May et al., 2009). However, the SC has a relatively poor representation of the visual environment and is specialised in rapidly detecting transient visual events (such as movement induced changes in luminance within a spatial area) rather than identifying objects or familiar surroundings. Therefore SC indicates that something has occurred, where in the visual field this is, to a certain extent

whether it was predicted, but it does not identify *what* it is. SC responses are also related to reward and predictability – they rapidly habituate to repeated neutral events but increase their response when the stimulus coincides with reward delivery (Ikeda and Hikosaka, 2003). If this is the main source of visual input to midbrain DA it is unclear how a refined reward predicting signal could be calculated on the basis of such limited information. Rather than midbrain DA system being reward predicting error calculators, it is instead argued that midbrain DA signal might be a "sensory prediction error" (May et al., 2009) which converges in the striatum with concurrent contextual and motor signals and reinforces the repetition of actions which immediately preceded an unpredicted salient environmental event. Thus, the agent will soon discover if any of the recently executed actions caused the unexpected event (including reward) to occur. This system is made efficient by working at a short latency: the more time that passes the more likely it is that unrelated motor events will have been performed leading to interference in discovering the actual casual action.

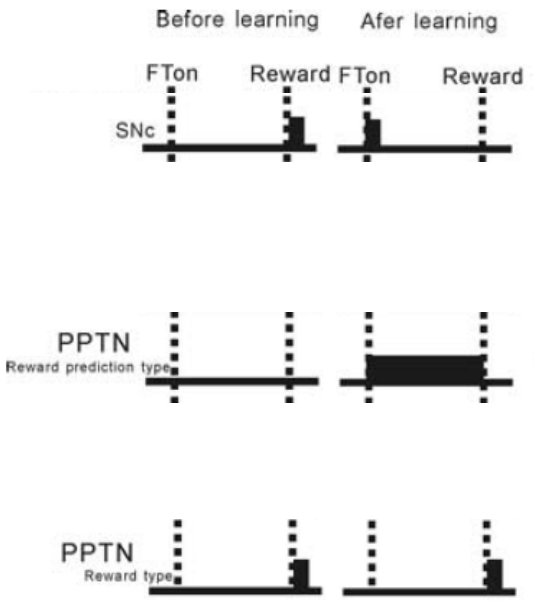
This theory highlights a fundamental prerequisite for the reward prediction error hypothesis: for the theory to be valid, there *must* be enough information present in DA systems, and present at the right timepoint, for such a calculation to occur. The possibility of other short latency sources of inputs to midbrain DA systems will be returned to shortly.

What the RPE and determination of agency theories have in common is that the firing of midbrain DA systems drives learning. Be it a detailed reward prediction signal that propagates throughout brain or a sensory prediction error that triggers the instruction to repeat immediately those motor actions recently executed, learning and discovering the consequences of actions and what stimuli in the environment do and do not lead to salient changes, is driven by it. Moreover, absence or abnormality of this signal would disrupt normal learning.

The relationship between the PPTg and the midbrain DA phasic firing

It has long being hypothesised that the cholinergic and glutamatergic projections from PPTg to midbrain DA neurons have a modulatory or controlling influence, but the exact nature of this has remained unclear (Charara et al., 1996; Maskos, 2008; Mena-Segovia et al., 2008a). ACh plays a key role in the regulation of DA burst firing: in mice genetically altered to lack the nicotinic acetylcholine receptor (nAChR) β2 receptor subunit (therefore blocking some of the effects of endogenous ACh) VTA burst firing was virtually absent. However, in a clever experimental setup, the knocked out nAChRs were re-expressed in the VTA by means of a lentiviral vector and subsequently VTA DA burst firing developed (Maskos et al., 2005; Maskos, 2007). Uwe Maskos used these findings to hypothesise that the cholinergic neurons of the mesopontine tegmentum (one of their major sources of cholinergic innervation) serve as a "master modulator" of the midbrain dopaminergic system, essential for glutamate driven switching to burst firing (Maskos, 2008). Indeed, stimulation of the PPTg reliably leads to an increase burst firing activity of midbrain DA neurons, with this increase being restricted to already active neurons (PPTg stimulation does not seem to be able to activate inactive neurons, something LDTg stimulation can do) (Scarnati et al., 1984; Floresco et al., 2003) and there is also some evidence that the PPTg driven increase in bursting is predominantly mediated by stimulation of glutamatergic projections (Scarnati et al., 1986). Inactivation of the neighbouring LDTg blocks the ability of PPTg stimulation to elicit DA burst firing (Lodge and Grace, 2006), leading to the hypothesis that LDTg is a 'gate' which can enable of block the ability of PPTg to drive DA bursting (Grace et al., 2007). These studies clearly show a close functional link between PPTg/LDTg and midbrain DA systems, raising the question of what function might this link have? In rats trained to predict what cues signal upcoming reward delivery, a reliable VTA DA phasic firing pattern developed in response to these cues. Unilateral inactivation of the PPTg then suppressed the conditioned phasic DA response without having

an effect on the overall rate of spontaneously active VTA neurons. That is, PPTg inactivation selectively attenuated the phasic DA response without altering baseline DA firing rates (Pan and Hyland, 2005). In a primate visually guided reward based task, analysing the firing characteristics of PPTg neurons revealed that different populations of PPTg neurons fired in response to stimuli predicting reward and to actual reward delivery itself (Okada et al., 2009). Furthermore, firing rate was dependent on reward magnitude – stimuli predicting large reward led to greater firing than stimuli predicting small reward. There was no evidence of habituation or reduction in PPTg responses over time, but adaptation to a reversal of the stimulus-reward size pairing occurred rapidly after reversal (conforming that PPTg was responding to the magnitude of the reward, rather than simply having different firing patters to different stimuli) (Okada et al., 2011). Populations of PPTg neurons responded to reward predicting stimuli and reward itself at latencies shorter than those of midbrain DA neurons (Pan and Hyland, 2005; Okada et al., 2009), which is consistent with other studies showing PPTg has very short latency responses to sensory inputs (Reese et al., 1995; Dormont et al., 1998). It is therefore reasonable to consider that PPTg may be supplying midbrain DA systems with salient aspects of sensory information (reward predicting stimuli, reward itself, magnitude of reward) which in turn is used to generate phasic DA responses to reward predicting stimuli (see fig 1.5).



Reward prediction error midbrain DA neuron

Before training these neurons fire in response to the reward itself and not to the reward predicting stimuli, after training the neurons fire to the reward predicting stimuli and not to the reward itself.

Reward prediction type PPTg neuron

Silent before training, then fires in response to reward predicting stimuli. Firing is proportional to reward magnetite and sustained until reward delivery

Reward type PPTg neuron

Fires in response to the reward both before and after training.

Figure 1.5: Schematic of the firing pattern of midbrain DA (top), PPTg "reward prediction type" (middle) and PPTg "reward type" (bottom) neurons, with responses before training on the left and after training on the right. Image adapted from: Kobayashi and Okada, (2007).

The PPTg and attention

Attention is a notoriously difficult term to define. In the psychological literature it has been split into many sub-types (including top down, bottom up, split, divided, sustained, selective, voluntary, executive as well as numerous sensory divisions such as auditory, visual, spatial) which only in some cases map easily onto neuroscientific findings. A general consensus is that attention refers to the ability for selective perception, such that an organism with limited resources can focus on particular (relevant) aspects of the environment while ignoring other (irrelevant) aspects of it. Clearly, the ability to decide what is relevant (and therefore worth attending to) rather than irrelevant is key for successful adaptive behaviour. Despite the lack of agreement over the exact types of attention, there are some clear common components and brain systems strongly linked to these. One of the most enduring classifications is the distinction between top down (deliberately maintaining attention on

particular stimuli) and bottom up (where stimuli interrupt ongoing behaviour and direct attention onto them). Most further sub divisions of attention can be assigned to one of these categories. Of course it is also the case that bottom up processes can trigger the engagement of top down attentional systems. Top down attention is believed to originate in prefrontal cortical areas, with bottom up beginning with the sensory areas associated with the stimulus modality and subsequently progressing by various routes through brainstem and midbrain areas into thalamus and cortical systems (see: Sarter et al., 2001; Meck and Benson, 2002; Saalmann et al., 2007; Wykowska and Schubo, 2010; Knudsen, 2011). Indeed, while once regarded as simply a relay station, the thalamus, with intricate modality specific sub regions and extensive cortical, subcortical and sensory inputs, is now regarded as a 'hub' for both bottom up and top down sensory processing and as the sensory gateway to cerebral cortex (McAlonan et al., 2000; Petrof, 2007; Baluch and Itti, 2011). The patterns of deficits observed in PPTg lesioned rats during tasks of sustained attention have several possible interpretations. The cholinergic innervation of thalamus is a key route for possible disrupted attention after PPTg lesion. PPTg lesion could block the input of sensory information into thalamus and subsequent thalamo-cortical (or, for that matter, thalamo-striatal or thalamo-cortico-striatal) loops. Additionally, ACh is believed to depolarise and disinhibit thalamic neurons, directly increasing thalamic activity and facilitating sensory processing (Steriade et al., 1991; Parent and Descarries, 2008). In support of this, stimulation of brainstem innervation to the lateral geniculate nucleus (LGN) has been shown to enhance its response to sensory stimuli (Uhlrich et al., 1995). It is therefore also reasonable to consider that loss of cholinergic innervation could impair thalamic activity and sensory processing. Projections from PPTg to the NbM (a structure with heavy cholinergic cortical projections which are critical for sustained attention (McGaughy et al., 1996)) initially appeared a likely route of interest in the role of the PPTg in attentional processing (Kozak et al., 2005). However, in the same behavioural task, the deficits

observed after PPTg lesion were different to those produced by NbM lesion, suggesting that the PPTg effects are not simply due to disrupted input into NbM (Rostron et al., 2008). At a more global level, the role of cholinergic PPTg systems in cortical activation has also been hypothesised to have an influence on the level of top down attention (Rostron et al., 2008): Electrical stimulation of PPTg induced a 350% increase in cerebral cortex ACh levels (Rasmusson et al., 1994) which is likely to enhance cortical inputs and induce a general increase in cortical activity (McCormick and Prince, 1986; Dringenberg and Olmstead, 2003) and therefore possibly subsequent increased cortical top-down control of behaviour/attentional processes. Finally, while not always being presented in attentional terms, the effects of PPTg lesion on PPI can, at least in part, be considered attentional in nature in that PPI is a measure of responses to the environment / vigilance. The role of the PPTg in attention remains rather unclear, and indeed it is not certain to what extent deficits observed can be explained by strictly attentional impairments or other factors such as action selection. For example, is it the case that PPTg lesioned rats perform poorly on attentional tasks due to deficits in the selective perception of salient stimuli (attentional impairment) or due to failure in executing the correct behaviour in response to salient stimuli (action-selection impairment)? One promising avenue of future work is the use of selective cholinergic PPTg lesions. Combining targeted lesions with refined attentional tasks (separating out attentional, learning and action selection aspects) will hopefully reveal the nature of the contributions of the PPTg to attentional processing.

The PPTg and action selection

Action selection (also known as response selection) is the processes of selecting one action out of a repertoire of many possible actions. Generally these possible actions will be mutually exclusive (turn left or turn right?) and in many cases the consequences of the action

executed (freeze or flee?) will have dire repercussions if the less favourable action is chosen. The ability to only execute one action at a time has been referred to as "the final common motor path" (Redgrave et al., 1999) and selecting the correct path is an evolutionary ancient problem faced by brains. Indeed, any creature that can execute more than one movement is faced with the recurrent question of what to do and when to do it. It is believed that the basal ganglia and connected structures have evolved to address this problem (see: Kamali Sarvestani et al., 2011; Redgrave et al., 2011; Stephenson-Jones et al., 2011; Stephenson-Jones et al., 2012). Evidence for this is strong: (1) the basal ganglia are as old as the problem itself, and, in a similar manner to the problem, have remained well conserved through evolution, which suggests a stable need for these structures; (2) the basal ganglia have the necessary anatomical connections to fulfil such a function; (3) disorders of motor selection (that is, disorders where the primary deficit is in selecting the right motor action at the right time, rather than for example, motor disorders such as paralysis) are closely linked to basal ganglia dysfunction. The most notable of these being Parkinson's Disease (Obeso et al., 2008), addiction (Pierce and Vanderschuren, 2010) and obsessive-compulsive disorder (Cavedini et al., 2006). A full review of basal ganglia in motor control is beyond the scope of this thesis and would largely be redundant due to the excellent existing reviews which should be consulted for further reading (see: Steiner and Tseng, 2010). Briefly, the basal ganglia are a collection of interconnected nuclei at the base of the forebrain and midbrain. Long standing descriptions describe the BG as being comprised of the striatum (further subdivided into caudate and putamen in primate with corresponding caudate-putamen in non-primate brains), the globus pallidus (further subdivided into external and internal segments, GPe and GPi), the subthalamic nucleus and substantia nigra (sub divided into pars compacta (SNc) and pars reticulata (SNr)) (Anton, 2010; Gerfen and Bolam, 2010). See figure 1.6. This classical definition of the BG has been extended to include the PPTg (Mena-Segovia et al., 2004a) and, while not

formally argued for inclusion, the VTA and NAcc are built into some authors descriptions of the BG (eg: Yin and Knowlton, 2006). While there is a highly organised set of interconnections between BG structures (see fig 1.6) there are relatively few entry and exit points from this system. The major entry points include the striatum and the STN (receiving projections from cerebral cortex, thalamus and limbic structures) and to a lesser extent the SNc/VTA. The major output sites of the BG are the SNr and GPi, which project to thalamus, PPTg and brainstem motor output sites.

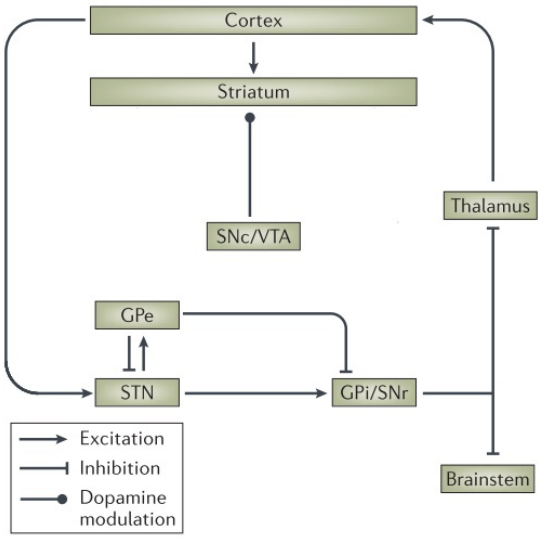


Figure 1.6: Illustration of the structures and main connections of the classical basal ganglia architecture. Image adapted from Yin et al. (2006). See fig 1.1 for PPTg connections to BG nuclei.

The ability of information to enter BG from cortex, process through BG and be output to thalamus and subsequently return back to cortex leads to the formation of functional "loops". These are known as cortico-basal ganglia-thalamic-cortical loops and it has been proposed that, depending on the information they process and the particular sites in BG and cortex which they target, that they can be split into largely segregated loops in a tripartite

manner (Alexander et al., 1986; Joel and Weiner, 2000; Voorn et al., 2004; Wilson et al., 2009b): (1) “limbic loops” connecting medial and orbital prefrontal cortex to ventromedial striatum and are involved in the processing of motivational information; (2) “cognitive loops” connecting dorsolateral prefrontal cortex to central and dorsal striatum which are involved in so called “executive processes” such as working memory, top-down attention and planning; (3) “motor loops” which connect premotor, presupplementary motor and cingulate cortical areas to dorsolateral striatum. These loops mirror the distinction made previously between action-outcome and stimulus response processing, with “motor loops” processing stimulus response information to and from the DLS and cognitive/limbic loops conveying information through the DMS and NAcc. Loops are bi-directional and there is also a degree of transfer across loops (both by convergence of neurons at loop end points and crossing of axons along loops). It has been argued that loops are in competition for final motor control with organised decision making having control pass from limbic loops (what do I want to do?) to cognitive loops (how do I do that?) and finally into motor loops which initiate the downstream process of execution of the appropriate behaviour. However, in certain situations this neat hierarchy breaks down and motor loops can seize final control more easily (Everitt and Robbins, 2005; Pierce and Vanderschuren, 2010). Again this mirrors the distinction between A-O and S-R control, with behaviour dominated by S-R processes being an example of motor loops executing behaviour without contributions from limbic or cognitive loops. In addition to these cortical loops, there are also closed loops to subcortical structures which link the PPTg, SC, periaqueductal grey, cuneiform and parabrachial nuclei to BG structures (McHaffie et al., 2005; Redgrave and Coizet, 2007; Winn et al., 2010). These loops give BG access to and influence over brainstem sensory and motor structures independently of thalamo-cortico-striatal loops. The exact nature of BG loops and the methods by which information can transfer across and between them is a topic of much debate. Therefore, these descriptions are used here to serve as

general framework - rather than a strict definition – from which to consider BG function. The role of the PPTg in action selection is closely linked to the notion that PPTg can control BG systems, interface information between BG and brainstem structures, and interact with brainstem independently of other BG structures. More specifically, PPTg can: (1) control and gate information entering BG and subsequent transfer of it through BG and cortical loops. PPTg can integrate fast sensory information into midbrain DA systems and thalamus (which may in turn affect the level of cortico-basal ganglia-cortical looped activity). Moreover, PPTg can exercise control role over other BG sites, for example altered modulation of STN which may affect the "breaking" of on-going BG activity; (2) control and gate information leaving BG. SNr is a major BG output site, not just to thalamocortical neurons but also extensively to midbrain and brainstem structures, many of which in turn project to the reticular formation and other brainstem motor output sites. The SNr output is predominantly inhibitory and it is believed that release from this inhibition triggers motor action. PPTg (which receives this outflow from SNr) is in a position to control this as it leaves BG; (3) potentially function independently of other major BG sites and interact with brainstem nuclei to initiate and select actions. Evidence for this comes from decerebrate animals (where all major BG sites except PPTg and depending on decerebration method, SN, are removed). These animals are able to engage in and switch between different simple behaviours such as orienting to sound, grooming and performing lever press actions that are previously known to lead to reward (actions which while basic, are considerably more sophisticated than simple reflexes) (see: Humphries et al., 2007; Wilson et al., 2009b). While these animals may be rather clumsy in these actions and the ability to switch between them, this ability clearly shows a degree of action selection independent of a fully intact BG. This has been argued to occur via the medial reticular formation which, due to having neuronal clusters representing components of co-ordinated action (rather than of a sensory or muscular-motoric topographic arrangement)

could be able to execute reasonably complex action sequences through co-activation of a small set of clusters. This has been proposed as a possible "brainstem substrate for action selection" (Humphries et al., 2007). PPTg, with extensive output to the mRF, is in a position to induce and control such activation (Wilson et al., 2009b; Winn et al., 2010). The idea that there is a brainstem substrate for action selection 'underneath' that of the BG speaks strongly for a layered evolutionary development of action selection systems (Prescott et al., 1999; Humphries et al., 2007; Wullimann, 2011). What is critically important to consider is that rather than overriding and rendering them obsolete, advanced levels build on and interact with still functioning lower levels (Anton, 2010; Stephenson-Jones et al., 2011). Therefore, it is essential to consider the functions, contributions and interactions of early systems of action selection even when considering seemingly complex and advanced processes. It is also important to consider the role of different PPTg subregions in action selection processes. Sensory input to PPTg target predominantly the posterior region, and it is this region which outputs to VTA (and subsequently onwards into ventral striatal and cognitive/limbic circuitry). In contrast to this, PPTg projections to the mRF arise from the aPPTg, which is also the region bi-directionally connected to the SN which in turn is closely associated with dorsolateral striatum (DLS) and motor cortices. This leads to the conclusion that manipulation of pPPTg is likely to affect input to BG and the processing of sensory events, whereas aPPTg damage would affect BG outflow, interactions with mRF and have a more direct effect on motor processing.

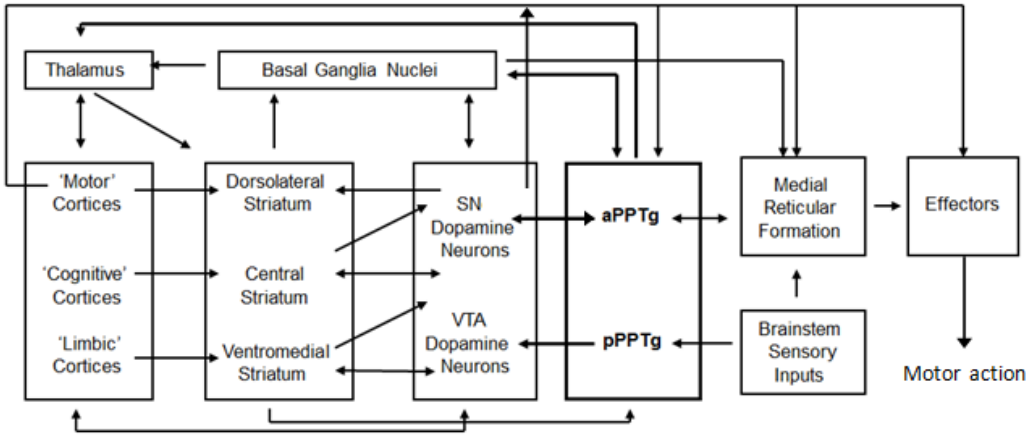


Figure 1.7: Illustration of the main connections between the PPTg and cortico-striatal circuitry, the basal ganglia, brainstem sensory inputs and effectors. Arrows which project into a box indicate the pathway targets a specific area of the structure, arrows which target the edge of a box indicate the pathway projections to all of (or to undefined areas of) the whole structure. Image modified from figure 1 of Wilson, MacLaren and Winn (2009).

Summary and aims

Concise summary

Rats bearing excitotoxic lesions of the PPTg have various separable yet potentially related deficits in operant tasks. To summarise briefly: (1) there is a clear learning impairment in naïve rats and in trained rats when testing schedules change; (2) attention may be impaired in tasks with a high attentional component; (3) while the ability to execute the motor actions required by operant and maze tasks appears intact, the ability to select the correct action or inhibit incorrect actions may be disrupted. Largely due to the lack of ability to manipulate different neuronal populations within PPTg, very few studies have been able to relate the deficits observed in behaviour to one of the three main neuronal populations within PPTg (cholinergic, glutamatergic and GABAergic). However, there has been progress in establishing the functions of different PPTg subregions: lesions of the posterior portion impair operant learning while anterior lesions do not affect learning rate but do produce signs of behavioural

disinhibition. These differing effects are believed to be the result of disruption of the different pattern of connections of PPTg subregions: aPPTg targets SNc midbrain DA neurons, receives output from SNr and targets motor output sites in the brainstem. In contrast, pPPTg is in receipt of fast sensory information and targets the midbrain DA systems.

Specific aims

The aims of this thesis are to advance the conclusions reached above by establishing the nature of the learning impairment seen after PPTg lesion and relating this to the functions of cholinergic and non-cholinergic PPTg neurons.

The learning impairments seen after pPPTg (and indeed PPTg) lesion have been interpreted as being most likely to be an impairment in forming and updating associations between actions and outcomes. However, none of the behavioural tests have explicitly assessed this. Moreover, on the basis of the conclusions of Yin et al (2008) one could hypothesize that the main impairment seen after pPPTg lesion would be expected to be in the formation of Pavlovian rather than instrumental associations (because much of the focus of pPPTg is on projections to the VTA and subsequent NAcc systems which are viewed as having a more restricted role in Pavlovian associations), whereas aPPTg lesions are more likely to disrupt instrumental learning (due to aPPTg projections to SNc which subsequently targets DMS and DLS - structures critically involved in instrumental learning). This conclusion is not immediately compatible with the previous behavioural results and interpretations. Due to the design of the previously used learning tests, learning and performance could be impaired (at least to some extent) by deficits in the formation of Pavlovian associations and PIT. Therefore, this is clearly a point requiring clarification and in turn explanation. Chapter 3 discuses

methods of assessing action-outcome (A-O) learning and implements two of these in order to fully assess the role of the pPPTg in action outcome learning and performance.

From this point on the focus of the experimental work is on investigating the role of the cholinergic pPPTg neurons in instrumental learning and interactions with the VTA. There are strong cholinergic projections from PPTg to midbrain DA (forming a topographical gradient where aPPTg targets SNc, pPPTg targets both VTA and SNc and the neighbouring LDTg targets VTA) which have been hypothesised to be a 'master modulator' of midbrain DA systems. In chapter 4 a protocol is refined for using the newly developed Dtx-UII fusion toxin to create highly selective bilateral lesions of the cholinergic pPPTg. The feasibility of these lesions and their effects on locomotion and sucrose consumption are then assessed. Following on from this, in chapter 5 the ability of rats with selective bilateral lesions in cholinergic pPPTg to learn and perform a range of fixed and variable operant reinforcement schedules is assessed. In chapter 6, the relationship between the cholinergic pPPTg and VTA is probed by assessing the rate of nicotine sensitisation in rats with selective cholinergic lesions in pPPTg. The last experimental study, in chapter 7, investigates the possibility of causing further disruption to cholinergic innervation of midbrain DA systems by assessing the feasibility of combined pPPTg and LDTg selective cholinergic lesions.

Finally, the results of all the experimental studies are discussed in chapter 8. The implications of the findings are related to the broader literature on the source of input to midbrain DA systems. A theory of PPTg as being a structure which extracts salient aspects from incoming sensory information and interfaces these with appropriate basal ganglia and brainstem systems is discussed. Additionally, the possible functions of cholinergic and non-cholinergic projections to midbrain DA systems are discussed with reference to instrumental learning and the role of Ach agonists in reinforcement enhancement.

Chapter 2: General methods

Subjects

All experiments were carried out on experimentally naive adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK). Rats were housed in a temperature and humidity controlled room where lights were on a 12-h light/dark cycle. All behavioural testing was conducted during the light phase. Rats were housed in solid base plastic cages with a wire mesh top, measuring either 25 cm × 45 cm × 15 cm or 36 cm × 56 cm × 26 cm (size depending on availability of cages, with a preference for the larger ones). Wherever possible rats were pair housed; the main exception to this being rats with cannula implant assemblies (chapter 3) which were always single housed. Cages were environmentally enriched with a perspex tube and disposable toy (changed twice weekly). However, during the days after headcap implantation, tubes were temporarily removed while the rats became accustomed to having the implant. Water was available ad-libitum in the homecage. Food restriction was used in chapters 3 and 5 to motivate rats to work for food reward (details of restriction given in the chapters) and apart from these times standard lab chow was also available ad-libitum in the homecage. Group sizes and weight ranges are stated in each chapter; in general the target surgery weight for lesion experiments (chapters 4 - 7) was 330 g and for cannulae implantation (chapter 3) it was 400 g. The heavier weights used for cannulae implants was an attempt to increase headcap stability by using rats with thicker skulls. All experiments were carried out in compliance with the Animals (Scientific Procedures) Act 1986, the European Communities Council Directive of 24/11/86 (86/609/EEC) and, where appropriate, the local ethical review policies of St Andrews University and the University of Strathclyde.

Surgical procedures

Anesthesia

Rats were anaesthetized using isoflurane (Abbot Laboratories Ltd, Maidenhead, UK) by placing them in an induction box being fed with 4 L / min O_2. After 1 min isoflurane was added to the O_2. This was started at a concentration of 1% and increased by 1% approximately every minute until the final concentration of 5% was reached. This was maintained for 1.5 min before the rat was removed from the induction box. The timings were approximate and adjusted such that total loss of righting reflex was observed before increasing the concentration beyond 3% and deep steady breathing observed before removing the rat from the induction box. Once in the stereotaxic frame (David Kopf, Tujunga CA, USA) anaesthesia was maintained via a facemask mounted on the incisor bar. During scalp incision and cleaning, 2.5% isoflurane in 1.4 L/min O_2 was delivered through the facemask, after which maintenance was achieved with 2.0% isoflurane in 1.2 L/min O_2. This was adjusted appropriately if reflexes were observed or if breathing became very shallow. Death from anesthesia was rare (<1% of surgeries).

Stereotaxic surgery

All stereotaxic surgery was performed on “Model 900” Kopf stereotaxic frames (David Kopf, Tujunga, CA, USA) in a clean surgical environment.

Cannulae implantation

Once anaesthetized and secured in the stereotaxic frame, a pre-surgical analgesic dose of Rimadyl (0.05 mL/rat; 5% w/v carprofen; Pfizer Ltd, Kent, UK) was injected subcutaneously. The scalp was shaved with electric clippers, a midline incision made with a scalpel and the skull cleaned of tissue before the incision was held open with artery clamps. The height of the

incisor bar was then adjusted such that the dorsoventral (DV) measurements at lambda and bregma were equal (flat skull position). Craniotomies were made with a hand held dental drill at the following co-ordinates: +0.4 mm from interaural line (IAL); ±1.9 mm from the midline (measured from skull surface). Six stainless steel round-head machine screws with sharply cut threads (0-80 x 1/16 or 0-80 x 3/32 or 0-80 x 1/8; see chapter 3 for details; Plastics One, Roanoke, VA, USA) were fixed onto the skull, two behind the craniotomies, two in front and a further two in front of lambda. Bilateral guide cannulae (22 ga, 3.8 mm apart, protruding 5.0 mm below skull surface; Plastics One) were lowered in place using a mounting holder on the stereotaxic arm (Plastics One) and fixed onto the skull and screws using a methyl methacrylate based dental acrylic (Simplex Rapid, Kemdent Works, Wiltshire, UK). Internal dummy cannula (3.8 mm apart, protruding 1.0 mm from the guide cannulae; Plastics One) were inserted into the implanted cannulae and a dust cap (Plastics One) screwed on top. The wound behind the headcap assembly was closed with sutures (Ethicon, Edinburgh, UK). Once removed from the stereotaxic frame rats were given an i.p injection of Hartmann's solution to aid recovery (1 mL, Baxter Healthcare Ltd, Norfolk, UK). Rats were placed in a heated recovery cage and monitored until they had recovered from anesthesia before being returned to their homecage. Every 1 - 2 days, dummy cannulae were changed with clean replacements. Rats were given at least 7 days to recover prior to the start of behavioural training.

Toxin microinfusion

Once anaesthetized and secured in the stereotaxic frame, a pre-surgical analgesic dose of Rimadyl (0.05 mL/rat; 5% w/v carprofen; Pfizer Ltd, Kent, UK) was injected subcutaneously. The scalp was shaved with electric clippers, a midline incision made with a scalpel and the skull cleaned of tissue before the incision was held open with artery clamps. The height of the incisor bar was then elevated such that the horizontal angle between the incisor bar and the

interaural line was 8°29′(achieved by multiplying the distance between the IAL and the back of the incisors by the sine of 8°29′(0.147), as described by Whishaw et al., 1977). Craniotomies were made with a hand held dental drill above the location of the final infusion co-ordinates (appropriate co-ordinates given in individual chapters). Dtx-UII toxin (or sterile phosphate buffer (PB) in for sham controls) was backfilled into a fine tipped (40 - 50 µm) glass pipette which was then secured into an electrode holder on the stereotaxic frame (pipettes were manufactured in-house from borosilicate capillary tubes (1.16 – 1.19 mm o.d.; 0.49 mm i.d.) pulled into pipettes with a pipette puller (Model 720 Needle Pipette Puller, David Kopf, Tujunga, CA, USA) before having tips manipulated to 40 – 50 µm diameters under a light microscope). Measurements were then re-taken to allow the pipette to be advanced to the required co-ordinate. After measurement, dura was cut with the bent tip of a 29 gauge needle to ensure that penetration of dura did not damage the pipette tip. Toxin was delivered by pressure injection from a 10 mL plastic syringed connected to the pipette by polythene tubing (containing air). Pressure was applied to the syringe by hand. Manually covering a small hole drilled in the syringe maintained air pressure during infusion and enabled the immediate release of pressure from the infusion system when the required toxin volume (indicated by a scale attached to the pipette) had been infused. After infusion the pipette was left *in-situ* for 5 min to allow diffusion from the tip before being slowly removed. After all infusions had been made, the skull was cleaned with saline and the wound closed with Michel clips. Once removed from the frame rats were given 1 mL of Hartmann's solution (Baxter Healthcare Ltd, Norfolk, UK) to aid recovery. Rats were placed in a heated recovery cage and monitored until they had recovered from anesthesia before being returned to their homecage.

Behavioural procedures

Operant training and testing

Operant testing was conducted in 12 Med-PC operant chambers individually housed in sound and light attenuating boxes (Med-Associates, St Albans, Vermont, USA). These chambers were monitored and controlled by a computer system running Med-PC software (Med-Associates, St Albans, Vermont, USA). Each operant chamber had two retractable levers either side of a reward magazine. This magazine contained a trough into which pellets could be delivered from a dispenser located outside the chamber and a cup (0.1 mL) which could be filled with liquid reward by a dipper mechanism which lowered the cup into a reservoir below the hopper. One of the levers had a light above it (1.5W) and there was a houselight located on the upper central portion of the opposing wall. A fan installed inside the sound attenuating boxes provided airflow and also served as low level constant background noise.

Locomotor testing

Locomotor monitoring was conducted in 6 perspex cages (45.7 cm x 24.1 cm) situated inside "SmartFrame™ Cage Rack Stations" (LED rearing 7x15 High Density, Hamilton Kinder LLC, Poway CA, USA). These contained a 7 x 15 grid of infra-red beams at the height of the rats body and an additional 7 x 0 grid of beams at a height only reached when the rat reared. All stations were interfaced with a computer system running "Motor Monitor" software (Hamilton Kinder LLC, Poway CA, USA) which recorded all beam breaks made in the cages. The following measures were recorded by the software: basic movements (total of all recorded beam breaks at the rats body level); fine movements (number of beam breaks caused while rat not changing its whole body position – that is, as would be caused by forepaw grooming) and rearing

(number of beam breaks across the higher level of infra-red beams). Locomotor testing was conducted in a dimly lit room.

Microinfusion of muscimol/saline into the pPPTg

Prior to surgery rats in microinfusion experiments (chapter 3) were handled and habituated to the light restraint that would subsequently be used during the microinfusion procedure. This involved holding the rat to the experimenters chest such that all paws were supported and body movement greatly restricted without forceful pressure or restraint being necessary. Microinfusions into the pPPTg were made via bilateral cannulae (3.8 mm apart, protruding 7.5 mm from the base; Plastics One) that were inserted into the previously implanted guide cannulae while the rats were lightly restrained. Injectors were attached by polyethylene tubing (PE50 thin wall; Plastics One) to 2 syringes (1 µL, 23 ga needle, SGE Analytical Science, Victoria, Australia) driven by a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA). The pump setup was placed and tubing cut such that tubing length could be of as short as possible, and all infusions were done with tubing of the same length. This was to try and prevent pressure buildup in the tubing and to ensure that if this did occur, it was as constant as possible. Infusions of 0.3 µL of muscimol (0.05 µg muscimol; Tocris Bioscience, Bristol, UK in 0.3 µL saline; Baxter Healthcare Ltd) or saline (0.3 µL; Baxter Healthcare Ltd.) were made over 1 min and injectors were left in situ for 1 min post-infusion before being removed and replaced with dummy cannulae.

Histological procedures

Transcardial perfusion with fixative

Rats were prepared for perfusion by being given a lethal i.p. injection of sodium pentobarbitone (0.6 - 0.8 mL per rat; 200 mg/mL sodium pentobarbitone. Depending on

availability either: Dolethal; Univet Ltd, Oxford, UK or Euthatal; Merial Animal Health Ltd, Harlow, UK). Once deeply anaesthetized they were immediately transcardially perfused with phosphate buffered saline followed by approximately 300 mL of fixative (4 % paraformaldehyde in 0.1 M phosphate buffer) at a flow rate of ~14 mL / min. Completion of perfusion and successful fixation was judged by inspection of the rat rather than the the quantity of fixative used. Following perfusion brains were left to post-fix in situ for around 60 min before being removed and stored in sucrose solution (20 % sucrose in 0.1 M PB) in a refrigerator.

Tissue preparation and sectioning

Coronal 30 µm sections were cut on a freezing microtome. The left side of the brain was marked by penetration with a needle though an area not considered relevant to the studies being conducted. Sections to be processed immediately were stored in PBS in a refrigerator; remaining sections were stored in ethylene glycol based cryoprotectant at -20 C.

Immunohistochemical staining

All immunohistochemistry was performed at room temperature on free floating sections on a flatbed shaker. Both choline acetyltransferase (ChAT) and neuron specific nuclear protein (NeuN) staining followed the same protocol. To reduce the risk of cross-contamination of antibodies (details below) the two stains were not conducted in parallel.

NeuN/cresyl

In the central nervous system (CNS) antibodies against neuron-specific nuclear protein (NeuN) react strongly with neuronal nuclei and to a lesser extent with cytoplasm. This reaction occurs regardless of the neurotransmitter type expressed by neurons. Therefore, NeuN can be

successfully used as a non-selective marker of the presence (and density) of neurons. It is used here (see details below) to assess the selectivity of cholinergic mesopontine tegmentum lesions. Cresyl violet staining marks both Nissl bodies and reactive gliosis (which develops in response to CNS injury) a dark purple colour. While reactive gliosis is frequently observed after excitotoxic lesions, it is seldom detected after Dtx-UII lesions. However, track damage from the pipettes used for microinfusion can cause small amounts of gliosis. The primary use of the cresyl counterstain here is to aid the detection of pipette tracks and final location of the pipette tip.

ChAT

Choline acetyltransferase (ChAT) is an enzyme required for the synthesis of ACh, it is found in both the nuclei and terminals of ACh expressing (cholinergic) neurons and, importantly, not in neurons that do not express Ach. Immunohistochemical staining with ChAT antibodies is therefore an effective and reliable method for selectively staining cholinergic neurons. It is used here (see details below) to quantify the extent of cholinergic neurons within the PPTg/LDTg.

Immunohistochemical protocol

Sections were washed by being rinsed for > 5 min in each of > 3 trays of clean PBS solution. They were then placed in blocking solution (20 % normal serum; 79.9 % PBS; 0.1 % Triton X-100) for 60 min. After washing (as before) sections were placed in antibody diluting solution (ADS) (98.9% PBS; 1% Triton X-100; 1% normal serum) containing the primary antibody and incubated overnight. The following day sections were processed using a Vector Labs Elite ABC kit (Vector Laboratories, Peterborough, UK). The first step of this process was to wash sections (as before) and incubate them for 90 min in a biotinylated secondary antibody (IgG,

1:200 concentration in ADS) and then (after washing, as before) incubate them in avidin-biotin complx (ABC, 1:50 concentration in ADS) for 45 min. After washing (as before) the staining was revealed with 3,3 diaminobenzidine tetrahydrochloride (Sigma Fast DAB; Sigma-Aldrich, Gillingham, UK) before a final set of washing. ChAT stained sections were then mounted on glass slides and coverslipped using glass coverslips and DPX mounting medium (Sigma-Aldrich, Gillingham, UK). NeuN stained sections were mounted onto gelatin coated glass slides and counterstained with cresyl-violet (see below).

Antibodies used

ChAT

The serum used for the blocking solution and ADS construction in the ChAT stain was normal rabbit serum. The primary antibody was a goat derived polyclonal antibody (Chemicon International Inc, Temecula, CA, USA) diluted in ADS to a final concentration of 1:500. The goat version of the Vector Labs Elite ABC kits (Vector Laboratories, Peterborough, UK) was used for the secondary antibody and avidin-biotin complex. Sections were stained in the Sigma Fast DAB peroxidase substrate (Sigma-Aldrich, Gillingham, UK) for 10 min.

NeuN

The serum used for the blocking solution and ADS in the NeuN stain was normal goat serum. The primary antibody was a mouse derived monoclonal antibody (Chemicon International Inc, Temecula, CA, USA), diluted in ADS to a final concentration of 1:20,000. The mouse version of the Vector Labs Elite ABC kits (Vector Laboratories, Peterborough, UK) was used for the secondary antibody and avidin-biotin complex. Sections were stained in the Sigma Fast DAB (Sigma-Aldrich, Gillingham, UK) peroxidase substrate for 7 min.

Cressyl violet counterstain

NeuN stained sections mounted onto gelatin coated slides were dried overnight in a gas chamber containing paraformaldehyde. Slides were placed in xylene for 3 min and rehydrated through graded alcohol (100%; 50%; 0% ethanol in water) before being thoroughly rinsed in running tap water. Slides were then placed in cresyl violet stain for 60 sec, rinsed in running tap water for 5 min and dehydrated through graded alcohol (0%; 50% 100% ethanol in water). After soaking in xylene for < 3 min slides were coverslipped with glass coverslips and DPX mounting medium (Sigma-Aldrich, Gillingham, UK).

Microscopy

Slides were examined under a light microscope. This was either a Leica DM LB2 microscope connected to a desktop computer system by a Leica DFC320 high resolution camera or a Leica Diaplan microscope (both: Leica Microsystems, Milton Keynes, UK) connected to a high resolution monitor with a Sony DXC-300P camera.

Cannula tip location

Cannulae tip location in microinjection experiments (chapter 3) was determined by examining guide cannulae location and the evidence of injector track marks protruding from these.

Lesion analysis

Analysis of selective cholinergic pPPTg (chapters 4,5,6) and LDTg/pPPTg (chapter 7) lesions was performed by examining the NeuN stain to assess the selectivity of the lesion and

quantifying (see below) the number of surviving cholinergic PPTg neurons visible on the ChAT stained sections.

NeuN/cresyl assessment

The NeuN/cresyl stained sections were examined for possible damage in and around the location of the PPTg (the area from and including the trigeminal of the motor nucleus through to and including substantia nigra was examined). Cell loss on the NeuN stain is evident by a lack of or reduction in the density of stained neuronal nuclei. Within the pPPTg the cholinergic neurons are highly interdigitated with the non-cholinergic neurons. Therefore, selective loss of cholinergic pPPTg neurons (which comprise 31% of pPPTg and 23% of aPPTg neurons) causes only a slight reduction in overall level of NeuN staining which, due to the larger size of cholinergic rather than non-cholinergic neurons, should be most marked in large stained nuclei. Importantly, any indication of an area with no stained nuclei is a clear sign of non-selective damage.

PPTg ChAT+ cell counting

In order to quantify the number of ChAT+ neurons within the PPTg, software assisted manual counts of ChAT+ PPTg neurons were performed. Each ChAT stained PPTg section was photographed with the Leica DM LB2 microscope connected to a computer system with the Leica DFC320 camera. Images were then loaded into the Image-J (U. S. National Institutes of Health, Bethesda, Maryland, USA) analysis program with the additional Cell Counter plugin installed. Once the image was loaded, every ChAT+ neuron within the PPTg was manually tagged by the experimenter and the total number of neurons on each section was was recorded by the program. This enabled the subsequent creation of a spreadsheet containing the number of ChAT+ neurons along the anterior-posterior plane of the PPTg in each

hemisphere. As the distance between the 1:4 series of sections (120 µm) is considerably greater than the diameter of mesopontine cholinergic neurons (believed to be no larger than 50 µm (Takakusaki et al., 1996)) the risk of counting the same neuron twice was considered low and therefore no correction to the raw count values was performed. It should be noted that cell counting was to provide an estimate of lesion size, not an exact number of neurons present. While photographing and counting the ChAT+ sections with the experimenter blind as to whether the rat was a lesion or sham appears a tight control, due to the lesions generally being extensive it would quickly be apparent from the sections themselves if the rat had a successful lesion or not. Therefore, while during counting no deliberate attempt was made to match the rat number to treatment group, slides and images were also not camouflaged with respect to rat number.

Behavioural Data Analysis

Statistical analysis was performed in SPSS versions 14.0 - 18.0 (SPSS Inc., Chicago, Illinois USA). The exact details of particular tests used are given in each chapter. For operant data (chapters 3 and 5) and locomotor data (chapters 4 and 6) various univariate and repeated measures ANOVAs were performed. Significant interactions were further investigated with a variety of post hoc tests or planned pairwise comparisons, where appropriate. The Huynh-Feldt correction was used to decrease the effect of heterogeneity of variance (assessed by Levene's test) and log 10 transformations were used to adjust for skew in the data (identified by the Shapiro-Wilk test). When used, these adjustments are always stated. T-tests were used to compare smaller single data sets (for example sucrose solution consumption in chapter 4), with particular details again always being given in individual chapters. All effects were considered statistically significant when $p \leq 0.05$.

Chapter 3: An examination of the role of the pPPTg in the learning and performance of action-outcome associations

Chapter introduction

Concise summary of action-outcome learning

Action-outcome (A-O) learning is the ability to form and update a relationship between the actions one performs and the outcomes (events) these actions produce. Once these associations have been formed, the actions can then be re-executed with the goal-directed aim of obtaining the outcome. As discussed in the general introduction this is an essential kind of learning (indeed, it is perhaps what is thought of first when considering learning). However, it is only one of several interacting and competing learning types, the main other one being stimulus-response (S-R) learning. S-R learning is the formation of automatically executed associations between stimuli and actions. Once the association is formed, the stimuli trigger the habitual execution of the actions without influence from factors such as the expected consequence or desirability of the outcomes these actions produce. Many brain systems are known to be involved in A-O learning and performance, central to which are the basal ganglia (Balleine et al., 2009) (of which the posterior dorsomedial striatum is critical (Yin et al., 2005b)), prelimbic cortex (Corbit and Balleine, 2003); mediodorsal thalamus (Corbit et al., 2003) and entrorhinal cortex (Corbit et al., 2002). These are all parts of an extended cortico-striatal circuitry critical for developing appropriate responses to new stimuli as well as the maintenance of habitual responding and the selection of appropriate actions in situations where there are multiple choices (Everitt and Robbins, 2005; Redgrave et al., 2011).

The PPTg and action-outcome learning

While numerous studies have reported learning deficits after PPTg lesion (see general introduction, pp20-24), two studies in particular are relevant in terms of action-outcome learning. Alderson et al. (2004) showed that rats with excitotoxic PPTg lesions and no prior

experience of operant tasks did not learn to lever press to receive an intravenous infusion of d-amphetamine. However, they showed no impairment if they had learned that lever pressing was rewarded (with food pellet) prior to lesion (Alderson et al., 2004). Wilson et al. (2009) found that pPPTg lesioned (but not aPPTg lesioned) rats were impaired at learning a FR1 schedule of reinforcement for food pellet reward, but that with significantly more training sessions than sham rats they were ultimately able to learn this task. However, when the reinforcement schedules changed to FR5, pPPTg lesioned rats were again slow to adjust their rates of lever pressing (Wilson et al., 2009a). What these results have in common is that the main deficits seen were at points where the formation (the naive group in the Alderson et al. (2004) study; the first FR1 testing sessions in the Wilson et al. study (2009)) and updating (immediately after schedule change in the Wilson et al study) of associations occurred. This strongly suggests that the core deficit in these rats is impairment in the formation and updating of action-outcome associations. However, these tasks are not well designed specifically to assess action-outcome learning. For example, both tasks (and indeed the majority of all instrumental leaning operant tasks) employ a light above the active lever which illuminates simultaneously with reward delivery. This light is deliberately used to aid learning by causing a salient change in the testing environment and signaling that the reward is now available. However, additions of cues such as this complicate the interpretation of the results as, for example, impairment in forming a stimulus-outcome association between the light and reward delivery would impair learning of the whole task. Indeed, the pPPTg lesioned rats in the Wilson et al. (2009) study showed increased 'late presses' (presses made after illumination of the lever light but before collecting the reward), perhaps suggesting that they were not successfully utilizing or responding to the light cue. Therefore, while these results strongly suggest an action-outcome impairment, the possibility that impairments other than those of action-outcome might have contributed to the poor performance of pPPTg lesioned rats

means clear assessment of the role of the PPTg in action-outcome learning cannot be drawn from these studies alone.

Assessing action-outcome learning

Within the rodent (and also used in primate and human) literature (eg: Shanks and Dickinson, 1991; Yin et al., 2005b; Liljeholm et al., 2011) two experimental paradigms have become standard use in the assessment of action-outcome learning. These are referred to as 'outcome devaluation' and 'contingency degradation'.

Outcome devaluation

In outcome devaluation experiments the aim is to assess the effects of manipulating the value of a reward on the level of behavior performed in order to obtain the reward. This is based on the premise that if behaviour is governed by goal directed action-outcome processes then devaluation of the reward will lead to a subsequent reduction in the performance of behaviour to obtain the reward. However, if this behaviour is governed by stimulus-response processes (and therefore not performed with the intent of obtaining the reward) then the behaviour will be insensitive to changes in reward value. There are many different versions of this paradigm, but they all share common features. Typically subjects are trained to perform an operant response (lever press) to receive an outcome (reward pellet or liquid). The value of this outcome is then reduced, either through satiation (induced by a free exposure session) or pairing with an aversive outcome (associating the reward with lithium chloride induced illness for example). Subjects' willingness to work for the reward is then re-tested; a reduction in lever pressing is taken as demonstrating intact action-outcome processing, whereas unchanged lever pressing is indicative of behaviour which is governed by stimulus-response mechanisms. A particularly elegant version of this paradigm involves training the subjects to

press on two different levers, each of which delivers a different and distinct reward (Yin et al., 2005b). One of these rewards is then devalued immediately before giving the subjects a free choice extinction session where the rates of lever pressing for both the devalued and valued (not manipulated) rewards are measured. This design allows for a tight within-subjects control: rather than comparing the effects of devaluation between a devalued and control group, the effects can be compared between the devalued and control reward within each subject.

Contingency degradation

The aim of contingency degradation is to manipulate the strength of the relationship between action and outcome, typically by delivery of rewards not contingent on action performance. This is based on the premise that if behaviour is governed by action-outcome processes then the delivery of 'free' rewards not dependent on any action will cause a reduction in actions associated with being required to obtain that reward. In other words: realizing that the reward no longer needs work to obtain it leads to a reduction in the amount of work done. As with outcome devaluation, there are many subtly different versions of this task. Typically subjects are trained to lever press for reward delivery on a very variable schedule of reinforcement (which maintains a degree of uncertainty between action and outcome). Once stable levels of performance are reached, the experimental manipulation is performed (for example lesion or, more commonly, temporary inactivation) and the subjects tested under conditions of reduced contingency between lever press and reward delivery (for example, by the random delivery of rewards not dependent on action). Subjects are then tested in an extinction test. The hypothesis here is that subjects with intact monitoring of the actions they perform and the outcomes they produce will be sensitive to this disjunction between action and outcome and accordingly reduce the number of lever presses they make. Subjects with impaired action-outcome updating will continue pressing the lever and continue

getting the outcome, but not notice that these two events are no longer linked. Again, and pioneered by the same laboratory as the outcome devaluation test described above, a particularly appealing version of this task exists. In this version subjects are trained with two levers, each giving one distinctive reward. The contingency between one lever and reward is then degraded, while the other lever-reward association is maintained (Yin et al., 2005b). Comparison of rates of lever pressing on the contingent versus non-contingent lever allows for a tight within-subjects assessment of performance.

In both of these paradigms attempts are made to reduce possible contributions from stimulus-response and other learning types. This involves using no programmed conditioned stimuli (for example lights or retraction of levers upon reward delivery) and keeping the testing and training regime as succinct as possible. The emphasis on keeping the training period short is due to extended or distributed training being known to induce habitual responding (Adams, 1982). As both of these paradigms require training to be conducted before the brain region of interest is manipulated, they are more suited to temporary inactivation (for example by the direct microinjection of the GABA agonist muscimol) than permanent lesions. Cannulae can be permanently implanted prior to any training and infusions of inactivating agents then made at the appropriate points within the testing regime. While it is possible to pre-train animals prior to lesion surgery and recovery, this introduces a delay period in an otherwise quick training protocol.

Chapter aims

The aim of this chapter was to specifically assess the role of the pPPTg in action-outcome learning using the outcome devaluation and contingency degradation paradigms developed by Balleine and colleagues (Yin et al., 2005b). Inactivation of the pPPTg was

achieved by means of direct microinjection of muscimol. The data for experiment 1 (outcome devaluation) were collected with Dr David Wilson; experiment 2 (contingency degradation) was conducted independently.

Inactivating the pPPTg

Various pharmacological agents have been reported successfully to inactivate the PPTg. Of these, the local anesthetics lidocaine (Pan and Hyland, 2005) and procaine (Nowacka et al., 2002) and the GABA agonist muscimol (Samson and Chappell, 2001; Diederich and Koch, 2005) are the most commonly used. There is concern that local anesthetics inactivate fibers of passage (Levy et al., 2001) which are unaffected by muscimol (Majchrzak and Di Scala, 2000). In addition muscimol also has a more constant and stable field of action than anesthetic agents (Martin, 1991). Muscimol, therefore, is a clear preference for use. There is a wide range of doses of muscimol reported in the PPTg literature (for example within one paper both 0.05 μg and 0.5 μg are used (Diederich and Koch, 2005)). This laboratory has recently developed a muscimol pPPTg infusion procedure which causes over-consumption of 20% sucrose solution but no detectable locomotor impairment (measured in photocell cages) (Wilson and MacLaren, unpublished data, (2008)). This dose of 0.05 ug in 0.3 uL saline is at the conservative end of the range of previously reported doses. However, as the effects on sucrose consumption and locomotion mirror those seen after ibotenic pPPTg lesion (Wilson et al., 2009a) and as higher doses caused a clear motor and orientating impairment (not seen after ibotenic lesion), this dose is believed to be the most effective and will be used here. Because muscimol is a full GABA-A, full GABA-C and partial GABA-B receptor agonist its effects are generally believed non-specific for neuronal type within PPTg. However, one laboratory has reported the interesting claim that within PPTg GAGA agonists may predominantly affect non-cholinergic neurons (Torterolo et al., 2002). They support this claim with reference to another

paper from their laboratory. Despite extensive searching of online and physical libraries, the journals archive and the personal webpages of the authors, no other record of this paper or another paper with a similar title has been found. Contacting all the authors resulted in no response. The current Editor-in-Chief of the journal (*Sleep*) confirmed that the referenced paper is not in the journal archives (Dinges DF, Personal communication, (2010)). In contrast to this, work in slice preparation (albeit from juvenile rather than adult rat) has shown that muscimol affects both cholinergic and non-cholinergic PPTg neurons (Bay et al., 2007). Other authors have also concluded that muscimol is likely to affect all PPTg neuronal sub-populations (Diederich and Koch, 2005). Therefore the evidence suggests that muscimol should inactivate the pPPTg without preference for one neuronal type.

Chapter 3, experiment 1: outcome devaluation

Experiment overview:

The behavioural training and testing protocol is illustrated in figure 3.1. Rats were implanted with guide cannulae aimed at the pPPTg prior to operant training. They were then trained to press on two levers (left and right) which both delivered the same low value pellet reward from the same reward magazine within the operant box. Once stable performance was reached they were infused with either saline or muscimol prior to an acquisition session where each lever lead to the delivery of a distinctive high value reward (sweet pellets and 20% sucrose solution). The following day one of these rewards (counterbalanced across rats) was devalued by giving free exposure to it in the homecage for 60 min. Immediately after this rats were tested (without microinjection) in an extinction session where no rewards were delivered. The rationale is that prior to the acquisition session rats learned that both levers were rewarded equally. They then had only one session (the acquisition session itself where

muscimol was active) to learn that the lever-reward associations had changed and each lever now delivered a unique reward. Devaluing one of the rewards should, in rats who had learned the lever-reward association and were governed by action-outcome processes, cause them to have higher rates of pressing on the lever associated with the non-devalued reward.

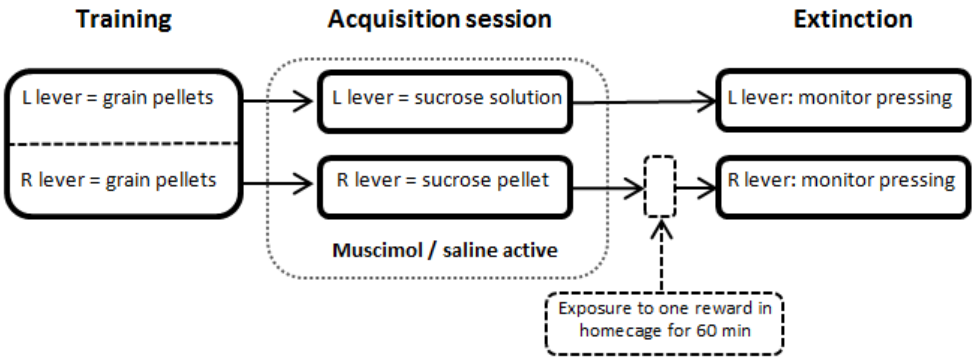


Figure 3.1: Schematic of the outcome devaluation paradigm.

Materials and Methods

Subjects

24 male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used in this experiment. Rats had a mean weight of 440g at the start of the experiment and were housed as described in the general methods. Seven days prior to behavioural testing food was restricted to 15 - 16 g/rat/day standard lab chow, 7 days a week. Throughout testing bodyweight was monitored daily to ensure it did not fall to below 85% of free-food weight.

Surgery

Rats were anaesthetized with isoflurane and placed in a stereotaxic frame as described in the general methods. Bilateral guide cannulae (22 ga, 3.8 mm apart, protruding 5.0 mm from

pedestal base; Plastics One, Roanoke, VA, USA) aimed at the pPPTg were implanted (as described in the general methods) at the following co-ordinates: +0.4 mm from the IAL; ±1.9 mm from the midline; 5.0 mm ventral from the dorsal surface of skull. Every 1 - 2 days following surgery dummy cannulae were removed and changed with clean replacements. Rats were given at least 7 days to recover from surgery prior to the start of operant training.

Operant Training

Operant training was conducted in the operant chambers described in the general methods. The features of the chambers used in these experiments were the 2 retractable levers on either side of the reward magazine, the reward magazine itself (which contained a food trough and a liquid solution cup allowing the delivery of both pellets and solution rewards) and the houselight on the top of the wall opposite to the levers and reward magazine.

Three days prior to testing rats were given 2 g of the reward which would be used as the low value training reward (grain pellets; Noyes Precision rodent food pellets, Formula A/I, 45 mg, Research Diets Inc., Brunswick, NJ, USA) in a small bowl in their homecage. This exposure was an attempt to reduce neophobia to the pellets. Operant training began the next day; the first session lasted 30 min and during this time pellets were delivered randomly at a rate of 1 pellet per min (Random Time [RT] 60). This was to familiarize the rats with the operant box and learn the location of the reward magazine. The following day instrumental training began. Rats were trained in 2 30 minute sessions a day (morning and afternoon) during which only 1 lever was present (counterbalanced across sessions). Starting with fixed-ratio 1 (FR1) schedule (where 1 lever press always produced a food pellet) they were advanced (once having met criteria, see table 3.1) onto a RR5 (Random Ratio 5: 0.2 probability of reward per lever press) schedule and then onto a RR10 schedule.

Schedule		Probability of pellet delivery upon lever press		Criteria to reach before advancing to next schedule
RT60		No programmed relation (1/60 probability of pellet delivery every second)		1 session
FR1		1		1 session of > 60 rewards earned
RR5		0.2		2 sessions of > 10 rewards earned
RR10		0.1		2 sessions of > 10 rewards earned
L lever = novel high value reward 1	R lever = novel high value reward 2	0.1	0.1	1 session (90 min)
Extinction		No pellets delivered		End of experiment

Table 3.1: Reinforcement schedules and criteria for advancement through schedules.

Learning of novel action-outcome associations

In an attempt to reduce neophobia to the high value rewards which would be used in the novel outcome acquisition session, the day before this session rats were given both of the novel outcomes (15 mL sucrose solution [20% sugar in water] and 2 g sweetened food pellets [Test Diet purified rodent tablet 5TUL, Sandown Scientific, Middlesex, UK]) in their homecage.

On the day of the novel action-outcome acquisition session rats received microinfusions into the pPPTg 20 min prior to the start of the operant session. Bilateral infusions of either muscimol (0.05µg in 0.3µL saline; Tocris Bioscience, Bristol, UK) or saline (0.3µL; Baxter Healthcare Ltd.) were made via bilateral internal cannulae injectors (3.8mm apart, protruding 7.5 mm from the base; Plastics One) using the infusion method described in the general methods. Following microinjection, rats were placed in their homecage (in the infusion room) for 20 min during which time they had no access to food or water before being taken to their operant box for the acquisition session.

During the acquisition session only 1 lever was extended at any time with left and right levers being automatically alternated every 5 min. Both levers were programmed on a RR10

schedule of reinforcement, one lever delivered 20% sucrose solution (0.1 mL) and the other a sweet reward pellet. The acquisition session lasted for 90 min, after which rats which had previously been infused with muscimol were infused bilaterally with saline, and rats previously infused with saline were infused bilaterally with muscimol (using the infusion procedure described in the general methods). Thus all rats received muscimol in the acquisition day; however half received it before, and half after, the acquisition session (for convenience, the group who received muscimol before the acquisition session and saline after it are referred to as the muscimol group; the group who received saline before and muscimol after the acquisition session are referred to as the saline group).

Outcome Devaluation

The following day rats were given 1 h of unlimited exposure to one of the high value rewards in their homecage. No infusions were given on this day: half of each previous treatment group (muscimol or saline) received 20% sucrose solution, the other half received sweet pellets. Pellets were given in full small bowl and sucrose solution in a full glass drinking bottle. Consumption of each of the rewards was measured by weighing the bottle/bowl before and after exposure. Immediately following exposure to one reward, rats were tested in a 5 min operant test under conditions of extinction where both levers were extended and no outcomes delivered. The number of lever presses on each lever was recorded and allowed both the measurement of presses on levers associated with sweet pellets and sucrose solution as well as the calculation of presses on levers associated with the devalued and the valued reward.

Histology

All histological procedures were performed as described in the general methods. After completing behavioural testing rats were transcardially perfused with fixative and brains stored

in sucrose solution. Subsequently, 30μm coronal sections were cut through the area of the PPTg and ~1mm beyond in anterior-posterior plane. Parallel 1:4 series were processed immunohistochemically for ChAT reactivity and mounted onto glass slides. Slides were viewed under a light microscope (Leitz Diaplan) and cannulae tip locations judged with reference to the densely packed cholinergic neurons of the pPPTg. Only rats where injector tips were located within the pPPTg were included in the subsequent analysis.

Behavioural Data Analysis

Statistical analysis was performed in PSAW 18.0 (SPSS Inc., Chicago, Illinois USA). ANOVAs were performed to compare lever pressing rates for pellet and solution levers, consumption during the free exposure session and pressing for valued versus devalued rewards (all within-subjects factors). These were compared across the between subjects factor of infusion group (muscimol versus saline) and outcome devalued (sweet pellets versus sucrose solution). When necessary the Huynh-Feldt correction was used to decrease the effect of heterogeneity of variance. Where significant interactions were found, these were investigated with post-hoc t-test. All effects were considered statistically significant when $p \leq 0.05$.

Results:

Histology

Of the rats that completed the behavioural paradigm all injector tips were found to be located within the pPPTg. Figure 3.2 shows the location of injector tips. Ten rats were excluded from analysis for either loss of headcap (n=6); due to developing erratic movements post-surgery (n=3); or due to being tested with faulty equipment for an unknown number of

sessions (n = 1). The high loss of headcaps is discussed later and successfully addressed in the next experiment.

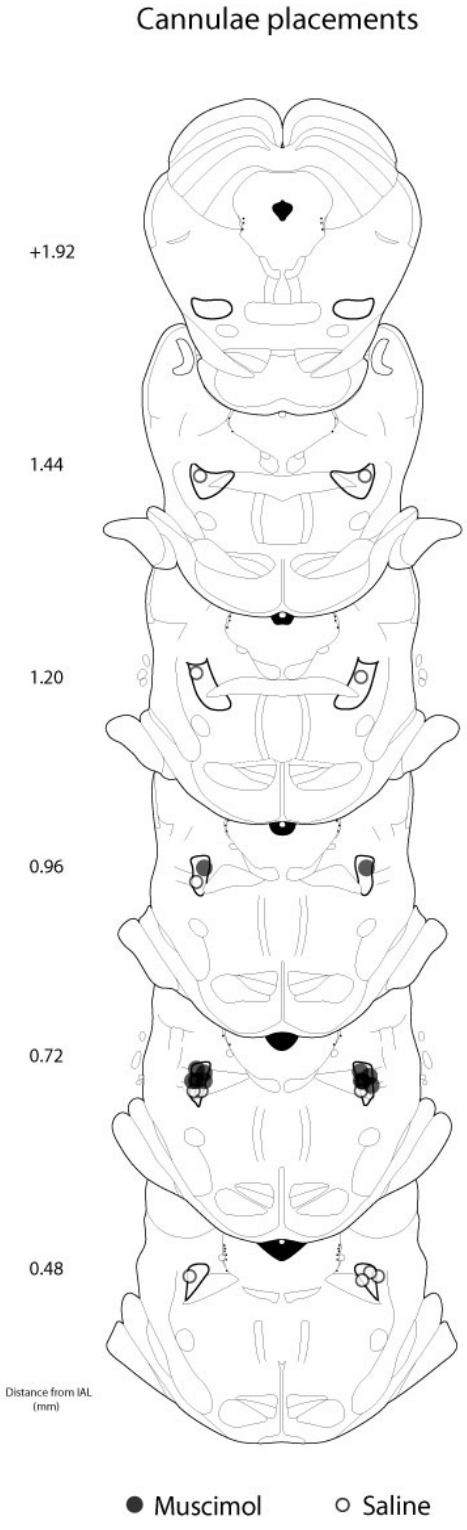


Figure 3.2: Illustration of the location of the bilateral injector tips, shown on coronal sections adapted from the stereotaxic atlas of Paxinos and Watson (2005). The location of each saline injector tip is represented by an open circle and each muscimol injector tip by a closed circle. The PPTg is outlined in bold, numbers indicate distance from the IAL (mm).

Training to lever press on low value rewards

During initial training all rats quickly learnt to lever press for grain pellets and reached criterion level of performance on the RR10 with a mean total training of 8 days. Once assigned to groups (saline, muscimol) multivariate ANOVA confirmed there were no pre-existing pre-infusion significant differences in the overall rate of lever pressing between groups or in the preference for either lever (effect of *lever* ($F_{1,12}$ = 1.44; p = 0.254); *lever * group* ($F_{1,12}$ = 0.47; p = 0.51); *group* ($F_{1,12}$ = 0.03; p = 0.96).

Lever pressing during acquisition session

During the acquisition session, where muscimol was active and the new rewards (sweet pellets and sucrose solution) were assigned to individual levers, a two way mixed ANOVA revealed a significant of *lever* ($F_{1,12}$ = 11.99; p = 0.005) and a *lever x group* interaction ($F_{1,12}$ = 4.98; p = 0.046) and no main effect of *group* ($F_{1,12}$ = 0.228; p = 0.64). Post hoc t-tests confirm that saline treated rats pressed significantly more on the pellet versus solution lever (t_6 = -2.98; p = 0.048) whereas muscimol treated rats showed no significant differential pressing (t_6 = 5.39; p = 0.17). See figure 3.3.

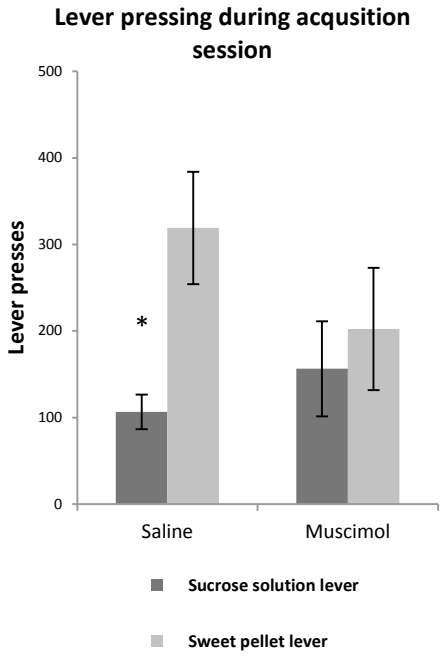


Figure 3.3: Lever pressing during the acquisition session. Graph shows group means ± SEM. See text for statistical analysis.

Reward devaluation

The amount of each reward consumed during the 1 hour free exposure session in the homecage is shown in figure 3.4. One way ANOVAs found no significant difference between the saline and muscimol groups in consumption of either sweet pellets ($F_{1,5}$ = 0.62; p = 0.46) or sucrose solution ($F_{1,5}$ = 1.39; p = 0.29).

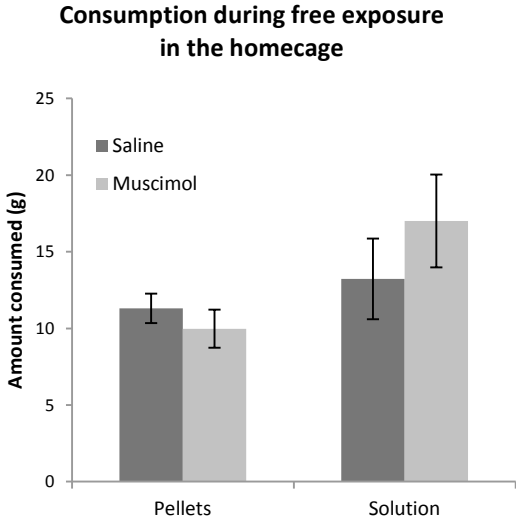


Figure 3.4: Consumption (in g) during the free exposure session. Graph shows group means ± SEM. See text for statistical analysis.

Extinction session

Rates of lever pressing during the extinction session were calculated for both pressing on the lever associated with particular rewards (sweet pellet and sucrose solution) and reward value (devalued = free exposure to reward in homecage; valued = no free exposure in homecage). These results are shown in figure 3.5. For the analysis of pressing for reward type (sweet pellet and sucrose solution), a two way mixed ANOVA revealed a significant of *lever* ($F_{1,12}$ = 14.37; p = 0.003) a significant *lever x group* interaction ($F_{1,12}$ = 9.02; p = 0.011) and no

main effect of *group* ($F_{1,12}$ = 0.13; p = 0.73). Post hoc t-tests confirm that rats in the saline group pressed significantly more on the pellet versus solution lever (t_6 = -3.63; p = 0.01) in contrast to rats in the muscimol group which showed no significant differential pressing (t_6 = -1.13; p = 0.30). For the analysis of pressing for reward value (valued and devalued), a two way mixed ANOVA found no significant effects (*lever value* ($F_{1,12}$ = 0.00; p=1.0) *lever value x group* ($F_{1,12}$ = 0.411; p = 0.53) *group* ($F_{1,12}$ = 0.13; p = 0.73). Additional t-tests confirm that neither rats in the saline or muscimol group pressed significantly more on either lever (saline: t_6 = 0.32; p = 0.76); (muscimol: t_6 = -1.72; p = 0.14). See figure 3.5.

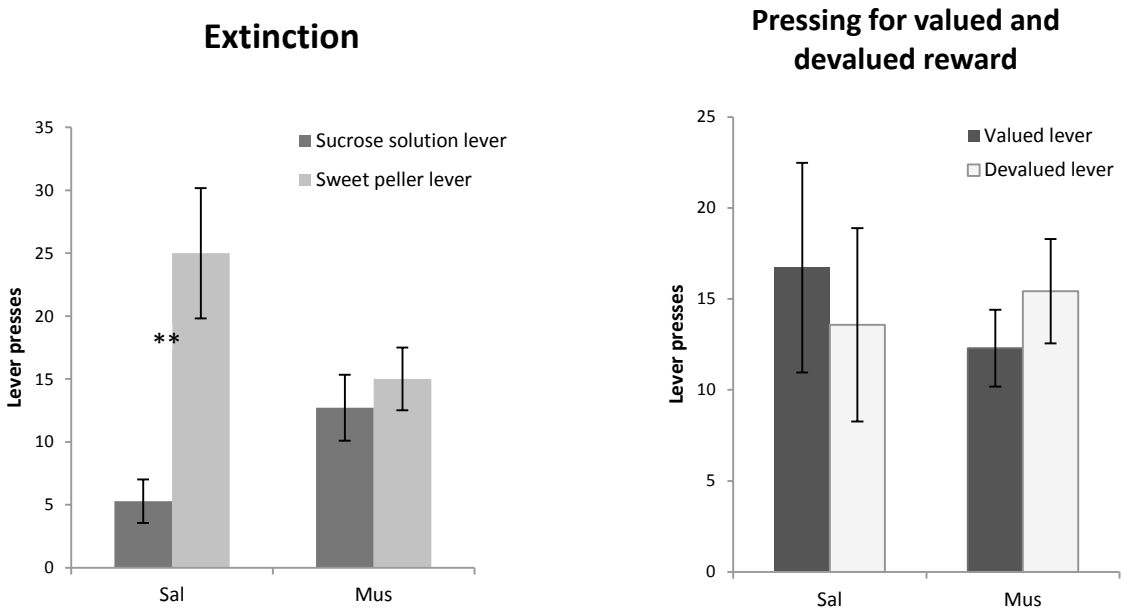


Figure 3.5: Lever pressing during the extinction session. The left graph shows pressing on the lever associated with sweet pellets or sucrose solution, the right graph shows pressing on the lever associated with either the valued or devalued reward. Graph shows group means ± SEM. See text for statistical analysis.

Summary of results

During the acquisition session saline treated rats developed significantly higher rates of pressing on the lever associated with the pellets compared to sucrose solution. Inactivation of the pPPTg had no effect on the overall rate of lever pressing but blocked the development of differential lever pressing: muscimol treated rats pressed equally on both levers. During the extinction session (when all rats were drug free) the same pattern was found, there was no effect of previous infusion treatment on the overall rate of lever pressing but saline treated rates again pressed significantly more on the sweet pellet associated lever compared to the sucrose solution lever, whereas muscimol treated rats displayed no difference between rate of pressing on either lever. Exposure to one reward in the homecage did not alter subsequent rates of lever pressing for valued or devalued rewards in either the saline or muscimol group.

Experiment discussion:

No effect of outcome devaluation

The most striking result is that the attempt to devalue one of the outcomes by giving free exposure in the homecage prior to the extinction test did not create an outcome devaluation effect in the saline treated rats. The standard interpretation of insensitivity to outcome devaluation is that the behavior has become habitual action governed by stimulus-response rather than action-outcome processes. However, a careful examination of the saline treated group suggests this is unlikely here. Prior to the acquisition session, rats were trained to press each of the two levers for a low value grain pellet reward and at this point they had equal rates of pressing on both levers. It was only during the 90 min acquisition session and subsequent extinction test that the pattern of preferential pressing on the lever now associated with the sweet pellets developed. If this were a stimulus-response habit then then

it must have formed for the pellet associated lever in the 90 min acquisition session. The rapid formation of a habit during this session but not during previous sessions seems highly unlikely, but cannot be excluded from the data here. However, another interpretation is that the rats preferred the sweet pellets over the sucrose solution, and once they had learned which lever delivered which reward, pressed significantly more on the lever associated with the sweet pellet reward. There are some arguments to suggest that the rats would prefer the sweet pellets to the sucrose solution. The sweet pellets were the same shape, size and texture as the low value pellet rewards used throughout training. They were simply a nice version of what the rats were used to. Moreover, they were delivered from the same location in the reward magazine as the training pellets. The sucrose solution was a new reward type (that the rats have only briefly experienced once in the homecage prior to the acquisition session) delivered from a different mechanism within the reward magazine. In the operant boxes used here, the rat must reach with his head into the entry of the reward magazine to collect the pellet or access (in a different location within the magazine) the recessed cup containing liquid. It has been shown that rats prefer to consume food from a familiar rather than novel food container (Mitchell, 1976) and observation of the rats when learning the reward location revealed that when equipped with a cannulae headcap assembly there was very little room for the rat to easily collect the reward without collision between the headcap and top of the reward magazine (this anecdotal evidence is supported by 3 rats losing their headcap assemblies in the operant boxes). Therefore, it is possible that rats were “anxious” about (or even did not notice) the new reward location and preferred to continue collecting the reward type they were familiar with collecting. The MED-PC equipment used does not have a means of verifying that the solution was consumed when delivered, so it is unknown if the rats found the solution after every (or any) delivery. Stronger evidence that the rats formed a preference for the reward type they were trained with comes from earlier unpublished work from this laboratory

(Wilson, D and MacLaren, D; unpublished results). We attempted the same experiment described here using a different reward combination. Rats were initially trained to lever press on both levers for 20% sucrose solution reward (rather than the grain pellets used here) and subsequently we used blackcurrant flavored Ribena and sweet pellets as the novel high value rewards. During the extinction test we found a significant preference for the lever that was associated with Ribena, and this preference was not affected by the free exposure to Ribena in the homecage prior to the extinction test. Thus, we saw the same preference for the reward type used during training and a lack of effect of attempted reward devaluation, but for the opposite (liquid rather than dry) reward type.

Why did the devaluation effect not replicate?

There are 2 main possible reasons for the lack of devaluation effect. (1) It is possible that the preference formed for the pellets during training and the acquisition session was strong enough to override the effects of devaluation through exposure in the homecage. While the use of two distinct rewards is a powerful paradigm, it requires that the consumption which occurs during the devaluation exposure session in the homecage satiates the rat specifically on that one reward (sensory specific satiation) rather than inducing general satiation for all calorific/sweet rewards. Moreover, it requires that sensory specific satiation induced in the homecage is maintained in the operant box. From the early days of assessment of outcome devaluation, issues with sensory specific satiation have been evident. It has been found that in rats trained to lever press for saccharin reward, free exposure to saccharin in the homecage had no effect on the subsequent rates of lever pressing on a saccharin associated lever, however, free exposure to dextrose (which had never been presented in the operant box, these rats were also trained on saccharin) significantly reduced rates of lever pressing on the saccharin lever (Holman, 1975). This effect cannot be explained by general satiation induced

by the calorific dextrose, as when saccharin consumption was measured in the homecage, previous pre-exposure to either saccharin or dextrose both reduced (and reduced equally) the amount of saccharin subsequently consumed. These results show that satiation can reduce levels of lever pressing, but highlight that the specificity of the satiation is hard to predict. (2) It is possible that the rewards used in this experiment were not sufficiently different to induce sensory specific satiation. The exact rewards used in the original experiment are not available in the United Kingdom (hence the substitution for seemingly similar rewards) which may have inadvertently compromised the reliability of the devaluation effect. However, the finding of development of a reward preference for the reward type used during training in this and the earlier attempt using different rewards suggests it is a systematic problem of the experimental paradigm rather than an unfortunate choice of mal-matched rewards.[1]

Effects of inactivation of the pPPTg

Notwithstanding the attempted devaluation having no effect on sham rats subsequent rates of lever pressing, these rats did develop clear differential pressing in both the acquisition and extinction test, an effect that was completely blocked by inactivation of the pPPTg. Three interpretations of this effect are possible: (1) inactivation blocked the formation of a S-R habit in response to the sweet pellet associated lever; (2) inactivation blocked the preference for sweet pellets, rendering both rewards equally desirable; and (3) inactivation blocked learning that the action of lever pressing now led to the outcome of different rewards being delivered. Option (1) can be discounted using the argument above – that the formation of a S-R habit during the acquisition session does not appear to be a satisfactory explanation of the effect seen in the saline treated rats. Option (2) is possible, but previous lesion data show that PPTg

[1] At the 2010 (San Diego) Society for Neuroscience conference I spoke with 1 and at the 2011 (Washington DC) Society for Neuroscience conference I spoke with 2 other researchers who were attempting the same outcome devaluation behavioural paradigm and had also encountered the problem of control rats forming preferential lever pressing that was insensitive to reward devaluation.

lesion does not affect reward discrimination, perceived reward value or contrast effects (Olmstead et al., 1999; Taylor et al., 2004) suggesting that PPTg inactivation is unlikely to affect reward preference formation. The third interpretation – that pPPTg inactivation blocked learning that the levers were now associated with different rewards – is the interpretation most in keeping with previous studies showing clear learning impairment after pPPTg lesion. If rats with pPPTg inactivation were unable to learn that the lever-outcome associations had changed they would continue pressing equally on each lever (as they had done during training) – which is exactly what was found here.

Experiment conclusions

A full assessment of the role of the pPPTg in action-outcome learning cannot be made on the basis of the results of this experiment. However, these results show that inactivation of the pPPTg blocks the behavioural changes in response to modifications in the relationship between actions and outcomes without affecting the expression of previously learnt action-reward associations. The most parsimonious interpretation of this being that rats with pPPTg inactivation were unable to learn that the lever-reward associations had now changed.

In order to address fully the role of the pPPTg in action-outcome learning, two possibilities emerge: either attempting to modify the outcome devaluation paradigm, or running another behavioural task (for example a contingency degradation experiment). It is possible that with different rewards and even a different reward delivery mechanism that no preferential lever pressing would develop and the standard outcome devaluation effect would be seen. In order to enhance the devaluation effect an additional option would be to pair one reward with illness induced by lithium chloride (Adams and Dickinson, 1981). However, subjecting rats to lithium induced malaise when other measurements of action-outcome

learning exist seems an unnecessary step. In contingency degradation experiments all the training and manipulations are conducted in the operant box so there is no need to induce sensory specific satiation in the homecage. While the version of this task used by Balleine and colleagues again has two levers associated with two distinct rewards (Yin et al, 2005), it should be possible to modify this experiment by using one reward and a between-subjects rather than within-subjects design. In this modification all rats are trained with one reward, and in certain groups of rats the contingency between lever pressing and reward delivery is degraded before all rats are tested in an extinction test. As this successfully addresses the two main issues experienced with the outcome devaluation experiment, and as contingency degradation is a valid and widely used measure of action-outcome learning, it was considered preferable to attempting to modify the outcome devaluation paradigm.

Chapter 3, experiment 2: contingency degradation

Experiment overview:

The contingency degradation paradigm used here is a modification of that used by Yin et al. (2005). Rats are implanted with guide cannulae before operant training begins. After recovery from surgery, training starts with a FR1 schedule and, once criterion performance has been reached (see table 3.2 for details) rats are advanced through ever increasing RR schedules until stable performance on a RR20 schedule is reached. At this point rats are split into control and inactivation groups and further split into contingent and non-contingent groups (see figure 3.6 for schematic of experimental design). Rats are then tested in 3 contingency degradation sessions where they are infused with muscimol or saline prior to being trained on a contingent or non-contingent reinforcement schedule (according to group designation). The contingent schedule is the same RR20 schedule as the final training session,

in the non-contingent schedule a comparable number of pellets are delivered throughout the session, but delivered in a non-contingent manner such that the probability of pellet delivery in each second is equally likely whether the rat has responded appropriately or not. Finally, all rats are tested (without infusion) in an extinction session where no rewards are delivered.

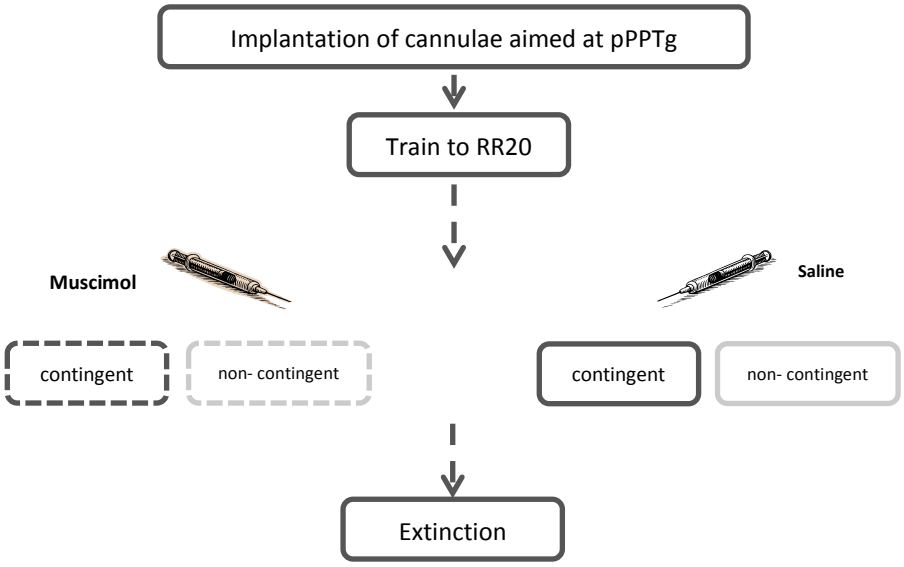


Figure 3.6: Schematic of the contingency degradation paradigm.

Materials and Methods

Subjects

48 male Lister Hooded rats (Harlan Olac Ltd, Bicester, UK) with a mean surgery weight of 404g were used in this experiment. Rats were maintained as described in the general methods. Prior to surgery and during recovery rats had free access to food and water. Four days prior to behavioural testing food was restricted to 15 g/rat/day standard lab chow, 7 days

a week. Throughout testing bodyweight was monitored daily to ensure it did not fall to below 85% of free-food weight.

Surgery

Rats were anaesthetized with isoflurane and placed in a stereotaxic frame as described in the general methods. Bilateral guide cannulae (22 ga, 3.8 mm apart, protruding 5.0 mm from pedestal base; Plastics One, Roanoke, VA, USA) aimed at the pPPTg were implanted (as described in the general methods) at the following co-ordinates: +0.4 mm from the IAL; ±1.9 mm from the midline; 5.0 mm ventral from the dorsal surface of skull. Every 1 - 2 days following surgery dummy cannulae were removed and changed with clean replacements. Rats were given at least seven days to recover from surgery prior to the start of operant training. In order to try and overcome the high loss of headcaps experienced in experiment 1 of this chapter, new screw types were used in an attempt to add more support to the headcap assembly. These were 2 x 0-80 X 3/32 stainless steel mounting screws (Plastics One, Roanoke, VA, USA) in front and behind the cannula pedestal and 2 x 0-80 X 1/8 stainless steel mounting screws (same supplier) in front of bregma. Despite being longer than the previous screws, care was taken to ensure they did not penetrate further through the skull. This modification appeared successful as no further headcaps were lost.

Operant Training

Operant testing was conducted in the operant chambers described in the general methods. The features of the chambers used in this experiment were the 2 retractable levers either side of the reward delivery magazine and the houselight on the top of the wall opposite the levers and magazine.

Two days prior to training each rat twice received 1 g of testing pellets (Test Diet purified rodent tablet 5TUL, Sandown Scientific, Middlesex, UK) in a small bowl in their homecage in an attempt to reduce neophobia to the pellets. On the first day of operant training, 25 pellets were freely available in the reward magazine, no levers were extended and the doors to the sound and light attenuating boxes were left open. Once all the pellets were consumed (~20min) rats were returned to their homecage. This allowed the rats to become familiar with the operant box and learn the location of the reward delivery. Daily training sessions (40 min duration) began the following day, starting with a FR1 schedule. Once rats had met the criteria for performance on FR1 (see table 3.2) they were advanced onto a random-ratio 5 schedule (RR5; a 1:5 probability that a single pellet would be delivered per lever press), then onto random-ratio 10 (RR10) and random-ratio 20 (RR20) schedules. Pressing on the inactive lever (side counterbalanced across rats) was recorded but had no programmed consequence.

Contingency degradation testing

Once rats had met criteria on the RR20 schedule they were randomly assigned to either the saline or muscimol group, then further assigned to a contingent or non-contingent subgroup. Thus final groups were: (1) Saline – contingent. (2) Saline – non-contingent. (3) Muscimol – contingent. (4) Muscimol – non-contingent. The 'contingent' groups were subsequently trained in a regime where pellet delivery was still dependent on lever pressing on a RR20 schedule (i.e. no change in contingency from previous training). The 'non-contingent' groups were trained in a regime where pellet delivery was not dependent on lever pressing. Each second there was a 1:34 probability of pellet delivery whether the rat responded appropriately or not. Training sessions lasted 20 min and were started 15 min after the appropriate saline / muscimol infusion. Infusions were made as described in the general

methods. Training during these sessions was conducted every other day to ensure full clearance of drug.

Schedule		Probability of pellet delivery upon lever press		Criteria to reach before advancing to next schedule
FR1		1.0		2 sessions of > 70 rewards earned
RR5		0.2		2 sessions of > 60 rewards earned
RR10		0.1		2 sessions of > 20 rewards earned
RR20		0.05		2 sessions of > 20 rewards earned
Contingent	Non-contingent	0.05	No relation	3 sessions
Extinction		No pellets delivered		End of experiment

Table 3.2: Training regime and criteria for advancing through schedules.

Extinction test

In the session after the third contingency training session rats were tested, without infusion, in a 20 min extinction test. In the same manner as all training and testing sessions both levers were extended and the houselight illuminated, however no rewards were delivered.

Histology

All histological procedures were performed as described in the general methods. After completing behavioural testing rats were transcardially perfused with fixative and brains stored in sucrose solution. Subsequently, 30 µm coronal sections were cut through the area of the PPTg and ~1mm beyond in anterior-posterior plane. Parallel 1:4 series were processed immunohistochemically for ChAT reactivity and mounted onto glass slides. Slides were viewed under a light microscope (Leica DM LB2) connected to a desktop computer system by a high resolution camera (Leica DFC320). Cannulae tip locations were judged with reference to the

densely packed cholinergic neurons of the pPPTg. Only rats where injector tips were located within the pPPTg were included in the subsequent analysis.

Behavioural Data Analysis

Statistical analysis was performed in PASW 18.0 (SPSS Inc., Chicago, Illinois USA). Repeated measures ANOVA were performed across the degradation training sessions. Univariate ANOVAs were performed to compare pressing rates between the saline and muscimol contingent and non-contingent groups on the final day of pre-training and during the extinction test. In cases of significant group differences or interactions, these were investigated with univariate ANOVAs, protected planned pairwise comparisons and Bonferroni corrected t-tests, where appropriate. All effects were considered statistically significant when $p \leq 0.05$.

Results

Histological results

Of the rats which completed behavioural testing, 29 were found to have cannulae tips located within the pPPTg, giving final group sizes of: saline n = 14 (contingent n = 7; non-contingent n = 7); muscimol n = 15 (contingent n = 8; non-contingent n = 7) The location of cannulae tips is depicted in fig 3.7. The remaining rats were excluded from all analysis due to: complications with infusions (n = 4); tissue damage causing lesion (n = 4); cannulae missing the pPPTg (n = 2).

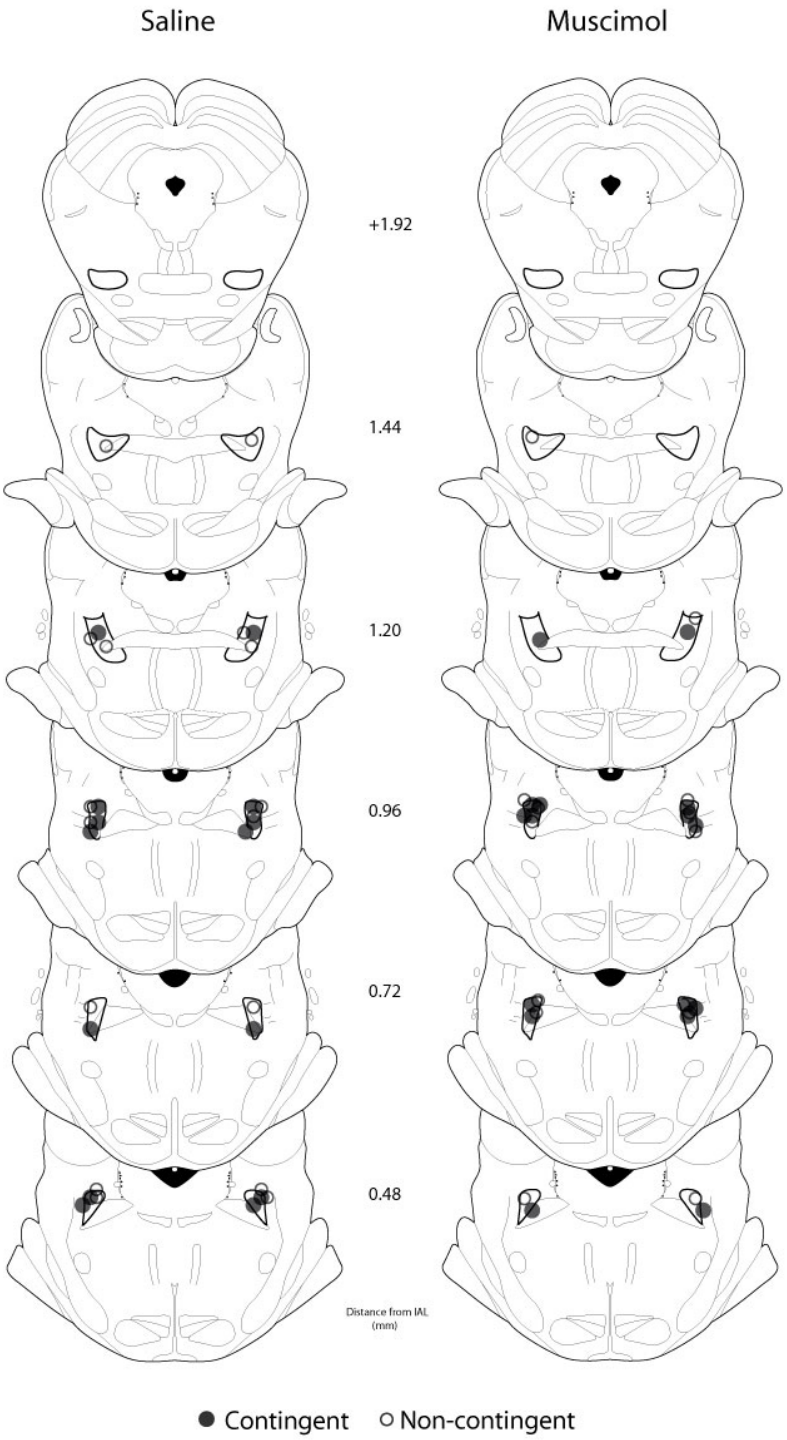


Figure 3.7: Illustration of the location of the bilateral injector tips, shown on coronal sections adapted from the stereotaxic atlas of Paxinos and Watson (2005). The location of each injector tip is represented by a closed circle (contingent group) or an open circle (non-contingent group) with saline treated rats in the left column and muscimol treated rats on the right. The PPTg is outlined in bold, numbers indicate distance from the IAL (mm).

Behavioural results

Training

Rats reached criteria level on the RR20 training schedule in a mean of 11 (S.E. 0.27) sessions. Multivariate ANOVA confirmed there were no group differences at this pre-infusion point.

Contingency degradation testing

Repeated measures ANOVA across the three degradation training sessions found no main effect of group, but a significant effect of *session* ($F_{2,50}$ = 3.56; p = 0.036) and a *group x session* interaction ($F_{6,50}$ = 3.30; p = 0.008). To investigate the effect of session, corrected paired samples t-tests were performed comparing rates of lever pressing on the first and last session. These found that pressing in the saline non-contingent group was significantly less during session 3 compared to 1 (t_6 = 5.39; p = 0.008), but that no other groups had a significant change in rate of lever pressing. Univariate ANOVA of the last session found a main effect of *group* ($F_{3,25}$ = 3.53, p = 0.029) and restricted pairwise comparisons revealed that the difference between saline contingent and non-contingent lever pressing was significant (p = 0.005) whereas the difference between the muscimol contingent and non-contingent pressing was not significant (p = 0.294). Furthermore, the difference between saline and muscimol contingent pressing was also not-significant (p = 0.399). Results are summarized in table 3.3.

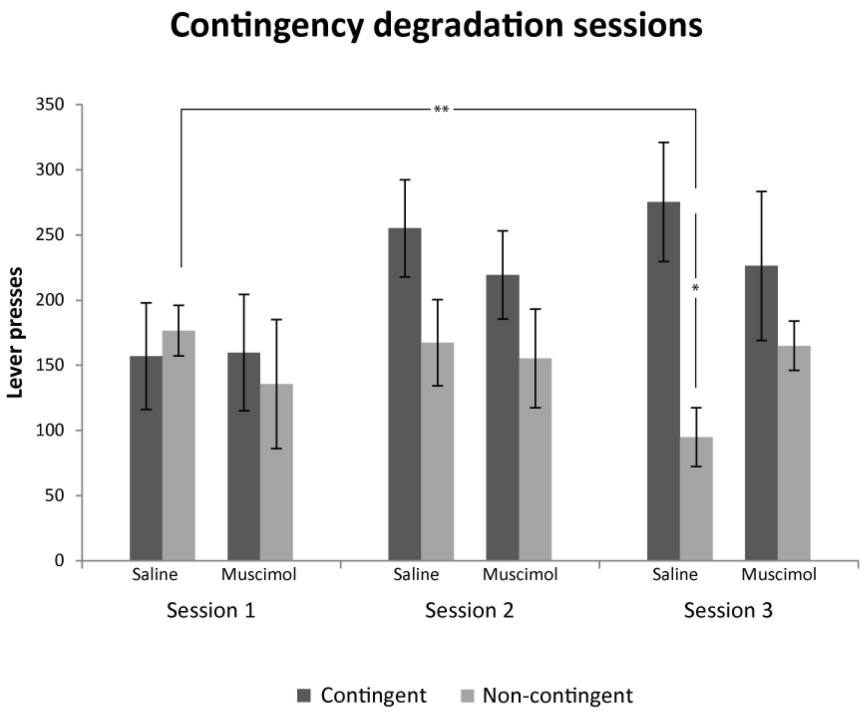


Figure 3.8: Mean number of lever presses on the active lever for each group during each of the three contingency degradation training sessions. Error bars show SEM.

Extinction test

Performance during the extinction test is shown in figure 3.9. Univariate ANOVA showed a main effect of *group* ($F_{3,25}$ = 4.61, p = 0.011), restricted pairwise comparisons confirm that saline non-contingent lever pressing was significantly lower than saline contingent (p = 0.018) and that in muscimol treated rats the difference between contingent and non-continent pressing was not significant (p = 0.906). The difference between saline contingent and muscimol contingent pressing was also not significant (p = 0.488).

Performance during the extinction test

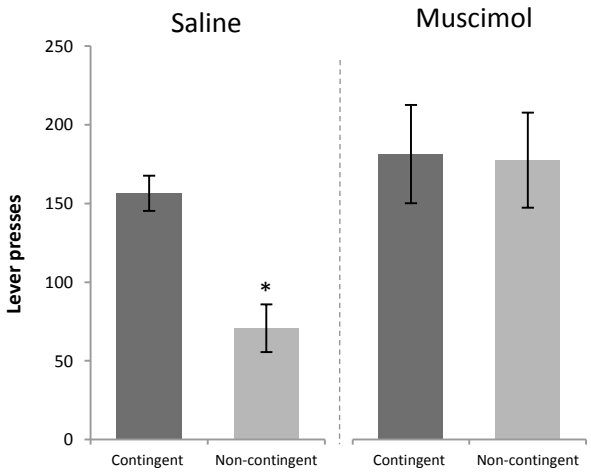


Figure 3.9: Mean number of lever presses on the active lever for each group during the extinction test. Error bars shown SEM.

Results summary

		Change during degradation training	Performance on last day of degradation	Performance in the extinction test
Saline	Contingent	—	Significant difference (p=0.005)	Significant difference (p=0.018)
	Non-contingent	Significant reduction (p=0.008)		
Muscimol	Contingent	—	—	—
	Non-contingent	—		

Table 3.3: summary of main results, "—" indicates no significant effect.

These results show that inactivation of the pPPTg blocks sensitivity to contingency degradation, both during degradation training and the extinction test, while having no effect on performance of previously learnt contingent lever pressing.

Chapter discussion

This chapter was conducted with the aim of assessing the role of the pPPTg in action-outcome learning. Experiment 1 used an outcome devaluation paradigm. While this failed to show an outcome devaluation effect in control rats, it successfully demonstrated that inactivation of the pPPTg had no effect on overall rates of lever pressing but did block the development of preferential lever pressing in response to changes in lever-reward associations. The most parsimonious explanation of this is that inactivation of the pPPTg blocked the formation of new associations between lever pressing and reward delivery. However, the lack of reward devaluation effect in the control rats and possibility of other explanations meant that conclusive interpretations about the role of the pPPTg in action-outcome learning could not be made solely on the results of that experiment. Experiment 2 used a contingency degradation paradigm. Saline treated rats were highly sensitive to degradation of instrumental contingency, reducing their rates of non-contingent lever pressing across degradation training sessions and in the extinction test. Inactivation of the pPPTg completely blocked this sensitivity to degradation of contingency, with rates of lever pressing in pPPTg inactivated rats remaining unchanged across degradation training sessions and in the extinction test. As in experiment 1, inactivation of the pPPTg had no effect on overall levels of lever pressing, and there was no difference between saline contingent and muscimol contingent lever pressing. This shows that pPPTg inactivation did not produce a general deficit in lever pressing but instead specifically blocked the modification of an established pattern of lever pressing in response to changes in the relationship between lever pressing and reward delivery. More specifically, inactivation of the pPPTg blocked the updating of action-outcome associations, but had no effect on task performance in conditions where these associations remained unchanged.

Having established the involvement of the pPPTg in action-outcome learning, before fully interpreting these results in the broader literature a further question will be addressed: which neuronal sub-populations of pPPTg neurons are involved in learning? As discussed in chapter 1 (pp5-10) the pPPTg is comprised of an interdigitated collection of cholinergic, glutamatergic and GABAergic neurons. Of particular interest in the assessment of learning are the projections to midbrain DA, which are predominantly cholinergic and glutamatergic. As the muscimol induced inactivation used in these experiments is likely to have affected all neuronal populations, conclusions about neuronal type are not possible. No agent for creating a selective transient inactivation of one neuronal PPTg subtype exits. However, the development of the fusion toxin Dtx-UII has enabled the creation of selective cholinergic PPTg lesions (see general introduction, p15). The contingency degradation paradigm used here, while successful for assessing action-outcome learning, is not well suited for lesion studies due to the need to create the lesion at a specific point in the testing protocol. Moreover, as the Dtx-UII selective cholinergic lesion takes 21 days to develop fully, the interruption in testing would therefore be longer than the overall testing regime. Given that one aim of the assessment of action-outcome learning is to keep behavioural training short (to reduce the possibility of interference from other learning systems which have been shown to develop over distributed training (Adams, 1982)) this delay would compromise the interpretation of the results.

While not suited for contingency degradation studies, the Dtx-UII lesions are suited for assessing instrumental learning in more conventional paradigms where the lesion is performed before any behavioural training begins and rats subsequent performance in learning is assessed. The behavioural protocol of that used by Wilson et al. (2009) has several advantages over others: (1) This protocol involves initially training rats (post-surgery) on an FR1 reinforcement schedule, and then once criteria levels of performance have been reached, advancing them through increasing fixed and variable ratio schedules. This allows for a

detailed assessment of initial learning rate; the rate of behavioural change in response to changes in the relationship between lever pressing and reward delivery and the effects of the systematic introduction of variability into this relationship; (2) This is one of the few published studies with separate lesions of pPPTg and aPPTg. pPPTg (but not aPPTg) lesions produced a clear learning impairment during FR1 and after the switch FR5. While pPPTg lesioned rats did ultimately perform this schedule at the same level as shames, when they were then switched to higher variable schedules (VR10-30) persistently low levels of performance were evident. By replicating this behavioural assessment with pPPTg Dtx-UII lesions, direct comparison between selective cholinergic and the previous results with non-selective excitotoxic lesions can be made and from that conclusions about the role of the cholinergic neurons in learning may be drawn; (3) I conducted the Wilson et al. (2009) experiment with Dr David Wilson as part of my Research Assistant position at the University of St Andrews, therefore I am very familiar with the experimental procedure and the data.

However, before conducting a behavioural experiment with Dtx-UII lesions, it was necessary to modify the lesion technique. As is discussed in the next chapter, published results with Dtx-UII lesions show a successful unilateral lesion of the whole (anterior and posterior) PPTg (Clark et al., 2007). Results of attempts at bilateral lesions were mixed, with cell loss being low and often unequal between hemispheres (Rostron et al., 2007). In the subsequent experiment this technique was refined with the aim of creating highly selective bilateral cholinergic lesions of the pPPTg in group sizes large enough for behavioural testing.

Chapter 4: Development of a procedure for making bilateral selective cholinergic neuron lesions in the pPPTg

Selective cholinergic lesions within PPTg

As discussed in the general introduction (p15), the fusion of diphtheria toxin to urotensin-II created a toxin (Dtx-UII) which, when microinjected directly into the PPTg, selectively destroyed (through internalisation and protein synthesis inhibition) cholinergic neurons while leaving surrounding non-cholinergic neurons intact (Clark et al., 2007). This is the toxin which will be used in subsequent experiments in this thesis. However, before conducting any behavioural tests the correct infusion technique for creating selective bilateral cholinergic pPPTg lesions needs to be established.

The need for bilateral lesions with a high neuronal loss

Current lesion methods

The Clark et al. (2007) study reported highly selective unilateral lesions of around 85% of cholinergic PPTg neurons with minimal non-selective damage. There are currently no published reports of bilateral Dtx-UII lesions. Prior experience from this laboratory of making bilateral lesions using the same technique of multiple infusions along the length of PPTg has resulted in substantial cholinergic PPTg loss, but also often considerable non-selective damage. Adjustment of the technique by reducing the number of infusions and concentration of toxin reduced the extent of non-selective damage, but also the amount of cholinergic loss (with 30-50% cholinergic survival frequently found) and increased the proportion of rats which had no indication of any lesion. Unpublished studies (conducted as part of my undergraduate project) found that rats with lesions of this size had no impairment in the delayed spatial win shift task on the 8 arm radial maze (MacLaren et al., 2007). This task is badly affected by bilateral ibotenate lesions of the PPTg (Keating and Winn, 2002). However, because of the level of cholinergic survival and small final group sizes (a result of many rats having no sign of lesion)

the results of the study were largely inconclusive. It could not be determined if these lesions produced no deficit or if behavioural changes were masked by compensatory mechanisms as a result of only partial cell lesions and/or low statistical power due to the small post-histology group size. Consequently, in order to assess more fully the role of cholinergic pPPTg neurons it is essential that the lesions are highly destructive to cholinergic pPPTg neurons (while maintaining selectivity) and the possibility of functional compensation should be considered.

The need for bilateral lesions

One solution to the problem of creating selective cholinergic bilateral PPTg lesions would be to return to the Clark et al. (2007) method and create unilateral PPTg lesions. However, there are very few reports of behavioural changes after unilateral PPTg lesion. Where reported (eg Dunbar et al., 1992) these changes are in drug induced locomotion rather than cognitive tasks. It has been shown that unilateral ibotenic lesion of the PPTg had no effect on learning the 2-way active avoidance task, but the addition of a contralateral (but not ipsilateral) MPTP lesion of the SNc produced a pronounced impairment (Bortolanza et al., 2010). In cases where authors intending bilateral PPTg lesions have reported a detailed assessment of both histological and behavioural results, this has also often revealed that essentially unilateral PPTg lesions (due to failure to achieve a bilateral lesion, for example by missing PPTg in one hemisphere) produced no deficit in a reinforcement based operant task (Olmstead et al., 1998) and only a mild impairment on the 5-choice serial reaction time task (Inglis et al., 2001); tasks which were both affected by bilateral PPTg lesions. This is not entirely surprising, the extensive contralateral projections within PPTg and to afferent and efferent structures mean that a unilateral PPTg lesion is likely to leave no target structure entirely without connection to contralateral PPTg (Winn, 2006). Thus, while the lesion may be nearly

complete in one hemisphere of the PPTg, in terms of effects on function it may be comparable to a bilateral partial lesion.

The need for extensive lesions

The effects of compensatory mechanisms as a result of incomplete lesion are of considerable concern. Little is known about compensation as a result of PPTg cholinergic loss. However, using another brain system – the dopaminergic nigrostriatal pathway – as a model, the likelihood of compensation can be considered. The dopaminergic system has been widely studied due in substantial part to its involvement in Parkinson's Disease (PD). PD is characterised by progressive loss of DA cells in the SNc, and while far from the only pathology of this condition, it is the most studied (Halliday et al., 2011). One striking feature is the extent of DA cell loss before behavioural effect is evident. It is estimated that in humans symptoms do not show until >80% of SNc DA cells have degenerated (Singh et al., 2007). Animal models confirm this, selective depletion of the DA nigrostriatal pathway (with the catecholamine selective neurotoxin 6-hydroxydopamine (6-OHDA)) produces a condition very similar to idiopathic PD (bradykinesia, sensorimotor neglect, aphagia, adipsia, short-step locomotion, postural abnormalities, and cognitive dysfunction) only when cell loss is near complete (Castaneda et al., 1990; Perez et al., 2008). Smaller lesions produce a transient deficit after which the animal recovers many behavioural functions (Robinson et al., 1994). Three stages of lesion and compensation have been suggested (Robinson et al., 1990): (1) lesions smaller than 80% have been found to leave striatal DA levels no different to controls, with the proposed compensatory mechanism being an increase in DA output from remaining cells. (2) Lesions in the range of 80-95% produced more marked and long lasting behavioural effects and, while there was some recovery of function, there was also persistent impairment. This recovery is believed to be a result of both pre-synaptic (increased DA output) and post-synaptic

(supersensitivity of DA receptors, reduction in DA reuptake and increased permeability of DA) compensation, leading no near normal levels of post-synaptic DA. (3) Lesions greater than 95% showed little evidence of successful compensation: behaviourally rats never regained normal function and neurochemically DA levels were significantly below those of smaller lesions and sham controls. It is believed that with lesions of this size no amount of pre- and post-synaptic plasticity can compensate for the number of DA cells lost. Less is known about compensatory mechanisms in the cholinergic system. However, evidence from selective cholinergic lesions of the cholinergic basal forebrain suggests that very similar compensatory mechanisms might develop. Small and large lesions of cholinergic NbM resulted in much smaller loss of Ach in efferent structures than would be predicted from lesion size alone, with the compensation developing over time and with more effective compensation arising from smaller rather than substantial lesions (de Lacalle et al., 1998; Waite and Chen, 2001). These studies highlight the need for substantial lesions in the region of >80% loss of neurons and for the proper consideration of compensation even to large lesions. Moreover, they emphasise that surviving cell number in the lesion site is not necessarily a reliable indicator of post-synaptic neurotransmitter level. Direct measures of assessing residual transmitter level include microdialysis (Robinson et al., 1994), voltammetry (Blaha et al., 1996; Perez et al., 2008), and tissue punching (Jenkins et al., 2002). Frequently used indirect measures of upregulation and other compensatory changes include analysing the response to a drug challenge, for example by administering a receptor agonist and comparing the behavioural changes in sham and lesioned rats (Hudson et al., 1993; Alderson et al., 2008) . Chapter 6 utilises such a paradigm to investigate the possibility of upregulation after Dtx-UII pPPTg lesion.

Area of PPTg to target

Rather than creating selective cholinergic lesions of the whole PPTg, there are compelling reasons to target only the pPPTg: (1) the pPPTg is the region of particular interest in this thesis: behaviourally it is known that the aPPTg and pPPTg are involved in different functions, the behaviours which will subsequently be investigated with Dtx-UII lesions are affected by ibotenate lesions of the posterior, but not anterior PPTg (Alderson et al., 2008; Wilson et al., 2009a). The subsequent experiments also relate to and extend the results of chapter 3, which again focused on the pPPTg. (2) With ibotenic acid the pPPTg can be targeted with a single toxin infusion in each hemisphere (Wilson et al., 2009a). There is some (anecdotal) evidence from this laboratory that part of the problem with non-selective damage experienced after Dtx-UII lesions is due to the overlapping of infusions and subsequent high concentrations of toxin when delivered into several sites in close proximity along the PPTg. Targeting the pPPTg with one infusion removes this possibility of toxin accumulation.

Chapter aim

The following experiments are an attempt to further refine the selective cholinergic PPTg lesion procedure. The specific aim is to establish a method of creating bilateral selective cholinergic pPPTg lesions in the region of 90+% loss of ChAT cells with group sizes large enough for rigorous behavioural analysis.

Toxin pilot study 1

Recent experience with creating only very small cholinergic PPTg lesions with the stock of Dtx-UII held in St Andrews raised concern about its potency. Having been stored at -20^{o}C rather than the recommended -70^{o}C (due to lack of suitable equipment) for a period of over 2

years it was considered to be in too poor condition to use. A fresh supply of freeze-dried Dtx-UII was acquired from SD Clark (Department of Pharmacology, University of California, Irvine, CA, USA) reconstituted as per the recommended method and stored at -70^{o}C. The co-ordinates for the pPPTg that will be used are those which we also used for ibotenic acid in the Wilson et al. (2007) study. These co-ordinates are somewhat unusual in the PPTg literature in that rather than fixing the rat in the flat skill position they involve angling the rats nose upwards in the stereotaxic frame. This builds on pilot work conducted by Dr Claire Rostron and involves calculating the height to raise the incisor bar to by measuring the distance between the IAL and the incisor bar and multiplying this value by the sine of 8˚ 29' (as described by Whishaw et al., 1977). Determining the height to raise the incisor bar individually for each rat maintains a constant angle of elevation across rats of varying sizes. The main advantage of infusing toxin from this angle is that the craniotomy and toxin infusion are further away from the superior sagittal sinus which sits above the pPPTg in the flat skull position, and therefore reduces the risk of surgical complications arising from severe blood loss. However, it is also hypothesised that because of the shape of the pPPTg, targeting it from this angle increases the likelihood of being able to destroy the entire pPPTg (several years after conducting these pilot studies another laboratory directly investigated the differences between the flat skull and raised nose co-ordinate systems and concluded that the raised nose system does indeed lead to greater cholinergic cell loss and fewer surgical complications (Cyr et al., 2011)). In order to establish an effective concentration and volume of toxin, the first experiment of this chapter tested a range of volumes and concentrations covering and extending those used by Clark et al. (2007). When using excitotoxins, the PPTg is generally targeted in two unilateral procedures in an attempt to reduce post-surgery mortality during the recovery period (Wilson et al., 2009a). As Dtx-UII is a protein synthesis inhibitor based toxin, cell death occurs gradually and does not progress rapidly until several days after surgery (Clark et al., 2007). Therefore, the surgical recovery

period is not noticeably different to that of a sham infused animal and so with Dtx-UII both hemispheres are infused during the same surgical procedure. It is plausible that during the period of maximal cell loss (7-14 days post-surgery) adverse health effects would become evident and therefore the rats were monitored closely during this period.

Toxin pilot study 1: methods

12 adult male Lister-Hooded rats were used with a mean surgery weight of 364g (S.D. 22.2; range 330-397g). Rats were housed, anaesthetised and lesion surgery performed as described in the general methods. The co-ordinate for the pPPTg was: -0.8 mm from the IAL, ± 1.9 mm from the midline, -6.5 mm below dura and the order of infusion (left hemisphere, right hemisphere) was alternated across rats.

Doses of toxin

The following concentrations and volumes of toxin were used:

Toxin concentration (diluted in sterile PB)	Volume infused	Group size
3.0%	400nL	2
3.0%	600nL	2
4.0%	400nL	3
5.0%	400nL	3
Control	No infusion	2

Table 4.1: Volumes and concentrations of Dtx-UII toxin used in toxin pilot study 1.

Recovery

All rats recovered well from the surgical procedure. By 7 days post-surgery several rats were losing bodyweight and required additional wet mash and sucrose solution to maintain weight at >85% of pre-surgery weight. All of these rats were from the 4.0% and 5.0% Dtx-UII group. At 14 days post-surgery these rats were unable to maintain a stable body weight and were displaying clear signs of stress (porphyrin discharge around the eyes). At this point all rats were perfused (as described in the general methods). The initial aim was to perfuse at 21 days post-surgery as is it is reported that the lesion is not fully formed until this point (Clark et al., 2007). However, due to problems maintaining bodyweight and the ethical concerns of the stress signs it was necessary to euthanize these rats before day 21. While it would have been possible to only perfuse the rats with low weight, in order to directly compare the effects of different doses of toxin it is necessary that all rats are perfused at the same time point post-surgery, otherwise the effects of a longer lesion formation period cannot be separated from the effects of a higher concentration of toxin with shorter lesion formation period.

Histology

Following the procedures described in the general methods, rats were transcardially perfused with fixative and brains stored in sucrose solution. Subsequently, 30 μm coronal sections were cut through the area of the PPTg and ~1mm beyond in anterior-posterior plane. Parallel 1:4 series were processed immunohistochemically for ChAT and NeuN reactivity. NeuN stained sections were then counterstained with cresyl violet and all stained sections mounted onto glass slides and viewed under a light microscope. Software assisted counts of ChAT+ neurons throughout the PPTg enabled quantification of the cholinergic lesions. Non-cholinergic damage was assessed by visual inspection of the NeuN/cresyl stained sections.

Results

Of the 20 infusions, 17 hit the pPPTg; the remaining 3 were too posterior (n = 2) or too dorsal (n = 1). A clear relationship between concentration of toxin and lesion was found and is illustrated in figure 4.1. In the 5.0% toxin group extensive non-selective damage was seen in and around the pPPTg on the NeuN/cresyl stain, more extensive that that seen after ibotenic pPPTg lesions (180nL of 0.12M concentration) (Wilson et al., 2009a) indicating a highly non-specific lesion. In these rats the ChAT stain revealed that the cholinergic pPPTg was almost entirely destroyed (estimated <2% cells surviving). The 4% Dtx-UII group displayed signs of less severe but nonetheless extensive non-selective damage and again near total loss of ChAT+ neurons. Within the 3% toxin group, the 600nL volume produced considerably less damage on the NeuN stain than the higher concentrations, and the 400nL volume produced only small or no signs of non-selective damage. Analysis of the ChAT stain showed that, as with the higher concentrations, there was extensive, near total, destruction of the cholinergic pPPTg.

Toxin concentration	ChAT	NeuN	Conclusions
3% (400 nL)	Near total pPPTg loss	Occasional small indications of neuronal loss, but cell survival throughout PPTg	Selective cholinergic lesion
3% (600 nL)	Near total pPPTg loss	Non-selective damage evident	Unsuitable due to non-selective damage
4% (400 nL)	Near total pPPTg loss	Non-selective damage evident	Unsuitable due to non-selective damage
5% (400 nL)	Near total pPPTg loss	Clear non-selective damage	Unsuitable due to non-selective damage
Control	pPPTg present	pPPTg present	Control rats

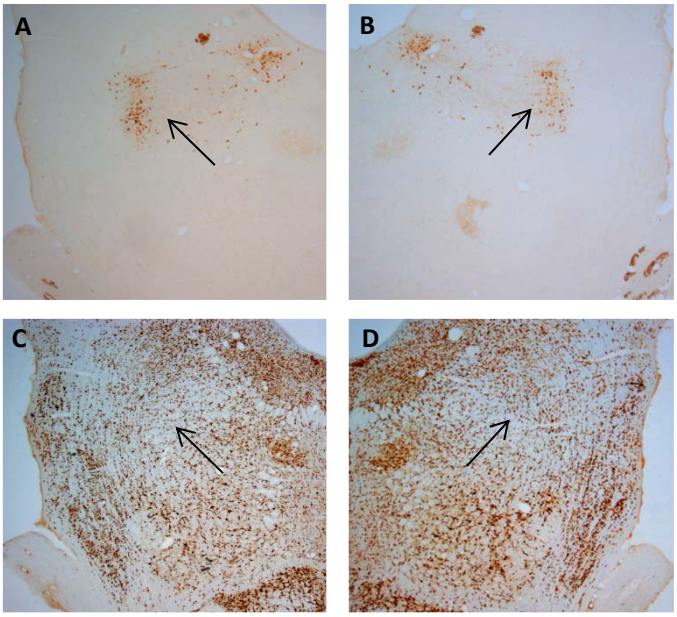
Table 4.2: Summary of histological results from pilot experiment 1.

Control

Images from a control brain.

The top row (A,B) shows ChAT stained and the bottom row (C,D) NeuN/Cresyl stained sections.

Arrows indicate the location of the pPPTg

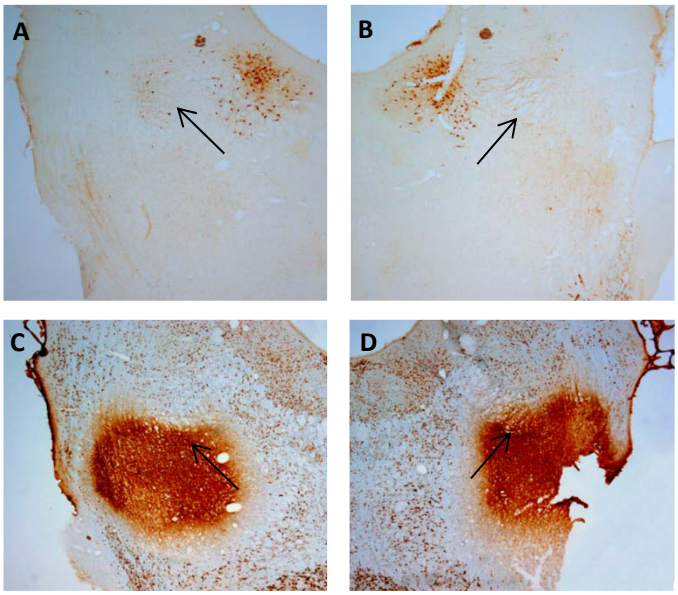


5% Dtx-UII

Images from a brain infused with 400nL of 5% Dtx-UII into the pPPTg.

The top row (A,B) shows ChAT stained and the bottom row (C,D) NeuN/Cresyl stained sections.

Note the near total loss of cholinergic pPPTg neurons and the extensive neuronal damage on the NeuN/Cresyl sections.



4% Dtx-UII

Images from a brain infused with 400nL of 4% Dtx-UII into the pPPTg.

The top row (A,B) shows ChAT stained and the bottom row (C,D) NeuN/Cresyl stained sections.

Note the near total loss of cholinergic pPPTg neurons and clear signs of cell loss on the NeuN/Cresyl sections.

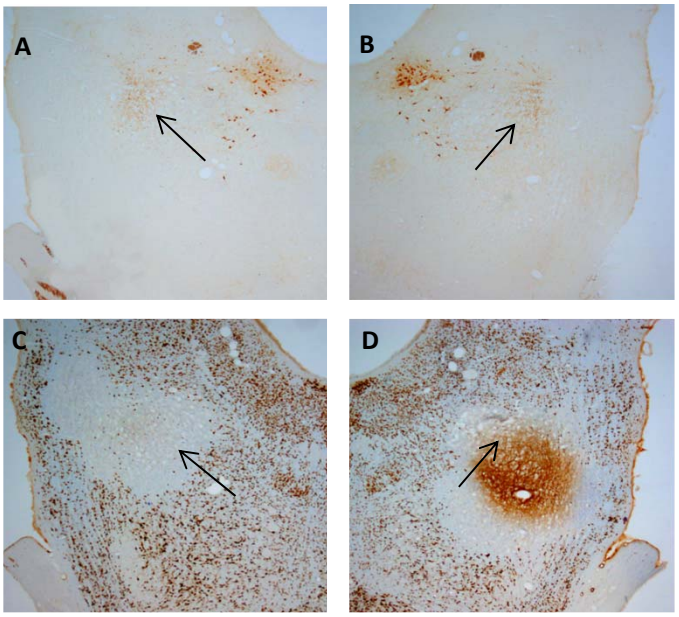


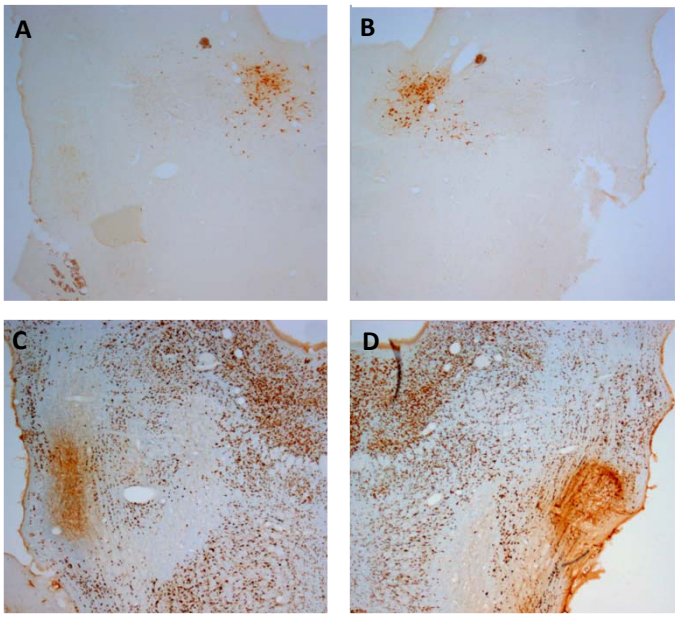
Figure 4.1: Effects of varying the concentration and volume of Dtx-UII

3% Dtx-UII – 600 nL

Images from a brain infused with 600nL of 3% Dtx-UII into the pPPTg.

The top row (A,B) shows ChAT stained and the bottom row (C,D) NeuN/Cresyl stained sections.

Note the near total loss of cholinergic pPPTg neurons and the damage on the NeuN/Cresyl sections.

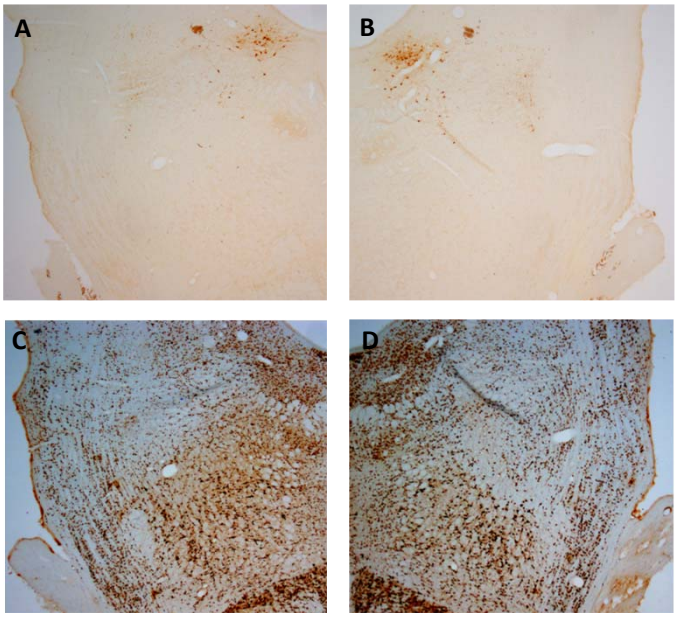


3% Dtx-UII – 400 nL

Images from a brain infused with 400nL of 3% Dtx-UII into the pPPTg.

The top row (A,B) shows ChAT stained and the bottom row (C,D) NeuN/Cresyl stained sections.

Note the near total loss of cholinergic pPPTg neurons and lack of clear indications of lesion on the NeuN stain.



Characterisation of the lesion

The number of cholinergic neurons throughout the PPTg of the 400nL 3% Dtx-UII group (ie the group with selective cholinergic lesions) was quantified (as described in the general methods) and compared to that of a control group (comprised of the 2 control rats in this pilot study and 2 shams from the subsequent pilot in this chapter). The distribution of surviving cholinergic neurons along the anterior-posterior plane is shown in figure 4.2. The 3.0% 400 nL group were found to have a 96% loss of ChAT+ pPPTg neurons (when calculated for the whole PPTg, this gives an overall ChAT+ loss of 78%).

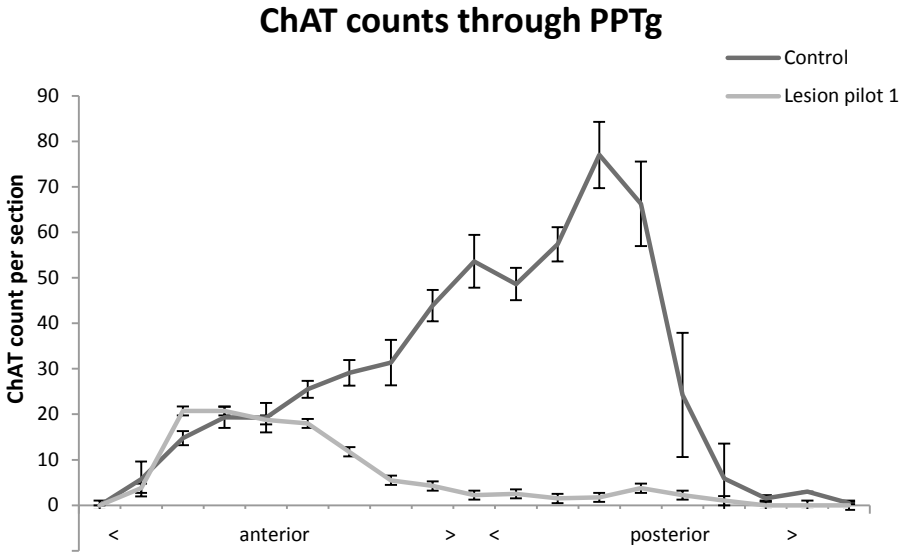


Figure 4.2: Distribution of ChAT+ neurons along the anterior-posterior plane of the 3.0%/400nL lesion and control groups. ChAT counts = Numbers of ChAT+ neurons. Graph shows group means ±SEM

Conclusions of pilot study 1

This pilot study verified that the new batch of Dtx-UII, delivered from a glass pipette with rats' noses elevated in stereotaxic frame, can successfully create selective bilateral cholinergic pPPTg lesions. That total near total ChAT+ pPPTg cell loss (96% in the 3.0% group which had selective lesions) was seen at 14 days post-surgery suggests this method is more successful at destroying cholinergic neurons than previous attempts, which only reduced unilateral cell count by ~85% after 21 days (Clark et al., 2007). Moreover, this study confirms that bilateral infusions of 3.0% toxin can be performed in the same surgical procedure without adverse effects during recovery (the problems with significant weight loss in the lesion formation period were only in rats with non-selective lesions in the 4.0% and 5.0% toxin groups). Having established an effective concentration of toxin, the next aim was to verify that this can be reproduced in a larger group size.

Toxin pilot study 2

Pilot study 1 revealed that 3.0% toxin can create selective cholinergic pPPTg lesions. It also suggested that the volume of toxin infused was important, with 600nL causing evidence of non-selective damage and 400nL producing largely selective lesions. However, due to the small group sizes and short (14 day) survival period a full analysis of the lesion and the effects of volume infused was not possible. To establish the reliability and reproducibility of these lesions the second pilot study used a larger group size and ensured the lesion formation period was > 21 days. To further investigate the effects of the infusion volume and to try and eliminate non-selective damage the previous 400nL and a new 300nL volume were used. Furthermore, simple behavioural tests (consumption of sucrose solution and measurement of spontaneously generated locomotion) were performed. It has been shown that excitotoxic pPPTg and whole

PPTg lesions caused overconsumption of 20% sucrose solution and no locomotor impairment (measured in photocell cages) (Alderson et al., 2001; Winn, 2006; Wilson et al., 2009a) and it was of interest if the same pattern emerged after selective cholinergic pPPTg lesions. It was predicted that locomotion would be unaffected by the lesion; there were no clear predictions as to whether sucrose consumption would be affected.

Methods

28 adult male Lister-Hooded rats were used in this experiment, with a mean surgery weight of 313.4g (S.D. 13.9; range 286-339g). Rats were housed, anaesthetised and lesion surgery performed as described in the general methods. The co-ordinate for the pPPTg was: -0.8 mm from the IAL, ± 1.9 mm from the midline, -6.5 mm below dura (the same as pilot experiment 1) and the order of infusion (left hemisphere, right hemisphere) was alternated across rats.

Doses of toxin

The following concentrations and volumes of toxin were used:

Toxin concentration (diluted in sterile PB)	Volume infused	Group size
3.0%	300nL	10
3.0%	400nL	10
Vehicle only control	300nL	4
Vehicle only control	400nL	4

Table 4.3: Concentrations and volumes of toxin used in the second Dtx-UII pilot study

Recovery

All rats recovered well from the surgical procedure. During the 21 day lesion formation period 3 rats (2 from the 400nL group and 1 from the 300nL) required wet mash to maintain a stable body weight. Inspection of these rats revealed that their lower incisor teeth were considerably longer than normal and were pressing into their upper jaw. The named veterinary surgeon was contacted and clipped elongated teeth back to normal length. Two of these rats then started eating wet mash and maintained a stable weight; the third rat (which had the most severe tooth growth and weight loss) failed to maintain bodyweight and was perfused 6 days later.

Locomotor testing

Spontaneously generated locomotion was measured in the photocell cages described in the general methods. Testing sessions lasted 40 min and each rat was tested on 2 consecutive days. Testing was conducted under dim illumination during the rats light phase.

Sucrose solution consumption test

Rats were individually housed and given access to a bottle containing 20% w/v sucrose solution (in tap water) alongside their standard water bottle. Sucrose solution and water consumption were measured over 18 h.

Histology

Following the procedures described in the general methods, rats were transcardially perfused with fixative and brains stored in sucrose solution. Subsequently, 30µm coronal sections were cut through the area of the PPTg and ~1mm beyond in anterior-posterior plane.

Parallel 1:4 series were processed immunohistochemically for ChAT and NeuN reactivity. NeuN stained sections were then counterstained with cresyl violet and all stained sections mounted onto glass slides and viewed under a light microscope. Software assisted counts of ChAT+ neurons throughout the PPTg enabled quantification of the cholinergic lesions. Non-cholinergic damage was assessed by visual inspection of the NeuN/cresyl stained sections.

Behavioural data analysis

Behavioural data were statistically analysed using SPSS 14.0 for Windows (SPSS Inc., Chicago, Illinois USA). Locomotor data were square root transformed to correct for positive skew and analysed with a repeated measures ANOVA with *day* and *group* as factors. Sucrose solution consumption data were analysed with a one way ANOVA. All results were considered statistically significant when $p \leq 0.05$.

Results

Histological results

6 rats had bilateral cholinergic lesions of the pPPTg with either very small or no indications of non-selective damage on the NeuN sections. Within these 6 rats there was considerable variance in the extent of the lesions which ranged from 96% to 55% cell loss. 4 of these rats had received 300nL of toxin per hemisphere and 2 had received 400nL per hemisphere (see table 4.4 for summary). A more detailed analysis revealed that rather than forming a gradient of different lesion sizes across the 55-96% range, two clear patterns of lesion emerged: lesions were either highly destructive (destroying ~95% ChAT pPPTg neurons) or were only moderately destructive (destroying 20-55% ChAT+ pPPTg neurons). See figures 4.4 and 4.5. Of the remaining rats, 11 had bilateral pPPTg cholinergic lesions with non-selective

damage either in or immediately behind the pPPTg, of these 8 had received 400nL of toxin per hemisphere and 3 had received 300nL. 3 rats had a unilateral or partial lesion, all had received 300nl of toxin. There was no indication of lesion of in any sham operated rat. Only data from the six rats with selective cholinergic pPPTg lesions were used in the behavioural analyses reported.

Histological results summary

Toxin concentration	ChAT	NeuN	Conclusions
3% (300 nL)	7/10 rats had near total pPPTg loss. 3/10 had partial or unilateral lesions.	4/10 rats had little or no evidence of non-selective damage. 3/10 had evidence of non-selective damage 3/10 had unilateral or no damage	4/10 successful bilateral selective cholinergic pPPTg lesions. 3/10 cholinergic lesions also with non-selective damage. 3/10 partial lesions or missed infusions.
3% (400 nL)	10/10 rats had near total loss of pPPTg.	2/10 rats had little or no evidence of non-selective damage. 8/10 had evidence of non-selective damage.	2/10 successful selective cholinergic bilateral pPPTg lesions. 8/10 cholinergic lesions which also had non-selective damage.
Control (300nL and 400nL)	pPPTg present	pPPTg present	Control rats

Table 4.4: Summary of toxin pilot 2 histological results.

Characterisation of the lesion

The distribution of ChAT+ neurons throughout the PPTg of the rats with successful bilateral cholinergic lesions is shown in figure 4.3 and 4.4.

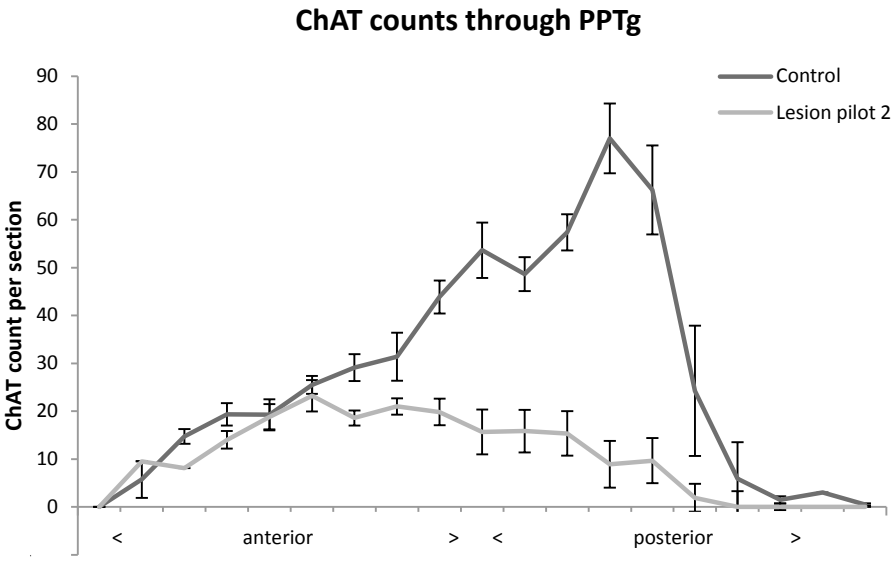


Figure 4.3: Distribution of ChAT+ neurons along the anterior-posterior plane of the 3.0% / 400nL lesion and control groups. ChAT counts = Numbers of ChAT+ neurons. Graph shows group means ±SEM.

The mean pPPTg ChAT cell loss in the lesion group was 62%. However, the lesion group was further split into lesions which produced substantial ChAT+ cell loss (>90) or only partial ChAT loss (20-55%). The results of this subdivision are shown in figures 4.4 and 4.5.

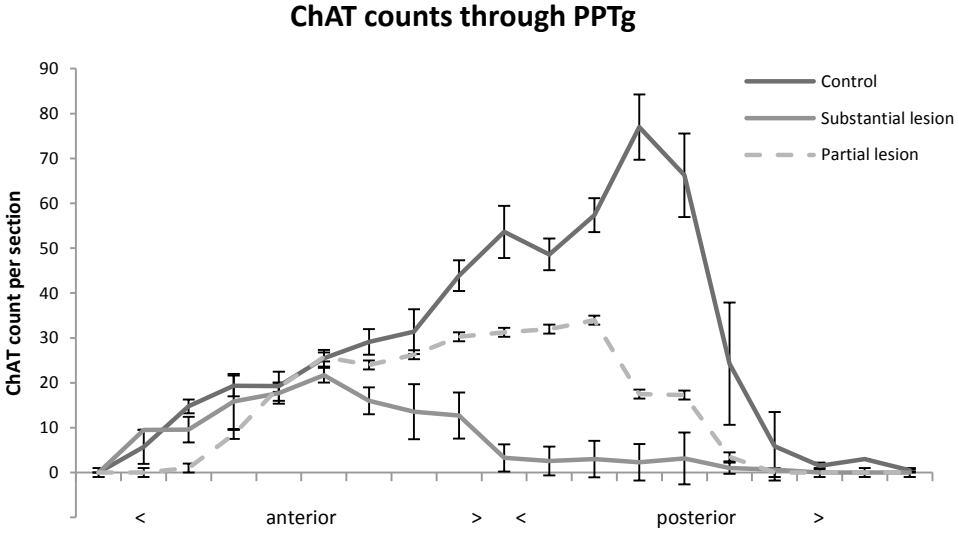


Figure 4.4: Distribution of ChAT+ neurons along the anterior-posterior plane of substantial, partial and control lesions. ChAT counts = Numbers of ChAT+ neurons. Graph shows group means ±SEM.

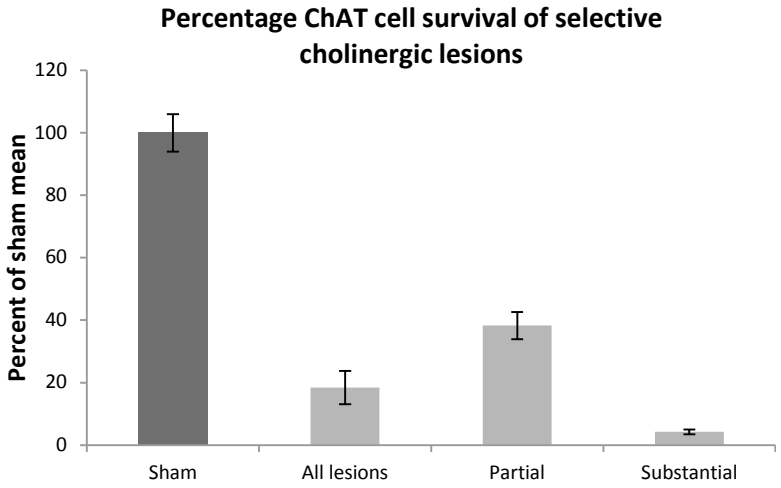


Figure 4.5: Percentage of ChAT+ pPPTg neurons in the partial, substantial and combined (all lesion) groups. Graph shows group means ±SEM.

Behavioural results:

Locomotor testing

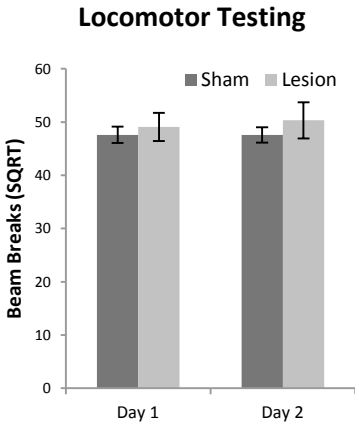


Figure 4.6 Mean number of beam breaks (square root transformed). Graph shows group means ±SEM.

The mean number of beam breaks (SQRT transformed) made during 2 locomotor testing sessions are shown in figure 4.6. Repeated measures ANOVA found no effect of *day* ($F_{(1,12)}$ = 0.126, p = 0.729) *group* ($F_{(1,12)}$= 0.683, p = 0.425) or *day x group* interaction ($F_{(1,12)}$ = 0.124, p=0.731). Rats with selective cholinergic pPPTg lesions had the same levels of spontaneous locomotion as sham controls.

Sucrose solution consumption

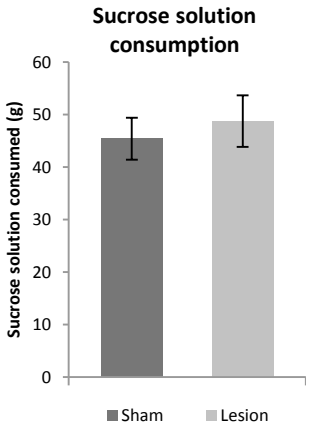


Figure 4.7: Consumption of 20% sucrose solution in the homecage. Graph shows group means ±SEM.

The amount of sucrose solution consumed during exposure in the homecage is shown in figure 4.7. A univariate ANOVA confirmed that there were no significant differences between the groups ($F_{(1,12}$ = 0.284, p = 0.604).

Conclusions from pilot study 2

Feasibility of selective bilateral pPPTg lesions

Of the rats which had bilateral selective cholinergic lesions of the pPPTg all recovered well from surgery and maintained health throughout the lesion formation and testing period,

confirming that that bilateral selective cholinergic lesions can be made without adverse effects on health. The 3 rats which struggled to maintain bodyweight and developed abnormal tooth shape were all found to have non-selective damage in and around the pPPTg. The tooth growth was a curious effect. Apart from the long teeth and weight loss these rats displayed no other signs of ill health or stress (confirmed by the unit NACWO). One possible explanation is that through reduced orofacial movement (caused by reduced eating and tooth grinding) the teeth grew without being worn away. However, the named veterinary surgeon advised that he has never seen tooth growth that rapid, even in rats that are not eating. That 2/3 of the rats resumed eating as soon as the teeth were clipped also suggests that the long teeth were preventing eating rather than the lack of desire to eat causing the teeth to grow. A second explanation was that the teeth were inadvertently damaged in surgery when fixing the rat in the raised incisor bar. However, that this tooth growth was only seen after Dtx-UII infusion (moreover only in rats with non-selective Dtx-UII lesions), and never after sham (or in separate experiments not reported here, ibotenic acid) infusion using the same procedure suggests that it was the lesion rather than surgery itself which was the cause. This tooth growth was seen to some extent in subsequent batches of surgery, but in parallel with also creating lesions with less non-selective damage it became less frequent and less severe. It does however remain an unexplained phenomenon.

Extent of partial lesions and non-selective damage

While the second pilot study confirmed that 3.0% Dtx-UII can reliably create highly selective cholinergic lesions of the pPPTg, the persistence of occasional partial lesion and non-selective damage in some rats was a disappointment. There is a clear pattern for 300nL of toxin per hemisphere to produce less non-selective damage than 400nL, however even in this group 30% of rats had unusably high levels of damage on the NeuN stain. In order to try and

further reduce the amount of non-selective damage future experiments used 300 nL and a lower volume of 200 nL. There is concern that at some point the volume will be too low to cover the entire pPPTg, however as 180 nL of ibotenic acid can destroy the pPPTg (Wilson et al., 2009a) 200 nL, at least in terms of spread, should still be sufficient to cover the pPPTg. There is no clear explanation as to why some lesions produced a partial loss (20-55%) of ChAT+ neurons rather than the extensive loss (~95%) seen in the remainder of the rats. It is possible that this was due to mis-placement of the pipette, but, where detectable, the pipettes appear to have hit the pPPTg. There appears to be no solution to the conflicting problems of non-selective damage and partial lesions – increasing the volume or concentration to reduce the number of partial lesions is likely to increase the degree of non-selective damage and conversely decreasing the concentration in an attempt to reduce the non-selective damage is likely to increase the occurrence of partial lesions. Nonetheless, the majority of 3.0% concentration infusions resulted in extensive cholinergic pPPTg lesions, making this a successful lesion technique. However, it is essential when designing experiments to consider that a small yet nonetheless appreciable proportion of rats are likely to be excluded from analysis on the basis of histology, therefore the initial lesion group sizes should be large enough to incorporate this subsequent loss after histological analysis.

Behavioural effects

Ibotenic acid lesions restricted to the pPPTg have been shown to cause rats to over-consume 20% sucrose solution (Wilson et al., 2009a), this is a curious effect also repeatedly found after whole-PPTg lesions (Olmstead et al., 1999; Alderson et al., 2001; Keating et al., 2002; Ainge et al., 2006) with no clear explanation, though it has been suggested to be the result of disordered response control or perseveration (Ainge et al., 2006; Walker and Winn, 2007). The finding that selective cholinergic pPPTg lesions caused no change in the amount of

sucrose consumed suggests that the process underlying overconsumption in ibotenic lesioned rats are not cholinergic mediated. As pPPTg ibotenic lesions do not affect spontaneously generated locomotion (Alderson et al., 2008; Wilson et al., 2009a) the finding that selective cholinergic pPPTg lesions also do not produce locomotor changes was expected.

Overall chapter conclusions

The experiments conducted in this chapter established that 300nL of 3.0% Dtx-UII, delivered from a glass pipette with the rats nose elevated in the stereotaxic frame, can create highly selective bilateral cholinergic pPPTg lesions. No surgical or recovery complications were encountered with successful selective lesions even when the toxin was infused in both hemispheres in the same surgical procedure. Moreover, these lesions are reliable enough for creating groups suitable for behavioural analysis. Problems with non-selective damage were not eliminated, but it is hoped use of a lower volume of toxin in subsequent experiments with reduce this problem. Having established the lesion procedure, the next chapter assesses the effects of these lesions on instrumental learning.

Chapter 5: Operant learning after selective bilateral lesions of cholinergic neurons in the pPPTg

Chapter Introduction

As discussed in chapter 1, rats bearing excitotoxic lesions of the PPTg have various separable yet potentially related deficits in operant tasks. To summarise briefly: (1) there is a clear learning impairment in naïve rats and in trained rats when testing schedules change. The results of chapter 3 show that part of this deficit is a specific impairment in the updating of associations between actions and outcomes; (2) attention may be impaired in tasks with a high attentional component; (3) while the ability to execute the motor actions required by these tasks appears intact, the ability to select the correct action or inhibit incorrect actions may be disrupted.

In terms of subregions within the PPTg involved in these deficits, the learning impairments are closely associated with the posterior rather than anterior PPTg. Excitotoxic lesions of pPPTg but not aPPTg impaired initial instrumental learning and adaptation to changes in reinforcement schedules (Wilson et al., 2009a) and the impairment in action-outcome learning found in chapter 3 was during inactivation centred on the pPPTg. Despite these advances in functionally dissecting subregions within the PPTg, because of the lack of manipulation specific for one neuronal type, these previous studies cannot address one key question: in what ways are cholinergic, glutamatergic and GABAergic PPTg neurons involved in learning? This chapter specifically addresses this question by assessing operant learning in rats bearing highly selective bilateral cholinergic lesions of the pPPTg.

The operant task

The operant task used here is a replication of the instrumental learning regime used by Wilson et al. (2009). It involves training rats on an FR1 schedule of reinforcement, and once they have learned this to a predefined criterion level, advancing them through increasing fixed

and variable rate schedules of reinforcement before testing the rats under conditions of extinction where no rewards are delivered. This experimental design enables the assessment of initial instrumental learning, adaptation to changes in previously learned associations and also the response to the systematic introduction of variability in the association between lever press and reward delivery. By replicating a paradigm which produced clear impairment in excitotoxic pPPTg lesioned rats a comparison between any deficits seen after selective cholinergic pPPTg lesions and excitotoxic lesions can be made. Moreover, as the Wilson et al. (2009) study comprised part of my work as a research assistant at the University of St Andrews, I am very familiar with the details of both the experimental protocol and the behavioural results.

Methods

Subjects

Twenty four adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used, with a mean weight of 338g (range 310-361g) at the time of surgery. Animals were housed and maintained as described in the general methods. Three days prior to behavioural testing food was restricted to 17-19g/rat/day standard lab chow. Weights were monitored to ensure they never fell below 85% of free food weight at any point in the experiment. The food restriction was to motivate the rats by ensuring they were hungry at the time of testing. This is achievable without causing a dramatic reduction in bodyweight and therefore after an initial adjustment to food restriction the rats continued to put on weight throughout the behavioural testing.

Surgery

Anaesthesia was induced and lesion surgery performed as described in the general methods. The co-ordinate for the pPPTg was: -0.8 from IAL; ± 1.9 from midline; -6.5 from dura (the same as used in chapter 4) and the order of infusion (left hemisphere, right hemisphere)

was counterbalanced across rats. In the lesion group rats were infused bilaterally with either 200 nL (n = 8) or 300 nL (n = 8) of 3.0% Dtx-UII and in the sham group (n = 8) rats were infused with sterile PB (300nL and 200 nL; 4 of each). 300 nL of 3.0% Dtx-UII is the volume of toxin which proved most successful in chapter 4, the introduction of the 200 nL group was an attempt to further reduce non-selective damage. During the lesion formation period rats were monitored daily for signs of ill health or other lesion-related complications.

Behavioural testing

To allow for lesion formation, behavioural testing began >21 days post-surgery. Testing was conducted in the operant testing apparatus described in the general methods. Each rat was always tested in the same operant box and testing was conducted at approximately the same time every day. Food restriction began 3 days prior to behavioural testing. Two days prior to testing rats were given 2 g of 45 mg testing pellets (Test Diet purified rodent tablet 5TUL, Sandown Scientific, Middlesex, UK) in a bowl in their homecage. The following day rats were placed in operant boxes where 40 reward pellets were available in the pellet dispenser, no levers were extended and the sound attenuating doors were left open. Once all pellets were consumed (~20 min) rats were returned to their homecage. These procedures were used to reduce neophobia to the reward pellets and allow rats to familiarise themselves with reward delivery location in the operant testing chamber. Rats were then tested daily in 40 min testing sessions where pressing on the 'correct lever' (the side of which was counterbalanced across rats) the required number of times led to a pellet being delivered. All presses on the second lever (the 'incorrect lever') and approaches to the reward delivery magazine were recorded but had no programmed consequence. At the start of the testing session both levers were extended and the houselight illuminated. Initially rats were trained on FR1 where one press on the correct lever triggered pellet delivery and the simultaneous illumination of the

light located above the active lever. This light remained illuminated for 10 sec and during this time (defined as the inter-trial-interval, ITI) pressing on the correct lever had no programmed consequence. After the 10 sec ITI the lever-light was extinguished and the next trial began. Rats were advanced through a variety of fixed ratio (FR) and variable ratio (VR) testing schedules (see table 5.1) depending on their individual performance. Each trial in every session followed the same format, except that correct presses up to the final press in the schedules above FR1 did not trigger pellet delivery. In the extinction schedule the trials were programed in the format of VR30 but no pellets were delivered.

Schedules of reinforcement

Schedule	Number of correct presses before reward delivery	Criteria to be met before advancing to the next schedule
FR1	1	Two consecutive sessions of > 80 trials completed
FR5	5	Two consecutive sessions of > 60 trials completed, or 5 sessions.
VR5	1-9 (mean 5)	5 sessions completed
VR10	1-19 (mean 10)	2 sessions completed
VR15	1-29 (mean 15)	2 sessions completed
VR30	1-59 (mean 30)	7 sessions completed
Extinction	No rewards delivered	7 sessions completed

Table 5.1: schedules of reinforcement and criteria for advancement.

Behavioural measures

Throughout all sessions all lever presses and approaches to the food hopper were recorded, allowing the following behavioural measurements to be calculated: c*orrect presses* – the total number of presses on the correct lever during a schedule; *incorrect presses* – the total number of presses on the incorrect lever; *late pressing ratio* - the ratio of presses on the correct lever between reward delivery and approach to the food hopper to *correct presses*;

reward collection latency - the latency to collect the pellet after delivery; *early pressing ratio* - the ratio of correct lever presses between reward collection and the start of the next trial to *correct presses*; *post-reinforcement pause* - the latency from the start of the trial to first lever press; *approaches* – number of approaches to the reward delivery hopper.

Histology

All histological procedures followed the methods described in the general methods. Briefly, following completion of behavioural testing rats were transcardially perfused with fixative and brains stored in sucrose solution. Subsequently, 30 µm coronal sections were cut through the area of the PPTg and ~1mm beyond in anterior-posterior plane. Parallel 1:4 series were processed immunohistochemically for ChAT and NeuN reactivity. NeuN stained sections were then counterstained with cresyl violet and all stained sections mounted onto glass slides and viewed under a light microscope. Software assisted counts of ChAT+ neurons throughout the PPTg enabled quantification of the cholinergic lesions, non-cholinergic damage was assessed by visual inspection of the NeuN/Cresyl stained sections. A lesion was considered acceptable if >~80% of ChAT+ pPPTg neurons were destroyed bilaterally and there was minimal, if any, non-selective damage evident on the NeuN/cresyl sections. Due to poor fixation and subsequent poor staining, one sham rat was excluded from the ChAT cell count analysis, but included in all other analyses.

Behavioural data analysis

Data were statistically analysed using PASW 18 for Windows (SPSS Inc., Chicago, Illinois USA). Various univariate and repeated measures ANOVAs were performed across *schedule* (reinforcement schedule; within subjects factor) and between *lesion group* (lesion, sham; between subjects factor). Details of each test, subsequent post hoc tests (Sidak corrected planned pairwise comparisons) and investigations of interactions are given in the appropriate

results sub-section. The Huynh-Felt correction was applied where the data were not homogeneous; skew in the data was identified by the Shapiro-Wilk test and corrected by log10 transformation (reported in the text when performed). Details of other tests are reported where used. Results were considered statistically significant when $p \leq 0.05$.

Results

Recovery

All rats initially recovered well from the surgical procedure. During the 21 day lesion formation period 3 rats struggled to maintain stable bodyweight. One of these rats responded well to wet mash and a few days later was able to consume dry pellets again. The remaining 2 rats continued to lose weight and developed the rapid tooth growth described in chapter 4 (p112 + p118). The named veterinary surgeon clipped their teeth 3 times; despite this (and additional hand feeding with babyfood (various flavours; Boots PLC, Nottingham, UK)) they still failed to maintain a stable bodyweight. These 2 rats were perfused (as described in the general methods) 29 and 31 days post-surgery. Subsequent examination of ChAT and NeuN stained PPTg sections revealed both had bilateral, large, non-selective lesions centred on the pPPTg. Of the rats which were subsequently found to have bilateral selective cholinergic pPPTg lesions all recovered well from surgery with the only noticeable effect being a small transient, yet significant, change in growth rate. See figure 5.1 and subsequent analysis for details.

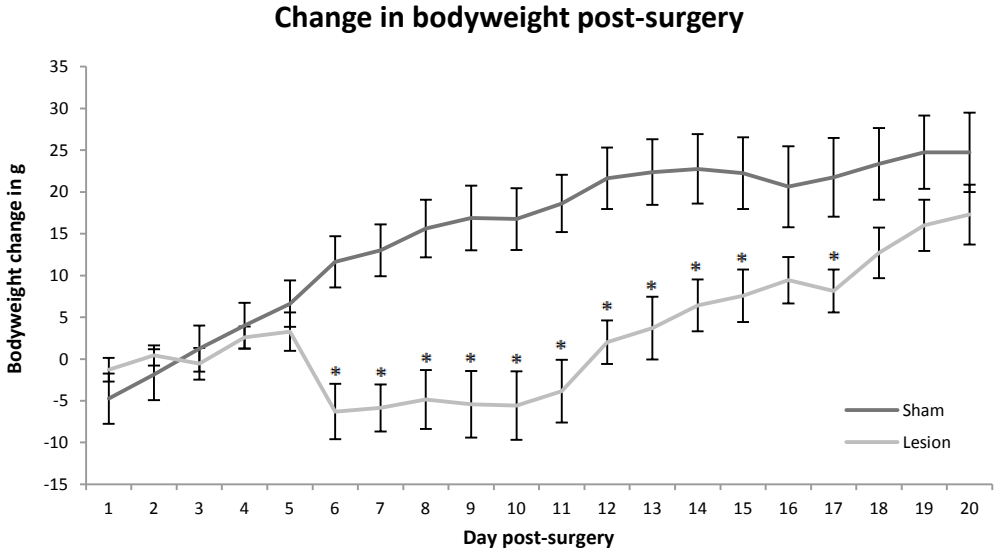


*Figure 5.1: Post-surgery bodyweight of sham control and successful selective cholinergic pPPTg lesioned rats. Graph shows group means ± SEM. * indicates significant difference between sham and lesion group on that day. See text for full statistical analysis.*

Analysis of the change in post-surgery bodyweight by repeated measures ANOVA found a significant effect of *day post-surgery* ($F_{19,247}$ = 35.72 $p < 0.001$) a significant effect of *lesion group* ($F_{1,13}$ = 8.17 $p = 0.013$) and a significant *lesion group x day post-surgery* interaction ($F_{19,247}$ = 12.41 $p < 0.001$). Significant differences between sham and lesion groups within each day are indicated by a * on figure 5.1.

Lesion

Seven rats had selective bilateral lesions of the cholinergic pPPTg. These lesions destroyed a mean of 93% of ChAT+ pPPTg neurons (range 87.8% to 98.2%). Five of these rats had evidence of some level of non-selective damage on the NeuN/Cresyl stain, generally this was a small area on the NeuN stain present on around 2 sections which displayed signs of having fewer than expected neurons present. In 3 of these rats these areas were located outside the pPPTg and were in a non-consistent pattern across animals: there was no single

structure affected or standard pattern of non-selective damage in the lesion group. In 2 of these rats there were trace amounts of unilateral non-selective damage within the PPTg (see figure 5.2; this shows the type of non-selective damage found and the largest amount deemed acceptable within pPPTg). The remaining rats in the lesion group were excluded from all analysis due to having no clear sign of any lesion (n = 2); unilateral or partially unilateral lesions (n = 3); due to having partial non-selective damage in or around pPPTg, predominantly unilaterally (n = 2) or due to having extensive non-selective damage destroying the entire pPPTg and spreading into surrounding structures in both hemispheres (n = 2). These 2 rats with extensive non-selective damage destroying the entire pPPTg and encroaching on surrounding structures were the 2 rats which were perfused prior to behavioural testing due to ill health and weight loss (see p127). This is unfortunate as their behavioural data would have made a valuable control group allowing a direct comparison between the effects of Dtx-UII induced damage to the pPPTg which was selective for cholinergic neurons verses that which had become non-selective and destroyed all neuronal types.

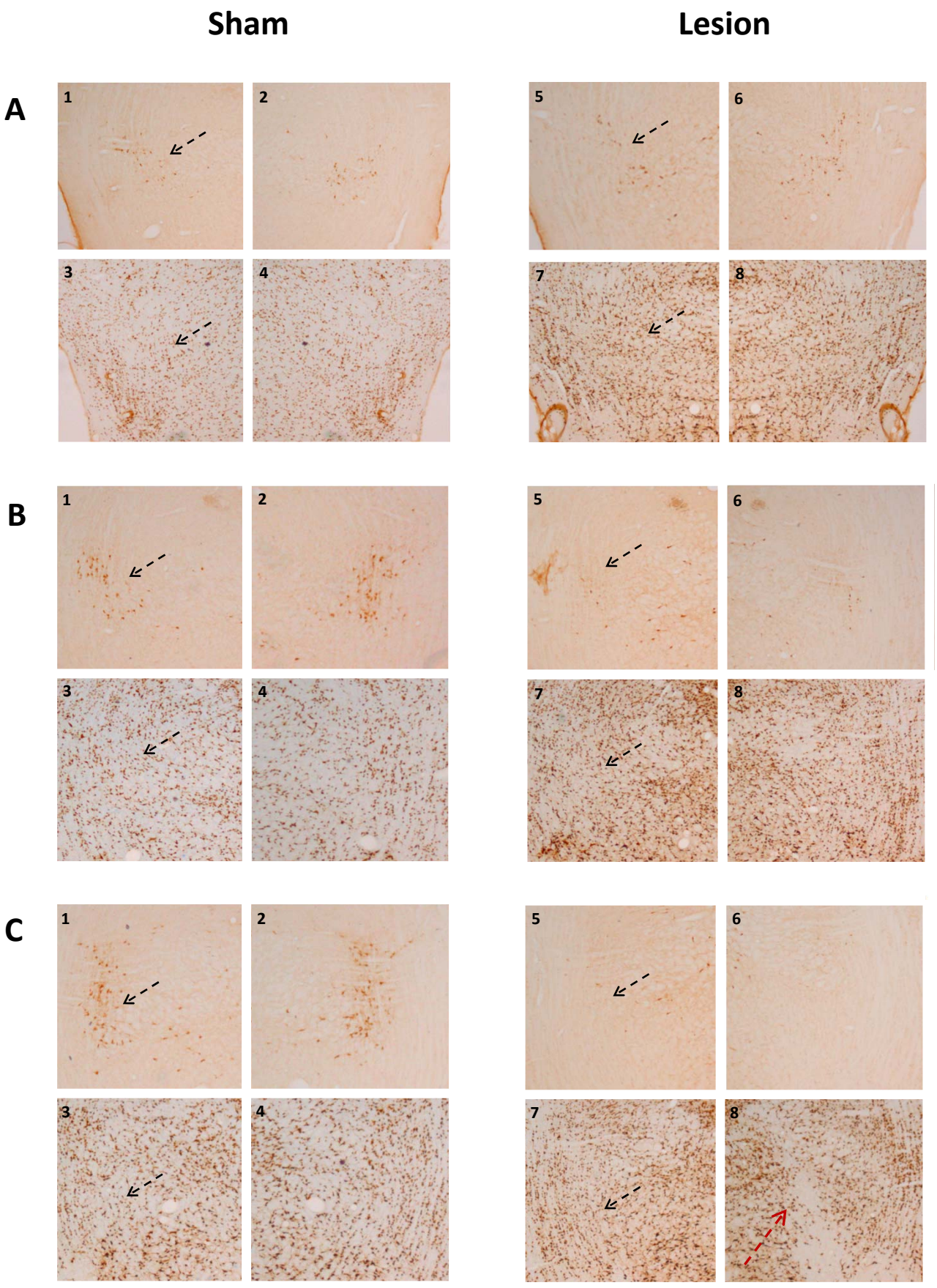


Figure 5.2:

Example sections from a Dtx-UII and a sham lesioned rat. Rows A – C show ChAT (boxes 1,2,5,6) and NeuN/Cresyl (boxes 3,4,7,8) stained sections of anterior PPTg (rows A), central PPTg (rows B) and posterior PPTg (rows C). Black dotted arrows indicate the location of the PPTg. The red arrow in box C8 indicates the maximum level of non-selective damage within the area of the PPTg.

Characterisation of lesion

The number of ChAT cells at each level of the PPTg for the sham and lesion group is shown in figure 5.3. The percentage of surviving pPPTg neurons in the lesion group is shown in figure 5.4.

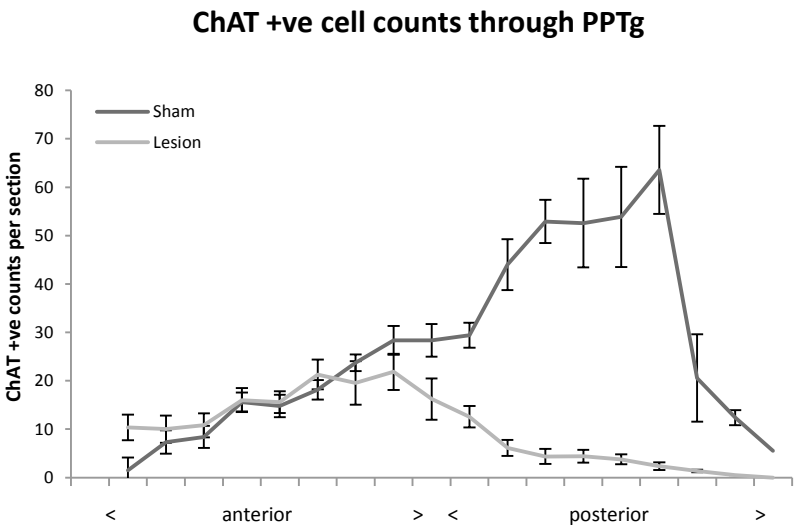


Figure 5.3: Counts of ChAT+ neurons throughout the PPTg along the anterior-posterior axis. ChAT counts = Numbers of ChAT+ neurons. Graph shows group means ± SEM.

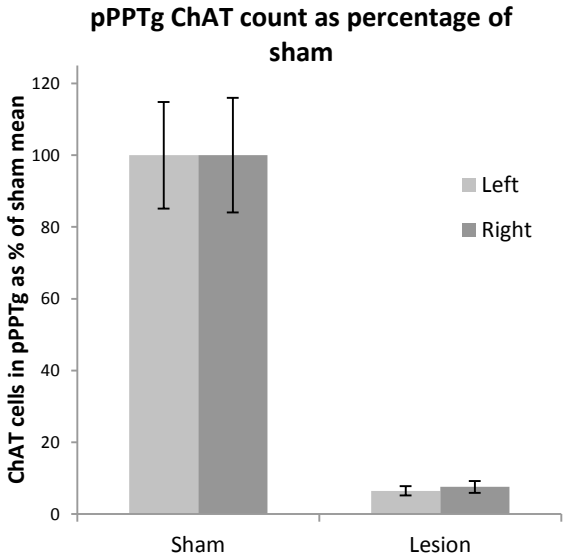


Figure 5.4: Remaining ChAT+ pPPTg neurons in the lesion group displayed as a percentage of the sham group. Left = left hemisphere, Right = right hemisphere. Graph shows group means ± SEM.

Analysis of lesion

T-tests (on raw count values, not percentages) confirm that the lesion group had significantly fewer ChAT+ neurons than sham controls in both the left ($t_{6.9}$ = 17.75 $p < 0.001$) and right ($t_{7.8}$ = 16.58 $p < 0.001$) hemispheres. A comparison between the lesioned rats which received 300 nL and 200 nL of toxin found no detectable difference between the levels of cholinergic cell loss nor the number of cases of non-selective damage. However, where non-selective damage did occur it was more extensive in the 300nL lesion group.

Behavioural results

Operant learning

The primary question addressed was whether selective cholinergic lesions of the pPPTg caused impairment in operant learning. This was evaluated in two ways: firstly by performing a detailed analysis of behavioural measures during initial operant learning of the FR1 schedule, then by subsequently analysing the behavioural changes in response to the systematic increase in reinforcement schedules and during extinction. Rather than presenting every dependant variable recorded by the operant software, particular attention was paid to the following variables: number of correct lever presses; days to reach criterion level of performance; reward collection latency; post-reinforcement pause and the number of approaches to the reward delivery magazine. These variables were chosen as they allow a rigorous assessment of both the learning rate (correct pressing and days to criteria) and provide a general assessment of the rats behaviour (time to collect reward, delay before starting a trial, number of approaches to the food hopper). They are also the variables most affected by ibotenic acid pPPTg lesions (Wilson et al., 2009a). Analysis of the remaining dependant variables (data not shown) revealed no significant differences between sham and lesion groups.

Initial learning of FR1

Figure 5.5 shows the mean number of correct lever presses when learning FR1. Repeated measures ANOVA showed a significant effect of *session* ($F_{2,26}$ = 100.38 p < 0.001) no significant effect of *lesion group* ($F_{1,13}$ = 1.94 p = 0.187) and no significant *lesion group x session* interaction ($F_{2,26}$ = 0.45 p = 0.643). Planned pairwise comparisons found that the overall rate of correct pressing in session 2 was higher than session 1 (p < 0.001) and higher in session 3 than session 2 (p = 0.001). Fig 5.5 shows the number of days required to reach criterion level of performance (two consecutive days of >80 correct trials) on FR1. A T-test found no significant difference between the sham and lesion groups (t_{13} = -1.31, p = 0.211) in the number of days taken to reach criterion level.

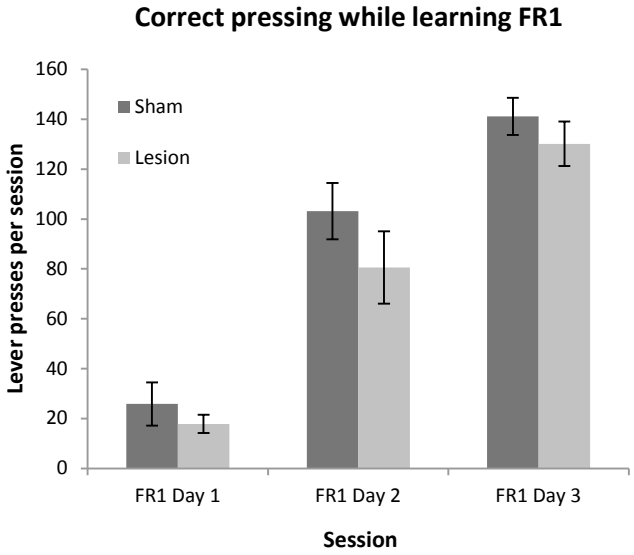


Figure 5.5: Number of correct lever presses made in each session while learning FR1. Graph shows group means ± SEM. See text for statistical analysis.

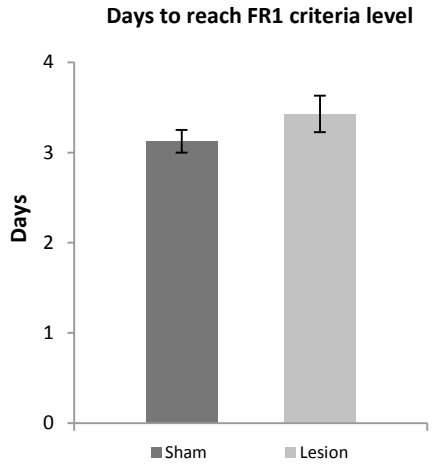


Fig 5.6: Number of days taken to reach criterion level on FR1. Graph shows group means ± SEM. See text for statistical analysis.

Reward collection latency while learning FR1

Figure 5.7 shows the reward collection latency (RCL) of each group during FR1 (log10 transformed to correct for positive skew). Repeated measures ANOVA found a significant effect of *session* ($F_{2,26}$ = 181.00 p < 0.001) no significant effect of *lesion group* ($F_{1,13}$ = 0.163 p = 0.693) and no significant *lesion group x session* interaction ($F_{2,26}$ = 0.088 p = 0.916). Planned pairwise comparisons found that the overall RCL in session 2 was lower than session 1 (p < 0.001) and lower in session 3 than session 2 (p < 0.001).

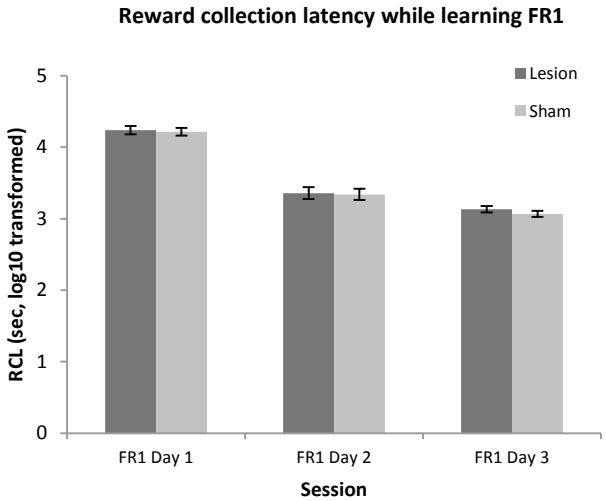


Figure 5.7: Reward collection latencies during FR1. Graph shows group means ±SEM. See text for statistical analysis.

Post-reinforcement pause when learning FR1

Figure 5.8 shows the post reinforcement pause (PRP) for each group during FR1 (log10 transformed to correct for positive skew). Repeated measures ANOVA found a significant effect of *session* ($F_{2,15}$ = 118.04 p < 0.001) no significant effect of *lesion group* ($F_{1,13}$ = 1.58 p = 0.231) and no significant *lesion group x session* interaction ($F_{2,26}$ = 0.437 p = 0.651). Planned pairwise comparisons found that the overall PRP in session 2 was lower than session 1 (p < 0.001) and lower in session 3 than session 2 (p = 0.003).

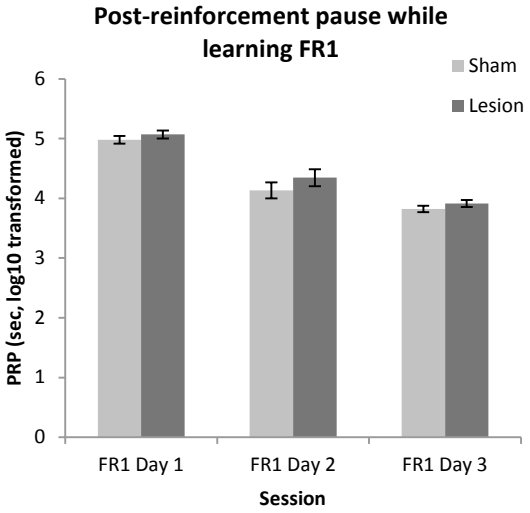


Figure 5.8: Post reinforcement pause during FR1. Graph shows group means ± SEM. See text for statistical analysis.

Approaches when learning FR1

Figure 5.9 shows the number of approaches to the food hopper made during the testing schedule by each group during FR1. Repeated measures ANOVA found a significant effect of *session* ($F_{2,26}$ = 15.49 p < 0.001) a significant effect of *lesion group* ($F_{1,13}$ = 6.13 p = 0.028) and no significant *lesion group x session* interaction ($F_{2,26}$ = 0.399 p = 0.675). Planned pairwise comparisons found that the overall number of approaches in session 1 was lower

than session 2 or 3 ($p < 0.001$ and $p = 0.007$ respectively) and that the lesion group made fewer approaches than the sham group ($p = 0.028$).

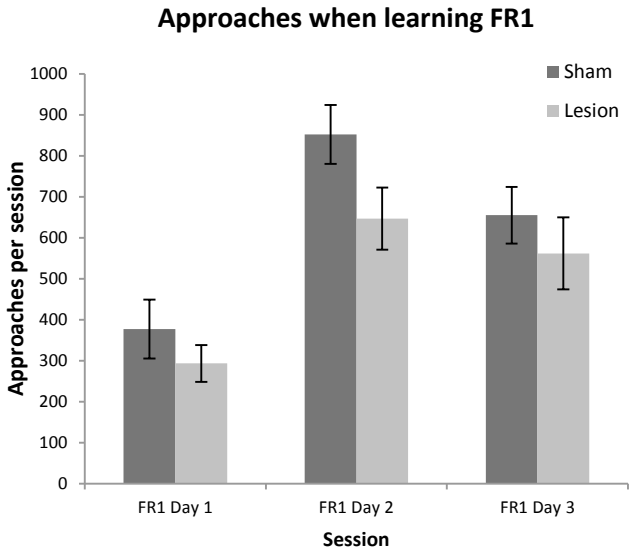


Figure 5.9: Number of approaches made during FR1. Graph shows group means ± SEM. See text for statistical analysis.

In order to investigate whether the number of approaches per session correlates strongly with the number of trials completed, the number of approaches per trial was calculated and analysed. This is shown in figure 5.10. Repeated measures ANOVA found a significant effect of *session* ($F_{1.4,17.6} = 77.50$ $p < 0.001$) no significant effect of *lesion group* ($F_{1,13} = 0.018$ $p = 0.896$) and no significant *lesion group x session* interaction ($F_{1.4.17.6} = 0.143$ $p = 0.784$). Planned pairwise comparisons found the overall number of approaches per session in session 1 was higher than session 2 ($p < 0.001$) and is higher in session 2 than session 3 ($p < 0.001$).

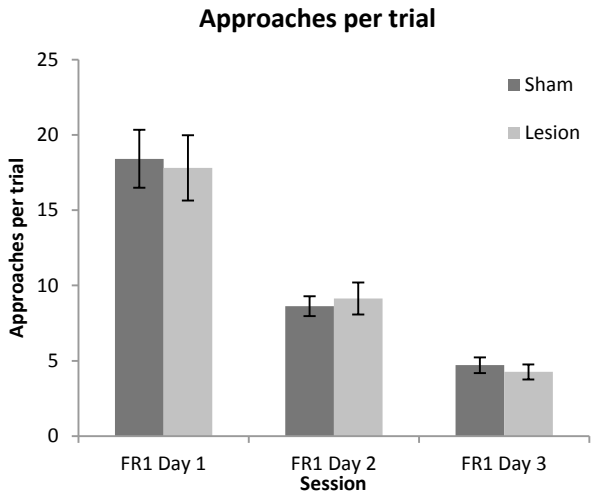


Figure 5.10: Number of approaches made per trial during FR1. Graph shows group means ±SEM. See text for statistical analysis.

Learning of new fixed and variable schedules of reinforcement

Having completed the analysis of learning during FR1, the analysis now turned to the subsequent learning of new fixed and variable ratio schedules. This was achieved by assessing performance on the first and last day of each schedule. This gives an assessment of response to a new schedule (change from last day of one to 1st day of next schedule) and adaptation within a schedule (change between 1st and last day of the same schedule).

Correct lever pressing

The total number of presses on the correct lever during the first and last session of each schedule is shown in graph 5.11

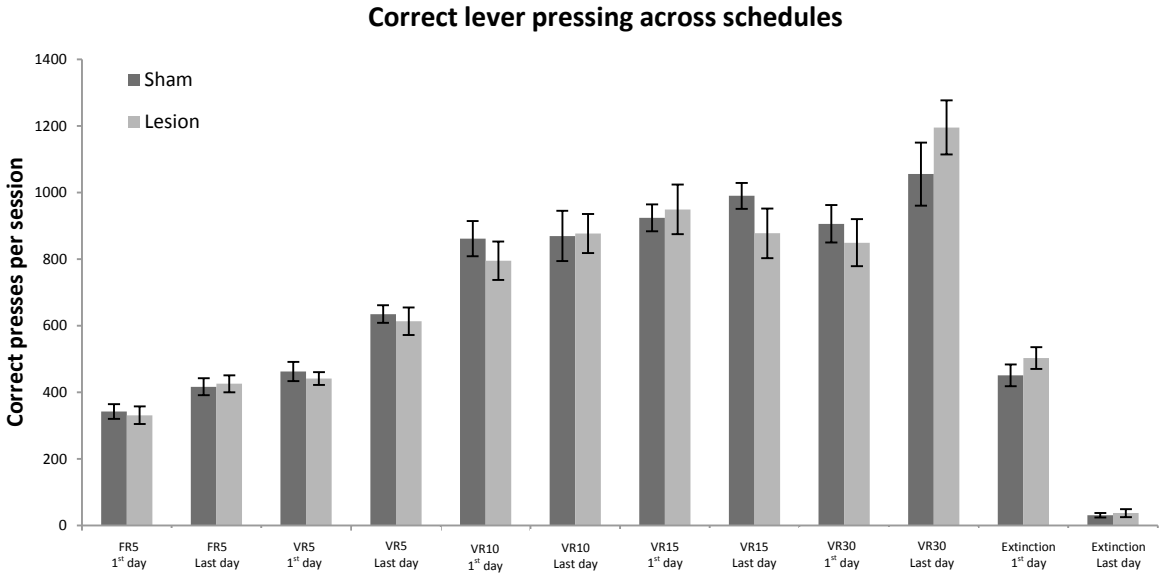


Figure 5.11: Rate of correct lever pressing across schedules. Graph shows group means ± SEM. See text for statistical analysis.

Repeated measures ANOVA found a significant effect of *schedule* ($F_{6.9,90.4}$ = 96.92 p < 0.001) no significant effect of *lesion group* ($F_{1,13}$ = 0.01 p = 0.924) and no significant *lesion group x schedule* interaction ($F_{6.9,90.4}$ = 0.96 p = 0.468). The results of the planned pairwise comparisons investigating the overall effect of *schedule* are summarised in table 5.2

Schedule	Change from previous schedule	Change within schedule
FR5	-	↑ p = 0.02
VR5	No sig change	↑ p < 0.001
VR10	↑ p < 0.001	No sig change
VR15	No sig change	No sig change
VR30	No sig change	No change (p = 0.09)
Extinction	↓ p < 0.001	↓ p < 0.001

Table 5.2: Table of significant changes in overall levels of correct lever pressing across schedules.

Reward collection latency

The reward collection latency (log10 transformed to correct for positive skew) during the first and last session of each schedule is shown in figure 5.12.

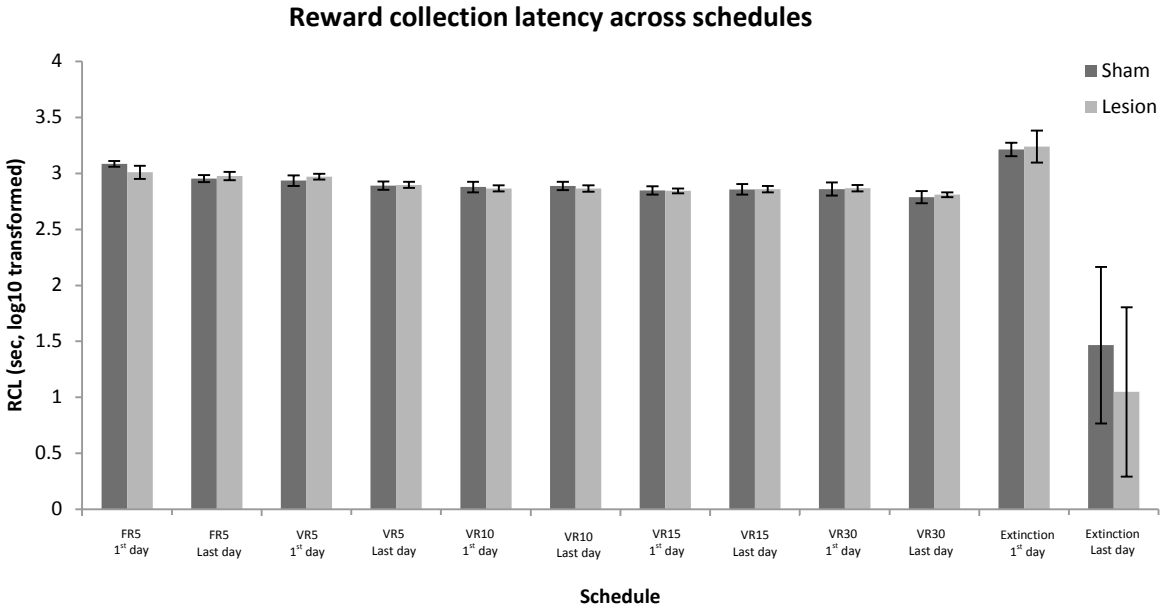


Figure 5.12: Reward collection latency across schedules. Graph shows group means ± SEM. See text for statistical analysis.

Repeated measures ANOVA found a significant effect of *schedule* ($F_{1.2,15.0}$ = 10.66 p = 0.004) no significant effect of *lesion group* ($F_{1,13}$ = 0.113 p = 0.743) and no significant *lesion group x schedule* interaction ($F_{1.6,15.0}$ = 0.17 p = 0.725). The results of the planned pairwise comparisons investigating the overall effect of *schedule* are summarised in table 5.3

Table 5.3: Table of significant changes in overall reward collection latencies across sessions.

Schedule	Change from previous schedule	Change within schedule
FR5	-	No sig change
VR5	No sig change	No sig change
VR10	No sig change	No sig change
VR15	No sig change	No sig change
VR30	No sig change	No sig change
Extinction	↑ p < 0.008	No sig change

Post-reinforcement pause

The post-reinforcement pause (log10 transformed to correct for positive skew) during the first and last session of each schedule is shown in graph 5.13.

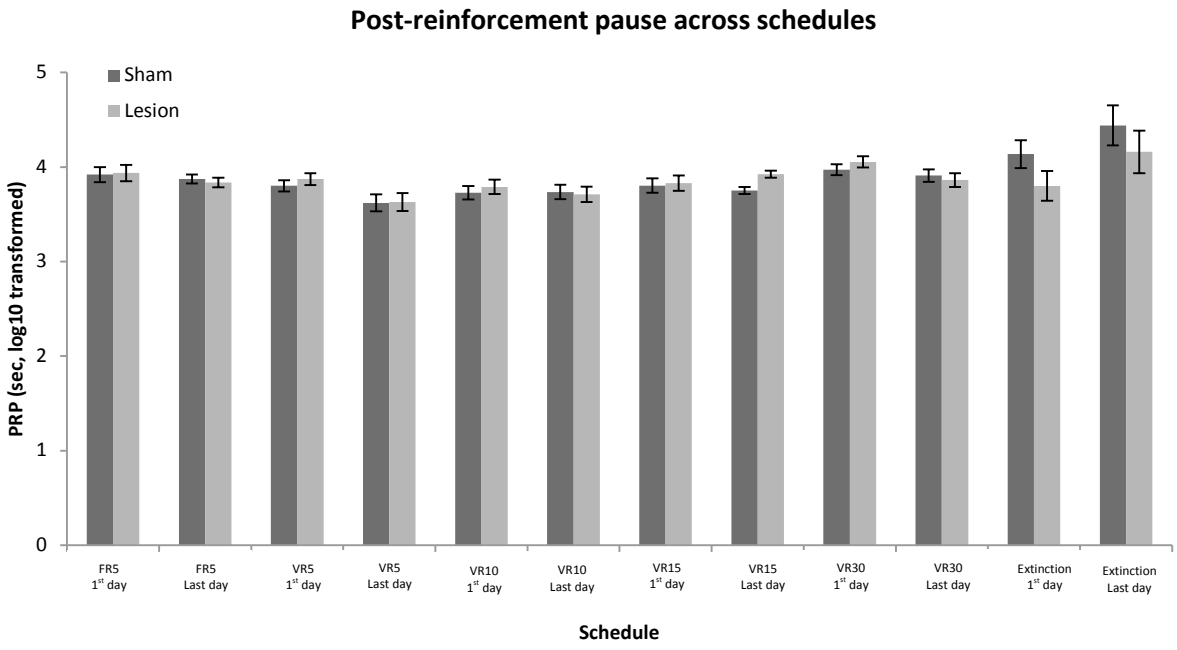


Figure 5.13: Post-reinforcement pause across schedules. Graph shows group means ± SEM. See text for statistical analysis.

Repeated measures ANOVA found a significant effect of *schedule* ($F_{4.2,54.3}$ = 7.70 p < 0.001) no significant effect of *lesion group* ($F_{1,13}$ = 0.159 p = 0.697) and no significant *lesion group x schedule* interaction ($F_{4.2,54.3}$ = 1.44 p = 0.232). The results of the planned pairwise comparisons investigating the effect of *schedule* are summarised in table 5.4.

Table 5.4: Table of significant changes in post-reinforcement pause across schedules.

Schedule	Change from previous schedule	Change within schedule
FR5	-	No sig change
VR5	No sig change	↓ p < 0.007
VR10	↑ p < 0.004	No sig change
VR15	No sig change	No sig change
VR30	↑ p < 0.041	No sig change
Extinction	No sig change	No sig change

Approaches

The number of approaches made during the testing session on the first and last session of each schedule is shown in graph 5.14.

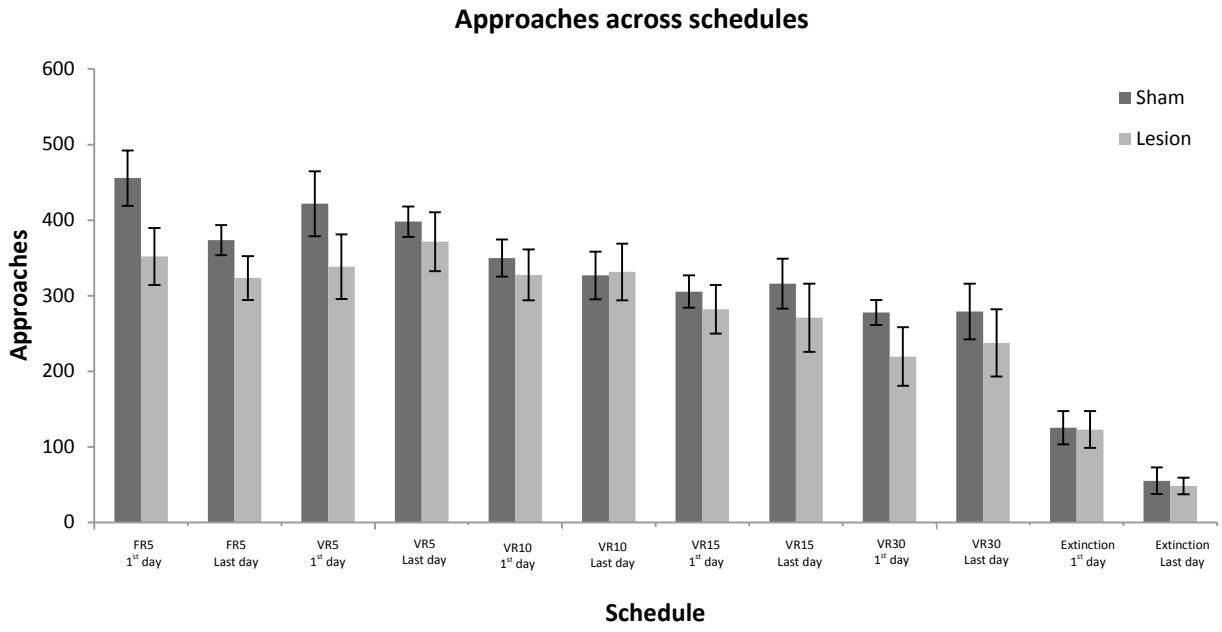


Figure 5.14: Total number of approaches across schedules. Graph shows group means ± SEM. See text for statistical analysis.

Repeated measures ANOVA found a significant effect of *schedule* ($F_{8.0,104.5}$ = 38.8 p < 0.001) no significant effect of *lesion group* ($F_{1,13}$ = 1.52 p = 0.239) and no significant *lesion group x schedule* interaction ($F_{8.0,104.5}$ = 0.905 p = 0.516). The results of the planned pairwise comparisons investigating the overall effect of *schedule* are summarised in table 5.5.

Table 5.5: Table of significant overall differences in number of approaches across schedules.

Schedule	Change from previous schedule	Change within schedule
FR5	-	No sig change
VR5	No sig change	No sig change
VR10	No sig change	No sig change
VR15	No sig change	No sig change
VR30	No sig change	No sig change
Extinction	↓ p < 0.001	↓ p = 0.045

Chapter discussion

Summary

This experiment was conducted in order to investigate the effects of highly selective bilateral lesions of the cholinergic pPPTg on operant learning. Lesions, made using Dtx-UII toxin, were highly selective for cholinergic pPPTg neurons, destroying a mean of 93% of ChAT+ neurons within the pPPTg while causing little or no detectable non-selective damage. Lesioned rats learned the simple FR1 task at the same rate and in the same number of sessions as sham operated controls. The only detectable difference between sham and lesioned rats was a slight reduction in the total number of approaches made to the food hopper during FR1 sessions. However, when performing a more detailed analysis and assessing the number of approaches made per trial rather than per session, the effect completely disappeared. At this stage of training rats make on around 9-17 approaches per trial. Therefore if the number of approaches per trial is constant but the rats make a slightly different number of trials, this difference in the number of trials is amplified when assessing the number of approaches per session. As the effect dissapeard in the analysis per trial, it is perhaps more reflective of the lesion group completing slightly fewer (but not significantly fewer) trials during the early FR1 sessions rather than a true indication of a reduction in approach behaviour. When switched to higher fixed ratio, variable ratio and finally extinction schedules there was no significant difference between the lesioned and sham rats on any measure taken. Moreover, in addition to there being no significant differences, there was no indication of a near significant effect or underlying trend within the data: on these schedules lesioned rats were indistinguishable from sham controls.

Comparison to ibotenic acid pPPTg lesions

As discussed in the introduction, a considerable volume of research has shown that destruction of inactivation of the PPTg without selectivity for neuronal type (for example with excitotoxic ibotenic acid lesions or transient muscimol induced inactivation) causes impairment in operant learning paradigms and the updating of action-outcome associations. The behavioural testing regime utilised in this chapter is a repetition of that used by Wilson et al. (2009), the main results of which are summarised in table 5.6. Wilson et al. found substantial impairment in ibotenic acid pPPTg lesioned rats when learning FR1, characterised by lesioned rats having markedly reduced rates of correct lever pressing, taking significantly more training sessions (5.6 versus 3.2) to reach criterion level of performance, having reduced number of approaches, an increased RCL and an increased PRP. When advanced through higher testing schedules the reduced rate of correct lever pressing persisted throughout all schedules except extinction, the increased RCL and PRP were present in FR5 but were largely absent in later schedules. It is also worth noting that while ibotenic pPPTg lesioned rats did eventually learn FR1 and FR5, despite extensive training they never performed VR10, VR15 or VR30 to the same level as sham controls. Clearly, ibotenic pPPTg lesions caused a severe impairment in this testing regime, an impairment that could be overcome by extensive training in low demand schedules, but which persistently affected performance in high demand schedules. The results of this chapter are in sharp contrast to the effects of ibotenic acid pPPTg lesions. Dtx-UII lesioned rats at no point had significantly reduced rates of lever pressing, nor impairment in learning of or adaption to new reinforcement schedules.

Schedule of reinforcement	Correct lever presses	Approaches	Reward Collection Latency	Post-Reinforcement Pause
FR1	↓	↓	↑	↑
FR5	↓	-	↑	↑
VR5	↓	-	-	-
VR10	↓	-	-	-
VR15	↓	-	-	-
VR30	↓	-	↑	-
Extinction	-	-	-	-

Table 5.6: Table of behavioural results from the ibotenic acid pPPTg lesioned rats in the Wilson et al. (2009) study. Arrows indicate significant difference and direction of difference (compared to sham controls). "-" indicates no significant difference.

Possible reasons for no effect of Dtx-UII lesion on operant learning

Methodological considerations

Lesion size

One possibility for the lack of behavioural effect is that the Dtx-UII lesion was not destructive enough within the cholinergic pPPTg to cause an effect – is higher cholinergic cell loss needed? The lesions in this chapter destroyed a mean of 93% of ChAT+ pPPTg neurons (range: 88% to 98%). Ibotenic acid lesions of the PPTg typically have some cholinergic cell survival, indeed in the Wilson et al. (2009) experiment ChAT+ cell loss was a mean of 64% (range 58-72%) which is consistent with other studies (see: Blaha and Winn, 1993; Inglis et al., 2001) which typically find between 20-50% PPTg cholinergic cell survival after ibotenic acid lesion. The Dtx-UII lesions here are considerably more destructive to ChAT+ neurons than standard ibotenic acid lesions and therefore it is unlikely that ibotenic lesions are causing a cholinergic mediated effect that the Dtx-UII lesions are failing to achieve due to not destroying sufficient numbers of neurons.

Timecourse of lesion formation and possible compensatory mechanisms

The Dtx-UII toxin is a protein synthesis inhibitor based toxin, once internalised into the neuron cell death occurs slowly over a period of days. Clark et al. (2007) report that 21 days is needed to achieve maximum cell loss, while in chapter 4 (which used the same lesion procedure as this chapter) extensive (96%) cell loss was observed at 14 days. In contrast to this, ibotenic acid is an excitotoxin which binds to glutamate receptors, locking them open and causing unregulated calcium influx which becomes neurotoxic. The effects are rapid, the process leading to cell death begins immediately as is extensive in under 72 hours (Kohler et al., 1979; Garey and Hornung, 1980). Thus Dtx-UII lesions form considerably more slowly than ibotenic acid lesions. Is it possible that during the Dtx-UII lesion formation period efferent targets of the pPPTg compensate for the gradual reduction in cholinergic outflow from the pPPTg, but given the quick nature of the ibotenic lesions and proximity between lesion formation and behavioural testing (typical experiments start testing 7 days post-surgery) there is not the same opportunity for compensatory mechanisms to develop? While Wilson et al. (2009) did start testing ibotenic pPPTg lesioned rats around 7 days post-surgery, because of the length of the testing regime these rats were still being tested 28 days post-surgery, with no sign that the rats performance was improving with time (indeed, performance was poorest in the last schedules) suggesting that gradual recovery of full function does not occur after PPTg damage. However, this does not rule out the possibility that the different timecourse of lesion formation allows for the development of different compensatory mechanisms. Assessment of compensatory changes after lesion generally involve either direct measurement of transmitter level (by for example microdialysis, electrochemistry, tissue punching), direct assessment of changes in receptors (histological visualisation and characterisation of receptors) or administration of pharmacological agents to detect alternations in transmitter synthesis and/or receptor sensitivity (for example administration of receptor agonists to detect receptor

supersensitivity) (see pp100-101). If compensatory mechanisms were to develop after Dtx-UII pPPTg lesions, candidate structures for up-regulation of AChRs in response to reduction of endogenous innervation would be the midbrain DA systems and the thalamus. Projections from the pPPTg to the VTA and SNc DA neurons are a key candidate route for the learning impairment observed after excitotoxic PPTg lesions, making it a region of interest in this study. Therefore, the possibility of functional compensation is a concern for interpreting these results. The possibility of compensatory mechanisms within VTA DA systems will be investigated as part of the following chapter.

Theoretical implications

The most parsimonious interpretation of the results from this chapter is that cholinergic pPPTg neurons and connected systems are not essential for operant learning or performance. While much focus has been placed on the role of the cholinergic PPTg in the modulation of midbrain DA systems, recent histological studies show that in actuality there are considerably more glutamatergic than cholinergic projections to the midbrain from the PPTg (Wang et al., 2010). It is very possible that loss of these glutamatergic projections or a combined loss of both cholinergic and glutamatergic transmission underlies the learning impairments after non-selective PPTg manipulation. The implications of this, in combination with the results of subsequent chapters, are fully discussed in the general discussion (chapter 8).

Chapter conclusions

The experiment in this chapter was conducted to assess the effects of selective cholinergic lesions of the pPPTg on instrumental learning. This objective was successfully met. In sharp contrast to previous studies using non-selective excitotoxic lesions, no impairment in

learning of operant schedules was observed after highly selective cholinergic lesions of the pPPTg. The interim conclusion from this is that the cholinergic pPPTg and connected structures appear not to be substantially involved in operant learning. This conclusion will be further corroborated by investigating the possibility of compensatory mechanisms in the following chapter.

Chapter 6: Nicotine sensitisation after bilateral selective lesion of cholinergic neurons in the pPPTg

Nicotine and the CNS

Nicotine is an agonist of the nicotinic acetylcholine receptor (nAChR) family of acetylcholine receptors (AChRs). While endogenous Ach activates both muscarinic (mAChR) and nicotinic Ach receptors, nicotine is selective for nAChR subtypes. nAChRs are formed as combinations of 5 α(2-10) and β(2-4) subunits or as 5 repetitions of the single α7 subunit (Gotti et al., 2006; Miwa et al., 2011). Each combination of subunits has a different set of pharmacological properties. For example, the seemingly subtle difference between $\alpha4_2\beta2_3$ and $\alpha4_3\beta2_2$ results in the former having a 10 fold higher sensitivity to nicotine than the latter (Miwa et al., 2011). The distribution of nAChRs is widespread across brain, reaching from the spinal cord to the olfactory bulb (Gotti et al., 2006). Figure 6.1 summarises the distribution of the most frequently found arrangements of receptor subunits and their sensitivity to nicotine. Activation of an nAChR induces an ion channel response in the microsecond to sub-microsecond range and so nicotine can rapidly induce cellular response (Albuquerque et al., 2009). Once nicotine has activated a nAChR, the receptor is typically desensitised for a period of time which is dependent on the receptor subtype and level of previous exposure to nicotine (Dani and Heinemann, 1996; Vezina et al., 2007; Govind et al., 2009; Miwa et al., 2011). Long term exposure to nicotine (a difficult term to define, but it could be as short as several days (Miwa et al., 2011) for certain doses / concentrations) or even perhaps a single exposure (Govind et al., 2009) causes a process that appears to be able to extend this period of desensitisation into "persistent inactivation" in certain subsets of nAChRs (particularly α4β2)(Gentry and Lukas, 2002; Govind et al., 2009). Increased expression of nAChRs have been found after chronic nicotine exposure, with the greatest increases believed to occur also in α4β2 subtypes (Albuquerque et al., 2009). This may be a functional means of maintaining a stable balance in conditions of drug induced extended receptor inactivation. Perhaps most interestingly, different subtypes of nAChR upregulate to different levels (for example α4β4

nAChRs have poor levels of upregulation (Albuquerque et al., 2009)) and some actually downregulate (e.g. α6β2)(Mugnaini et al., 2006). Thus, repeated exposure to nicotine does not simply cause a rightwards shift in the dose-response curve in all brain systems, but results in complex and differential alterations. Because upregulated and downregulated nAChRs will respond differently to reduced nicotine levels after chronic drug exposure, this may in part explain the diffuse range and timescale of symptoms experienced by smokers upon cessation of smoking (Dani and Heinemann, 1996).

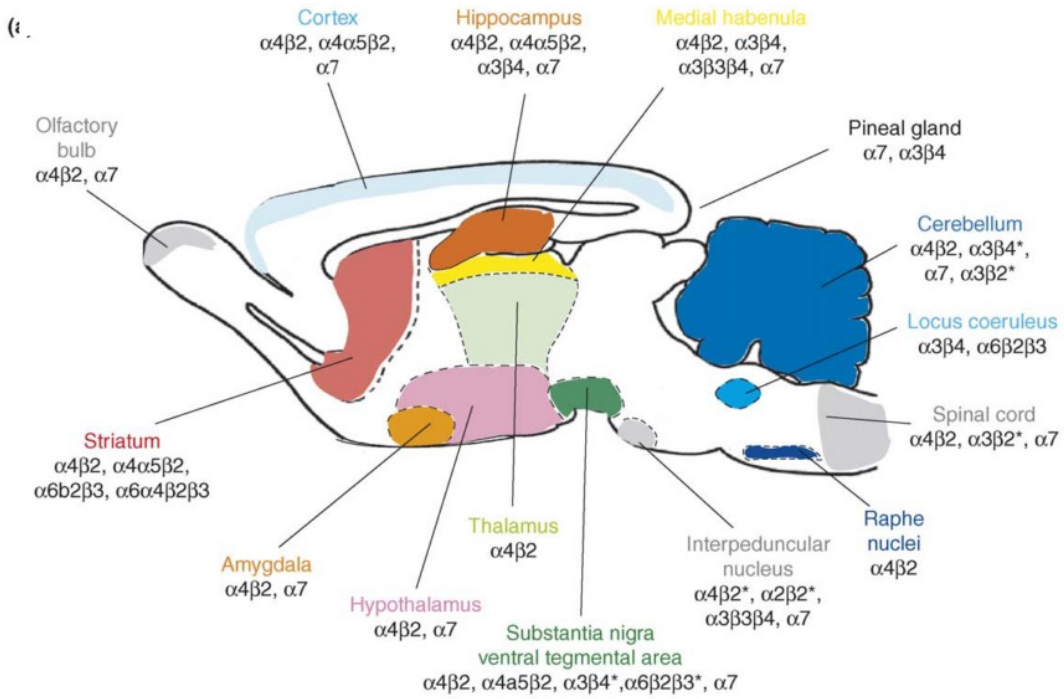


Figure 6.1: Distribution of major locations of known nAChRs throughout the CNS. Image reproduced from: Gotti et al., (2006).

Nicotine induced locomotion

In rats, systemically administered nicotine alters locomotion in a predictable manner. After the first administration, doses within the range of 0.1 – 0.8 mg/kg induce locomotor depression, which, after repeated administration, develops into hyperlocomotion (Benwell and Balfour, 1992; Porter, 2007; Vezina et al., 2007). The effects on locomotion are believed to be a result of nicotine binding to nAChRs in the VTA which causes activation of the mesoacumbens pathway and in turn leads to increased DA levels in the NAcc (Benwell and Balfour, 1992; Rose and Corrigall, 1997). Support for this hypothesis is strong: administration of the nAChR mecamylamine blocks the locomotor effects of nicotine (Clarke and Kumar, 1983), and 6-OHDA lesions of the NAcc also block the locomotor effects (Clarke et al., 1988). However, infusion of nicotine directly into the NAcc has no effect on locomotion, whereas infusion into the VTA (but no other site tested in that study) has the same effects as systemically administered nicotine (Reavill and Stolerman, 1990 ; for review see: Vezina et al., 2007). The majority of nAChRs on VTA DA neurons contain either the β2 or β4 subunit in combination with α3-7 subunits. However, the β3 subunit and homomeric α7 receptors are also present (Wooltorton et al., 2003). Upregulation of VTA nAChRs after repeated nicotine exposure is believed to be a key part of the process resulting in sensitisation and the increased locomotor response to the same dose of drug (Govind et al., 2009). Nicotine also activates nAChRs on VTA glutamate neurons (Grillner and Svensson, 2000) and GABAergic neurons (Mansvelder et al., 2002). It has been proposed that nicotine has a prolonged action on glutamatergic VTA and VTA projecting neurons which in turn increases glutamate driven VTA DA activation. In addition to this, nicotine's action on VTA GABAergic (inhibitory) neurons has been shown to be short transient activation followed by prolonged desensitisation leading to depression of inhibition (Mansvelder et al., 2002). Therefore, the action of nicotine in the VTA is more complex than simply acting directly upon DA neurons and driving DA output, but

instead may involve several parallel events: immediate excitatory action on DA neurons, prolonged activation of DA neurons mediated by glutamatergic activity and persistent depression of inhibitory GABAergic input (Mansvelder et al., 2002; Fowler et al., 2008), with the net result being rapid and sustained increase in mesoaccumbens DA levels.

The pPPTg and nicotine induced locomotion

Non-selective, ibotenic acid lesions of the pPPTg have been shown to alter the standard locomotor response to nicotine (Alderson et al., 2008). After pPPTg lesion, the initial locomotor depressant effects of the drug (seen during the first 2 sessions in control rats) were absent; subsequent locomotor sensitisation developed in both control and pPPTg lesioned rats, but first evidence of hyperlocomotion was seen on session 3 in pPPTg lesioned rats and not until session 7 (the final session) in sham controls (Alderson et al., 2008). The VTA receives strong cholinergic and glutamatergic innervation from the pPPTg, which can modulate and enhance midbrain DA activity (Charara et al., 1996; Parent et al., 1999; Floresco et al., 2003; Maskos, 2008; Wang et al., 2010 ; See general introduction pp36-38 for full discussion). The altered locomotor response and change in rate of sensitisation to systemic nicotine in pPPTg lesioned rats was interpreted to be the result of compensatory changes in the VTA leading to supersensitivity of nAChRs as a result of loss of endogenous cholinergic innervation from the pPPTg. As a consequence of this supersensitivity nicotine would have an enhanced effect (reduced initial locomotor depressant and advanced rate of sensitisation) which is what was observed in pPPTg lesioned rats.

An alternative explanation is that nicotine has a direct effect on pPPTg and disruption of this action, either alone or in combination with supersensitivity of VTA receptors, may account for the altered locomotor response to nicotine observed after pPPTg lesion. For

example nicotinic activation of glutamatergic or cholinergic pPPTg neurons may alter VTA activity and the subsequent release of DA in the mesoaccumbens pathway. The results obtained by Alderson et al. (2008) are unable to distinguish between these two interpretations.

If it were the case that supersensitivity of nAChRs within the VTA had developed in response to loss of endogenous cholinergic innervation from the pPPTg, the same physiological changes, and therefore the same behavioural effect, would be predicted after selective cholinergic pPPTg lesions. If, however, it is the case that the previously observed changes in the locomotor response to nicotine are a result of loss of a pPPTg to VTA glutamatergic pathway, or the combined loss of cholinergic and glutamatergic innervation of the VTA, no behavioural effect of selective cholinergic pPPTg lesions would be predicted.

Investigating possible functional compensation after Dtx-UII pPPTg lesion

In chapter 5 it was found that highly selective lesions of the cholinergic pPPTg produced no deficits in the learning of simple or complex operant reinforcement schedules. One possibility for the lack of behavioural effect is that compensatory mechanisms developed in response to the long Dtx-UII lesion formation and testing period. Could it be possible that the remaining ˜10% of cholinergic cells were able to support normal behaviour? This is unlikely (primarily because ibotenic acid lesions sparing more than 10% of cholinergic cells produced strong deficits in the same behavioural task over a prolonged period of testing). However, due to the different method of lesion formation this possibility should be investigated before being excluded. As discussed in Chapter 4 (pp100-101), loss of neurons can lead to dramatic compensatory changes whereby normal level of post-synaptic transmitter and behavioural function are maintained despite substantial neuronal loss. One key candidate for maintaining

behavioural function is the upregulation and development of supersensitivity of post-synaptic receptors. Upregulation of VTA AChRs is believed to occur after excitotoxic PPTg lesion (Blaha and Winn, 1993; Blaha et al., 1996) which is thought to be responsible for the enhanced locomotor response to nicotine (Alderson et al., 2008). Utilising this VTA mediated behaviour offers a means of assessing the effects of selective cholinergic pPPTg lesions on VTA nAChRs.

Chapter aims

In this chapter, the methodology of Alderson et al. (2008) is repeated with rats bearing bilateral selective cholinergic Dtx-UII pPPTg lesions. This has two aims: (1) to investigate if the changes in nicotine induced locomotion seen after non-selective ibotenic acid lesions are also seen after selective cholinergic Dtx-UII lesions. (2) to investigate the possibility of upregulation developing in VTA nAChRs after loss of pPPTg cholinergic innervation. Experimentally naive rats were given Dtx-UII lesions of the pPPTg. Subsequent extensive habituation to the locomotor testing cages and injection procedure was conducted before systemic nicotine was administered in a day-on day-off regime (saline on off days) for 7 sessions. With the exception that different locomotor testing cages are used (commercially available chambers with an array of 15 infrared light beams rather than in-house made chambers (of a similar size) with 2 light beams) this was an exact replication of the testing protocol used by Alderson et al. (2008). Enhanced response to nicotine after selective cholinergic pPPTg lesion (revealed by increased locomotion in response to the drug, compared to sham controls) would reveal supersensitivity in the VTA; no change in response to nicotine would support the view that VTA nicotinic receptors had not supersensitised after loss of endogenous innervation from pPPTg. The results are discussed with comparison to non-selective ibotenic pPPTg lesions and the effects of nicotine on the mesopontine tegmentum and midbrain dopamine systems.

Methods

Subjects

Twenty-four adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used, with a mean weight of 355g (range 331 – 389g) at time of surgery. Animals were housed and maintained as described in the general methods.

Surgery

Lesion surgery was performed as described in the general methods. Sham pPPTg lesioned rats (n = 8) were infused with 200 nL sterile PB, lesioned rats (n = 16) were infused with 200 nL 3% Dtx-UII (in sterile PB). The co-ordinates for the pPPTg were: -0.8 mm from the IAL, ± 1.9 mm from the midline, -6.5 mm below dura (as used in chapter 4 and 5) and the order of infusion (left hemisphere, right hemisphere) was alternated across rats.

Behavioural testing

To allow full formation of the lesion, behavioural testing began 21-24 days post-surgery. During this time animals were monitored daily for signs of ill health or other lesion related complications. Locomotor testing was conducted in the locomotor monitoring photocell cages described in the general methods. Daily testing sessions were 60 min in duration, conducted in a dimly illuminated room and each session had a proportionally equal number of sham and lesioned rats. Rats were given three habituation sessions where they were placed in the locomotor cages without any injections, this was followed by seven sessions where rats were injected with 0.9% w/v saline (s.c.; 1mL/kg) immediately prior to testing. After completing this habituation period nicotine testing began. Nicotine sensitisation was performed in a day-on day-off routine whereby rats received nicotine (s.c.; 0.4 mg/kg in 0.9%

saline; nicotine hydrogen tartrate, Sigma–Aldrich, UK; dose refers to salt) or saline (0.4 mg/kg 0.9% saline) on alternating days for 14 days. The order of testing was counterbalanced so that on any given day half the rats received nicotine and half saline. All injections were performed in a procedure room opposite the locomotor testing room, each rat was individually taken to the procedure room, injected, then taken to and placed in the locomotor testing cage, which started recording beam breaks immediately. Thus each rat was always injected in the same room, and no rat was ever in the presence of another rat receiving an injection.

Histology

All histological procedures followed the methods described in the general methods. Briefly, after completing behavioural testing rats were transcardially perfused with fixative and brains stored in sucrose solution. Subsequently, 30 µm coronal sections were cut through the area of the PPTg and ~1mm beyond in anterior-posterior plane. Parallel 1:4 series were processed immunohistochemically for ChAT and NeuN reactivity. NeuN stained sections were then counterstained with cresyl violet and all stained sections mounted onto glass slides and viewed under a light microscope. Software assisted counts of ChAT+ neurons throughout the PPTg enabled quantification of the lesions. A lesion was considered acceptable if >~80% of ChAT+ pPPTg neurons were destroyed bilaterally and there was minimal, if any, non-selective damage evident on the NeuN/cresyl sections.

Behavioural data analysis

Data were analysed using PASW 18 for Windows (SPSS Inc., Chicago, Illinois USA). A range of repeated measures ANOVAs with appropriate post hoc tests were performed. Details of particular factors analysed are reported in the text. For locomotor data the number of beam

breaks per session were SQRT transformed to correct for positive skew in the data (identified by the Shapiro-Wilk test). Results were considered significant when $p \leq 0.05$.

Results

All animals recovered well from the surgical procedure. During the 21 day lesion formation period a small yet significant decrease in bodyweight growth was observed. See figure 6.2. Repeated measures ANOVA found a main effect of *group* ($F_{1,13} = 6.0$, $p = 0.029$), *day* ($F_{20,260} = 67.4$, $p < 0.001$) and *a day x group* interaction ($F_{20,260} = 1.8$, $p = 0.022$).

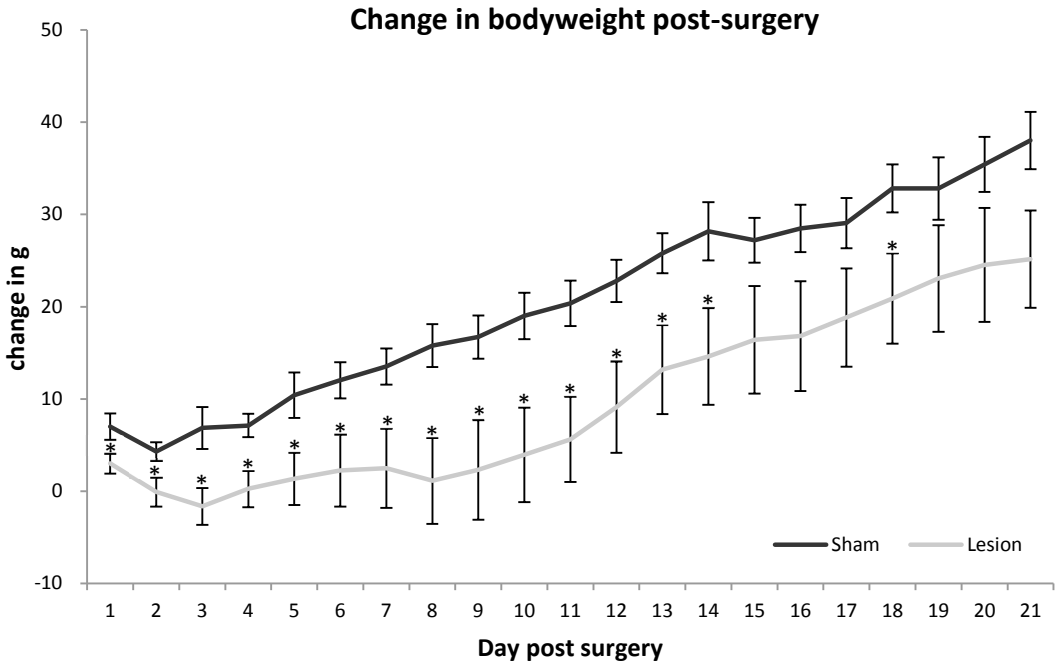


*Figure 6.2: change in bodyweight in the 21 day post-surgery period. * indicates significant difference between sham and lesion on that day. Graph shows group means ± SEM.*

Lesion analysis

Seven rats had selective bilateral lesions of the cholinergic pPPTg. These lesions destroyed a mean of 89.5% of ChAT+ pPPTg neurons (range 78.8% to 94.8%) with only very small, if any, evidence of non-selective damage on the NeuN/Cresyl stain. Figure 6.3 (p160) shows representative lesion and sham tissue. The remaining rats in the lesion group were excluded from all analysis due to having unilateral lesions (n = 2); partial ChAT+ lesions (range ˜34-70% cell loss, n = 5); having unilateral non-selective damage (n = 1) or death before being processed for histology (n = 1). This rat was found dead in the homecage after completing behavioural testing but before histological processing began. This was 8 weeks post-surgery, before the death (which happened when the rat was unattended in the home cage) the rat had appeared healthy (stable weight and no indications of stress) and so it was not believed to be related to surgery or testing (a view reached after consultation with the unit NACWO).

Characterisation of lesion

The distribution of cholinergic neurons throughout the PPTg is shown in figure 6.4. The number of remaining pPPTg ChAT+ cells in the lesion group was calculated and expressed as a percentage of the sham mean. This is shown in figure 6.5. T-tests (on raw data, not percentages) confirm that lesioned rats had significantly fewer ChAT+ pPPTg cells than sham rats in both the left ($t_{13} = 9.1$ $p < 0.001$) and right ($t_{13} = 9.8$ $p < 0.001$) hemispheres.

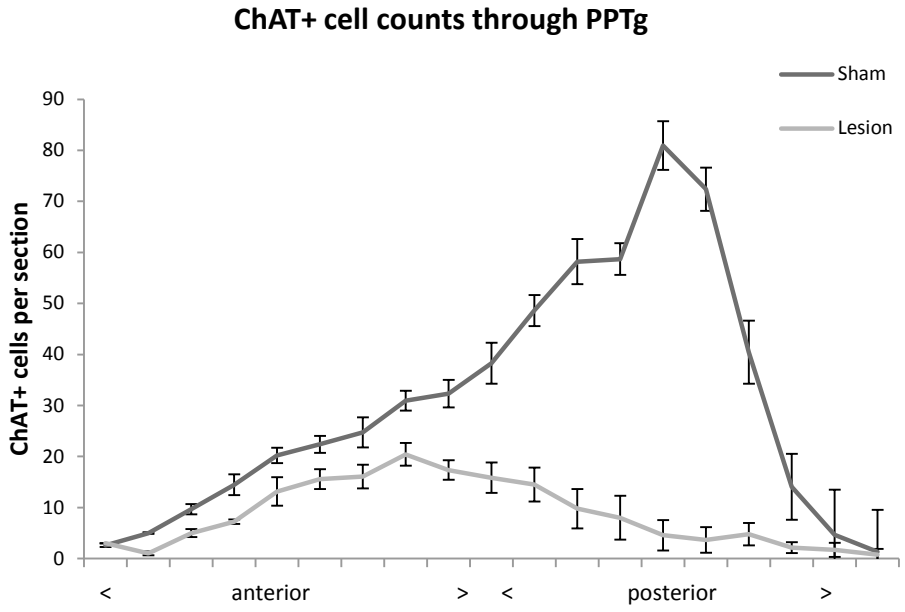


Figure 6.4: Distribution of ChAT+ neurons throughout the PPTg. ChAT counts = Numbers of ChAT+ neurons. Graph shows group means ± SEM.

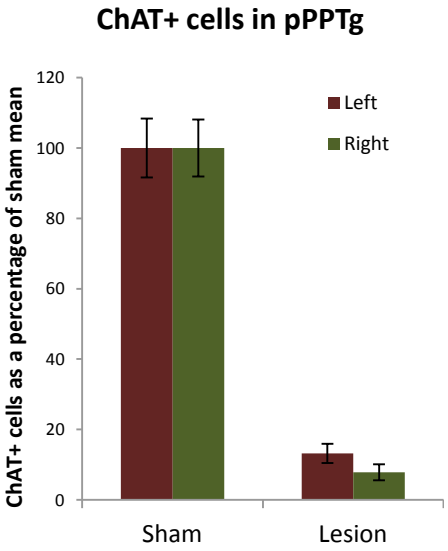


Figure 6.5: Number of ChAT+ pPPTg cells as a percentage of sham mean. Left = left hemisphere; right = right hemisphere. Graph shows group means ± SEM.

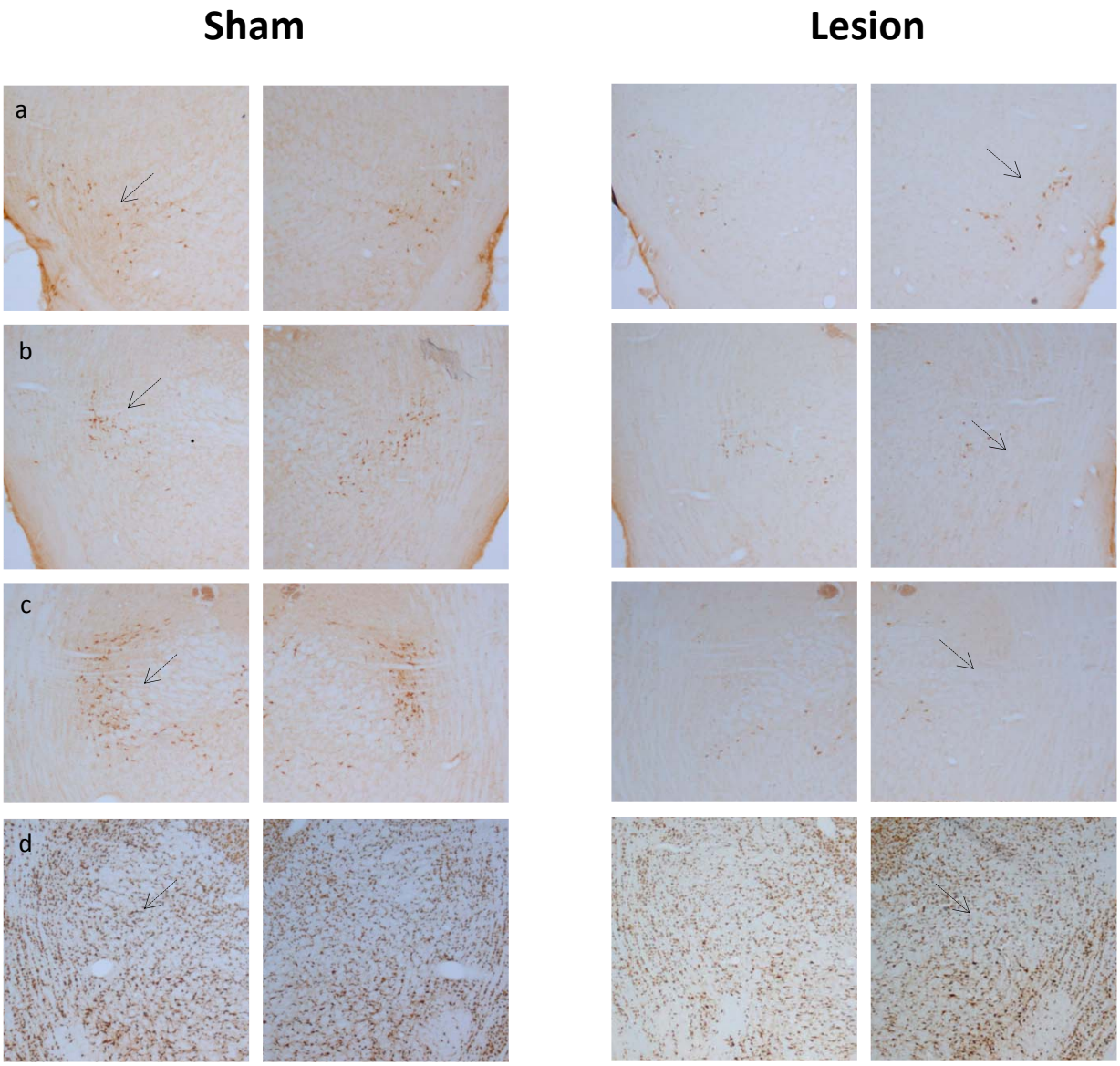


Figure 6.3: Example sections of a sham and a Dtx-UII lesioned rat. Rows a – c show ChAT stained sections of anterior PPTg (row a), central PPTg (row b) and posterior PPTg (row c). Row d shows a NeuN / Creyl double stained section immediately parallel to row c, at the level of the posterior PPTg and greatest ChAT cell loss. Dotted arrow indicates the location of the PPTg

Behavioural results

Habituation sessions

The rate of locomotion and rearing during the habituation sessions (where rats had 3 sessions of no injections followed by 7 sessions with saline injections) is shown in figure 6.6 and 6.7. For beam breaks during the daily habituation sessions a repeated measures ANOVA found a main effect of *session* ($F_{6.41,83.40} = 6.46$, $p < 0.001$) but not *group* ($F_{1,13} = 0.63$, $p = 0.44$) and no *session x group* interaction ($F_{6.41,83.40} = 1.27$, $p = 0.28$). Restricted planned pairwise comparisons found that sessions 1, 2 and 3 differed from some, but not all, later sessions (1 from 6 and 8; 2 and 3 from 8) and that from session 4 onwards there were no differences between sessions. The same analyses on the rearing data found a main effect of *session* ($F_{6.56,85.32} = 3.86$, $p = 0.001$) but not *group* ($F_{1,13} = 2.09$, $p = 0.17$) and no *session x group* interaction ($F_{6.56,85.32} = 0.96$, $p = 0.46$). Restricted planned pairwise comparisons failed to find any significant differences between sessions, however habituation sessions 1 and 3 did approach significance compared to the final session ($p = 0.095$ and 0.057 respectively). These data show that Dtx-UII lesions of pPPTg had no effect on spontaneously generated locomotion or rearing during the habituation sessions. Moreover, they show that by the end of the habituation sessions rats performance was stable, confirming habituation to the testing environment had been achieved.

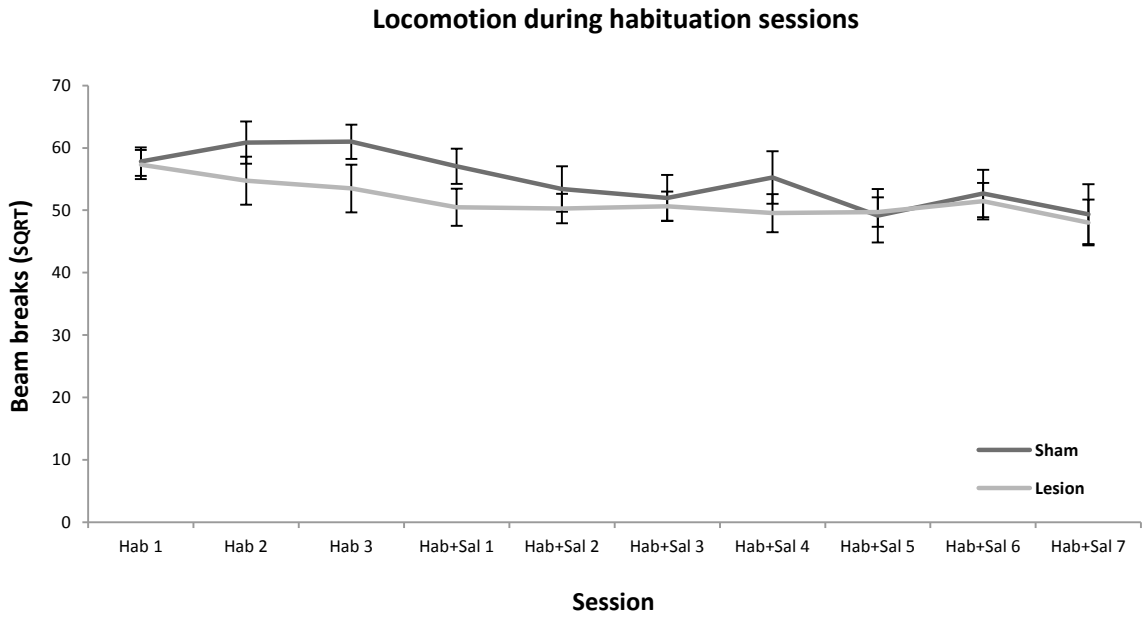


Figure 6.6: Beam breaks (SQRT transformed) made during the habituation sessions. Hab = habituation session; Hab+sal = saline injection and habituation session. Graph shows group means ± SEM.

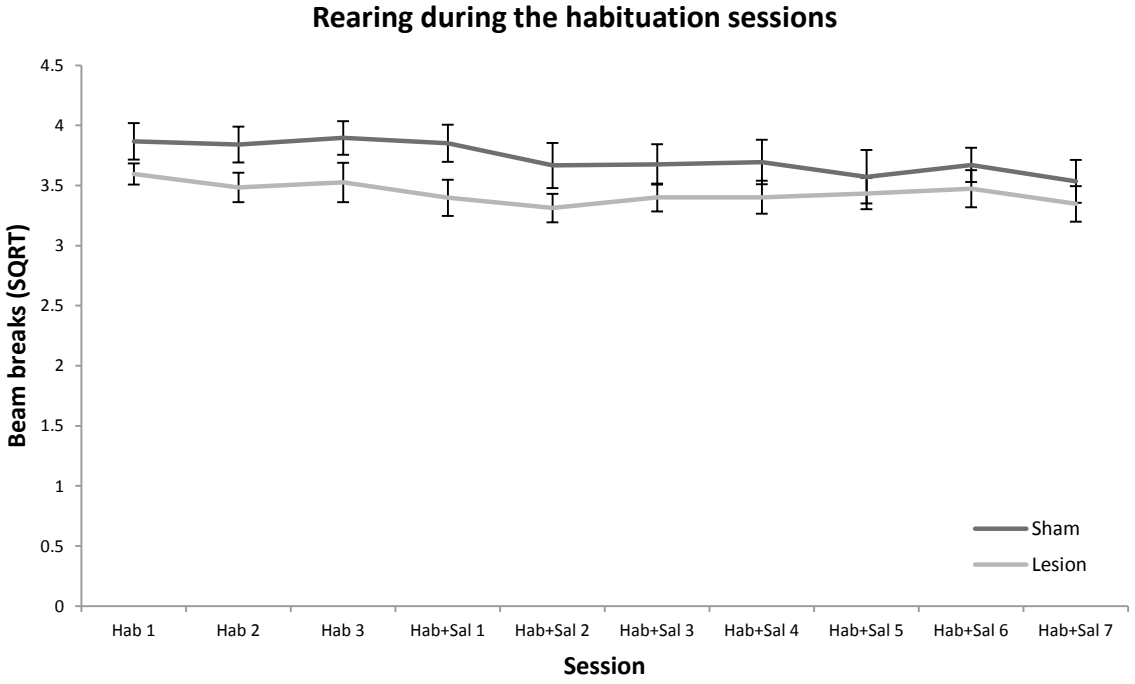


Figure 6.7: Rearing (SQRT transformed) during the habituation sessions. Hab = habituation session; Hab+sal = saline injection and habituation session. Graph shows group means ± SEM.

Nicotine testing sessions

Figure 6.8 shows the mean number of beam breaks (basic movements) during the nicotine and saline testing sessions. Repeated measures ANOVA found a significant effect of *session* ($F_{6,78}$ = 27.39, p < 0.001) a *drug x session* interaction ($F_{6,78}$ = 44.52, p < 0.001) a near significant effect of *drug* ($F_{1,78}$ = 4.58, p = 0.052) and that all effects involving *group* were non-significant (*group* ($F_{1,13}$ = 0.58, p = 0.46); *drug x group* ($F_{1,78}$ = 2.76, p = 0.121); *group x session* ($F_{6,78}$ = 1.75, p = 0.122); *drug x group x session* ($F_{6,78}$ = 0.76, p = 0.601)). Bonferroni corrected paired sample t-tests comparing the effect of nicotine and saline during each session found that during the first session both the lesion and sham groups displayed hypolocomotion (sham t_7 = -4.82, p = 0.014; lesion t_6 = -8.56, p < 0.01) which developed into hyperlocomotion during the later testing sessions (sham session 5: t_7 = -6.33, p < 0.01. Lesion session 6: t_6 = -6.10, p = 0.007).

Nicotine induced locomotion

To further assess whether nicotine induced a different level of change in locomotion in the lesion compared to the sham group, for each rat the beam breaks made in each saline session was subtracted from the corresponding nicotine session. Group means are shown in fig 6.9. Repeated measures ANOVA found a main effect of *session* ($F_{6,78}$ = 46.02, p < 0.01) but no effect of *group* ($F_{1,13}$ = 1.61, p = 0.226) or *session x group* interaction ($F_{6,78}$ = 0.40, p = 0.88).

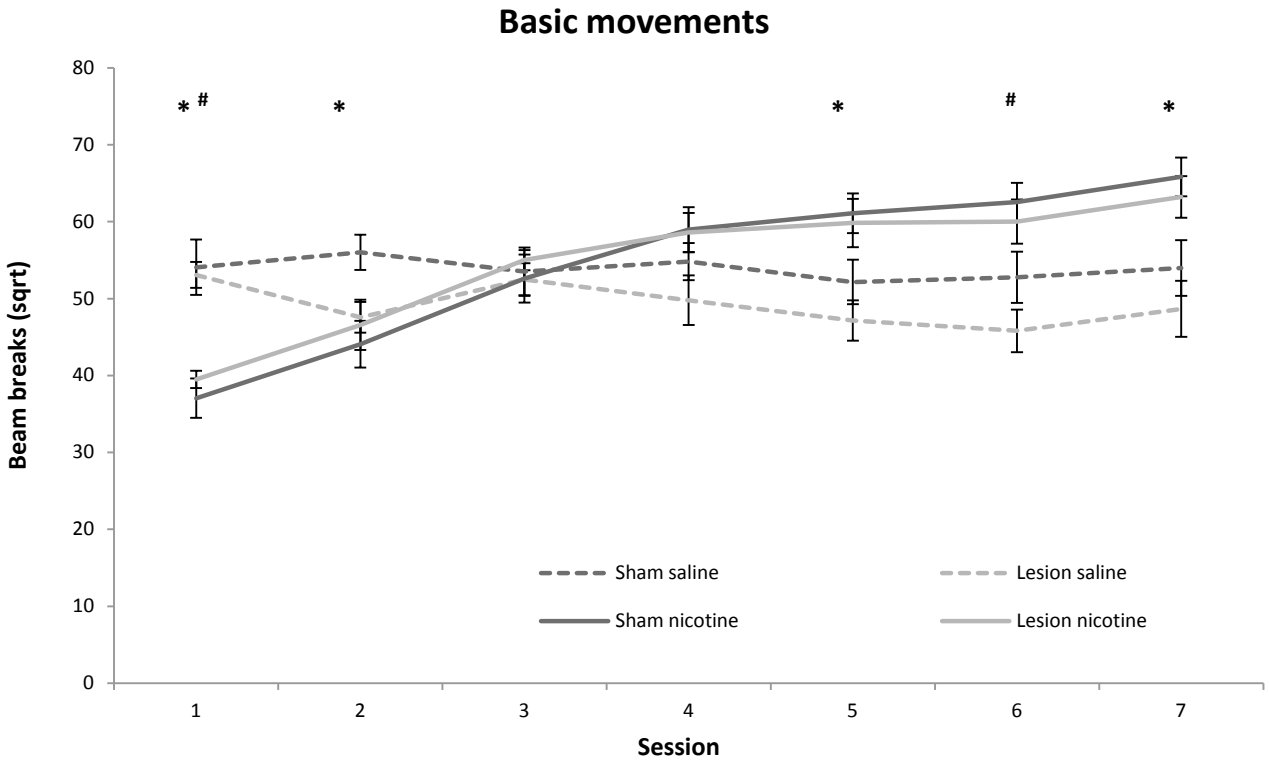


*Figure 6.8: Basic movements (SQRT transformed) made during the nicotine testing sessions. Graph shows group means ± SEM. * indicates significant difference between saline and nicotine in the sham group in the corresponding session; # indicates difference between saline and nicotine in the lesion group in the corresponding session.*

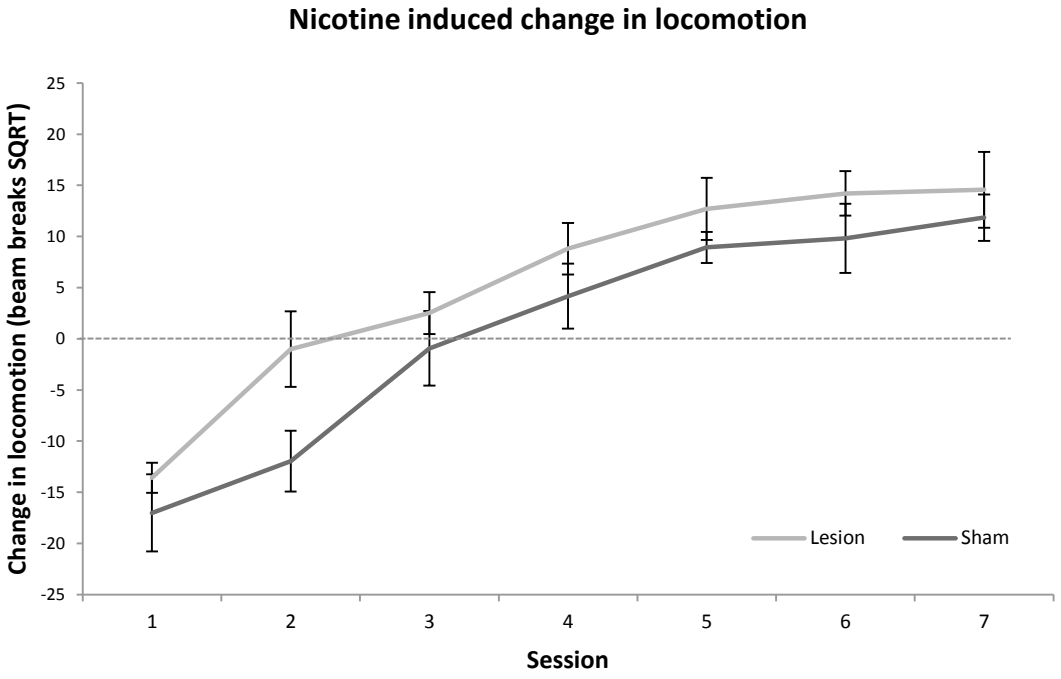


Figure 6.9: Nicotine induced change in locomotion. Graph shows group means ± SEM.

Individual responses to nicotine

In order to investigate whether there was a pattern within each group (for example a small number of outliers affecting the group mean or a particular pattern displayed by individual rats) the response to nicotine of each rat was plotted and inspected. No clear pattern emerged; all rats displaced similar locomotor patterns and there was no sign of a large degree of variance within the lesion group, indeed the sham group often had higher variance and a greater spread on individual days than the lesion group. Figure 6.10 shows the rates of locomotion of each rat on the first and last nicotine testing days, data from other days (not shown) reveals a similar pattern.

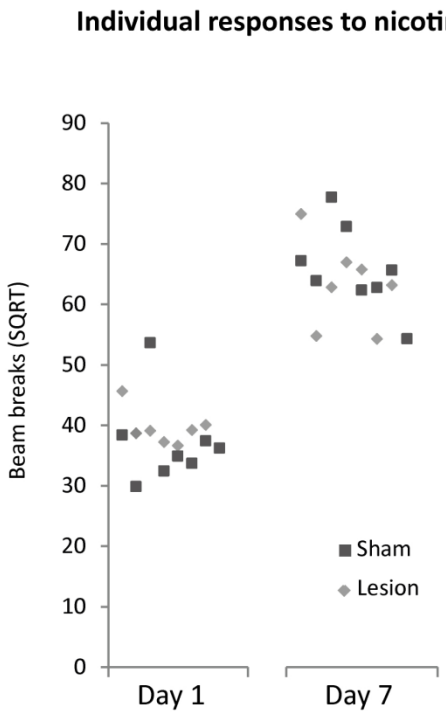


Figure 6.10: Responses to nicotine of every rat on the first and last day of nicotine injections. Each point represents one rat.

Initial response to nicotine

The lack of significant group effect in the analyses performed suggests that supersensitivity to nicotine had not developed in VTA nAChRs. However, as the effects of repeated nicotine are complex and long lasting, it is likely that by the second administration of nicotine the chain of changes the drug itself produces on nAChRs had already started. This could potentially mask any more subtle effects caused by the effects of the Dtx-UII pPPTg lesion. An additional analysis restricted to the first nicotine testing session was therefore performed to assess the effects of nicotine in Dtx-UII lesioned rats before any long term nicotine induced changes had developed. Figure 6.11 shows the level of locomotion after initial nicotine administration and the nicotine induced change in locomotion. Univariate ANOVA comparing the effect of nicotine on the level of basic movements finds no effect of *group* ($F_{1,13} = 0.69$, $p = 0.422$). Likewise, univariate ANOVA finds no effect of *group* on nicotine induced locomotion ($F_{1,13} = 0.71$, $p = 0.414$).

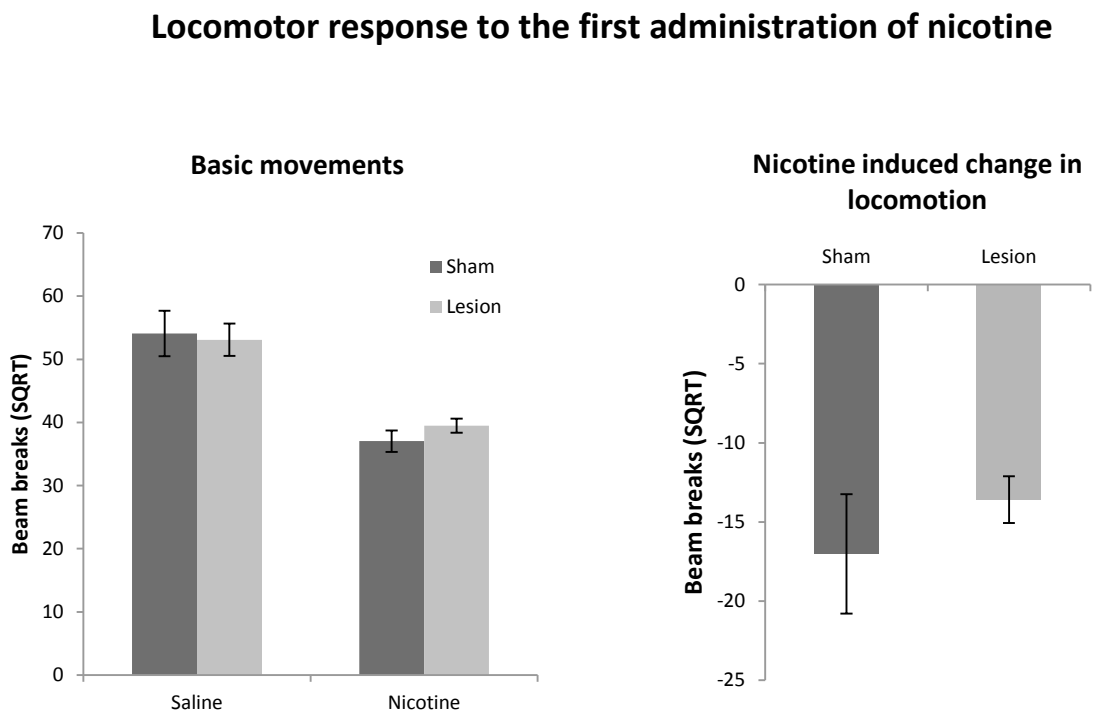


Figure 6.11: Basic movements (left) and nicotine induced change in locomotion (right) during the first testing session. Graph shows group means ± SEM.

Chapter discussion

This experiment was conducted to investigate the effects of selective cholinergic pPPTg lesions on the locomotor response to repeated systemic nicotine administration. Dtx-UII lesions destroyed ~90% of pPPTg ChAT+ neurons. Extensive habituation to both the testing environment and injection procedure was performed prior to the start of alternating nicotine (0.4mg/kg) and saline testing sessions. No differences in baseline levels of locomotion or rearing were found during the habituation sessions. During the testing sessions, nicotine caused hypolocomotion after first administration, which developed into hyperlocomotion across the testing sessions. The same levels of locomotion and the same pattern of sensitisation were observed in both the sham and lesion group: selective cholinergic lesions of

the pPPTg produced no changes in nicotine induced locomotion or the rate of sensation to nicotine.

Relation to previous work

The results of this study are in contrast to those of Alderson et al. (2008) who found that non-selective ibotenic acid lesions of the pPPTg altered the locomotor response to nicotine. The locomotor depression normally seen after initial administration was absent and hyperlocomotion to repeated nicotine was accelerated, developing on session 3 rather than session 7. The authors offer several interpretations, the primary one being that their result would be predicted if supersensitivity of VTA nAChRs had developed in response to loss of endogenous cholinergic innervation from the pPPTg. Support for supersensitivity of DA nAChRs after PPTg loss also comes from an older study finding an increase in nicotine induced DA efflux in dorsalmedial striatum (measured with chronoamperometry and microdialysis) after nicotine infusion directly into the SNc of whole-PPTg lesioned rats (Blaha and Winn, 1993). The findings from the current study are not compatible with the hypothesis that upregulation of VTA nAChRs occurs after loss of endogenous PPTg cholinergic innervation and that this underlies the alterations of nicotine induced locomotion observed after ibotenic pPPTg lesions. One methodological consideration is that the Dtx-UII lesion might not be as destructive even to cholinergic neurons as ibotenic lesions, resulting in less compensatory upregulation. Alderson et al. (2008) do not quantify the size of cholinergic loss in their study, but experience of the ibotenic lesion technique and other published results normally find that ibotenic lesions are not markedly destructive to cholinergic PPTg neurons and call loss in the region of 50-70% is quite normal (Blaha et al., 1996; Wilson et al., 2009a). The selective cholinergic lesions in this experiment had a mean cell loss of 90%, which is generally higher than that obtained with ibotenic acid. It is therefore unlikely that the difference in behavioural effects is the result of

smaller cholinergic lesions in this study. Consequently, it is reasonable to conclude that the different results are a consequence of the different type of lesion (no major loss of glutamatergic or GABAergic neurons after selective cholinergic lesion).

Does nicotine have a direct effect on PPTg?

A question raised by these results is where the locus of the effect is after non-selective pPPTg lesion: is it due to loss of nicotine's direct action on non-cholinergic pPPTg, or is it the result of changes in VTA after pPPTg lesion (for example, alterations in nicotine induced glutamatergic activation of DA neurons caused by loss of glutamatergic input from pPPTg). At the most fundamental level, in order for nicotine to have a direct effect on PPTg, PPTg neurons must express appropriate nicotinic receptors. This is indeed the case - both cholinergic and non-cholinergic PPTg neurons express mRNA for α7 and β2 nAChR subtypes; while non-cholinergic neurons also extensively express α4 receptors, only around 5% of cholinergic neurons express this subtype (Azam et al., 2003). As the α4β2 receptor subtype is considerably more susceptible to upregulation by nicotine than the α7β2 subtype (Azam et al., 2003; Albuquerque et al., 2009) the differential pattern of receptor expression suggests that upregulation is more likely to develop in non-cholinergic rather than cholinergic PPTg neurons. Behaviourally there is also evidence that nicotine has a direct effect on PPTg. In a conditioned place preference paradigm, nicotine infused directly into the PPTg (but not in control infusions 2 mm dorsal) caused a place preference for the side of the CPP apparatus paired with drug infusion (Iwamoto, 1990).

The PPTg and nicotine self-administration

Much of the literature on nicotine and the PPTg addresses the potential role of the PPTg in nicotine intravenous self-administration (IVSA). In rats pre-trained to intravenously

self-administer drug, infusion of the GABA agonist muscimol into PPTg reduced the rate of nicotine, but not cocaine, self-administration (Corrigall et al., 2001). The contrast between drugs suggests that the reduction in IVSA was specific to nicotine and not a general reduction in level of reward related performance. Likewise, lesions made with the ethylcholine mustard aziridinium ion AF64A which were reported to be selective for PPTg cholinergic neurons also reduced rates of nicotine self-administration (Lanca et al., 2000b). While this toxin was claimed to make selective cholinergic lesions, this and other laboratories have found it causes non-selective neuronal and also physical damage (including to fibres of passage) in the area of the PPTg (Rodriguez et al., 1998; Alderson et al., 2006 ; see chapter 1, p15 for full discussion of AF64A). In contrast to these findings it has been shown that ibotenic acid lesions restricted to the pPPTg enhanced nicotine self-administration (Alderson et al., 2006). Differences in experimental design may explain this apparent inconsistency: In the studies finding a reduction in nicotine IVSA after PPTg inactivation there were no additional conditioned stimuli programmed to accompany the drug delivery (however the houselight did extinguish for 60 sec). In the lesion study finding enhanced responding after pPPTg lesions, animals were pre-trained to high levels of lever pressing for food reward and drug delivery was simultaneous with illumination of a bright light above the lever and retraction of both levers for 20 seconds. Thus in this experiment reward delivery was accompanied with many conditioned stimuli. It is known that in conditions where nicotine delivery is paired with prominent stimuli (CS light or tone) robust self-administration can develop, but lack of any CS leads to considerably lower and unstable rates of administration (Liu et al., 2007). This fits current views of nicotine having reinforcement enhancing properties rather than being a particularly strong reinforcer in its own right, with drug delivery associated with mildly rewarding CS significantly enhancing rates of self-administration compared to conditions with no CS (Palmatier et al., 2006; Farquhar et al., 2011). Therefore, the seemingly contradictory findings of PPTg lesion reducing lever

pressing in some studies (Lanca et al., 2000b; Corrigall et al., 2001) and enhancing it in others (Alderson et al., 2006) may in actuality be the result different training and experimental IVSA protocols (particularly the use of CS lights) and the way these interact with PPTg lesion. Regardless of the nature of what underlies this inconsistency, these studies show that PPTg manipulation affects responding for nicotine. As the same manipulations do not affect levels of responding for cocaine or food pellet reward (Corrigall et al., 2001; Alderson et al., 2006) this change is not a global deficit in reward related lever pressing, but is instead selective for performance of lever pressing when it is associated with nicotine. This body of work confirms that the PPTg has a close functional relationship with nicotine, it does not, however, show that this is due to nicotine having a direct effect on PPTg. These results could still be the explained by the consequence of, for example, alterations in the effect of nicotine in PPTg afferent structures after loss of endogenous cholinergic innervation (as is suggested by Alderson et al., 2006). To confirm that nicotine can and does activate PPTg neurons a direct measurement of this is required.

C-fos expression in the PPTg after nicotine administration

C-fos is an immediate early gene transiently expressed by many neurons upon activation, due to the relative ease of immunohistochemically marking the protein produced by the gene, it has become a frequently used indicator of neuronal activation in response to experimental manipulations (for review see: Okuno, 2011). Single administration of systemic nicotine induces c-fos expression in PPTg over and above that of control rats (Lanca et al., 2000a). Interestingly, the same study found that over 95% of nicotine activated PPTg cells were non-cholinergic. Further analysis speculated that the number of activated non-cholinergic neurons represented the entire non-cholinergic PPTg population. Therefore all non-cholinergic and less than 5% of cholinergic neurons were activated by systemic nicotine.

NADPHd (a selective stain for nNOS expressing neurons) was used as a histochemical marker of cholinergic neurons, while a reliable and often used marker (eg Alderson et al., 2008), combining it with c-fos immunohistochemistry is less common. Histochemical NADPHd is a very dark stain and could potentially mask double labelled neurons. Porter (2007) repeated and extended the Lanca et al study, using a double labelled double immunohistochemical stain to mark nNOS (for cholinergic neurons) and c-fos. Double labelling of ChAT and c-fos would be the ideal solution, however antibody compatibility issues make this unfeasible with the c-fos antibody used in those studies (Latimer MP, Personal communication; 2009). Porter's results confirm and extend those of Lanca et al. (2000a): acute and chronic (5 days of daily injections) nicotine induced c-fos expression in PPTg, with levels of fos and cholinergic co-expression around 1% in both condition (Porter, 2007). These studies strongly suggest that within PPTg nicotine preferentially acts upon non-cholinergic rather than cholinergic neurons. However, the very low expression of c-fos in cholinergic neurons may not be a reliable indicator of no activation. One intriguing finding when reading the literature on c-fos expression in PPTg cholinergic neurons is that while studies have found c-fos expressed in these neurons (confirming that they can express the c-fos protein) doubled labelled fos and cholinergic cells are almost always a small minority of all c-fos labelled PPTg neurons, typically around 5% (see: Shiromani et al., 1992; Shiromani et al., 1996; Yamuy et al., 1998; Maloney et al., 1999; Lanca et al., 2000a; Nakahara et al., 2001; Torterolo et al., 2001; Hayward and Castellanos, 2004; Mena-Segovia et al., 2004b; Verret et al., 2005; Deurveilher et al., 2006; Heise and Mitrofanis, 2006; Brudzynski et al., 2011). The only condition to show the reverse pattern (more cholinergic than non-cholinergic activation) is in a REM sleep rebound study, this found a relatively small number of PPTg c-fos cells (n = 64, considerably smaller than the numbers reported in other studies) and 75% of these were also ChAT+ (Datta et al., 2009). This reinforces that PPTg ChAT cells can express c-fos, however, taking all of these studies together

the general finding of very low level c-fos expression in PPTg cholinergic cells raises questions about how to interpret the results. The induction threshold for c-fos is now considered high in comparison to other immediate early genes (such as *zif268*) (Okuno, 2011), some neurons do not reliably express c-fos and there are also conditions where activation does not lead to c-fos expression (for example relative increases in activation of already active neurons and realise from chronic inhibition (Kovacs, 2008)). These features of c-fos expression have led many authors to reinforce the view that no staining for c-fos should not be used as evidence of no activation (Cullinan et al., 1995; Kovacs, 2008; Okuno, 2011). Nakahara et al. (2001) analyse PPTg co-expression of c-fos and ChAT and c-fos and GABA and discover that even in control animals ChAT and c-fos co-expression is remarkably low compared to GABA and c-fos co-expression (5% of c-fos+ neurons were also ChAT+ 50% of c-fos+ were GABA+), which they claim is evidence that PPTg cholinergic neurons do not easily or reliably express c-fos and so is not a good marker of activation of these neurons.

It is possible that the low level of co-expression of c-fos and ChAT in behavioural studies gives insight into the function of these neurons, for example indicating that acute behavioural changes do not activate this neuronal population whereas longer lasting more global processes (such as REM rebound sleep) do. However, until additional analysis (for example with other immediate early genes or electrophysiological characterisation of neuronal responses to nicotine) add further support, the nicotine c-fos studies should be taken as a potential indication that nicotine does not activate PPTg cholinergic neurons rather than a final conclusion.

Support for the view that nicotine does indeed activate PPTg cholinergic neurons (in apparent contrast to the *c-fos* studies) comes from a slice preparation study and a microdialysis study. In slice preparation application of nicotine has been shown to depolarise

both cholinergic and non-cholinergic mesopontine tegmentum neurons (Ishibashi et al., 2009). Unfortunately, this study cannot give insight into whether nicotine preferentially acts upon non-cholinergic neurons, but as 100% of the cholinergic neurons tested were affected by nicotine it does suggest that this neuronal population is readily susceptible to the drug. Nakahara et al. (2001) used microdialysis to measure ACh levels in the VTA after ICSS of the MFB and compared this with levels of ChAT and c-fos co-expression in the PPTg and LDTg (a major source of ACh in the VTA). They found 1 h of ICSS produced a significant increase in ACh levels in the VTA and a significant increase in non-cholinergic Fos expression in PPTg and LDTg. However, levels of c-fos expression in cholinergic PPTg/LDTg neurons were low (again around 5% in control rats and 7% in the ICSS group). Given that PPTg was clearly activated by the MFB ICSS, that VTA ACh levels rose sharply and the most likely source of this ACh is the PPTg/LDTg cholinergic output, they argue that the most reasonable explanation for the low expression of c-fos in PPTg/LDTg cholinergic neurons was due to an inability of these neurons to reliably express c-fos rather than due to not being activated. However, they cannot rule out the possibility that the ACh was released presynaptically in the VTA or that it came from other less prominent sources.

Reanalysis of the role of the pPPTg in nicotine induced locomotion

The results of the experiment conducted in this chapter show that selective bilateral loss of cholinergic pPPTg neurons does not affect the locomotor response to nicotine or the rate of nicotine sensitisation. This suggests that the changes in nicotine induced locomotion previously reported after non-selective ibotenic acid lesions are the result of glutamatergic or GABAergic pPPTg loss. There are strong glutamatergic projections from pPPTg to VTA which from excitatory synapses with both DA and non-DA neurons, while less is known about GABAergic projections (and there are considerably fewer GABAergic than glutamatergic or

cholinergic neurons in pPPTg) these also project to the VTA (Charara et al., 1996; Winn, 2006; Wang and Morales, 2009; Wang et al., 2010). As discussed in the chapter introduction, within the VTA the effects of nicotine are more complex than simple activation of DA neurons by binding to nAChRs expressed on DA containing neurons and is hypothesised to consist of three components: direct activation of DA neurons; activation of glutamatergic neurons which subsequently enhances glutamatergic mediated drive of DA neurons; activation followed by long term inhibition of GABAergic signalling leading to a reduction of GABAergic inhibition of DA neurons. Disruption of the balance between these processes could result from loss of glutamatergic innervation from pPPTg. If compensatory upregulation of glutamate receptors developed after excitotoxic pPPTg lesion the nicotine induced VTA glutamate release would subsequently lead to enhanced activation of the DA mesoaccumbens pathway and increased locomotion. This hypothesis is in fitting with the results of Alderson et al. (2008). In addition to considering the effects in VTA, as discussed above, nicotine clearly has an effect on the PPTg and changed activity within the PPTg or in the projections to VTA and elsewhere rather than changed activity of receptors in the VTA may explain some of the effects of pPPTg lesions on nicotine induced locomotion. Support for this comes from studies finding changes in midbrain DA meditated increases in striatal DA levels following manipulation of PPTg and LDTg mAChRs (Forster and Blaha, 2000, 2003).

Further investigation of pPPTg-VTA interactions and the role these play in the effects of nicotine could be achieved with selective manipulation of receptors (for example intra-VTA infusion of the ionotropic glutamate receptor antagonist kynurenate prior to nicotine administration) and targeted rather than systemic nicotine delivery (delivering nicotine directly into VTA and pPPTg rather than s.c.). Establishing the locus of nicotine's effect (does nicotine in the pPPTg have any effect on locomotion? What effect does excitotoxic pPPTg lesion have on intra-VTA nicotine infusion?) and the effects of manipulation of signalling within the VTA

(what happens to nicotine induced locomotion when VTA glutamate signalling is depressed?) would disassociate some of the multiple effects of nicotine on these brain regions.

No evidence of upregulation of VTA nAChRs after bilateral selective cholinergic pPPTg lesion

A sub-aim of this chapter was to specifically investigate whether there was evidence of upregulation of VTA nAChRs after loss of endogenous cholinergic innervation from the pPPTg. One interpretation of the lack of behavioural effects of selective cholinergic lesions on operant learning (chapter 5) was that the slow formation of the Dtx-UII lesion and delay between lesion and testing enabled greater functional compensation to develop compared to the quick nature of ibotenic acid lesions and testing. Based on previous studies showing increased striatal DA levels after cholinergic stimulation of midbrain DA neurons in PPTg lesioned rats, and of an altered response to VTA mediated nicotine induced locomotor changes in PPTg lesioned rats, it was hypothesised that upregulation would be revealed by an increased response to nicotine in the lesion group. That no such increase was observed, both when analysed across all nicotine sessions or when the analysis was restricted to the first nicotine session, suggests that no substantial upregulation and therefore no extensive functional compensation had developed. It is possible that upregulation at a level not high enough to produce a measurable behavioural change had occurred, this could be further analysed with a more direct measure of ACh levels in the VTA (for example with electrochemistry) but such low levels of change are unlikely to enable a complete recovery of behavioural function (see chapter 4, pp100-101 for full discussion).

Chapter conclusions

This aim of this chapter was to establish if selective cholinergic lesions of the pPPTg produced the same changes in nicotine induced locomotion as non-selective excitotoxic lesions and attempt to integrate the results into the broader literature on the PPTg and nicotine. The Dtx-UII lesions were highly selective for cholinergic pPPTg neurons and reduced pPPTg ChAT+ neuron count but an average of ˜90%. In the behavioural response to nicotine there was no significant difference between sham and lesioned rats: nicotine did not induce higher levels of locomotion in the lesion group and the rate of sensitisation was unchanged. This supports the view that the behavioural changes seen after excitotoxic pPPTg lesions were the result of loss of glutamatergic and GABAergic (with or without the combined loss of cholinergic) neurons rather than solely the loss of cholinergic neurons. Further support for the non-involvement of the cholinergic pPPTg in nicotine induced locomotion comes from c-fos studies showing that both acute and chronic nicotine induces c-fos expression in non-cholinergic, but not cholinergic PPTg neurons. However, as there is evidence that nicotine directly acts upon cholinergic mesopontine tegmentum neurons the results of the *c-fos* studies need to be treated with caution.

Chapter acknowledgements

Mr Nicholas Scott assisted with the behavioural data collection as part of an undergraduate summer project at the University of St Andrews. I would like to express my gratitude to Dr David Wilson, Dr Morag Farquhar and Mrs Mary Latimer for continuing the behavioural testing during my unforeseen absence from St Andrew: without their immediate help and assistance the completion of the data collection would not have been possible.

Chapter 7: Combined selective cholinergic lesions in the pPPTg and LDTg

Introduction

The laterodorsal tegmental nucleus

The laterodorsal tegmental nucleus (LDTg) is located below the fourth ventricle, and is largely embedded within the periaqueductal gray. LDTg neurons are present as far back as the locus coeruleus and extend forward to the level of the pPPTg and dorsal raphe nucleus; at the anterior level, the LDTg extends out of the periaqueductal gray to merge, below the superior cerebellar peduncle with the pPPTg to form the so-called subpeduncular tegmental nucleus (Mesulam et al., 1983; Paxinos and Watson, 2005 ; for review see: Maskos, 2008). It is thus both medial and dorsal to the PPTg, with some overlap in the anterior-posterior plane. In terms of composition and connectivity, the LDTg shares several key features with the PPTg, particularly possession of cholinergic neurons. LDTg cholinergic neurons were designated the Ch6 group by Mesulam et al. (1983) - the PPTg cholinergic neurons being the Ch5 group - and it is the second major cholinergic structure in the mesopontine tegmentum.

Structure of LDTg

In a manner comparable to the PPTg, the LDTg is comprised primarily of an interdigitated collection of cholinergic, glutamatergic and GABAergic neurons with a heterogeneous distribution of these neuronal types along the anterior-posterior plane (Wang and Morales, 2009). Glutamatergic and GABAergic neurons comprise the bulk of the LDTg and are roughly equal in proportion (38% and 40% of total neuronal population, respectively) with cholinergic neurons in a more distant third place (22%) (Wang and Morales, 2009). However, like the PPTg, much of the older literature on the LDTg refers to it as a prominently cholinergic structure and it is only recent histological studies which have highlighted the need to change this view. Cholinergic neurons are most densely packed in the region of the dorsal medial LDTg

(mLDTg) with those extending outside this region along the superior cerebellar peduncle forming a more sparse and scattered arrangement (Inglis and Semba, 1997; Wang and Morales, 2009). LDTg cholinergic neurons share many common properties with PPTg cholinergic neurons: The use of *in situ*-hybridisation has shown both LDTg and PPTg cholinergic neurons have very low rates of co-expression of either glutamate or GABA (<3%) (Wang and Morales, 2009); both LDTg and PPTg cholinergic neurons express the UII receptor (Clark et al., 2001); both also express NOS (Vincent and Kimura, 1992) (almost non-existent in non-cholinergic mesopontine tegmentum neurons); cholinergic neurons of both structures have very low levels of expression of the calcium binding proteins calbindin, parvalbumin and calretinin (Dun et al., 1995; Fortin and Parent, 1999) (which are expressed by non-cholinergic mesopontine tegmentum neurons) and cholinergic neurons of neither structure express the P75 low affinity nerve growth factor found on basal forebrain cholinergic neurons (Richardson et al., 1986; Knusel and Hefti, 1988).

Connectivity of LDTg (and comparison to PPTg)

The connectivity of the PPTg has been extensively discussed previously (chapter 1, pp5-11) and will only be mentioned here in comparison to LDTg connections. The literature on mesopontine tegmentum connections is extensive and there are aspects common to many of these reports which should be mentioned before reviewing it. As in the case with the PPTg, there is no reliable histochemical or immunohistochemical marker of LDTg glutamatergic or GABAergic neurons. Therefore, before the frequent use of *in-situ* hybridisation (which does have reliable markers of the three main neuronal types) any distinction between neuronal type is generally not made or only considered in terms of “cholinergic” versus “non-cholinergic”. The LDTg makes both ipsilateral and contralateral projections, but the distinction between the two is not always assessed, making a comparison of ipsilateral and contralateral not possible

for all projections. Finally, many studies only address either the PPTg or the LDTg. Therefore, if a projection between the LDTg and another structure is characterised with no mention of the PPTg, it is unclear if this is because there was no projection originating in the PPTg, or if the PPTg was not assessed. Consequently distinctions between PPTg and LDTg projections will only be mentioned when they have been explicitly documented.

Inputs to the LDTg are not well characterised, with many articles referring to several key studies from the 1980s and 1990s (Satoh and Fibiger, 1986; Cornwall et al., 1990; Semba and Fibiger, 1992). These provide an extensive list of structures containing neurons showing positive for tracer after retrograde injection into the LDTg (some structures were also verified with anterograde tracer). While excellent studies for their time, the techniques and methods used are now somewhat dated and unfortunately mean a detailed assessment of connections cannot be ascertained from them. More recently, a small selection of detailed investigations looking at particular structures have been conducted. A brief summary of the most relevant inputs from all of these studies will be given; however the original research articles should be consulted for the full details of the tracing studies (in particular see: Cornwall et al., 1990; Semba and Fibiger, 1992).

Inputs to LDTg arise from a broad range of brain regions extending from the brainstem to the prefrontal cortex. Both the LDTg and PPTg receive “massive inputs” from the reticular formation and entire brainstem, with no clearly defined differential pattern of connections (Semba and Fibiger, 1992). In terms of midbrain systems, both the LDTg and PPTg receive input from SNr (with an apparent dominance of caudal SNr projections to LDTg, whereas PPTg receives output from the entire SNr). In addition to this, PPTg receives input from other BG structures that do not appear to project so heavily to LDTg (Semba and Fibiger, 1992; Mena-Segovia et al., 2004a; Kita and Kita, 2011). The lateral habenula, lateral hypothalamus,

midbrain central grey and rostromedial tegmental nucleus all project to LDTg, with the habenula appearing to have preferential projections to LDTg over PPTg (Cornwall et al., 1990; Semba and Fibiger, 1992; Jhou et al., 2009a; Jhou et al., 2009b). The amygdala projects to PPTg and LDTg, with an apparent dominance to PPTg (Semba and Fibiger, 1992; Zahm et al., 2001) and frontal and medial prefrontal cortex project more strongly to LDTg than PPTg (Cornwall et al., 1990; Semba and Fibiger, 1992).

The outputs of the LDTg are somewhat better characterised and can be classified as projecting to: (1) other brainstem sites, (2) midbrain, BG and thalamus, (3) other subcortical and cortical areas. (1) LDTg innervates other brainstem and pontine nuclei, including contralateral LDTg and PPTg, PnO, RMTg, interpeducular nucleus, and structures involved in early visualmotor processing (interstitial nucleus of the medial longitudinal fasciculus, paramedian regions of the pontine reticular formation and medial terminal nucleus) (Cornwall et al., 1990; Semba and Fibiger, 1992; Jhou et al., 2009b). (2) Both the LDTg and PPTg send strong cholinergic and non-cholinergic projections to midbrain DA neurons. As discussed, these form a topographical gradient whereby aPPTg preferentially projects to SNc, pPPTg projects to VTA and SNc and the LDTg essentially only innervates the VTA with only very few projections to SNc (Satoh and Fibiger, 1986; Gould et al., 1989; Cornwall et al., 1990; Oakman et al., 1995a ; for review see: Maskos, 2008). Both structures innervate the thalamus (indeed essentially all thalamic nuclei receive innervation from the cholinergic mesopontine tegmentum (Hallanger et al., 1987; Hallanger and Wainer, 1988), again with what appears to be a differential (but not well defined) pattern of projections: For example LDTg innervates the mediodorsal and anterior thalamic nuclei (which in turn is not substantially innervated by the PPTg) (Shibata, 1992) but LDTg also innervates other thalamic nuclei and the hypothalamus which are conjointly innervated by PPTg. While the LDTg projects to the STN, this is to a considerably smaller extent than the projections arising from PPTg (Bevan and Bolam, 1995;

Kita and Kita, 2011). LDTg sends cholinergic and possibly non cholinergic projections to the superior colliculus (however projections from the PPTg are twice as numerous) and this projection is predominantly ipsilateral (Motts and Schofield, 2009). LDTg and PPTg connections to the PnC place the cholinergic mesopontine tegmentum in part of the primary startle circuitry (Fendt et al., 2001; Bosch and Schmid, 2008). (3) LDTg and PPTg innervate the cholinergic basal forebrain (Woolf and Butcher, 1986) and LDTg innervates the prefrontal cortex (Semba and Fibiger, 1992), "hippocampal cortex" and septum (Cornwall et al., 1990) (which do not appear to be innervated by PPTg; however PPTg manipulation does affect hippocampal theta activity, but this is believed to be mediated through secondary structures such as RPO (Nowacka et al., 2002)). Within these projections there are a large number of collateral projections: single LDTg (especially cholinergic) neurons form collateral projections to several of the key output structures (Bolton et al., 1993), which is also the case for single PPTg cholinergic neurons (Mena-Segovia et al., 2008b).

While both LDTg and PPTg share many prominent connections, a subtle divergence between them emerges. In addition to the common connections, PPTg is far more intricately connected to basal ganglia circuitry than LDTg and in turn the LDTg is more integrated to limbic and visuomotor control circuits than the PPTg. This has led some authors to speculate that PPTg and LDTg may share common functions but that PPTg may be more specialised for 'motor' functions whereas LDTg would have a stronger contribution to 'limbic' processing (Nemcova et al., 2000; Jenkins et al., 2002). The definition of motor is ambiguous here and could lead to confusion (for example, does it mean simple motor movement, or include motor processes such as action selection?). However, if it is replaced with 'BG interfaced' the distinction is still useful in highlighting the preferential interconnection between the PPTg and BG and LDTg to limbic structures.

Functions of the LDTg

Historically the LDTg (along with the PPTg) has been considered to form a major component of the ascending reticular activating system and contemporary research finds that both structures are associated with changes in the generation of cortical EEG across behavioural states and are active during REM sleep (Maloney et al., 1999; Verret et al., 2005; Mena-Segovia et al., 2008b). However, given its diverse connections, the LDTg is in a position to influence far more than cortical EEG. Bilateral non-selective excitotoxic LDTg lesions have been shown to reduce baseline levels of locomotion (measured in photocell cages) and abolish the locomotor changes induced by systemically administered nicotine (Alderson et al., 2005). In a different study, measurement of locomotion by observational scoring over a 2 min period found excitotoxic LDTg lesions did not affect locomotion, but increased amphetamine induced stereotypy and decreased morphine induced stereotypy (Forster and Blaha, 2000). Chronoamperometric measurement of NAcc DA release in the same experiment found diminished DA release to morphine and enhanced DA release to amphetamine in the lesion group. This firmly linked LDTg manipulation to altered striatal DA activity, believed to be mediated through efferent connectivity to VTA. In fitting with LDTg having a functional connection to the VTA, inactivation of the LDTg by combined muscimol and baclofen infusion has been shown to essentially block VTA burst firing (Lodge and Grace, 2006). Several recent reviews have stressed the importance of cholinergic modulation of the VTA (Maskos, 2008; Mena-Segovia et al., 2008a) claiming it is a 'master modulator' of the DA system. There is good evidence from slice preparations that midbrain DA neurons cannot switch to burst firing mode without cholinergic innervation, a substantial proportion of which originates in the LDTg and pPPTg (see general introduction, pp36-38 for further discussion). Based on this it would be expected that combined depletion of LDTg and pPPTg cholinergic neurons would cause severe VTA dysfunction with behavioural consequences including learning impairment and altered

response to dopaminergic manipulating drugs. Given the extensive innervation of thalamus and the VTA, combined with the theoretical reasoning for involvement of these systems in DA modulation, it is surprising there are not more cognitive behavioural assessments of animals bearing LDTg manipulations (indeed, there are more studies on the LDTg and bladder state (Koyama et al., 1999) than there are on LDTg and instrumental learning). Excitotoxic lesions of the LDTg do affect the startle response – reducing PPI but not affecting startle amplitude (Jones and Shannon, 2004) and similar results have been shown after PPTg inactivation (Diederich and Koch, 2005 ; see general introduction, pp18-19 for discussion of PPTg and PPI). The types of behaviour discussed above show that there is a striking similarity between behaviours associated the LDTg and those associated with the PPTg, and given the common neuronal compositions and connections, this is not surprising. However, the similarities between the LDTg and PPTg should not mask the subtle yet important differences, which can be broadly summarised as: (1) LDTg to midbrain DA connections are generally restricted to the VTA, whereas the PPTg projects to both VTA and SNc; (2) While the LDTg is connected to some major BG sites, the PPTg is much more closely integrated into the BG; (3) While both structures innervate the thalamus, the pattern of nuclei targeted changes across the PPTg and LDTg; (4) There are no reported assessments of the effects of LDTg manipulation on learning; it is unclear if this is due to having not being investigated or due to the non-publication of negative results; (5) In the literature the PPTg is discussed in terms of locomotion far more than the LDTg. This is largely due to the notion that the PPTg forms part of the functionally defined mesencephalic locomotor region. Strong evidence stands against considering the PPTg as a motor structure and indeed the finding that LDTg and *not* PPTg lesion affects baseline levels of locomotion immediately highlights the lack of robustness in this distinction between PPTg and LDTg. As LDTg and PPTg share many common properties while also retaining features unique to each structure, this has led some authors to suggest that some functions may be distributed

– or shared – across the entire PPTg-LDTg system, with the combined effects being a "dynamic balance" of function (Nemcova et al., 2000; Jenkins et al., 2002). The experiments conducted in chapter 5 and 6 addressed the possible functional role of cholinergic pPPTg to VTA projections and found that selective cholinergic lesions of the pPPTg were without behavioural effect. The next logical step in the investigation of mesopontine tegmentum to midbrain DA cholinergic projections is therefore to cause greater disruption of the cholinergic projection to midbrain by lesioning the cholinergic LDTg in addition to the pPPTg. A near total lesion of both structures would remove the most substantial cholinergic input to midbrain DA, an input believed to be a 'master modulator' of these systems and essential for the switch of DA neurons from baseline to burst firing.

Dtx-UII in the LDTg

There are no published studies of selective cholinergic lesions of the LDTg or reports using Dtx-UII in the LDTg. However, because LDTg cholinergic neurons selectively express the UII receptor (Clark et al., 2001) there is good reason to believe Dtx-UII should be as effective in the LDTg as it is in the PPTg.

Specific chapter aims

The aims of this chapter are to establish the feasibility and parameters for creating selective cholinergic lesions of the LDTg, and combined selective cholinergic lesions of the pPPTg and LDTg. The feasibility applies to both the surgical procedure itself and effects on the rats health during the lesion formation period. While selective cholinergic pPPTg lesions are without ill effect on health, it was unknown what the effects of combined LDTg and pPPTg lesions would be.

Getting at the LDTg

In the rat, because of its location ventral to the fourth ventricle and proximity to both the lambdoid and midline suture, surgically targeting the LDTg is challenging. Fixing the rat in the flat skull position (bregma and lambda at equal elevations) and lowing a syringe or pipette down the dorsal-ventral plane can lead to extensive bleeding from the major midline blood vessels and penetration of the ventricle, which in turn can lead to seeping of toxin up the syringe needing tack into the ventricle itself. There are reports of the LDTg being successfully lesioned with excitotoxins in this manner (Alderson et al., 2005). However, most laboratories use an approach where the arm of the stereotaxic frame is angled in the mediolateral plane, which results in the LDTg being targeted from a dorsolateral position and the needle (or pipette) passing beside the ventricle rather than through it (Blaha et al., 1996; Inglis and Semba, 1997; Jones and Shannon, 2004) (It should be noted that this technique angles the infusion in a different plane to the technique used in chapters 4, 5 and 6 in which the pipette approaches the target in the anteroposterior plane). This, however, does not avoid the problems with bleeding from around the lambdoid suture. As one aim here is to create combined LDTg and pPPTg lesions, doing this in the flat skull position would require extensive drilling above the lambdoid and midline sinus (essentially removal of the entire skull over lambda and ~2 mm lateral on either side). Instead the decision was taken to target the LDTg in the same manner as had been successfully used for the PPTg in previous chapters – keeping the arm of the stereotaxic frame vertical and raising rats' noses to an elevated position such that the angle between the horizontal plane of the IAL and the incisor was 8˚ 29'. Thus the craniotomies are drilled in front of lambda and the risk of disruption of major blood vessels in combined pPPTg/LDTg surgeries reduced. Keeping the arm vertical does not avoid proximity to the ventricle and the possibility of diffusion of toxin into it, but it is hoped that the use of fine tipped glass pipettes and a delay between infusion and removal of the pipette will help

overcome this. The study which targeted the LDTg directly through the ventricle used glass pipettes and was successful in creating well placed excitotoxic lesions (Alderson et al., 2005). A third option, being piloted in at least one laboratory, involves angling the arm of the stereotaxic frame in both the mediolateral and anteroposterior plane, this avoids both the lambdoid blood vessel and the ventricle (Clark SD, personal communication, 2010). However this "double angle" method introduces another margin for error as well as testing the accuracy of the stereotaxic frame, and so this approach was decided against at this stage.

Area of the LDTg to target

When lesioned with excitotoxins it has been reported that two small infusions along the length of the LDTg produce lesions with less damage to surrounding structures than one larger infusion (Inglis and Semba, 1997). However, as the majority of LDTg cholinergic neurons are located within the mLDTg, and as Dtx-UII should be selective for UII expressing neurons (making possible damage to surrounding structures less likely) the initial experiments were conducted with a single infusion aimed at the mLDTg. A subsequent second and third experiment attempted to use two infusions to cover more of the LDTg.

LDTg lesion experiment 1

Methods

Subjects

Twelve adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used with a mean weight of 444g (range 410-489g) at the time of surgery. Animals were housed and maintained as described in the general methods.

Co-ordinate calculation

As there are no published reports targeting the LDTg with the rats nose raised in the stereotaxic fame, there are no previous studies to base an infusion co-ordinate on. Therefore, a co-ordinate was estimated based on that used for the pPPTg and consultation of the rat brain atlas of Paxinos and Watson (2005). In order to verify and refine this, an infusion of cresyl violet dye was performed under terminal anaesthesia (using the surgical procedure described in the general methods) in 2 rats. After transcardial perfusion with fixative (following the procedure in the general methods) sections were cut through the LDTg, mounted on glass slides and examined under a light microscope. See figure 7.1 for example placement. Based on the results of this analysis the following co-ordinates were considered acceptable possibilities for toxin infusion: (1) -1.0 mm from IAL; ±0.4 mm from midline; -5.9 mm from dura, (2) -1.1 mm from IAL; ±0.4 mm from midline; -5.9 mm from dura, (3) -1.2 mm from IAL; ±0.4 mm from midline; -5.9 mm from dura.

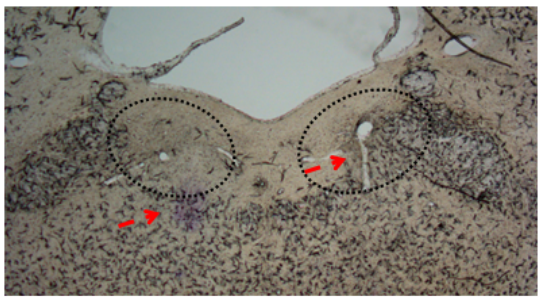


Figure 7.1: Photograph of tissue from a brain infused with cresyl violet dye for co-ordinate refinement. The approximate location of the mLDTg is indicated by the black dashed spheres. The left red arrow indicates the presence of cresyl violet immediately ventral to the mLDTg, the right red arrow indicates possible pipette track within the mLDTg.

Surgery

Anaesthesia was induced and lesion surgery performed as described in the general methods. Rats were bilaterally infused (order of infusion counterbalanced across rats) with 300nL of Dtx-UII at the co-ordinates stated above. Four rats were infused at co-ordinates (1) and (2) and 2 rats at co-ordinate (3). No sham surgeries were performed, analysis of previous sham rats from pPPTg experiments served as the control group.

Histology

All histological procedures followed the methods described in the general methods. Briefly, after allowing >21 days for lesion formation (during which time no adverse effects were noticed), rats were transcardially perfused with fixative and brains stored in sucrose solution. Subsequently, 30 µm coronal sections were cut through the area of the LDTg, PPTg and ~1mm beyond in anterior-posterior plane. Parallel 1:4 series were processed immunohistochemically for ChAT and NeuN reactivity. Sections were mounted onto glass slides, NeuN stained sections were then counterstained with cresyl violet and all sections viewed under a light microscope. Cholinergic cell loss was judged by assessment on the ChAT stained sections, non-selective damage was assessed by inspection of the NeuN/Cresyl stained sections.

Results

Lesion results

Four rats had evidence of damage to the LDTg cholinergic neurons: in 2 cases these were selective cholinergic lesions (no evidence of damage on NeuN stain over and above loss of cholinergic neurons) and the remaining two had some evidence of non-selective damage. The location of the non-selective damage below the LDTg in one of these rats suggested that

the centre of the infusion was below the LDTg. In all cases the lesions were often asymmetrical, causing considerable damage on one side and only moderate or small amounts of damage on the other, again the infusions appeared to be too ventral and possibly cantered at the most ventral areas of or even just below the LDTg. However, 1 rat had no evidence of ChAT or NeuN damage but on the NeuN stain had what appeared to be evidence of pipette tips located within the LDTg. The 5 remaining rats had no evidence of lesion at all. In these rats it was generally not possible to definitively confirm the end of the pipette track. Given the small size of the 40 - 50 µm tipped glass pipettes and angle of insertion meaning the track is only visible briefly on each section, it is not uncommon to be unable to follow the track damage to a certain end point. The vicinity to the ventricle proved establishing the end of the track even harder for the LDTg infusions. No clear pattern emerged when comparing to the co-ordinates used, one lesion was from the AP IAL -1.0 mm group, 2 from the IAL -1.1 mm group and 1 from the IAL -1.2 mm group.

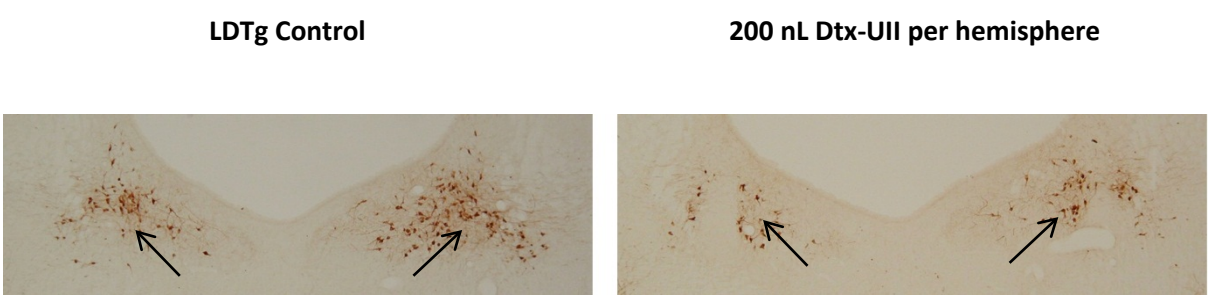


Figure 7.2: Photograph of ChAT stained sections form a control rat (left panel) and a rat infused with Dtx-UII into the LDTg (right panel). Arrows indicate the location of the LDTg.

Conclusions of LDTg lesion experiment 1

This experiment established that the Dtx-UII toxin can destroy cholinergic LDTg neurons and moreover, can destroy these neurons without creating extensive non-selective damage. However it failed to created successful, extensive bilateral selective cholinergic LDTg lesions. While the co-ordinates used placed the infusion in the vicinity of the LDTg, there was an apparent trend for the infusion be too ventral and possibly even missing the LDTg in some rats. Based on this, a second lesion experiment was performed with two additional co-ordinates: -1.1 mm from IAL; ±0.4 mm from midline; -5.7 mm from dura and -1.1 mm from IAL; ±0.4 mm from midline; -5.5 mm from dura (the most successful co-ordinate used here with the DV raised by 0.2 mm and 0.4 mm respectively). It was also decided to attempt combined pPPTg and LDTg lesions by infusing DTx-UII into the pPPTg (using the same method as that used in chapter 4) in addition to the LDTg in a subset of these rats.

LDTg lesion experiment 2

Subjects

Twelve adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used, with a mean weight of 345g (range 308-371g) at the time of surgery. Animals were housed and maintained as described in the general methods.

Surgery

Anaesthesia was induced and lesion surgery performed as described in the general methods. Rats were split into the infusion groups described in table 7.1 and had 300 nL of 3% DTx-UII infused bilaterally at the appropriate stereotaxic co-ordinate, the order of infusion (left hemisphere, right hemisphere, LDTg first, pPPTg first) was counterbalanced across rats. No

sham surgeries were performed, analysis of previous sham rats form pPPTg experiments served as the control group.

Target	Co-ordinate(s)	Number of rats
LDTg	**-1.1;+-0.4;-5.9** (same as lesion experiment 1)	(n = 3)
LDTg	**-1.1;+-0.4;-5.7** (most successful co-ordinate from lesion experiment 1 with DV +0.2)	(n = 2)
LDTg	**-1.1; ±0.4;-5.5** (most successful co-ordinate from lesion experiment 1 with DV +0.4)	(n = 2)
Combined LDTg + pPPTg	**-1.1; ±0.4;-5.9** (most successful co-ordinate from LDTg lesion experiment 1) **-0.8; ±1.9; -6.5** (pPPTg co-ordinate used in chapters 4, 5 and 6)	(n = 2)
Combined LDTg + pPPTg	**-1.1; ±0.4;-5.7** (most successful co-ordinate from LDTg lesion experiment 1 with DV changed by +0.2) **-0.8; ±1.9; -6.5** (pPPTg co-ordinate used in chapters 4, 5 and 6)	(n = 3)

Table 7.1: Surgical groups in LDTg lesion experiment 2. All co-ordinates are measured in mm and are in the format of: distance from IAL; midline; dura.

Histology

All histological procedures followed the methods described in the general methods. Briefly, after allowing >21 for lesion formation (during which time no adverse effects were noticed), rats were transcardially perfused with fixative and brains stored in sucrose solution. Subsequently, 30 µm coronal sections were cut through the area of the LDTg, PPTg and ~1mm beyond in anterior-posterior plane. Parallel 1:4 series were processed immunohistochemically

for ChAT and NeuN reactivity. Sections were mounted onto glass slides and NeuN sections were then counterstained with cresyl violet and all sections viewed under a light microscope. Cholinergic cell loss was judged by assessment on the ChAT stained sections, non-selective damage was assessed by inspection of the NeuN/Cresyl stained sections.

Results

Lesion results

In a very similar manner to LDTg lesion experiment 1, damage to the LDTg was either only partial or unilateral. Four rats had partial selective cholinergic lesions with evidence of ChAT cell loss in both hemispheres, but this cell loss never approached a near complete level (see figure 7.3). These rats were from the new co-ordinate groups DV +0.2 mm (n = 2) and DV +0.4 mm (n = 2). 2 rats had unilateral LDTg damage (both from the original LDTg co-ordinate) with only low levels of non-selective damage. Of the combined LDTg and pPPTg lesions, 1 rat had a successful pPPTg lesion and a partial LDTg lesion which, at both targets, was selective for cholinergic neurons. The remaining combined LDTg/pPPTg rats had low levels of pPPTg cholinergic cell loss (n = 2) or no clear pPPTg cell loss (n = 2) and in all cases no extensive LDTg damage. The 1 remaining rat (LDTg only, co-ordinate from previous LDTg experiment used) had no evidence of a lesion.

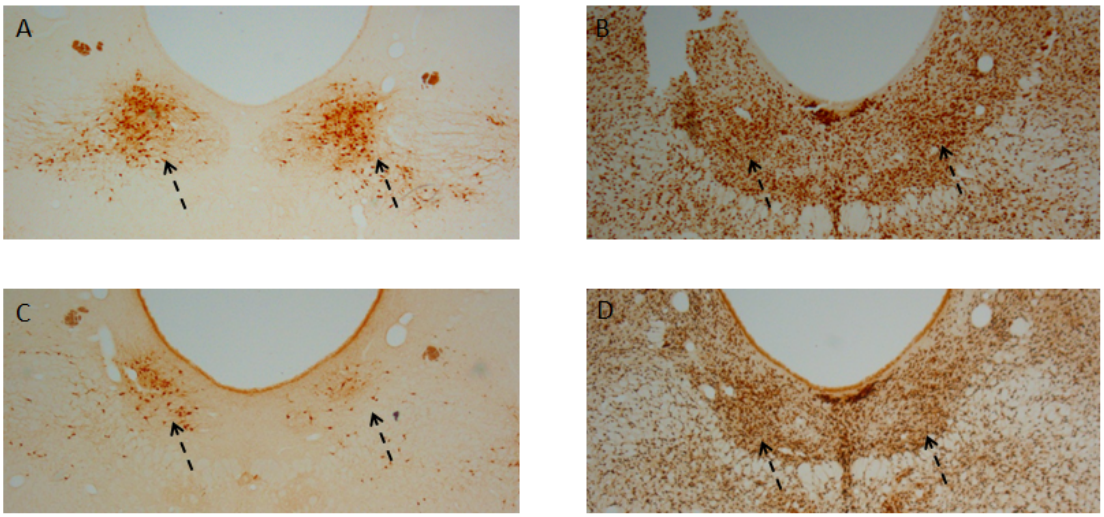


Figure 7.3: Photograph of ChAT (left panels) and NeuN/cresyl (right panels) stained sections form a control rat (top panels) and a rat infused with Dtx-UII into the LDTg (bottom panel). Arrows indicate the location of the LDTg.

Conclusions of LDTg lesion experiment 2

Lesion experiment 2 resulted in a higher proportion of rats having some signs of LDTg damage, which was nearly always selective for cholinergic neurons. The altered co-ordinate -1.1 mm from IAL; ±0.4 mm from midline; -5.7 mm from dura appeared to be the most successful. However the small group sizes meant establishing a clear difference between this co-ordinate and the second new co-ordinate -1.1 mm from IAL; ±0.4 mm from midline; -5.5 mm from dura (DV 0.2 mm higher) was not possible. The original LDTg co-ordinate had the lowest success rate and often resulted in unilateral damage. That only 1/4 rats in the combined LDTg/pPPTg lesion groups had a good pPPTg lesion (with the rest having small or no signs of lesion) was surprising and the low levels of LDTg loss in these rats was disappointing. It was feared that combining the LDTg and pPPTg lesions might lead to extensive non-selective damage (due to the large volume of Dtx-UII being infused into the mesopontine tegmentum). There is no clear explanation for the low levels of cell loss seen in the pPPTg other than (based

on the lesions in chapter 5 and 6) Dtx-UII does not have a 100% success rate (even when the target structure is hit) and in each batch of surgery a small yet considerable proportion of rats have had to be excluded from the analysis for having no or small signs of lesion. However, the success rate here of 1/4 is considerably less than normal (around 75% of rats normally have at least some signs of lesion). One explanation for the LDTg lesions being partial rather than complete may be that not enough toxin is infused. The area of LDTg being targeted (mLDTg) has considerably (46%) more cholinergic neurons than the pPPTg, and they may also be more densely packed than pPPTg cholinergic neurons (Wang and Morales, 2009). Therefore, it is possible the dose of toxin used for the pPPTg is insufficient for the LDTg. In order to investigate this, it was decided to run another lesion study increasing the amount of toxin infused into the LDTg. This was achieved in two ways: the first was to infuse 300 nL into two co-ordinates within the LDTg (at each of the new co-ordinates used in this study, it was feared that a single of infusion of 600 nL would lead to non-selective damage); the second was to increase the toxin concentration from 3.0% to 3.2%. The seemingly cautious increase of toxin concentration by 0.2% was based on the results of chapter 4 which show that a Dtx-UII concentration of 4.0% causes substantial non-selective damage in the pPPTg.

LDTg lesion experiment 3

Subjects

Eight adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used, with a mean weight of 323g (range 313-330g) at the time of surgery. Animals were housed and maintained as described in the general methods.

Surgery

Anaesthesia was induced and lesion surgery performed as described in the general methods. Rats were split into the infusion groups described in table 7.2 and had 300nL of Dtx-UII infused bilaterally at the appropriate stereotaxic co-ordinate and toxin concentration. The order of infusion (left hemisphere, right hemisphere, LDTg first, pPPTg first, was counterbalanced across rats). No sham surgeries were performed, analysis of previous sham rats form pPPTg experiments served as the control group. One rat died the night after surgery: this was from the combined 3.0% LDTg/pPPTg infusion group. There had been extensive bleeding from the midline sinus during surgery and this rat was noticeably quieter than the other rats during recovery.

Target	Co-ordinate and toxin concentration	Number of rats
LDTg	**-1.1; ±0.4;-5.7** **-1.1; ±0.4;-5.5** **300nL of 3.2% Dtx-UII**	(n = 2)
Combined LDTg + pPPTg	**-1.1; ±0.4;-5.7** **-1.1; ±0.4;-5.5** **-0.8; ±1.9; -6.5** (pPPTg) **300nL of 3.0% Dtx-UII**	(n = 4)
Combined LDTg + pPPTg	**--1.1; ±0.4;-5.7** **-1.1; ±0.4;-5.5** **-0.8; ±1.9; -6.5** (pPPTg) **300nL of 3.2% Dtx-UII**	(n = 2)

Table 7.2: Infusion groups in LDTg lesion experiment 3. All co-ordinates are measured in mm and are in the format of: distance from IAL; midline; dura.

Histology

All histological procedures followed the methods described in the general methods. Briefly, after allowing >21 for lesion formation (during which time no adverse effects were

noticed), rats were transcardially perfused with fixative and brains stored in sucrose solution. Subsequently, 30 µm coronal sections were cut through the area of the LDTg, PPTg and ~1mm beyond in anterior-posterior plane. Parallel 1:4 series were processed immunohistochemically for ChAT and NeuN reactivity. Sections were mounted onto glass slides and NeuN sections were then counterstained with cresyl violet and all sections viewed under a light microscope. Cholinergic cell loss was judged by assessment of the ChAT stained sections, non-selective damage was assessed by inspection of the NeuN/Cresyl stained sections.

Results

Lesion results

The results of the third pilot study are very similar to the first two. Four out of the seven rats which had LDTg infusions had evidence of selective cholinergic damage to the LDTg, again this was only partial (estimated at not more than 50% ChAT cell loss) and the remaining 3 rats had no clear signs of any LDTg lesion. Of the 5 rats which received combined LDTg/pPPTg infusions, 3 had partial selective cholinergic lesions with the remaining 2 having no clear sign of damage. None of the pPPTg lesions resulted in near-total loss of ChAT+ neurons and had an estimated 25-60% of ChAT+ cells remaining. Two of the partial pPPTg lesions were in rats which also had partial LDTg lesions (shown in figure 7.4). The 3.2% concentration produced more reliable cell loss than the 3.0% (every rat infused with 3.2% had some damage in either LDTg or pPPTg, whereas 2 of the 3.0% group had no signs of lesions) however it should be noted that none of the pPPTg lesions (even those infused with 300nL of 3.2% toxin) were as extensive as the lesions obtained in chapters 4, 5 and 6.

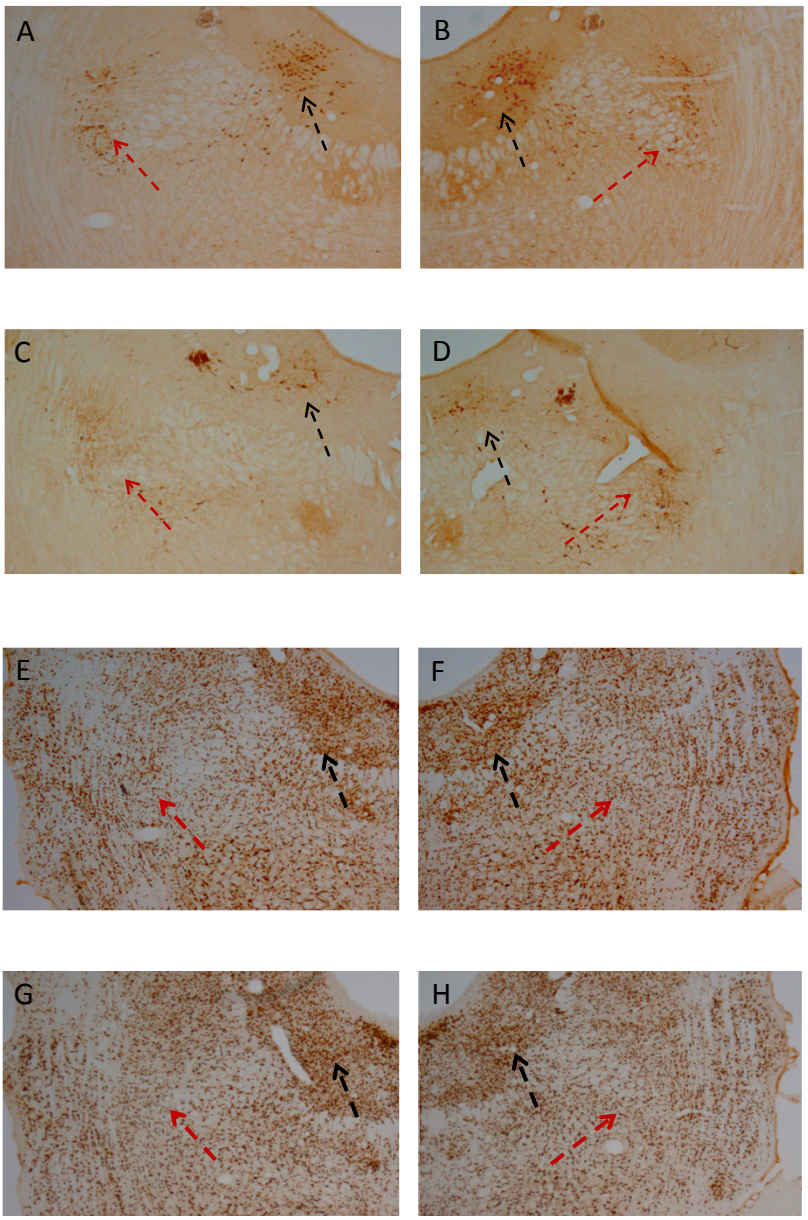


Figure 7.4: Photograph of ChAT (panels A-D) and NeuN/cresyl (panels E-H) stained sections form a control rat (panels A,B,E,F) and a rat infused with Dtx-UII into the LDTg and pPPTg (panels C,D,G,H). Black arrows indicate the location of the mLDTg and red arrows the pPPTg.

Conclusions of LDTg lesion experiment 3

Despite doubling the volume and increasing the concentration of Dtx-UII used, the third pilot study failed to produce extensive lesions of the LDTg. Moreover, the pPPTg lesions were smaller and less reliable than in previous chapters. This is true even for the rats which had 300 nL of 3.2% toxin infused into the pPPTg. As this is a stronger concentration and a higher volume than has successfully produced pPPTg lesions in previous chapters (chapters 5 and 6 both had extensive selective cholinergic lesions with 200 nL of 3.0% toxin) it is a somewhat surprising finding. Taken together the continued finding of very low levels of cholinergic cell loss despite evidence of successful pipette placement suggests that there is now a systematic failure in the lesion procedure.

Chapter discussion

The aim of this chapter was to verify that the Dtx-UII toxin is effective at selectively lesioning cholinergic LDTg neurons and to establish the feasibility and parameters for creating combined selective bilateral lesions of both the pPPTg and LDTg. These aims were partially met: bilateral delivery of toxin into the LDTg from a glass pipette was successful, the Dtx-UII toxin destroyed cholinergic neurons without creating significant non-selective damage, confirming that this toxin is effective in the LDTg. However, lesions in general were small and unreliable, rats receiving infusions into both the pPPTg and LDTg showed only small lesions in each structure which was not overcome by increasing both the concentration and volume of toxin infused.

Possible reasons for partial lesions

There are several explanations to be considered for the lack of substantial cholinergic lesions within the LDTg. (1) It is possible that the toxin is ineffective in this structure, though this is unlikely because the cholinergic neurons express the UII receptor to which the toxin binds (Clark et al., 2001). Moreover, some cholinergic cell loss is seen, confirming that the toxin can destroy these neurons. (2) It is possible that the amount of toxin delivered was insufficient to destroy the sheer number of cholinergic neurons within the LDTg. This was addressed by the third lesion experiment which systematically increased both the volume and concentration of toxin used and found that with over double the amount of toxin infused into the LDTg the lesions were not substantially different to smaller infusions. While the density of cholinergic neurons within LDTg is likely to affect the amount of toxin required to destroy them, it seems this alone is not an explanation for the small lesions. (3) It is possible that the toxin is not being delivered into the LDTg or is diffusing away rapidly (for example into the ventricle). This is a serious concern: the presence of signs of pipette tips within the LDTg suggests that the toxin is being delivered correctly, but the possibility of seepage into the ventricle is harder to asses. Circumstantial support for this not being a problem comes from an LDTg excitotoxic lesion study, where 400 nL of toxin (i.e. less than the total volume of some of the infusions performed here), again delivered into the LDTg from a glass pipette which went through the ventricle, was sufficient to create extensive non-selective LDTg lesions (Alderson et al., 2005). However, it should be noted that while the Alderson et al. (2005) study succeeded in creating excitotoxic LDTg lesions, examination of all the histological results from that and other unpublished studies in this laboratory, together with reports from other laboratories, show that this is a problematic and often unsuccessful procedure (Inglis and Semba, 1997). A way of reducing the possibility of diffusion into the ventricle would be to target the LDTg at an angle along the mediolateral plane (Inglis and Semba, 1997). However,

none of these concerns address the second finding from these experiments: the very low levels of cholinergic cell loss observed in the pPPTg of rats which had Dtx-UII infused into both the pPPTg and LDTg. In the second pilot study (there were no combined lesions in the first pilot) 1/4 pPPTg lesions was successful, in the third pilot study 1/6 pPPTg lesions were successfully bilaterally, this was despite using stronger toxin (3.2% rather than 3.0%) and larger infusions (300 nL rather than 200 nL) than had proved successful in previous chapters. One possibility is that the addition of the LDTg infusion renders the pPPTg infusion unsuccessful, however, this seems very unlikely. A second possibility is that the stock of Dtx-UII had deteriorated and lost potency or become inactive for another unknown reason. A period of 14 months elapsed between the last successful pPPTg lesions (chapter 6 – nicotine locomotion) and the combined pPPTg and LDTg infusions attempted here. In contrast to this, all the successful pPPTg lesions (chapters 4, 5 and 6) were conducted within an 11 month period before the 14 month gap. It is unclear how long the Dtx-UII should maintain potency, *ex vivo* testing of the toxin to assess its potency requires cell lines not available here (Clark SD, personal communication). One solution would be to continue the experiments with a new batch of Dtx-UII. The toxin is not commercially available, being manufactured and supplied through collaboration with Dr Clark at University of California, Irvine (now at SUNY, University at Buffalo) who is willing and able to construct fresh toxin. However, the manufacturing time is around 3 months (Clark SD, personal communication). Given that a new batch of toxin would also need *en vivo* testing (which would take approximately 6 weeks), due to the time constraints of this thesis it was not possible to continue using Dtx-UII.

Chapter update

Since conducting these experiments a poster presentation at the 2011 annual Society for Neuroscience meeting (Washington DC) reported that Dtx-UII toxin had successfully been used to destroy around 80% of LDTg cholinergic neurons bilaterally (Steidl et al., 2011). While the histological assessment of non-selective damage had not been completed, this confirms that the Dtx-UII is able to cause extensive lesions to the LDTg. This gives further support to the view that if the co-ordinates and procedures reported here were repeated with new Dtx-UII, substantial combined selective lesions of LDTg and pPPTg cholinergic neurons lesions could be obtained.

Chapter 8: General discussion

Summary of aims and results:

The aim of this work was to examine the role of the PPTg in associative learning, with particular emphasis on: (1) establishing if a functioning PPTg is required for normal action-outcome learning and (2) examining the contribution of cholinergic pPPTg neurons to learning.

Using a contingency degradation paradigm, it was shown in chapter 3 that a functioning pPPTg is essential for the updating of associations between actions and outcomes. In this paradigm the relationship between action (lever press) and outcome (pellet delivery) was degraded such that, in the degraded condition, pellets were delivered with the same probability whether the rat executed a correct response or not. Rats with intact monitoring of the actions they perform and the outcomes these produce are highly sensitive to this manipulation and accordingly reduce the number of actions they perform. This pattern was evident in saline treated rats: rates of lever pressing significantly reduced across degradation sessions and were significantly lower than contingent controls in the extinction test. In contrast, pPPTg inactivation (by direct microinfusion of muscimol) blocked sensitivity to contingency degradation: lever pressing was not significantly different between the contingent and non-contingent groups at any point in contingency training or the extinction test. This was not a deficit in ability to lever press, for rats in the muscimol contingent and non-contingent groups pressed at the same rate as the saline contingent group. Rather, it was a specific deficit in adapting behaviour in response to the change in contingency, a defining characteristic of impairment in updating the association between actions and outcomes. In addition to this main finding, the finding that muscimol contingent and saline contingent rats continued to lever press at the same rate, reinforces two additional points suggested by previous studies: (1) loss of the pPPTg has no impact on the ability to lever press; (2) inactivation of the pPPTg does not affect the motivation to work for or the incentive salience value of rewards.

These results extend significantly the previous studies showing learning impairments after PPTg lesion (for review see: chapter 1, pp20-25). Rats bearing bilateral excitotoxic PPTg lesions were unable to learn or perform radial maze tasks in which reward location varied on every trial, making the relationship between action (which arm of the maze to enter) and outcome (successful reward retrieval) unpredictable (Keating and Winn, 2002; Taylor et al., 2004). Alderson and colleagues (2004) found that rats with PPTg lesions were impaired at learning to lever press on a FR2 schedule of reinforcement for intravenous amphetamine, but unimpaired if they had learned prior to lesion that lever pressing was rewarded. However, both naïve and trained PPTg lesioned rats were unable to respond properly when on a progressive ratio schedule of reinforcement in which the relationship between outcomes and actions (the number of presses required) constantly changed (Alderson et al., 2004). More recently, it has been shown that pPPTg lesioned rats were slow to learn to lever press during the initial stages of simple operant learning, and though with extended training they did learn the task, they were subsequently again slow to adapt their rates of lever pressing in response to changes in reinforcement schedule (Wilson et al., 2009a). These findings can be explained by a deficit in A-O learning: it is during initial learning and when situations change that the updating of A-O association is performed and where impairment would slow learning.

In chapter 4 the parameters for and feasibility of creating bilateral lesions highly selective for cholinergic pPPTg neurons were established. Subsequently, the effect of these lesions on locomotion and sucrose consumption was assessed. Substantial loss of cholinergic pPPTg neurons (~95%) was achieved, with little or no evidence of non-selective damage. These lesions produced no overt health consequences during (of after) the 21 day lesion formation period - the only detectable effect during this time was a small (yet significant) transient reduction in bodyweight growth rate. Analysis of rates of spontaneously generated locomotion (measured in photocell cages) and consumption of 20% sucrose solution (measured in

homecage) revealed no differences between sham control rats or rats with selective cholinergic lesions in pPPTg.

An assessment of the ability of rats bearing highly selective lesions of cholinergic pPPTg neurons to learn various fixed and variable ratio schedules of reinforcement was assessed in chapter 5. Selective lesions of cholinergic pPPTg neurons, created prior to any operant training, had no effect on initial learning of an FR1 reinforcement schedule or the subsequent adaption to and performance of various fixed and variable ratio schedules up to and including VR30. Lesioned rats learned, performed and adapted to changes in the reinforcement schedules in a manner indistinguishable from sham operated controls. An extinction test showed they also had a normal pattern of extinction. Using the same operant testing protocol, this laboratory has previously shown that non-selective ibotenic acid lesions of the pPPTg produced a clear and persistent learning impairment: pPPTg lesioned rats took significantly longer to learn FR1, and, despite learning it to criterion level, were slow to adapt rates of lever pressing in response to increases and changes in the reinforcement schedules. Despite extensive training they never performed VR30 at the same rate as sham controls (Wilson et al., 2009a). The results of chapter 5 show that the cholinergic portion of the pPPTg is not required for normal instrumental learning. Much emphasis has been placed on the role of cholinergic PPTg and LDTg neurons in modulating the activity of midbrain DA neurons. This is point is returned to and discussed in subsequent sections.

In chapter 6 an assessment of nicotine sensitization was performed in rats with selective lesions of cholinergic pPPTg neurons. This had 2 aims: (1) to assess the effects of loss of cholinergic pPPTg neurons on sensitization to nicotine. It has previously been shown that ibotenic acid pPPTg lesions reduced the initial locomotor depression caused by nicotine and enhanced subsequent hyperlocomotion (Alderson et al., 2008), which was hypothesized to be

a result of upregulation of nAChRs in the VTA; (2) to assess whether there is evidence of development of compensatory mechanisms in response to the formation of selective cholinergic lesions of pPPTg neurons. The results showed that there was no difference between sham and lesioned rats in either baseline levels of locomotion, response to nicotine induced changes in locomotion or the rate of sensitisation to nicotine. A further restricted analysis was performed on the response to the first administration of nicotine. This was to rule out the possibility that the effects of repeated administration of nicotine were masking any subtle effects caused by the lesion. Once again no difference was found between rats with selective cholinergic lesions in pPPTg and sham operated controls. These results suggest that the previously observed changes in the locomotor response to nicotine after ibotenic acid pPPTg lesions (Alderson et al., 2008) were not the direct result of loss of endogenous cholinergic innervation of the VTA. Instead, it would appear the involvement of the pPPTg in the locomotor response to nicotine is more complex than originally thought. These results are discussed fully in chapter 6 (pp168-177) which should be consulted for more details.

In order to try and achieve greater disruption to the cholinergic mesopontine tegmentum, in chapter 7 an attempt was made to create selective lesions of cholinergic neurons in both pPPTg and the neighboring LDTg. Together these structures form the bulk of the cholinergic innervation of midbrain DA systems (and thalamus). While there was clear evidence that the Dtx-UII toxin had been delivered into both structures, the combined pPPTg – LDTg lesions were only partially successful. Cholinergic cell loss was observed throughout the pPPTg – LDTg complex, but it never approached near total cell loss. Due to the proximity of the LDTg to the fourth ventricle there was concern that toxin was seeping out of the LDTg before it had been internalized by cholinergic neurons. However, the finding that cholinergic cell loss in the pPPTg was only partial and did not achieve nearly as substantial levels as had previously

been obtained (with the same procedure) suggests that at least part of the reason for partial lesions was due to reduced potency of the stock of Dtx-UII toxin.

Further discussion and wider interpretation of results

How might pPPTg be involved in A-O learning?

The pPPTg is well positioned to contribute to action-outcome learning. Of particular interest are the strong cholinergic and non-cholinergic projections to the midbrain VTA and SNc DA neurons (Oakman et al., 1995a; Charara et al., 1996; Wang et al., 2010 ; see: Mena-Segovia et al., 2008a). Phasic activity of these DA neurons in response to reward related sensory input leads to adaptation of firing patterns during learning about the events leading up to reward acquisition: initial phasic firing in response to unpredicted reward declines as phasic firing in response to stimuli that predict the reward develops. Subsequently, absence of the expected reward after a reward-predicting stimulus leads to decreased firing at the time of expected reward delivery (Schultz, 1999, 2010). As discussed in chapter 1 (pp32-35), this phasic firing pattern is regarded by many as a "reward prediction error" signal, which together with subsequent projections to the striatum, is considered to form the basis of associative reward related learning. However, this view is not unanimous and alternative explanations focus on the hypothesis that the phasic DA response might severe to function as a signal used to determine if, and which, self-initiated motor actions led to unpredicted salient changes in the environment (including gaining a reward) (Redgrave and Gurney, 2006; Redgrave et al., 2008). The shortness of the short latency phasic DA response raises the question of what is the neural source of information used for generating the phasic DA signal. As reviewed by Redgrave et al. (2008) and discussed in chapter 1 (pp33-35), cortical and striatal regions which subserve object identification and reward information do not reliably respond to stimuli until

80 - ~200 ms after presentation, and therefore cannot be used in the generation of the phasic DA signal at a typical latency of 70 – 100 ms . Subcortical inputs are deemed to be the likely source of such rapid input. The SC, involved in early visual processing and which responds (at least in part) in a novelty dependent manner to spatially located movement and luminance changes, has direct projections to midbrain DA neurons (May et al., 2009). However, as SC responds to visual transients rather than static features or object identification, it is therefore considered to contain insufficient information for the calculation of a DA firing pattern based on the predication of an identified reward (Redgrave and Gurney, 2006; Redgrave et al., 2008). Given the anatomical connections between the PPTg and midbrain DA neurons, the type of information that the PPTg may be conveying to these midbrain systems must be considered. Studies from primate electrophysiological recordings have revealed that, during a two-value reward-driven behavioural task, different populations of PPTg neurons fired in response to stimuli that predicted the reward and to the delivery of the actual reward itself. Furthermore, firing rate was dependent on reward magnitude – stimuli predicting large reward led to greater firing than stimuli predicting small reward (Kobayashi and Okada, 2007; Okada et al., 2009; Okada et al., 2011). Moreover, PPTg neurons rapidly adapted to reversal of the stimulus-reward size pairing, confirming they were indeed responding to reward magnitude information rather than solely physical aspects of the stimuli (Okada et al., 2011). These findings show that PPTg responds to salient aspects of sensory inputs. Of particular interest and relevance here are the responses to reward prediction stimuli, reward delivery and information regarding reward magnitude. This raises the hypothesis that PPTg can extract these components from incoming sensory information and send them into midbrain DA systems for use in a reward prediction error calculation. This is supported by the response latencies of PPTg neurons: responses to auditory and visual stimuli have been reported within the range of 8 – 100 ms (Dormont et al., 1998; Pan and Hyland, 2005) therefore placing them at or before the firing

latencies of the phasic DA response (Pan and Hyland, 2005; Kobayashi and Okada, 2007). Direct evidence for the involvement of the PPTg in the generation of the phasic DA signal is shown in a study where, in rats which have developed a reliable phasic DA response to reward predicting stimuli, unilateral inactivation of PPTg suppressed the phasic response of midbrain DA neurons without affecting baseline levels of DA firing (Pan and Hyland, 2005). However, in order fully to consider the possible contribution of PPTg short latency input to midbrain DA systems, it is necessary to assess what other information may be being supplied concurrently and therefore establish what is unique about PPTg input. In addition to the PPTg and SC already described, there are currently only a few identified and characterized sources of short latency input to midbrain DA systems: (1) the lateral habenula, which displays an almost 'inverse' RPE signal in that it is inhibited by reward predicting stimuli and exited by stimuli predicting that there will be no reward (Matsumoto and Hikosaka, 2007). While once thought to project directly to VTA, it now appears that the habenula in a large part relays to VTA via the RMTg (Balcita-Pedicino et al., 2011; Hong et al., 2011); (2) In addition to this relay of habenula output, RMTg is a direct source of predominantly GABAergic innervation of midbrain DA neurons (Jhou et al., 2009b; Lavezzi and Zahm, 2011). RMTg is activated by aversive stimuli such as footshock and by cues which predict footshock, inhibited by reward and reward predicting stimuli (Jhou et al., 2009b). As it has a predominantly inhibitory output, this has led to the hypothesis that it feeds aversive information into VTA to inhibit motor activation; (3) The parabrachial nucleus (Zahm et al., 2011) which has been shown to transmit information regarding noxious stimuli into midbrain DA systems (Coizet et al., 2010); (4) Two other inputs midbrain DA systems - the BNST and LH orexinergic ouput, will be mentioned and discussed here, for, while it is unclear if they have short latency responses to sensory stimuli, they influence midbrain DA activity in an ongoing manner which is dependent on internal state and therefore directly affect the ability of concurrent input to midbrain DA systems to elicit a

response. Part of the extended amygdala the BNST projects to the VTA (Georges and Aston-Jones, 2002) and processes information related to stress levels (for review see: Hammack et al., 2010) and is involved in stress-induced relapse to drug seeking behaviour in stressful situations where drug cues are present (Erb and Stewart, 1999; Erb et al., 2001). Orexinergic (excitatory neuropeptide hormone) innervation arrives at the VTA (and PPTg/LDTg) from the LH (Boutrel et al., 2010) and potentiates the effects of other DA inputs as a function of the circadian state – increased DA activation occurs during waking compared to resting periods (Moorman and Aston-Jones, 2010) and when there are higher metabolic needs such as hunger (Boutrel et al., 2010). This gives a mechanism for increasing reward related behaviour at times when the animal is suitably awake and aroused (Moorman and Aston-Jones, 2010). While this discussion has focused on only the best characterized inputs to midbrain DA systems that can influence short latency input (for more see: Geisler and Zahm, 2005; Zahm et al., 2011) it reveals that DA systems are a convergence point for multiple sources and types of input – sensory signaling novelty, reward, reward prediction, aversion as well as information regarding the ongoing internal sate – stress levels, circadian rhythm, deprivation/hunger levels and presumably many more. This high level of integration enables midbrain DA systems simultaneously to monitor several ongoing factors, with convergent inputs modulating, enhancing and inhibiting one another, leading, only in certain combinations to the generation of a phasic DA signal used for salience signaling and reward prediction. So far, the PPTg is only identified input which contains reliable polymodal sensory information and responds excitatorily to reward prediction and reward delivery information. The exact functions and nature of interactions of each of these inputs remains to be discovered. However, in terms of PPTg input to midbrain DA systems, the behavioral results from the contingency degradation study – that rats with pPPTg inactivation could continue to perform an already learned task but did not update associations between actions and outcomes – adds a behavioural component

to the view that pPPTg supplies midbrain DA systems with predictive and actual reward delivery information which can subsequently be used to form a phasic DA response which drives associative learning. Moreover, the contrast in effects on learning between non-selective excitotoxic pPPTg lesions and selective cholinergic lesions of pPPTg reveals that the information conveying projection from pPPTg appears to be non-cholinergic in nature.

Cholinergic versus non-cholinergic projections to midbrain DA: the role of acetylcholine

The finding that selective loss of the cholinergic neurons within pPPTg has no effect on the learning or performance of fixed and variable ratio operant reinforcement schedules (chapter 5) is in sharp contrast to the persistent learning impairment seen in the same behavioural task after non-selective excitotoxic pPPTg lesions (Wilson et al., 2009a). As discussed in chapter 1 (pp36-38), the cholinergic neurons of the mesopontine tegmentum are believed to form a "master modulator" of midbrain DA neurons (Maskos, 2008; Mena-Segovia et al., 2008a). Activation of AChRs decreases the input resistance of inactive DA neurons, increases the firing rate of active neurons (Futami et al., 1995; Sorenson et al., 1998) and, in a smaller proportion of neurons, leads to calcium channel dependent increase in burst firing (Zhang et al., 2005). In mice genetically engineered to lack the β2 nAChR subunit (and therefore be largely insensitive to the effects of endogenous cholinergic innervation), midbrain DA burst firing was virtually absent (Mameli-Engvall et al., 2006) but was restored by lentiviral induced re-expression of β2 nAChRs (Mameli-Engvall et al., 2006; Maskos, 2007). While cholinergic innervation is necessary for, regulates and facilitates increases in midbrain DA activity, the actual primary trigger to increase firing appears to be convergence of several inputs and predominantly driven by concurrent excitatory glutamatergic input (Futami et al., 1995; Sorenson et al., 1998; Kitai et al., 1999; Maskos, 2008; Mao et al., 2011) and by the

facilitation of pre-synaptic release of glutamate (Schilström et al., 2003; Mameli-Engvall et al., 2006; Mao et al., 2011). Glutamatergic inputs target midbrain DA systems from a wide variety of areas from the brainstem to the prefrontal cortex: these structures include (but are not restricted to): central gray; cuneiform nucleus; dorsal raphe; lateral hypothalamic area; lateral habenula; medial hypothalamus; medial septum; parabrachial nucleus; prefrontal cortex; PPTg/LDTg; reticular formation and ventral pallidum (Sesack and Pickel, 1992; Charara et al., 1996; Parent et al., 1999; Geisler et al., 2007; Omelchenko and Sesack, 2007). In contrast to this, as previously described, cholinergic input to midbrain DA arises principally from the mesopontine tegmentum and follows a clear topographical gradient: aPPTg targets SNc, pPPTg targets both SNc and VTA while LDTg predominantly targets the VTA (Oakman et al., 1995a ; see chapter 1 and: Mena-Segovia et al., 2008a). Taking these differences between cholinergic and glutamatergic function this into consideration, the contrast between the effects of selective cholinergic and excitotoxic pPPTg lesions on learning are easier to interpret. Despite near total-loss of pPPTg cholinergic neurons, midbrain DA systems are likely to still receive considerable cholinergic innervation. Indeed, loss of pPPTg cholinergic neurons results in an overall loss of only 20% of cholinergic mesopontine tegmentum neurons. The number of neurons projecting to the SNc would remain largely unchanged (as this projection arises primarily from the unlesioned aPPTg), and the loss of the pPPTg would decrease the possible number of cholinergic neurons projecting to VTA by a maximum of around 26% (numbers used for calculation taken from: Wang and Morales, 2009). If this input is predominantly modulatory in nature then the rather subtle loss of pPPTg input may have led to no overt changes in DA functionality. However, after ibotenic acid lesions, both the cholinergic and glutamatergic inputs are destroyed, leading not only to a reduction in modulatory cholinergic input, but also loss of the excitatory glutamatergic signal. This can also be considered in the context of studies on the reinforcing effects of self-administered nicotine. Animals will not

reliably self-administer nicotine either systemically (Dougherty et al., 1981; Palmatier et al., 2006) or directly into the VTA (Farquhar et al., 2011) showing that it is not a strong primary reinforcer. However, if nicotine is concurrent with another mildly rewarding stimuli (for example conditioned light stimulus) the overall rewarding properties of both are enhanced and lead to greater responding for the combination of stimuli than either presented individually (Palmatier et al., 2006). Likewise, sub-reinforcing doses of cocaine can become reinforcing if delivered concurrently with a non-reinforcing dose of nicotine (Zachariou et al., 2001). These findings have led to the hypothesis that the effects of nicotine are primarily reinforcement enhancing rather reinforcing in themselves. One intriguing finding is that this reinforcement enhancing effect occurs even when nicotine is delivered non-contingently with the additional reinforcer. Indeed, it has been shown that contingent and non-contingent nicotine (yoked control to the contingent group) administration have equal levels of reinforcement enhancing abilities (Chaudhri et al., 2006). What this shows is that simply the presence of nicotine, rather that the simultaneous delivery with another stimuli, is enough to enhance the reinforcing aspects of other primary or conditioned reinforces. Add in to this that administration of cholinergic antagonists reduces the rewarding properties of other (non-cholinergic) drugs (Zachariou et al., 2001) and these studies point to a simple pattern: the level of cholinergic innervation sets the level at which other stimuli will drive learning: nicotinic antagonists reduce reinforcing effects and nicotinic agonists enhance reinforcing effects. From this it is reasonable to propose a model where cholinergic innervation sets the 'volume' of learning, but it is the non-cholinergic excitatory inputs which signal what is actually learnt about. The relatively subtle loss of cholinergic innervation after Dtx-UII lesions of the pPPTg may be insufficient to cause a noticeable effect in a simple learning paradigm. It is also very likely that different AChR subtypes play appreciatively different roles in this process – there is some evidence that cholinergic reinforcement enhancing effects are limited to nAChRs whereas

stimulation of muscarinic (alone or in combination with nicotinic) AChRs may have stronger primary reinforcing effects. This explains the finding that carbachol (a mAChR agonist) has stronger primary reinforcing effects than nicotine (a nAChR agonist) (Farquhar et al., 2011).

How has knowledge of PPTg function been advanced?

Advances have been made in two directions: (1) pPPTg has been shown to be crucially involved in the formation of action-outcome associations, making it the deepest known structure in the brain to be essential for this process; (2) a clear distinction between the functions of pPPTg cholinergic and non-cholinergic systems has been shown.

In recent years it has become firmly established that the PPTg can be "functionally dissected" into discrete components. The different composition and connections of anterior and posterior portions map onto behavioural function. Briefly: pPPTg receives rapid polymodal sensory input and projects into VTA and SNc DA neurons; in contrast to this, aPPTg preferentially receives cortico-striatal and extended amygdala outflow, has comparably restricted projection the DA neurons of the SNc, but in addition projects to motor control sites lower the brainstem (Winn, 2008; Wilson et al., 2009b). Behavioural results are entirely consistent with this distinction: ibotenic pPPTg lesions impair instrumental learning (indicative of disrupted input of sensory information into BG / cortico-striatal systems) whereas aPPTg lesions have no effect on learning rate but produce evidence of behavioural disinhibition (suggestive of altered inhibitory BG outflow) (Wilson et al., 2009a; Wilson et al., 2009b; Winn et al., 2010). The work presented here further scrutinizes the functional nature of the pPPTg input to BG systems, reaching the conclusion that a parsimonious interpretation of one function of pPPTg is to process incoming sensory information, extract specific salient aspects of it and interface this with appropriate systems. It is important to note that while the work here

has been primarily focused on connections to BG and brainstem systems, PPTg has a not-to-be neglected projection to all areas of the thalamus. The discovery that loss of cholinergic pPPTg neurons has no discernible effect on the rate of instrumental learning adds a new dimension to the functional dissection of the PPTg. It shows that even within pPPTg there are two separable functional systems - the non-cholinergic which is directly involved in instrumental learning and the cholinergic, which either has no involvement in instrumental learning or performs as part of a wider cholinergic modulatory system. As glutamatergic pPPTg neurons are nearly 3 times as numerous as GABAergic (Wang and Morales, 2009; Wang et al., 2010) and as the excitatory pathway from PPTg to midbrain DA appears to be glutamatergic (Scarnati et al., 1986), the evidence strongly suggests, but cannot conclusively show, that the pPPTg component critical for learning is glutamatergic.

It is clear that PPTg is more than a simple relay station or part of an extended modulatory system. Indeed, going in the other direction, it is possible to speculate as to the extent to which PPTg could function as an independent controller. PPTg is a highly integrated part of the basal ganglia family (Mena-Segovia et al., 2004a) but it is also hard wired into brainstem structures including those involved in motor control (Humphries et al., 2007; Wilson et al., 2009b; Winn et al., 2010) and startle response (Koch et al., 1993; Swerdlow and Geyer, 1993b; Fendt et al., 2001; Bosch and Schmid, 2008). This makes it a uniquely placed component of both BG and brainstem systems and in a pivotal position to influence and execute information transfer into and between these systems. Furthermore, the ability of PPTg to recognize the significance of external sensory events shows that a degree of analysis is performed. It is interesting to wonder what a structure with these connections and analytical ability may be capable of. There are situations where an immediate rapid response to a sensory event is beneficial, for example, escaping signs of imminent danger where there is insufficient time fully to process the stimuli and consider all options. In this situation PPTg

could elicit escape by executing motor actions in the reticular formation at a very short latency, perhaps even before conscious awareness of the stimuli has been developed. However, in situations where more detailed processing or integration with wider brain systems and internal information is required (for example execution of instrumental actions based on hunger state or long term planning) this sensory information could be interfaced into cortico-striatal and thalamic circuitry. Furthermore, basal ganglia output mediated through PPTg gives PPTg access to the entire domain of processed information going through and leaving BG, making it the last fully integrated BG structure before this information leaves the system. This gives PPTg the ability to relate this outflow to immediate ongoing sensory input and brainstem events, then allow the information flow to continue out of BG, block it, or even re-enter it back into basal ganglia, cortico-striatal or thalamic systems for further processing (Wilson et al., 2009b; Winn et al., 2010). While simply a conceptual model of possible PPTg function, a framework in general agreement with this offers an explanation of the wide range of behavioural changes which occur after damage to the PPTg.

The ability of PPTg to operate both as part of a sensorimotor gating mechanism and a modulator of dopamine systems can lead to speculation about a relationship between PPTg and the symptoms of schizophrenia. PPTg is involved in pre-pulse inhibition (chapter 1, pp18-19; Swerdlow and Geyer, 1993b; Diederich and Koch, 2005) and the auditory P50 (P13 in the rat) (Miyazato et al., 1999), both measures of sensorimotor gating which are abnormal in schizophrenia and have been considered as endophenotypes of the disease (Turetsky et al., 2007). DA function is abnormal in schizophrenia, however the nature of this is far from clear. The original 'dopamine hypothesis' of schizophrenia has not withstood the test of time (or scientific scrutiny) (see: Moncrieff, 2009) and instead current interpretations of the role of DA in schizophrenia do not view the disease as a direct result of abnormal DA levels but focus instead on interactions between DA and other closely connected systems (Willner, 1997),

including mesopontine tegmentum cholinergic regulation of midbrain DA systems (Lester et al., 2010). Indeed, there is an apparent link between schizophrenia and Ach: schizophrenic patients have high rates of cigarette smoking (de Leon et al., 2002; Zhang et al., 2012) which has been argued to be a form of 'self-medication' of cholinergic agonists (Kumari and Postma, 2005) and genes linked to α7 nAChRs have been associated with the disease (Freedman et al., 1997). Forming inappropriate (or non-existent) associative relationships has been proposed as an explanation of the development of delusions in schizophrenia (Kapur, 2003; Romaniuk et al., 2010) and unusually increased activation of midbrain in response to neutral cues (measured with fMRI) has been correlated with the severity of delusional symptoms in schizophrenic patients (Romaniuk et al., 2010). Taking into consideration that PPTg neurons have a role in sensorimotor gating, the cholinergic modulation of DA systems, and the formation of associations, one can wonder if aberrant functioning of the PPTg may lead to some of the cardinal symptoms of the disease.

Future directions

The conclusions reached here present some testable hypotheses. The first is that substantial loss of PPTg and LDTg cholinergic neurons should disrupt innervation of midbrain DA systems, impairing their ability to switch firing patterns and consequently impairing instrumental learning. This could be further investigated and tested by: (1) electrochemical measurement of Ach levels in the VTA / SNc after combined PPTg / LDTg lesion. A correlation between PPTg / LDTg lesion size, level of Ach and behavioural impairment would establish the amount of cholinergic innervation required to maintain normal function (see chapter 3, pp100-101, for discussion of compensatory mechanisms and the non-linear relationship between cell loss and post-synaptic transmitter level); (2) assessing the ability of artificial cholinergic innervation to restore normal DA function. The hypothesis proposed is that cholinergic

innervation sets the 'volume' of learning, whereas glutamatergic input signals what is leaned about. If this is the case, then direct infusion of cholinergic agonists into midbrain DA systems of rats with combined cholinergic lesions in PPTg / LDTg should be able to temporarily restore cholinergic DA innervation and support normal function. This manipulative rather than correlative assessment would also explicitly link behavioural impairment after cholinergic PPTg / LDTg lesion to midbrain DA dysfunction.

Clearly a toxin selective for PPTg glutamatergic neurons would be helpful; currently no such toxin exists. Moreover, as these neurons are not known to uniquely express any unique receptor type there is also as of yet no immediate way to hypothesize how such a toxin could be created. Given the current enthusiasm in optogenetic control of neuronal function – described as the Nature Methods "Method of the Year 2010" (Nature Methods Editorial, 2011) - it is worth considering the possible use of such a technique in the PPTg. Optogenetics is the "*integration of optics and genetics to achieve gain-or loss-of-function of well-defined events within specific cells"* (Yizhar et al., 2011) with the two techniques of most relevance here being channelrhodopsin induced excitation or halorhodopsin induced inhibition (Deisseroth, 2011). Thus, once cells are modified to contain the specific light sensitive proteins (for example after infection with a lentiviaral vector) neuronal activity can be controlled with precise temporal precision by illumination of an implanted LED light source (Pastrana, 2011; Yizhar et al., 2011). A recent advancement to this technique is the development, in rat, of a two part process which enables the selective optogenetic targeting of a specific neuronal type while leaving other interdigitated neurons unaffected. This process involves the stereotaxic injection of a Cre-dependent opsin-expressing viral vector into a rat strain where the neuronal type of interest has been modified to selectively express Cre recombinase (currently ChAT and TH strains exist) (Witten et al., 2011). Therefore, only the neurons expressing the Cre recombinase cells in the vicinity of the viral injection site should become light sensitive. This

offers a method for selectively enhancing cholinergic output from PPTg (and, indeed LDTg, however it should be noted that as this technique involves a stereotaxic injection and light source implantation, it does not overcome the problematic location of the LDTg). While currently this technique is limited to activation of ChAT and TH cells, the possibility of development of specific targeting of other neuronal populations offers intriguing future prospects. Moreover, as it is possible to have combinations of several optogenetic techniques in the same organism (Pastrana, 2011), the prospect of multimodal control of neuronal activity raises the possibility of, for example, blue light induced activation of glutamate cells and concurrent yellow light induced deactivation of cholinergic cells (Deisseroth, 2011), and vice versa. Combining this with the precise temporal control offered by optogenetics would enable a detailed assessment of the effects of specific alternations in cholinergic and glutamatergic PPTg output during particular points of a learning paradigm.

Final conclusions and thoughts

There are two clear results from this work: (1) the pPPTg is critical for forming associations between actions and outcomes, but not the performance of previously learned associations; (2) loss of pPPTg cholinergic neurons has no discernible effect on instrumental learning. The disruption of action-outcome learning is hypothesized to be due to loss of PPTg signaling to midbrain DA neurons, with electrophysiological evidence revealing that this information is likely to be regarding reward prediction and reward magnitude aspects of stimuli. The contrast in effects on learning between ibotenic acid and selective cholinergic Dtx-UII pPPTg lesions shows that it is the non-cholinergic (presumed glutamatergic) projections that play a crucial role in learning; a finding compatible with the notion that the cholinergic projections are modulatory in nature. The PPTg has long been hypothesized to facilitate and

control basal ganglia output, it is now evident that PPTg also plays a crucial role in facilitating the transfer of information into and through basal ganglia and cortico-striatal circuitry. Indeed, PPTg is a highly integrated part of both basal ganglia and brainstem circuitry, making it uniquely placed to perform a 'first pass' analysis of incoming sensory information and interface salient aspects of this with the appropriate circuitry. In addition, the ability of PPTg to monitor information exiting basal ganglia enables it to assess its relevance in relation to concurrent sensory and brainstem events – a 'last pass' analysis - before sending it into motor output structures lower in the brainstem or re-entering it back into basal ganglia circuitry.

References

Adams CD (1982) Variations in the sensitivity of instrumental responding to reinforcer devaluation. The Quarterly Journal of Experimental Psychology Section B 34:77-98.

Adams CD, Dickinson A (1981) Instrumental responding following reinforcer devaluation. The Quarterly Journal of Experimental Psychology Section B 33:109-121.

Ainge JA, Jenkins TA, Winn P (2004) Induction of c-fos in specific thalamic nuclei following stimulation of the pedunculopontine tegmental nucleus. Eur J Neurosci 20:1827-1837.

Ainge JA, Keating GL, Latimer MP, Winn P (2006) The pedunculopontine tegmental nucleus and responding for sucrose reward. Behav Neurosci 120:563-570.

Albuquerque EX, Pereira EF, Alkondon M, Rogers SW (2009) Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev 89:73-120.

Alderson HL, Latimer MP, Winn P (2005) Involvement of the laterodorsal tegmental nucleus in the locomotor response to repeated nicotine administration. Neuroscience letters 380:335-339.

Alderson HL, Latimer MP, Winn P (2006) Intravenous self-administration of nicotine is altered by lesions of the posterior, but not anterior, pedunculopontine tegmental nucleus. Eur J Neurosci 23:2169-2175.

Alderson HL, Latimer MP, Winn P (2008) A functional dissociation of the anterior and posterior pedunculopontine tegmental nucleus: excitotoxic lesions have differential effects on locomotion and the response to nicotine. Brain Struct Funct 213:247-253.

Alderson HL, Jenkins TA, Kozak R, Latimer MP, Winn P (2001) The effects of excitotoxic lesions of the pedunculopontine tegmental nucleus on conditioned place preference to 4%, 12% and 20% sucrose solutions. Brain Res Bull 56:599-605.

Alderson HL, Latimer MP, Blaha CD, Phillips AG, Winn P (2004) An examination of d-amphetamine self-administration in pedunculopontine tegmental nucleus-lesioned rats. Neuroscience 125:349-358.

Alderson HL, Brown VJ, Latimer MP, Brasted PJ, Robertson AH, Winn P (2002) The effect of excitotoxic lesions of the pedunculopontine tegmental nucleus on performance of a progressive ratio schedule of reinforcement. Neuroscience 112:417-425.

Alexander GE, DeLong MR, Strick PL (1986) Parallel Organization of Functionally Segregated Circuits Linking Basal Ganglia and Cortex. Annual Review of Neuroscience 9:357-381.

Anton R (2010) Chapter 2 - The Conservative Evolution of the Vertebrate Basal Ganglia. In: Handbook of Basal Ganglia Structure and Function (Heinz S, Kuei YT, eds): Academic Press.

Azam L, Winzer-Serhan U, Leslie FM (2003) Co-expression of alpha7 and beta2 nicotinic acetylcholine receptor subunit mRNAs within rat brain cholinergic neurons. Neuroscience 119:965-977.

Balcita-Pedicino JJ, Omelchenko N, Bell R, Sesack SR (2011) The inhibitory influence of the lateral habenula on midbrain dopamine cells: ultrastructural evidence for indirect mediation via the rostromedial mesopontine tegmental nucleus. J Comp Neurol 519:1143-1164.

Balleine BW, Liljeholm M, Ostlund SB (2009) The integrative function of the basal ganglia in instrumental conditioning. Behav Brain Res 199:43-52.

Baluch F, Itti L (2011) Mechanisms of top-down attention. Trends Neurosci 34:210-224.

Bay KD, Beck P, Skinner RD, Garcia-Rill E (2007) GABAergic modulation of developing pedunculopontine nucleus. Neuroreport 18:249-253.

Belin D, Everitt BJ (2011) Drug Addiction: the Neural and Psychological Basis of a Compulsive Incentive Habit. IN:. Steiner, H & Tseng, K Handbook of Basal Ganglia Structure and Function Volume 20 pp571-592.

Beninato M, Spencer RF (1986) A cholinergic projection to the rat superior colliculus demonstrated by retrograde transport of horseradish peroxidase and choline acetyltransferase immunohistochemistry. J Comp Neurol 253:525-538.

Benwell ME, Balfour DJ (1992) The effects of acute and repeated nicotine treatment on nucleus accumbens dopamine and locomotor activity. Br J Pharmacol 105:849-856.

Berridge KC (2007) The debate over dopamine's role in reward: the case for incentive salience. Psychopharmacology (Berl) 191:391-431.

Berridge KC, Robinson TE (1998) What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? Brain Res Rev 28:309-369.

Bevan MD, Bolam JP (1995) Cholinergic, GABAergic, and glutamate-enriched inputs from the mesopontine tegmentum to the subthalamic nucleus in the rat. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:7105-7120.

Blaha CD, Winn P (1993) Modulation of dopamine efflux in the striatum following cholinergic stimulation of the substantia nigra in intact and pedunculopontine tegmental nucleus-lesioned rats. J Neurosci 13:1035-1044.

Blaha CD, Allen LF, Das S, Inglis WL, Latimer MP, Vincent SR, Winn P (1996) Modulation of dopamine efflux in the nucleus accumbens after cholinergic stimulation of the ventral tegmental area in intact, pedunculopontine tegmental nucleus-lesioned, and laterodorsal tegmental nucleus-lesioned rats. J Neurosci 16:714-722.

Bolton RF, Cornwall J, Phillipson OT (1993) Collateral axons of cholinergic pontine neurones projecting to midline, mediodorsal and parafascicular thalamic nuclei in the rat. J Chem Neuroanat 6:101-114.

Bortolanza M, Wietzikoski EC, Boschen SL, Dombrowski PA, Latimer M, Maclaren DA, Winn P, Da Cunha C (2010) Functional disconnection of the substantia nigra pars compacta from the pedunculopontine nucleus impairs learning of a conditioned avoidance task. Neurobiol Learn Mem 94:229-239.

Bosch D, Schmid S (2008) Cholinergic mechanism underlying prepulse inhibition of the startle response in rats. Neuroscience 155:326-335.

Boutrel B, Cannella N, de Lecea L (2010) The role of hypocretin in driving arousal and goal-oriented behaviors. Brain Res 1314:103-111.

Brantley RK, Bass AH (1988) Cholinergic neurons in the brain of a teleost fish (Porichthys notatus) located with a monoclonal antibody to choline acetyltransferase. J Comp Neurol 275:87-105.

Brudzynski SM, Iku A, Harness Nee Savoy A (2011) Activity of cholinergic neurons in the laterodorsal tegmental nucleus during emission of 22kHz vocalization in rats. Behav Brain Res.

Capozzo A, Florio T, Cellini R, Moriconi U, Scarnati E (2003) The pedunculopontine nucleus projection to the parafascicular nucleus of the thalamus: an electrophysiological investigation in the rat. J Neural Transm 110:733-747.

Castaneda E, Whishaw IQ, Robinson TE (1990) Changes in striatal dopamine neurotransmission assessed with microdialysis following recovery from a bilateral 6-OHDA lesion: variation as a function of lesion size. J Neurosci 10:1847-1854.

Cavedini P, Gorini A, Bellodi L (2006) Understanding obsessive-compulsive disorder: focus on decision making. Neuropsychology review 16:3-15.

Charara A, Smith Y, Parent A (1996) Glutamatergic inputs from the pedunculopontine nucleus to midbrain dopaminergic neurons in primates: Phaseolus vulgaris-leucoagglutinin anterograde labeling combined with postembedding glutamate and GABA immunohistochemistry. J Comp Neurol 364:254-266.

Chaudhri N, Caggiula A, Donny E, Palmatier M, Liu X, Sved A (2006) Complex interactions between nicotine and nonpharmacological stimuli reveal multiple roles for nicotine in reinforcement. Psychopharmacology 184:353-366.

Clark SD, Nothacker HP, Wang Z, Saito Y, Leslie FM, Civelli O (2001) The urotensin II receptor is expressed in the cholinergic mesopontine tegmentum of the rat. Brain Res 923:120-127.

Clark SD, Alderson HL, Winn P, Latimer MP, Nothacker HP, Civelli O (2007) Fusion of diphtheria toxin and urotensin II produces a neurotoxin selective for cholinergic neurons in the rat mesopontine tegmentum. J Neurochem 102:112-120.

Clarke PB, Kumar R (1983) The effects of nicotine on locomotor activity in non-tolerant and tolerant rats. Br J Pharmacol 78:329-337.

Clarke PB, Fu DS, Jakubovic A, Fibiger HC (1988) Evidence that mesolimbic dopaminergic activation underlies the locomotor stimulant action of nicotine in rats. J Pharmacol Exp Ther 246:701-708.

Coizet V, Dommett EJ, Klop EM, Redgrave P, Overton PG (2010) The parabrachial nucleus is a critical link in the transmission of short latency nociceptive information to midbrain dopaminergic neurons. Neuroscience 168:263-272.

Conde H, Dormont JF, Farin D (1998) The role of the pedunculopontine tegmental nucleus in relation to conditioned motor performance in the cat. II. Effects of reversible inactivation by intracerebral microinjections. Exp Brain Res 121:411-418.

Corbit LH, Balleine BW (2003) The role of prelimbic cortex in instrumental conditioning. Behav Brain Res 146:145-157.

Corbit LH, Muir JL, Balleine BW (2001) The role of the nucleus accumbens in instrumental conditioning: Evidence of a functional dissociation between accumbens core and shell. J Neurosci 21:3251-3260.

Corbit LH, Ostlund SB, Balleine BW (2002) Sensitivity to instrumental contingency degradation is mediated by the entorhinal cortex and its efferents via the dorsal hippocampus. J Neurosci 22:10976-10984.

Corbit LH, Muir JL, Balleine BW (2003) Lesions of mediodorsal thalamus and anterior thalamic nuclei produce dissociable effects on instrumental conditioning in rats. Eur J Neurosci 18:1286-1294.

Cornwall J, Cooper JD, Phillipson OT (1990) Afferent and efferent connections of the laterodorsal tegmental nucleus in the rat. Brain research bulletin 25:271-284.

Corrigall WA, Coen KM, Zhang J, Adamson KL (2001) GABA mechanisms in the pedunculopontine tegmental nucleus influence particular aspects of nicotine self-administration selectively in the rat. Psychopharmacology (Berl) 158:190-197.

Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ (1995) Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience 64:477-505.

Cyr M, MacLaren DA, Bedard M-A, Clark SD, Mechawar N, Rochford J, Winn P (2011) Highly selective lesion of the cholinergic pedunculopontine neurons using a minimally-invasive angular stereotaxic surgery with the Diphteria-Urotensin-II neurotoxin in rat. Program No 3706/C47 2011 Neuroscience Meeting Planner Washington, DC: Society for Neuroscience, 2011 Online

Dalley JW, Lääne K, Theobald DEH, Armstrong HC, Corlett PR, Chudasama Y, Robbins TW (2005) Time-limited modulation of appetitive Pavlovian memory by D1 and NMDA receptors in the nucleus accumbens. Proceedings of the National Academy of Sciences 102:6189-6194.

Dani JA, Heinemann S (1996) Molecular and cellular aspects of nicotine abuse. Neuron 16:905-908.

Datta S, Siwek DF, Stack EC (2009) Identification of cholinergic and non-cholinergic neurons in the pons expressing phosphorylated cyclic adenosine monophosphate response element-binding protein as a function of rapid eye movement sleep. Neuroscience 163:397-414.

de Borchgrave R, Rawlins JN, Dickinson A, Balleine BW (2002) Effects of cytotoxic nucleus accumbens lesions on instrumental conditioning in rats. Exp Brain Res 144:50-68.

de Lacalle S, Kulkarni S, Wiley RG (1998) Lesion-induced transneuronal plasticity of the cholinergic innervation in the adult rat entorhinal cortex. Eur J Neurosci 10:1054-1062.

de Leon J, Tracy J, McCann E, McGrory A, Diaz FJ (2002) Schizophrenia and tobacco smoking: a replication study in another US psychiatric hospital. Schizophrenia Research 56:55-65.

Deisseroth K (2011) Optogenetics. Nat Meth 8:26-29.

DeRusso A, Fan D, Gupta J, Shelest O, Costa RM, Yin HH (2010) Instrumental uncertainty as a determinant of behavior under interval schedules of reinforcement. Frontiers in Integrative Neuroscience 4.

Deurveilher S, Hennevin E (2001) Lesions of the pedunculopontine tegmental nucleus reduce paradoxical sleep (PS) propensity: evidence from a short-term PS deprivation study in rats. Eur J Neurosci 13:1963-1976.

Deurveilher S, Lo H, Murphy JA, Burns J, Semba K (2006) Differential c-Fos immunoreactivity in arousal-promoting cell groups following systemic administration of caffeine in rats. J Comp Neurol 498:667-689.

Diederich K, Koch M (2005) Role of the pedunculopontine tegmental nucleus in sensorimotor gating and reward-related behavior in rats. Psychopharmacology (Berl) 179:402-408.

Dormont JF, Conde H, Farin D (1998) The role of the pedunculopontine tegmental nucleus in relation to conditioned motor performance in the cat. I. Context-dependent and reinforcement-related single unit activity. Exp Brain Res 121:401-410.

Dougherty J, Miller D, Todd G, Kostenbauder HB (1981) Reinforcing and other behavioral effects of nicotine. Neurosci Biobehav Rev 5:487-495.

Dringenberg HC, Olmstead MC (2003) Integrated contributions of basal forebrain and thalamus to neocortical activation elicited by pedunculopontine tegmental stimulation in urethane-anesthetized rats. Neuroscience 119:839-853.

Dun NJ, Dun SL, Hwang LL, Forstermann U (1995) Infrequent co-existence of nitric oxide synthase and parvalbumin, calbindin and calretinin immunoreactivity in rat pontine neurons. Neuroscience letters 191:165-168.

Dunbar JS, Hitchcock K, Latimer M, Rugg EL, Ward N, Winn P (1992) Excitotoxic lesions of the pedunculopontine tegmental nucleus of the rat. II. Examination of eating and drinking, rotation, and reaching and grasping following unilateral ibotenate or quinolinate lesions. Brain Res 589:194-206.

Erb S, Stewart J (1999) A role for the bed nucleus of the stria terminalis, but not the amygdala, in the effects of corticotropin-releasing factor on stress-induced reinstatement of cocaine seeking. J Neurosci 19:RC35.

Erb S, Salmaso N, Rodaros D, Stewart J (2001) A role for the CRF-containing pathway from central nucleus of the amygdala to bed nucleus of the stria terminalis in the stress-induced reinstatement of cocaine seeking in rats. Psychopharmacology 158:360-365.

Eschenko O, Magri C, Panzeri S, Sara SJ (2011) Noradrenergic Neurons of the Locus Coeruleus Are Phase Locked to Cortical Up-Down States during Sleep. Cerebral Cortex.

Everitt BJ, Robbins TW (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nat Neurosci 8:1481-1489.

Farquhar MJ, Latimer MP, Winn P (2011) Nicotine self-administered directly into the VTA by rats is weakly reinforcing but has strong reinforcement enhancing properties. Psychopharmacology (Berl).

Fay RA, Norgren R (1997a) Identification of rat brainstem multisynaptic connections to the oral motor nuclei using pseudorabies virus. I. Masticatory muscle motor systems. Brain Res Brain Res Rev 25:255-275.

Fay RA, Norgren R (1997b) Identification of rat brainstem multisynaptic connections to the oral motor nuclei in the rat using pseudorabies virus. II. Facial muscle motor systems. Brain Res Brain Res Rev 25:276-290.

Fay RA, Norgren R (1997c) Identification of rat brainstem multisynaptic connections to the oral motor nuclei using pseudorabies virus. III. Lingual muscle motor systems. Brain Res Brain Res Rev 25:291-311.

Featherstone RE, McDonald RJ (2004) Dorsal striatum and stimulus-response learning: lesions of the dorsolateral, but not dorsomedial, striatum impair acquisition of a simple discrimination task. Behav Brain Res 150:15-23.

Featherstone RE, McDonald RJ (2005) Lesions of the dorsolateral striatum impair the acquisition of a simplified stimulus-response dependent conditional discrimination task. Neuroscience 136:387-395.

Fendt M, Li L, Yeomans JS (2001) Brain stem circuits mediating prepulse inhibition of the startle reflex. Psychopharmacology 156:216-224.

Floresco SB, West AR, Ash B, Moore H, Grace AA (2003) Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. Nat Neurosci 6:968-973.

Florio T, Capozzo A, Puglielli E, Pupillo R, Pizzuti G, Scarnati E (1999) The function of the pedunculopontine nucleus in the preparation and execution of an externally-cued bar pressing task in the rat. Behav Brain Res 104:95-104.

Forster GL, Blaha CD (2000) Laterodorsal tegmental stimulation elicits dopamine efflux in the rat nucleus accumbens by activation of acetylcholine and glutamate receptors in the ventral tegmental area. Eur J Neurosci 12:3596-3604.

Forster GL, Blaha CD (2003) Pedunculopontine tegmental stimulation evokes striatal dopamine efflux by activation of acetylcholine and glutamate receptors in the midbrain and pons of the rat. Eur J Neurosci 17:751-762.

Fort P, Luppi PH, Jouvet M (1993) Glycine-immunoreactive neurones in the cat brain stem reticular formation. Neuroreport 4:1123-1126.

Fortin M, Parent A (1999) Calretinin-immunoreactive neurons in primate pedunculopontine and laterodorsal tegmental nuclei. Neuroscience 88:535-547.

Fowler CD, Arends MA, Kenny PJ (2008) Subtypes of nicotinic acetylcholine receptors in nicotine reward, dependence, and withdrawal: evidence from genetically modified mice. Behav Pharmacol 19:461-484.

Freedman R et al. (1997) Linkage of a neurophysiological deficit in schizophrenia to a chromosome 15 locus. Proceedings of the National Academy of Sciences 94:587-592.

Fuller PM, Saper CB, Lu J (2007) The pontine REM switch: past and present. J Physiol 584:735-741.

Futami T, Takakusaki K, Kitai ST (1995) Glutamatergic and cholinergic inputs from the pedunculopontine tegmental nucleus to dopamine neurons in the substantia nigra pars compacta. Neuroscience Research 21:331-342.

Garey LJ, Hornung JP (1980) The use of ibotenic acid lesions for light and electron microscopic study of anterograde degeneration in the visual pathway of the cat. Neurosci Lett 19:117-123.

Geisler S, Zahm DS (2005) Afferents of the ventral tegmental area in the rat-anatomical substratum for integrative functions. J Comp Neurol 490:270-294.

Geisler S, Derst C, Veh RW, Zahm DS (2007) Glutamatergic afferents of the ventral tegmental area in the rat. J Neurosci 27:5730-5743.

Gentry CL, Lukas RJ (2002) Regulation of nicotinic acetylcholine receptor numbers and function by chronic nicotine exposure. Curr Drug Targets CNS Neurol Disord 1:359-385.

Georges F, Aston-Jones G (2002) Activation of Ventral Tegmental Area Cells by the Bed Nucleus of the Stria Terminalis: A Novel Excitatory Amino Acid Input to Midbrain Dopamine Neurons. The Journal of Neuroscience 22:5173-5187.

Gerfen CR, Bolam JP (2010) Chapter 1 - The Neuroanatomical Organization of the Basal Ganglia. In: Handbook of Basal Ganglia Structure and Function (Heinz S, Kuei YT, eds), pp 3-28: Academic Press.

Gillies AJ, Willshaw DJ (1998) A massively connected subthalamic nucleus leads to the generation of widespread pulses. Proceedings Biological sciences / The Royal Society 265:2101-2109.

Gotti C, Zoli M, Clementi F (2006) Brain nicotinic acetylcholine receptors: native subtypes and their relevance. Trends Pharmacol Sci 27:482-491.

Gould E, Woolf NJ, Butcher LL (1989) Cholinergic projections to the substantia nigra from the pedunculopontine and laterodorsal tegmental nuclei. Neuroscience 28:611-623.

Govind AP, Vezina P, Green WN (2009) Nicotine-induced upregulation of nicotinic receptors: underlying mechanisms and relevance to nicotine addiction. Biochem Pharmacol 78:756-765.

Grace AA, Floresco SB, Goto Y, Lodge DJ (2007) Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. Trends in Neurosciences 30:220-227.

Grillner P, Svensson TH (2000) Nicotine-induced excitation of midbrain dopamine neurons in vitro involves ionotropic glutamate receptor activation. Synapse 38:1-9.

Gut NK, Winn P (2011) The Role of the Pedunculopontine Tegmental Nucleus in Motor Disorders. In: Animal Models of Movement Disorders, pp 321-342.

Hall WC, Fitzpatrick D, Klatt LL, Raczkowski D (1989) Cholinergic innervation of the superior colliculus in the cat. J Comp Neurol 287:495-514.

Hallanger AE, Wainer BH (1988) Ascending projections from the pedunculopontine tegmental nucleus and the adjacent mesopontine tegmentum in the rat. The Journal of comparative neurology 274:483-515.

Hallanger AE, Levey AI, Lee HJ, Rye DB, Wainer BH (1987) The origins of cholinergic and other subcortical afferents to the thalamus in the rat. J Comp Neurol 262:105-124.

Halliday GM, Barker RA, Rowe DB (2011) Non-dopamine lesions in Parkinson's disease. New York: Oxford University Press.

Hamaguchi-Hamada K, Fujisawa Y, Koizumi O, Muneoka Y, Okado N, Hamada S (2009) Immunohistochemical evidence for the existence of novel mammalian neuropeptides related to the <i>Hydra GLW-amide neuropeptide family. Cell and Tissue Research 337:15-25.

Hammack S, Roman C, Lezak K, Kocho-Shellenberg M, Grimmig B, Falls W, Braas K, May V (2010) Roles for Pituitary Adenylate Cyclase-Activating Peptide (PACAP) Expression and Signaling in the Bed Nucleus of the Stria Terminalis (BNST) in Mediating the Behavioral Consequences of Chronic Stress. Journal of Molecular Neuroscience 42:327-340.

Hayward LF, Castellanos M (2004) Activation of the dorsal periaqueductal gray in the rat induces Fos-like immunoreactivity in select non-cholinergic mesopontine neurons. Neurosci Lett 360:5-8.

Heckers S, Geula C, Mesulam MM (1992) Cholinergic innervation of the human thalamus: dual origin and differential nuclear distribution. J Comp Neurol 325:68-82.

Heise CE, Mitrofanis J (2006) Fos immunoreactivity in some locomotor neural centres of 6OHDA-lesioned rats. Anat Embryol (Berl) 211:659-671.

Higo S, Matsuyama T, Kawamura S (1996) Direct projections from the pedunculopontine and laterodorsal tegmental nuclei to area 17 of the visual cortex in the cat. Neuroscience Research 26:109-118.

Hikosaka O, Sakamoto M, Usui S (1989) Functional properties of monkey caudate neurons. II. Visual and auditory responses. Journal of Neurophysiology 61:799-813.

Hilario MRF, Costa RM (2008) High on habits. Frontiers in Neuroscience 2.

Holman EW (1975) Some conditions for the dissociation of consummatory and instrumental behavior in rats. Learning and Motivation 6:358-366.

Holmes NM, Marchand AR, Coutureau E (2010) Pavlovian to instrumental transfer: a neurobehavioural perspective. Neurosci Biobehav Rev 34:1277-1295.

Holmstrand EC, Sesack SR (2011) Projections from the rat pedunculopontine and laterodorsal tegmental nuclei to the anterior thalamus and ventral tegmental area arise from largely separate populations of neurons. Brain Struct Funct 216:331-345.

Homs-Ormo S, Coll-Andreu M, Satorra-Marin N, Arevalo-Garcia R, Morgado-Bernal I (2003) Effects of pedunculopontine tegmental nucleus lesions on emotional reactivity and locomotion in rats. Brain Res Bull 59:495-503.

Honda T, Semba K (1995) An ultrastructural study of cholinergic and non-cholinergic neurons in the laterodorsal and pedunculopontine tegmental nuclei in the rat. Neuroscience 68:837-853.

Hong LE, Wonodi I, Lewis J, Thaker GK (2008) Nicotine effect on prepulse inhibition and prepulse facilitation in schizophrenia patients. Neuropsychopharmacology 33:2167-2174.

Hong S, Jhou TC, Smith M, Saleem KS, Hikosaka O (2011) Negative Reward Signals from the Lateral Habenula to Dopamine Neurons Are Mediated by Rostromedial Tegmental Nucleus in Primates. The Journal of Neuroscience 31:11457-11471.

Hudson JL, van Horne CG, Stromberg I, Brock S, Clayton J, Masserano J, Hoffer BJ, Gerhardt GA (1993) Correlation of apomorphine- and amphetamine-induced turning with nigrostriatal dopamine content in unilateral 6-hydroxydopamine lesioned rats. Brain Res 626:167-174.

Humphries MD, Gurney K, Prescott TJ (2007) Is there a brainstem substrate for action selection? Philosophical Transactions of the Royal Society B: Biological Sciences 362:1627-1639.

Ikeda T, Hikosaka O (2003) Reward-Dependent Gain and Bias of Visual Responses in Primate Superior Colliculus. Neuron 39:693-700.

Inglis WL, Semba K (1996) Colocalization of ionotropic glutamate receptor subunits with NADPH-diaphorase-containing neurons in the rat mesopontine tegmentum. J Comp Neurol 368:17-32.

Inglis WL, Semba K (1997) Discriminable excitotoxic effects of ibotenic acid, AMPA, NMDA and quinolinic acid in the rat laterodorsal tegmental nucleus. Brain research 755:17-27.

Inglis WL, Olmstead MC, Robbins TW (2001) Selective deficits in attentional performance on the 5-choice serial reaction time task following pedunculopontine tegmental nucleus lesions. Behav Brain Res 123:117-131.

Inglis WL, Allen LF, Whitelaw RB, Latimer MP, Brace HM, Winn P (1994) An investigation into the role of the pedunculopontine tegmental nucleus in the mediation of locomotion and orofacial stereotypy induced by d-amphetamine and apomorphine in the rat. Neuroscience 58:817-833.

Ishibashi M, Leonard CS, Kohlmeier KA (2009) Nicotinic activation of laterodorsal tegmental neurons: implications for addiction to nicotine. Neuropsychopharmacology 34:2529-2547.

Iwamoto ET (1990) Nicotine conditions place preferences after intracerebral administration in rats. Psychopharmacology (Berl) 100:251-257.

Jenkins TA, Latimer MP, Alderson HL, Winn P (2002) Determination of acetylcholine and dopamine content in thalamus and striatum after excitotoxic lesions of the pedunculopontine tegmental nucleus in rats. Neurosci Lett 322:45-48.

Jhou TC, Fields HL, Baxter MG, Saper CB, Holland PC (2009a) The Rostromedial Tegmental Nucleus (RMTg), a GABAergic Afferent to Midbrain Dopamine Neurons, Encodes Aversive Stimuli and Inhibits Motor Responses. Neuron 61:786-800.

Jhou TC, Geisler S, Marinelli M, Degarmo BA, Zahm DS (2009b) The mesopontine rostromedial tegmental nucleus: A structure targeted by the lateral habenula that projects to the ventral tegmental area of Tsai and substantia nigra compacta. J Comp Neurol 513:566-596.

Jia HG, Yamuy J, Sampogna S, Morales FR, Chase MH (2003) Colocalization of gamma-aminobutyric acid and acetylcholine in neurons in the laterodorsal and pedunculopontine tegmental nuclei in the cat: a light and electron microscopic study. Brain Res 992:205-219.

Joel D, Weiner I (2000) The connections of the dopaminergic system with the striatum in rats and primates: an analysis with respect to the functional and compartmental organization of the striatum. Neuroscience 96:451-474.

Jones CK, Shannon HE (2004) Lesions of the laterodorsal tegmental nucleus disrupt prepulse inhibition of the acoustic startle reflex. Pharmacology, biochemistry, and behavior 78:229-237.

Kamali Sarvestani I, Lindahl M, Hellgren-Kotaleski J, Ekeberg O (2011) The arbitration-extension hypothesis: a hierarchical interpretation of the functional organization of the Basal Ganglia. Front Syst Neurosci 5:13.

Kapur S (2003) Psychosis as a state of aberrant salience: a framework linking biology, phenomenology, and pharmacology in schizophrenia. Am J Psychiatry 160:13-23.

Keating GL, Winn P (2002) Examination of the role of the pedunculopontine tegmental nucleus in radial maze tasks with or without a delay. Neuroscience 112:687-696.

Keating GL, Walker SC, Winn P (2002) An examination of the effects of bilateral excitotoxic lesions of the pedunculopontine tegmental nucleus on responding to sucrose reward. Behav Brain Res 134:217-228.

Kinney GG, Vogel GW, Feng P (1998) Brainstem carbachol injections in the urethane anesthetized rat produce hippocampal theta rhythm and cortical desynchronization: a comparison of pedunculopontine tegmental versus nucleus pontis oralis injections. Brain Research 809:307-313.

Kita T, Kita H (2011) Cholinergic and non-cholinergic mesopontine tegmental neurons projecting to the subthalamic nucleus in the rat. The European journal of neuroscience 33:433-443.

Kitai ST, Shepard PD, Callaway JC, Scroggs R (1999) Afferent modulation of dopamine neuron firing patterns. Curr Opin Neurobiol 9:690-697.

Knudsen EI (2011) Control from below: the role of a midbrain network in spatial attention. Eur J Neurosci 33:1961-1972.

Knusel B, Hefti F (1988) Development of cholinergic pedunculopontine neurons in vitro: comparison with cholinergic septal cells and response to nerve growth factor, ciliary neuronotrophic factor, and retinoic acid. J Neurosci Res 21:365-375.

Kobayashi Y, Okada K (2007) Reward prediction error computation in the pedunculopontine tegmental nucleus neurons. Ann N Y Acad Sci 1104:310-323.

Koch M, Schnitzler HU (1997) The acoustic startle response in rats--circuits mediating evocation, inhibition and potentiation. Behav Brain Res 89:35-49.

Koch M, Kungel M, Herbert H (1993) Cholinergic neurons in the pedunculopontine tegmental nucleus are involved in the mediation of prepulse inhibition of the acoustic startle response in the rat. Exp Brain Res 97:71-82.

Kohler C, Schwarcz R, Fuxe K (1979) Intrahippocampal injections of ibotenic acid provide histological evidence for a neurotoxic mechanism different from kainic acid. Neurosci Lett 15:223-228.

Kolmac CI, Mitrofanis J (1998) Patterns of brainstem projection to the thalamic reticular nucleus. The Journal of Comparative Neurology 396:531-543.

Koob G (1982) The dopamine anhedonia hypothesis: a pharmacological phrenology. Behav Brain Sci 5:63–64.

Kovacs KJ (2008) Measurement of immediate-early gene activation- c-fos and beyond. J Neuroendocrinol 20:665-672.

Koyama Y, Imada N, Kawauchi A, Kayama Y (1999) Firing of putative cholinergic neurons and micturition center neurons in the rat laterodorsal tegmentum during distention and contraction of urinary bladder. Brain research 840:45-55.

Kozak R, Bowman EM, Latimer MP, Rostron CL, Winn P (2005) Excitotoxic lesions of the pedunculopontine tegmental nucleus in rats impair performance on a test of sustained attention. Exp Brain Res 162:257-264.

Kumari V, Postma P (2005) Nicotine use in schizophrenia: The self medication hypotheses. Neuroscience & Biobehavioral Reviews 29:1021-1034.

Lanca AJ, Sanelli TR, Corrigall WA (2000a) Nicotine-induced fos expression in the pedunculopontine mesencephalic tegmentum in the rat. Neuropharmacology 39:2808-2817.

Lanca AJ, Adamson KL, Coen KM, Chow BL, Corrigall WA (2000b) The pedunculopontine tegmental nucleus and the role of cholinergic neurons in nicotine self-administration in the rat: a correlative neuroanatomical and behavioral study. Neuroscience 96:735-742.

Lavezzi HN, Zahm DS (2011) The mesopontine rostromedial tegmental nucleus: an integrative modulator of the reward system. Basal ganglia 1:191-200.

Lavoie B, Parent A (1994a) Pedunculopontine nucleus in the squirrel monkey: cholinergic and glutamatergic projections to the substantia nigra. J Comp Neurol 344:232-241.

Lavoie B, Parent A (1994b) Pedunculopontine nucleus in the squirrel monkey: distribution of cholinergic and monoaminergic neurons in the mesopontine tegmentum with evidence for the presence of glutamate in cholinergic neurons. J Comp Neurol 344:190-209.

Leonard CS, Kerman I, Blaha G, Taveras E, Taylor B (1995) Interdigitation of nitric oxide synthase-, tyrosine hydroxylase-, and serotonin-containing neurons in and around the laterodorsal and pedunculopontine tegmental nuclei of the guinea pig. J Comp Neurol 362:411-432.

Leri F, Franklin KBJ (1998) Learning impairments caused by lesions to the pedunculopontine tegmental nucleus: an artifact of anxiety? Brain Research 807:187-192.

Lester DB, Rogers TD, Blaha CD (2010) Acetylcholine–Dopamine Interactions in the Pathophysiology and Treatment of CNS Disorders. CNS Neuroscience & Therapeutics 16:137-162.

Levy R, Lang AE, Dostrovsky JO, Pahapill P, Romas J, Saint-Cyr J, Hutchison WD, Lozano AM (2001) Lidocaine and muscimol microinjections in subthalamic nucleus reverse Parkinsonian symptoms. Brain 124:2105-2118.

Lex B, Hauber W (2010a) Disconnection of the entorhinal cortex and dorsomedial striatum impairs the sensitivity to instrumental contingency degradation. Neuropsychopharmacology 35:1788-1796.

Lex B, Hauber W (2010b) The role of nucleus accumbens dopamine in outcome encoding in instrumental and Pavlovian conditioning. Neurobiol Learn Mem 93:283-290.

Lex B, Sommer S, Hauber W (2011) The role of dopamine in the dorsomedial striatum in place and response learning. Neuroscience 172:212-218.

Liljeholm M, Tricomi E, O'Doherty JP, Balleine BW (2011) Neural correlates of instrumental contingency learning: differential effects of action-reward conjunction and disjunction. J Neurosci 31:2474-2480.

Liu X, Palmatier MI, Caggiula AR, Donny EC, Sved AF (2007) Reinforcement enhancing effect of nicotine and its attenuation by nicotinic antagonists in rats. Psychopharmacology (Berl) 194:463-473.

Ljungberg T, Apicella P, Schultz W (1992) Responses of monkey dopamine neurons during learning of behavioral reactions. Journal of Neurophysiology 67:145-163.

Lodge DJ, Grace AA (2006) The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. Proceedings of the National Academy of Sciences of the United States of America 103:5167-5172.

Lokwan SJ, Overton PG, Berry MS, Clark D (1999) Stimulation of the pedunculopontine tegmental nucleus in the rat produces burst firing in A9 dopaminergic neurons. Neuroscience 92:245-254.

Losier BJ, Semba K (1993) Dual projections of single cholinergic and aminergic brainstem neurons to the thalamus and basal forebrain in the rat. Brain Res 604:41-52.

Lovibond PF (1983) Facilitation of instrumental behavior by a Pavlovian appetitive conditioned stimulus. J Exp Psychol Anim Behav Process 9:225-247.

MacLaren DA, Isherwood A, Latimer M, Winn P (2007) Assessing the effect selective cholinergic lesions of the pedunculopontine tegmental nucleus in performance of the delayed spatial win-shift radial maze task. In. Unpublished senior honours dissertation, Department of Psychology, University of St Andrews.

Majchrzak M, Di Scala G (2000) GABA and muscimol as reversible inactivation tools in learning and memory. Neural Plast 7:19-29.

Maloney KJ, Mainville L, Jones BE (1999) Differential c-Fos expression in cholinergic, monoaminergic, and GABAergic cell groups of the pontomesencephalic tegmentum after paradoxical sleep deprivation and recovery. J Neurosci 19:3057-3072.

Mameli-Engvall M, Evrard A, Pons S, Maskos U, Svensson TH, Changeux J-P, Faure P (2006) Hierarchical Control of Dopamine Neuron-Firing Patterns by Nicotinic Receptors. Neuron 50:911-921.

Manaye KF, Zweig R, Wu D, Hersh LB, De Lacalle S, Saper CB, German DC (1999) Quantification of cholinergic and select non-cholinergic mesopontine neuronal populations in the human brain. Neuroscience 89:759-770.

Mansvelder HD, Keath JR, McGehee DS (2002) Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. Neuron 33:905-919.

Mao D, Gallagher K, McGehee DS (2011) Nicotine Potentiation of Excitatory Inputs to Ventral Tegmental Area Dopamine Neurons. The Journal of Neuroscience 31:6710-6720.

Marcotte ER, Pearson DM, Srivastava LK (2001) Animal models of schizophrenia: a critical review. Journal of psychiatry & neuroscience : JPN 26:395-410.

Martin JH (1991) Autoradiographic estimation of the extent of reversible inactivation produced by microinjection of lidocaine and muscimol in the rat. Neurosci Lett 127:160-164.

Martinez-Gonzalez C, Bolam JP, Mena-Segovia J (2011) Topographical organization of the pedunculopontine nucleus. Frontiers in neuroanatomy 5:22.

Martinez-Gonzalez C, Wang H-L, Micklem BR, Bolam JP, Mena-Segovia J (2012) Subpopulations of cholinergic, GABAergic and glutamatergic neurons in the pedunculopontine nucleus contain calcium-binding proteins and are heterogeneously distributed. European Journal of Neuroscience:no-no.

Maskos U (2007) Emerging concepts: novel integration of in vivo approaches to localize the function of nicotinic receptors. Journal of Neurochemistry 100:596-602.

Maskos U (2008) The cholinergic mesopontine tegmentum is a relatively neglected nicotinic master modulator of the dopaminergic system: relevance to drugs of abuse and pathology. Br J Pharmacol 153 Suppl 1:S438-445.

Maskos U, Molles BE, Pons S, Besson M, Guiard BP, Guilloux JP, Evrard A, Cazala P, Cormier A, Mameli-Engvall M, Dufour N, Cloez-Tayarani I, Bemelmans AP, Mallet J, Gardier AM, David V, Faure P, Granon S, Changeux JP (2005) Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. Nature 436:103-107.

Matsumoto M, Hikosaka O (2007) Lateral habenula as a source of negative reward signals in dopamine neurons. Nature 447:1111-1115.

Matsumura M, Nambu A, Yamaji Y, Watanabe K, Imai H, Inase M, Tokuno H, Takada M (2000) Organization of somatic motor inputs from the frontal lobe to the pedunculopontine tegmental nucleus in the macaque monkey. Neuroscience 98:97-110.

May PJ, McHaffie JG, Stanford TR, Jiang H, Costello MG, Coizet V, Hayes LM, Haber SN, Redgrave P (2009) Tectonigral projections in the primate: a pathway for pre-attentive sensory input to midbrain dopaminergic neurons. Eur J Neurosci 29:575-587.

McAlonan K, Brown VJ, Bowman EM (2000) Thalamic reticular nucleus activation reflects attentional gating during classical conditioning. J Neurosci 20:8897-8901.

McCormick DA, Prince DA (1986) Mechanisms of action of acetylcholine in the guinea-pig cerebral cortex in vitro. J Physiol 375:169-194.

McGaughy J, Kaiser T, Sarter M (1996) Behavioral vigilance following infusions of 192 IgG-saporin into the basal forebrain: selectivity of the behavioral impairment and relation to cortical AChE-positive fiber density. Behav Neurosci 110:247-265.

McHaffie JG, Stanford TR, Stein BE, Coizet V, Redgrave P (2005) Subcortical loops through the basal ganglia. Trends Neurosci 28:401-407.

Meck WH, Benson AM (2002) Dissecting the Brain's Internal Clock: How Frontal–Striatal Circuitry Keeps Time and Shifts Attention. Brain and Cognition 48:195-211.

Medina L, Reiner A (1994) Distribution of choline acetyltransferase immunoreactivity in the pigeon brain. J Comp Neurol 342:497-537.

Mena-Segovia J, Bolam JP (2011) Phasic modulation of cortical high-frequency oscillations by pedunculopontine neurons. Prog Brain Res 193C:85-92.

Mena-Segovia J, Bolam JP, Magill PJ (2004a) Pedunculopontine nucleus and basal ganglia: distant relatives or part of the same family? Trends Neurosci 27:585-588.

Mena-Segovia J, Favila R, Giordano M (2004b) Long-term effects of striatal lesions on c-Fos immunoreactivity in the pedunculopontine nucleus. Eur J Neurosci 20:2367-2376.

Mena-Segovia J, Winn P, Bolam JP (2008a) Cholinergic modulation of midbrain dopaminergic systems. Brain Res Rev 58:265-271.

Mena-Segovia J, Sims HM, Magill PJ, Bolam JP (2008b) Cholinergic brainstem neurons modulate cortical gamma activity during slow oscillations. The Journal of physiology 586:2947-2960.

Mesulam MM, Mufson EJ, Wainer BH, Levey AI (1983) Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1-Ch6). Neuroscience 10:1185-1201.

Mirenowicz J, Schultz W (1994) Importance of unpredictability for reward responses in primate dopamine neurons. Journal of Neurophysiology 72:1024-1027.

Mirenowicz J, Schultz W (1996) Preferential activation of midbrain dopamine neurons by appetitive rather than aversive stimuli. Nature 379:449-451.

Mitchell D (1976) Experiments on neophobia in wild and laboratory rats: a reevaluation. J Comp Physiol Psychol 90:190-197.

Miwa JM, Freedman R, Lester HA (2011) Neural systems governed by nicotinic acetylcholine receptors: emerging hypotheses. Neuron 70:20-33.

Miyazato H, Skinner RD, Garcia-Rill E (1999) Neurochemical modulation of the P13 midlatency auditory evoked potential in the rat. Neuroscience 92:911-920.

Moncrieff J (2009) A Critique of the Dopamine Hypothesis of Schizophrenia and Psychosis. Harvard Review of Psychiatry 17:214-225.

Moorman DE, Aston-Jones G (2010) Orexin/Hypocretin Modulates Response of Ventral Tegmental Dopamine Neurons to Prefrontal Activation: Diurnal Influences. The Journal of Neuroscience 30:15585-15599.

Moriizumi T, Hattori T (1992) Separate neuronal populations of the rat globus pallidus projecting to the subthalamic nucleus, auditory cortex and pedunculopontine tegmental area. Neuroscience 46:701-710.

Moruzzi G, Magoun HW (1949) Brain stem reticular formation and activation of the EEG. Electroencephalography and Clinical Neurophysiology 1:455-473.

Motts SD, Schofield BR (2009) Sources of cholinergic input to the inferior colliculus. Neuroscience 160:103-114.

Mugnaini M, Garzotti M, Sartori I, Pilla M, Repeto P, Heidbreder CA, Tessari M (2006) Selective down-regulation of [(125)I]Y0-alpha-conotoxin MII binding in rat mesostriatal dopamine pathway following continuous infusion of nicotine. Neuroscience 137:565-572.

Nakahara D, Ishida Y, Nakamura M, Furuno N, Nishimori T (2001) Intracranial self-stimulation induces Fos expression in GABAergic neurons in the rat mesopontine tegmentum. Neuroscience 106:633-641.

Nakamura Y, Tokuno H, Moriizumi T, Kitao Y, Kudo M (1989) Monosynaptic nigral inputs to the pedunculopontine tegmental nucleus neurons which send their axons to the medial reticular formation in the medulla oblongata. An electron microscopic study in the cat. Neuroscience Letters 103:145-150.

Nature_Methods_Editorial (2011) Method of the Year 2010. Nat Meth 8:1-1.

Nemcova V, Petrovicky P, ten Donkelaar HJ (2000) The effect of electrolytic thalamic lesions on the NADPH-diaphorase activity of neurons of the laterodorsal tegmental and pedunculopontine nuclei in rats. J Chem Neuroanat 17:227-232.

Norton AB, Jo YS, Clark EW, Taylor CA, Mizumori SJ (2011) Independent neural coding of reward and movement by pedunculopontine tegmental nucleus neurons in freely navigating rats. Eur J Neurosci 33:1885-1896.

Nowacka A, Jurkowlaniec E, Trojniar W (2002) Microinjection of procaine into the pedunculopontine tegmental nucleus suppresses hippocampal theta rhythm in urethane-anesthetized rats. Brain Res Bull 58:377-384.

Oakman SA, Faris PL, Kerr PE, Cozzari C, Hartman BK (1995a) Distribution of pontomesencephalic cholinergic neurons projecting to substantia nigra differs significantly from those projecting to ventral tegmental area. J Neurosci 15:5859-5869.

Oakman SA, Faris PL, Kerr PE, Cozzari C, Hartman BK (1995b) Distribution of pontomesencephalic cholinergic neurons projecting to substantia nigra differs significantly from those projecting to ventral tegmental area. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:5859-5869.

Obeso JA, Marin C, Rodriguez-Oroz C, Blesa J, Benitez-Temiño B, Mena-Segovia J, Rodríguez M, Olanow CW (2008) The basal ganglia in Parkinson's disease: Current concepts and unexplained observations. Annals of Neurology 64:S30-S46.

Okada K, Nakamura K, Kobayashi Y (2011) A Neural Correlate of Predicted and Actual Reward-Value Information in Monkey Pedunculopontine Tegmental and Dorsal Raphe Nucleus during Saccade Tasks. Neural Plast 2011:579840.

Okada K, Toyama K, Inoue Y, Isa T, Kobayashi Y (2009) Different pedunculopontine tegmental neurons signal predicted and actual task rewards. J Neurosci 29:4858-4870.

Okuno H (2011) Regulation and function of immediate-early genes in the brain: beyond neuronal activity markers. Neurosci Res 69:175-186.

Olmstead MC, Franklin KB (1994) Lesions of the pedunculopontine tegmental nucleus block drug-induced reinforcement but not amphetamine-induced locomotion. Brain Res 638:29-35.

Olmstead MC, Munn EM, Franklin KB, Wise RA (1998) Effects of pedunculopontine tegmental nucleus lesions on responding for intravenous heroin under different schedules of reinforcement. J Neurosci 18:5035-5044.

Olmstead MC, Inglis WL, Bordeaux CP, Clarke EJ, Wallum NP, Everitt BJ, Robbins TW (1999) Lesions of the pedunculopontine tegmental nucleus increase sucrose consumption but do not affect discrimination or contrast effects. Behav Neurosci 113:732-743.

Omelchenko N, Sesack SR (2007) Glutamate synaptic inputs to ventral tegmental area neurons in the rat derive primarily from subcortical sources. Neuroscience 146:1259-1274.

Palmatier MI, Evans-Martin FF, Hoffman A, Caggiula AR, Chaudhri N, Donny EC, Liu X, Booth S, Gharib M, Craven L, Sved AF (2006) Dissociating the primary reinforcing and reinforcement-enhancing effects of nicotine using a rat self-administration paradigm

with concurrently available drug and environmental reinforcers. Psychopharmacology (Berl) 184:391-400.

Pan WX, Hyland BI (2005) Pedunculopontine tegmental nucleus controls conditioned responses of midbrain dopamine neurons in behaving rats. J Neurosci 25:4725-4732.

Parent A, Parent M, Charara A (1999) Glutamatergic inputs to midbrain dopaminergic neurons in primates. Parkinsonism Relat Disord 5:193-201.

Parent M, Descarries L (2008) Acetylcholine innervation of the adult rat thalamus: distribution and ultrastructural features in dorsolateral geniculate, parafascicular, and reticular thalamic nuclei. J Comp Neurol 511:678-691.

Parkinson JA, Willoughby PJ, Robbins TW, Everitt BJ (2000) Disconnection of the anterior cingulate cortex and nucleus accumbens core impairs Pavlovian approach behavior: further evidence for limbic cortical-ventral striatopallidal systems. Behav Neurosci 114:42-63.

Pastrana E (2011) Optogenetics: controlling cell function with light. Nat Meth 8:24-25.

Paxinos G, Huang X (1995) Atlas of the Human Brainstem: San Diego Academic Press.

Paxinos G, Franklin KB (2003) The Mouse Brain in Stereotaxic Coordinates: Compact Second Edition: Academic Press.

Paxinos G, Watson C (2005) The rat brain in stereotaxic coordinates, 5th Edition. Amsterdam ; Boston: Elsevier Academic Press.

Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates, 6th Edition. Amsterdam ; Boston ;: Academic Press/Elsevier.

Perez XA, Parameswaran N, Huang LZ, O'Leary KT, Quik M (2008) Pre-synaptic dopaminergic compensation after moderate nigrostriatal damage in non-human primates. J Neurochem 105:1861-1872.

Petrof I (2007) Behavioural analysis of the role of caudal thalamic reticular nucleus in attention. PhD thesis, School of Psychology, University of St Andrews. In.

Pielock SM, Lex B, Hauber W (2011) The role of dopamine in the dorsomedial striatum in general and outcome-selective Pavlovian-instrumental transfer. Eur J Neurosci 33:717-725.

Pierce RC, Vanderschuren LJMJ (2010) Kicking the habit: The neural basis of ingrained behaviors in cocaine addiction. Neuroscience & Biobehavioral Reviews 35:212-219.

Podhorna J, Franklin KB (1999) Long-lasting increase in anxiety after electrolytic lesions of the pedunculopontine tegmental nucleus. Behav Neurosci 113:550-557.

Podhorna J, Franklin KBJ (2000) Pontine Tegmentum Lesions Increase Anxiety-Like Behavior in Rats: A Comparison With Anxiety Produced by β-CCE. Pharmacology Biochemistry and Behavior 65:267-273.

Porter A (2007) Immediate early gene expression in the mesopontine tegmentum and midbrain after acute or chronic nicotine administration. In: School of Psychology: University of St Andrews.

Prescott TJ, Redgrave P, Gurney K (1999) Layered Control Architectures in Robots and Vertebrates. Adaptive Behavior 7:99-127.

Rasmusson DD, Clow K, Szerb JC (1994) Modification of neocortical acetylcholine release and electroencephalogram desynchronization due to brainstem stimulation by drugs applied to the basal forebrain. Neuroscience 60:665-677.

Reavill C, Stolerman IP (1990) Locomotor activity in rats after administration of nicotinic agonists intracerebrally. Br J Pharmacol 99:273-278.

Redgrave P, Gurney K (2006) The short-latency dopamine signal: a role in discovering novel actions? Nat Rev Neurosci 7:967-975.

Redgrave P, Coizet V (2007) Brainstem interactions with the basal ganglia. Parkinsonism Relat Disord 13 Suppl 3:S301-305.

Redgrave P, Mitchell IJ, Dean P (1987) Descending projections from the superior colliculus in rat: a study using orthograde transport of wheatgerm-agglutinin conjugated horseradish peroxidase. Experimental Brain Research 68:147-167.

Redgrave P, Prescott TJ, Gurney K (1999) The basal ganglia: a vertebrate solution to the selection problem? Neuroscience 89:1009-1023.

Redgrave P, Gurney K, Reynolds J (2008) What is reinforced by phasic dopamine signals? Brain Res Rev 58:322-339.

Redgrave P, Coizet V, Reynolds J (2010a) Chapter 31 - Phasic Dopamine Signaling and Basal Ganglia Function. In: Handbook of Basal Ganglia Structure and Function (Heinz S, Kuei YT, eds), pp 549-559: Elsevier.

Redgrave P, Vautrelle N, Reynolds JN (2011) Functional properties of the basal ganglia's re-entrant loop architecture: selection and reinforcement. Neuroscience 198:138-151.

Redgrave P, Rodriguez M, Smith Y, Rodriguez-Oroz MC, Lehericy S, Bergman H, Agid Y, DeLong MR, Obeso JA (2010b) Goal-directed and habitual control in the basal ganglia: implications for Parkinson's disease. Nat Rev Neurosci 11:760-772.

Reese NB, Garcia-Rill E, Skinner RD (1995) Auditory input to the pedunculopontine nucleus: I. Evoked potentials. Brain Res Bull 37:257-264.

Rescorla RA, Wagner AW (1972) A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement. In: Classical Conditioning II:

Current Research and Theory (Black AH, Prokasy WF, eds), pp 64-99: Appleton-Century-Crofts.

Richardson PM, Issa VM, Riopelle RJ (1986) Distribution of neuronal receptors for nerve growth factor in the rat. The Journal of neuroscience : the official journal of the Society for Neuroscience 6:2312-2321.

Robinson TE, Castaneda E, Whishaw IQ (1990) Compensatory changes in striatal dopamine neurons following recovery from injury induced by 6-OHDA or methamphetamine: a review of evidence from microdialysis studies. Can J Psychol 44:253-275.

Robinson TE, Mocsary Z, Camp DM, Whishaw IQ (1994) Time course of recovery of extracellular dopamine following partial damage to the nigrostriatal dopamine system. J Neurosci 14:2687-2696.

Rodriguez M, Mantolan-Sarmiento B, Gonzalez-Hernandez T (1998) Effects of ethylcholine mustard azirinium ion (AF64A) on the choline acetyltransferase and nitric oxide synthase activities in mesopontine cholinergic neurons of the rat. Neuroscience 82:853-866.

Rolland AS, Karachi C, Muriel MP, Hirsch EC, Francois C (2011) Internal pallidum and substantia nigra control different parts of the mesopontine reticular formation in primate. Movement disorders : official journal of the Movement Disorder Society 26:1648-1656.

Romaniuk L, Honey GD, King JR, Whalley HC, McIntosh AM, Levita L, Hughes M, Johnstone EC, Day M, Lawrie SM, Hall J (2010) Midbrain activation during Pavlovian conditioning and delusional symptoms in schizophrenia. Archives of general psychiatry 67:1246-1254.

Ros H, Magill PJ, Moss J, Bolam JP, Mena-Segovia J (2010) Distinct types of non-cholinergic pedunculopontine neurons are differentially modulated during global brain states. Neuroscience 170:78-91.

Rose JE, Corrigall WA (1997) Nicotine self-administration in animals and humans: similarities and differences. Psychopharmacology (Berl) 130:28-40.

Rostron CL, Farquhar MJ, Latimer MP, Winn P (2008) The pedunculopontine tegmental nucleus and the nucleus basalis magnocellularis: do both have a role in sustained attention? BMC Neurosci 9:16.

Rostron CL, Clark SD, Farquhar MJ, Isherwood A, Latimer M, Hothacker H, Winn P (2007) Selective bilateral cholinergic lesions of the pedunculopontine tegmental nucleus in rats by diphtheria toxin/urotensin II. Program No 62213/SS30 Neuroscience Meeting Planner San Diego, CA: Society for Neuroscience, 2007 Online.

Rousselet GA, Thorpe SJ, Fabre-Thorpe M (2004) How parallel is visual processing in the ventral pathway? Trends in Cognitive Sciences 8:363-370.

Rugg EL, Dunbar JS, Latimer M, Winn P (1992) Excitotoxic lesions of the pedunculopontine tegmental nucleus of the rat. I. Comparison of the effects of various excitotoxins, with

particular reference to the loss of immunohistochemically identified cholinergic neurons. Brain Res 589:181-193.

Ryan MC, Gundlach AL (1995) Anatomical localisation of preproatrial natriuretic peptide mRNA in the rat brain by in situ hybridisation histochemistry: novel identification in olfactory regions. J Comp Neurol 356:168-182.

Saalmann YB, Pigarev IN, Vidyasagar TR (2007) Neural mechanisms of visual attention: how top-down feedback highlights relevant locations. Science 316:1612-1615.

Saitoh K, Hattori S, Song WJ, Isa T, Takakusaki K (2003) Nigral GABAergic inhibition upon cholinergic neurons in the rat pedunculopontine tegmental nucleus. Eur J Neurosci 18:879-886.

Samson HH, Chappell A (2001) Injected muscimol in pedunculopontine tegmental nucleus alters ethanol self-administration. Alcohol 23:41-48.

Sandberg K, Schnaar RL, McKinney M, Hanin I, Fisher A, Coyle JT (1985) AF64A: an active site directed irreversible inhibitor of choline acetyltransferase. J Neurochem 44:439-445.

Saper CB, Loewy AD (1982) Projections of the pedunculopontine tegmental nucleus in the rat: evidence for additional extrapyramidal circuitry. Brain Research 252:367-372.

Sarter M, Givens B, Bruno JP (2001) The cognitive neuroscience of sustained attention: where top-down meets bottom-up. Brain Res Brain Res Rev 35:146-160.

Satoh K, Fibiger HC (1986) Cholinergic neurons of the laterodorsal tegmental nucleus: efferent and afferent connections. The Journal of comparative neurology 253:277-302.

Scarnati E, Campana E, Pacitti C (1984) Pedunculopontine-evoked excitation of substantia nigra neurons in the rat. Brain Res 304:351-361.

Scarnati E, Proia A, Campana E, Pacitti C (1986) A microiontophoretic study on the nature of the putative synaptic neurotransmitter involved in the pedunculopontine-substantia nigra pars compacta excitatory pathway of the rat. Exp Brain Res 62:470-478.

Schilström B, ouml, rn, Rawal N, Mameli-Engvall M, Nomikos GG, Svensson TH (2003) Dual effects of nicotine on dopamine neurons mediated by different nicotinic receptor subtypes. The International Journal of Neuropsychopharmacology 6:1-11.

Schofield BR (2010) Projections from auditory cortex to midbrain cholinergic neurons that project to the inferior colliculus. Neuroscience 166:231-240.

Schultz W (1998) Predictive Reward Signal of Dopamine Neurons. Journal of Neurophysiology 80:1-27.

Schultz W (1999) The Reward Signal of Midbrain Dopamine Neurons. News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society 14:249-255.

Schultz W (2010) Dopamine signals for reward value and risk: basic and recent data. Behavioral and brain functions : BBF 6:24.

Schwabe L, Wolf OT (2011) Stress-induced modulation of instrumental behavior: From goal-directed to habitual control of action. Behavioural Brain Research 219:321-328.

Semba K, Fibiger HC (1992) Afferent connections of the laterodorsal and the pedunculopontine tegmental nuclei in the rat: a retro- and antero-grade transport and immunohistochemical study. The Journal of comparative neurology 323:387-410.

Semba K, Reiner PB, Fibiger HC (1990) Single cholinergic mesopontine tegmental neurons project to both the pontine reticular formation and the thalamus in the rat. Neuroscience 38:643-654.

Semba K, Reiner PB, McGeer EG, Fibiger HC (1988) Brainstem afferents to the magnocellular basal forebrain studied by axonal transport, immunohistochemistry, and electrophysiology in the rat. J Comp Neurol 267:433-453.

Sesack SR, Pickel VM (1992) Prefrontal cortical efferents in the rat synapse on unlabeled neuronal targets of catecholamine terminals in the nucleus accumbens septi and on dopamine neurons in the ventral tegmental area. The Journal of Comparative Neurology 320:145-160.

Shanks DR, Dickinson A (1991) Instrumental judgment and performance under variations in action-outcome contingency and contiguity. Mem Cognit 19:353-360.

Shibata H (1992) Topographic organization of subcortical projections to the anterior thalamic nuclei in the rat. The Journal of comparative neurology 323:117-127.

Shik ML, Severin FV, Orlovskii GN (1966) [Control of walking and running by means of electric stimulation of the midbrain]. Biofizika 11:659-666.

Shiromani PJ, Winston S, McCarley RW (1996) Pontine cholinergic neurons show Fos-like immunoreactivity associated with cholinergically induced REM sleep. Brain Res Mol Brain Res 38:77-84.

Shiromani PJ, Kilduff TS, Bloom FE, McCarley RW (1992) Cholinergically induced REM sleep triggers Fos-like immunoreactivity in dorsolateral pontine regions associated with REM sleep. Brain Res 580:351-357.

Silveira MCL, Sandner G, Graeff FG (1993) Induction of Fos immunoreactivity in the brain by exposure to the elevated plus-maze. Behavioural Brain Research 56:115-118.

Singh N, Pillay V, Choonara YE (2007) Advances in the treatment of Parkinson's disease. Prog Neurobiol 81:29-44.

Skinner RD, Kinjo N, Henderson V, Garcia-Rill E (1990a) Locomotor projections from the pedunculopontine nucleus to the spinal cord. Neuroreport 1:183-186.

Skinner RD, Kinjo N, Ishikawa Y, Biedermann JA, Garcia-Rill E (1990b) Locomotor projections from the pedunculopontine nucleus to the medioventral medulla. Neuroreport 1:207-210.

Sorenson EM, Shiroyama T, Kitai ST (1998) Postsynaptic nicotinic receptors on dopaminergic neurons in the substantia nigra pars compacta of the rat. Neuroscience 87:659-673.

Steidl S, Wang H, Morales M, Wise RA (2011) Effects of laterodorsal tegmental nucleus cholinergic neuron lesions on cocaine self-administration in rats Program No 30015/WW58 Neuroscience Meeting Planner Washington, DC: Society for Neuroscience, 2011 Online.

Steiner H, Tseng K (2010) Handbook of Basal Ganglia Structure and Function. Academic Press; 1st edition.

Steiniger B, Kretschmer BD (2004) Effects of ibotenate pedunculopontine tegmental nucleus lesions on exploratory behaviour in the open field. Behav Brain Res 151:17-23.

Stephenson-Jones M, Ericsson J, Robertson B, Grillner S (2012) Evolution of the basal ganglia; Dual output pathways conserved throughout vertebrate phylogeny. The Journal of Comparative Neurology:n/a-n/a.

Stephenson-Jones M, Samuelsson E, Ericsson J, Robertson B, Grillner S (2011) Evolutionary conservation of the basal ganglia as a common vertebrate mechanism for action selection. Current biology : CB 21:1081-1091.

Steriade M, McCarley RW (1990) Brainstem control of wakefulness and sleep. New York: Plenum Press.

Steriade M, Dossi RC, Paré D, Oakson G (1991) Fast oscillations (20-40 Hz) in thalamocortical systems and their potentiation by mesopontine cholinergic nuclei in the cat. Proceedings of the National Academy of Sciences 88:4396-4400.

Steven R V (2000) The ascending reticular activating system — from aminergic neurons to nitric oxide. J Chem Neuroanat 18:23-30.

Sugase-Miyamoto Y, Richmond BJ (2005) Neuronal Signals in the Monkey Basolateral Amygdala during Reward Schedules. The Journal of Neuroscience 25:11071-11083.

Swerdlow NR, Geyer MA (1993a) Prepulse inhibition of acoustic startle in rats after lesions of the pedunculopontine tegmental nucleus. Behav Neurosci 107:104-117.

Swerdlow NR, Geyer MA (1993b) Prepulse inhibition of acoustic startle in rats after lesions of the pedunculopontine tegmental nucleus. Behavioral neuroscience 107:104-117.

Takakusaki K, Shiroyama T, Yamamoto T, Kitai ST (1996) Cholinergic and noncholinergic tegmental pedunculopontine projection neurons in rats revealed by intracellular labeling. J Comp Neurol 371:345-361.

Taylor CL, Kozak R, Latimer MP, Winn P (2004) Effects of changing reward on performance of the delayed spatial win-shift radial maze task in pedunculopontine tegmental nucleus lesioned rats. Behav Brain Res 153:431-438.

Torterolo P, Morales FR, Chase MH (2002) GABAergic mechanisms in the pedunculopontine tegmental nucleus of the cat promote active (REM) sleep. Brain Res 944:1-9.

Torterolo P, Yamuy J, Sampogna S, Morales FR, Chase MH (2001) GABAergic neurons of the laterodorsal and pedunculopontine tegmental nuclei of the cat express c-fos during carbachol-induced active sleep. Brain Res 892:309-319.

Turetsky BI, Calkins ME, Light GA, Olincy A, Radant AD, Swerdlow NR (2007) Neurophysiological endophenotypes of schizophrenia: the viability of selected candidate measures. Schizophr Bull 33:69-94.

Uhlrich DJ, Tamamaki N, Murphy PC, Sherman SM (1995) Effects of brain stem parabrachial activation on receptive field properties of cells in the cat's lateral geniculate nucleus. Journal of Neurophysiology 73:2428-2447.

Verret L, Leger L, Fort P, Luppi PH (2005) Cholinergic and noncholinergic brainstem neurons expressing Fos after paradoxical (REM) sleep deprivation and recovery. Eur J Neurosci 21:2488-2504.

Vezina P, McGehee DS, Green WN (2007) Exposure to nicotine and sensitization of nicotine-induced behaviors. Prog Neuropsychopharmacol Biol Psychiatry 31:1625-1638.

Vincent SR, Kimura H (1992) Histochemical mapping of nitric oxide synthase in the rat brain. Neuroscience 46:755-784.

Vincent SR, Satoh K, Armstrong DM, Panula P, Vale W, Fibiger HC (1986) Neuropeptides and nadph-diaphorase activity in the ascending cholinergic reticular system of the rat. Neuroscience 17:167-182.

Voorn P, Vanderschuren LJ, Groenewegen HJ, Robbins TW, Pennartz CM (2004) Putting a spin on the dorsal-ventral divide of the striatum. Trends Neurosci 27:468-474.

Waite JJ, Chen AD (2001) Differential changes in rat cholinergic parameters subsequent to immunotoxic lesion of the basal forebrain nuclei. Brain Res 918:113-120.

Walker SC, Winn P (2007) An assessment of the contributions of the pedunculopontine tegmental and cuneiform nuclei to anxiety and neophobia. Neuroscience 150:273-290.

Wang HL, Morales M (2009) Pedunculopontine and laterodorsal tegmental nuclei contain distinct populations of cholinergic, glutamatergic and GABAergic neurons in the rat. Eur J Neurosci 29:340-358.

Wang HL, Chakraborti A, NG T, Yamaguchi T, Morales M (2010) Ventral tegmental input from the pedunculopontine and laterodorsal tegmental nuclei is dominated by glutamatergic and GABAergic, rather than cholinergic neurons. Program No 3664/FF8

2010 Neuroscience Meeting Planner San Diego, CA: Society for Neuroscience, 2010 Online.

Whishaw IQ, Cioe JD, Previsich N, Kolb B (1977) The variability of the interaural line vs the stability of bregma in rat stereotaxic surgery. Physiol Behav 19:719-722.

Williams JA, Reiner PB (1993) Noradrenaline hyperpolarizes identified rat mesopontine cholinergic neurons in vitro. J Neurosci 13:3878-3883.

Willner P (1997) The dopamine hypothesis of schizophrenia: current status, future prospects. International Clinical Psychopharmacology 12:297-308.

Wilson DI, MacLaren DA, Winn P (2009a) Bar pressing for food: differential consequences of lesions to the anterior versus posterior pedunculopontine. Eur J Neurosci 30:504-513.

Wilson DIG, MacLaren DAA, Winn P (2009b) On the Relationships Between the Pedunculopontine Tegmental Nucleus, Corticostriatal Architecture, and the Medial Reticular Formation. In: The Basal Ganglia IX, pp 143-157: Springer New York.

Wiltgen BJ, Law M, Ostlund S, Mayford M, Balleine BW (2007) The influence of Pavlovian cues on instrumental performance is mediated by CaMKII activity in the striatum. Eur J Neurosci 25:2491-2497.

Winn P (2006) How best to consider the structure and function of the pedunculopontine tegmental nucleus: evidence from animal studies. J Neurol Sci 248:234-250.

Winn P (2008) Experimental studies of pedunculopontine functions: are they motor, sensory or integrative? Parkinsonism Relat Disord 14 Suppl 2:S194-198.

Winn P, Wilson DIG, Redgrave P (2010) Chapter 23 - Subcortical Connections of the Basal Ganglia. In: Handbook of Basal Ganglia Structure and Function (Heinz S, Kuei YT, eds), pp 397-408: Academic Press.

Winn P, Rostron PR, Latimer MP, Zahm DS (2005) Afferents of the rat pedunculopontine tegmental nucleus: investigation with fluorogold. Program No 3018 2005 Neuroscience Meeting Planner Washington, DC: Society for Neuroscience, 2005 Online

Witten IB, Steinberg EE, Lee SY, Davidson TJ, Zalocusky KA, Brodsky M, Yizhar O, Cho SL, Gong S, Ramakrishnan C, Stuber GD, Tye KM, Janak PH, Deisseroth K (2011) Recombinase-driver rat lines: tools, techniques, and optogenetic application to dopamine-mediated reinforcement. Neuron 72:721-733.

Woolf NJ, Butcher LL (1986) Cholinergic systems in the rat brain: III. Projections from the pontomesencephalic tegmentum to the thalamus, tectum, basal ganglia, and basal forebrain. Brain research bulletin 16:603-637.

Wooltorton JRA, Pidoplichko VI, Broide RS, Dani JA (2003) Differential Desensitization and Distribution of Nicotinic Acetylcholine Receptor Subtypes in Midbrain Dopamine Areas. The Journal of Neuroscience 23:3176-3185.

Wu MF, Krueger J, Ison JR, Gerrard RL (1984) Startle reflex inhibition in the rat: its persistence after extended repetition of the inhibitory stimulus. J Exp Psychol Anim Behav Process 10:221-228.

Wullimann Mario F (2011) Basal Ganglia: Insights into Origins from Lamprey Brains. Current biology : CB 21:R497-R500.

Wykowska A, Schubo A (2010) On the temporal relation of top-down and bottom-up mechanisms during guidance of attention. J Cogn Neurosci 22:640-654.

Yamuy J, Sampogna S, Morales FR, Chase MH (1998) c-fos Expression in mesopontine noradrenergic and cholinergic neurons of the cat during carbachol-induced active sleep: a double-labeling study. Sleep Res Online 1:28-40.

Yasui Y, Cechetto DF, Saper CB (1990) Evidence for a cholinergic projection from the pedunculopontine tegmental nucleus to the rostral ventrolateral medulla in the rat. Brain Res 517:19-24.

Ye M, Hayar A, Strotman B, Garcia-Rill E (2010) Cholinergic modulation of fast inhibitory and excitatory transmission to pedunculopontine thalamic projecting neurons. J Neurophysiol 103:2417-2432.

Yin HH, Knowlton BJ (2006) The role of the basal ganglia in habit formation. Nat Rev Neurosci 7:464-476.

Yin HH, Knowlton BJ, Balleine BW (2004) Lesions of dorsolateral striatum preserve outcome expectancy but disrupt habit formation in instrumental learning. Eur J Neurosci 19:181-189.

Yin HH, Knowlton BJ, Balleine BW (2005a) Blockade of NMDA receptors in the dorsomedial striatum prevents action-outcome learning in instrumental conditioning. Eur J Neurosci 22:505-512.

Yin HH, Ostlund SB, Balleine BW (2008) Reward-guided learning beyond dopamine in the nucleus accumbens: the integrative functions of cortico-basal ganglia networks. Eur J Neurosci 28:1437-1448.

Yin HH, Ostlund SB, Knowlton BJ, Balleine BW (2005b) The role of the dorsomedial striatum in instrumental conditioning. Eur J Neurosci 22:513-523.

Yizhar O, Fenno LE, Davidson TJ, Mogri M, Deisseroth K (2011) Optogenetics in neural systems. Neuron 71:9-34.

Zachariou V, Caldarone BJ, Weathers-Lowin A, George TP, Elsworth JD, Roth RH, Changeux JP, Picciotto MR (2001) Nicotine receptor inactivation decreases sensitivity to cocaine. Neuropsychopharmacology 24:576-589.

Zahm DS, Williams EA, Latimer MP, Winn P (2001) Ventral mesopontine projections of the caudomedial shell of the nucleus accumbens and extended amygdala in the rat: double dissociation by organization and development. J Comp Neurol 436:111-125.

Zahm DS, Cheng AY, Lee TJ, Ghobadi CW, Schwartz ZM, Geisler S, Parsely KP, Gruber C, Veh RW (2011) Inputs to the midbrain dopaminergic complex in the rat, with emphasis on extended amygdala-recipient sectors. The Journal of Comparative Neurology 519:3159-3188.

Zhang L, Liu Y, Chen X (2005) Carbachol induces burst firing of dopamine cells in the ventral tegmental area by promoting calcium entry through L-type channels in the rat. J Physiol 568:469-481.

Zhang QJ, Liu J, Wang Y, Wang S, Wu ZH, Yan W, Hui YP, Ali U (2008) The firing activity of presumed cholinergic and non-cholinergic neurons of the pedunculopontine nucleus in 6-hydroxydopamine-lesioned rats: an in vivo electrophysiological study. Brain Res 1243:152-160.

Zhang XY, Liang J, Chen DC, Xiu MH, He J, Cheng W, Wu Z, De Yang F, Haile CN, Sun H, Lu L, Kosten TA, Kosten TR (2012) Cigarette Smoking in Male Patients with Chronic Schizophrenia in a Chinese Population: Prevalence and Relationship to Clinical Phenotypes. PLoS One 7:e30937.

Zrinzo L, Zrinzo LV (2008) Surgical anatomy of the pedunculopontine and peripeduncular nuclei. Br J Neurosurg 22 Suppl 1:S19-24.