Mesopontine mechanisms of behavioural control: An anatomical and functional analysis of the pedunculopontine tegmental nucleus.

James A. Ainge

University of St Andrews

PhD Thesis

September 2002

DECLARATION

i) I, James Ainge, hereby certify that this thesis, approximately 45,000 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.

Date:

Signature of Candidate:

ii) I was admitted as a research student in September 1998 and as a candidate for the degree of PhD in September 1998; the higher study for which this is a record was carried out in the University of St Andrews between 1998 and 2001.

Date:

Signature of Candidate:

iii) 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:

COPYRIGHT

Unrestricted

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 the copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker.

Date:

Signature of Candidate:

CONTENTS

Chapter 2: Investigation of the role of the PPTg in reward related behaviour

Chapter 3: Investigation of the psychological processes underlying disinhibited behaviour in PPTg lesioned rats

Section B: Functional anatomy of the mesopontine tegmental projections to the thalamus

Chapter 1: Introduction

Chapter 2: Thalamic activation following PPTg stimulation I: Pilot work

Acknowledgements

I would like to thank my supervisor, Professor Philip Winn, for his help and support throughout and for talking me out of doing the radial arm maze PhD.

I am also extremely grateful to Dr Ian Varndell and Dr Paul Sheppard from AFFINITI research products for their technical and financial assistance.

I would also like to thank Mary Latimer for her help in the lab and constructive advice throughout this PhD. Thanks also to Wendy Taylor, Heather Bowman and Steve Laing for looking after my animals.

Many thanks to Dr Helen Alderson and Dr Trisha Jenkins for endless help in the lab and during the writing-up process. Thanks also to Professor Scott Zahm for teaching me tract tracing techniques.

Thanks to the numerous friends that have helped me get through the whole process and remain reasonably sane. Especially Dr Jenni Birrell, Dr Andy Blackwell, Dr Richard Dyde, Dr Janice Phillips, Jo Hart and Dr John Hammond.

Finally very special thanks to my family. Thanks to my brothers for enabling me to escape St Andrews once in a while, and to my parents for their endless support and not asking me how the writing was going in the last few months.

Abstract

The pedunculopontine tegmental nucleus (PPTg) is a group of neurons in the mesopontine tegmentum traditionally associated with the neural circuits that control sleep and motor function. However, anatomical and functional evidence suggest that the PPTg may have a role to play in higher cognitive processes such as executive function, reward related behaviour and attention. Experimental work in section A of this thesis examined the claim that excitotoxic lesions of the PPTg result in a decreased level of motivation. A simple runway test was used to examine this suggestion and it was found that PPTg lesioned rats were slower to adjust their behaviour in response to a change in the level of reward. In further experiments the underlying psychological causes of the well documented over-consumption of high concentrations of sucrose were studied. This was done by examining the consumption of quinine and saccharin solutions in PPTg lesioned rats. It was found that these rats consumed normal levels of quinine but showed an increased consumption of high concentrations of saccharin compared to sham lesioned animals. These results are discussed in terms of PPTg lesions producing a disruption of executive function and attention.

Section B of the thesis examined the functional anatomy of the projection from the PPTg to the thalamus. This was done by examining fos immunoreactivity in the thalamus following stimulation of the PPTg using a number of different techniques. It was found that glutamate uptake blockade within the PPTg produced clear and consistent changes in activity in some sensorimotor thalamic nuclei and the

thalamic reticular nucleus. These results are discussed with reference to the position of the PPTg in the neural systems controlling high-level cognitive processes.

List of Abbreviations

2-DG – 2-deoxyglucose
aCSF – artificial cerebrospinal fluid
ACh - acetycholine
AChE – acetylcholinesterase
AMPA - α-amino-3-hydroxy-5methyl-4-isoxazolepropionate
ANOVA – analysis of variance
ARAS – ascending reticular activating system
ATP – adenosine triphosphate
AV – anteroventral nucleus
BDA - biotinylated dextran amine
ChAT – choline acetyl transferase
CL – centrolateral nucleus
CM – centromedial nucleus
COase – cytochrome oxidase
CnF – cuneiform nucleus
CP – caudate putamen
CPP – conditioned place preference
CTb – cholera toxin subunit B
DLG - – dorsolateral geniculate nucleus
EEG - Electroencephalogram
EP – entopeduncular nucleus
GABA - gamma-aminobutyric acid
GP – globus pallidus
HRP – horse radish peroxidase
i.p. – intra peritoneal
IVSA – intravenous self-administration
LC – locus coeruleus
LDN – long duration neurons
LDTg – laterodorsal tegmental nucleus
MD – mediodorsal nucleus
MEA – midbrain extrapyramidal area
MG – medial geniculate nucleus
MLR –mesencephalis locomotor region
MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAcc – nucleus accumbens
NADPH – nicotinamide adenine dinucleotide phosphate
NMDA – N-methyl-D-asparate
NO – nitric oxide
PB – phosphate buffer
PD – Parkinson's disease
PDC – L-trans-pyrrolidine 2,4-dicarboxylic acid
PGO-waves – ponto-geniculo-occipital waves
PHA-L - *Phaseolus vulgaris*-leukoagglutinin
PPTg – pedunculopontine tegmental nucleus
PPTg-pc - pedunculopontine tegmental nucleus pars compacta
PPTg-pd - pedunculopontine tegmental nucleus pars dissipatus
Po - posterior nucleus
PR – progressive ratio

PS – paradoxical sleep
REM – rapid eye movement
RN – raphe nucleus
Rt – reticular nucleus
SDN – short duration neuorns
SE – standard error
SN – substantia nigra
SNc – substantia nigra pars compacta
SNr – substantia nigra pars reticulata
SNP – supranuclear palsy
STN – subthalamic nucleus
SWS – slow wave sleep
TH - tyrosine hydroxylase
VAChT – vesicular acetylcholine transporter
VL – ventrolateral nucleus
VM - ventromedial nucleus
VP – ventral pallidum
VPL/VPM - vetroposterior lateral/ ventroposterior medial nuclei
VTA – ventral tegmental area
WGA – wheat germ agglutinin

General Introduction

The pedunculopontine tegmental nucleus (PPTg) is a group of cells found in the mesopontine tegmentum. It is made up of a population of large cholinergic cells interdigitated with a group of smaller and more numerous non-cholinergic cells. Traditionally it has been associated with two particular neural processes. It has been implicated in the control of behavioural state (Leonard & Llinas 1990) and was studied with reference to its role in sleep and the neural mechanisms involved in changes of behavioural state. It has also been studied as a site through which motor information from higher neural structures can output to the brainstem and spinal cord (Garcia-Rill 1991).

In recent years, however, the increased anatomical understanding of the brain and the acceptance of the need to examine structures within the brain as part of integrated neural systems has led researchers to look more closely at the PPTg. Several lines of research, including those examining Parkinson's disease, executive functions such as working memory and attention, and reward related behaviours, have demonstrated the PPTg is involved in the systems controlling high level cognitive functions previously thought to be entirely localised in structures much higher in the neuraxis. This thesis will review work examining the anatomical and functional characteristics of the PPTg. Experimental data looking at specific aspects of the PPTg will then be presented in an attempt to demonstrate how areas low down along the neuraxis are involved in the neural systems concerned with the control of higher order behavioural processes. Before examining the PPTg in terms of its

relationship to other structures, however, a definition of what and where the PPTg is must be presented.

Cytoarchitecture and neuronal identity

The PPTg is a heterogeneous group of cells in the mesopontine tegmentum. While these are not exclusively cholinergic, by far the most striking feature of the PPTg is a group a large cholinergic neurons found throughout the nucleus and forming a densely packed cluster in its caudal section. In the rodent brain there are approximately 1700 of these neurons in each hemisphere (Rye et al., 1987). Immunohistochemical studies using antibodies directed against choline acetyltransferase (ChAT), the enzyme responsible for the biosynthesis of acetylcholine, were carried out in the early 1980s to visualise cholinergic neurons throughout the brain (Kimura et al., 1981; Armstrong et al 1983). Previous studies of the cholinergic system employed methods that suffered from a lack of specificity such as acetylcholinesterase (AChE) histochemistry. However, studies using the ChAT antibody were able to provide information regarding the exact morphology and distribution of cholinergic neurons within the brain. They described the presence of a large cluster of loosely aggregated ChAT immunoreactive neurons within the mesopontine tegmentum which incorporated the PPTg. These neurons are part of a very striking group of cholinergic cells termed the "caudal cholinergic column" by Sato et al. (1983) which also includes the cholinergic cells of the laterodorsal tegmental nucleus (LDTg). More recently studies using antibodies directed against

the vesicular acetylcholine transporter (VAChT) have also demonstrated cholinergic cell groups in this region (Ardvisson et al., 1997; Roghani et al., 1998). These stains complement each other well with the ChAT enzyme, found largely in the soma, being targeted by the ChAT antibody and the VAChT antibody targeting neuronal processes where vesicles are most abundant, thus clearly labelling all aspects of the cholinergic neurons in this area.

The first systematic classification of these neurons was published by Mesulam et al in 1983. In this paper, Mesulam described six groups of neurons which made up the major ascending cholinergic pathways[1]. The cholinergic innervation of the thalamus, as revealed by retrograde tracing combined with ChAT immunohistochemistry and acetylcholinesterase (AChE) histochemistry, was shown to originate in the cholinergic cells of the PPTg (Ch5 neurons) and LDTg (Ch6 neurons). These cholinergic PPTg neurons extend rostrally from the lateral tip of the superior cerebellar peduncle toward the caudal pole of the substantia nigra. The caudal section of the nucleus is densely packed with cholinergic neurons and is thus referred to as the PPTg pars compacta (PPTg-pc, Rye et al., 1987; Lee et al., 1988; Rye et al., 1988; Steininger et al., 1997). The PPTg-pc is bordered caudally by the parabrachial nucleus and dorsally by the cuneiform nucleus in its caudal section, and by the microcellular tegmental nucleus and deep mesencephalic nucleus as the PPTg progresses forward. In the anterior section of the nucleus the cholinergic neurons become more diffuse, such that in the rostral portion of the PPTg, situated ventral to the retrorubral fields, they are well dispersed and consequently this part of the

[1] While only six cholinergic groups were reported in the 1983 article, further work by the same group of researchers reclassified the ascending cholinergic pathways including a further two separate groups (Ch7 and Ch8).

nucleus is referred to as the PPTg pars dissipatas (PPTg-pd). Quantitative neuroanatomical techniques were used by Manaye et al (1999) to demonstrate in the human brain that of the total number of cholinergic cells within the mesopontine tegmentum 30% were in the PPTg-pc and 57% in the PPTg-pd, the remaining making up the LDTg. While the vast majority of the Ch5 neurons are found within the boundaries of the PPTg there are a few ectopic cells found within its surrounding structures including the substantia nigra, cuneiform nucleus and parabrachial nucleus.

The Ch5 neurons are defined in terms of their cholinergic nature but recent studies have shown acetylcholine to be co-localised with other neurotransmitters within these neurons. The neuromodulator nitric oxide (NO) has been shown to be present in both the Ch5 and Ch6 neurons (Vincent et al., 1986; Vincent & Kimura 1992; Datta et al., 1997; Vincent 2000). Staining for NADPH-diaphorase, an enzyme responsible for the synthesis of NO from arginine, is so clear in these neurons that it is now commonly used as a histological tool with which to visualise them. Other neurotransmitters present include the neuropeptide substance P, shown to be present in approximately 15% of PPTg neurons (Vincent et al., 1986) and glutamate (Bevan & Bolam 1995; Inglis & Semba 1996; Parent et al., 1999). In the squirrel monkey glutamate was demonstrated to be co-localised in as many as 40% of the cholinergic Ch5 neurons at the level of the trochlear nucleus using double immunohistochemical labelling for ChAT and glutamate (Lavoie & Parent, 1994). Morphological studies of PPTg neurons in organotypic culture have demonstrated similar findings with all PPTg cultured neurons staining for NADPH and 10% of these cells showing double

labelling with antibodies directed against glutamate (Ichinohe et al, 2000). The role of glutamate in the neurochemistry of the system was further characterised by Inglis & Semba who demonstrated colocalisation of NADPH-diaphorase with various ionotropic glutamate receptor subtypes including AMPA receptor subunits GluR1, GluR2/3 and GluR4, kainate receptor subunits GluR5/6/7 and the NMDA receptor subunit NMDAR1. The proportion of the NADPH-diaphorase positive neurons displaying double labelling with one of the receptor subunits was variable. The most common receptor subunit was the GluR1, which was present in 43% of NADPH-diaphorase positive PPTg neurons while the least common were the GluR 5/6/7 which were only present in 12% of the NADPH-diaphorase positive cells. Clearly glutamatergic inputs to these cholinergic cells are an important feature of their neural activity.

Interdigitated with the Ch5 neurons are a number of smaller and yet more numerous non-cholinergic neurons. The cytoarchitecture and relative densities of these neurons compliments the Ch5 neurons in that they are most numerous in the PPTg-pd where they make up 25-75% of the total number of neurons in that area in humans (Mesulam et al, 1989) and less numerous in the PPTg-pc where they make up only 10% of the total number of neurons. Ultrastructural studies of these neurons have described a number of significant differences between these populations (Spann & Grofova, 1992; Honda & Semba, 1995; Steininger et al., 1997). Spann & Grofova (1992) described three anatomically distinct populations of neurons within the two subdivisions of the PPTg previously defined. The first were the ChAT positive neurons found mostly in the PPTg-pc which correspond to Mesulam's Ch5 neurons.

These neurons were described as having fusiform or polygonal somata that ranged between 20 to 60 μm in length. They were found in both the pars compacta and pars dissipatus of the PPTg but larger polygonal cells were found to be more numerous in the pars dissipatus. The PPTg-pd ChAT positive neurons had long dendritic processes (300-425μm) which were relatively straight and smooth, while the PPTg-pc ChAT positive neurons had slightly shorter dendritic processes measuring approximately 250μm which were more undulated and branched closer to the somata.

The non-cholinergic cells examined in the PPTg-pd were found to be separable into two equally represented populations. The first of these were of a similar size, shape and subcellular composition to ChAT positive neurons, while a second population were smaller with a more invaginated nucleus and thinner layer of cytoplasm. This organisation based on a number of populations of cells with differing characteristics fits well with electrophysiological data which will described later in the chapter.

The non-cholinergic cells described have been the subject of some debate between groups investigating the anatomical and neurochemical nature of the PPTg. Wainer, Rye and colleagues published a number of studies examining the cytoarchitecture, cytochemistry and connectivity of the PPTg (Rye et al., 1987; Lee et al., 1988; Hallanger & Wainer, 1988; Rye et al., 1988; Steininger et al., 1992). They defined the PPTg solely in terms of the Ch5 neurons and went on to suggest that the non-cholinergic neurons directly medial to the Ch5 neurons be classified separately as part of the midbrain extrapyramidal area (MEA). This conclusion is

based principally on the patterns of connectivity of the two sets of neurons. Rye et al (1987) demonstrated that the smaller non-cholinergic MEA neurons receive the majority of the input from the basal ganglia and associated structures that had previously been attributed to the PPTg itself (Nauta, 1979; Jackson & Crossman, 1983). These included the inputs from the globus pallidus, entopeduncular nucleus and substantia nigra.

This classification based on the cholinergic and non-cholinergic neurons of the PPTg being described as anatomically distinct nuclei has been called into question by a number of other researchers. The undisputed fact that the cholinergic and non-cholinergic neurons are intermingled coupled with the presence of Ch5 neurons outside the boundaries of the PPTg led Spann and Grofova to suggest that "..the definition of the PPTg as consisting only of large, cholinergic neurons is hardly tenable. Similarly, the concept of a non-cholinergic MEA did not receive support from anatomical and physiological studies." (Spann & Grofova, 1992. p. 223). In direct support of this Lavoie and Parent (1994) examined the structure of the PPTg in the squirrel monkey and concluded that no equivalent of the MEA could be clearly identified in the mesopontine tegmentum of this animal. In an attempt to reconcile these two arguments Inglis & Winn, in their 1995 review, used the substantia nigra (SN) as a model for how the PPTg may be described. Histological studies demonstrate clearly the SN is made up two distinct populations of cells; the SN pars compacta (SNc) and the SN pars reticulata (SNr). These two populations are anatomically, morphologically and electrophysiologically distinct and yet they are considered to be part of the same structure as the interactions between the two

groups of cells play a key role in the computational processes that occur within the structure.

As emphasised by Inglis & Winn (1995) the cholinergic and non-cholinergic cells of the PPTg are clearly anatomically connected. Ultrastructurally it can be seen that ChAT positive somata receive many synapses from ChAT negative dendritic processes. Similarly ChAT positive axons are seen in direct apposition to somata of unlabelled neurons in the area (Honda & Semba, 1995). This would seem to add weight to the suggestion that a classification based on two functionally and anatomically distinct sets of cells, as suggested by Rye and his colleagues, is untenable. On closer examination of the literature it is clear that the precise neurochemical identity of these non-cholinergic cells is poorly understood. Surprisingly while a number of studies have identified a population of non-cholinergic cells in this area and even studied their sub-cellular composition (Rye et al., 1988; Spann & Grofova, 1992; Lavoie & Parent, 1994; Honda & Semba, 1995; Takakusaki et al., 1996; Steininger et al., 1997) and structure their neurochemical properties remain relatively poorly defined. As described earlier studies examining the Ch5 neurons have shown glutamate to be co-localised in at least some of these cholinergic cells and these studies have also reported non-cholinergic neurons that are glutamate immunoreactive (Clements & Grant, 1990; Lavoie & Parent, 1994). Evidence for the presence of neurotransmitters other than glutamate in these non-cholinergic cells is less clear. Some researchers have suggested that at least some of these neurons are GABAergic (Jones, 1991; Ford et al., 1995; Charara et al., 1996; Winn, 1998; Torterolo et al., 2001), while others reported a complete lack of staining

for GABA in the PPTg (Lavoie & Parent, 1994). Other studies have reported the existence of the peptide neurotransmitter galanin (Gai et al., 1993) and the amino acid neurotransmitter glycine (Mineff et al., 1998). What is clear is that further studies are needed to allow a meaningful classification of these non-cholinergic neurons to be made.

These studies demonstrate that while the PPTg is clearly a heterogeneous structure the neurons in this area are closely anatomically connected. If researchers look beyond the initial obvious distinction between the cholinergic and non-cholinergic cells and examine the levels of interdigitation and synaptic connectivity between these neuronal populations it seems more plausible to suggest that these neurons be classified together as part of the same structure. Consequently for the purpose of this thesis the term PPTg will refer to a combination of the cholinergic Ch5 neurons and the interdigitated non-cholinergic neurons including at least some of those described by Rye et al as the MEA. The terms PPTg-pc and PPTg-pd are still useful anatomical terms when describing the structure and thus will be retained and used when appropriate. These neuroanatomical data are complimented nicely by electrophysiological investigations of the PPTg examining the response profiles of PPTg neurons.

Electrophysiology

The electrophysiological properties of PPTg neurons have been examined in a number of studies, in particular those attempting to understand the role of the PPTg

in control of behavioural state, and especially REM sleep (Leonard & Llinas, 1990; Kang & Kitai, 1990; Takakusaki et al., 1997). *In vivo* studies examining neuronal firing patterns in the mesopontine tegmentum of the cat have described a number of different classes of cells corresponding to different stages of sleep (for review see Leonard & Llinas, 1990; Datta, 1995). These included PGO-burst neurons defined by their bursts of three to five action potentials that show a close temporal relationship to ponto-geniculo-occipital (PGO) waves, the major phasic event of REM sleep. Other subpopulations of neurons associated with the start and finish of REM or paradoxical sleep have been termed PS-on and PS-off neurons.

In order to gain a fuller understanding of the intrinsic electrophysiological properties of PPTg neurons Leonard & Llinas (1990) recorded from brain slice preparations of the dorsolateral pontine reticular formation. Using intracellular recording techniques they identified three types of neurons on the basis of their reaction to direct current injection. Type I neurons made up about 10% of the recorded cells and were found in both the PPTg and LDTg. These cells were characterised by repetitive firing following the direct application of depolarising current and on cessation of the hyperpolarizing current a prominent overshoot of membrane potential sufficient to elicit a subsequent burst of action potentials. This type of responding is similar to that produced by low threshold calcium spikes (LTS) and demonstrates that these cells have phasic, burst firing patterns. Type II neurons also produced repetitive firing on application of depolarising current but unlike type I neurons on cessation of hyperpolarizing current displayed a delay in returning to baseline potential rather than rebound excitation. These neurons made up the

majority of the recorded cells and were characterised by this prominent transient outward current (similar to A-type potassium conductance)[2]. Type III neurons were similar to type II neurons in that they showed the same pattern of A-type conductance. When the hyperpolarizing current was increased to examine the A-type conductance, however, a rebound excitation was reported. This underlying LTS response pattern, similar to type I neurons, combined with the A-type conductance characterised the type III neurons as being capable of both tonic and phasic responding.

Similar results to these were found using electrical stimulation from the SNr. Kang and Kitai (1990) used intracellular recording to examine PPTg activity following SNr stimulation and found three types of neuronal firing pattern. The first were characterised by the LTS response reported by Leonard and Llinas in their type I cells. The second type corresponded to a combination of types II and III in the Leonard and Llinas account with all cells showing the A-type conductance in this group, while the third type of cell response was characterised by a lack of either the LTS pattern or the A-type conductance. This type of neuron was not described by Leonard and Llinas.

The electrophysiological classification was complimented in these studies by immunohistochemical and tracing methods used to identify the morphology of the cells recorded from. Leonard and Llinas used injections of the retrograde tracing agent rhodamine into the thalamus combined with injections of the fluorescent dye Lucifer yellow into the electrophysiologically defined cell groups to elucidate the

[2] This pattern of firing suggests that these cells are tonically active as compared to the phasic nature of the type I cells.

patterns of connectivity of these neurons. Type I neurons did not project to the thalamus and were found to have small ovoid somas giving rise to three or four primary dendrites. Types II and III were both labelled following retrograde tracer injections in the thalamus but could not be distinguished from each other by their morphology and were found to have large fusiform somas giving rise to five to nine dendrites. Neurochemical characteristics were studied by examining colocalisation of Lucifer yellow in electrophysiologically defined cell groups with NADPH-diaphorase staining. The results from this combined with previous reports of PPTg cell morphology suggested that the type I cells correspond to the small non-cholinergic neurons of the PPTg, while types II and III correspond to the large cholinergic neurons. Kang and Kitai (1990) used ChAT immunohistochemistry and intracellular biocytin labelling to examine the morphological characteristics of their PPTg cell groups. They confirmed previous findings by reporting the type I neurons characterised by the LTS responding as being smaller with fewer dendrites than the type II and III neurons. ChAT positive staining was demonstrated in approximately 50% of the type II neurons but was completely absent from type I and III neurons.

More recently Takakusaki et al (1997) have defined two electrophysiologically distinct populations of cholinergic neurons within the PPTg. The type II neurons described by Kang & Kitai (1990) have been further divided into two separate populations based on the length of the spikes recorded from these cells. One set of cells found throughout the PPTg have short duration spikes of 0.7-1.5ms and are thus referred to as short duration neurons (SDN). While another group of cells found predominately in the PPTg-pc have long duration spikes of between 1.6-

2.9ms and consequently are referred to as long duration neurons (LDN). These data fit nicely with morphological characterisation of the cholinergic neurons described by Spann & Grofova (1992) who showed that cholinergic neurons of the PPTg-pc and PPTg-pd had differing ultrastructural properties. However it remains unclear as to whether these two cholinergic subpopulations are analogous to the type II and III neurons described by Leonard & Llinas.

Taken together the morphological and physiological descriptions of the PPTg provide a relatively coherent account of the structure in terms of four interdigitated subpopulations of neurons. This is summarised in Table 1. The cholinergic neurons can be split into two groups; the SDN, which correspond to the cholinergic neurons found throughout the PPTg, and the LDN which correspond to morphologically distinct cholinergic neurons of the PPTg-pc (Spann & Grofova, 1992). This could fit nicely with Leonard and Llinas' account as these subpopulations probably correspond to their type II and III groups, although this is purely speculative as the position of the Type II and III neurons within the PPTg were not reported. The non-cholinergic neurons are also subdivided into two groups. The larger non-cholinergic cells that are morphologically similar to the Ch5 neurons (Spann & Grofova, 1992) would appear to correspond to the type III cells of Kang & Kitai. One possible explanation for the failure of Leonard & Llinas to report neurons with these characteristics could be that they recorded solely from the PPTg-pc where the large non-cholinergic neurons are very scarce. This is consistent with the fact that only 10% of the cells recorded from were non-cholinergic. Had they recorded throughout the PPTg the proportion of non-cholinergic cells reported would be expected to be

higher. The smaller non-cholinergic cells correspond to the type I neurons described by both Leonard & Llinas (1990) and Kang and Kitai (1990).

Table 1: Classification of PPTg neurons by morphology and electrophysiology

	Electrophysiology			Morphology
Population	Leonard & Llinas (1990)	Kang & Kitai (1990)	Takakusaki et al (1997)	Spann & Grofova (1992)
1	Type I (LTS)	Type I (LTS)	-	Non-cholinergic (small)
2	Type II (A-conductance)	Type II (A-conductance)	SDN	Cholinergic (throughout PPTg)
3	Type III (LTS & A-conductance)	Type II (A-conductance)	LDN	Cholinergic (PPTg-pc)
4	-	Type III	-	Non-cholinergic (large)

Abreviations: LTS – Low threshold calcium spikes, SDN – short duration neurons, LDN – long duration neurons

While these studies have provided very useful information regarding neuronal firing patterns of the PPTg they fail to address the question of how neurons within this nucleus respond in awake behaving animals. This question has been asked in two studies using different behavioural paradigms. Matsumura et al. (1997) examined single unit activity in primates during voluntary arm movements to assess the role of the PPTg in motor behaviour. It was demonstrated that around half of the neurons in the PPTg fired during voluntary arm movements with 53% of PPTg neurons fired during a contralateral arm movement and 48% fired during an ipsilateral arm movement. However, the distinct categories identified in the above studies were not seen. The authors suggested that this failure to find the patterns of

neuronal firing reported previously might be due to either comparative differences of PPTg neurons between species or methodological problems. These results were interpreted in terms of the role of the PPTg in motor functioning which will be discussed later in the chapter.

In a separate and perhaps more enlightening study Dormont et al. (1998) examined the electrophysiological properties of the PPTg neurons in the cat in a reaction time task where animals were trained to press and release and lever in order to gain a reward. Cells recorded were split into two categories based on the duration of their spikes. Brief spike neurons had a mean duration of 0.7ms while broad spike neurons had a mean of duration 2ms. On closer examination of the firing patterns it was reported that the majority of the brief spike neurons showed very early excitation after the stimulus, with a mean onset activation latency of 8.6ms, signalling to the animal that it must make a response (in this case releasing the lever and collecting a reward). It was also demonstrated that the vast majority (19/20) of the broad spike neurons fired in response to reinforcement related activity. The authors suggested that the broad spike neurons might correspond to the Ch5 neurons of the PPTg while the brief spike neurons may be the non-cholinergic neurons of the nucleus. While this is intuitively attractive, especially in light of suggestions that the Ch5 neurons may be involved in reward related behaviour (see section A for detailed analysis of this claim), it does not fit entirely with other findings. Specifically, in their 1997 study Takakusaki et al. reported the presence of both cholinergic and non-cholinergic brief and broad spike neurons. It is clear that more studies are needed incorporating recording from awake animals combined with immunohistochemical

labelling to allow the exact electrophysiological properties of the different subpopulations of neurons within the PPTg to be defined. However, the demonstration that PPTg neurons fire in direct response to reward and in response to conditioned stimuli point towards it being involved in high level cognitive functions. This will be discussed in section A of this thesis.

Having looked at what the PPTg has previously been described as and formed a reasonable definition of it in terms of cytoarchitecture, neurochemical and neurophysiological make-up it is important to examine the patterns of connectivity between the PPTg and other structures of the brain. This will help elucidate the functional characteristics of the structure by describing which neuronal circuits the PPTg is part of.

Anatomical Connections

The PPTg has extensive ascending and descending connections with a variety of structures and neural systems. The main categories of efferent and afferent connections are summarised in Figures 1 and 2.

Efferent connections

Structures that receive innervation from the PPTg can be split into four main categories. The first of these, as mentioned previously in the chapter, is the thalamus which is the principle structure receiving innervation from the cholinergic neurons of

Figure 1: Efferent connections of the PPTg

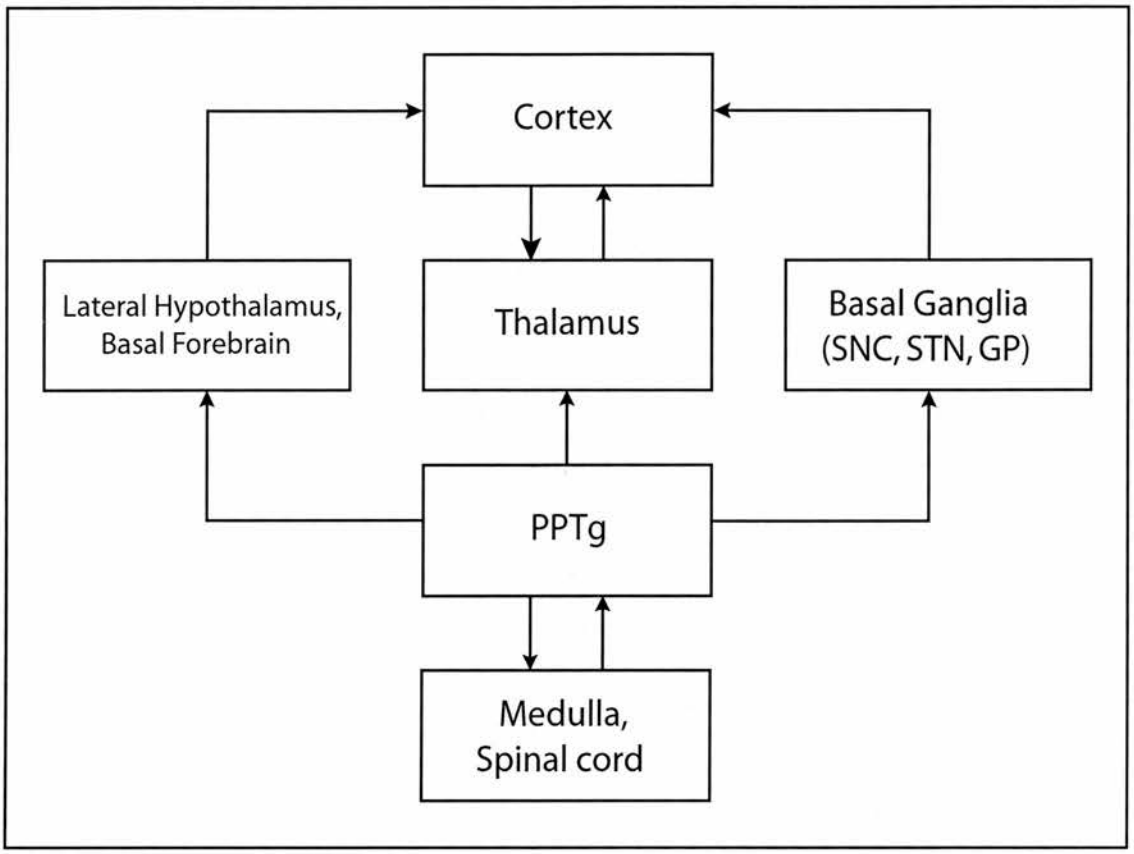
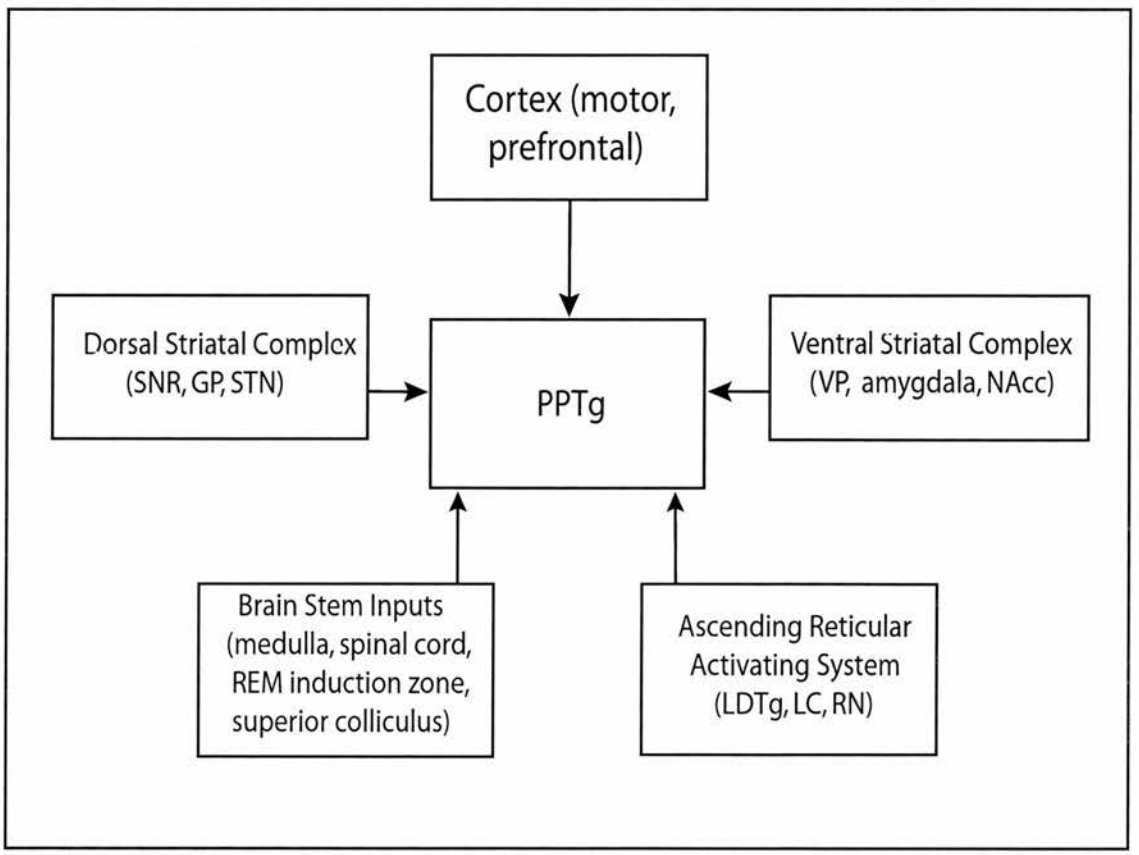


Figure 2: Afferent Connections of the PPTg



the PPTg. The Mesulam et al., (1983) article, in which the nomenclature for classifying ascending cholinergic pathways in the brain was set out, classified the Ch5 neurons of the PPTg and the Ch6 neurons of the LDTg on the basis of their efferent connections to the thalamus.

This study used injections of the tracing agent horseradish peroxidase conjugated covalently to wheat germ agglutinin (HRP-WGA) into the thalamus combined with AChE histochemistry[3] to examine the structures providing cholinergic innervation to the thalamus. It was shown that following injections in the thalamus the vast majority of retrogradely labelled cells that also stained well for AChE were found in the PPTg and LDTg. Unfortunately while this study provided the first real evidence of the origins of the cholinergic input to the thalamus the injections used large quantities of tracer resulting in an average injection site diameter of up to 2mm. Injections were reported as involving several different thalamic nuclei including mostly the anterior, lateral and reticular nuclei. Thus conclusions regarding the efferent connections of the PPTg to specific thalamic nuclei were unavailable. These findings were verified using HRP tracing and ChAT immunohistochemistry by Sofroniew et al (1985). However, once again the patterns of staining allowed only limited conclusions to be drawn due to the relatively large size of the injection sites reported.

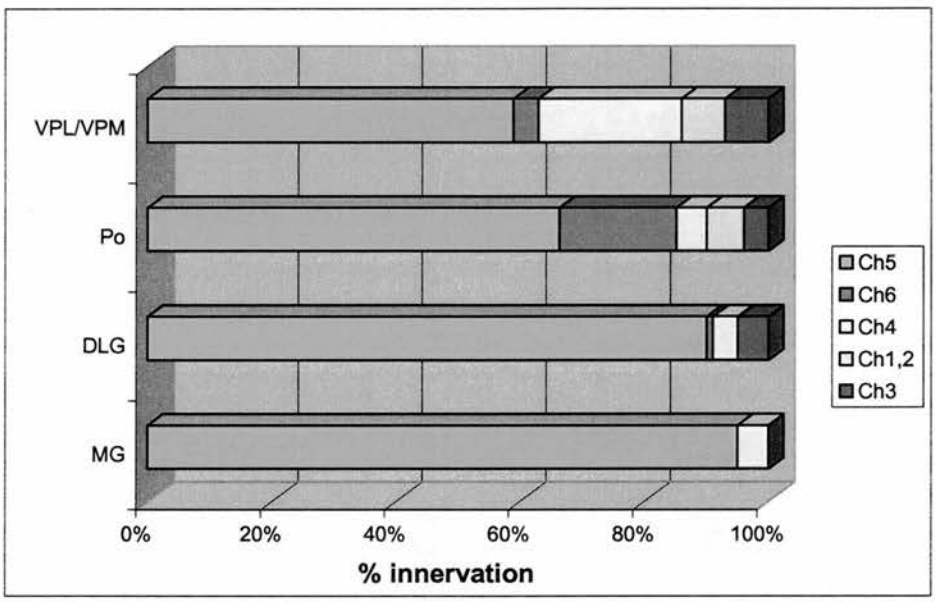
Following on from these studies Hallanger et al (1987) used the same methodologies, but with much smaller quantities of tracer to examine the patterns of

[3] Although AChE was used as a cholinergic marker in this study, later research revealed that it was not specific to cholinergic neurons. However, the general picture presented in this study regarding cholinergic innervation of the thalamus has since been verified using more specific histological tools.

retrograde tracing from specific thalamic nuclei. In total nine thalamic sites were chosen including examples of sensory, motor, association and intralaminar nuclei. Retrograde labelling was reported in the PPTg following injections in all thalamic sites. Looking more specifically at the pattern of innervation form the PPTg to the various thalamic nuclei it is clear that the proportion of the total input to each nucleus originating in the PPTg varies across the sites chosen. These patterns are demonstrated in the following Figures adapted from Inglis & Winn (1995) that illustrate the percentage of the total cholinergic innervation of the thalamus that arises from the ascending cholinergic pathways. The figures use the nomenclature set out by Mesulam et al., (1983) in which the major ascending cholinergic pathways were described. Briefly the Ch1 and Ch2 neurons are found in the medial septal nucleus and the vertical limb nucleus of the diagonal band respectively, the Ch3 neurons are contained within the lateral portion of the horizontal limb nucleus of the diagonal band. The Ch4 neurons are mainly found in the nucleus basalis of Meynert, while the Ch5 and Ch6 neurons are largely contained within the mesopontine tegmentum as previously described. The graphs are based on data collected by Hallanger et al (1988) in a study examining the efferent connections of the PPTg.

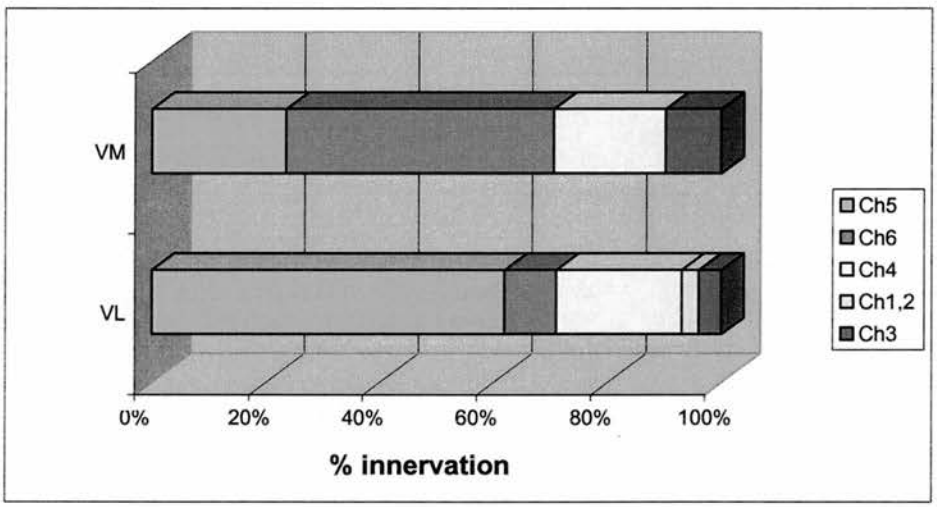
Figure 3 shows clearly that the vast majority of the cholinergic input to the sensory nuclei examined arises in the PPTg with the other cholinergic cell groups making relatively small innervations.

Figure 3: Cholinergic innervation of the sensory thalamic nuclei



Abbreviations: VPL/VPM – vetroposterior lateral/ ventroposterior medial nuclei, Po – posterior nucleus, DLG – dorsolateral geniculate nucleus, MG – medial geniculate nucleus.

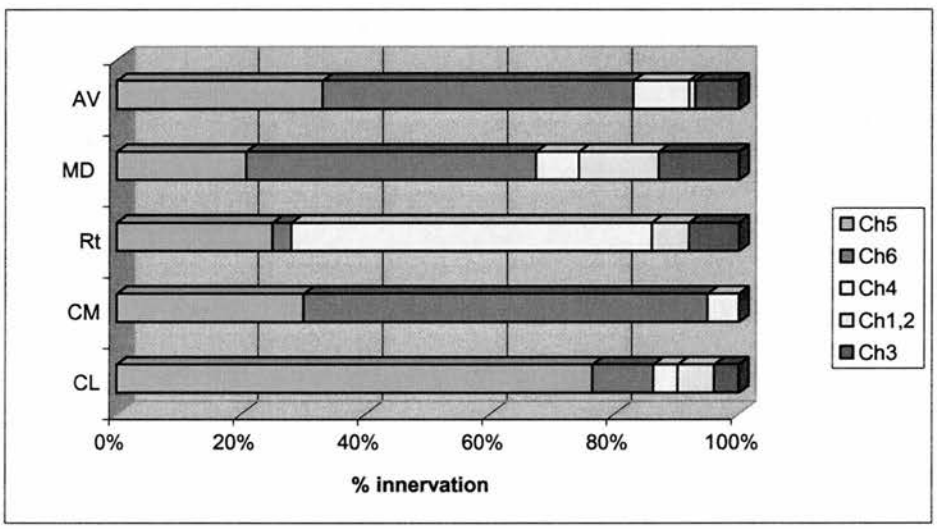
Figure 4: cholinergic innervation of the motor thalamic nuclei



Abbreviations: VM - ventromedial nucleus, VL – ventrolateral nucleus

The pattern of innervation to the motor thalamic nuclei, however, is not as clear. Figure 4 demonstrates that the PPTg still provides a large amount of the cholinergic input, especially to the ventrolateral nucleus (VL), but other substantial cholinergic inputs are also apparent particularly those from the LDTg.

Figure 5: cholinergic innervation of the intralaminar, association and reticular nuclei



Abbreviations: AV – anteroventral nucleus, MD – mediodorsal nucleus, Rt – reticular nucleus, CM – centromedial nucleus, CL – centrolateral nucleus

Input to the remaining thalamic injection sites was equally as varied. In general the association nuclei sampled (anteroventral and mediodorsal) received a relatively small proportion of their input from the PPTg with only 15-30% of the cholinergic innervation originating there. The reticular nucleus received 25% of its innervation from the PPTg while the two intralaminar nuclei examined had vastly differing patterns of cholinergic input. The centrolateral nucleus received 78% of its cholinergic input from the PPTg in contrast to the centromedial that received only

30%. While it is clear that PPTg does not provide a uniform pattern of innervation to the thalamus, the important observation from this study remains that the PPTg innervates all of the thalamic nuclei sampled providing an anatomical substrate for possible mechanisms by which it may influence thalamic activity. More specifically the PPTg appears to send strong innervation to the sensory nuclei as well as some of the motor and intralaminar nuclei while the LDTg has a greater input to the association or limbic nuclei.

These results fit neatly with a further study by Hallanger & Wainer (1988). This study looked at the same anatomical connections from the opposite viewpoint by injecting the anterograde tracer *Phaseolus vulgaris*-leukoagglutinin (PHA-L) into the PPTg. In all cases where injections were centred in the PPTg-pc three sets of axons were reported ascending into the forebrain. The thalamic targets of these axons were very much what would have been predicted from the retrograde tracing study. The sensory thalamic nuclei including the DLG, VLG, Po and VPL/VPM complex all received input as well as the motor nuclei (VL, VM), the reticular nucleus and a number of intralaminar nuclei including the CL.

This cholinergic innervation of the thalamus from PPTg has been widely studied since these articles were published as interest in the role of the PPTg in the thalamocortical and basal ganglia function has increased. Specifically studies have reported projections to the ventral lateral geniculate nucleus (Kolmac & Mitrofanis, 2000), reticular nucleus (Kolmac & Mitrofanis, 1998), lateralis medialis-suprageniculate nucleus (Hoshino et al., 2000) and the ventrobasal complex (Usonoff et al., 1999). Many other studies have reported the existence of collateral projections

from the PPTg to a number of forebrain sites within and outside the thalamus (Shiromani et al., 1990; Losier & Semba, 1993; Spreafico et al., 1993; Oakman et al., 1999). These studies will be discussed in greater detail later in the chapter when the position of the PPTg in the cortico-thalamo-striatal circuitry is discussed.

Moving on from the thalamus the other main target of ascending projections from the PPTg is the basal ganglia and associated structures. The most well studied of these is the projection to the substantia nigra. (Jackson & Crossman, 1983; Rye et al., 1987; Beninato & Spencer, 1987; Gould et al., 1989; Bolam et al., 1991; Lavoie & Parent, 1994a; Lavoie & Parent, 1994b; Blaha & Winn, 1993; Futami et al., 1995; Oakman et al., 1995; Charara et al., 1996; Oakman et al., 1999; Parent et al., 1999). Initial studies used tracing injections in the SN combined with ChAT immunohistochemistry to examine cholinergic inputs to the structure. Beninato & Spencer (1987) used large injections of HRP-WGA into various parts of the SN, all injections affecting both the pars compacta (SNc) and the pars reticulata (SNr) subdivisions of the structure. Neurons displaying double labelling for ChAT and HRP were observed throughout the PPTg with a predominance in the rostral portion (PPTg-pd). Interestingly a population of ChAT immunonegative neurons containing HRP was observed in the PPTg-pd, thus demonstrating both cholinergic and non-cholinergic PPTg projections.

Gould et al (1989) expanded on these findings using separate smaller injections of fluorescent tracing agents into the two subdivisions of the SN combined with ChAT immunohistochemistry. In these studies it was demonstrated that injection into the SNc produced 2-3 times the number of double labelled neurons in

the PPTg than similar injections into the SNr. However, relative distribution of cholinergic cells within the PPTg and presence of fluorescent labelled ChAT immunonegative neurons were not reported. In a closer examination of the cholinergic input to the SN, Bolam et al (1991) used a double immunohistochemical study combining ChAT and tyrosine hydroxylase (TH) staining to examine the interaction of the cholinergic innervation with the dopaminergic SN neurons. The cholinergic axons were reported to be closely linked with TH positive dendrites giving rise to as many as 10 boutons closely apposed to a single TH positive dendrite. Although the origins of the cholinergic axons were not explored in this study it was suggested that previous research in this area would implicate the PPTg as the likely source. More recently these findings were corroborated by Lavoie & Parent (1994) who used the more sensitive anterograde tracing agent PHA-L combined with autoradiographic techniques to examine the pathway in the squirrel monkey. Injection into the central portion of the PPTg produced strong autoradiographic and PHA-L staining in the SNc with weaker staining in the SNr. More rostral injections produced weaker staining in both SN subdivisions. Moreover it was shown that PPTg axons establish close pericellular contacts with the soma and proximal dendrites of the dopaminergic neurons of the SNc suggesting that the cholinergic innervation from PPTg exerts strong influence over the nigrostiatal dopamine system.

In a subsequent study Lavoie & Parent (1994) went on to describe in detail the neurochemistry of this innervation in the squirrel monkey. It was shown that only 25% of retrogradely labelled cells were cholinergic. The distribution of the

cholinergic cells followed the gradient within the PPTg with the caudal, middle and rostral thirds containing 15%, 25% and 35% of the double labelled cells respectively. It was also demonstrated that glutamate played a significant part in the pedunculonigral projection. Although no quantitative data were reported glutamate was shown to make up a significant part of the non-cholinergic projection as well as being co-localised within some of the cholinergic neurons. Charara et al (1996) went on to further characterise the neurochemistry of the pathway and quantify the levels of the different neurotransmitters present. They described a similar pattern of anterograde tracing with the densest labelling found in the SNc and went on to demonstrate that 40% of this innervation was glutamatergic and 30-40% were GABAergic. This glutamatergic component of the pathway has been confirmed in a number of other studies thus demonstrating that ACh, while obviously an important part of the pathway, is by no means the only neurotransmitter involved (Parent et al 1999, Futami et al 1995). These data give a clear indication that the glutamatergic component of the PPTg is a key constituent of the pedunculonigral innervation. This adds further weight to the description of the PPTg as a heterogeneous group of cells made up of subpopulations of neurons, containing a number of neurotransmitters acting as a functional unit. The idea of a purely cholinergic PPTg as described by Rye et al is not consistent with these studies showing frequent occurrences of multiple neurotransmitters in common efferent pathways.

The PPTg also has efferent connections with other areas of the basal ganglia including the subthalamic nucleus (STN), globus pallidus (GP), ventral tegmental area (VTA) and entopeduncular nucleus (EP). Subthalamic projections have been

demonstrated using HRP-WGA injections (Hammond et al, 1983) although the relative size of the projection was not reported. More recently the connection was confirmed using the more sensitive tracing agents PHA-L and BDA in both the rat (Bevan & Bolam, 1995; Erro et al., 1999) and the squirrel monkey (Lavoie & Parent, 1994). PHA-L fibres originating in the central portion of the PPTg were reported entering the STN at its medial tip and arborizing profusely throughout the nucleus in the squirrel monkey. Bevan & Bolam (1995) examined the origins and neurochemistry of the innervation in the rat using a variety of tracing and immunohistochemical methods. They identified three populations of mesopontine tegmental neurons projecting to the STN including the non-cholinergic neurons described by Rye et al as the MEA, the cholinergic Ch5 neurons and a smaller projection form the Ch6 neurons of the LDTg. Examination of the amino acid content of the projection revealed that separate populations of glutamate rich and GABA rich neurons. Double labelling studies demonstrated that cholinergic neurons showed very little GABA immunoreactivity, which is consistent with previous studies of mesopontine GABAergic content (Jones, 1993). Unfortunately the fixation methods used prevented the direct examination of glutamate and ChAT co-localisation but previous studies examining the mesopontine cholinergic efferent connections suggest that glutamate enrichment of the cholinergic neurons is likely (Lavoie & Parent, 1994). This problem was addressed in a later study where glutamate enrichment of the cholinergic projection to the STN was clearly demonstrated (Clarke et al., 1997).

Recent studies have demonstrated similar patterns of efferent connection from the PPTg to the entopeduncular nucleus (the rat homologue of the internal section of the globus pallidus) in the squirrel monkey (Lavoie & Parent, 1994; Charara & Parent, 1994) and the rat (Clarke et al., 1996; Clarke et al., 1997; Erro et al., 1999). Once again tracing studies have shown the origin of the mesopontine innervation to be in the Ch5 neurons and the non-cholinergic neurons directly medial to them. The projection definitely contains a glutamatergic component with the origin of the projecting neurons suggesting that at least some of them are also cholinergic. While the innervation to the EP is not as strong as the innervation of the SN and STN it does appear to share the heterogeneous nature of these stronger ascending pathways. Direct innervation of the external segment of the globus pallidus has also been demonstrated in the squirrel monkey (Lavoie & Parent, 1994) with labelled neurons reported in the ventral third of the structure. The other target of PPTg outflow in the basal ganglia is the VTA. This receives a small innervation from the caudal part of the PPTg but the main cholinergic input from the mesopontine tegmentum comes from the Ch6 neurons of the LDTg (Oakman et al., 1995). Other ascending projections from the PPTg include those to the basal forebrain (Jones & Cuello, 1989; Losier & Semba, 1993), lateral hypothalamus (Woolf & Butcher, 1986, Hallanger & Wainer, 1988, Ford et al., 1995), superior colliculus (Krauthamer et al., 1995) and the zona incerta (Kolmac et al., 1998).

The PPTg also has efferent connections with areas lower down the neuraxis in the brainstem and spinal cord. These pathways have been of particular significance to researchers examining the role of the PPTg as a possible output

station for motor information from the cortex and midbrain to areas in the brainstem and spinal cord as well as those examining the neural systems underlying sleep. Systematic reviews of descending cholinergic and non-cholinergic pathways from the mesopontine tegmentum have been carried out to examine these connections. David Rye and colleagues (1988) used anterograde autoradiographic techniques combined with retrograde HRP tracing and ChAT immunohistochemistry to identify the patterns of descending cholinergic pathways. One important aspect of this study is the definition of the PPTg used by Rye et al. In keeping with previous reports from the same group this study defines the PPTg purely in terms of the Ch5 neurons, choosing to classify the non-cholinergic neurons as part of the MEA.

Anterograde studies used injections of large quantities of autoradiographic tracers into various parts of the dorsolateral pontine tegmentum, each injection thus affecting a number of the surrounding nuclei. Injections including the PPTg produced staining in the ventromedial branch of Probst tract which diverged from the main body of the tract at the pontomedullary junction and coursed through the gigantocellular field. While this study provides useful information regarding the general pattern of the descending pathways the injection sites were too large to make specific conclusions about PPTg output sites. The retrograde studies using smaller injections of HRP demonstrated retrograde staining in the PPTg originating primarily in the medullary reticular formation with lighter staining being reported following injections in the nucleus of the solitary tract. One interesting point pertains to the definition of the PPTg used in this study. It was reported that mesopontine projections to spinal cord, specifically the upper thoracic region, originated almost

exclusively in the MEA going on to describe a number of non-cholinergic cells admixed with the Ch5 also providing outflow to the spinal cord. The definition of the PPTg used in this thesis includes these non-cholinergic cells of the MEA and consequently these projections to spinal cord can be classified as originating in the non-cholinergic cells of the PPTg.

Woolf & Butcher (1989) used retrograde tracing injections in a variety of brainstem and spinal cord locations to examine descending cholinergic projections. Neurons in the mesopontine tegmentum were found to be retrogradely labelled following tracer injections into the majority of hindbrain sites. More specifically PPTg neurons were found to project to; the motor nuclei of the cranial nerves 5, 7 and 12; the dorsal raphe, median raphe and raphe magnus; oral pontine, caudal pontine and medullary reticular nuclei of the reticular formation; locus coeruleus and the deep cerebellar nuclei. Other studies have reported similar patterns of descending projection to the medulla and spinal cord (Semba et al., 1990; Yasui et al., 1990; Spann & Grofova, 1991; Ruggiero et al., 1997; Lai et al., 1999). A separate set of descending projections from the PPTg target the REM sleep induction zone described by Semba (1993) with 30% of the total input to this area originating in the PPTg. These descending projections will be discussed later in this chapter.

Afferent connections

The PPTg receives extensive afferent connections from various parts of the brain and these are summarised in Figure 2. The descending inputs can be divided

into three groups: those from the dorsal striatal complex, ventral striatal complex and cortical systems. The dorsal striatal complex comprises the subcortical structures concerned with motor control including the caudate putamen (CP), substantia nigra, subthalamic nucleus and globus pallidus (GP). Direct inputs from the CP have not been widely reported although one recent study has shown inputs from the ventrolateral portion of the nucleus (Zahm et al., 2001). Despite the relatively small number of studies demonstrating a direct innervation there has been a substantial amount of research demonstrating innervation from the outflow sites of the CP. The strong innervation of the PPTg from the SN, described earlier, has lead a number of researchers to examine the reciprocal pathway from SN to PPTg (Edley & Graybiel, 1983; Rye et al., 1987; Kang & Kitai, 1990; Spann & Grofova, 1991; Semba & Fibiger, 1992; Steininger et al., 1992; Grofova & Zhou, 1998). Rye et al (1987) used HRP-WGA tracing and ChAT immunohistochemistry to examine inputs to the PPTg from the basal ganglia. As described earlier, the patterns of connectivity between these structures were used by these authors to suggest that the Ch5 neurons and interdigitated non-cholinergic neurons be described separately as the PPTg and MEA respectively. Specifically, it was demonstrated that the majority of the innervation from the SN and entopeduncular nucleus terminated in the MEA with very few of the cholinergic cells expressing retrograde labelling. Clearly this differs from the definition of the PPTg used in this thesis but as previously stated the levels of co-localisation of neurotransmitters combined with the obvious interconnectivity of these two sets of neurons is not consistent with a definition in terms of two distinct neuronal populations. If the PPTg is defined as a heterogeneous structure it is clear

that the nigral innervation of the mesopontine tegmentum terminates in the non-cholinergic neurons of the PPTg.

This was confirmed by Spann & Grofova (1991) who used PHA-L injections in the SNr to identify the pattern of nigralpedunculopontine projections. The densest area of labelled fibres was seen in the medial half of the PPTg-pd. Using smaller quantities of tracing agent a degree of topographical organisation of the nigral input was also demonstrated and this will be discussed later in the chapter. This pathway was further characterised by Grofova & Zhou (1998) who used PHA-L and ChAT methods to examine its ultrastructural properties. It was demonstrated that the majority of the nigral innervation is centred on the non-cholinergic cells of the PPTg-pd although some labelled cells in the PPTg-pc were seen. Interestingly 12% of the PHA-L labelled descending nigral cells to the PPTg-pd made synaptic connections with cholinergic cells. This provides further support for the definition of the PPTg used in this thesis by countering the suggestion that the nigral innervation is centred solely on non-cholinergic cells. At an ultrastructural level the subdivisions of the nucleus were still distinguishable with 34% of labelled terminals in the PPTg-pd synapsed to cell bodies and only 15% of labelled terminals within the PPTg-pc impinged on perikarya. The vast majority of perikarya receiving PHA-L labelled terminals from SNr were non-cholinergic with both types of non-cholinergic cell described by Spann & Grofova (1991, see Table 2) identified as targets of this descending projection. This study went on to examine the neurochemistry of the PPTg cells receiving nigral innervation and reported that while a small subpopulation

of cholinergic cells received input the majority of the descending axons targeted glutamatergic neurons.

Other descending innervation from the dorsal striatal complex include those from the globus pallidus (GP) and entopeduncular nucleus (EP), the rat homologue of the internal segment of the GP (Moriizumi & Hattori, 1992; Semba & Fibiger, 1992; Steininger et al., 1992; Shammah-Lagnado et al., 1996; Shink et al., 1997). Innervation from the globus pallidus was found to originate in a population of neurons in the caudal third of the structure using retrograde fluorogold labelling (Moriizumi & Hattori, 1992). While this projection is clearly not as substantial as that from the SNr the existence of the pathway has been confirmed in a number of other studies (Steininger et al., 1992; Shammah-Lagnado et al., 1996) using both anterograde and retrograde tracing. Steininger et al (1992) described the projection from the GP and EP as targeting the MEA, but as explained earlier in this section this can be seen as being, at least in part, analogous to the non-cholinergic neurons of the PPTg-pd. This pathway was further characterised by Shink et al (1997) in a systematic study of the PPTg projecting neurons of the internal segment of the GP in the squirrel monkey. This study used a series of injections of biotinylated dextran amine (BDA) in different parts of the internal GP to see if the topographical organisation of the GP was reproduced at the level of the PPTg. The results showed that all the injections in the GP produced anterograde labelling in the PPTg with the densest staining occurring in the middle third of the nucleus. It was also demonstrated using double labelling studies involving NADPH-diaphorase that the projections from GP specifically targeted the non-cholinergic neurons of the area.

The level of topography will be discussed later but briefly it was demonstrated that the regions of the PPTg innervated by the different parts of the internal GP overlapped to some degree.

The only other structure associated with the basal ganglia to provide a significant innervation of the PPTg is the subthalamic nucleus (STN). A number of studies have reported a projection from STN to the PPTg using retrograde tracing but the projection is clearly not as strong as those from the SN and GP (Hammond et al., 1983; Moriizumi & Hattori, 1992; Semba & Fibiger, 1992). In a study examining the anatomical connections of the Ch5 neurons and the MEA Steininger et al (1992) reported strong retrograde labelling in the STN following injections in sites including the MEA. This, however, is not a universal finding as other reports of retrograde tracing from the PPTg have described much smaller projections from the STN (Semba & Fibiger, 1992)

Descending innervation from the ventral striatal complex originates in the limbic structures involved in reward related behaviours including the nucleus accumbens and the ventral pallidum / substantia innominata (VP; Heimer et al., 1991; Semba & Fibiger, 1992; Groenewegen et al., 1993). Direct innervation from the accumbens has been reported from both the core and shell (Groenewegen et al., 1993). Specifically injections of the retrograde tracer cholera toxin subunit B (CTb) in the caudal mesencephalon that included the PPTg produced labelled cells in the medial part of the core and, more caudally, the ventromedial part of the shell. These direct projections are, however, relatively sparse. Stronger projections are reported from the primary output site of the accumbens, namely the ventral

pallidum/substantia innominata. CTb labelled neurons were found to be numerous throughout the rostrocaudal extent of the VP with the exeception of the most dorsal part of the subcomissural VP (Semba & Fibiger, 1992; Steininger et al., 1992; Groenewegen et al., 1993). Indeed the projections to the VP were found to be much more dense than those to the GP reported earlier. These findings have recently been extrapolated by Zahm et al (2001) who have suggested that the majority of the limbic innervation of the mesopontine tegmentum originates in the extended amygdala rather than the accumbens. Retrograde fluorogold labelling was demonstrated in the central extended amygdala following injections into the mesopontine tegmentum with very little labelling seen in the accumbens itself. Other limbic projections to the PPTg have been described from the lateral hypothalamus (Semba & Fibiger, 1992; Steininger et al., 1992).

The final category of descending inputs to the PPTg originate in various parts of the cortex. Recently a number of studies have reported direct projections from the cortex to the PPTg (Leergaard et al., 2000; Matsumura et al., 2000). Matsumura et al (2000) examined the input from the motor cortex to the PPTg in the macaque and demonstrated input from a number of areas including the primary motor cortex, supplementary and presupplementary areas, the dorsal and ventral divisions of the premotor cortex and the frontal eye field. As neurotransmission from the cortex to lower brain regions is mediated by glutamate this provides evidence of an excitatory input to the PPTg. This is particularly interesting as the majority of the inputs to the PPTg, especially those from the SNr and GP, are inhibitory GABAergic projections. The study used anterograde tracing from areas of the motor cortex that had been

mapped using microstimulation to examine the somatotopy of the PPTg. The implications of this study for the position of the PPTg in looped neural systems will be discussed later. Leergaard et al (2000) examined the topography of projections of the barrel cortex of the rat to the pontine nuclei demonstrating direct cortical innervation of the pons. Unfortunately the exact target of the innervation was not defined and consequently conclusions regarding specific input to the PPTg were unavailable. Preliminary studies by Zahm & Winn (personal communication) examining retrograde fluorogold labelling following injections into the PPTg have also described direct corticopontine projections with labelled cell bodies reported in the medial wall of the prefrontal cortex.

The PPTg also receives ascending innervation from the constituent nuclei of the ascending reticular activating system (ARAS). This includes serotonergic projections from the raphe nucleus, which have been identified using both tracing and electrophysiological studies, (Honda & Semba, 1994; Leonard & Llinas, 1994; Steininger et al., 1997) noradrenergic input form from the locus coerulus, (Williams & Reiner, 1993; Aston-Jones et al., 1995) as well as cholinergic input from the LDTg and contralateral PPTg (Semba & Fibiger, 1992). One other source of input comes from the superior colliculus (SC; Redgrave et al., 1987).

PPTg and neural organisation

Before moving on to examine the functional characteristics of the PPTg it is important to assimilate the extensive anatomical research and to consider how its

connections may define the position of the PPTg in general neural circuitry. It is clear that the PPTg cannot be simply described in terms of one specific pattern of connections and consequent functional role. It has extensive connections with both the motor systems of the dorsal striatal complex and the reward related systems of the ventral striatal complex as well as direct input from the cortex and connections with brainstem and spinal cord. This pattern of extensive and varied anatomical links has lead to a number of theories regarding its role in neural functioning. Parent & Hazrati (1995) reviewed the anatomical organisation of the PPTg and suggested that it be included in the looped organisation of the brain proposed by Alexander et al (1986) as part of a subsidiary circuit. This proposed system is based on a series of parallel, functionally distinct and topographically organised loops within the brain. This system is organised such that a given area of striatum receives input from a number of areas of cortex. This input is integrated and then funnelled through the pallidum or SN before passing to the thalamus and back to cortex thus providing feedback loops whereby information passing through the system can be refined and processed by the relevant area of cortex. The PPTg is ideally placed to influence cortical activity through its innervation of the thalamus as well as basal ganglia function through its innervation of the SNc. When this is combined with the direct input from the cortex as well as motor and limbic striatal systems it is clear that the PPTg could be a constituent part of a long loop. A further possibility is that the PPTg could provide an escape route for information from the loops via its connection with the brainstem and spinal cord. Thus allowing the passage of information from the

loops to areas controlling motor and autonomic processes without having to refer back to the cortex.

It is of course quite conceivable that the PPTg could fulfill both of these possible roles simultaneously. The extensive anatomical research carried out in the area has uncovered two specific anatomical characteristics that could help elucidate the functional position of the PPTg. The first of these is the level of collateralisation that occurs in the efferent projections from the PPTg. Several studies focusing on different aspects of the efferent connections have described PPTg neurons sending axons to more then one location. Oakman et al (1999) reported cholinergic cells in the PPTg sending collaterals to the SNr and the thalamus, Semba et al (1990) reported single neurons projecting to both the thalamus and pontine reticular formation in the brainstem and Losier & Semba (1993) reported projections to both the basal forebrain and the thalamus from individual neurons. Perhaps most significantly Takakusaki et al (1996) described neurons producing three way collaterals that innervated the SNr, thalamus and brainstem. This demonstrates clearly that individual neurons in the PPTg have the capacity to influence activity in multiple sites within the looped organisation and of the brain. This collateralisation combined with the extensive reciprocal anatomical connections are consistent with this nucleus playing a full part in the computational activity that takes place in these systems.

Other studies have used small injections of tracing agents within subdivisions of the structures that provide input to the PPTg to investigate the level of topographical organisation present within the nucleus. Matsumura et al (2000)

examined labelling in the PPTg following tracing injections into functionally distinct areas of the motor cortex. It was demonstrated that while a certain degree of topographical organisation was maintained with inputs from orofacial, forelimb and hindlimb representations in the primary motor cortex arranged orderly from medial to lateral in the PPTg the terminal zones in the PPTg did overlap. Similar findings were demonstrated for the inputs from the SNr (Spann & Grofova, 1991) and the internal segment of the GP (Shink et al., 1997).

These findings demonstrate that the level of topographical organisation and the segregation of pathways from different functional loops is not fully maintained at the level of the pons. All these findings, however, could still be consistent with a definition of the PPTg in terms of a dual role incorporating a position in a long loop combined with a possible pathway for information from the loops to access the brain stem and spinal cord. The level of collateralisation combined with the connection patterns of the PPTg point towards it being involved in the computational processing that occurs within the loops. The overlapping of functionally distinct inputs, however, demonstrates that the functional segregation of different pathways is not fully maintained at the level of the pons. This overlapping of functional pathways, however, would allow motor, limbic and sensory information from the various inputs to influence one another at a subcortical level. This would seem to be consistent with models of brain circuitry based around the idea of funnelling information from the cortex through the midbrain and onto the brainstem. In this system areas of cortex would produce large numbers of behavioural strategies and these would be integrated and outputted through areas of the midbrain and brainstem. Purely in terms of the

numbers of neurons it is clear that cortical and midbrain neurons will vastly outnumber brainstem cells through which signals must ultimately pass on the way to motor and autonomic structures. With this in mind it is not surprising that a degree of anatomical and possibly functional overlapping between systems will occur at this level. Taken as a whole it would seem sensible to suggest that the PPTg incorporates aspects of both of these possible types of neural organisation. As mentioned earlier the collateralisation and reciprocal anatomical connections would suggest it is a part of the looped structure while the overlapping of previously segregated systems suggests that functional information is being integrated before output signals to brainstem and spinal cord are sent. It could well be the case that both of these roles are subserved by this structure.

One final hypothesis that is worth noting before moving on is the idea put forward by Inglis and Winn (1995) that the PPTg may be structurally analogous to the substantia nigra. This suggests that the two populations of neurons clearly differing in neurochemical, electrophysiological and morphological properties could be part of different functional circuits that overlap in this area. With this in mind the Ch5 neurons, and in particular their innervation of the thalamus, could be seen as part of the ascending reticular activating system (ARAS) while the non-cholinergic cells could be more involved in the fronto-striatal looped circuitry. This is not to say that these populations are completely functionally distinct as much of the anatomical evidence presented in this introduction would argue against this. Rather it would suggest that the PPTg provides a possible neural substrate through which these functional circuits could overlap. This is clearly purely speculative, but the question

of where exactly in the neural circuitry the PPTg fits is one of the key questions that experimental work in this thesis will attempt to answer.

The next section will describe functional characteristics of the PPTg focusing initially on its accepted role in maintenance and change of behavioural state as well as its role in simple motor functioning.

Sleep

One of the first functional attributes of the PPTg to be described was its role in changes of behavioural state especially with reference to rapid eye movement (REM) or paradoxical sleep (PS). Numerous studies have examined various areas of the brain in an attempt to delineate the neural substrates responsible for the production and maintenance of PS. Early studies used fairly crude neuroanatomical interventions, including complete brain transactions, to confirm that the brainstem was particularly important for this process (Jouvet, 1962). It was later demonstrated that the mesopontine tegmentum and particularly its cholinergic component were important components of the system. Using microinjections of carbachol, a long lasting cholinergic agonist, it was demonstrated that increased cholinergic activity in the anterodorsal pontine tegmentum produced a state that was behaviourally and physiologically indistinguishable from natural PS (Yamamoto et al., 1990). The area which produced the most marked occurrence of PS following carbachol microinjections included the dorsal part of the subcoeruleus nucleus as well as the adjacent dorsomedial tegmental area, which has been termed the REM sleep

induction zone (Yamamoto et al., 1990; Semba, 1993). Clearly the cholinergic innervation of this area will play a significant role in the system which controls PS, by activating these neurons and initiating the physiological responses associated with it. Semba (1993) used a variety of retrograde tracing techniques to demonstrate that while the cholinergic input to the area only made up approximately 39% of the total innervation the majority of this cholinergic input originated in the PPTg (approximately 77%).

In addition to these connections with the REM sleep induction zone the cholinergic projection to the thalamus from the PPTg has also been studied with reference to its role in PS. Electrophysiological studies examining firing patterns in the PPTg, thalamus and cortex have suggested that the PPTg is responsible for initiating and maintaining thalamic response patterns associated with PS and waking. Thalamus activity during slow wave sleep (SWS) is characterised by burst firing where clusters of high frequency action potentials form burst discharges thus preventing the accurate relaying of sensory information to the cortex[4]. During PS and waking this burst firing is inhibited allowing desynchronised single spike activity to convey accurate sensory information through the thalamus to the cortex (McCormick & Feeser, 1990). PPTg neurons have been shown to be most active during waking and PS. Indeed the electrophysiological response profile suggests that the PPTg/LDTg complex is the best candidate for triggering and maintaining thalamocortical activation as not only are these neurons most active during the relevant behavioural state their activity is increased 20-60 seconds preceding the

[4] This pattern of synchronised burst thalamic responding has lead to SWS also being referred to as EEG-synchronised sleep.

onset of EEG desynchrony (Steriade et al., 1990). This is to say that PPTg neurons increase activity before changes between SWS and either PS or waking suggesting the cholinergic innervation of the thalamus may be the trigger for this change in behavioural state.

One other very interesting possibility has recently been suggested by Sherman & Guillery (2001). They noted that the burst firing is not only seen during slow wave sleep, but is also present in awake animals. Indeed a recent study by Ramcharan et al. (2000) demonstrated burst firing in awake monkeys in both the visual and somatosensory thalamus. This finding is supported by other recent studies showing burst firing in the human thalamus in awake patients (Lenz et al., 1998) and in rats (Nicolelis et al., 1995). Sherman & Guillery (2001) went on to point out that studies examining responses to visual stimuli have demonstrated that approximately equal quantities of information are conveyed by these two modes of thalamic firing (Reinagel et al., 1999). They went on to suggest that tonic and burst firing in the awake thalamus might be concerned with the processing of different types of information regarding incoming sensory stimuli. They suggested that the electrophysiological properties of thalamic neurons in burst firing, including increased signal detection and non-linearity, would suggest that this mode of firing may be concerned with detection of sudden or novel stimuli. Equally the properties of tonic firing would suggest that this mode of firing may be involved in the accurate relaying of stimulus characteristics. Thus these two modes of firing allow the thalamus to gain information regarding novel, potentially interesting or dangerous stimuli as well as gaining precise sensory information regarding stimuli that are

being attended to. This is obviously of particular interest for two reasons. Firstly the research cited above implicating the PPTg in changes of behavioural state provide strong support for its involvement in the changing of firing modes in the thalamus from burst firing to tonic firing. Secondly experimental data from McCormick (1989, 1992) has demonstrated that application of acetylcholine (ACh) *in vitro* causes bursting cells to fire in a tonic fashion. Taken as a whole these data suggest that the PPTg may have an integral part to play in the mode of firing of thalamic neurons. Consequently activity within PPTg could well be of great importance during the waking state as well as during sleep.

In an attempt to examine further the neural system responsible for the control of behavioural state Thakkar et al (1998) looked at the effects of serotonergic inhibition on the mesopontine tegmentum. It was demonstrated that increased serotonergic activity produced an almost complete inhibition of the electrophysiologically defined REM-on neurons. Intriguingly the same level of serotonergic activity had almost no effect on the wake/REM-on class of neurons. This would suggest that the serotonergic input from the raphe nucleus is the key to behavioural state control mediated through the mesopontine tegmentum.

Other studies examining the PPTg and its role in PS have described at least two lines of evidence which implicate the PPTg in the production of ponto-geniculo-occipital (PGO) waves, a major phasic event of REM sleep which probably underlie the mental states involved in dreaming (Steriade, 1992). These include the demonstration of a sharp PGO wave in the DLG nucleus of the thalamus following stimulation of the PPTg and secondly the close temporal relationship of firing

patterns in the PPTg, thalamus and cortex during PGO wave activity (Steriade et al., 1990). Clearly electrophysiological characteristics of the PPTg combined with its extensive anatomical connections point towards it being the structure responsible for the production of PS[5]. To this effect Steriade concluded his 1992 article by saying; "At this time, it can be accepted that most REM sleep phenomena, implicated in the tonic and phasic activation of forebrain structures (EEG desynchronisation and PGO waves) are generated and transferred rostrally by the PPTg/LDTg cholinergic nuclei." (Steriade, 1992. p 16).

In direct support of this strong physiological evidence other recent studies have used different methodologies to examine the PPTg and its role in PS. Datta et al (1997) demonstrated that microinjections of the excitatory amino acid L-glutamate into the PPTg produced increased levels of REM sleep at low doses and increased wakefulness at higher doses. Increased activity of the PPTg during PS has also been demonstrated using immunohistochemical staining for the immediate early gene *c-fos*. Increased *c-fos* expression was reported in both the PPTg and LDTg following REM sleep induced by carbochol microinjections into the REM sleep induction zone (Torterolo et al., 2001).

One other interesting development has been the demonstration of the neuromodulatory function of nitric oxide (NO) and the effect that this has on the cholinergic neurons of the PPTg (Datta et al., 1997; Leonard & Lydic, 1997; Hars, 1999). Datta et al (1997) used separate microinjections of a NO donor and a NO inhibitor into the PPTg to show that increased levels of NO produced increased

[5] For reviews of the neural substrates of PS see Jones (1991) and Datta (1995).

levels of PS and slow wave sleep (SWS), while decreased NO activity produced a decreased level of PS and SWS. Similar results were found by Hars (1999) who reported decreased levels of sleep after microinjections of a NO synthase inhibitor into the PPTg and increased levels of sleep following microinjections of a NO precursor. Although in this study the NO donor used had little effect the general pattern of results is the same in that facilitation of NO activity in the PPTg produces prolonged bouts of sleep while inhibition of NO activity decreases normal sleep levels. Taken as a whole it is clear that the cholinergic cells of the PPTg play an important role in the production of PS and that extracellular NO within the nucleus may well modulate this function.

Interestingly recent lesion studies in this area have confirmed that discrete lesions of neurons within the PPTg do not disrupt normal PS. It was originally thought that lesions of the cholinergic neurons of the PPTg produced deficits in the normal patterns of PS. Studies using either large excitotoxic or electrolytic lesions of the mesopontine tegmentum (including the LDTg) reported deficits in PS (Webster & Jones, 1988; Shouse & Siegel, 1992). However, recent studies using more discrete ibotenic acid lesions of the PPTg have reported no lasting changes in PS (Inglis et al., 1995; Deurveilher & Hennevin, 2001). Inglis et al (1995) reported disturbances in PS that lasted for four days post surgery where after rats returned to normal with only slight disturbances in entering and maintaining PS. Deurveilher & Hennevin (2001) allowed rats two weeks recovery before any measurement of PS was made and consequently no changes in the normal baseline PS were observed. There are two possible explanations for this; firstly the lesions reported in these studies left at

least 20% of the cholinergic neurons of the PPTg intact. It is possible that the remaining cholinergic neurons were able to adapt over the post surgery period to provide the necessary physiological signals to allow normal PS to take place. Alternatively it may be the case that the Ch6 neurons of the LDTg, which remained undamaged in both the studies mentioned, were able to provide the necessary cholinergic input to the thalamus in the absence of the Ch5 neurons. What seems most likely is that a combination of the two processes take place where by the LDTg plays a greater role than usual in PS as well as the remaining PPTg neurons adapting to the loss of the cells lesioned. The functional significance of these lesion data will be discussed in later chapters with reference to behavioural deficits produced by PPTg lesions similar to those reported here.

Motor control

The other area with which the PPTg has traditionally been associated is motor control. The anatomical connections of the PPTg with its direct projections to the spinal cord as well as its extensive connections with the dorsal striatal complex obviously implicate it in the system concerned with generating motor responses to external stimuli from the environment. A lot of the interest in the PPTg as a motor structure stemmed from work on the precollicular-postmamillary transection preparation in cats reported by Garcia-Rill and colleagues in which it was included in the functionally defined mesencephalic locomotor region (MLR, Shik et al., 1966; Garcia-Rill & Skinner, 1987 I; Skinner & Garcia-Rill, 1984). Using this model it was

demonstrated that stimulation of the MLR produced controlled stepping movements on a treadmill. Anatomical studies looking at the functionally defined MLR neurons reported direct projections to areas of the brainstem and spinal cord which, combined with the well defined PPTg output to spinal cord, further strengthened the argument for the inclusion of the PPTg in the MLR (Garcia-Rill & Skinner, 1987 II). In an attempt to describe the systems responsible for locomotion in more detail a number of studies using direct intervention by way of lesion or stimulation were carried out. Brudzynski & Mogenson (1985) used two different methods to examine the effect of deactivating the PPTg, namely excitoxic kainic acid lesions and microinjections of the local anaesthetic procaine. In both of these cases a decrease in NAcc stimulated locomotion was demonstrated suggesting that the PPTg is involved in the output of striatal motor information. Both of the methodologies used in this experiment, however, have problems associated with them. Kainate lesions in the mesopontine tegmentum have been demonstrated to be large and non-specific (Rugg et al, 1992), while the use of local anaesthetics like procaine have the effect of the deactivating fibres of passage that run through an area as well as the neurons that make up the structure in question. The specificity of both of these methods for examining PPTg function is clearly debatable as they are also affecting surrounding structures.

Other studies using electrical stimulation or microinjections of low doses of the glutamate agonist NMDA have, however, provided direct support for this hypothesis by demonstrating increased locomotion following PPTg excitation (Milner & Mogenson, 1988; Garcia-Rill et al., 1990). Intriguingly, microinjections of GABAergic antagonists in the same study also produce increased levels of

locomotion (Milner & Mogenson, 1988). This is interesting because it suggests that the two neurochemical interventions described are acting on different aspects of the PPTg to produce the same behavioural outcome. These data are best reconciled by assuming that the NMDA acts upon the non-cholinergic cells of the PPTg to produce excitation by facilitating glutamate activity and inducing locomotion while the inhibition of GABA activity deactivates the inhibitory input from the SNc thus facilitating locomotion through a disinhibition of the nucleus. Further evidence for the involvement of the PPTg in striatal motor outflow was presented by Brudzynski & Wang (1996) who demonstrated increased *c-fos* activity in the PPTg following locomotion induced by disinhibition of the subpallidal region.

These findings have, however, been called into question by a number of other researchers. Studies using smaller and more selective ibotenate lesions of the PPTg have failed to find any changes in either spontaneous (Inglis et al., 1994a) or drug induced locomotion induced by injection of amphetamine either directly into NAcc or via non-specific systemic injection (Inglis et al., 1994b). Similarly other studies examining the effect of quisqualic acid lesions (Dellu et al., 1991) and NMDA lesions (Olmstead & Franklin, 1994) of the PPTg found no significant change in levels of spontaneous or amphetamine induced locomotion in lesioned rats. Swerdlow & Koob (1987) demonstrated that the supersensitive response to apomorphine seen in rats with dopaminergic depletion of the NAcc was also unaffected by lesions of the PPTg.

These two sets of studies would appear to present contradictory reports of the role of the PPTg in the neural systems involved in striatal motor output. However

two possible explanations for these apparently conflicting accounts have been proposed by Inglis & Winn (1995). The first is a methological point which pertains to the area of the MLR which was targeted by the studies which demonstrated either increased locomotion on excitation of the PPTg (Milner & Mogenson, 1988; Garcia-Rill et al., 1990) or decreased locomotion following lesions or injections of local anaesthetic (Brudzynski & Mogenson, 1985). On closer examination of the area in which interventions were centred it was demonstrated that only those that were aimed at the PPTg-pc produced the relevant behavioural outcome implicating the PPTg in locomotor control. While this is most densely packed part of the nucleus it is also adjacent to the cuneiform nucleus (CnF) which would appear to be a better approximation of the functionally defined MLR than the PPTg (Shik et al., 1966). This raises the possibility that excitation of the CnF rather than other areas of the MLR produces the patterns of increased locomotion observed in these studies.

The second possible explanation is based on the fact that a number of possible pathways through the brainstem and down to the spinal cord are probably responsible for the modulation of motor activity. If this is the case then these two conflicting sets of studies can be reconciled. If the PPTg were one of a number of possible sites controlling outflow of motor information then studies involving stimulation in this area would be expected to report increased levels of locomotion. However if there were indeed a number of sites responsible for the generation of motor signals then the functional integrity of the PPTg would not be a prerequisite for locomotion per se. Studies reporting no effects of PPTg lesions on patterns of locomotion could be explained by suggesting that other possible routes for motor

outflow from the striatum are compensating for the lesions in this area. This would be supported by a closer examination of the method used in the original Garcia-Rill studies which defined the MLR. It is clear from this work that the locomotion is not produced following low level stimulation of the MLR, and indeed a sequence of high frequency stimulation was needed to produce the stepping pattern reported. This suggests that stimulation was not acting at a single locus but rather was recruiting a number of sites to produce the motor outcome (Garcia-Rill, 1991). To summarise it would appear that with the exception of the early lesion work using kainate and procaine, which has been demonstrated to be methodologically unsound, the data presented forms a fairly consistent account of the role of the PPTg in motor control. It would seem sensible to suggest that while the PPTg is clearly part of the system it is only one of a number of sites involved in the output of motor information.

This suggestion of the role of the PPTg in motor control is consistent with the reported degeneration of the structure in Parkinson's disease. There have been many reports of a loss of neurons in this area from post-mortem studies of the brains of sufferers of Parkinson's disease. The interactions of the nucleus with other areas of the basal ganglia has been studied in greater detail with reference to possible treatments for the disease and this will be discussed in the next section.

Neurodegenerative diseases

In recent years increasingly sophisticated histological techniques have allowed researchers to examine in detail the pathology of neurodegenerative

disorders such as Parkinson's disease and Alzheimer's disease. A number of these studies have reported that loss of neurons in the PPTg is a common feature of the pathology of both Parkinson's disease (PD) and progressive supranuclear palsy (PSP; Hirsch et al., 1987; Zweig et al., 1987; Jellinger, 1988; Halliday., et al 1990; Henderson et al., 2000). The extent of the degeneration in this area is severe with an average loss of 60% of PPTg-pc neurons in SNP and 50% loss in PD (Jellinger, 1988). Other studies have reported even larger neuronal losses. Hirsch et al (1987) examined similar post-mortem tissue and found an average of only 21% of PPTg neurons remaining in SNP and 43% remaining in PD.

The general prevalence of the two disorders has meant that while a lot of research has been carried out examining the role of the PPTg in PD, research into SNP has been reasonably scarce and confined to the types of study reported examining the extent of neuronal loss in post-mortem examinations. The research into PD has been more wide ranging and included a number of studies using animal models of the disease to examine specific surgically induced brain trauma. Parkinson's disease is characterised primarily by the loss of dopamine containing neurons from the substantia nigra pars compacta. The progressive functional degeneration of these neurons is accompanied by the degeneration of many other structures within, and associated with, the basal ganglia including the PPTg. As expected from the neuropathology the cardinal symptoms of PD affect motor systems and these include muscular rigidity, tremor and disorders of posture and volitional movement. To examine the neural basis of these symptoms a number of recent studies have used unilateral kainic acid lesions in the PPTg to examine the

effects on locomotion in primates (Kojima et al., 1997; Munro-Davies et al 2001). Unsurprisingly the results are very similar to those studies reported previously which used the same methodology in rats to show a disruption of normal locomotion. However, the same methodological problems are also present in that kainate lesions in this area tend to be large and non-specific thus making conclusions regarding specific properties of the PPTg hard to make.

Perhaps more interesting are two studies that have used regional 2-deoxyglucose (2-DG) uptake to measure metabolic activity throughout the brain following surgically induced Parkinsonian symptoms. Mitchell et al (1989) produced 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced lesions of the SNc in primates and then examined changes in synaptic activity by mapping regional changes in 2-DG uptake. Changes in activity were reported in the GP, VA/VL complex of the thalamus and the STN. However the most dramatic change of metabolic activity was seen in the PPTg with an increase of up to 69% in 2-DG uptake reported. In a similar study in rats Carlson et al (1999) reported significantly increased 2-DG activity in the PPTg following unilateral 6-hydroxydopamine lesions of the SNc. When these results are combined with the research implicating the PPTg as one of a number of possible sites involved in motor control they strongly suggest that altered PPTg function may be involved in the motor symptoms associated with the disorder. A number of studies have addressed this increase in PPTg 2-DG activity and suggested that the likely cause is an overactivity of the inhibitory pallidal inputs (Lee et al., 2000). This would result in increased inhibition of the PPTg and a consequent aggravation of the motor symptoms produced by nigral

dopamine depletion by suppressing the excitatory input to the SN from the PPTg as well as blocking a possible route for transfer of motor information from basal ganglia to the spinal cord.

Having examined the anatomical, neurochemical, physiological and basic functional properties of the structure the thesis will now split into two parts. The first will review evidence of PPTg involvement in high level cognitive processes such as executive function and motivation and go on to present experimental work furthering our understanding in this area. The second part will examine the possible anatomical substrates through which the PPTg may affect structures higher in the neuraxis and present functional anatomical data demonstrating one possible route through which PPTg functioning may influence cognition.

Section A: Functional Characteristics of the PPTg

1.0 Introduction

This section of the thesis will deal with the work to date examining the functions of the PPTg and will go on to present experimental data furthering our understanding of the role of the PPTg in the systems controlling high level cognitive functions. The majority of the studies carried out in this area have used surgically placed lesions to examine the behavioural deficits produced by loss of neurons from the PPTg. It should be noted at this point that the lesions carried out in the studies that will be reviewed always affect both the Ch5 neurons and the interdigitated non-cholinergic cells. The possibility of making specific cholinergic lesions[1]and the functional consequences that this may have will be discussed later in the thesis.

Before moving on to discuss studies involving lesions of the PPTg and the consequent disruption to high level cognitive processes it is important to examine the effect that damage to cells in this area may have on low level behavioural processes. Tests used to examine cognitive functions such as planning, memory and attention usually involve direct observation of animals in specifically designed behavioural paradigms. Deficits in low-level behavioural processes could seriously affect behaviour in such tests and result in cognitive deficits being implied where non exist. Given that the PPTg has been implicated in the systems controlling sleep processes and motor functioning it is important to examine the effects of lesions of the PPTg

[1] Specific cholinergic lesions in the basal forebrain have been produced using the neurotoxin IgG192 saporin. Unfortunately mesopontine tegmental neurons do not have the nerve growth factor receptors to which the IgG192 binds and consequently similar lesions in the PPTg are not possible.

on these low level behavioural functions to ensure that any behaviour observed implying cognitive deficits are not simply an artefact of sleep deprivation or motor dysfunction.

Given its accepted role in sleep and control of behavioural state, relatively few studies have examined the effect of lesions of the PPTg on sleep patterns. Only two studies using discrete excitotoxic lesions have examined the effect of cells loss in this area on sleep. The first of these reported a disruption of REM sleep for up to four days following surgery but thereafter sleep patterns return to normal with no difficulties in entering or maintaining PS reported (Inglis et al., 1995). The other study reported no deficit at all in normal patterns of REM sleep following lesions in this area, although it should be noted that rats were not examined until two weeks after surgery so no conclusions on sleep patterns directly following surgery could be reached (Deurveilher & Hennevin, 2001). These studies would suggest that the structural integrity of the PPTg is not essential for the formation of normal sleep patterns. Possible reasons for why this might be the case are covered in the introduction.

Studies examining locomotion following lesions of the PPTg have produced some contradictory results. However, as explained in the introduction it is clear that experiments reporting deficits in locomotor activity have been shown to involve large non-specific lesions (Brudzynski & Mogenson, 1985). More recent studies involving more specific lesion methods have reported no change in either spontaneous or drug induced locomotion (Inglis et al., 1994a; Inglis et al., 1994b, Olmstead et al., 1994). Lesioned animals have also been shown to have normal

feeding, drinking and grooming patterns (Allen & Winn, 1995). It would seem, therefore, that there are no overt changes in basic motor and regulatory processes following ibotenate lesions of the PPTg.

There are, however, a number of more subtle deficits produced and these have been interpreted in a number of ways. A large amount of research has examined the possibility that the PPTg is a constituent part of the neural circuitry involved in the processing of reward and that lesions in this area produce a deficit associated with either a change in the perception of reward or motivation. Other researchers have suggested that the PPTg may be involved in the neural systems controlling executive functions and that PPTg damage results in a breakdown of these cognitive processes. Further studies have suggested that behavioural problems following PPTg damage are a result of different underlying mechanisms including increased levels of anxiety, decreased reaction time or deficits in attention. Clearly the deficits produced have been examined from a number of different theoretical perspectives and the consequent body of literature produced is somewhat confusing. The following sections will summarise the findings of research to date from each perspective before presenting experimental work designed to further our knowledge of the functional aspects of the PPTg and the consequent behavioural abnormalities produced by lesions of this nucleus.

1.1 Executive functions

The study of executive function was at least initially predominantly associated with the study of damage to the frontal lobe and the resulting behavioural deficits that this produced. This set of symptoms has been termed frontal syndrome. In his 1995 article Duncan describes the problems associated with frontal lobe damage stating "..the normal structure of goal-directed behaviour is disturbed producing activity that seems fragmented, irrelevant or bizarre". The term executive function is derived from Baddeley's working memory model in which a mechanism termed the central executive combines information from slave systems, which process sensory information, to select and operate goal-orientated strategies. Unfortunately the research carried out on the working memory model has concentrated almost exclusively on the slave systems leaving the central executive as a poorly defined catch-all term including any cognitive process that is involved in the implementation and integration of memory into systems concerned with behavioural control. As a consequence of this, the term executive function is equally poorly defined and has been used in various ways by different researchers in the field. For the purpose of this thesis it was decided to use a definition of executive function in terms of its original conception as a classification of frontal deficits in an attempt to be consistent with previous research. For this reason the following definition by Mulder et al (2000) who were describing executive dysfunction in patients with frontal damage has been used. They stated that following frontal damage "These deficits in executive functions include poor strategy planning, response inhibition

and response flexibility." While it is clear that this term will need to be broken down when examining specific behavioural abnormalities it can still have value when discussing the neural systems co-ordinating goal orientated behaviour.

When examining the neural circuitry associated with executive functions it is important to realise that the looped organisation of the brain described in the introduction ensures that the functional integrity of the frontal cortex is dependent on the other structures that make up the loop. Consequently, if constituent parts of the loop lower down the neuraxis in areas of the midbrain are selectively lesioned this will also produce behavioural deficits associated with frontal damage and executive dysfunction. That is not to say that damage to subcortical structures will produce identical behavioural outcomes to frontal damage, but rather that both types of damage will disrupt the system which controls this set of cognitive processes. By way of example Owen et al. (1993) examined the cognitive deficits of a group of patients with frontal lobe damage compared to a group of patients with Parkinson's disease. The experiment examined executive functional problems by testing the ability of the two groups of patients to perform an attentional set-shifting task. The experiment involved two variations on a basic attentional set-shifting task in which subjects were tested on their ability to switch their attention from a previously rewarding category of stimuli to a new category. It was demonstrated that while both groups showed deficits on the task, the mechanisms underlying the impairment were different. Parkinson's patients had difficulty in learning that a previously irrelevant stimulus could now be correct while patients with frontal damage showed greater levels of perseverative responding. It would appear that the two different patterns of

damage in the brain are affecting the same functional circuit, in different ways, to produce deficits on the same task. This is particularly interesting when the altered activity of the PPTg in Parkinson's described in the introduction is considered. It is possible that this change in activity may be a factor in the cognitive as well as the motor deficits seen in this disease.

Interestingly it has been suggested that damage to the PPTg produces behavioural deficits very similar to those associated with damage to the frontal lobe and associated structures (Winn, 1998). This is perhaps not surprising when its anatomical connections are taken into account. It was described in the introduction that the patterns of connectivity combined with the frequent occurrence of collateral projections from the PPTg to areas in the loops puts it in an ideal position to influence the corticostriatal circuitry. If this were the case then lesions of the PPTg would be expected to produce this type of behavioural outcome. Winn (1998) cites a number of studies in which excitotoxic lesions of the PPTg produce frontal type symptoms including disinhibition of behaviour, inappropriate behaviour and perseveration. To examine this claim the studies cited are examined below.

One form of behavioural deficit commonly associated with frontal damage is perseveration. Perseverative responding is seen when an animal is unable to disengage from a particular response and consequently shows a pattern of behaviour characterised by repetitive stereotyped responding. This type of behaviour has been demonstrated in PPTg lesioned rats in a number of different paradigms. As reported earlier the effect of PPTg lesions on locomotion has been examined to investigate the possibility that disruption of these neurons produces a general motor deficit (Inglis et

al., 1994a; Inglis et al 1994b, Allen & Winn, 1995). These studies used discrete ibotenate lesions and then examined levels of spontaneous and amphetamine induced locomotion. It was shown that spontaneous locomotion was unaffected in lesioned rats and low doses of amphetamine also failed to elicit any differential responding in the lesioned rats as compared with the sham animals. The importance of these findings has already been emphasised in the ruling out of low-level explanations for deficits produced by PPTg damage. Interestingly, however, the response to high doses of amphetamine was altered in lesioned animals. Systemic injections of amphetamine at high doses produced pronounced orofacial stereotopies including prolonged bouts of licking and biting. Two things should be emphasised about these data. Firstly, although this type of responding has been reported following systemic injections of apomorphine, there are no known reports of this type of behaviour following systemic amphetamine injections. Secondly this type of intense stereotopy is normally only seen following microinjections of amphetamine directly into the ventrolateral striatum (Delfs & Kelley, 1990). This suggests that the PPTg is directly involved in motor outflow from the dorsal striatal complex. This is perhaps not surprising when the strong connections between the PPTg and the SN, the principle output station of the dorsal striatum, are taken into account.

Other instances of perseverative responding in PPTg lesioned rats have been demonstrated using more complex behavioural paradigms (Winn et al., 1995; Keating & Winn, 2002). In their 2002 study, Keating & Winn examined PPTg lesioned animals performing spatial working memory tasks on an eight arm radial arm maze. The task involved a training phase in which rats were to retrieve food

pellets form four baited arms with the other four arms being blocked. Then followed a test phase in which the rats had to retrieve food pellets from the arms which were not accessible in the previous trial. Clearly the optimal strategy involves visiting each arm once to retrieve the reward. During this task lesioned rats showed peseverative responding in that they kept returning to previously baited arms from which they have already retrieved the reward (Keating & Winn, 1998). This is particularly interesting as deficits in this task had previously only been reported following lesions in areas involved in either learning and memory (Floresco et al., 1997) or executive function (Floresco et al., 1996; Seamans et al., 1995). This type of evidence provides strong support for the inclusion of the PPTg in the neural systems involved in executive processes.

Further examples of perseverative responding were reported in a task where rats are trained to depress a lever for a specific period of time following an auditory stimulus in order to gain a reward. It was reported that PPTg lesioned rats made significantly more late errors while frequency of early errors was not affected. This suggests that PPTg lesioned rats have no anticipatory deficit but have trouble in disengaging from a specific response. That is their bar pressing behaviour is perseverated such that once initiated they have trouble in disengaging a specific behaviour (Winn et al., 1995). At this point it is worth mentioning a more recent study that examined PPTg lesioned rats in a very similar behavioural task. Florio et al. (1999) examined the effects of excitotoxic lesions of the PPTg on the preparation and execution of an externally cued bar pressing task. Briefly lesioned rats were trained to depress a lever in response to a combined auditory and visual stimulus and

then release it following a trigger stimulus. As in the Winn et al. study PPTg lesioned rats made significantly more late errors. Interestingly this was interpreted in terms of a deficit in reaction time in these animals. This discrepancy illustrates nicely the point made earlier in the section that research in this field has been used to support a number of possible different roles or functions of the PPTg.

The next type of deficit in executive functioning reported by Winn (1998) in PPTg lesioned animals was inappropriate responding. This type of behavioural abnormality has been reported in tasks requiring rats to choose between two levers in an operant chamber only one of which was rewarded (Inglis et al., 1994a; Robertson, 1994). Inglis et al. (1994a) used a conditioned reinforcement paradigm to examine the effects of ibotenate lesions of the PPTg on behaviour shown to be stimulated by injections of amphetamine into the NAcc. The task involved training animals to associate reward (in this case food) with a previously neutral stimulus (in this case light) in a classic Pavlovian fashion. The test phase of the experiment involved presenting these animals with a choice of levers, one of which resulted in presentation of the conditioned reinforcer (the light), the other of which had no effect. Injections of amphetamine into the NAcc in normal animals stimulate responding on the lever producing the conditioned reinforcer. It was reported that rats with lesions of the PPTg, while showing a normal pattern of responding on the reinforced lever, showed a greatly elevated level of responding on the non-reinforced lever. Interestingly recent studies by Inglis et al. provide further evidence of inappropriate responding during conditioning studies by rats with PPTg damage. Inglis et al. (2000) demonstrated that PPTg lesioned rats show impaired learning in

an autoshaping paradigm. Rats were trained to associate a specific visual stimulus on a computer screen with a reward. In a following test phase rats were rewarded for approaching the conditioned stimulus. PPTg lesioned animals showed a tendency to approach the non-reinforced stimulus. This is very similar to the above cases of rats responding on the non-reinforced lever of an operant chamber.

This type of deficit was also reported by Alderson et al. (in press) who tested PPTg lesioned rats on fixed and progressive ratio responding for food. These tests involve rats being trained to press a lever in order to gain a food reward. Fixed ratio scales require the animal to make a fixed number of responses in order to gain each food reward, while progressive ratio scales require rats to increase the number of responses to gain reward on each successive trial. The point at which animals stop responding on the progressive ratio scale is called the breaking point. PPTg lesioned rats again showed an increase in responding on a non-reinforced lever which resulted in a decreased breaking point on the progressive ratio scale. In both these cases it appears that PPTg lesioned rats have been unable to suppress an inappropriate response.

Inappropriate responding has also been demonstrated in tasks using negative reinforcers (Fujimoto et al., 1992). In tests of passive avoidance PPTg lesioned animals showed significantly different patterns of behaviour to shams based on a similar inability to suppress an inappropriate response. This was tested by placing rats in a two chambered box where one of the chambers was dark and the other was light. On the first trial rats were placed in the light part of the box and allowed to enter the dark part at which point they were administered a small electric shock. The

second phase was carried out ten days later and involved placing the rats back in the light part of the box and measuring the length of time taken by the rats to enter the dark part. It was reported that PPTg lesioned animals took a significantly shorter length of time to re-enter the dark part of the box compared to shams (Fujimoto et al., 1992). Clearly in this case lesions of the PPTg have resulted in an inability to suppress an inappropriate action.

The final category of executive deficit cited by Winn (1998) following lesions of the PPTg is disinhibited behaviours. Once again this has been reported in a number of studies using different behavioural paradigms. Perhaps the most striking examples of this form of deficit have been reported in studies examining the effect of lesions of the PPTg on consummatory behaviour. It has been demonstrated on a number of occasions that PPTg lesioned rats given the opportunity to consume high concentrations of sucrose solution will overconsume compared to sham lesioned animals (Keating et al., in press; Ainge et al., 1999; Olmstead et al 1999). This was first reported when tests of conditioned place preference mediated by sucrose reward were examined. Since then the deficit has been more widely studied and it has been consistently shown that PPTg lesioned rats given access to low concentrations of sucrose solution (4%) will show normal patterns of consumption. However, when higher concentrations are presented (20%) PPTg lesioned rats show a disinhibited drinking response and overconsume compared to sham lesioned rats.

This is particularly interesting in light of research examining the effects of striatal damage on sucrose consumption. Eagle et al. (1999) made a systematic study of the effects of lesioning different parts of the striatum and the subsequent effects

on consumption of differing concentrations of sucrose. If the PPTg is functionally involved in the same neural circuitry as the striatum it might be expected that damage to the different areas of the striatum would produce similar deficits to those seen in PPTg lesioned animals. It was shown that lesions of the ventral striatum produced a shift of the intake–concentration function to the left. That is to say that lesioned rats drank more low concentration sucrose solution and less high concentration. Lesions of the medial and lateral sections of the striatum produced a general lowering of the volumes of sucrose consumed. These data show that while lesions of the PPTg are affecting the same types of behaviour as are disrupted by striatal lesions the behavioural outcomes are quite different.

Disinhibited responses are not solely restricted to consummatory responses, they have also been reported in tests of motor control. Perhaps the most well studied of these is pre-pulse inhibition of the startle reflex. The startle reflex can be induced in rats by the sounding of a sudden loud noise. This can be attenuated by presenting a stimulus of lesser intensity prior to the startle inducing stimulus. The consequent reduction in the magnitude of the startle reflex induced by the loud noise is known as the pre-pulse inhibition of the startle reflex. Studies have demonstrated that rats with either electrolytic (Swerdlow & Geyer, 1993) or excitotoxic (Koch et al., 1993) lesions of the PPTg fail to inhibit the startle reflex even after the warning stimulus. Their behaviour is disinhibited. In related experiments it has also been shown that the normal freezing response to overhead threat exhibited by rats is also decreased in PPTg lesioned animals (Allen, 1995), who failed to produce the appropriate

response, probably because of an inability to inhibit movement. Once again their behaviour was disinhibited.

It is worth noting at this point that these categories of executive dysfunction are far from being mutually exclusive. For example the orofacial stereotopies cited as an example of perseverative behaviour could also be used as an example of disinhibited behaviour. Equally the over-consumption of sucrose cited as an example of disinhibition could be seen as a perseverative response. The important point to take away from this review is that all three of these categories of behavioural deficit are indicative of a breakdown in executive function. If we examine the definition of executive function used in this thesis it is clear that lesions of the PPTg are producing deficits in exactly the processes described. Mulder et al. (2000) stated that frontal damage produced executive problems including poor strategy planning, response inhibition and response flexibility. This fits very well with the classification of the deficits produced by PPTg lesions proposed by Winn (1998). Inappropriate responding could easily be seen as poor strategy planning, disinhibition is clearly a problem associated with poor response inhibition and perseveration is one form of response inflexibility. On this basis it would seem that lesions of the PPTg are producing a set of behavioural deficits akin to frontal syndrome. This is perhaps the most compelling evidence presented thus far for including the PPTg in the anatomical circuitry responsible for high-level cognitive processes. One possible caveat to this are the data demonstrating remarkably different effects of lesions of the PPTg and various areas of the striatum on similar behavioural tasks, namely consumption of sucrose solution. However, this may simply be the result of the

difference between altered processing of reward information caused by striatal lesions compared to the effects of blocking the routes for this information to the muscles and spinal cord by lesioning the PPTg.

Moving on from executive functions another area in which the PPTg has been studied with a view to understanding its functional characteristics is reward related behaviour.

1.2 Reward related behaviour

A number of studies attempting to understand the neural systems involved in the processing of reward related behaviours have examined the functional properties of the PPTg. The hypothesis that the PPTg is involved in this system is based on the anatomical connections of the nucleus combined with experimental data implicating it in reward processing and output. As mentioned in the introduction the PPTg receives extensive input from the ventral striatal complex including the nucleus accumbens (Groenewegen et al., 1993) and the ventral pallidum/ substantia innominata (Semba & Fibiger, 1992; Steininger et al., 1992; Groenewegen et al., 1993). It also has efferent connections with the ventral tegmental area (Oakman et al., 1995) and the lateral hypothalamus (Woolf & Butcher, 1986, Hallanger & Wainer, 1988, Ford et al., 1995). Combined with the work implicating the PPTg in the output of motor information from the basal ganglia this has led some researchers to suggest that the PPTg may be an important site where information regarding the

reward and emotional significance of stimuli can gain access to and therefore modulate motor responses (Mogenson, 1987).

In response to this suggestion a number of researchers have examined the effect of direct manipulation of the PPTg on reward based paradigms. Lesions of the PPTg have been shown to disrupt rewarding brain stimulation responding mediated through the lateral hypothalamus (Lepore & Franklin, 1996; Buscher et al., 1989). Rats with electrodes implanted in their lateral hypothalamus will respond on a lever which delivers electric current to these electrodes. It has been suggested that the obviously rewarding nature of this stimulation is produced by stimulation of the ascending dopamine pathway from the ventral tegmental area (VTA) to the nucleus accumbens. The impairment of this behaviour by lesioning of the PPTg suggests that the cholinergic input from the mesopontine tegmentum to the VTA is an important part of the circuitry which supports this behaviour. This is further supported by studies examining the effects of altered cholinergic activity in the PPTg on rewarding brain stimulation. Yeomans et al. (1993) used microinjections of cholinergic agonists and antagonists into the PPTg to examine responding following activation and inhibition of the cholinergic aspect of the nucleus. They demonstrated that cholinergic agonists, which block cholinergic activity due to their action at inhibitory autoreceptors raised self-stimulation thresholds to 400% that of normal rats while cholinergic antagonists, that facilitate cholinergic activity, lowered the thresholds by 20-80%. However other researchers have demonstrated that the actual level of dopaminergic activity in the NAcc following VTA stimulation is unaffected

by PPTg lesions while it is greatly reduced following LDTg lesions (Blaha et al., 1996)[2].

The majority of the work examining the role of the PPTg in reward processing has centred around the conditioned place preference (CPP) paradigm. This test involves using a twin chambered box in which the two chambers have obviously different sensory properties. Common examples of differences between chambers include using different colours, floor textures and odours. Animals are trained such that a particular reward or drug is administered in one of the two chambers for a number of trials such that the sensory properties of that chamber become associated with the reward or drug. The test phase of the paradigm involves giving conditioned animals free access to both of the chambers and recording the length of time animals spend in each. It has been demonstrated that animals conditioned with positive reinforcers in one of the chambers will spend significantly longer in that chamber during the test period.

Unfortunately tests using PPTg lesioned animals in a CPP paradigm have produced, at best, confusing results. Van Der Kooy and colleagues used CPP experiments to examine the effects of state dependency on morphine and food supported conditioning and suggested that PPTg lesions block CPP when animals are drug naïve or non-deprived of food. That is to say they found that food deprived and morphine dependent rats with PPTg lesions formed normal CPP, while lesioned rats who were not food deprived or that were drug naïve rats did not form normal CPP (Bechara & Van Der Kooy, 1992). This state dependent deficit was also reported in

[2] This cholinergic modulation of midbrain dopamine will be discussed in greater detail in section B of this thesis.

other studies using morphine and amphetamine based CPP (Bechara & Van Der Kooy, 1989). Other studies, however, have produced conflicting results. Parker & Van Der Kooy (1995) reported that PPTg lesions did not affect acquisition of CPP to cocaine even in drug naïve animals, while CPP to saccharin was blocked in both deprived and non-deprived animals (Stefurak & Van Der Kooy, 1994). Studies in this laboratory have reported normal formation of CPP to sucrose in deprived and non-deprived animals, although a disinhibited drinking response to 20% sucrose was seen (Keating et al., in press; Alderson et al., 2001). These studies have produced a lot of conflicting results and accounting for these data en masse is clearly very difficult. In an attempt to further understand the role of the PPTg in reward it is necessary to examine other behavioural paradigms.

Intravenous self-administration (IVSA) of heroin and amphetamine has also been examined in PPTg lesioned rats (Olmstead et al., 1998; Alderson et al., 2001). In both of these studies it was reported that rats with lesions of the PPTg exhibited deficits in acquisition of fixed ratio responding and lower breaking points in progressive ratio responding. These data would appear to support the hypothesis that the PPTg is involved in the processing of reward information and that lesions in this area produce motivational deficits or problems in the perception of reward.

There are, however, a number of sources of evidence that suggest that the deficits seen in PPTg lesioned rats are not related to motivation or perception of reward. Firstly on closer examination of the amphetamine IVSA data it is clear that responding in PPTg lesioned rats was not generally decreased. As with the PR ratio responding for food mentioned earlier (Alderson et al., in press) lesioned animals in

this paradigm showed significantly higher rates of responding on the non-reinforced lever which could well account for the lower breaking point. Secondly tests examining rats ability to discriminate different levels of reward and show appropriate contrast effects to these differences have reported no effect of PPTg lesion on this behaviour (Olmstead et al., 1999). This suggests that these rats have no motivational problems or problems with perceiving different levels of reward. In addition to this in the study where PPTg lesioned animals showed decreased breaking point on PR schedules for food, latency to collect food after responses were made was unaffected which is not consistent with an explanation in terms of decreased motivation. Finally it is clear that an explanation of the deficit produced by lesions of the PPTg based upon a general motivational deficit does not explain a lot of the executive deficits described in the previous section. For example the orofacial stereotopy produced by high doses of amphetamine, nor the learning deficits produced on the radial arm maze can be explained in terms of decreased motivation.

Clearly the studies performed examining PPTg function in reward processing have produced mixed results. Before attempting to assimilate all this information it is worth noting that the differences in deficit produced by lesioning the PPTg may be related to the method of lesion used. Van Der Kooy and colleagues employ a single injection of ibotenate solution centred on the ventromedial portion of the structure to produce their lesion. These have tended to produce differing results from those using a twin injection procedure, which employ two smaller injections of ibotenate into the ventroanterior and dorsoposterior regions of the nucleus. It is interesting to note that those employing a twin injection procedure have produced complimentary results by

demonstrating no effect of PPTg damage on either CPP (Keating et al., 2002), reward discrimination or contrast effects (Olmstead et al., 1999).

By way of summary it would seem reasonable to suggest that these results can best be reconciled by expressing the deficit produced by PPTg damage in terms of a breakdown in executive function. Studies examining reward related behaviours using the twin injection lesion procedure have produced consistent results indicating no deficits in motivation (Keating et al., in press; Olmstead et al., 1999). Other studies using this lesion, including the PR responding for food and amphetamine, are easily explained with reference to a breakdown in executive function as explained in the previous section.

1.3 Anxiety

Other researchers have suggested that the behavioural deficits produced by PPTg damage are an artefact of a heightened level of anxiety. In their 1998 article Podhorna & Franklin suggested that changes in behaviours like the pre-pulse inhibition of the startle reflex could be interpreted as an increased level of anxiety. This was tested by examining the behaviour of PPTg lesioned rats on a well accepted test of anxiety; namely the elevated plus maze. The elevated plus maze is a platform in the shape of a cross which has two arms that are covered and two that are open. The test involves measuring the number of times an animal ventures onto the open arms and the amount of time they spend on them. Podhorna & Franklin reported that PPTg lesioned animals showed an increased level of anxiety like behaviour

manifested by a decreased number of entries to the open arms combined with a decrease in the time spent upon the open arms of the maze.

This argument was strengthened by Leri & Franklin (1998) who demonstrated that learning deficits on the delayed non-matching to position task produced by lesions of the PPTg could be reversed by pre-training injections of the anti-anxiolytic drug diazepam. Further experiments reported that PPTg lesions rats showed similar levels of performance on the elevated plus maze as sham lesioned animals treated with the anxiety inducing drug ethyl β-carboline-3-carboxlate (β-CCE). This series of experiments present a convincing argument for the hypothesis that PPTg damage produces heightened levels of anxiety like behaviour. Other researchers, however, have produced an alternative explanation for this apparently convincing evidence. The lesions made by Franklin and colleagues are centred on the caudal and ventral portion of the PPTg. Indeed histological analysis presented in these papers demonstrates fairly consistent damage to the cuneiform nucleus (CnF) directly dorsal to the PPTg in its caudal section. Ainge et al. (1999) demonstrated that lesions of the PPTg, which did not damage the CnF, had no effect on performance on the elevated plus maze. However, lesions centred on the CnF itself produced a significant decrease in exploration of the open arms. This would appear to demonstrate that lesions of the PPTg which do not damage the CnF do not produce heightened levels of anxiety and that the effects reported by Franklin and colleagues are a direct result of damage to the CnF.

1.4 Attention

The other hypothesis that has been proposed to account for the large amount of data demonstrating behavioural deficits following PPTg damage is that these deficits are a result of altered levels of attention. This hypothesis is particularly interesting as previously attention has been one of the terms included in the rather non-specific term executive function. Clearly research into attention as the major executive deficit in these rats is an important step towards a fuller understanding of the underlying causes of the behavioural problems reported. In a recent study Inglis et al. (2001) reported that bilateral damage to the PPTg produces a global deficit in attention as measured by performance on the 5-choice serial reaction time task. PPTg lesioned rats showed reduced accuracy, increased errors of omission and increased latency to correct errors. Attentional deficits have also been reported by Kozak et al. (2001) who demonstrated that PPTg lesioned rats showed an impairment in sustained attention. These studies are consistent with a study of normal healthy humans that showed that areas of the mesopontine tegmentum were part of a network involving cortical and thalamic areas that showed increased activation during a task designed to test attention (Sturm et al., 1999).

When the studies from the previous sections are examined it is clear that a large proportion of the findings can be accounted for by the hypothesis that PPTg damage produces a global deficit in attention. Much of the data cited as evidence of executive dysfunction fits equally well in a theory centring around attentional deficits. For example the tendency of PPTg lesioned rats to respond on a non-

reinforced lever in conditioning experiments as well as poor performance in the radial arm maze could easily be explained in terms of attentional abnormalities. However, this does not explain all of the executive problems. For example, the orofacial stereotopies following high doses of amphetamine cannot be explained by a deficit in attention.

What is clear from the research to date examining the functional properties of the PPTg is that the deficits produced from lesions to this structure are complex and not easily explainable in terms of one underlying cognitive process. The next section of the thesis will present experimental work carried out to further our understanding of the deficit produced by lesions of the PPTg. This will be done by attempting to delineate the psychological processes underlying one of the most striking deficits seen in these animals; that is the overconsumption of high concentrations of sucrose solution.

2.1 Method

Subjects

The subjects were 33 adult male Lister-hooded rats (average pre-surgery weight: 327.04g) housed individually in a temperature and light controlled (12-hr light-dark cycle) colony. All experimental and care procedures followed guidelines laid down by UK and European legislation (Animals [Scientific Procedures] Act, 1986, and European Communities Council Directive of 24 November 1986 [86/609/EEC]. All rats were given ad libitum access to food and water after surgery until they returned to their free feeding weight at which point they were deprived to 85% of their pre-surgery free feeding weight and maintained at that by 15-20g SDS maintenance #1 food pellets daily. Rats were tested on consecutive days in two groups of 12 animals.

Surgery

Animals were anaesthetised with 60 mg/kg Sagatal (sodium pentobarbitone), delivered at 1 ml/kg of body weight (combined with sterile water at a 50:50 ratio) and placed in the stereotaxic frame. Rats were checked for a satisfactory level of anaesthesia by ensuring that both the eye blink reflex and withdrawal reflex following tail pinch were not present. Lesions of the PPTg were made using two injections one in each of the following sets of co-ordinates. Set 1: anterior-posterior +0.8mm from interaural line (IAL), medial-lateral ± 1.6mm from midline, dorso-

ventral –7.0mm from skull surface, with skull level. Set 2: AP: 1.5mm from IAL, ML: ± 1.7mm from midline, DV: -7.8mm from skull surface.

The first group of 17 animals received infusions in each injection site of 0.2µl of 0.12M ibotenic acid (pH adjusted to 7.0), or sterile phosphate buffer, with the needle left in situ for 5 mins after infusion was finished to allow diffusion of solution away from the needle tip. The second group of animals received infusions of 0.2µl of 0.08M ibotenic acid, or sterile phosphate buffer, with needles left in situ for five minutes after infusions had finished. Bilateral lesions were performed in two separate unilateral surgeries with a gap of one week between each surgery. This was done as previous experience had proved that bilateral PPTg lesions performed in one surgery resulted in high mortality rates.

Apparatus:

The task employed a wooden runway measuring 3 m long x 30cm wide x 30cm high. The runway was painted grey and had a centrally located hole at one end 10cm from the bottom through which a drinking spout attached to a burette protruded.

Procedure

Following at least two weeks of post-operative recovery rats were given a ten minute habituation trial where no reward was available from the burette. The rats were then given four trials to habituate them to the availability of sucrose solution from the burette. The rats were placed facing the burette at progressively greater

distances from the burette, during the four trials, to habituate them to having to run to gain reward. Rats were allowed to drink for two minutes following first contact with the burette in these trials. Half of the rats from each group received 4% sucrose solution while the other half received 20% sucrose solution. Then followed seven days where rats were placed at the opposite end of the runway to the spout and the time taken to run the length of the runway and start drinking was measured. The rats were then allowed to drink for 30 minutes and the total amount of sucrose solution consumed was measured. The groups were then reversed such that the rats that had been receiving 20% sucrose now received 4% sucrose, and the rats that had been receiving 4% sucrose now received 20% sucrose. Then followed another seven trials where the time taken by the rat to run the length of the runway and start drinking was measured. Again the rats were given 30 minutes to drink and the total amount of sucrose solution consumed was measured.

Histology

Rats were humanely killed with barbiturate overdose (0.9ml i.p. injection of euthetal, 200mg/ml) and then transcardially perfused with 0.1M phosphate buffered saline for four minutes followed by 4% paraformaldehyde for a further 20 minutes. The brain was then quickly removed and placed in 20% sucrose in 0.1M phosphate buffer and stored at 4°C. Coronal sections were cut at 50 μm into 0.1M phosphate buffer on a freezing microtone taking one in four sections for processing. Sections were processed for NADPH-diaphorase and cresyl violet staining (see appendix for details). NADPH-diaphorase is an enzyme responsible for the synthesis of nitric

oxide and has been shown to be present in the Ch5 neuorns. All sections were inspected using a Leitz "Diaplan" microscope fitted with a Sony DXC-3000P video camera for visualization of sections on a high resolution colour monitor. Lesions were identified in cresyl violet stained sections by the presence of gliosis and degenerating neuronal somata.

Statistical Analysis

Consumption of sucrose data for the two weeks were analysed using repeated measures ANOVA with group (lesion vs. sham) and concentration (4% vs. 20%) as the between subjects factors and day as within subjects factor. Runway time data were also analysed using repeated measures ANOVA with group (lesion vs. sham) and concentration (4% vs. 20%) as the between subjects factors and day as within subjects factor. To investigate further the pattern of runway times a series of restricted analyses were performed comparing different pairs of groups using repeated measure ANOVAs. This procedure involves using the mean square error term and degrees of freedom from the original omnibus analysis in these repeat measures ANOVAs to protect against the possibility of type 1 errors.

2.2 Results

Lesion size was assessed by examining NADPH diaphorase stained sections as well as sections processed for cresyl violet staining. NADPH diaphorase is a good marker of the cholinergic Ch5 neurons in the PPTg while cresyl violet staining labels reactive gliosis and consequently the two stains complement each other demonstrating the extent of the cell damage within the PPTg. Lesion size was estimated as a percentage of the total area of the PPTg and lesions were accepted if the damage was symmetrical bilaterally and encompassed at least 70% of the nucleus. Figure 6 displays a schematic representation of the smallest and largest lesions made in this study and Figure 7 illustrates the lack of NADPH-diaphorase positive neurons in lesioned rats. As shown in the Figure the smallest lesions were almost entirely confined to the boundaries of the PPTg with approximately 70% of the neurons being destroyed. The largest lesions destroyed up to 90% of the neurons within the PPTg with some damage to the cuneiform nucleus in its caudal section and some damage to the retrorubral fields in the more rostral section. Six rats died either during or in the week prior to surgery. One lesion was found to be unilateral and excluded from future analysis and two further were excluded due to extensive damage reaching rostrally through the retrorubral fields and in one case into the substantia nigra. This left twelve rats in each group.

Quantities of sucrose consumed over the two weeks are displayed in Figure 8. This shows clearly that in both weeks rats given the opportunity to drink 20% sucrose consumed more than those drinking 4% sucrose. Further, PPTg lesioned rats

Figure 6: Schematic representation of largest and smallest lesions.

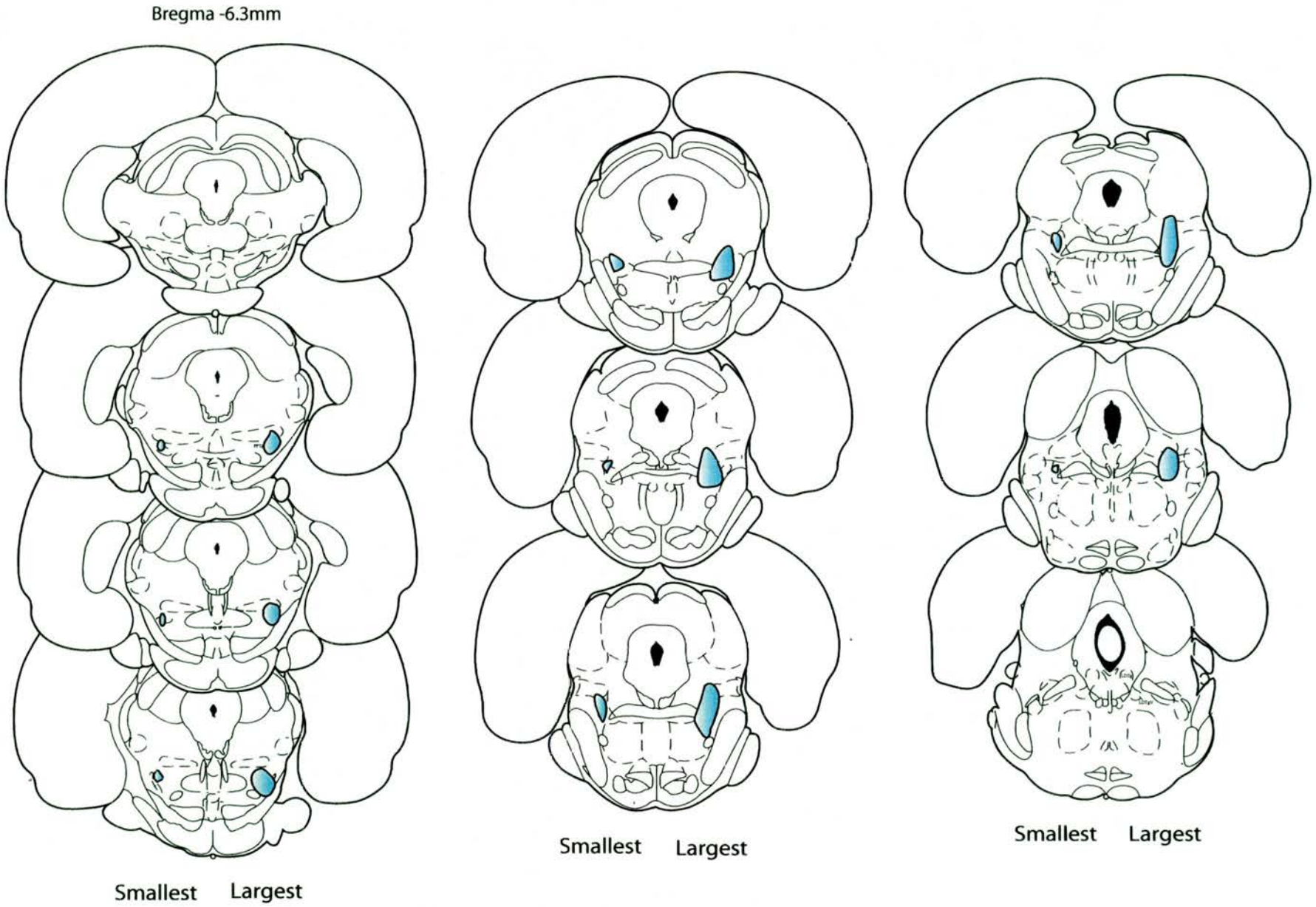
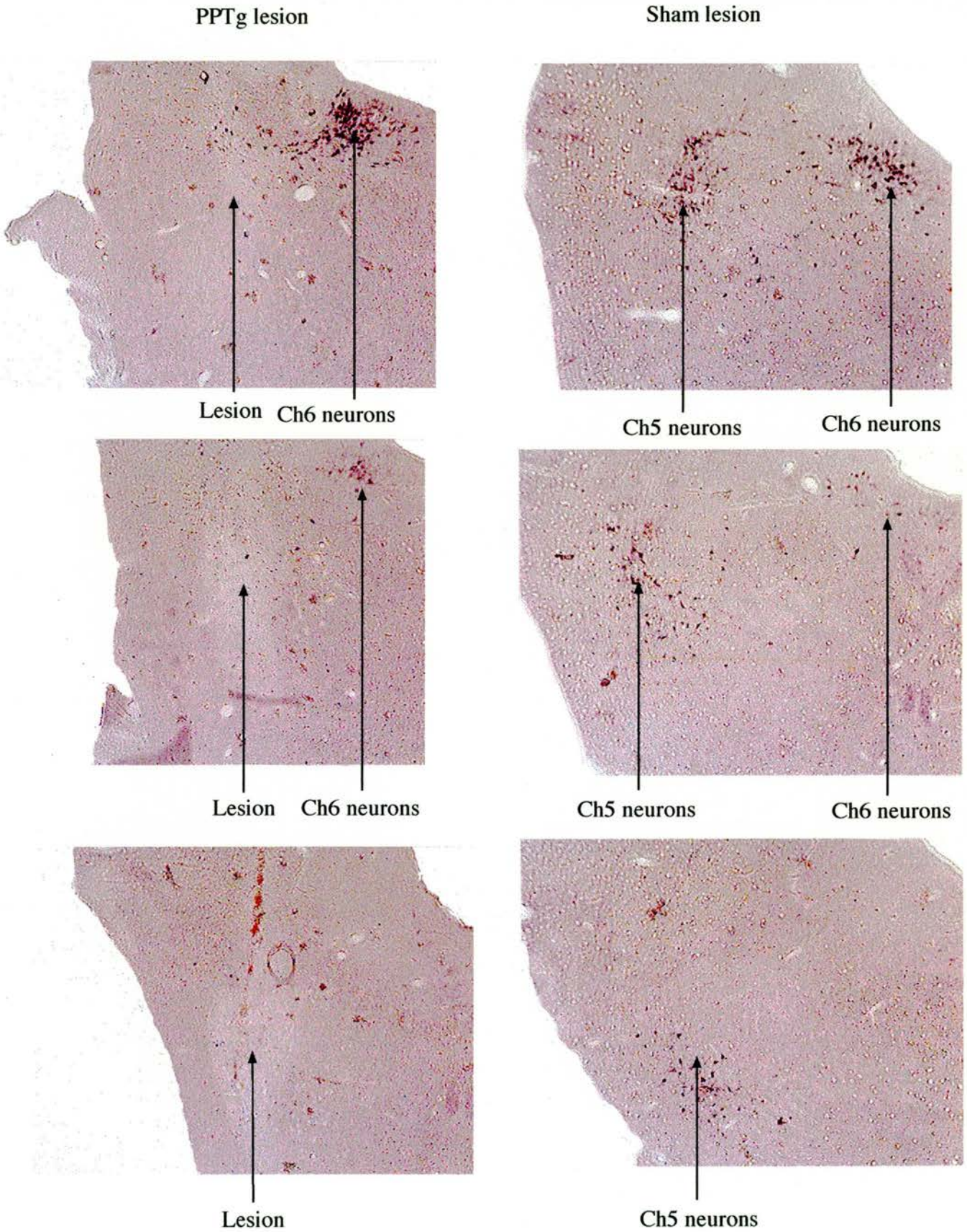
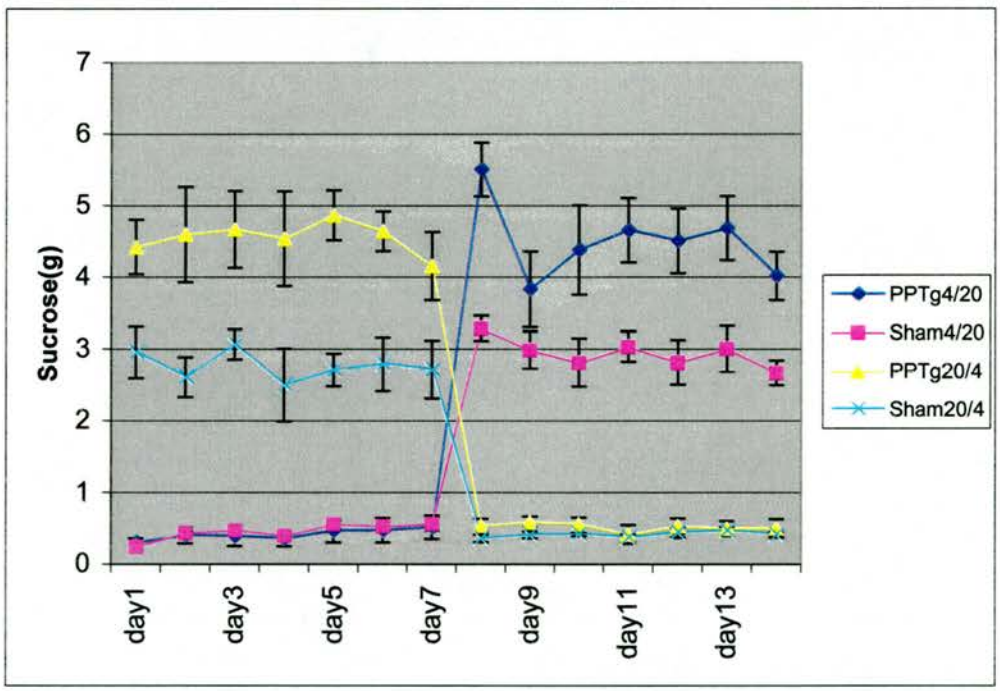


Figure 7: Comparison of PPTg lesioned brains and sham lesioned brains stained for NADPH-diaphorase



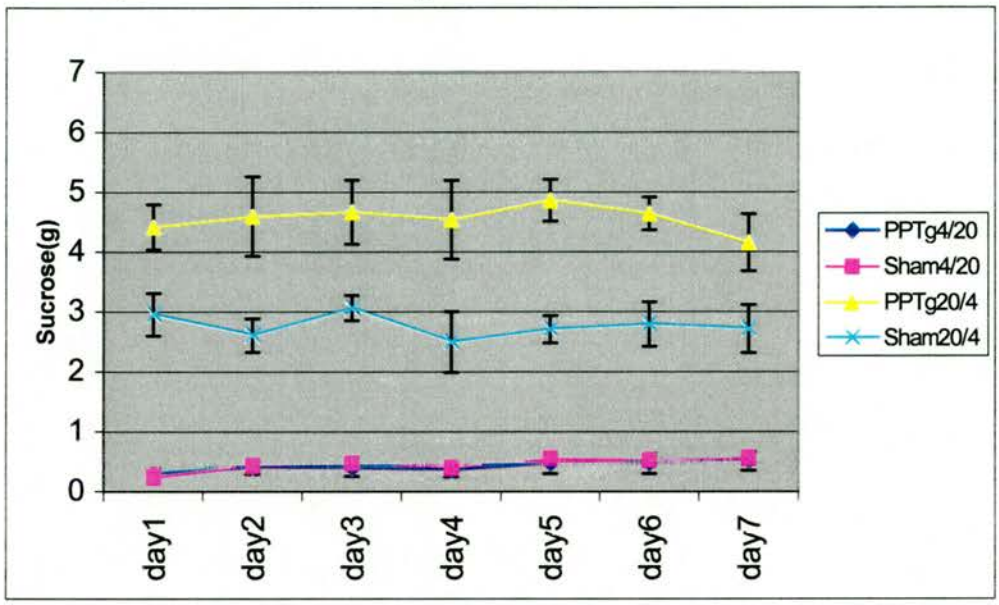
drinking 20% sucrose over-consumed when compared to shams drinking 20%. This pattern is demonstrated in Figure 8 by the dramatic changes in sucrose consumption at the end of the first week when the concentrations of sucrose received by each group are switched.

Figure 8: Sucrose consumption of PPTg and Sham lesioned rats over two weeks (mean sucrose consumption ± SE)



Analysis of amount of sucrose consumed was carried out using a repeated measures ANOVA on the data from the two weeks separately with days as the within subjects variable and group (PPTg vs sham) and concentration (20% or 4%) as the between subjects factors. These data are displayed in figures 9 and 10.

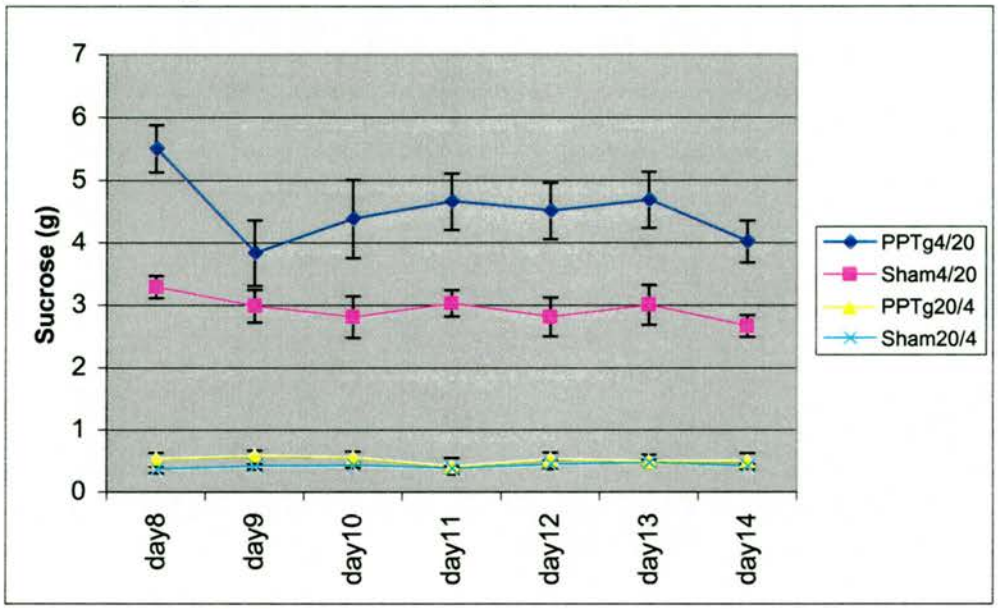
Figure 9: Sucrose consumption over the 30 min test period for days 1-7, pre-reversal (mean sucrose consumption ± SE)



Analysis of within subjects effects showed no main effect of days, no days x concentration or days x group interactions and no three-way interaction. However, between subjects effects showed significant main effects of group ($F_{1,20} = 12.31$, $p < 0.001$) and concentration ($F_{1,20} = 166.39$, $p < 0.001$) and a significant group x concentration interaction ($F_{1,20} = 13.41$, $p < 0.002$). This confirms that rats given 20% sucrose consumed more than those given 4%. It also demonstrates that PPTg lesioned rats given 20% sucrose drank more than shams receiving 20%.

Within subjects analysis from the second week show a main effect of day ($F_{6,120} = 4.27$, $p<0.001$) and a day x concentration interaction ($F_{6,120} = 4.98$, $p < 0.001$), but no days x group interaction or three way interaction.

Figure 10: Sucrose consumption over the 30 min test period for days 8-14, post-reversal (mean sucrose consumption ± SE)

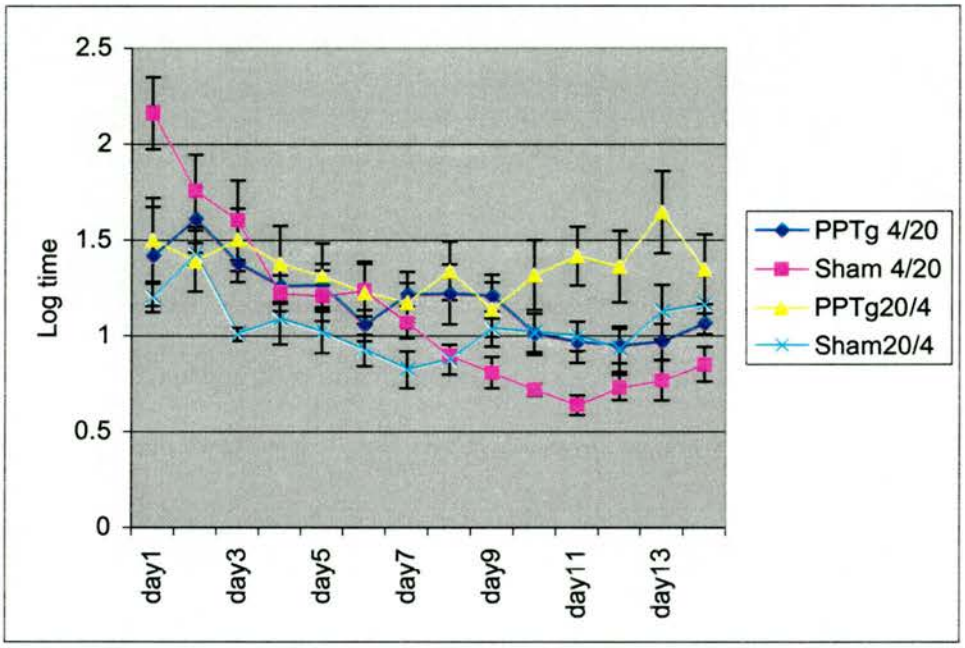


Between subjects effects show significant main effects of group ($F_{1,20} = 13.52$, $p < 0.001$) and concentration ($F_{1,20} = 205.21$, $p < 0.001$) and a significant concentration x group interaction ($F_{1,20} = 10.258$, $p<0.004$). Looking at Figure 9 it can be seen that the significant day effect and day x concentration interaction are due to the slightly variable nature of the PPTg 4/20 group. However, the same overall pattern as the first week exists with the rats given 20% sucrose drinking more than those given 4% and PPTg lesioned rats given 20% sucrose drinking more than shams given 20%.

The runway speed data was also analysed using a repeated measures ANOVA with days as the within subjects factor and group (lesion vs sham) and concentration (20% vs 4%) as the between subjects factors. The data was log transformed to

correct for positive skew and to reduce variance (Howell, 1996). Within subjects analysis revealed a significant main effect of day ($F_{13,260}$ = 11.32, p< 0.001), a significant days x concentration interaction ($F_{13,260}$ = 6.20, p< 0.001), a significant days x group interaction ($F_{13,260}$ = 2.47, p< 0.004) and a significant days x group x concentration interaction ($F_{13,260}$ = 2.116, p<0.014)

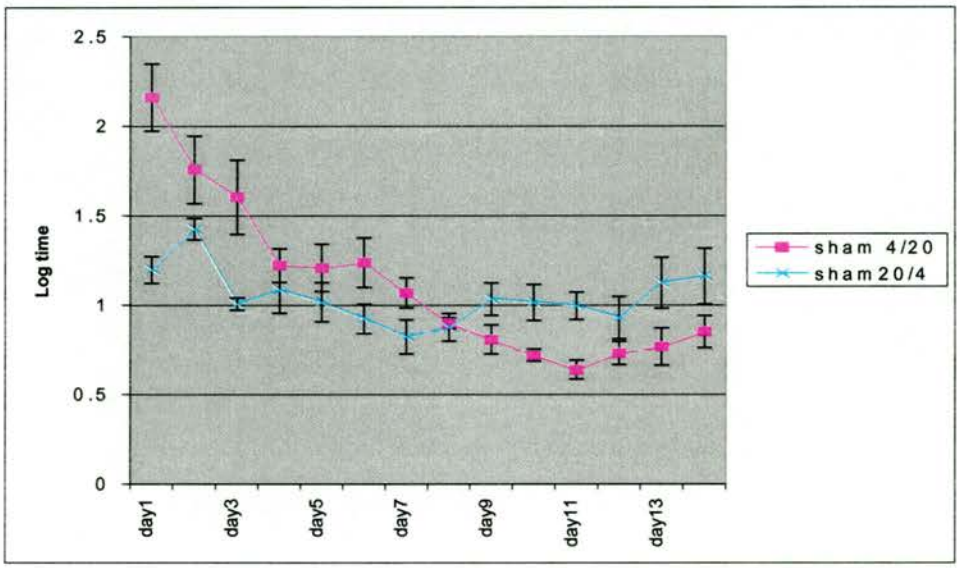
Figure 11: Log runway time for all groups (mean log time ± SE)



The data is displayed in Figure 11 which demonstrates that the relationship between the variables is far from straightforward. To gain a better understanding of the changes in runway times over the two weeks a series of restricted analyses were carried out to compare the patterns of runway times of individual groups with those of other groups. The restricted analyses involved slightly modifying the usual

ANOVA method by using the mean squares error term and degrees of freedom from the omnibus analysis to minimise the possibility of type 1 errors.

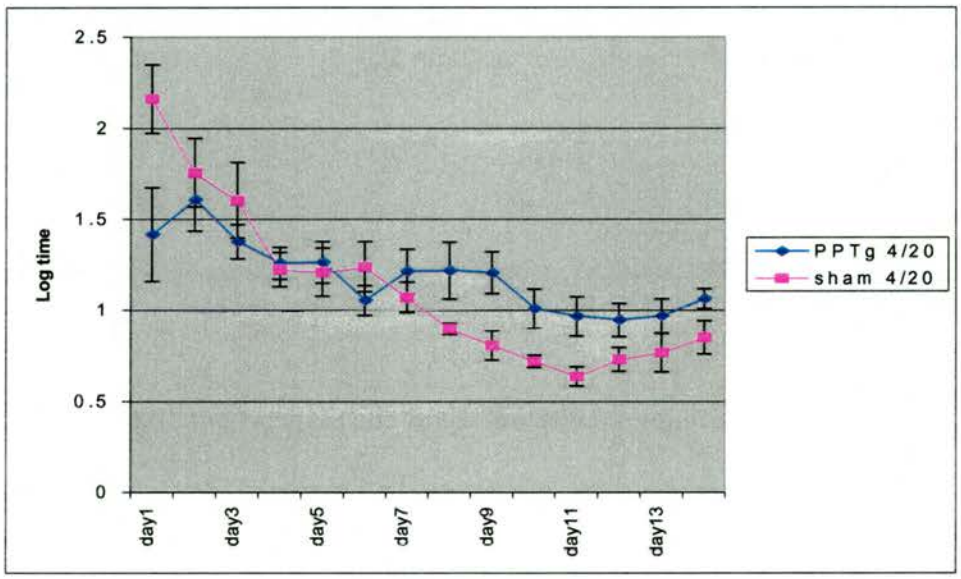
Figure 12: Comparison of runway times for the two sham lesioned groups (mean log time ± SE)



The first analysis looked at the relationship between the two sham groups. All the restricted analyses were repeated measures ANOVA with days as the within subjects variable and group as the between subjects variable with the relevant error term and degrees of freedom replaced from the omnibus analysis. Analysis revealed a significant main effect of days ($F_{13,260} = 11.52$, $p < 0.01$) and significant days x group interaction ($F_{13,260} = 6.20$, $p < 0.01$), but no main effect of group. Looking at Figure 12 it can be seen that initially the group receiving 4% sucrose solution were slower than the those receiving 20%, but in the second week when the concentrations were changed the rats whose reward increased to 20% sucrose decreased their

runway times while the times of the other group were unaffected by the change in reward.

Figure 13: Comparison of PPTg vs Sham lesioned rats changing from 4% sucrose to 20% sucrose rewards (mean log time ± SE)

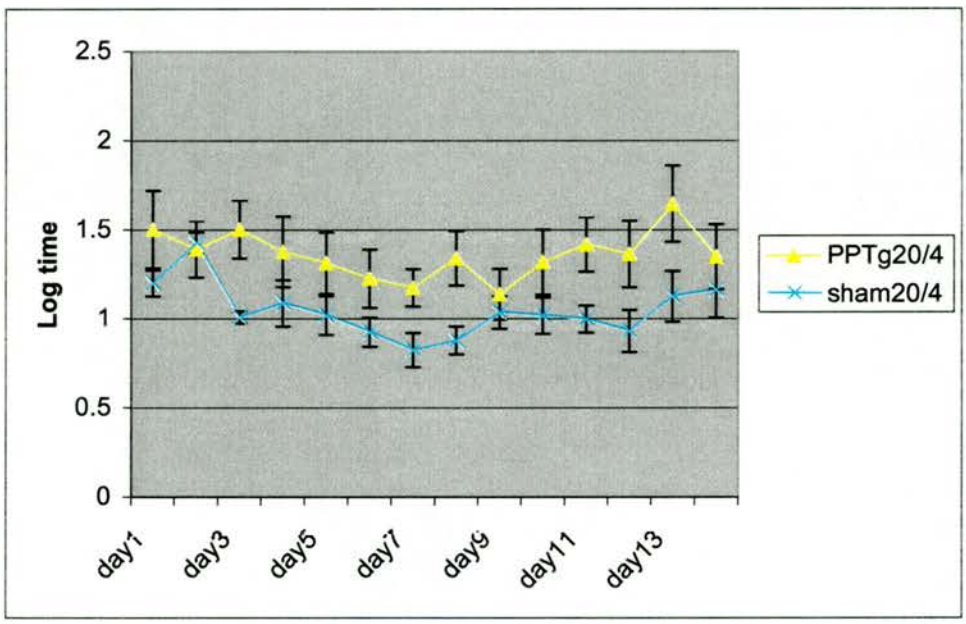


The next analysis examined the relationship between the sham and PPTg groups that received 4% sucrose in the first week and 20% in the second week. Analysis revealed a significant main effect of days ($F_{13,260} = 15.32$, $p < 0.001$) and a significant days x group interaction ($F_{13,260} = 3.66$, $p < 0.001$), but no main effect of group. The graph shows that both groups had very similar runway times during the first seven days, but in the second seven days the sham rats runway times decreased to a greater degree than the PPTg lesioned rats.

The third analysis looked at the difference between PPTg and sham lesioned animals that received 20% sucrose in the first week and 4% in the second week. This analysis revealed main effects of group ($F_{1,20} = 8.54$, $p < 0.001$) and day ($F_{13,260} =$

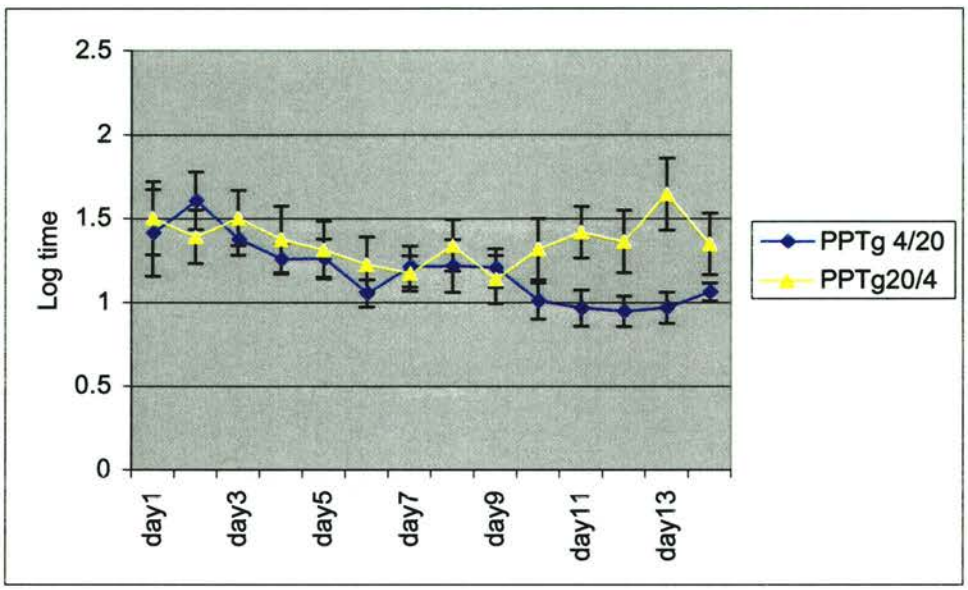
2.307, $p < 0.01$) but no group x day interaction. Figure 14 shows that while both groups did fluctuate across the two weeks the PPTg lesioned animals, with the exception of the second day, were always slower.

Figure 14: Comparison of PPTg vs Sham lesioned rats changing from 20% sucrose to 4% sucrose rewards (mean log time ± SE)



The final restricted analysis looked at the relationship between the two groups of PPTg lesioned rats. This analysis revealed a significant effect of days ($F_{13,260} = 2.26$, $p < 0.01$) and a significant group x day interaction ($F_{13,260} = 2.12$, $p < 0.01$), but no main effect of group. Looking at Figure 15 it is clear that two groups had very similar runway times until the last five days when the animals receiving 4% sucrose showed decreased runway times while those receiving 20% sucrose showed no change in runway times.

Figure 15: Comparison of runway times for the two PPTg lesioned groups (mean log time ± SE)



2.3 Discussion

The results of the study confirm the findings of Keating et al (in press) and Olmstead et al. (1999) in as much as the disinhibited drinking response to high concentrations of sucrose solution reported in these studies was once again observed in the PPTg lesioned rats in this experiment. That is to say that rats given the opportunity to drink 4% sucrose drank a fairly constant amount irrespective of lesion type. Rats given the opportunity to drink 20% sucrose consumed more than those drinking 4% and in addition to this PPTg lesioned rats drank more than sham lesioned rats. The change in sucrose concentrations at the end of the first week resulted in a reversal of the patterns of drinking response. Thus, once again, PPTg lesioned rats given 20% sucrose drank more than shams given 20% and both these groups consumed more than animals given 4%. The pattern of the sucrose drinking response was not affected in the second week by previous experience of a different level of reward. This is consistent with Olmstead et al. (1999) who showed no significant between sessions contrast effects for either sham or PPTg lesioned rats. It would appear, therefore, that the disinhibited drinking response to high concentration sucrose solution is a robust behavioural characteristic of animals with damage to this area.

The runway time data, however, are a good deal more complex. Initial inspection of the data from the sham lesioned animals demonstrates an effect that is directly predicted by the early work of Tolman and his colleagues (Tolman & Honzik, 1930b). While it is clear that all rats speeded up generally over the two

weeks of testing the patterns in change of runway time were determined by the level of reward that the animals were receiving. An interaction was demonstrated such that rats that received lower levels of rewards in the second week were relatively slower than those that went on receive higher levels of reward. It would appear that, as predicted by Tolman, rats will run relatively faster for larger rewards than for smaller rewards and indeed that the lack of effect reported by Keating (1998) was a result of rats having experience of only one concentration of sucrose reward.

If these data are now compared with that from the lesioned rats it is clear that damage to the PPTg is having some affect on the approach phase of this reward related behaviour. When PPTg and sham lesioned rats that move from 4% sucrose reward in the first week to 20% sucrose reward in the second week are compared it is clear that initially runway times were very similar. However, when the rewards were changed the sham lesioned rats started to run faster while the PPTg lesioned rats showed fairly constant runway times across the two weeks. A similar pattern is seen in the two groups that changed from high reward to low reward in that shams running for 20% sucrose ran faster than PPTg lesioned rats running for the same reward. This effect is carried through into the second week such that the PPTg lesioned animals appeared to be consistently slower of the two week testing period.

The data from the last two comparisons would appear to suggest that the approach phase of the runway behaviour is significantly altered in the PPTg lesioned rats. While sham lesioned rats appeared to find the 20% sucrose a more motivating factor than the 4%, PPTg lesioned rats showed little change in runway speed irrespective of the level of reward. This could be interpreted as demonstrating an

altered level of motivation such that the higher incentive properties of the 20% sucrose are not processed by rats with PPTg damage.

This would, however, be inconsistent with the studies cited in the introduction which suggested that PPTg lesions had no effect on either primary or incentive motivation (Keating et al., in press; Olmstead et al., 1999). These studies suggested that animals with PPTg damage have normal perceptions of the incentive values of different concentrations of sucrose and further more have the ability to use this information to shape their behavioural responses. An explanation in terms of levels of motivation would result in the curious and somewhat paradoxical conclusion that PPTg lesions decrease motivation while simultaneously increasing consumption of positively rewarding stimuli.

One possible way of reconciling these apparently contradictory conclusions is apparent when the data from the two groups of rats with PPTg lesions are directly compared. During the first ten days of the experiment the level of reward presented to the two groups produced no difference in the time taken to complete the runway task. However, if the final four days of testing are examined it is clear that eventually the level of reward did begin to predict the level of performance as measured by runway time. From day eleven onwards PPTg lesioned rats running for 20% sucrose began to run faster than those running for 4% sucrose in the same way that the sham lesioned rats did immediately after the change in reward was made. This could be interpreted as a deficit in either learning or attention such that PPTg lesioned rats take considerably longer than sham lesioned rats to learn that the level of reward has changed.

This explanation would appear to be much more consistent with other behavioural data from PPTg lesioned rats as this can also be seen as an impairment in response control and is entirely consistent with other experiments showing deficits in tasks involving learning and attention (Keating & Winn 1998, Dellu et al., 1991; Inglis et al., 2001). Thus the clear behavioural deficits seen in both the approach and consummatory phases of this behaviour can explained in terms of breakdown in executive function. Results from this study could be explained in terms of a decreased level of motivation in PPTg lesioned rats but this is not consistent with findings from the studies cited in the introduction or those examining other behavioural paradigms. For example in the radial arm maze PPTg lesioned rats have problems in performing the delayed spatial win shift task due to tendency to perseverate by revisiting arms from which the reward has already been retrieved (Keating & Winn, 2002). During this task, however, the time taken to make choices was not affected and rats retrieved all the pellets that were available, which is not consistent with a decreased level of motivation, either primary or incentive, for food. The next chapter will further examine this disinhibited drinking response by studying the consummatory behaviour of PPTg lesioned rats given the opportunity to drink differing concentrations of sucrose, saccharin and quinine. This will give an insight into the affects of PPTg damage on response control by examining the intake of non-nutritive rewards and aversive stimuli.

Investigation of the psychological processes underlying disinhibited behaviour in PPTg lesioned rats

3.0 Introduction

Experimental findings from the previous chapter have given some insight into the complex behavioural deficit produced by excitotoxic lesions of PPTg in the rat. The results appear to be consistent with the hypothesis put forward by Winn (1998) that lesions in this area produce a breakdown in executive processes that impair the rat in a number of subtle ways. The cause of these deficits, however, remains unclear. While it is unlikely that all experimental findings in this area will be explained in terms of one underlying deficit, it may be the case that the original hypothesis put forward by Winn (1998) could be refined to give a clearer understanding of the exact nature of the behavioural problems associated with PPTg damage. At present the only research involved in addressing this issue is the investigation of attention deficits in PPTg lesioned rats, but as stated in the introduction this type of deficit cannot account for all the behavioural abnormalities reported following damage to this nucleus.

The term executive function is very poorly defined. This can, at least in part, be explained by the ambiguity surrounding the central executive component of the working memory model from which the term is derived. This ambiguity has resulted in considerable overuse of the term to the point where its value in contemporary research is somewhat debatable. Thus it would appear that while the Winn review

served the useful purpose of suggesting a role for the PPTg in high level cognitive functions it is clearly only a beginning in the process of accurately describing the root of the behavioural deficit induced by PPTg damage. Further experiments must be carried out to examine the nature of the executive problems in PPTg lesioned rats and consequently break down the term executive function and into more meaningful components.

It is of course entirely conceivable that executive function may be a unitary construct made up of several interdependent cognitive processes. If this is indeed the case then isolating specific cognitive deficits in these animals may not be possible. However, these types of experiment will, at the very least, help describe the exact nature of the executive dysfunction in these rats and may reveal a more fundamental problem that has, as yet, gone undetected. This chapter aims to start to address this issue by investigating further the behavioural abnormalities observed in these rats. Findings from the previous chapter suggest that an explanation of these lesion effects in terms of levels of motivation or perception of reward do not adequately explain the experimental findings to date.

Experimental work in this chapter will attempt to examine further the consummatory phase of the behaviour studied in the previous chapter. That is the tendency of PPTg lesioned rats to overconsume high concentrations of sucrose solution. This disinhibited drinking response is not seen when examining consumption of low concentrations of sucrose and thus there must be some property of the high concentration sucrose solution that induces this behaviour. To attempt to ascertain the stimulus properties that induce this disinhibited drinking response

further experiments were carried out to examine the effect of PPTg lesions on the consumption of unpalatable stimuli, namely various concentrations of quinine solution. Further the effects on consumption of positive, yet non-nutritive, solutions were examined by investigating the effects of PPTg damage on saccharin intake. Two different experiments were carried out using the same solutions to examine the effects of time on the quantities of solution consumed. In the first phase of the experiment rats were allowed 6 hours to consume the test solution and in the second phase rats were given 24 hours access to the test solution.

Disinhibited behaviour in these animals has been previously examined using negative reinforces in the study of the pre-pulse inhibition of the startle reflex. In these studies it was demonstrated that the normal reduction of the startle reflex seen in rats facilitated by a warning tone is absent following PPTg damage (Koch et al., 1993). Interestingly it could be suggested that in both of the cited examples of disinhibtion, lesioned rats are incapable of inhibiting a previously appropriate response. If this is indeed the case then it may be expected that PPTg lesioned rats would consume less quinine than sham lesioned rats as this is the appropriate response in this situation. Further it might be predicted that patterns of saccharin consumption would be unchanged in these animals as saccharin is non-nutritive and consequently there is no benefit to increasing consumption.

In contrast to this prediction the increased consumption of high concentrations of sucrose may be due to increased finickiness in PPTg lesioned rats similar to that seen in animals with lesions of the ventral noradrenergic bunble (VNAB; Sahakian et al., 1983). Rats with VNAB lesions show normal feeding patterns when maintained

on lab chow, but show increased intake of novel palatable foods while simultaneously showing decreased intake of food adulterated by quinine. If the altered sucrose consumption is due to increased finickiness then an increase in saccharin consumption combined with a simultaneous decrease in quinine consumption would be predicted.

One further factor to be taken into consideration is that the disinhibition of sucrose drinking in PPTg lesioned rats was observed when they were in a state of high motivational excitement. That is in response to high concentration sucrose and not to low concentrations. While results from the previous chapter argue against the behavioural deficit in PPTg lesioned rats being a result of altered levels of motivation, it may be the case that an impairment in response control in these animals, manifested by disinhibtion of sucrose drinking, is only seen at high levels of motivational excitement. If this is the case then it would be predicted that quinine consumption would not be affected by damage to the PPTg as this is an aversive stimuli which would not produce motivational excitement, while saccharin consumption should be disinhibited at high concentrations.

3.1 Method

Subjects

The subjects were 20 adult male Lister-hooded rats (average pre-surgery weight: 297.30g) housed individually in a temperature and light controlled (12-hr light-dark cycle) colony. All experimental and care procedures followed guidelines laid down by UK and European legislation (Animals [Scientific Procedures] Act, 1986, and European Communities Council Directive of 24 November 1986 [86/609/EEC]. All rats were given ad libitum access to food and water after surgery until they returned to their free feeding weight at which point they were deprived to 85% of their free feeding weight and maintained at that by 15-20g SDS maintenance #1 food pellets daily. Rats were tested on consecutive days for the first part of the experiment and every other day in the second phase of the study.

Surgery

Surgery was identical to that performed on the second group of animals from the previous study. That is to say, that lesions were made using two separate injections of 0.12M ibotenic acid (pH adjusted to 7.0).

Procedure

The experiment was run in two stages. In each stage food deprived animals were presented with differing concentrations of either sucrose, saccharin or quinine in their home cage with their normal water supply being withdrawn. In the first phase

of the experiment rats were presented with the test solution for six hours during which the animals were free to drink as little or as much of the solution presented as they wanted. Different solutions were presented on successive days such that each animal was given the opportunity to drink each concentration of each solution on a different day. The orders of presentation of both solution and concentration were fully counterbalanced across and within groups. The concentrations of sucrose selected were 4%, 12% and 20%. These were chosen in an attempt to replicate previous research demonstrating disinhibited drinking in PPTg lesioned rats in response to 20% but not 4% sucrose (Alderson et al., 2001; Keating et al., in press; Olmstead et al., 1999). The concentrations of quinine and saccharin used were 0.01%, 0.1% and 0.3%. These concentrations were chosen as previous research had shown that control rats consumed sufficient quantities of each to allow meaningful comparisons across groups to be made in this study (Clark et al., 1990). Results from the first stage of the experiment, however, showed that rats were consuming very low quantities of quinine and consequently a second phase of the study was required. The second phase of the experiment was identical to the first except that animals were given the test solutions for 24 hours. This change in the procedure required testing to be carried out every second day to ensure that rats were allowed reasonable access to water.

Histology

At the end of the experiment rats were perfused as in the last experiment. Once again lesion size and placement was assessed using cresyl violet and NADPH-diaphorase histochemistry (see appendix for procedures).

Statistical Analysis

Quantities of solutions consumed were analysed using repeated measures ANOVA with concentration of solution as the within subjects factor and group (lesion vs. sham) as the between subjects factor. Further analysis to determine specific differences between sham and PPTg lesioned groups was done using planned comparisons.

3.2 Results

Lesion size was assessed as in the last experiment using NADPH-diaphorase and cresyl violet histochemistry. These stains compliment each other well by staining any surviving cholinergic cells of the PPTg as well as visualising reactive gliosis. Lesions were assessed to ensure that they destroyed at least 70% of PPTg neurons bilaterally without causing excessive damage to its surrounding structures as shown in Figure 16 and 17. Using these criteria one rat was excluded from the analysis as lesion damage extended rostrally from the PPTg causing damage to the retrorubral fields. Another rat died while recovering from surgery and consequently each group contained nine rats.

Data from the initial phase of the experiment in which rats were exposed to the test solutions for 6 hour periods were examined first. The patterns of consumption of the different solutions are illustrated in Figures 18 and 19. The data are presented both in terms of grams consumed and millilitres consumed. Analysis in terms of millilitres consumed illustrates the actual quantities of liquid drunk by the rats. Analysis in terms of grams consumed is most useful for the sucrose data as sucrose is a food source and so it may be more meaningful to analyse the amount of sucrose rather than the quantity of liquid consumed. Clearly analysis in terms of grams is less meaningful for quinine and saccharin as these solutions are non-nutritive but these analyses are included to compare with the sucrose data.

Figure 16: Comparison of PPTg lesioned and sham lesioned brains stained for NADPH-diaphorase

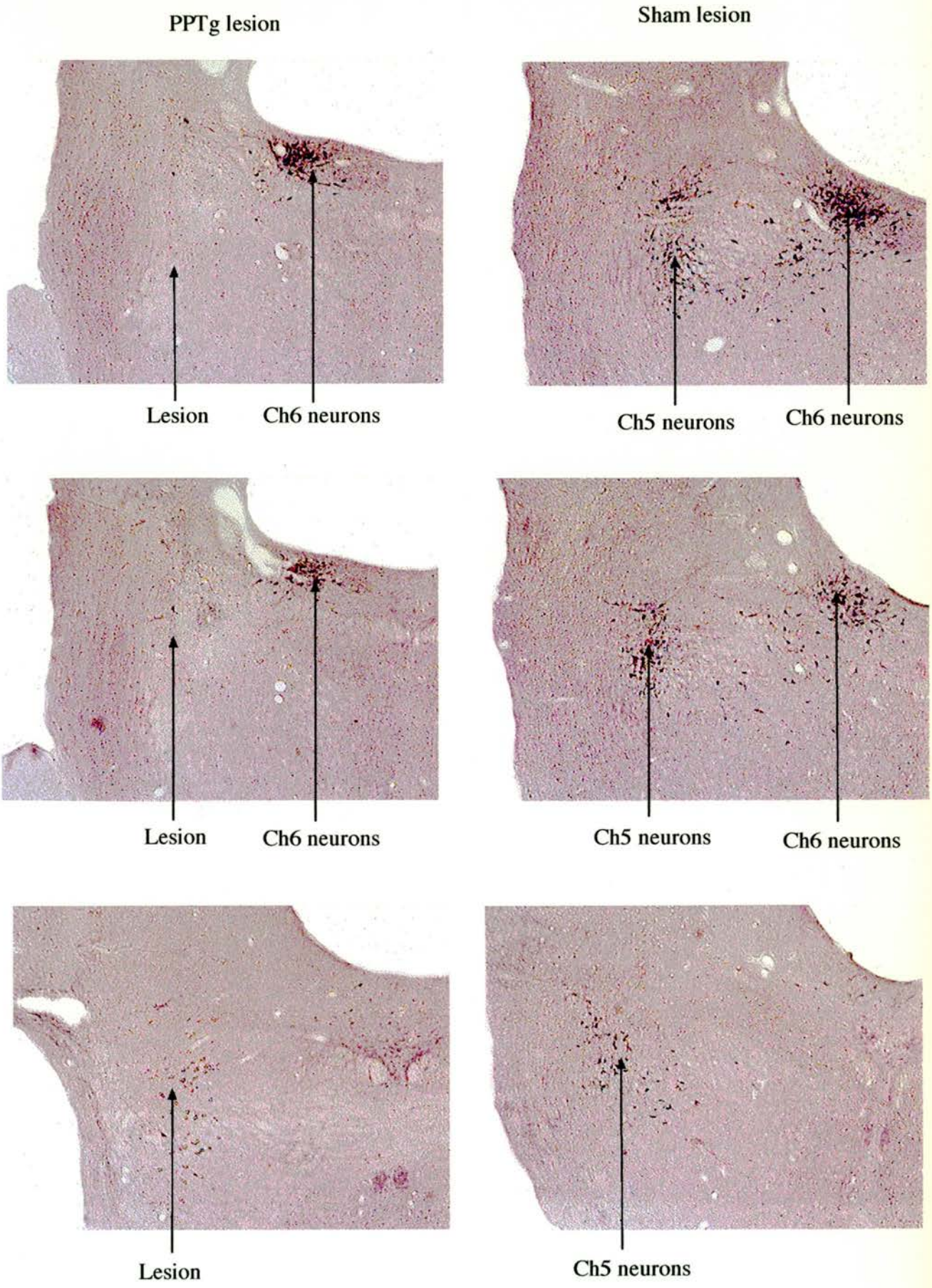


Figure 17: Schematic representation of largest and smallest lesions

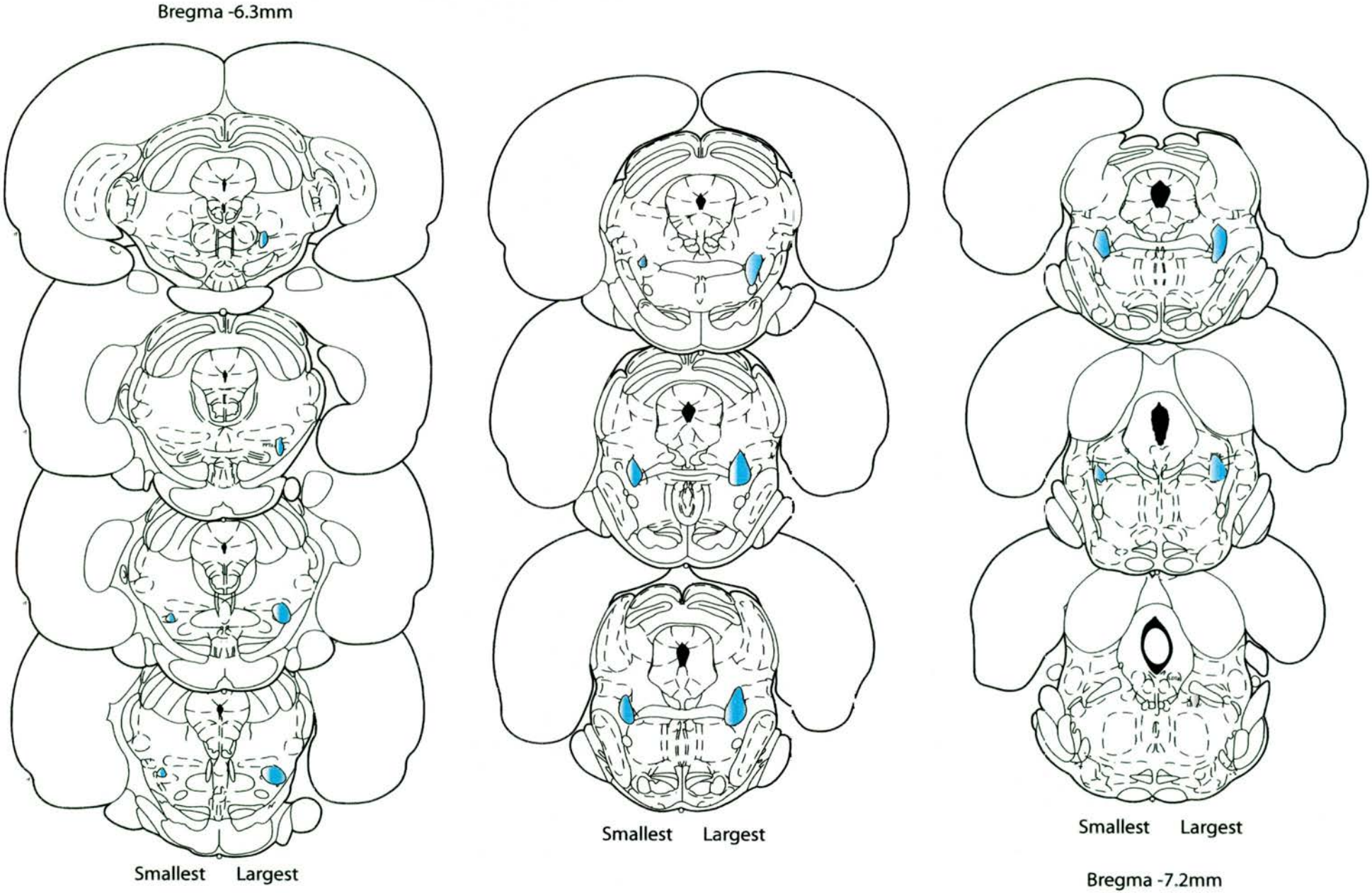


Figure 18: Grams of all solutions consumed over a 6 hour period (mean consumption ± SE)

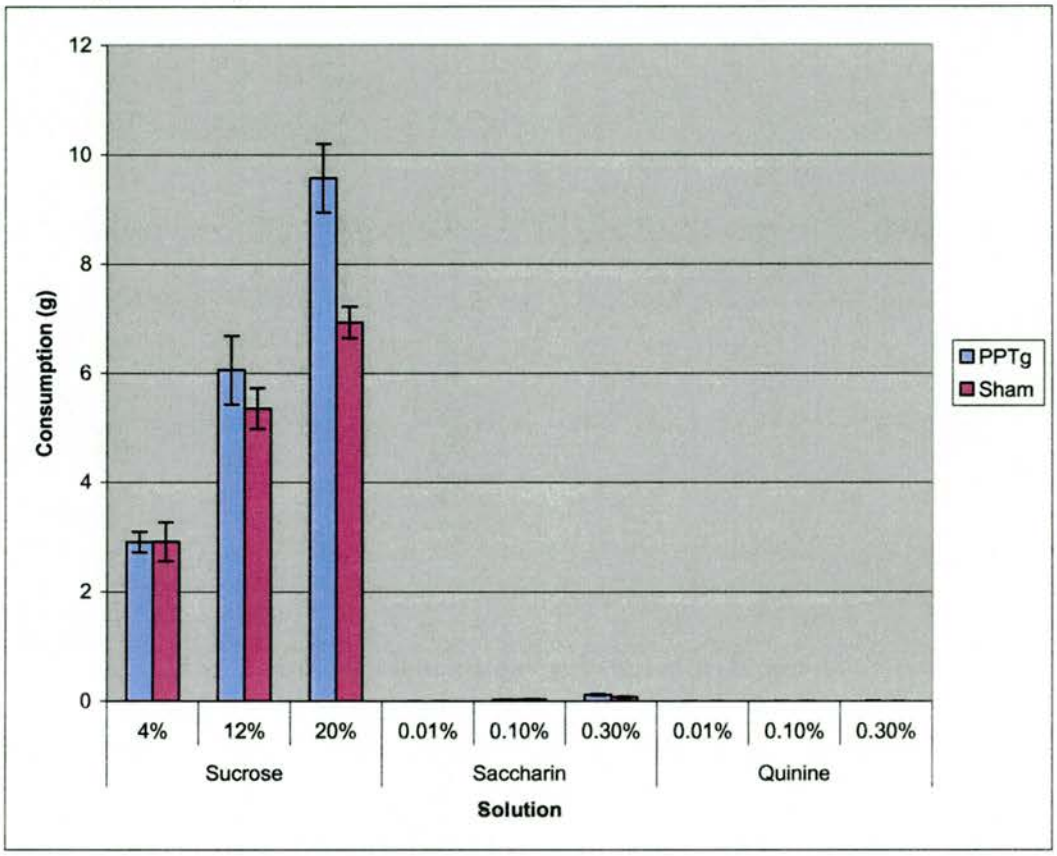
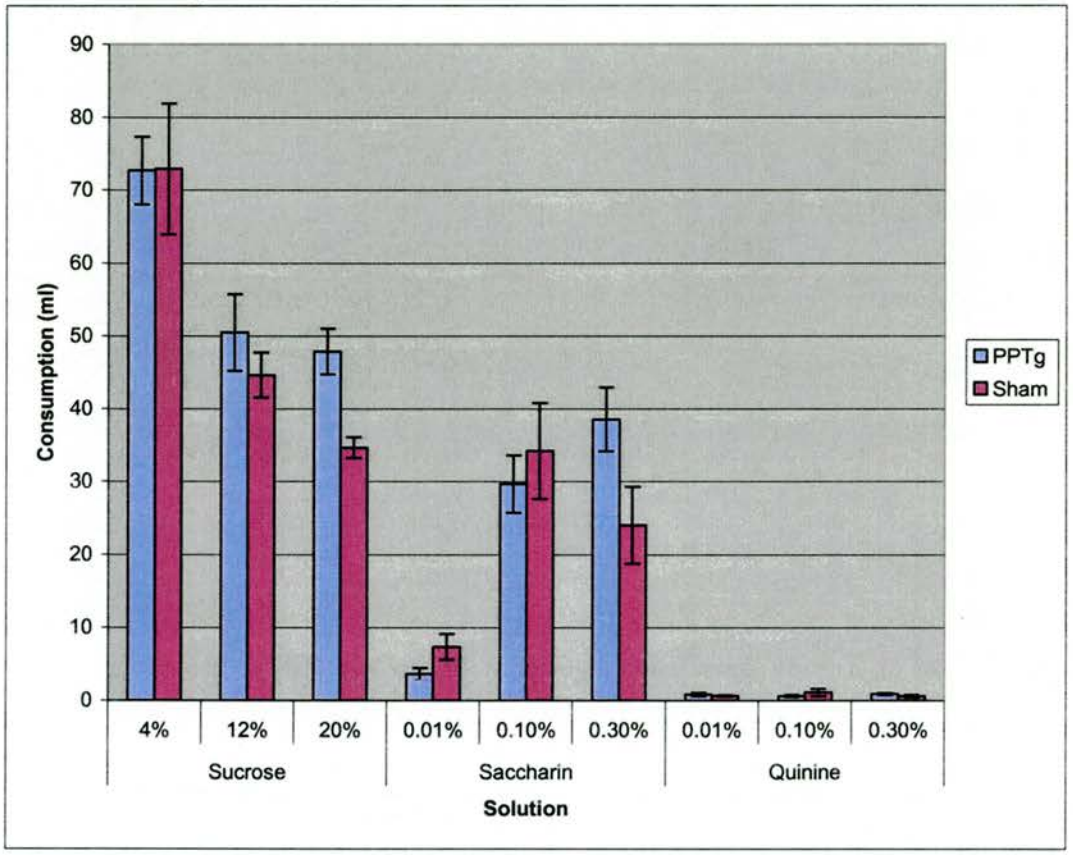


Figure 18 demonstrates that in terms of grams, rats consume a great deal more sucrose than either saccharin or quinine. This is what would be expected as the concentrations of the sucrose solutions used are much higher than the saccharin or quinine solutions and consequently an animal that drinks equal volumes of the three types of solution will be consuming more grams of sucrose than the other substances presented. Perhaps more enlightening are the data presented in Figure 19. This figure shows that rats consume more sucrose than either of the other two solutions and further consume more saccharin than quinine. While this is useful as an overview of

the effects in this study it tells us little about the effects of concentration of solution or lesion on consummatory behaviour, consequently a series of analyses on the data for each type of solution consumed were performed.

Figure 19: Millilitres of all solutions consumed over a 6 hour period (mean consumption ± SE)



Quantities of solutions consumed were analysed using repeated measures ANOVA with concentration of solution as the within subjects factor and group (lesion vs. sham) as the between subjects factor. Quantities of the different concentrations of quinine consumed by the groups are illustrated in Figures 20 and 21. Figure 21 demonstrates that irrespective of concentration or lesion type rats

consumed very low volumes of quinine. This is confirmed by the analysis that reported no significant main effects of either group or concentration or any significant group x concentration interaction. Analysis of the data from Figure 20 revealed a main effect of concentration ($F_{2,32} = 18.184$, $p<0.001$) but no main effect of group and no group x concentration interaction. This effect is a result of an increased level of intake in the high concentration quinine groups.

Figure 20: Grams of quinine consumed over a 6 hour period (mean consumption ± SE)

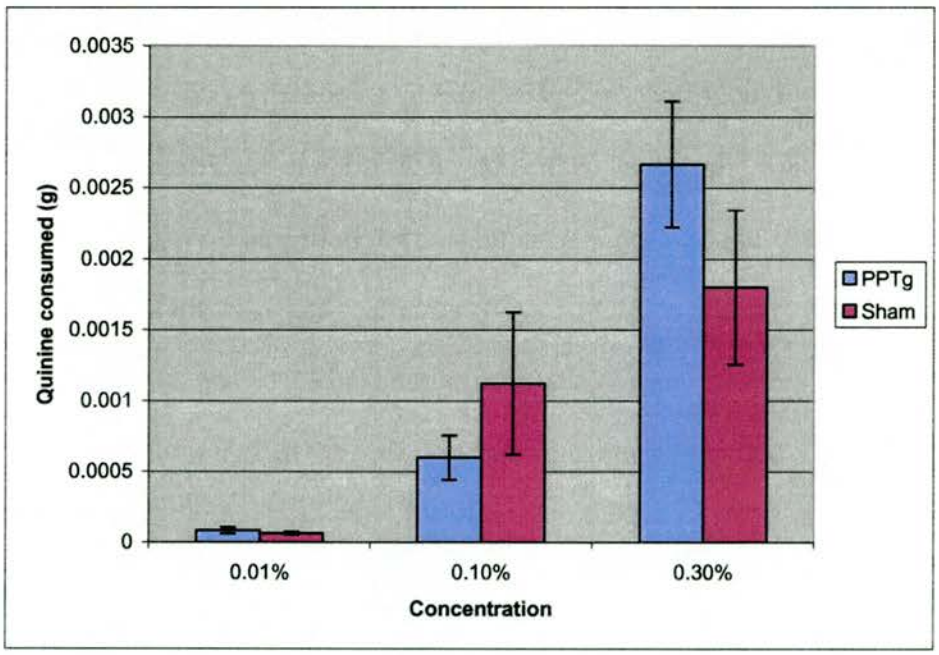
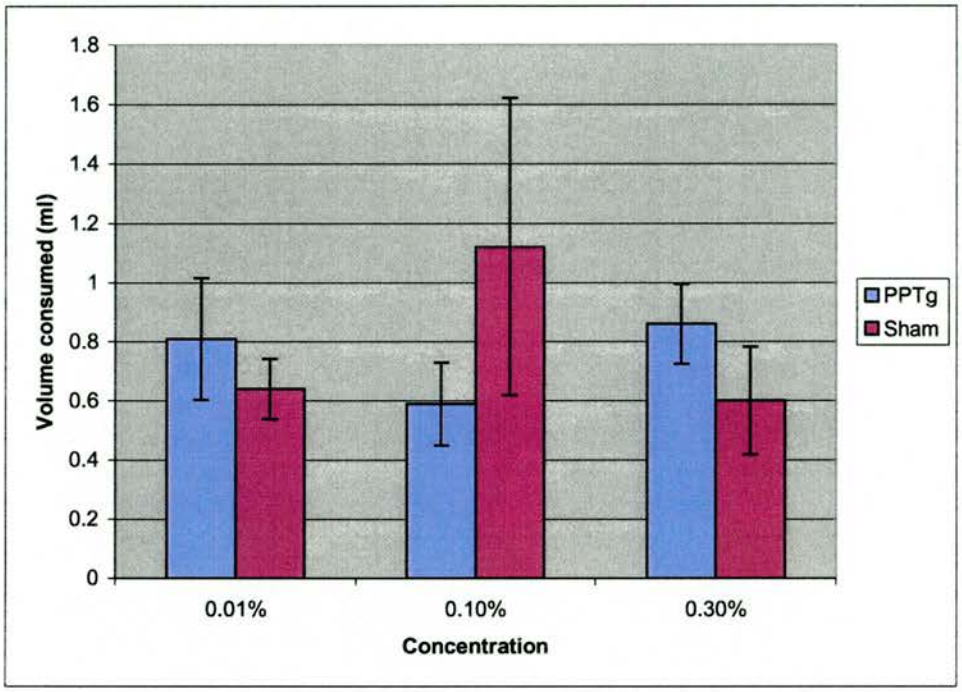


Figure 21: Millilitres of quinine consumed over a 6 hour period (mean consumption ± SE)



The pattern of sucrose consumption for the initial 6 hour exposure period is displayed in Figure 22. It can be clearly seen that rats given the opportunity to consume sucrose solution consume larger quantities when the concentration is high. That is to say that as the concentration of sucrose increases the amount of sucrose consumed by rats also increases. This is confirmed by the analysis. Analysis of within subjects effects revealed a significant main effect of concentration ($F_{2,32}$ = 92.807, $p<0.001$) and a significant concentration x group interaction ($F_{2,32}$ = 6.130, $p<0.006$). Analysis of between subjects effects revealed a main effect of group ($F_{1,16}$ = 5.795, $p< 0.028$). To examine the possibility that the disinhibited drinking effect reported in the last experiment was again present in this study, planned comparisons comparing the consumption of sucrose across groups for each concentration were carried out. These showed that while sham and PPTg lesioned rats did not consume

significantly different quantities of sucrose at low concentrations, PPTg lesioned rats again overconsumed compared to sham lesioned rats when given the opportunity to consume 20% sucrose ($t = 3.691$, $df = 16$, $p<0.002$).

Figure 22: Grams of sucrose consumed over a 6 hour period (mean consumption ± SE)

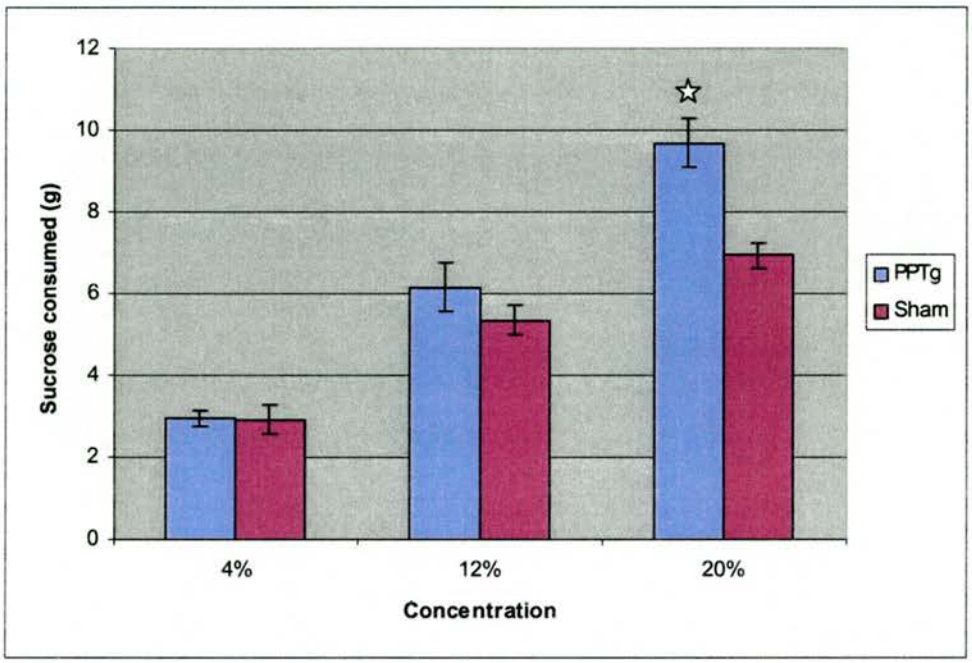


Figure 23 also demonstrates that PPTg lesioned animals overconsume high concentrations of sucrose solution. Interestingly the actual volume of solution consumed is highest in the low concentrations of sucrose although the pattern of consumption does not differ across lesion type. Analysis of between subjects effects revealed no significant main effect of group or group x concentration interaction. Within subjects effects analysis revealed a significant main effect of concentration was seen ($F_{2,32} = 24.865$, $p<0.001$). The lack of effect of group is slightly surprising and will be discussed further in the discussion.

Figure 23: Millilitres of sucrose consumed over a 6 hour period (mean consumption ± SE)

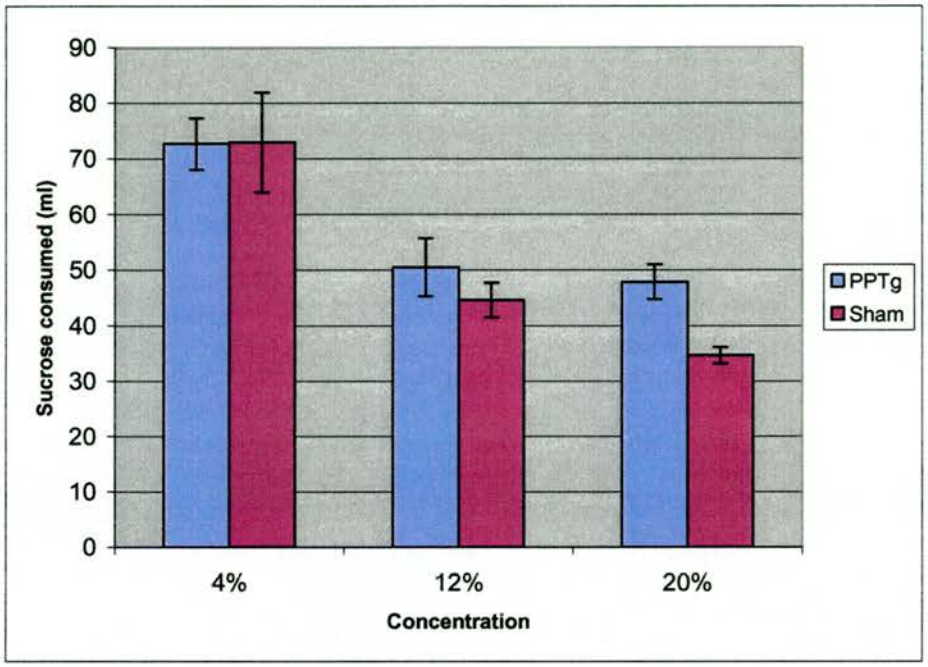


Figure 24 illustrates the pattern of consumption of saccharin in the initial phase of the study. At the lowest concentration both sham and PPTg lesioned rats consumed very small amounts of saccharin. However, at the higher concentrations both groups increased their intake. The analysis helps clarify what exactly was happening in this phase of the study. Analysis of within subjects effects revealed a significant main effect of concentration ($F_{2,32}$ = 66.585, $p<0.001$) as well as a significant group by concentration interaction ($F_{2,32}$ = 5.240, $p<0.011$). Between subjects analysis, however, demonstrated no main effect of group. From the figure it would appear that the significant interaction may be due to the increased consumption of the highest concentration of saccharin by the PPTg lesioned rats. To investigate the possibility that a disinhibited drinking response to the different concentrations of saccharin was present planned comparisons of the two groups

consumption for each concentration were performed. It was found that the volumes of saccharin consumed by the two groups were not significantly different at any of the different concentrations examined. However the difference in 0.3% saccharin intake approached significance ($t = 2.065$, $df = 16$, $p < 0.056$).

Figure 24: Grams of saccharin consumed over a 6 hour period (mean consumption ± SE)

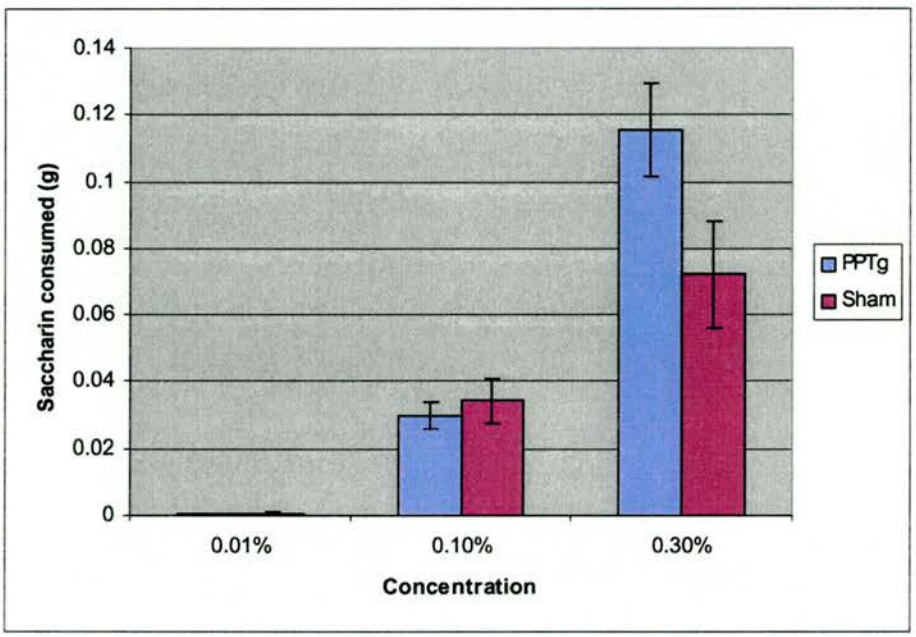
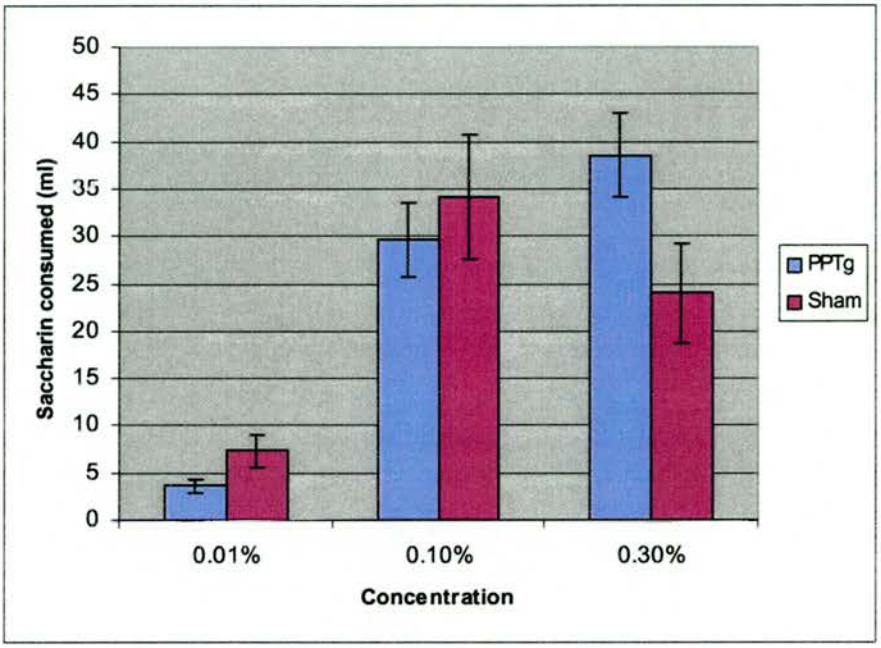


Figure 25 illustrates the actual volumes of saccharin consumed. Analysis of these data reveal the same pattern of results seen in the grams consumed data. That is to say there was a significant main effect of concentration ($F_{2,32} = 35.389$, $p < 0.001$) as well as a significant group x concentration interaction ($F_{2,32} = 4.507$, $p < 0.019$). Analysis of between subjects effects revealed a similar lack of effect of group. Further planned comparisons revealed no differences between any of the groups.

Figure 25: Millilitres of saccharin consumed over a 6 hour period (mean consumption ± SE)



Data from the second phase of the study, in which rats were given 24 hours to consume the test solutions are illustrated in Figures 26 and 27. Once again the pattern of consumption of solutions in grams shows that more sucrose was consumed than either of the other two solutions, at least in part due to the differing concentrations used. The pattern of consumption in terms of millilitres shows a similar pattern to that seen in the first phase of the study with rats consuming greater quantities of sucrose and saccharin than quinine. However, in this phase of the experiment the difference in consumption of sucrose and saccharin is reduced, especially at high concentrations.

Figure 26: Grams of solutions consumed over a 6 hour period (mean consumption ± SE)

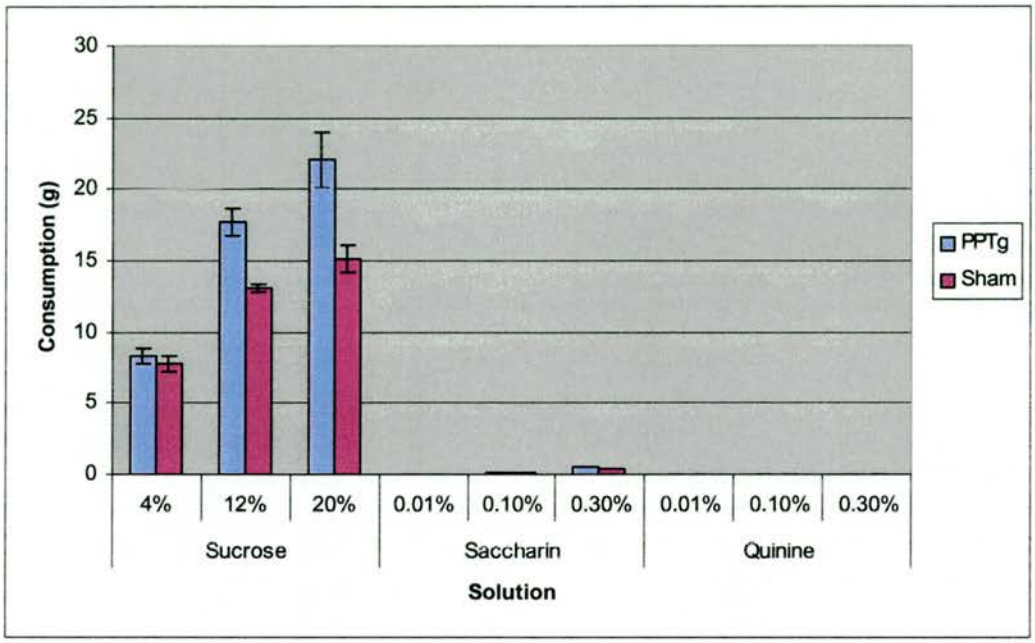
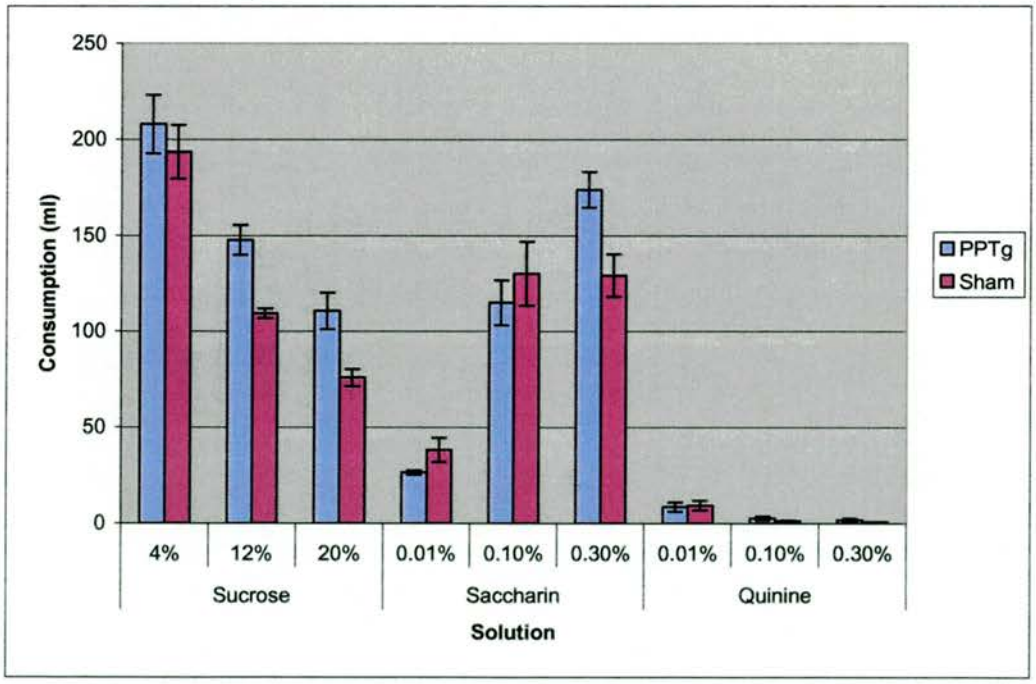


Figure 27: Millilitres of solutions consumed over a 6 hour period (mean consumption ± SE)



As in the first phase of the study the data were analysed using repeat measures ANOVA and planned comparisons. Figure 28 illustrates that pattern of quinine consumption in grams across the different concentrations used. As in the initial phase of the study consumption of the low concentrations of quinine was very low. However, consumption of the higher concentrations of quinine was considerably higher than in the previous phase. As expected from the figure analysis of the within subjects factors revealed a significant main effect of concentration ($F_{2,32} = 6.229$, $p<0.005$), but no interaction effect. Between subjects factor analysis revealed no main effect of group. The analysis suggests that consumption of the higher concentrations of quinine was greater than the lowest concentration, but that no difference in the pattern of consumption across groups was present.

Figure 28: Grams of quinine consumed over a 24 hour period (mean consumption ± SE)

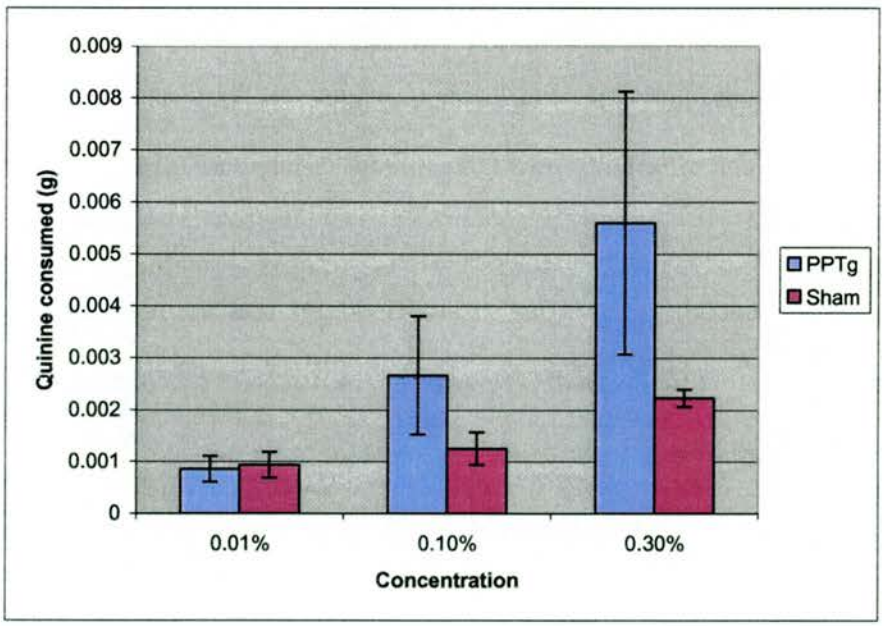
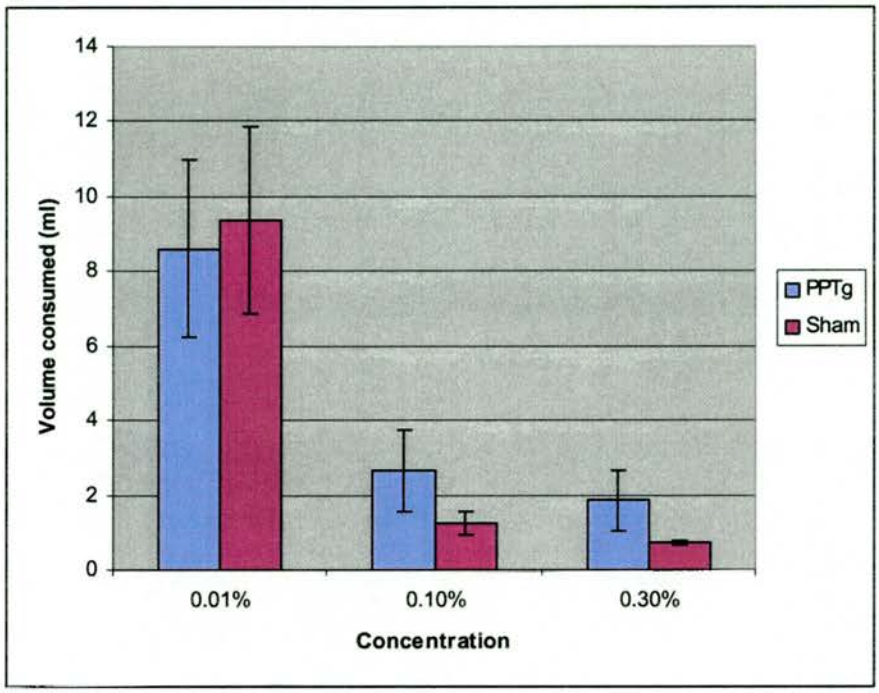


Figure 29: Millilitres of quinine consumed over a 24 hour period (mean consumption ± SE)

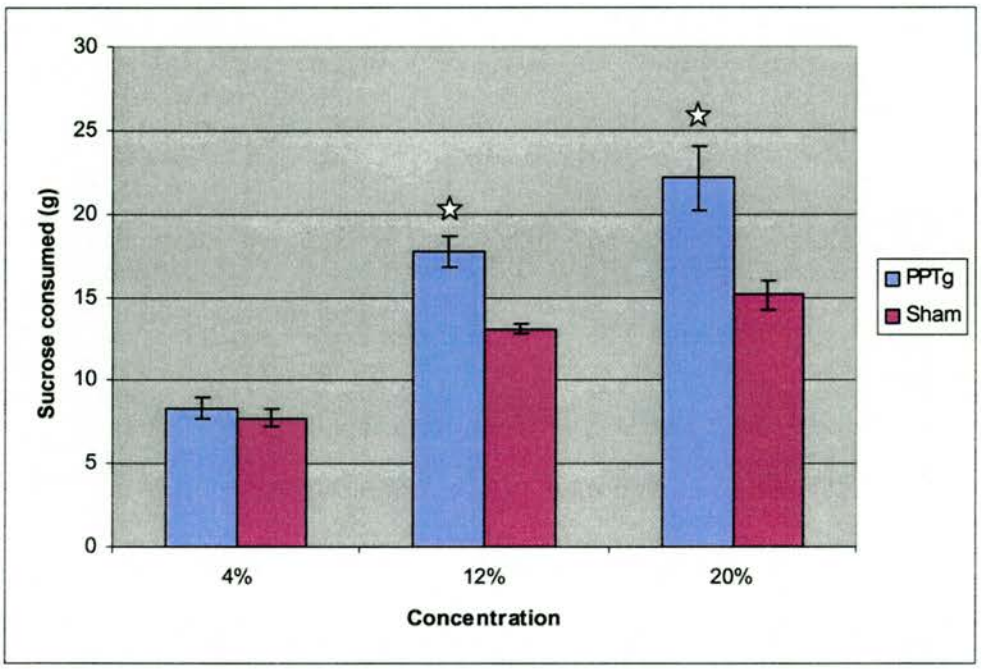


Analysis of the volumes of quinine consumed showed a similar lack of effect of group or any group x concentration interaction. However, a significant effect of concentration was seen ($F_{2,32}$ = 21.033, $p<0.001$), although the pattern of consumption appears to be reversed when the data are analysed in terms of volume consumed.

Figure 30 illustrates the grams of sucrose consumed in the 24 hour period. As in the previous phase of the study consumption of sucrose increased as the concentration increased. The figure would also suggest that the disinhibited drinking response seen in the PPTg lesioned rats was again present in the 12% sucrose condition as well as the 20% sucrose condition. Analysis of within subjects factors revealed a main effect of concentration ($F_{2,32}$ = 55.982, $p<0.001$) and a significant

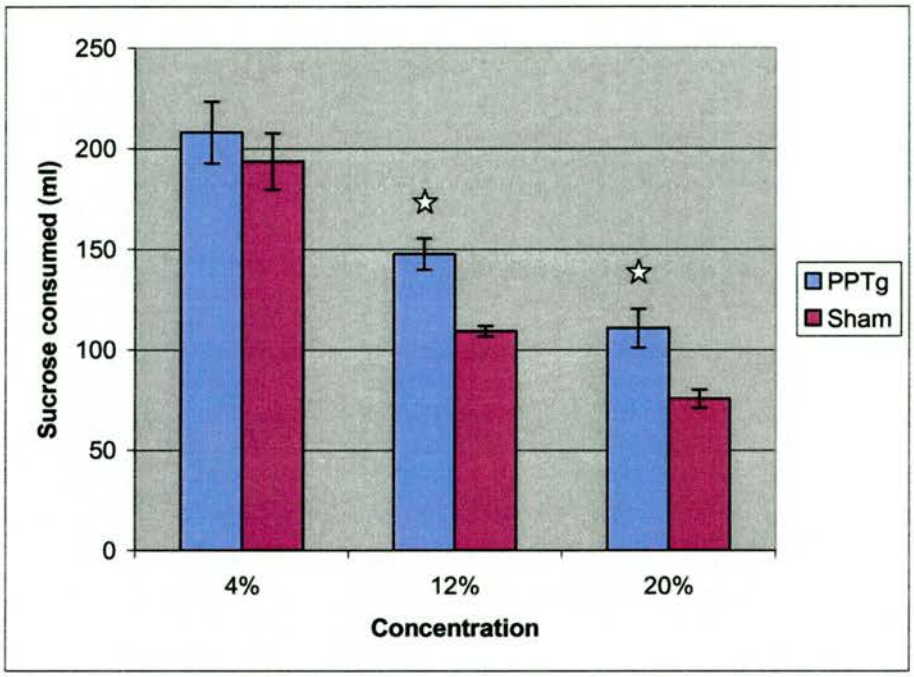
concentration x group interaction ($F_{2,32}$ = 4.979, $p<0.013$). Analysis of between subjects factors revealed a significant main effect of group ($F_{2,32}$ = 20.652, $p<0.001$)

Figure 30: Grams of sucrose consumed over a 24 hour period (mean consumption ± SE)



To examine the possibility that the disinhibited drinking response to high concentrations of sucrose was again present planned comparisons of the quantities of sucrose consumed by the two groups were examined for each sucrose concentration. These revealed that PPTg lesioned rats drank significantly more 12% (t = 4.521, df =16, p< 0.001) and 20% (t = 3.174, df=16, p<0.006) sucrose than sham lesioned rats, while no difference in consumption of 4% sucrose was seen. The actual volumes of the different sucrose solutions are illustrated in Figure 31.

Figure 31: Millilitres of sucrose consumed over a 24 hour period (mean consumption ± SE)



Analysis of these data produced a very similar pattern to that seen on the grams consumed data. Within subjects effects analysis revealed a significant main effect of concentration ($F_{2,32}$ = 59.531, $p<0.001$), but no group x concentration interaction while between subjects analysis revealed a significant main effect of group ($F_{2,32}$ = 10.614, $p<0.005$). Planned comparisons once again revealed that PPTg lesioned rats drank significantly more 12% ($t = 4.521$, $df = 16$, $p< 0.001$) and 20% ($t = 3.174$, $df=16$, $p<0.006$) sucrose than sham lesioned rats, while no difference in consumption of 4% sucrose was seen.

Finally the patterns of consumption of the various concentrations of saccharin by the two groups are displayed in Figures 32 and 33. As in the previous phase of the study rats consumed more of the higher concentrations of saccharin than the lower

concentrations. Analysis of the grams consumed data illustrated in Figure 32 suggests that the disinhibited drinking response seen in PPTg lesioned rats to high concentrations of sucrose is present when these rats are given 0.3% saccharin for 24 hours. Analysis of within subjects factors revealed a significant main effect of concentration ($F_{2,32}$ = 307.89, $p<0.001$) and a significant concentration x group interaction ($F_{2,32}$ = 9.562, $p<0.001$). Analysis of between subjects factors revealed a significant main effect of group ($F_{2,32}$ = 4.651, $p<0.047$). As in the previous phase planned comparisons of the volumes of saccharin consumed by the two groups were carried out for each concentration. These showed that there was no significant difference in the quantity of 0.01% or 0.1% saccharin consumed by the two groups. However, it was also shown that PPTg lesioned rats consumed significantly more 0.3% saccharin solution than sham lesioned rats ($t = 3.012$, $df = 16$, $p<0.008$).

This pattern was also seen in the volume consumed analysis. Within subjects factors analysis revealed a significant main effect of concentration ($F_{2,32}$ = 85.942, $p<0.001$)0 and a significant concentration x group interaction ($F_{2,32}$ = 6.275, $p<0.005$). Analysis of between subjects factors revealed no significant main effect of group. Planned comparisons showed that there was no significant difference in the quantity of 0.01% or 0.1% saccharin consumed by the two groups. However, it was again shown that PPTg lesioned rats consumed significantly more 0.3% saccharin solution than sham lesioned rats ($t = 3.012$, $df = 16$, $p<0.008$). Clearly the disinhibited drinking response to high concentrations of sucrose seen in these rats is also manifested when they are presented with high concentrations of saccharin.

Figure 32: Grams of saccharin consumed over a 24 hour period (mean consumption ± SE)

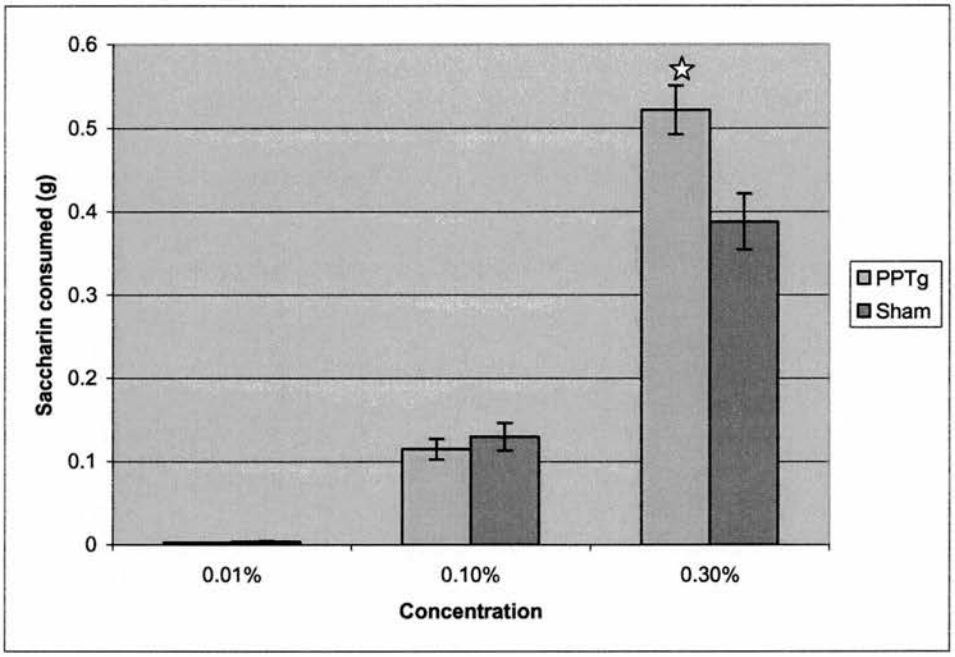
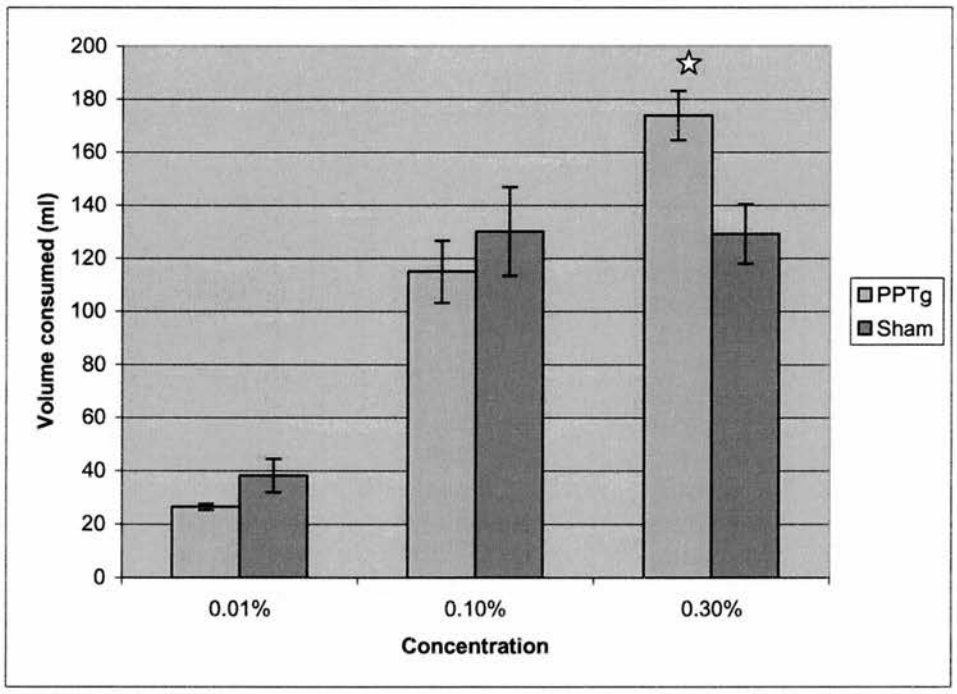


Figure 33: Millilitres of saccharine consumed over a 24 hour period (mean consumption ± SE)



3.3 Discussion

Results from this experiment reveal a number of interesting findings. The consumption data from the 6 hour phase of the experiment revealed that lesions of the PPTg had no effect on the consumption of either quinine concentration. The saccharin data, however, are of more interest. The initial ANOVA revealed a significant group x concentration interaction which would appear to be due to the PPTg lesioned rats drinking more of the high concentration saccharin than the shams. Unfortunately this did not come out as being significant when examined using planned comparisons. The pattern seen in these data are, however, very similar to that reported in the sucrose consumption data as the difference in levels of consumption of high concentration saccharin across groups approached significance in this phase of the study ($p<0.056$). As previously reported PPTg lesioned animals overconsume high concentrations of sucrose and this was again seen in this phase of the study.

Results from the 24 hour phase of the study were much more enlightening. The sucrose data, once again, showed disinhibited drinking of 20% sucrose by PPTg lesioned rats, but interestingly the effect was also seen for consumption of 12% sucrose solution in this stage of the study. As in the previous phase, no group differences in the levels of quinine consumption were found. Data from the saccharin consumption showed that at this time period the consumption of high concentration solutions was disinhibited in the PPTg lesioned animals.

These data provide an insight into the possible underlying psychological processes behind the disinhibited drinking response in these rats. The lack of group differences in the levels of quinine drinking is hard to explain in terms of the first two possible underlying mechanisms suggested in the introduction. The lack of effect of lesion of this area on consumption of quinine is not consistent with the hypothesis that PPTg lesions produce a disinhibition of appropriate responding. Were this the case the PPTg lesioned rats would be expected to show decreased levels of quinine consumption. However, neither are these data consistent with an explanation in terms of an increased level of finickiness, which would also predict a decrease in the levels of quinine consumption. It would appear that PPTg lesioned rats show no potentiation of the tendency to avoid food substances adulterated with quinine. Interestingly a recent study examining conditioned taste aversion in PPTg lesioned rats suggested that these animals are unable to use aversive properties of food substances to shape their behaviour (Mediavilla et al., 2000). That is to say that rats with PPTg lesions did not learn to avoid foods that had previously been paired with aversive stimuli. While this is not the case in this experiment, as lesioned animals showed different levels of consumption depending on the concentration of quinine presented, it is interesting that rats with damage in this area have been shown to have a problem interpreting aversive properties of novel stimuli. The findings from Mediavilla et al. should, however, be treated with caution as the lesions made in this study were electrolytic which cause a large amount of non-specific damage.

Moving on to examine the saccharin data, it is clear that lesions of the PPTg have had a significant effect on consumption levels. The pattern of saccharin

drinking over 24 hours was very similar to that seen in the sucrose drinking data. Once again this is not consistent with an explanation in terms of a disinhibition of appropriate responding. On the other hand, this is consistent with the hypothesis that these rats have an increased level of finickiness, although this is unlikely in light of the quinine data. It would appear that, at least in terms of consummatory behaviour, disinhibited responding is only seen in these animals in response to appetitve stimuli and further only in response to high concentrations of these rewarding solutions. This pattern of behaviour is consistent with the hypothesis put forward in the introduction that rats with lesions of the PPTg have an impairment in response control which is only manifested in states of high motivational excitement. This would explain the lack of effect of PPTg damage on quinine drinking while also providing an explanation for the increased consumption of saccharin at high concentrations.

4.0 Discussion

Experimental results from this chapter have produced some interesting insights into the behavioural deficits produced by excitotoxic lesions of the PPTg and the consequent functional role of this nucleus. Studies cited in the introduction to this section illustrate the volume of research that has attempted to understand what role the PPTg performs in higher cognitive functions. The research to date appears to suggest that lesions in this area produce a behavioural deficit best described in terms of a breakdown in executive functions and attention. However, it remained unclear as to whether this nucleus had a role to play in reward related behaviour. Previous studies had reported that the PPTg was involved in the neural circuit that supported brain stimulation responding mediated through the lateral hypothalamus (Lepore & Franklin, 1996; Buscher et al., 1989). Further evidence for a role in reward processing came from studies by Van Der Kooy and colleagues who showed that PPTg damage disrupted the formation of CPP to some conditioned reinforcers.

However, in contrast to these previous experiments, a series of studies using a standardised lesion method demonstrated that PPTg lesioned rats form normal CPP to sucrose (Keating et al., in press; Alderson et al., 2001) as well as showing normal discrimination and contrast effects (Olmstead et al., 1999). Interestingly while no clear effect on either primary or incentive motivation were reported one common finding from these studies was that PPTg damage produced a disinhibited drinking response to high concentrations of sucrose solution. In an attempt to examine the approach phase of this disinhibited behaviour Keating (1998) used a simple runway

paradigm as a very direct measure of motivation. PPTg and sham lesioned rats were trained to run down a runway to receive either a 4% or 20% sucrose solution reward. Early work by Tolman suggested that rats should run faster for a greater reward. However, Keating reported no effect of concentration of reward on either sham or PPTg lesioned rats. One possible explanation for this result is that the rats in this experiment only had experience of one concentration of sucrose. To examine this possibility the experiment was repeated incorporating a reversal at the end of the first week, such that those rats that had been receiving 20% sucrose now received 4% and vice versa. Rats were tested for a further week with the new level of reward. Further work by Tolman suggested that normal rats would increase their runway speed for higher levels of reward and decrease their speed for lower rewards and this was indeed found to be the case. PPTg lesioned rats, however, showed a different pattern of responding. Initially it appeared that PPTg lesioned rats showed generally slower runway speeds than the sham rats resulting in the curious conclusion that lesions in this area produced a decrease in motivation and a simultaneous increase in consumption of high concentration sucrose reward. However, it was shown that while the PPTg lesioned rats were slower after the concentrations were switched they did eventually alter their runway times according to the level of reward. This suggested that the behavioural deficit produced was best interpreted in terms of an impairment in response control following PPTg damage.

These data are consistent with the hypothesis that the PPTg is involved in the neural circuits associated with executive functions and attention. While some of the data could be explained in terms of an altered level of motivation, this hypothesis is

not consistent with many studies of rats with PPTg damage including those examining CPP (Keating et al., in press), reward discrimination and contrast effects (Olmstead et al., 1999). Further experiments examined the underlying psychological mechanisms of the disinhibited sucrose drinking by measuring the consumption of aversive and non-nutritive solutions. It was demonstrated that PPTg lesioned rats showed similar patterns of consumption of quinine solution to sham lesioned rats. Further these rats showed increased consumption of high concentrations of saccharin, compared to sham lesioned rats, when exposed to the solutions for 24 hours. The lack of effect of PPTg lesion on consumption of aversive stimuli argues against the deficit being a result of disinhibition of an appropriate response or an altered level of finickiness. While the over-consumption of high concentrations of saccharin solution combined with the well documented increase in consumption of high concentrations of sucrose solution suggests that this disinhibited drinking response is the manifestation of an impairment in response control only seen at high levels of motivational excitement.

These results suggest that the deficit seen in these lesioned rats are best explained in terms of either a breakdown in executive functioning or a deficit in attention. Explanations involving a deficit in the processing of reward cannot account for both the increased consumption of sucrose and saccharin solutions combined with the decreased runway speed. The implications of this work will be discussed later in the thesis. The following section will present work attempting to understand how altered activity in the PPTg impacts on higher neural systems with a

view to clarifying its position in the neural circuitry involved in higher cognitive function.

Section B: Functional anatomy of the mesopontine tegmental projections to the thalamus

1.0 Introduction

The studies described in last section explained the complex behavioural deficits produced by discrete lesions of the PPTg. These data strongly suggest that disruption of the circuits in which the PPTg are involved produces changes in reward related behaviour that perhaps are best understood in terms of response control. These findings are consistent with studies reported in the introduction that suggest a role for the PPTg in the systems controlling higher cognitive functions including executive function and attention. Having demonstrated that lesions of the PPTg produce this complex behavioural deficit it is important to address the issue of how and where the PPTg impacts on these higher neural systems. The anatomical connections of the structure would suggest a number of possible routes through which altered neural activity within the PPTg could affect the functioning of structures higher in the neuraxis but within the same functional network. The first of these is through its innervation of the substantia nigra and consequent role in the regulation of striatal dopamine systems. The second is through its innervation of the thalamus and consequent control over cortical functioning. The third is through its innervation of the lateral hypothalamus and basal forebrain which themselves have input to different areas of cortex. Finally it is also possible that altered PPTg activity could affect the outflow of information from higher structures like the striatum. The

next section will deal with the literature to date examining the effects of PPTg activity on higher brain structures and then go on to present experimental work examining activity in the thalamus following stimulation of the PPTg.

1.1 Substantia Nigra

One of the major ascending efferent connections of the PPTg is with the substantia nigra (SN; Jackson & Crossman, 1983; Rye et al., 1987; Beninato & Spencer, 1987; Gould et al., 1989; Bolam et al., 1991; Lavoie & Parent, 1994a; Lavoie & Parent, 1994b; Blaha & Winn, 1993; Futami et al., 1995; Oakman et al., 1995; Charara et al., 1996; Oakman et al., 1999; Parent et al., 1999). Many studies have reported a substantial projection to the pars compacta region of the SN from the PPTg including both cholinergic and non-cholinergic components (Lavoie & Parent, 1994a). While these studies provide a clear demonstration of the existence of the pathway they provide little information regarding its functional properties. There have, however, been a number of studies examining these properties using several different methodologies.

In a series of experiments Blaha & Winn (1993) used *in vivo* microdialysis and electrochemistry combined with pharmacological manipulation of the SN and lesions of the PPTg to examine the relationship between the mesopontine tegmentum and the striatal and nigral dopamine systems. Initially different cholinergic agents were microinjected into the substantia nigra and dopamine outflow in the dorsomedial striatum was measured. It was demonstrated that microinjections of

both nicotinic and muscarinic agonists into the SN produced substantial increases in extracellular dopamine in the dorsomedial striatum. They went on to show that inhibition of acetylcholinesterase (AChE) activity through application of neostigmine also produced an increase in striatal dopamine. These data are consistent with the suggestion that AChE is present within pars compacta neurons of the SN as well as clearly demonstrating that cholinergic agonists stimulate the neurons in this area.

In further experiments the effects of lesions of the PPTg on this cholinergic dependent dopamine efflux were examined. It was shown that in rats with PPTg damage, the levels of striatal extracellular dopamine following microinjections of neostigmine were significantly reduced, while levels of dopamine following microinjections of nicotine were significantly increased. This is most interesting as the dopamine efflux following nigral neostigmine application in non-lesioned rats was presumably dependent on the inhibition of AChE activity and the subsequent potentiation of endogenous ACh. Consequently removal of the cholinergic input to the SN would be expected to remove this endogenous ACh and decrease the resultant dopamine efflux. The decrease of neostigmine stimulated dopamine efflux following PPTg lesions reported in this study is therefore good evidence for suggesting that the cholinergic input to the SN-pc originates in the PPTg. Further support for this idea comes from the data showing that the dopamine efflux following nicotine application was potentiated in PPTg lesioned rats. Most probably the post-synaptic receptors have become supersensitised due to the lack of cholinergic input from the PPTg

resulting in increased activity within the SN-pc and an increased efflux of dopamine in the mediodorsal striatum.

Interestingly this series of experiments was repeated to examine the interactions between the mesopontine tegmental cholinergic neurons and the dopamine systems of the ventral tegmental area (VTA) and the nucleus accumbens (NAcc). Using the same methodologies it was demonstrated that both microinjections of cholinergic agonists into the VTA as well as inhibition of AChE activity in the VTA by microinjections of neostigmine produced pronounced and prolonged dopamine efflux in the NAcc. To examine further the effects of lesions of the different mesopontine tegmental cell groups on the dopamine efflux in the accumbens the experiments were repeated in PPTg and LDTg lesioned rats. The results produced a very neat reflection of those from the original study examining the PPTg and nigral dopamine systems. Lesions of the PPTg were shown to have no effect on dopamine efflux in the accumbens while lesions of the LDTg produced a profound drop in the efflux stimulated by microinjections of neostigmine into the VTA. This suggests that dopaminergic activity in the VTA and NAcc is controlled to some extent by the cholinergic input from the LDTg. Taken together these studies produce a coherent picture of the functional organisation of these cholinergic mesopontine neurons and the midbrain dopamine systems. It would appear that the cholinergic input from the PPTg controls activity within the SN-pc and thus affects activity in the caudate putamen, while cholinergic outflow from the LDTg controls dopamine activity in the VTA and consequently affects accumbens dopamine systems. These data are consistent with the LDTg being included in the systems of

the brain involved in the processing of reward information while suggesting that the PPTg is more involved in the processing of sensorimotor information.

In a further series of experiments the effect of direct cholinergic manipulation of the PPTg on striatal dopaminergic activity was examined. When reviewing these data it is important to first understand the effect that cholinergic drugs have in the mesopontine tegmentum. It has been demonstrated that cholinergic agonists have the effect of decreasing the activity of the cholinergic outflow from this area while cholinergic antagonists have the opposite effect and facilitate cholinergic activity (Yeomans et al., 1993; Chapman et al., 1997). This suggests strongly that cholinergic activity in this area is controlled by inhibitory autoreceptors on the cholinergic neurons. Chapman et al. (1997) examined the effects of cholinergic agents in this area on the dopaminergic outflow in the striatum. Using both systemic injections and specific microinjections into the PPTg it was demonstrated that scopolamine increased cholinergic activity through disinhibtion of PPTg neurons which resulted in increased dorsal striatal dopamine efflux. Further pre-treatment with carbachol attenuated the dopamine efflux in the dorsal striatum reinforcing the conclusion that cholinergic activity is driving the midbrain dopamine system.

In other studies electrophysiological and voltammetric measurements have been used to examine the relationship between the PPTg and the SN. Hernandez-Lopez et al. (1992) used *in vivo* voltammetry to measure the levels of dopamine metabolites in the dorsal striatum following inhibition of AChE activity in the SN and also following stimulation of the PPTg using kainic acid. In direct support of the previous studies inhibition of AChE in the SN resulted in dopamine efflux in the

dorsal striatum and stimulation of the PPTg also resulted in an increase in dopamine activity. Interestingly the demonstration of increased dopamine following kainic acid injections into the PPTg suggests that the glutamatergic component of the nucleus is also important in the control of nigral and striatal dopamine activity as kainic acid binds with glutamate receptors.

A number of other studies have examined the electrophysiological responding of midbrain dopamine neurons following stimulation of neurons within the PPTg (Clarke et al., 1987; Kelland et al., 1993; Meltzer et al., 1997; Lokwan et al., 1999). Clarke et al. (1987) performed a series of experiments helping to establish the existence of the pedunculonigral pathway. It was demonstrated that excitation of PPTg neurons using kainic acid microinjections produced increased firing in the dopamine neurons of the SN. Further it was shown that the level of responding was dose dependent such that increased doses of kainic acid produced increased responding in the SN.

In a similar study aiming to understand brainstem inputs to the SN and the effect that stimulation of these inputs would have on nigral functioning, electrophysiological responses in the SN and the NAcc were recorded following electrical stimulation of the PPTg (Kelland et al., 1993). It was reported that stimulation of PPTg produced an increase in nigral activity as reported in previous experiments. Interestingly it was also reported that accumbens neurons showed an equally large increase in activity following PPTg stimulation. This may be due to a general lack of specificity of the method of stimulation and the possibility that LDTg neurons were affected.

The electrophysiological response was further characterised by Lokwan et al. (1999). They used electrical stimulation of the PPTg and then recorded from cells in the SN-pc. It was reported that following PPTg stimulation burst firing in the SN was seen. This is particularly interesting as this mimics the natural firing patterns of cells in this area and thus implicates the PPTg in the production of these responses in natural situations.

It is clear from the studies reviewed that the PPTg has an important role to play in the regulation of dopaminergic activity within the SN-pc and consequently in the dorsal striatum. When combined with data implicating the LDTg in the control of mesoaccumbens dopamine it produces a consistent pattern where by mesopontine cells groups have considerable influence over the midbrain dopamine systems. The functional significance of this cholinergic control of the striatal dopamine system is, however, still unclear. Findings from the previous section argue against the PPTg being involved in reward related behaviours and this would fit with the data presented by Blaha et al. showing no effect of lesions of the PPTg on dopamine efflux in the NAcc. It is more conceivable that cholinergic activity in the PPTg is affecting the dopaminergic outflow from the SN and consequently dorsal striatal dopamine efflux. When these data are combined with studies demonstrating a clear projection from the SN back to the PPTg the altered nigral activity following PPTg manipulation could be further evidence of the PPTg being a constituent part of a long loop as mentioned in the introduction.

One point to consider at this point is that the pedunculonigral projection is by no means exclusively cholinergic. Both cholinergic and non-cholinergic neurons in

the PPTg have been reported to project to the SN (Lavoie & Parent 1994a). Further it has been shown that ACh and glutamate are co-localised in a least some of these nigral projecting neurons. It could be the case that the combined glutamatergic and cholinergic pedunculonigral projection serves to activate the SN, bringing it "on line" to produce behavioural responses to sensory stimuli. This suggestion is by no means new as this is the type of role suggested for the cholinergic neurons of the mesopontine tegmentum when they were included in the ascending reticular activating system. This is particularly interesting in light of the large projection from the PPTg to the thalamus. Clearly activity in the PPTg can have wide reaching affects on higher neural systems which would be consistent with it having a role in the activation of these systems. This projection to the thalamus will be examined in the next section.

1.2 Thalamus

While the pathway from the PPTg to SN has been the subject of a number of investigative studies the strong cholinergic projection to the thalamus has remained relatively poorly studied with only the anatomical characteristics and its role in sleep having been investigated. As explained in the introduction the PPTg sends projection to all the major thalamic nuclei with especially strong innervation of the sensorimotor nuclei (Hallanger & Wainer, 1988). The thalamus itself has extensive connections with the cortex and consequently it would seem that one possible explanation for the behavioural deficits described in the previous section following

lesions of the PPTg could be an alteration of thalamic and thus cortical function. Datta and colleagues have performed two studies examining the effects of stimulating PPTg on sleeping and wakefulness (Datta & Sweik, 1997; Datta et al., 2001). It was reported that in both rats and cats microinjections of glutamate into the PPTg produced increases in the normal levels of wakefulness and REM sleep at the expense of slow wave sleep. As explained in the introduction many studies have implicated the PPTg and in particular its innervation of the thalamus in the control of both the tonic and phasic activation of forebrain sites. While these studies do not measure thalamic activity directly it is clear that behavioural state, which in some respects can be seen as defined by thalamic activity, is clearly altered when PPTg neurons are stimulated.

Previous work in this laboratory has attempted to assess the impact of loss of cholinergic input to thalamus by measuring ACh levels in the thalamus following lesions of the PPTg (Jenkins et al., 2002). It was demonstrated using post-mortem punch studies that removal of cholinergic input from the PPTg produced differential changes in ACh levels throughout the thalamic nuclei. Interestingly the pattern of change mirrored that of the experiments examining striatal dopamine in that the motor nuclei examined had decreased levels of ACh while the limbic nuclei had increased levels of ACh. Clearly, decreased activity within the PPTg has reduced levels of ACh in the motor thalamic nuclei. Increased activity within the limbic nuclei may well be the result of disinhibited activity in the LDTg or possibly the Ch4 neurons of the basal forebrain. Mesopontine tegmental neurons are known to be involved in the control of behavioural state and it is therefore not surprising that

decreased activity in the PPTg produces a compensatory increase in LDTg activity resulting in higher levels of ACh in the thalamic projection sites of the LDTg. This reinforces the idea that the PPTg cholinergic cells are preferentially involved in the processing of sensorimotor information.

In an attempt to further our understanding of this pathway the following chapters report experimental work examining the relationship between the PPTg and thalamus. It is obviously of great interest to explore the possibility that the pattern of functional connections between the mesopontine cholinergic neurons and the striatal dopamine system may be reproduced in the pattern of connections between the mesopontine tegmentum and the thalamus.

Thalamic activation following PPTg stimulation I: Pilot work

2.0 Introduction

Work to date examining rats with PPTg lesions has shown clear and robust behavioural deficits produced by the disruption of functional circuits in the midbrain and cortex. However, it is still far from certain where exactly in these functional circuits that altered PPTg function has its impact on behavioural control. The last chapter described research examining the relationship between the mesopontine cholinergic system and the midbrain dopamine system. It is clear that activity within the PPTg, and specifically the neurons that project to the SN-pc, is a very important factor in the computational processes that occur within the SN, and consequently within the dorsal striatum. What remains less clear is the effect that activity within the PPTg has on the other structures to which it projects, in particular the thalamus. It has been demonstrated that cholinergic activity within the thalamus is an important factor in the changing of firing patterns of the thalamic neurons from burst firing to tonic firing (McCormick, 1989, 1992). This change in thalamic firing mode has been postulated to be important in the type of information that is passed through these cells and onto the cortex (Sherman & Guillery, 2001). Indeed it has been suggested that burst firing is involved in conveying information regarding sudden changes in sensory input while tonic firing is more involved in the relaying of accurate information regarding the sensory properties of stimuli that are being attended to.

These studies have done much to describe the role of ACh in the thalamus and its functions at a cellular level. However, it is remains unclear how the inputs to the thalamus are organised with respect to the role played by ACh in the thalamus. That is to say while it has been demonstrated that the mesopontine cholinergic neurons, as well as the Ch4 neurons of the basal forebrain, project to the thalamus, very little work has examined the functional significance of these projections. This section of the thesis aims to start to address this issue by examining the implications on thalamic activity of altered activity within the PPTg.

The next chapter will present pilot work aimed at finding a way to stimulate the PPTg and then adequately measure the impact of this stimulation on the neural functioning of the thalamus. Clearly the most critical parts of this study are the choice of stimulating agent in the PPTg and the method for recording activity within the thalamus. Two methods of stimulation are commonly used, namely pharmacological and electrical. However given the relatively small size of the PPTg and the problems already reported with non-specificity of electrical stimulation (Kelland et al., 1993) it was decided that pharmacological stimulation would be most suitable. While the choice of stimulating agent was far from straightforward it was also thought that driving the neurons in the PPTg with an endogenous excitatory neurochemical had greater validity than alternative methods. As explained previously, the cholinergic cells of the PPTg react to cholinergic drugs in the opposite fashion to other cholinergic cells due to the action of inhibitory autoreceptors on the cholinergic neurons. Some authors have reported that application of cholinergic antagonists like scopolamine into the PPTg have produced

decreased levels of activation as measured by the cholinergic dependent dopamine release in the dorsal striatum (Chapman et al., 1997). However, in other experiments examining the same striatal dopamine efflux following cholinergic stimulation from the PPTg it was reported that scopolamine reduced the levels of extra-cellular dopamine in the dorsal striatum (Hernandez-Lopez et al., 1992). Clearly the action of scopolamine in the PPTg is complex and consequently it would seem an inappropriate choice of stimulant.

When the neurochemistry of the PPTg is examined, the other obvious choice of stimulatory neurochemical is glutamate. The principle reason for this is that studies examining the neurochemistry of the PPTg have demonstrated that the cholinergic cells of the nucleus have a variety of glutamate receptor subunits clearly demonstrating they are excited by glutamatergic activity (Inglis & Semba, 1996). In support of this, lesions of the PPTg performed in this thesis and many of the studies cited use neurotoxins like ibotenic acid, which act at glutamate receptors, to lesion this nucleus. Clearly glutamate excites the cholinergic neurons of the PPTg, and as the projection from the PPTg to the thalamus has been shown to be predominantly if not exclusively cholinergic, glutamate stimulation will act on exactly the cells that project to the thalamus.

Having decided on a choice of stimulant it is also important to find a method of accurately demonstrating any functional changes that may have occurred in the thalamus. A number of methods have been used in recent years to examine activity in areas of the brain following various stimulating factors. The important factors to examine in the comparison of these methods are the levels of specificity or spatial

resolution that can be obtained when trying to assess levels of neural activity as well as the levels of sensitivity that these methods will pick up. The methods available for examining levels of neural activation include cytochrome oxidase histochemistry, 2-deoxyglucose (2-DG) mapping and fos immunohistochemistry. As a brief review, cytochrome oxidase (COase) is an endogenous protein of the inner membrane of all eukaryotic mitochondria. It acts as an enzyme in the electron transfer chain that is responsible for oxidative phosphorylation leading to the formation of adenosine triphosphate (ATP). Through this reaction alone COase accounts for more than 90% of oxygen consumption by living organisms on earth (Wong-Riley, 1989). ATP is the energy molecule that neurons use to drive ion-pumping mechanisms, facilitate fast axoplasmic transport and synthesise macromolecules and neurotransmitters (Papa et al, 1980). COase is the rate-limiting factor in the process that produces ATP and is thus tightly coupled to neuronal activity. This is the basis for using cytochrome oxidase staining as a marker for metabolic activity.

While it is clear that, theoretically, visualisation of this neurochemical is a valid method for measuring neural activity there remain a number of serious practical considerations to take into account. The major problem with this method is quantification of results. The histochemical procedure for visualising cytochrome oxidase is relatively straightforward. However, studies attempting to quantify differing levels of staining have had to resort to the use of homogenised brain tissue standards. These are used to quantify the relationship between levels of cytochrome oxidase and optical density. This process involves a number of stages that must be kept entirely constant throughout experiments to enable comparisons to be made

across animals to ensure that the method retains a reasonable level of sensitivity. This has proved to be more difficult than originally thought and consequently the use of cytochrome oxidase as a marker for neural activity in neuroscientific research is relatively scarce.

The next possible option for measuring neural activity is 2-DG mapping. This technique is based around the principle that active neural systems use more glucose than inactive systems. Consequently if a radioactive form of glucose is injected into an organism then the most active areas of the brain can be visualised by examining the areas where the largest quantities of radiation are collected. Unfortunately while this method does provide a valid technique for measuring activity within the brain the spatial resolution of the method is less precise than other methods. Results from 2-DG studies are reported in terms of areas that are activated while other methods, including fos immunohistochemistry, can be used to identify activity at a cellular level. As the present study aims to identify specific regions within the thalamus it was thought that the spatial resolution problem associated with the 2-DG method might cause problems when analysis of sections was carried out.

The other possible method used to visualise differential activation in the thalamus is fos immunohistochemistry. A lot of research has been carried out using this method to identify cells that have been recently active. *c-fos* is an immediate early gene (IEG) which are a group of genes that become active in response to extra-cellular stimuli. They are thought to be the first step in a chain of reactions that are responsible for changing a cells phenotype in response to external stimulation. In recent years antibodies directed against the fos protein, which is coded for by the *c-*

fos gene, have been developed and consequently a useful tool for identifying active cells in the process of responding to extra-cellular stimulation was made available. This method has the advantage that the staining is readily identifiable in the nuclear component of neurons thus enabling researchers to examine at a cellular level neurons that have been recently active.

Having reviewed the methods of measuring activation in the thalamus it was decided that fos immunohistochemistry[1] best suited the needs of the experiment. Thus pilot studies were carried out incorporating glutamatergic stimulation of the PPTg combined with measurement of levels of activation in the thalamus using fos immunohistochemistry. Initial work examined the affects of using different methods of glutamate application as well as the affects of anaesthetic on fos staining in the thalamus. In this series of experiments stimulation of the PPTg was carried out using reverse dialysis through a microdialysis probe of glutamate into the PPTg. Efficacy at stimulating thalamic fos was measured immunohistochemically.

[1] It should be noted that *c-fos* is not the only IEG that is used to visualise neurons that have recently been active and the decision to use c-fos rather than *c-jun, jun-B, zif/268* or any of the other IEGs was made due to reliability of the *c-fos* antibodies in common usage which has made it the method of choice for many researchers in the field.

2.1 Reverse dialysis

Reverse dialysis is a method of delivering chemicals directly into the brain via a microdialysis probe. In this procedure the usual theory behind microdialysis is reversed, such that instead of using osmotic pressure to collect neurochemicals from different areas of the brain, it is used instead to deliver them. This is achieved by using relatively concentrated solutions of the chemical to be delivered in the perfusate running through the probe and consequently creating an osmotic gradient where by the chemical in question moves across the semi-permeable membrane into the brain itself. This method allows the slow infusion of chemicals into the brain over a number of hours. This enables researchers to drive specific structures for long periods of time thus maximising any effects of stimulation of a structure on its efferent pathways.

In this first study glutamate was infused into the PPTg over two hours in an attempt to stimulate its efferent pathway to the thalamus.

2.1.1 Methods

Subjects

Subjects for this experiment were 12 adult male Lister hooded rats (Charles River, Kent, England, average pre-surgery weight 377.14g) All experimental and care procedures followed guidelines laid down by UK and European legislation

(Animals [Scientific Procedures] Act, 1986, and European Communities Council Directive of 24 November 1986 [86/609/EEC]). Rats were housed individually under a 12 hour light / dark cycle, and provided with food and water *ad libitum.*

Surgery

Animals were anaesthetised with 60 mg/ml, Sagatal (sodium pentobarbitone), delivered at 1 ml/kg of body weight (combined with sterile water at a 50:50 ratio) and placed in the stereotaxic frame. Rats were checked for a satisfactory level of anaesthesia by ensuring that both the eye blink reflex and withdrawal reflex following tail pinch were not present. Microdialysis probes, with 1mm exposed membrane, were implanted into the PPTg at the following co-ordinates: + 1.0mm anterior to interaural line, +/- 1.7mm from midline, -8.4mm from skull surface. Probes were secured in place with dental cement and skull screws. Animals were allowed at least 24 hours to recover before dialysis experiments were carried out.

Microdialysis

Microdialysis was performed upon rats under halothane anaesthesia. The study was run on a matched pair design. Thus for each rat receiving artificial cerebrospinal fluid (aCSF)[1]. through the probe another rat received glutamate (dissolved in aCSF). As the probes were implanted unilaterally, this provided two

[1] pH 7.4 made up with 127mM NaCl, 3mM KCl, 0.48mM NaH2PO4.2H2O, 2.6mM NaHCO3, 1.8mM CaCl2, 0.4mM MgSO4.7H2O, 3.6mM glucose and 3.3mM Urea.

control conditions for each glutamate infused hemisphere; firstly the contralateral hemisphere of that same rat and secondly both of the hemispheres from its matched pair which received an aCSF infusion. In the dialysis phase of the study rats were given halothane anaesthesia and then glutamate at a concentration of 1 $x10^{-4}$ M or aCSF was infused through the probe for two hours at a rate of 2µl/minute. Infusions were made with a Harvard Apparatus pump. At the end of this period rats were left for one hour in a dark quiet room to reduce sensory input.

Immunohistchemistry

At the end of procedures rats were humanely euthanised with i.p. injections of 0.9ml dolethol (sodium pentobarbitone, 20% weight by volume). They were then transcardially perfused with 50ml of phosphate buffered saline at a rate of approximately 20ml per minute followed by at least 250ml of 4% paraformaldehyde solution made up in 0.1% phosphate buffer. Brains were then extracted and placed in 20% sucrose solution (made up in 0.1% phosphate buffer) overnight. Brains were cut into 50µm sections on freezing microtone with one in four sections being taken for subsequent staining and analysis. Probe placement in the PPTg was confirmed using cresyl violet staining (see appendix for protocol), while thalamus sections were processed for fos immunohistochemistry

Sections were washed in PB before being placed in blocking solution (20% normal goat serum) for 60 minutes. Sections were then incubated in anti fos primary antibody at a concentration of 1:20000 (Oncogene Research Products, Calbiochem)

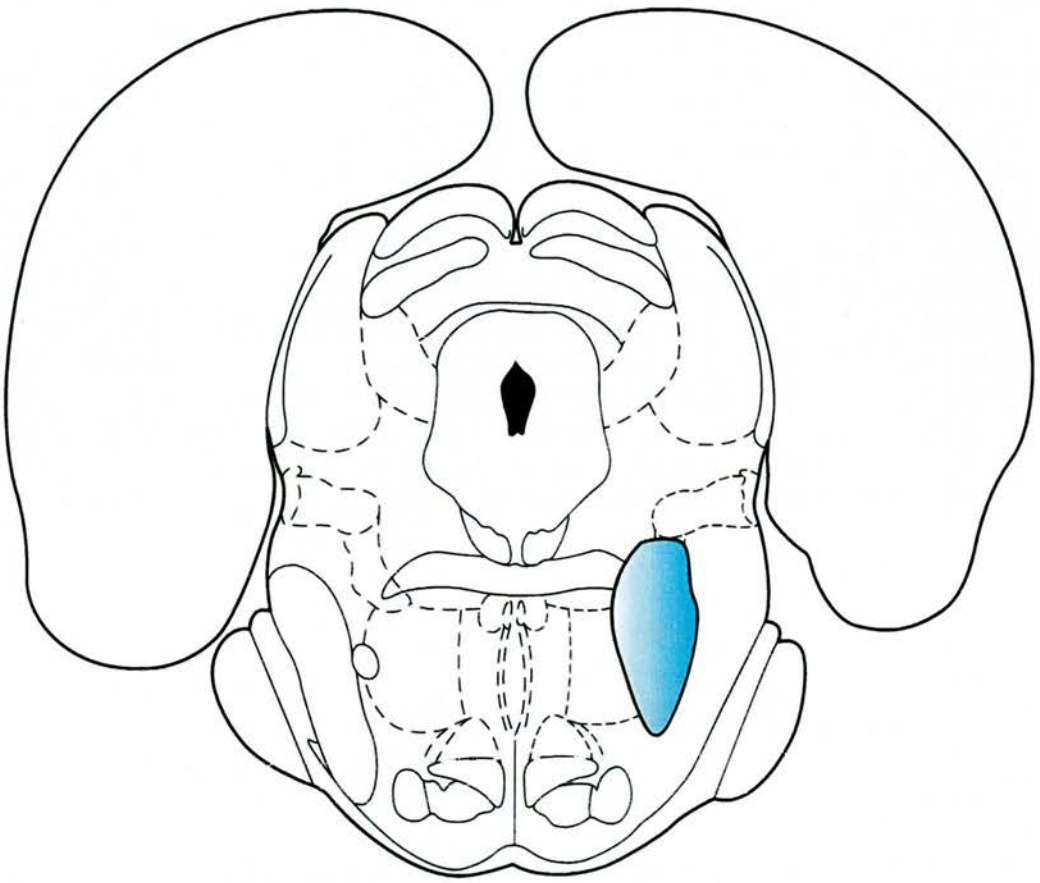
over two nights. Sections were then removed, washed in PB and placed in biotinylated IgG (anti rabbit, Vectastain Elite ABC kit) at a concentration of 1:200 for sixty minutes before finally being incubated in avidin-biotin complex (Vectastain Elite ABC kit) at a concentration of 1:50 for a further sixty minutes. Sections were then reacted with nickel enhanced 3,3-diaminobenzidine tetrahydrchloride (Sigma) before being mounted, dehydrated and coverslipped with DPX. Control experiments where antibodies were omitted were performed to ensure against non-specific staining.

Sections were analysed under the light microscope to examine levels of fos staining as compared to background staining. Specifically fos immunoreactive neurons in the thalamus were examined.

2.1.2 Results

Examination of probe placement revealed all probes to be at least partially within the boundaries of the PPTg. Average position on which the microdialysis probe was centred was estimated and the result displayed in Figure 34. The Figure demonstrates two things; firstly that the probes were centred within the area comprising the PPTg and secondly that the size of the probe was at least as big as the PPTg itself. This point is demonstrated in more detail in Figure 35, in which sections through a brain which had been implanted with a microdialysis probe are displayed. The area of damage produced by the probe includes nearly all of the PPTg as well as a substantial amount of tissue dorsal to the nucleus.

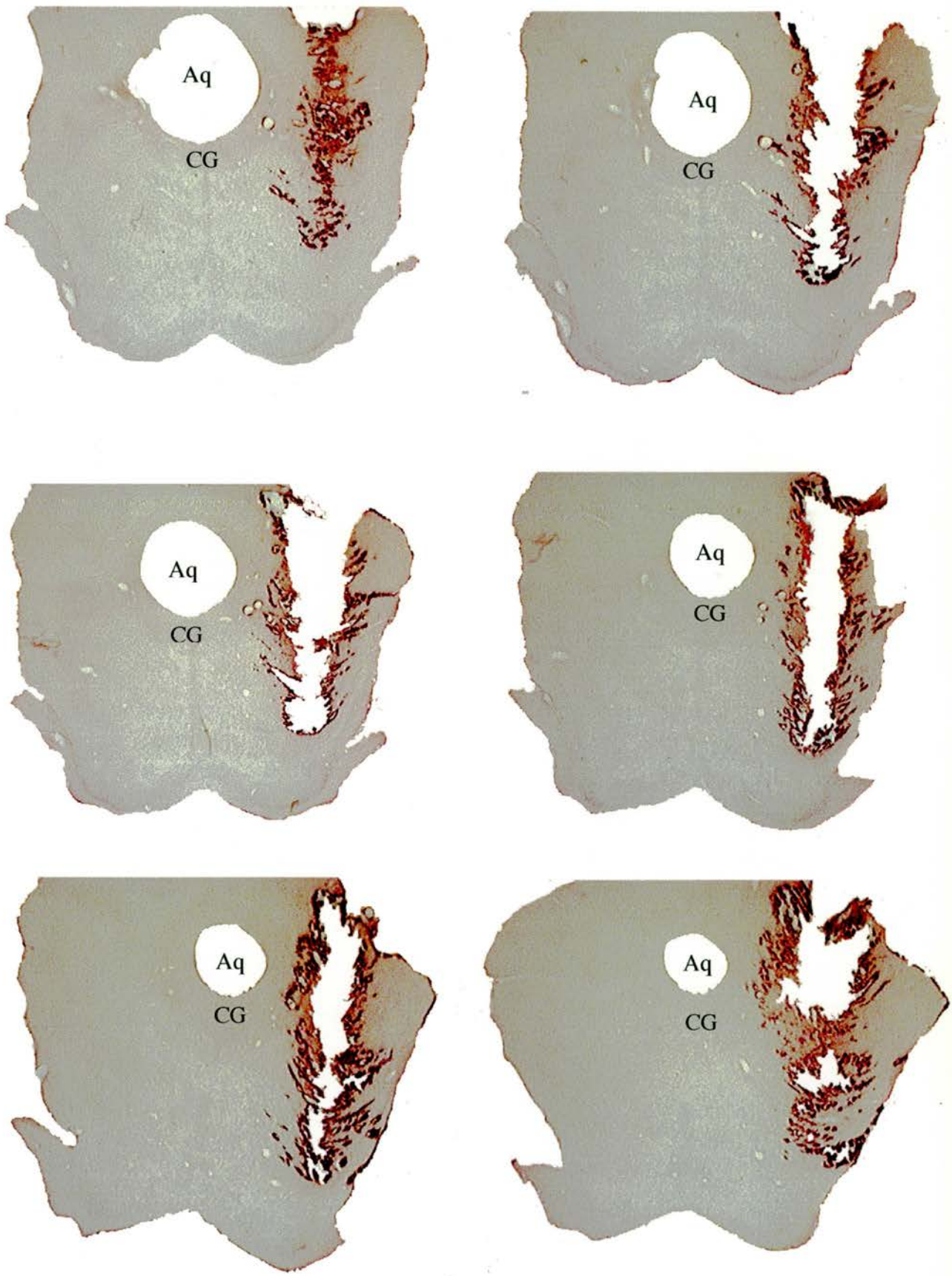
Figure 34: Schematic representation of average cannula placement



Interaural 1.20mm

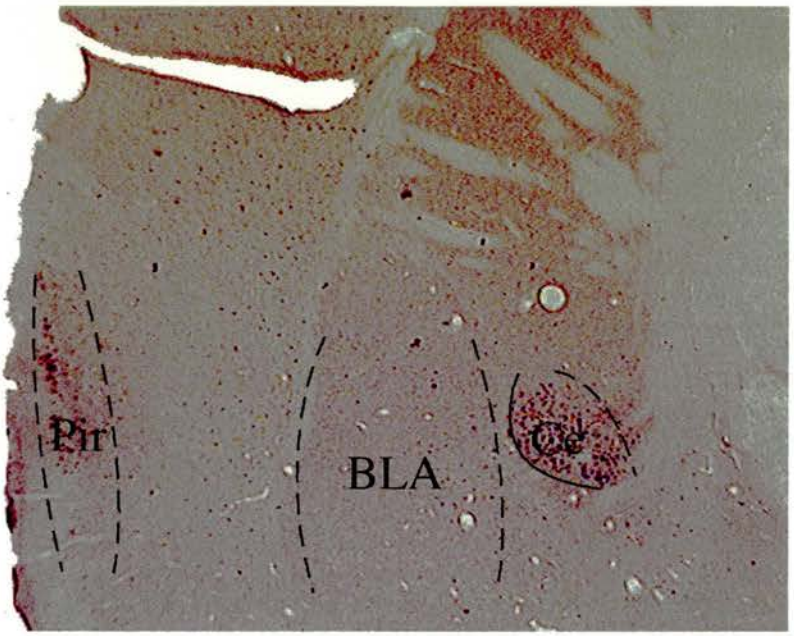
Bregma -7.80mm

Figure 35: Damage following implantation of microdialysis probe



Abreviations: Aq - aqueduct, CG - central gray

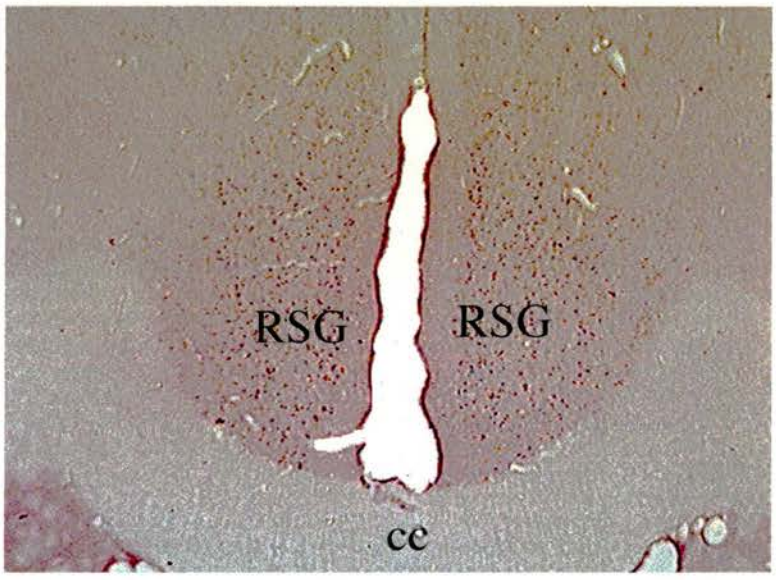
Figure 36: Background staining in the amygdala and piriform cortex



Abbreviations:

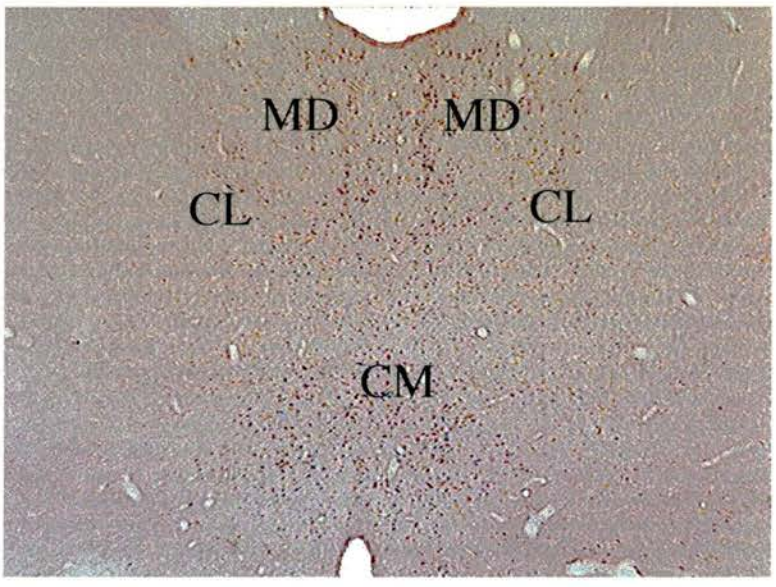
Ce - central amygdaloid nucleus
BLA - basolateral amygdaloid nucleus
Pir - piriform cortex

Figure 37: Background staining in the cortex



RSG - retrosplenial granular cortex
cc - corpus callosum

Figure 38: Background staining in the intralaminar thalamic nuclei



CL - centrolateral nucleus
CM - centromedial nucleus
MD - mediodorsal nucleus

Figures 36, 37 and 38 display background fos staining throughout the brain. Figure 36 illustrates staining in the amygdala and piriform cortex, while Figure 37 displays extensive fos staining in other areas of the cortex. Figure 38 demonstrates that the midline thalamic nuclei are showing clear and extensive non-specific staining in response to the dialysis protocol.

2.1.3 Discussion

The results of this pilot work illustrate a number of major problems with the method used in this study. Firstly, although the probe placement was good, with the majority of the dialysis probes being centred within the boundaries of the PPTg, it is clear that the probes themselves caused a lot of damage to the structure. Purely in terms of size the probe destroyed too many of the neurons that it was supposed to be exciting. Clearly a less intrusive method must be found that will decrease the level of physical damage that occurs within the PPTg itself.

Secondly the levels of background staining found throughout the thalamus and the rest of the brain was far too high to allow meaningful measures of differential fos activity to be detected. This could be the result of a number of different factors. Firstly the dialysis probe itself is very delicate and consequently rats cannot be given a long time to recover from surgery as this would inevitably mean that the probe would become damaged before testing could take place. It has previously been demonstrated that physical brain damage can induce fos expression (Dragunow & Robertson, 1988) and thus another method where fos induced by

surgical damage can be allowed to dissipate must be found. Secondly certain anaesthetics can induce general background fos activity and although halothane has been shown to better than some other anaesthetics (Takayama et al., 1994) this may still be a contributing factor. These methodological short-comings are addressed in the next set of studies.

2.2 Microinjections

To address the problems encountered in the previous set of experiments another set of pilot studies were carried out to in an attempt to decrease background fos staining to a level where reasonable measurements of thalamic activity could be taken. To this end a number of issues from the previous pilot studies were examined. Firstly it was clear that using microdialysis probes created far too much damage within the PPTg and consequently a less intrusive method was needed. With this in mind it was decided to stimulate the area using microinjections of glutamate directly into the PPTg. This has the advantage that guide cannulae can be implanted weeks before the injections take place allowing any fos stimulated by the surgery itself time to dissipate as well as causing much less physical damage due to the relatively small size of the needle. The microinjection procedure is also much easier to carry out in awake animals and consequently the problems associated with fos induced by anaesthesia can be eliminated. The disadvantage of this method, however, is that the injection procedure is reasonably stressful for the rat and stress is another factor that can induce background fos levels.

Finally one last issue to be addressed is the concentration of glutamate to be used for stimulation. Obviously the microinjection procedure can only provide a brief excitiation and with this in mind the concentration of glutamate will need to be higher to drive the PPTg sufficiently to induce activation in the thalamus. However, excess glutamate has been shown to be toxic in brain tissue, accounting for acute degenerative changes following ischemia and hypoglycemia as well chronic

degenerative changes in disorders such as Alzheimer's disease (Okazaki et al., 1996). The choice of dose is therefore critical to avoid neurotoxicity and yet still excite neurons within the nucleus. Nadler et al. (1981) carried out a series of experiments examining the relative toxicity of various excitatory amino acids and reported that at a concentration of 1.5M glutamate was 90 times less toxic than ibotenic acid. In an attempt to completely avoid lesion damage following microinjections it was initially decided to use 0.1M glutamate solution. In further experiments slightly higher concentrations were used in an attempt to establish whether a dose dependent increase in thalamic activity could be induced.

2.2.1 Method

Subjects

Subjects for this experiment were 26 adult male Lister hooded rats (Charles River, Kent, England, average pre-surgery weight 313g) All experimental and care procedures followed guidelines laid down by UK and European legislation (Animals [Scientific Procedures] Act, 1986, and European Communities Council Directive of 24 November 1986 [86/609/EEC]). Rats were housed individually under a 12 hour light / dark cycle, and provided with food and water *ad libitum.*

Surgery

Animals were anaesthetised with 60 mg/ml, Sagatal (sodium pentobarbitone), delivered at 1 ml/kg of body weight (combined with sterile water at a 50:50 ratio)

and placed in the stereotaxic frame. Rats were checked for a satisfactory level of anaesthesia by ensuring that both the eye blink reflex and withdrawal reflex following tail pinch were not present. Guide cannulae were implanted at the following co-ordinates: AP + 1.0 (from inter-aural line), ML +/- 1.6 (from midline), DV -4.0 (from skull surface). Guide cannulae were secured in place with dental cement and skull screws and occluded by stainless steel stylets to prevent blockage and infection. Cannulae were made from 23 gauge stainless steel needles and were 9mm in length. Cannula carriers were used to hold the cannula in position during implantation. These were made to fit inside the cannula and were also 9mm in length. Animals were given at least seven days to recover from surgery with stylets being changed every day to avoid infection and to habituate the animal to handling procedures.

Microinjection procedure

To minimise fos induced from non-specific stimuli rats were kept in a carefully controlled environment. This entailed minimising the visual and auditory input for 2 hours prior to the injection procedure by keeping the rats in an empty holding room with the lights switched off. Again, a matched pair design was used such that for each animal receiving a unilateral glutamate injection another received a unilateral aCSF injection. Three groups of rats were used each receiving a different concentration of glutamate dissolved in aCSF (0.1M, 0.2M or 0.4M). Injections of 0.3µl were made at a rate of 0.05µl/minute using a 0.5µl syringe and a Harvard

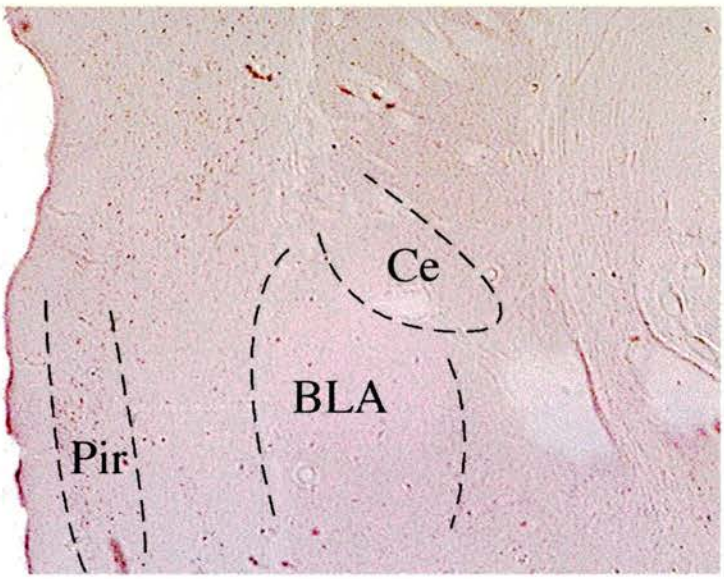
Apparatus pump with the needle being left *in situ* for 5 minutes at the end of the infusion to allow glutamate to diffuse away from the needle tip. Rats were then returned to the carefully controlled environment for one hour.

At the end of procedures rats were humanely euthanised with i.p. injections of 0.9ml dolethol (sodium pentobarbitone, 20% weight by volume). They were then transcardially perfused with 50ml of phosphate buffered saline at a rate of approximately 20ml per minute followed by at least 250ml of 4% paraformaldehyde solution made up in 0.1% phosphate buffer. Brains were then extracted and placed in 20% sucrose solution (made up in 0.1% phosphate buffer) overnight. Brains were cut into 50μm sections on freezing microtone with one in four sections being taken for subsequent staining and analysis. Cannula placement in the PPTg was confirmed using cresyl violet staining (see appendix for protocol), while thalamus sections were processed for fos immunohistochemistry. Fos immunohistochemistry was identical to the procedure described in the dialysis section of this chapter.

2.2.2 Results

Figure 39 illustrates the level of damage caused by the microinjection procedure. Clearly the level of tissue damage within and around the PPTg has been dramatically reduced when compared to the damage caused by the dialysis probes in the previous chapter.

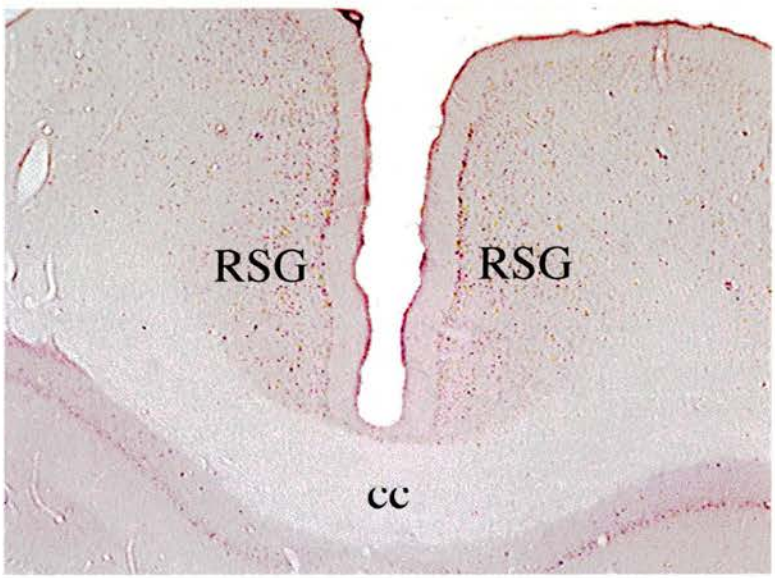
Figure 40: Decreased background staining in the amygdala and piriform cortex



Abbreviations:

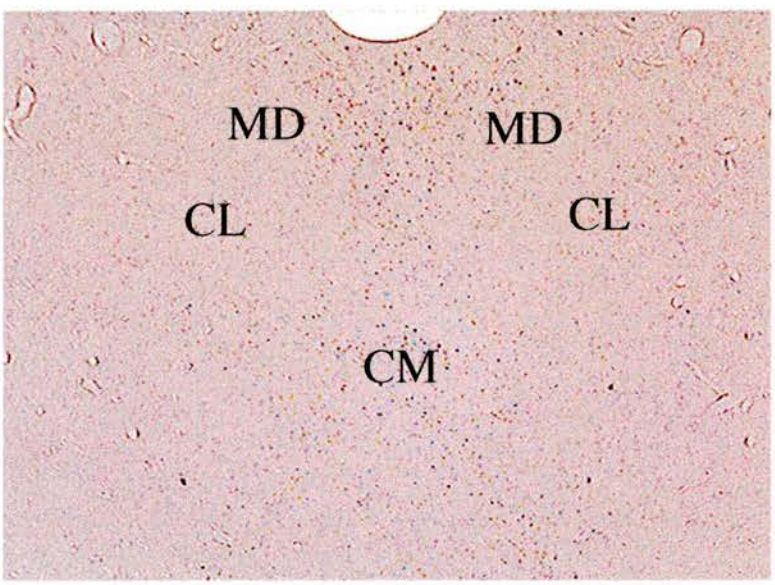
Ce - central amygdaloid nucleus
BLA - basolateral amygdaloid nucleus
Pir - piriform cortex

Figure 41: Background staining in the cortex



RSG - retrosplenial granular cortex
cc - corpus callosum

Figure 42 : Decreased background staining in the intralaminar thalamic nuclei



MD - mediodorsal nucleus
CL - centrolateral nucleus
CM - centromedial nucleus

Figure 43: Lack of staining in the posterior thalamus

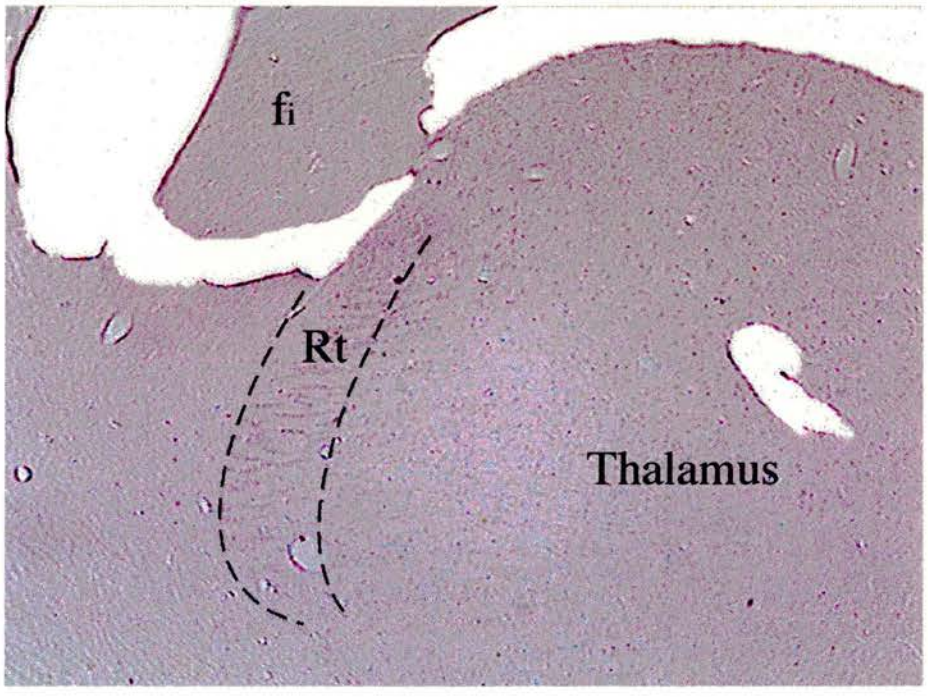
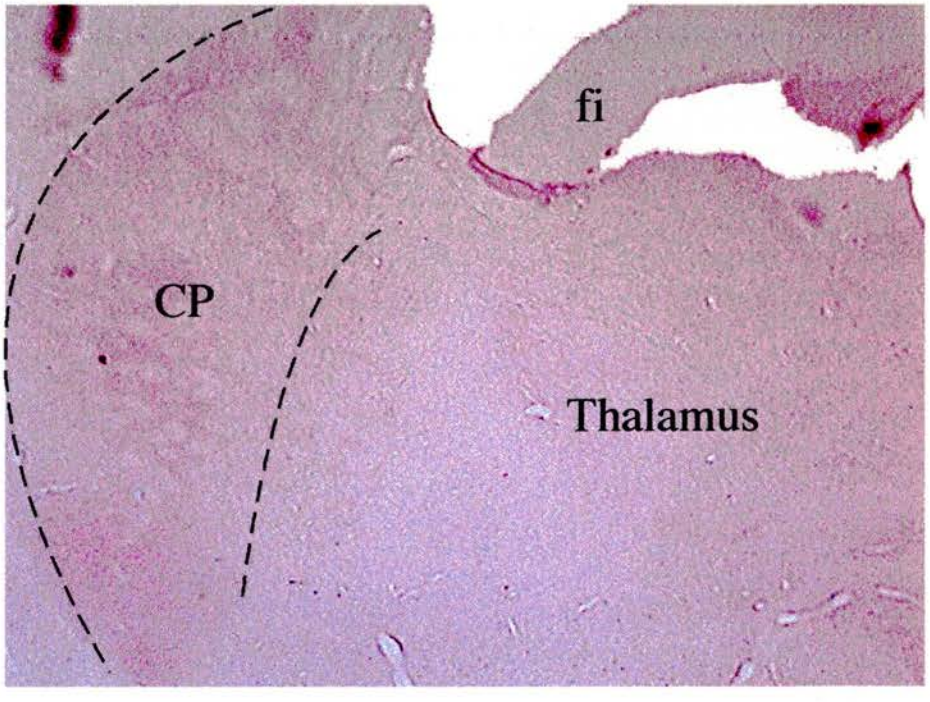


Figure 44: Lack of staining in anterior thalamus



Abbreviations: fi - fimbria, Rt - thalamic reticular nucleus,
CP - caudate putamen

Figures 40, 41 and 42 illustrate the level of background fos staining in these rats. Figure 40 demonstrates that background staining in the amygdala has been completely eliminated and staining in the piriform cortex has also been reduced. Figure 41, however, shows that staining is still clear in other areas of cortex. Figure 42 displays some staining in the midline thalamic nuclei but when this compared to the amount seen in Figure 38 it is clear that the background staining in thalamus has been significantly reduced with staining in the MD nucleus now not present. Levels of staining in the rest of thalamus are illustrated in Figures 43 and 44. These sections show that while the background levels of staining are low the number of fos immunoreactive neurons in both the anterior and posterior thalamus is also very low.

2.2.3 Discussion

The results of the study clearly illustrated that a lot of problems encountered in the earlier dialysis experiments had been dealt with resulting in greatly reduced background staining. Problems with physical damage to the PPTg itself had also been addressed and it is clear that the needles used to microinject the glutamate were producing greatly reduced damage to the neurons in the nucleus. However, while this method had reduced background fos levels to a point where changes in thalamic activity could be measured it was now apparent that stimulation of the PPTg using this method was not producing the consistent changes in thalamic activity that might be expected. As illustrated in Figures 40-44 large areas of the thalamus lacked staining completely with no fos-immunoreactive cells present. In the areas where fos

staining was seen it was found to be inconsistent across rats. Even at the highest dose, where neurotoxic damage was becoming a distinct possibility, thalamic fos levels were inconsistent. This made reporting cell counts meaningless as the staining that was found was not consistent to specific thalamic nuclei. Clearly the method of excitation used was having no real effect on levels of activity within the thalamus as measured by fos immunohistochemistry. Further the inconsistent nature of the staining suggests that the method could be refined further to reduce non-specific staining and increase accurate measurements of changes induced by excitation of the PPTg. These problems were addressed in the next set of studies.

One point that must be noted at this point is the potential problem of false negatives associated with the fos immunohistochemistry methodology. That is to say that the exact nature of the factors needed to induce the *c-fos* gene are far from fully understood and several cases of excitatory input to an area resulting in a complete lack of staining or very weak staining have been reported. Thus it is not possible to conclude that a lack of staining within an area is indicative of a complete lack of activity within said area. The possibility remains, therefore, that the lack of fos staining in the thalamus in this study is not due to an inability to excite the neurons but rather an inability to measure this excitiation.

Thalamic activation following PPTg stimulation II: Glutamate uptake inhibition

3.0 Introduction

Results from pilot work have demonstrated that while the microinjection procedure is a good way of keeping background staining to a minimum the method of excitation used previously has failed to produce consistent changes in neural activity in the thalamus. The next set of experiments aims to investigate different methods of stimulating the PPTg and also to further refine the procedure to try and eliminate background staining altogether. Studies from the previous chapter attempted to stimulate the PPTg with direct application of glutamate and this was shown to produce no consistent changes in thalamic activity. One possible explanation for this may be that the uptake mechanism in the PPTg is simply too efficient and that one dose of glutamate administered to the area will be removed before it can sufficiently excite the neurons of this nucleus. Termination of glutamatergic transmission is achieved by a family of high affinity glutamate transporters. These molecules are found predominantly in astrocytes and use the Na+/K+ electrochemical gradient to translocate glutamate molecules against a several thousandfold concentration gradient thus protecting the surrounding cells from glutamate induced neurotoxicity (Gegelashvili & Schousboe, 1998). One possible way of counteracting this mechanism would be to use a glutamate uptake inhibitor in an attempt to allow endogenous glutamate the opportunity to stimulate PPTg neurons.

In their 1991 article Bridges et al. examined a number of synthetic amino acids with regard to their impact on the glutamatergic system of the brain. It was demonstrated that one of these proteins, L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC), was a selective and potent inhibitor of the system that transports glutamate away from the synaptic cleft to avoid excess excitation. Further it was demonstrated that this protein acts selectively on the transport system itself without producing activation through glutamate receptors. Clearly this is an ideal tool to examine the possibility that the lack of thalamic activity following glutamate injection into the PPTg is a direct result of efficient reuptake of glutamate from the synaptic cleft. Other studies have gone on to show that application of PDC directly into the brain does indeed have behavioural and anatomical consequences in the rat. Kim & Vezina (1999) reported increased levels of locomotion following direct microinjections of PDC into the NAcc. However, other studies have reported that simultaneous application of glutamate and PDC can produce cell loss through glutamate induced neurotoxicity. The present study thus uses microinjections of PDC in an attempt to stimulate the PPTg and produce differential fos immunoreactivity within the thalamus.

The study also aims to reduce background staining further by refining the microinjection procedure. Three principle methodological changes were introduced; firstly rats were kept in a carefully controlled environment, with restricted visual and auditory stimulation, for a full 12 hours prior to the microinjections. Secondly the cannula carriers used to implant the cannulae were made an extra 2mm long. This means that during surgery the carrier will physically damage the cells that are

directly dorsal to the PPTg. This should lessen the stress of the microinjections themselves by minimising the cell damage produced by the needle when it is inserted through the guide cannula and thus minimising cell damage in the awake rat. Finally in a further attempt to reduce the stress of the animal during the procedure the level of handling was increased before and after surgery. As with the last set of studies three different doses of the stimulant were used in an attempt to produce a dose dependent increase in thalamic activity.

3.1 Method

Subjects

Subjects for this experiment were 45 adult male Lister hooded rats (Charles River, Kent, England, average pre-surgery weight 313g) All experimental and care procedures followed guidelines laid down by UK and European legislation (Animals [Scientific Procedures] Act, 1986, and European Communities Council Directive of 24 November 1986 [86/609/EEC]). Rats were housed individually under a 12 hour light / dark cycle, and provided with food and water *ad libitum.* Rats were handled everyday for at least two weeks prior to surgery.

Surgery

Animals were anaesthetised with 60 mg/ml, Sagatal (sodium pentobarbitone), delivered at 1 ml/kg of body weight (combined with sterile water at a 50:50 ratio)

and placed in the stereotaxic frame. Rats were checked for a satisfactory level of anaesthesia by ensuring that both the eye blink reflex and withdrawal reflex following tail pinch were not present. Guide cannulae were implanted at the following co-ordinates: AP + 1.0 (from inter-aural line), ML +/- 1.6 (from midline), DV -6.0 (from skull surface). Guide cannulae were secured in place with dental cement and skull screws and occluded by stainless steel stylets to prevent blockage and infection. Cannulae were made from 23 gauge stainless steel needles and were 9mm in length. Cannula carriers were 11mm in length. Animals were given at least seven days to recover from surgery with stylets being changed everyday to avoid infection and to habituate the animal to handling procedures.

Microinjection procedure

Rats were split into three groups each of which received a different dose of PDC dissolved in 0.3 µl aCSF (0.5,1 or 5nmol). Once again the experiment was run on a matched pair design such that for each rat receiving an injection of PDC another rat received an injection of acsf in the same test session. The microinjection procedure was the same as the last experiment except that rats were kept in the same carefully controlled environment for 12 hours prior to the injection to minimise sensory input. Perfusion and histology were as in the last experiment.

C-fos quantification

Changes in thalamic activity were quantified by cell counting within specific thalamic nuclei. Ensuring a constant level of illumination through the microscope, sections were counted blind such that the experimenter was unaware of the groups from which the sections came. Cell counts were taken from consecutive stained sections with 1:4 sections being stained. Section were cut at 50μm ensuring that consecutive sections were at least 150μm apart. Counts were taken from a minimum of eight sections for each structure depending on the size of the structure. Within each structure neuronal numbers were sampled in two ways. For nuclei whose boundaries were clearly stained by the fos antibody (reticular and ventrolateral geniculate), the total number of cells within the nucleus were counted. For nuclei whose boundaries were less clear samples were taken by estimating the centre of the structure under x25 magnification and projecting this image onto a 19" screen. Cells were counted by superimposing a 2" square grid upon the image and counting all the cells within the sampled area. Examples of size and position of the areas sampled from the centrolateral and ventrolateral nuclei are illustrated in Figure 45. Correction factors[1] (e.g. Abercrombie correction) are often applied to raw cell counts to avoid counting the same cell more than once, however in the present study sections counted were 150μm apart ensuring against this possibility as neuronal size was much smaller than the inter-section gap. Thus analysis was performed on the raw cell

[1] Correction factors are very important in experiments were researchers are concerned with the absolute number of cells in a structure, rather than an estimate as in this case. These types of study typically use every section cut in an attempt to count every cell. The inherent danger of this method is that cells will be counted more than once and it is for this reason that correction factors are applied.

counts recorded. This method almost certainly results in an underestimate of the number of positively labeled cells but guards against the possibility of amplifying small effects through counting the same cells more than once.

Thalamic nuclei sampled were chosen to assess the impact on the various types of nucleus within the structure (e.g. sensorimotor, limbic, intralaminar). The thalamic nuclei examined were the ventrolateral (VL), centrolateral (CL), reticular (Rt), ventrolateral geniculate (VLG), dorsolateral geniculate (DLG) and the mediodorsal (MD). Consistency was checked by recounting one in three rats and ensuring that counts did not differ by more than five percent. Finally cell counts were analysed by repeat measures ANOVA and restricted analysis.

3.2 Results

Cannula placement and needle tracks were confirmed using cresyl violet staining in sections taken through the PPTg. Needle track placement is illustrated in Figure 46. From the 45 animals implanted with cannula, 36 were accepted as having the needle track centred in the PPTg and these are depicted in the figure. Cell counts performed are illustrated in Figures 47-54. Cell counts for the DLG and MD are not reported as the majority of animals lacked any fos-immunoreactive staining in these areas and the staining that was present was sporadic and inconsistent. The data were analysed using repeated measures ANOVA with hemisphere (injected vs. non-injected) as the within subjects variable and group (drug/PDC vs. control) and dose (0.5, 1 or 5nmol) as the between subjects variables.

Figure 45: Schematic showing representative example of areas sampled for counting in the ventrolateral and centrolateral nuclei

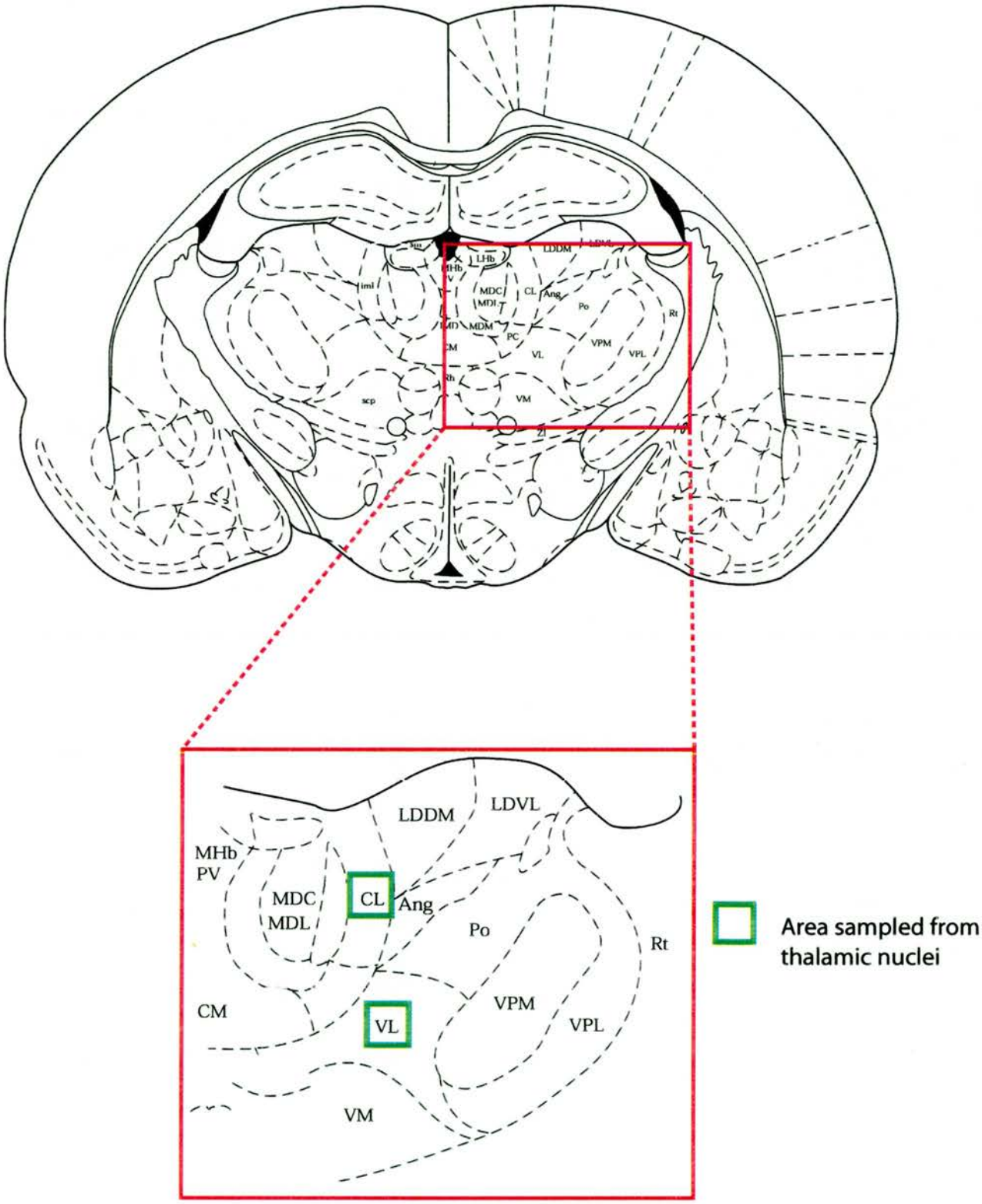


Figure 46: Schematic of cannula placement

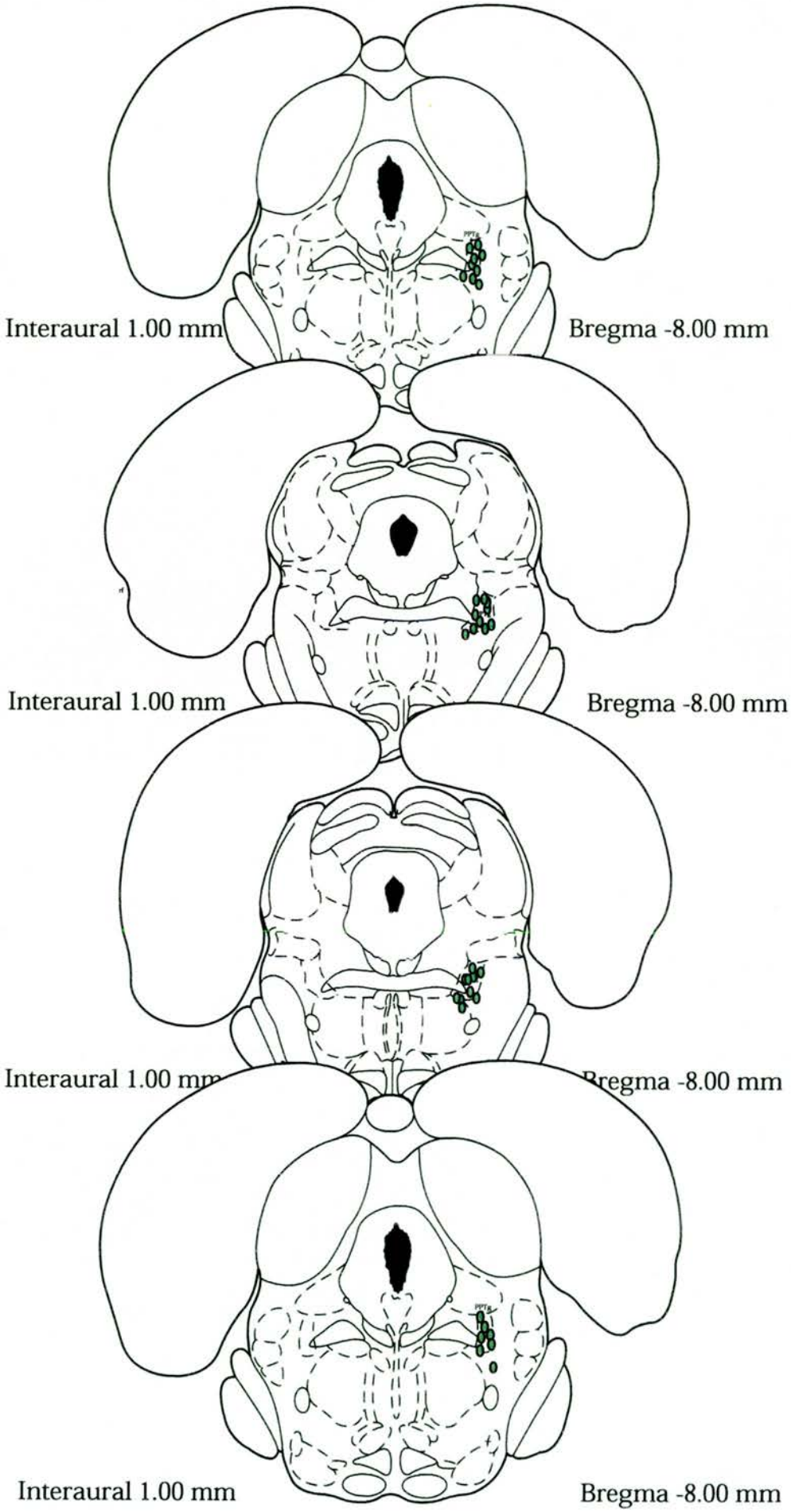
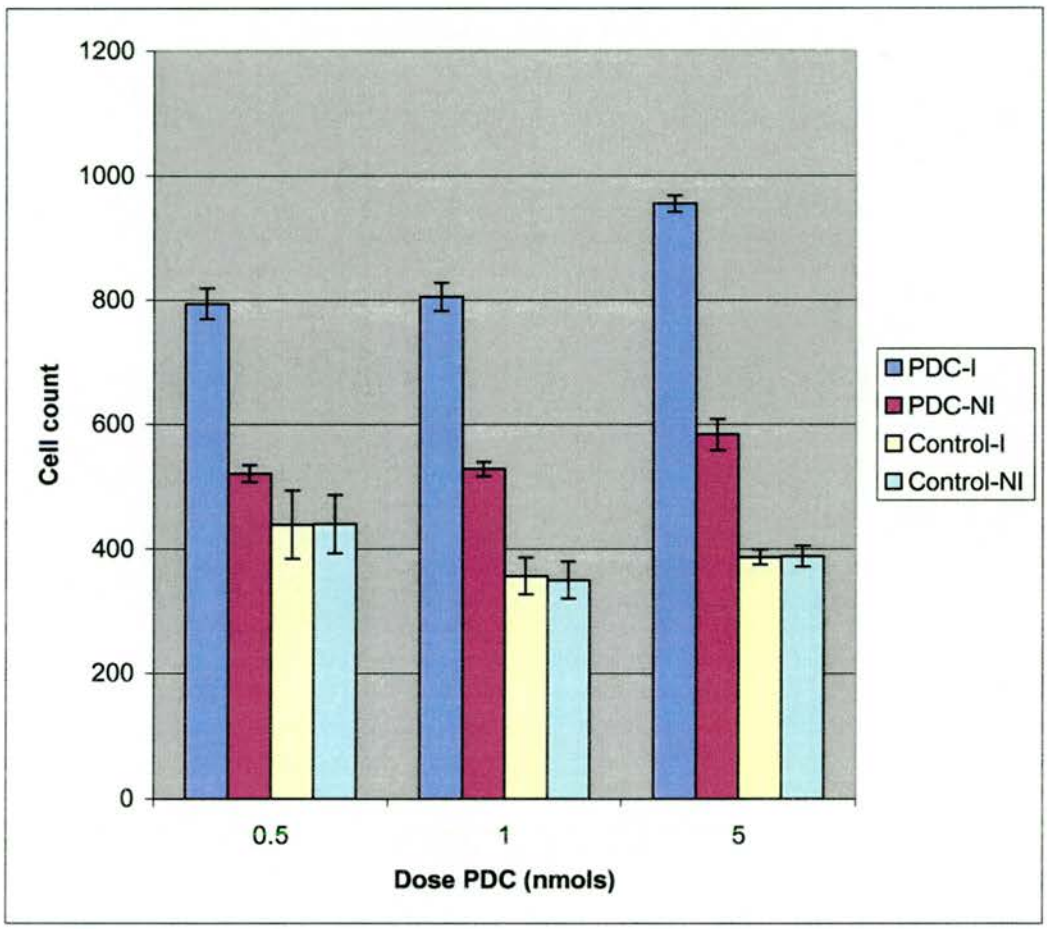


Figure 47: Mean (± SE) number of fos-immunoreactive neurons in the ventrolateral nucleus



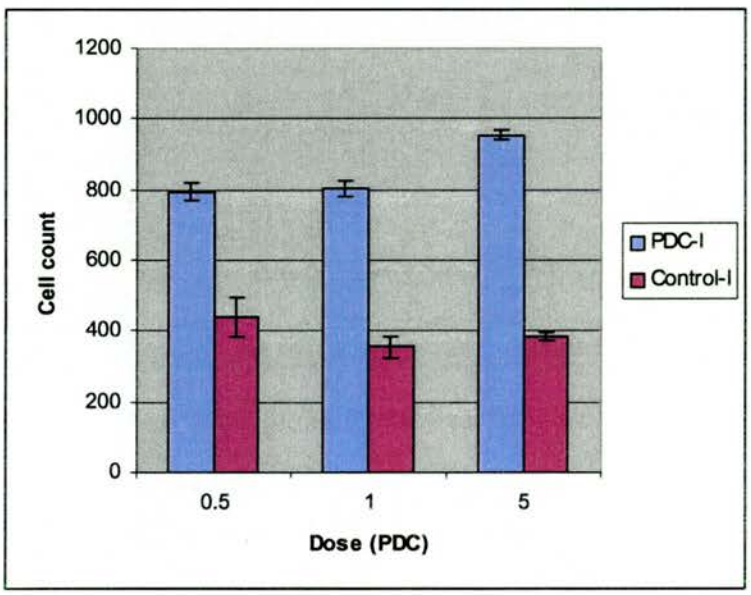
Abreviations: PDC-I – PDC injected hemisphere, PDC-NI – PDC non-injected hemisphere, Control –I - control injected hemisphere, Control-NI – control non-injected henisphere

Figure 47 illustrates the pattern of fos immunoreactive cells in the injected (I) and non-injected (NI) hemispheres of the ventrolateral nucleus in rats receiving PDC and control injections. It shows clearly that in rats receiving unilateral microinjections of the glutamate reuptake inhibitor PDC the number of fos-immunoreactive cells in the injected hemisphere was greater than in the non-injected hemisphere and this was the case for all doses of PDC. It was also the case that

counts from the non-injected hemispheres of rats receiving PDC were always greater than counts from either hemisphere of rats receiving aCSF injections. Counts from the two hemispheres of the control rats did not differ from each other. The effect of dose on the number of cells expressing fos-immunoreactivity was less clear but it was apparent that rats receiving the highest dose of PDC had higher cell counts than the two smaller doses. Results of the within subjects effects analysis revealed a significant main effect of hemisphere ($F_{1,26}$ = 444.561, $p<0.001$) as well as significant hemisphere x group ($F_{1,26}$ = 436.724, $p<0.001$), hemisphere x dose ($F_{2,26}$ = 4.724, $p<0.018$) and hemisphere x dose x group ($F_{2,26}$ = 5.597, $p<0.01$) interactions. Results of the between subjects effects analysis revealed main effects of group ($F_{1,26}$ = 211.703, $p<0.001$) and dose ($F_{2,26}$ = 3.678, $p<0.039$) as well as a group x dose interaction ($F_{2,26}$ = 5.222, $p<0.012$). Clearly the pattern of results is complex and further analysis was needed to clarify the effects that have occurred. The large effect of hemisphere in the PDC group (illustrated in figure 56) and the complete lack of effect of hemisphere in the control animals explains the hemisphere part of the interactions. However, what remained unclear is the effect of group and dose across hemispheres. To examine this relationship restricted analyses were performed examining the effect of group and dose on injected and non-injected hemispheres separately. The analyses were multivariate ANOVA with dose and group as between subjects factors. To ensure against type 1 errors the mean square error term and degrees of freedom from the original omnibus analysis were substituted into the restricted analysis. Restricted analysis of the injected hemisphere data revealed a main effect of group ($F_{1,26}$ = 238.99, $p<0.001$) and a significant group x dose

interaction ($F_{2,26}$ = 4.489, $p<0.025$) but no main effect of dose. Figure 48 shows that the main effect of group is a result of greater cell counts in the PDC injected rats than in control animals and the significant interaction is due to the increasing cell counts in the PDC injected rats as the does increases.

Figure 48: Comparison of cell counts for PDC injected and control animals in the ventrolateral nucleus of the injected hemispheres (mean ± SE)



Restricted analysis of the non-injected hemispheres revealed a significant main effect of group ($F_{1,26}$ = 50.751, $p<0.001$) but no main effect of dose and no dose x group interaction. Figure 49 shows that even in the non-injected hemispheres the PDC injected rats have higher cell counts than the control animals

Figure 49: Comparison of cell counts for PDC injected and control animals in the ventrolateral nucleus of the non-injected hemispheres (mean ± SE)

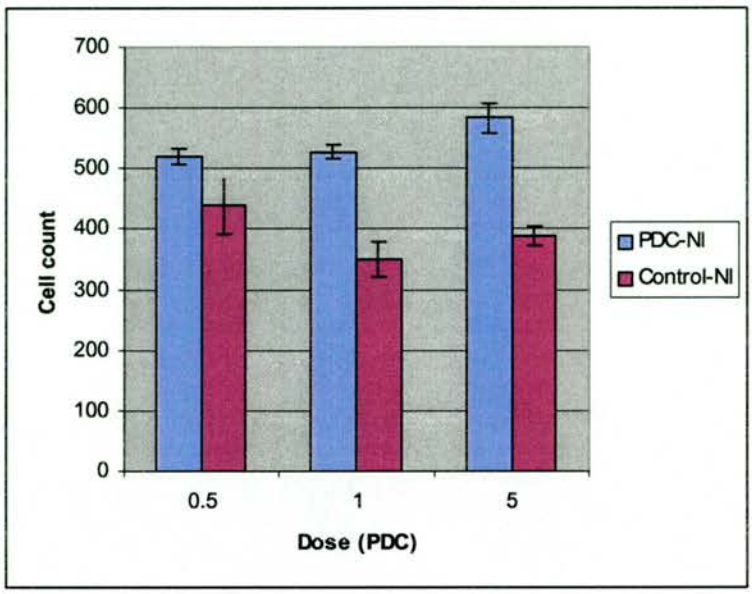
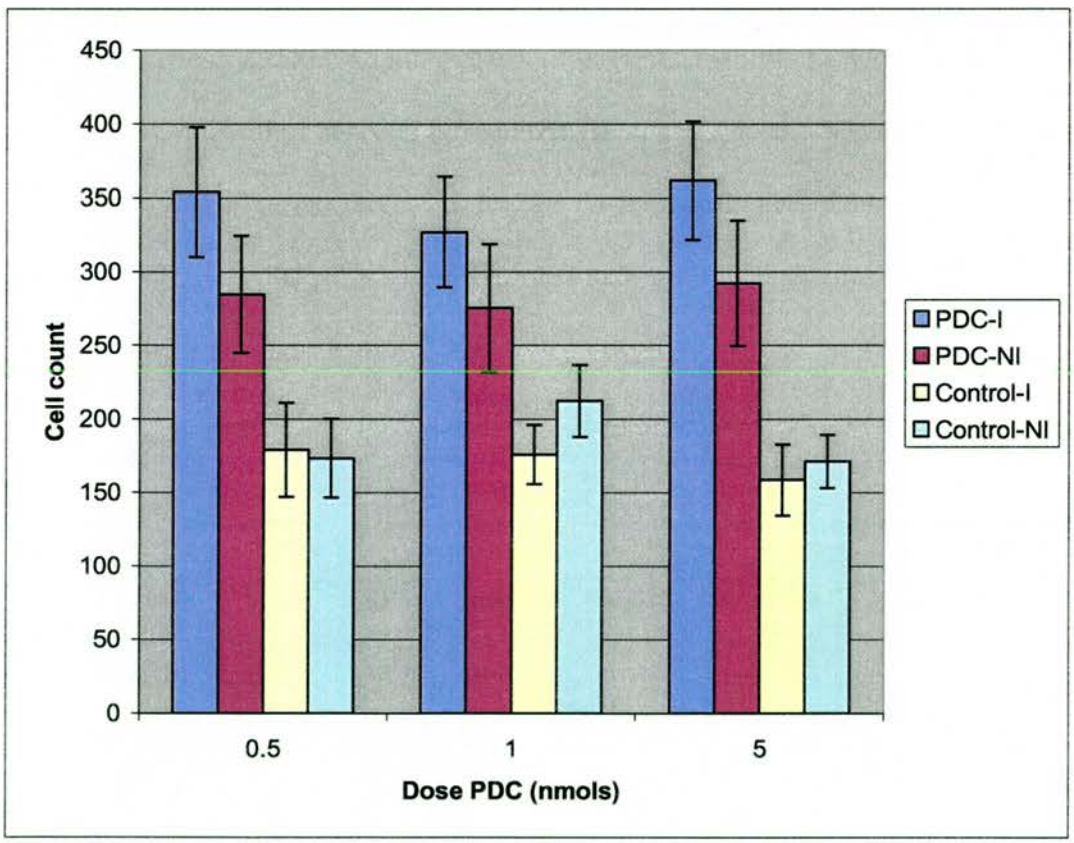


Figure 50 displays the pattern of fos-immunoreactive neurons in the thalamic reticular nucleus following microinjections of PDC. The pattern is similar to that found in the VL in that counts from the hemisphere injected with PDC were always higher than those from the non-injected hemisphere of the same rat (illustrated in Figure 55). Both hemispheres from these rats had higher fos counts than those from control animal in which the two hemispheres did not differ. In this case, however, the dose effect seen in the VL nucleus was not apparent with cell counts being similar across the doses of PDC. Results of within subjects analysis demonstrated a significant main effect of hemisphere ($F_{1,26}$ = 13.281, $p<0.001$) and a significant hemisphere x group interaction ($F_{1,26}$ = 33.178, $p<0.001$), but no hemisphere x dose or three way interaction. Between subjects analysis revealed a significant main effect of group ($F_{1,26}$ = 20.438, $p<0.001$) but no main effect of dose or group x dose

interaction. These data are slightly easier to interpret than the previous nucleus and consequently no further analysis is required. In contrast to the VL nucleus dose of PDC administered had no effect on the cell counts at all. The main effect of group shows clearly that the PDC injected rats had greater thalamic activation than controls, while the significant main effect of hemisphere and hemisphere x group interaction would seem to be a result of increased activation in the non-injected hemispheres of the rats receiving PDC injections.

Figure 50: Mean (±SE) number of fos-immunoreactive neurons in the reticular nucleus

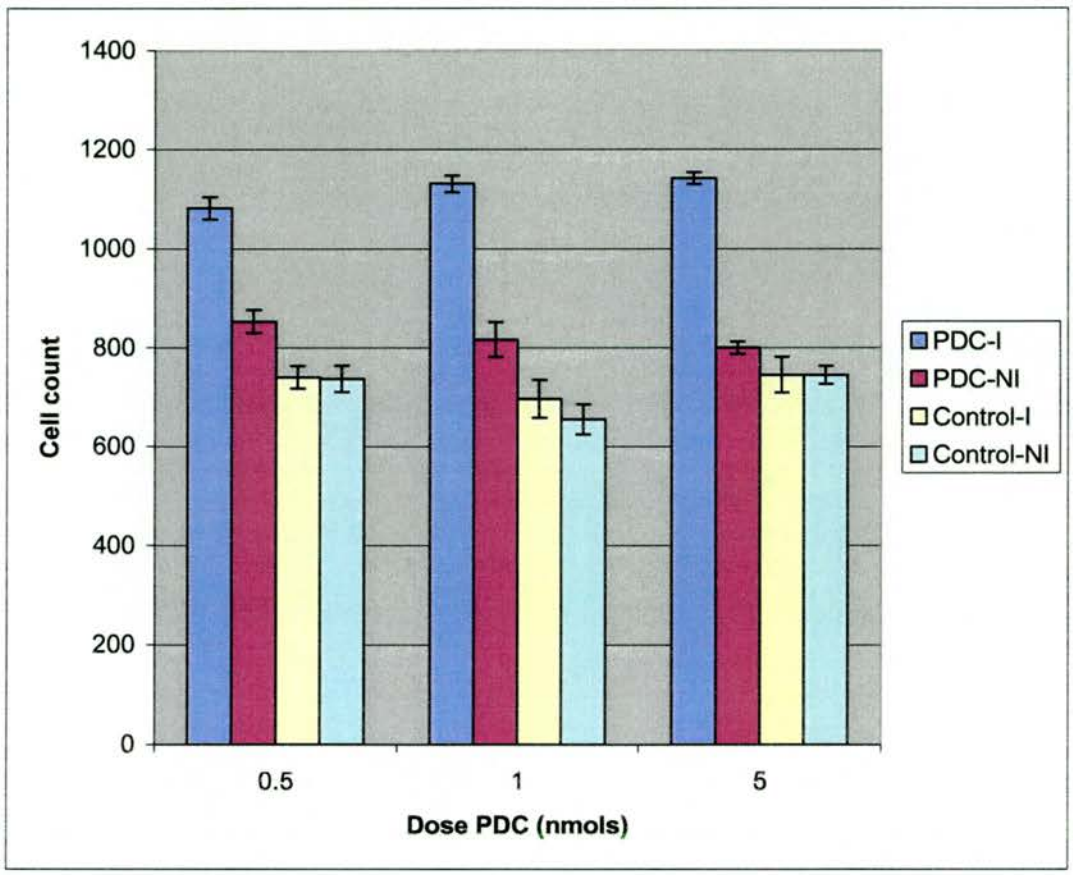


Abreviations: PDC-I – PDC injected hemisphere, PDC-NI – PDC non-injected hemisphere, Control –I - control injected hemisphere, Control-NI – control non-injected henisphere

Figure 51 illustrates the pattern of fos-immunoreactive cells in the CL nucleus. This pattern is very similar to that seen in the VL nucleus. Cell counts taken from the hemisphere injected with PDC are always higher than those taken in the non-injected hemisphere of the same rat (illustrated in Figure 57). Both of these scores are higher than counts taken from the matched pair rat that received an aCSF microinjection although this difference is less striking than in the VL nucleus. Results of within subjects analysis revealed a main effect of hemisphere ($F_{1,26}$ = 551.389, $p<0.001$) as well as significant hemisphere x group ($F_{1,26}$ = 483.339, $p<0.001$), hemisphere x dose ($F_{2,26}$ = 7.994, $p<0.002$) and hemisphere x group x dose ($F_{2,26}$ = 9.331, $p<0.001$) interactions.

Between subjects analysis revealed a significant main effect of group ($F_{1,26}$ = 160.976, $p<0.001$), but no effect of dose or group x dose interaction. While there was no main effect of dose it is clear from the interactions that the patterns of activation across the groups and hemispheres are different across the different doses and consequently further analysis is needed to explain the effects that have occurred. As in the VL analysis it would seem that the large effect of hemisphere in the PDC injected rats coupled with the complete lack of effect of hemisphere in the control rats accounts for the hemisphere part of the interactions. However the effect of group and dose across hemispheres was once again in need of further analysis. As with the VL restricted analysis in the form of multivariate ANOVA with dose and group as between subject variables were performed. Analysis of data from the injected hemispheres revealed a main effect of group ($F_{1,26}$ = 196.584, $p<0.001$) but no main effect of dose or dose x group interaction.

Figure 51: Mean (±SE) number of fos-immunoreactive neurons in the centrolateral nucleus



Abreviations: PDC-I – PDC injected hemisphere, PDC-NI – PDC non-injected hemisphere, Control –I - control injected hemisphere, Control-NI – control non-injected henisphere

Figure 52 shows that the significant group effect was a result of the PDC injected rats having much higher counts than the control rats. The restricted analysis fails to pick up any effect of dose or any interaction. Analysis of the data from the non-injected hemispheres also revealed a main effect of group ($F_{1,26}$ = 15.383, $p<0.001$) but no effect of dose or dose x group interaction. The pattern is very similar to data from the injected hemispheres and is illustrated in Figure 53.

Figure 52: Comparison of cell counts for PDC injected and control animals in the centrolateral nucleus of the injected hemispheres (mean ± SE)

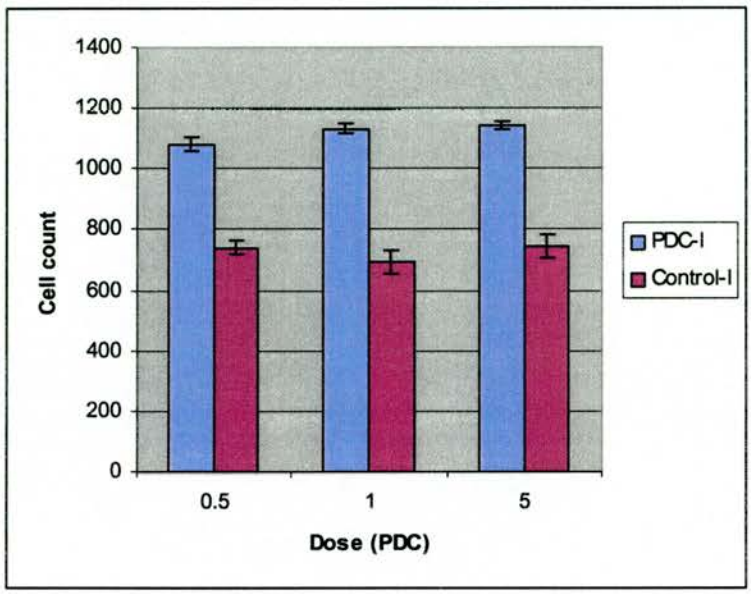


Figure 53: Comparison of cell counts for PDC injected and control animals in the centrolateral nucleus of the injected hemispheres (mean ± SE)

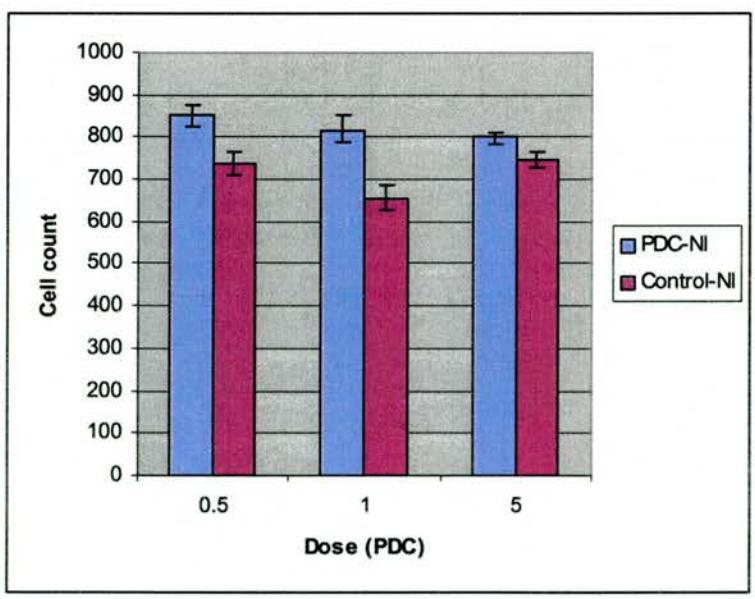
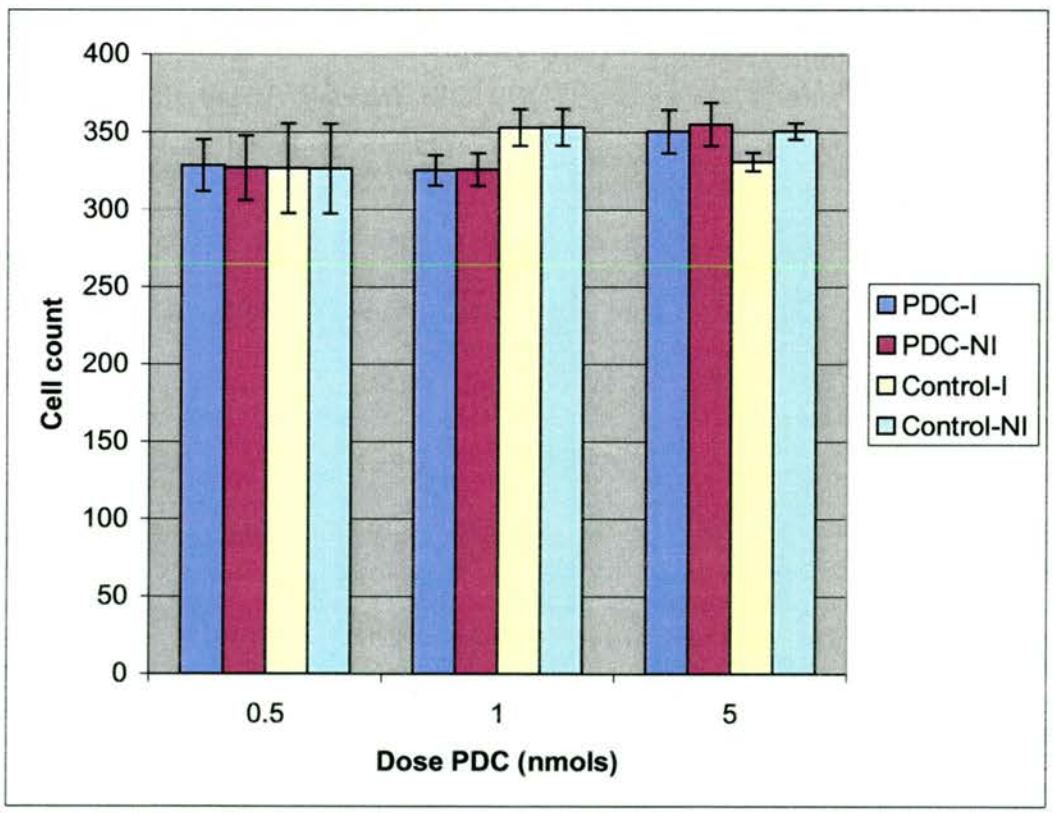


Figure 54 illustrates the pattern of fos-immunoreactive cells in the VLG nucleus. In direct contrast to the three previous sets of results the cell counts from the hemispheres of the rat injected with PDC showed very similar numbers of fos-immunoreactive neurons (illustrated in Figure 58). In addition to this the counts taken from the matched pair control rats were very similar to those reported in the rats receiving injections of the glutamate uptake blocker. Within subjects analysis of the data revealed no main effect of hemisphere or any significant interactions of hemisphere, group and dose. Similarly between subjects analysis failed to reveal either significant main effects of group or dose or any interactions between the two.

Figure 54: Mean (±SE) number of fos-immunoreactive neurons in the ventrolateral geniculate nucleus



Abreviations: PDC-I – PDC injected hemisphere, PDC-NI – PDC non-injected hemisphere, Control –I - control injected hemisphere, Control-NI – control non-injected henisphere

Figure 55: Fos staining in the reticular nucleus. An illustration of increased activity in the PDC injected hemisphere compared to the non-injected hemisphere

Injected

Non-injected

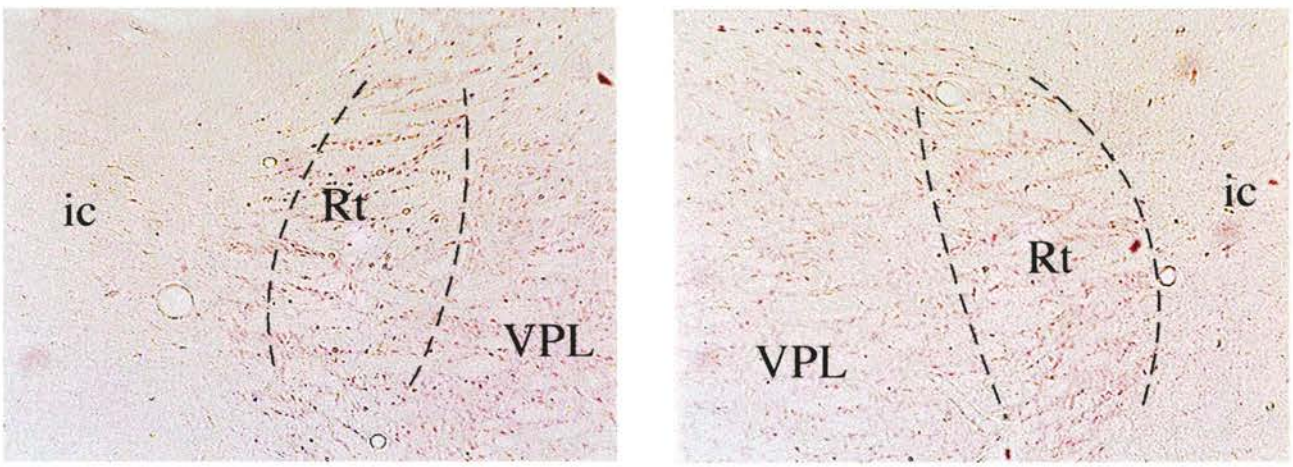
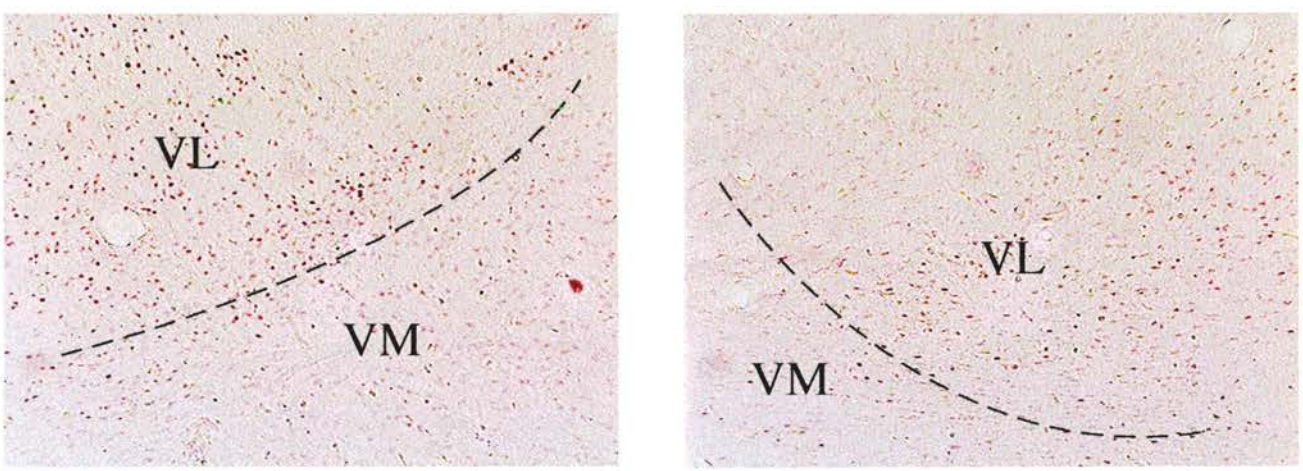


Figure 56: Fos staining in the ventrolateral nucleus. An illustration of increased activity in the PDC injected hemisphere compared to the non-injected hemisphere

Injected

Non-injected



Abbreviations: Rt - reticular nucleus, ic - internal capsule, VPL - ventral posterolateral nucleus, VL - ventrolateral nucleus, ventromedial nucleus

3.3 Discussion

The results of this study show clearly that blockade of the glutamate uptake mechanism within the PPTg produces specific and consistent changes in thalamic activity. More specifically, increased activity has been demonstrated in the CL, VL, and reticular nuclei while no difference was observed in the VLG nucleus. There are a number of aspects of this result which are particularly interesting and deserve further discussion. Firstly, the clear effect of glutamate uptake blockade on the levels of fos-immunoreactivity within the thalamus adds weight to the suggestion in the previous chapter that direct application of glutamate into the PPTg failed to excite the cells because of the efficiency of glutamate uptake system within the structure. It is of course possible that the glutamate did excite the neurons in this study but that the method used for detecting this increased activity was not sensitive enough to reveal these changes. This, however, would seem to be unlikely when the levels of background fos-immunoreactivity are considered. Background staining was consistently a problem throughout the study despite attempts to minimise fos-inducing factors. The fact that some background staining was still present after all the measures taken to minimise sensory input, stress and surgical damage suggests that fos is induced very easily in the brain and that the problem with the method would appear to be over-sensitivity rather than under-sensitivity. Thus the increased levels of activation reported in this chapter seem to suggest that the direct application of glutamate to the PPTg does not produce sufficient activation to directly alter thalamic activity, while the blockade of endogenous glutamate uptake does. It would

appear that sustained glutamatergic excitation is required to produce substantial changes in thalamic activity. This is perhaps not surprising when the neurotoxic effects of glutamate are considered. Clearly, it is vital that structures receiving glutamatergic input have an efficient mechanism by which the glutamate is removed before it causes damage to the cells of the structure in question. It may be the case that small quantities of glutamate can produce local changes in activity while more wide reaching effects require a more sustained glutamatergic excitation.

Secondly the pattern of excitation across the injected and non-injected hemispheres of the animals receiving the glutamate uptake blocker compared to those receiving control injections suggests that PPTg activation affects contralateral as well as ipsilateral activity. In the thalamic nuclei where effects of PDC were reported it was seen that activation was significantly higher in the ipsilateral hemisphere than the contralateral hemisphere as well as being higher than both the hemispheres from the control rats. What is particularly interesting, however, was that contralateral activation in drug treated rats was higher than the activation seen in the control animals. There are two possible explanations for this. It may be the case that ascending cholinergic projections from the PPTg target contralateral as well as ipsilateral thalamic targets. Alternatively the increased contralateral thalamic activation may be due to an increase in the activity of the PPTg in the contralateral hemisphere. This would be entirely consistent with anatomical studies which have described projections from PPTg to the PPTg in the contralateral hemisphere (Semba & Fibiger, 1992). An increase in activity in this pathway could affect the output from these neurons and consequently alter thalamic activity in this hemisphere.

Another intriguing result from the study concerns the effect of dose of PDC used to produce this differential thalamic activation. If the counts from the reticular nucleus are examined it would appear that a ceiling effect is present such that all doses of PDC administered produce a similar level of thalamic activation. However, results from the VL and CL nuclei would suggest that numbers of fos-immunoreactive neurons are affected in a dose dependent manner by the glutamate uptake inhibitor. This could perhaps be explained by examining the proportion of cholinergic input to these nuclei that originates in the PPTg. Figures 4 and 5 demonstrate that compared to the reticular nucleus that receives only 25% of its cholinergic input from the PPTg, the VL and CL nuclei receive a far greater proportion of their cholinergic input from the Ch5 neurons (62% and 78% respectively). It could be the case that the nuclei that receive their cholinergic input predominantly from the PPTg will be more affected by glutamate uptake blockade in this area. Were this the case, however, it would perhaps be expected that nuclei like the DLG, which is thought to receive 91% of cholinergic input from the Ch5 neurons, would be the most affected by changes in PPTg activity. This is clearly not the case as staining in the DLG was very light.

Another possible explanation for this inconsistency is the nature of the reticular nucleus compared to the VL and CL. The reticular nucleus, unlike other thalamic nuclei, is reciprocally connected to all other thalamic nuclei as well as receiving a large amount of cortical input. The nature of its anatomical connections suggests that the primary function of this nucleus is modulation of thalamocortical activity, unlike other thalamic sites which are generally involved in the processing of

a specific type of information. It could be the case that the large amount of input from other areas of the thalamus combined with the cortical input to Rt served to minimise the excitatory effects of stimulation from the ascending cholinergic pathway.

The VL and CL are both part of the system controlling motor activity within the brain. The fact that these nuclei are affected by changes in activity in the PPTg fits nicely with previous studies emphasising the role of the PPTg in the functioning of the SN and the dorsal striatum (Blaha & Winn, 1993). It would be interesting to examine activity in the limbic thalamic nuclei, like the MD, following LDTg stimulation. If the same dissociation between reward related and motor pathways modulated through the mesopontine tegmentum that was demonstrated by Blaha and colleagues in the striatum were present in the thalamus then it would be predicted that activity in reward related thalamic nuclei would be increased. Before moving on, however, it is important to address the fact that the majority of the changes seen in thalamic activity following PDC application are in areas associated with motor functioning. There are two alternative explanations for this pattern of excitation. Firstly it is possible that the activation seen in the thalamus is only indirectly related to PPTg activity. That is to say that stimulation of the PPTg may have activated another structure in the brain such as the SN which in turn is responsible for the activation of the thalamus. While this cannot be ruled out the pattern of anatomical connections and in particular the large projection from PPTg to thalamus would suggest that the most parsimonious explanation is direct excitation of the thalamus from the PPTg. Secondly this pattern of activation might be expected if an increase

in locomotion stimulated through increased glutamatergic activity in the PPTg was occurring following application of PDC. This issue is less easily dealt with and consequently is addressed in the next chapter.

Thalamic activation following PPTg stimulation III: Behavioural correlates of increased neural activation

4.0 Introduction

Experimental findings from the last chapter demonstrated clear and consistent changes in thalamic activation, as measured by fos immunohistochemistry, following stimulation of the PPTg. Blockade of the glutamate uptake process in this nucleus produced increased ipsilateral activation in specific thalamic nuclei and some evidence of increased contralateral activation. The thalamic sites which showed the clearest increases in activation were the VL and CL nuclei, both of which are involved in the processing of motor information. The nature of the method for invoking and demonstrating *c-fos* activation following PPTg stimulation involved injecting rats with a glutamate uptake blocker and then leaving them for an hour to allow the fos protein to be produced by the structures receiving excitatory afferent connections from the PPTg. It is thus conceivable that the increased level of fos-immunoreactivity in the thalamic motor nuclei is a direct result of increased motor activity caused by glutamate uptake blockade in the PPTg. To address this possibility it is important to examine the behavioural effects of microinjections of PDC in this area. To this end a further group of animals were given microinjections of PDC into the PPTg using the same procedure used in the previous study. These rats were then observed for the next hour and scored on a number of behavioural measures to assess whether an increase in gross motor function or some form of stereotopy had occurred. Although evidence for an effect of dose of PDC on c-fos activation was

minimal the largest dose was administered to these animals as this had produced the highest cells counts in the nuclei which did show a dose dependent response to the glutamate uptake blockade.

4.1 Method

Subjects

Subjects for this experiment were 8 adult male Lister hooded rats (Charles River, Kent, England, average pre-surgery weight 380.11g) All experimental and care procedures followed guidelines laid down by UK and European legislation (Animals [Scientific Procedures] Act, 1986, and European Communities Council Directive of 24 November 1986 [86/609/EEC]). Rats were housed individually under a 12 hour light / dark cycle, and provided with food and water *ad libitum.* Rats were handled everyday for at least two weeks prior to surgery. Cannula implantation procedure was as in the previous chapter.

Microinjection procedure and behavioural analysis

Rats were split into two groups. The first group received unilateral microinjections of PDC (5nmol) dissolved in 0.3 µl aCSF while the other group received control injections of aCSF. The protocol for the microinjection procedure was identical to the previous experiment. Immediately following the microinjection

procedure rats were placed in an open field environment and their behaviour was monitored for the next hour. The open field used was a larger version of the standard cage used to house the animals and measured 56cm x 40cm x 19cm. Behaviour was sampled every 30 seconds by observing the rat for 10 seconds and noting down behavioural responses that lasted for at least three seconds during the 10 seconds observation. The categories of behaviour that were observed were; still/asleep, pause, locomotion, rearing, sniffing, licking, gnawing, head down, fore paw grooming and other grooming. To briefly define the behavioural responses in each category; locomotion, sniffing, licking and gnawing are all self-explanatory. Still/asleep was defined as when the rat spent at least 3 secs lying down or asleep. Pause was defined as when the rat spent at least 3 secs stationary, but standing. Rearing was defined as when the rats reared up onto its hind legs for at least 3 secs. Head down was defined as when the rat spent at least 3 secs with its head below the horizontal axis of its body, that is to say facing the floor. Fore paw grooming was defined as when the rat spent at least 3 secs grooming its head with its fore paws and other grooming was defined as when it spent at least 3 secs grooming other areas of its body. When the hour was completed animals were perfused and brains extracted to examine cannula placement using cresyl violet staining (see appendix for method). Data was analysed by splitting the session into six 10 minute bins and then counting the number of times that each behaviour was observed in each of these bins. A floor effect was observed in three of the behavioural categories (still/asleep, licking and gnawing) and consequently the data from these measures were not analysed statistically as low counts and high variance meant that meaningful analysis was not possible. Those

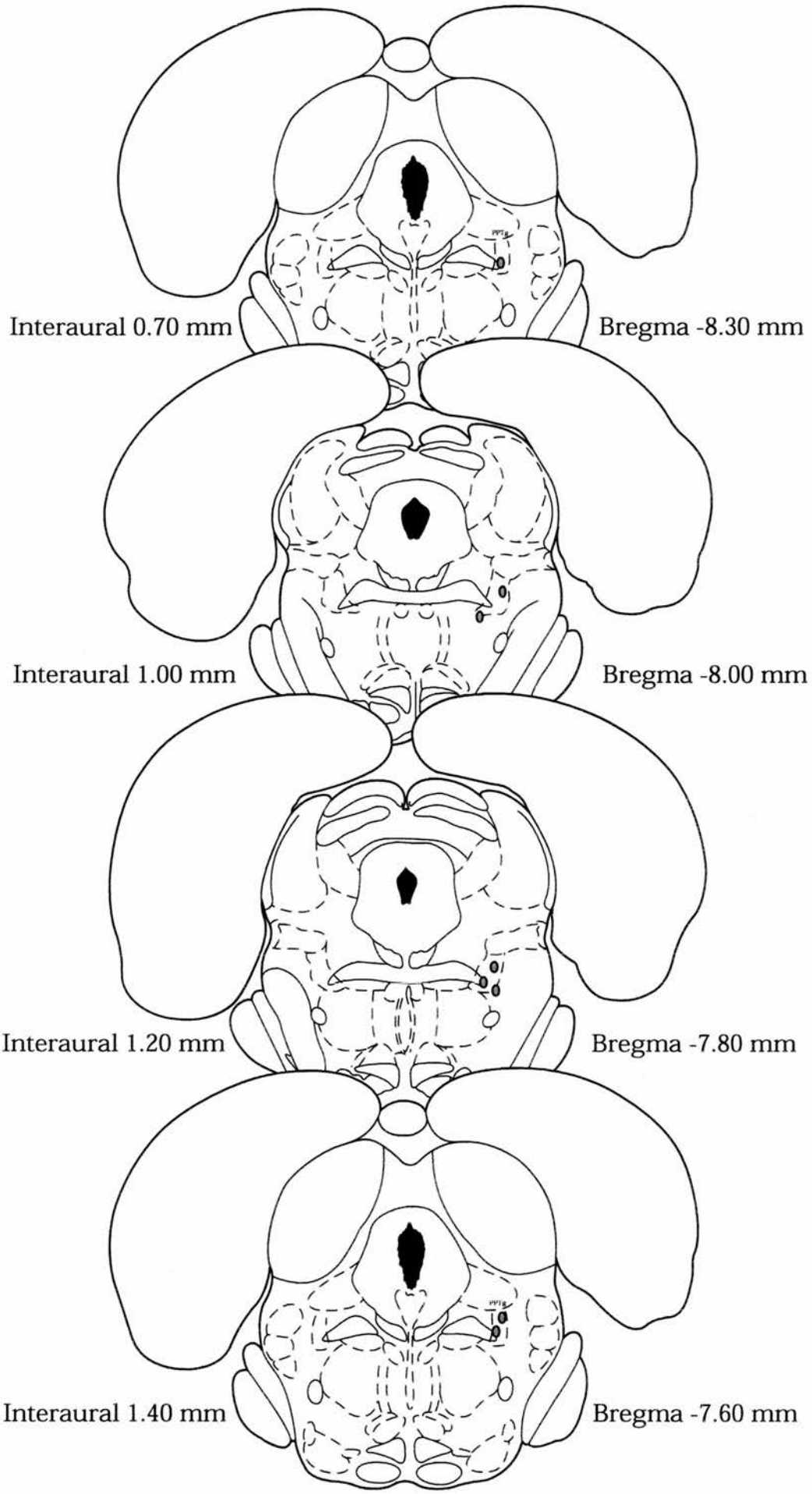
categories which did produce sufficient counts to allow meaningful analysis to be made were analysed using repeated measures ANOVA.

4.2 Results

Cannula placement and needle tracks were verified in the same way as in previous experiment. Results are displayed in Figure 59. The patterns of behavioural response to the glutamate uptake blockade within the PPTg are illustrated in Figures 60–66. Figure 60 shows the pattern of general locomotion across the hour following injections of the glutamate uptake inhibitor. It is clear that both groups initially showed high levels of locomotion, probably due to the animals exploring an unfamiliar environment. The levels of locomotion then decreased across the hour as the animals got used to their surroundings. Repeated measures ANOVA were performed on the data with bin as within subjects factor and group as between subjects factor. Analysis of within subjects factors revealed a significant main effect of bin ($F_{5,30}$ = 17.399, $p<0.001$) and no bin x group interaction. Between subjects factors analysis revealed no main effect of group. As shown in the figure, while levels of locomotion did change over the hour sampled the pattern of change across groups did not differ.

Similar analyses to those performed on the locomotion data were carried out for the other behavioural measures. These comprised repeated measures ANOVA with bin as within subjects factor and group as between subject factor. For the rearing data within subjects factor analysis revealed a significant main effect of bin

Figure 59: Schematic of cannula placement



($F_{5,30}$ = 8.785, $p<0.001$) resulting from rats exploring a novel environment at the beginning of the trial and exploring less as they got used to their surroundings. Similarly no bin x group interaction was observed and between subjects analysis revealed no main effect of group.

Figure 60: Comparison of levels of locomotion of PDC and acsf injected rats (mean ±SE)

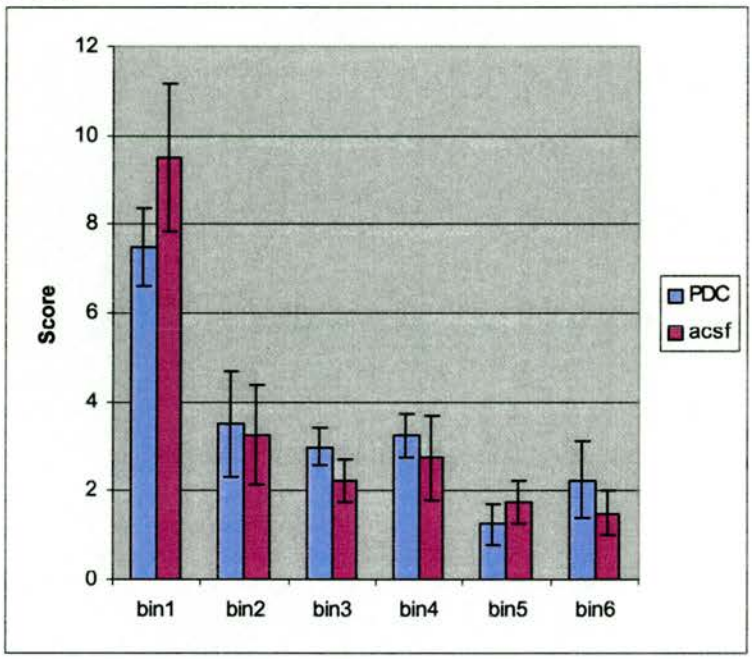
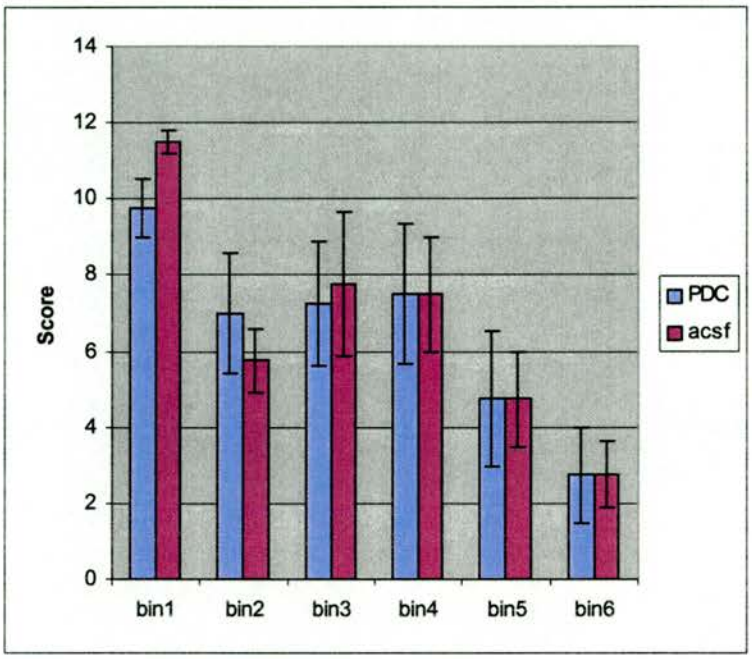


Figure 61: Comparison of levels of rearing of PDC and acsf injected rats (mean ±SE)



Sniffing data showed the same pattern with significant within subjects main effects of bin ($F_{5,30}$ = 4.405, $p<0.004$) and no group x bin interaction or between subjects main effect of group. Analysis of pausing, front paw grooming and other grooming data revealed no main effects of bin or group or any group x bin interaction. Finally analysis of head down data revealed a main effect of bin ($F_{5,30}$ = 3.506, $p<0.013$) but no main effect of group or bin x group interaction.

Figure 62: Comparison of levels of sniffing of PDC and acsf injected rats (mean ±SE)

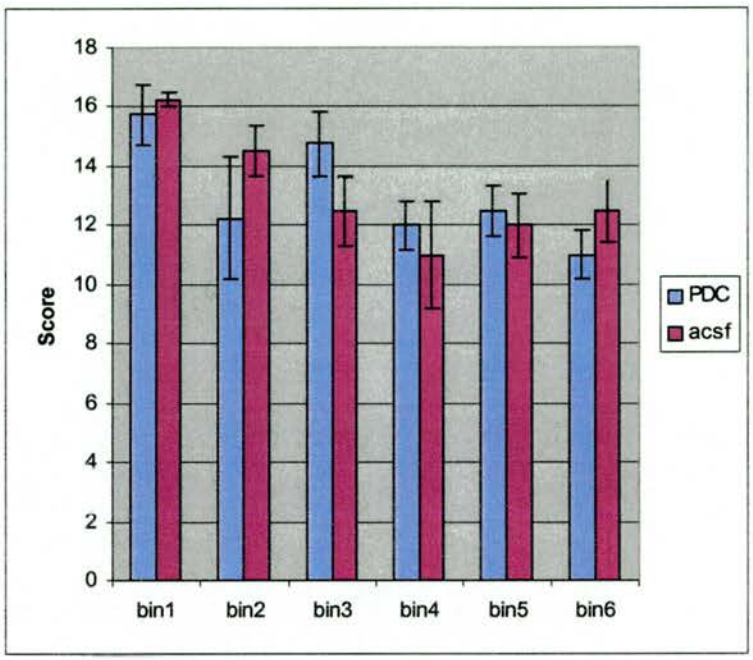


Figure 63: Comparison of levels of pausing of PDC and acsf injected rats (mean ±SE)

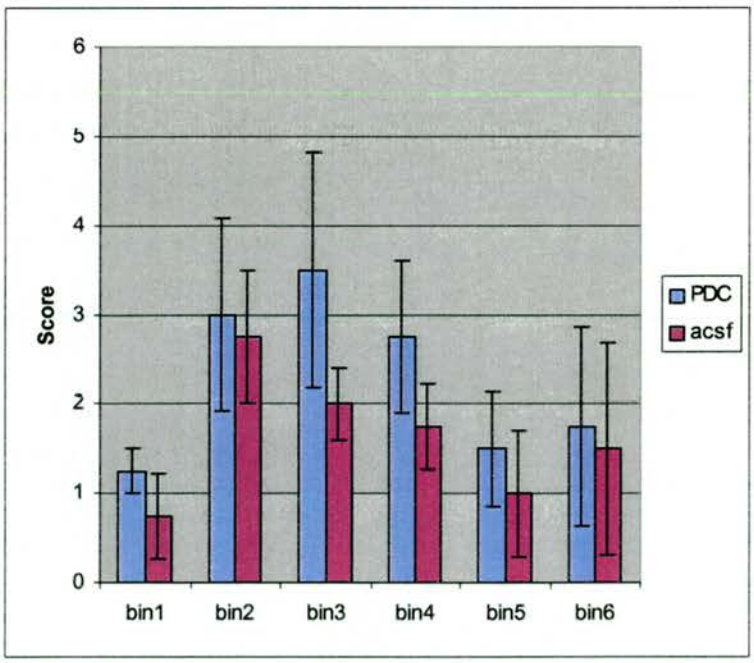


Figure 64: Comparison of levels of fore paw grooming of PDC and acsf injected rats (mean ±SE)

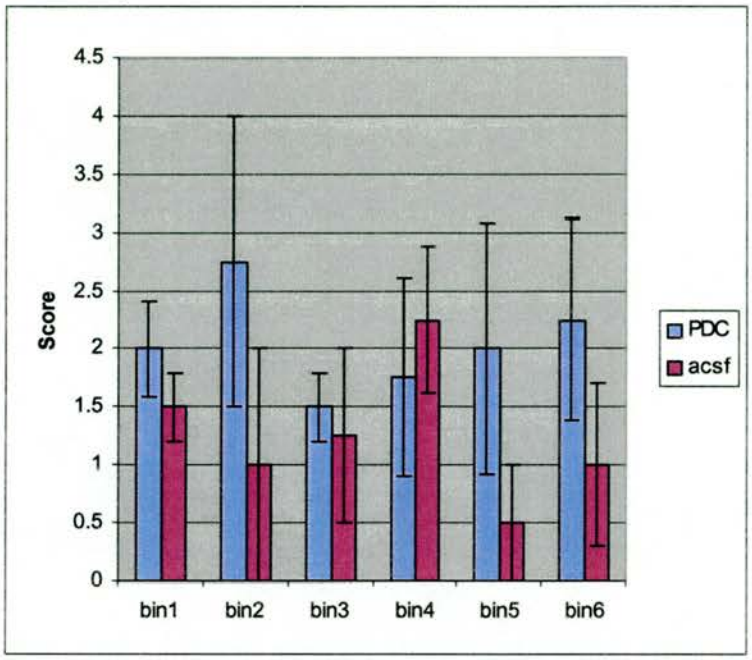


Figure 65: Comparison of levels of other grooming of PDC and acsf injected rats (mean ±SE)

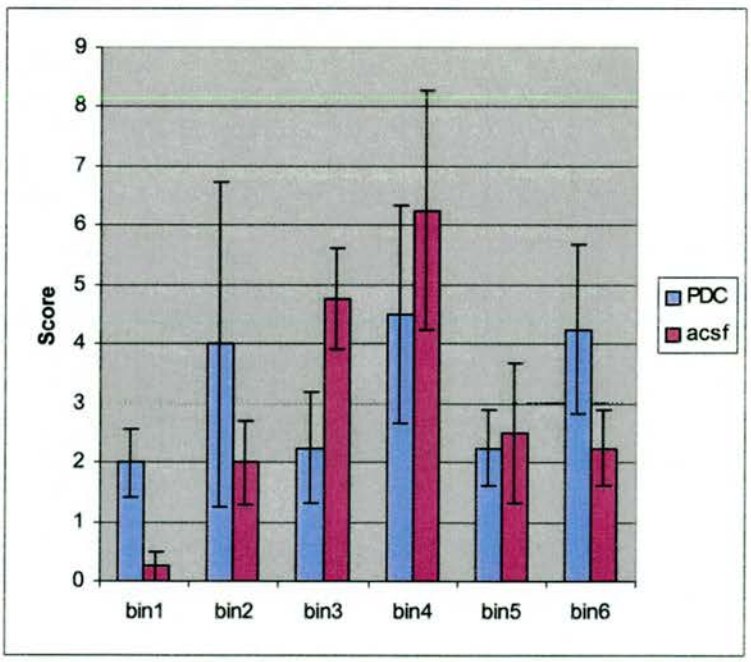
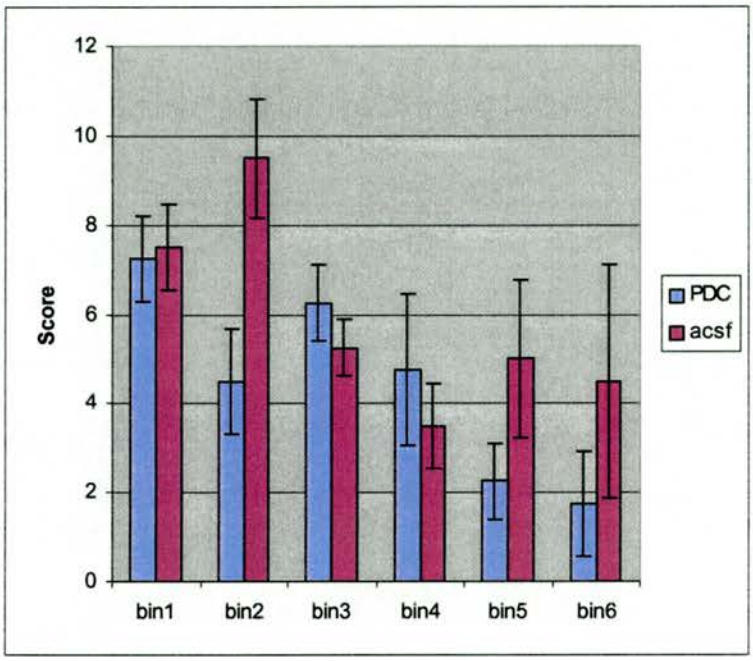


Figure 66: Comparison of levels of head down of PDC and acsf injected rats (mean ±SE)



4.3 Discussion

The results of this study show clearly that rats injected with the glutamate uptake inhibitor PDC show similar patterns of behaviour to those receiving control injections of aCSF. Significant main effects of bin were reported in four of behavioural categories, but these are easily explained in terms of rats having a tendency to explore a novel environment. The important result from this study is that no main effects of group or group x bin interactions were reported demonstrating that behavioural responses are not altered by the blockade of glutamate in the PPTg.

This is particularly important in light of the results from the previous chapter which reported an increase in activity in motor thalamic nuclei following blockade of glutamate uptake in the PPTg. The present study shows that this increase in thalamic activity cannot be attributed to an increase in either gross motor function or the formation of any type of stereotopy. Consequently these increases in thalamic function would appear to be a result of increased activity in the efferent pathway from the PPTg to the thalamus. The functional implications of these results will be discussed in the conclusion to this section.

5.0 Discussion

This section has dealt with the functional properties of the ascending efferent pathway from the PPTg to the thalamus. Earlier chapters dealt with the behavioural functions of PPTg and demonstrated that lesions of this nucleus produce deficits in high level cognitive functions that could be interpreted as a breakdown in executive function or attention. The main focus of the research from this chapter has been to try and identify an anatomical substrate through which the PPTg could have an effect on higher neural systems. Clearly if such anatomical substrates could be identified this would give a major insight into how lesions of the PPTg impact on the rest of the brain to produce these complex behavioural deficits. As stated in the introduction there are a number of possible pathways through which the PPTg could affect the rest of the brain including its innervation of the SN and its innervation of thalamus. Previous studies have shown that activity within the PPTg has a large influence on the SN and consequently on the dorsal striatum (Blaha & Winn, 1993), but very little research has examined the functional properties of the projection from PPTg to the thalamus. Experimental work from this chapter has attempted to show how and where the PPTg impacts on thalamus by stimulating the neurons of this nucleus and recording activity within the thalamus using fos immunohistochemistry.

Initial pilot work centred around refining a technique for producing adequate stimulation of the PPTg and gaining meaningful measurements of thalamic activity. This incorporated a series of studies using different methods of stimulation and a variety of techniques to reduce background fos staining to a point where activity

within the thalamus could be measured. Eventually it was found that blockade of glutamate uptake in the PPTg was the best method for stimulating these neurons and a series of experiments examining fos activation following microinjections of various doses of a glutamate uptake inhibitor (PDC) were carried out. Results from these showed that thalamic activation, as measured by fos-immunoreactivity, was significantly increased in animals receiving microinjections of PDC compared to those receiving control injections of aCSF. Further following unilateral injections it was shown that both ipsilateral and contralateral thalamic sites showed increased activity. Increased thalamic activity was demonstrated in the ventrolateral, centrolateral and thalamic reticular nuclei.

Before moving on to explore what this tells us about the position of the PPTg in neural circuitry there are two points pertaining to the nuclei where the biggest changes in activation were seen. The clearest changes were seen in the CL and VL nuclei which are both involved in the processing of motor information. Chapter four dealt with the possibility that these increases in thalamic function were merely an artefact of increased locomotion stimulated by glutamatergic activity within the PPTg. Behavioural measures demonstrated that blockade of glutamate in this nucleus produced no overt changes in behaviour. This suggests that any change in thalamic activation is a result of PPTg stimulation. One further possibility is that the changes in thalamic function are only indirectly caused by PPTg activity. It remains a possibility that activation of another structure to which the PPTg projects, such as the SN, is responsible for the change in thalamic function. However, as mentioned earlier, the patterns of connections between the PPTg and thalamus make it much

more likely that any increase in activation is a result of increased activity within the efferent pathway from the PPTg to the thalamus.

This result is consistent with previous literature examining the relationship of the PPTg with higher neural structures. A number of studies have demonstrated that cholinergic activity in the PPTg exerts control over dopaminergic function in the SN and consequently over dopamine activity in the dorsal striatum (Blaha & Winn, 1993; Clarke et al., 1987; Kelland et al., 1993; Meltzer et al., 1997; Lokwan et al., 1999). This result shows that the PPTg activation has its greatest effects in the motor nuclei of the thalamus as well as the thalamic reticular nucleus. Taken together these data present a coherent picture of the ascending cholinergic projections from the PPTg being involved in sensorimotor processing. Further experiments might aim to examine the pattern of activation in the thalamus following stimulation of the LDTg. It would be expected from this result and previous work examining the interaction of the LDTg with higher neural structures (Blaha et al., 1995) that stimulation in this area would produce activation in the limbic thalamic nuclei such as the mediodorsal.

Having examined how this fits in with previous literature it is now important to see what this result tells us about how the PPTg fits into the general circuitry of the brain. The experimental work from this section would suggest that activity in the PPTg has a major impact on the functioning of the thalamus and through this some influence on cortical activity. This reinforces the suggestion made in the introduction that the PPTg should be considered as part of a long loop of the frontostriatal system as it clearly impacts on higher neural structures as well providing an outflow for information from the striatum. Interestingly this result could also be interpreted as

providing support for including the PPTg in the ascending reticular activating system (ARAS). It has previously been suggested that the cholinergic neurons of the mesopontine tegmentum combined with the serotinergic neurons of the raphe nucleus and noradrenergic cells of the locus coeruleus are part of an ascending system of brainstem neurons involved in activation of higher neural systems through processes like attention and arousal.

At this point it is worth examining the suggestion of Sherman & Guillery (2001) that different modes of thalamic firing, controlled by its cholinergic input, are involved in conveying different types of sensory information to the cortex. It has been suggested that burst firing is involved in conveying information regarding sudden changes in sensory input while tonic firing is more involved in relaying accurate information regarding the sensory properties of stimuli that are being attended to. It would seem sensible to suggest that an animal that is no longer in control of what type of information is passed through its thalamic relays to the cortex would have severe behavioural deficits in tasks where the accurate conveying of sensory information is important, this could be manifested as an attentional deficit. Cholinergic activity within the thalamus has been demonstrated to be involved in changing of firing mode of thalamic neurons (McCormick, 1989,1992). When this is combined with the present data demonstrating clear changes in activity in specific thalamic nuclei following stimulation of the PPTg, as well as behavioural deficits that could be interpreted in terms of an attentional deficit, this is highly suggestive of a role for the PPTg in attention mediated through its projection to the thalamus.

With both of these suggestions being eminently plausible it is worth looking again at the hypothesis that the PPTg is functionally and structurally very similar to the SN. It is quite conceivable that the Ch5 neurons are part of the ARAS and, combined with the Ch6 neurons, are involved in the activation of the thalamus and consequent cortical control. Lesions of these cells could account for the attentional deficit reported by Olmstead et al (2001). The non-cholinergic cells of the PPTg, that have been shown to be in receipt of the majority of the input from the striatum, could be seen as being part of the frontostriatal system. Lesions of these cells may account for the more frontal or executive type deficits including the disinhibited behaviour examined in section A. Thus, as in the SN, the PPTg could be seen as a unitary structure comprising two separate but interdigitated populations of neurons with different functional properties. This explanation of PPTg organisation could be easily tested if it were possible to make specific lesions of the cholinergic cells in this nucleus and this possibility will be discussed in the general discussion.

One last point of interest to be discussed before moving on is how the neurotransmitters, other than ACh, in the projection to the thalamus from the PPTg effect the cells of the thalamic nuclei to which they project. Sherman and Guillery (2001) suggest that inputs to thalamic nuclei can be divided into two categories, namely drivers and modulators. An example of a driving input is the retinal input to the lateral geniculate nucleus (LGN), that is to say that the information in this input regards the function of the nucleus and in some way can be seen as defining this function. In this example the input from the retina is obviously visual and this means that the LGN is functionally involved in the processing of visual information before

it gets to cortex. Modulators are much more numerous than the driving inputs but they work through slower neurotransmitter systems compared to the ionotropic glutamate receptors through which the driving inputs have their effect. This means that driving inputs are much more likely to trigger firing in thalamic cells than modulators. It also means that in order for modulatory inputs to have an effect a large number of these inputs must be active.

It would seem sensible to suggest that the cholinergic input from the PPTg has a modulatory function on some thalamic nuclei. This would be consitent with the suggestion that this cholinergic projection is involved in the modulation of firing mode of the thalamic neurons. This aspect of PPTg function would be augmented by its effect on the activity of the thalamic reticular nucleus which is critically involved in the modulation of thalamic function. As suggested above, disruption of this pathway could result in large amounts of irrelevant material passing through thalamus to cortex that would otherwise have been filtered out. This type of problem would easily explain deficits in attentional processes. However, an explanation of the function of the projection of the PPTg to the thalamus purely in terms of this proposed modulatory role may not be entirely consistent with the anatomical evidence. The cholinergic neurons of the PPTg have been shown to have a number of other neurotransmittors co-localised within them, glutamate and GABA the principle examples of these. This raises the possibility that these neurons may provide a driving input to some thalamic nuclei. To examine this claim, further studies examining the precise anatomical nature of the efferent connection from the PPTg to the thalamus would be needed. It may be the case that while the cholinergic Ch5

neurons innervate all the thalamic nuclei in a modulatory role, a subset of these cells containing glutamate innervate a small number of specific thalamic nuclei providing a driving input. It is conceivable that the PPTg, using its inputs from the superior colliculus, could provide fast sensory information to motor thalamic sites to guide behaviour without having to pass through the areas of visual cortex. This could be hypothesis could be tested by examining the post-synaptic receptors at which these glutamatergic neurons terminate. Sherman & Guillery suggest that driving inputs act at ionotropic glutamate receptors and consequently detailed immunohistochemical studies of this pedunculothalamo projection may help elucidate the role of the PPTg in the functioning of the thalamus.

One final possibility is that single Ch5 neurons containing glutamate or GABA could act as both drivers and modulators depending on which neurotransmitter is released. The point of interest then would be under which conditions the PPTg neurons produce each neurotransmitter. Clearly all these suggestions require detailed examination of the post-synaptic receptors at the synaptic clefts where the PPTg afferents meet the thalamic neurons.

Having examined the functional properties of the projection from PPTg to thalamus, the implications of all experimental findings in this thesis will now be discussed.

General Discussion

Research carried out in this thesis has attempted to further characterise the functional and anatomical characteristics of the PPTg. The literature to date was summarised in the introduction to present a definition of the PPTg as a nucleus comprising a population of large cholinergic neurons interdigitated with another population of smaller, yet more numerous, non-cholinergic cells. Further investigation revealed that these two types of neuron shared some common neurochemical properties with glutamate and GABA being present in a proportion of both types of neuron. Along with a number of anatomical studies this evidence was used to suggest that these two neuronal populations be classified together as part of the PPTg. Having presented a definition of the nucleus it was of great interest to examine exactly what role the PPTg plays in neural circuitry. On further examination of the anatomical characteristics of the nucleus it was suggested that the patterns of connectivity with structures higher in the neuraxis put it in an ideal position to be considered as part of a long loop processing corticostriatal information. However, connections with the SNc and spinal cord also put it in an ideal position to be considered as an outflow site for information from the striatum. One other possibility is that it may be part of the ascending reticular activating system (ARAS) through its extensive connections with the thalamus. Clearly there is no need for these possible functions to be mutually exclusive and the PPTg may subserve more than one of these potential roles. Experimental work from this thesis aimed to further our understanding of how the PPTg interacts with higher neural systems.

Section A examined the functional characteristics of the nucleus. Traditionally the PPTg has been associated with sleep and motor function but more recently researchers have been examining it with reference to more high-level cognitive functions. Previous research had suggested that the PPTg be involved in a number of different functional systems including those involved with reward related behaviour, executive functions, anxiety and attention. Most of this research had involved direct manipulation of the PPTg either through the use of surgically placed lesions or pharmacological studies. A review of this literature suggested that the majority of the findings in this area were consistent with the PPTg being involved in the neural circuitry involved with executive function and attention. However, it remained unclear as to whether this nucleus played a role in reward related behaviour and this issue was addressed experimentally in section A. Previous research had demonstrated that excitotoxic lesions of the PPTg did not effect levels of motivation (Olmstead et al., 1999; Alderson et al., 2001; Keating et al., in press). In a study designed as a very direct test of incentive and primary motivation Keating (1998) used a simple runway paradigm to show that neither rats with PPTg nor sham lesions showed differing runway times for differing concentrations of sucrose reward. The possibility remained, however, that the reason for this lack of effect was due to the animals in this study only having experience of one concentration of sucrose. This was tested by incorporating a reversal into the experiment such that rats that had been running for a small reward were given a larger reward in a second week of testing and vise versa. Results showed that while PPTg lesioned rats took longer to

adjust their runway speed to a change in reward the level of reward did ultimately affect this runway speed. It was suggested that the PPTg lesioned rats were suffering from an impairment in response control, probably most consistent with either a learning deficit or attentional problem.

To investigate further the deficit induced by lesions of the PPTg a second study was run examining the consummatory phase of the behaviour examined in the first experiment of the section. Briefly PPTg lesioned rats have been repeatedly shown to over-consume high concentrations of sucrose compared to sham lesioned rats (Olmstead et al., 1999; Alderson et al., 2001; Keating et al., in press). In an attempt to explain the underlying psychological causes of this behaviour the effect of lesioning the PPTg on quinine and saccharin consumption was examined. If the underlying cause of the over-consumption of sucrose was a disinhibition of appropriate behaviour it might be expected that consumption of quinine solutions would be decreased, while the consumption of saccharin would be unaffected. However, if this behaviour arises through an inability to suppress a response in a state a high motivational then consumption of quinine would be unaffected while consumption of saccharin would increase. A third possibility is that the disinhibited drinking response is due to an increased level of finickiness which would predict an increased level of saccharin consumption combined with decreased consumption of quinine. The results were consistent with the second suggestion with PPTg lesioned rats showing an increased consumption of saccharin and normal levels of quinine consumption. Results from this section were consistent with the hypothesis that the PPTg is involved in the neural circuitry controlling executive function and attention.

Section B of the thesis attempted to address the question of how the PPTg impacts on structures higher in the neuraxis by examining the efferent projection from the PPTg to the thalamus. Anatomical studies have demonstrated that all cholinergic neurons in the PPTg project to the thalamus (Oakman et al., 1999) and further that all thalamic nuclei receive some of their cholinergic innervation from the PPTg (Hallanger & Wainer, 1988). Experimental work in this section attempted to stimulate the PPTg and then assess the impact of this stimulation on the various nuclei of the thalamus. Following a series of pilot studies it was found that the optimal method involved blocking glutamate uptake in the PPTg, using microinjections of a glutamate uptake blocker (PDC), and then examining the level of fos immunoreactivity within the thalamus. It was demonstrated that stimulation of the PPTg resulted in specific and consistent changes in some of the sensorimotor nuclei of the thalamus as well as in the reticular nucleus.

What remains to be seen is exactly what kind of input to these nuclei is being provided by the PPTg neurons. Work on the neural basis of sleep and control of behavioural state would suggest that the projection from the PPTg to the thalamus is providing a modulatory input which is concerned with turning the thalamus on and off when changing between waking and sleeping and between PS and slow wave sleep. Further it could well be the case that the PPTg is involved in changing the firing modes of thalamic cells in awake animals which would be entirely consistent with it having a role in attention. This is supported by the demonstration that the PPTg effects activity within the thalamic reticular nucleus which is involved in modulating thalamic function. However, it could that the PPTg also provides what

Sherman & Guillery (2001) referred to as a driving input to the thalamus. It may be the case that as part of the ARAS the PPTg is able to make use of information from its afferent connections from other brainstem nuclei, including early visual information from the superior colliculus, to activate or bring on-line higher structures including the thalamus and cortex. This type of explanation would be entirely consistent with the PPTg having a key role to play in the neural system involved in attention. Interestingly recent work in this laboratory has demonstrated that microinjections of PDC, as used to produce the thalamic activation in section B, have a detrimental effect on rats performing tasks requiring attentional processes. This result is consistent with the PPTg providing either a driving or modulatory input or indeed both. That is to say that a disruption of a driving signal carrying early visual information from the SC could produce deficits in attention. Equally a disruption in the system that controls what type of sensory information is let through the thalamic relays would also be expected to produce attentional deficits. It is of course eminently plausible that the PPTg could fulfil both of these functions depending on the neurotransmitter released by the neurons in question. The interesting factor would then be under what conditions PPTg neurons, containing for example glutamate and ACh, release the different neurotransmitters.

While this suggestion fits well with some of the behavioural data collected it is unfortunately not possible to account for all the lesion studies in terms of a deficit in attention. Deficits such as the orofacial stereotopies are difficult to account for in terms of attentional dysfunction. This type of deficit is more easily explained in terms of breakdown of executive function probably through a disruption of cortico-

striatal systems. It is at this point that it is worth remembering the suggestion that the PPTg may be similar in organisation to the SN. The deficits described could be explained by suggesting that the Ch5 neurons form part of the ARAS while the non-cholinergic cells within the PPTg, which receive the majority of the input from the SN, could be more involved in the processing of striatal information. Thus the attentional deficits would be a result of a disruption of the input to the thalamus while the more executive deficits could be a result of disruption of striatal processing.

There are a number of ways in which this suggested role for the PPTg can be tested experimentally. As mentioned in section B ultra structural analysis of the postsynaptic receptors on which the neurons from the PPTg terminate within the thalamus could help elucidate the nature of the input. Sherman and Guillery suggest that driving inputs usually operate through ionotropic glutamate receptors, as these are much faster than the metabatropic glutamate receptors. One other very interesting line of research would be concerned with the possibility of making specific cholinergic lesions within the nucleus. Recent studies have suggested that the PPTg Ch5 neurons contain receptors for the neuropeptide hypocretin/orexin. If this is indeed the case then it may be possible to create specific neurotoxins in the same way that IgG192 saporin was created to destroy the Ch4 neurons of the basal forebrain. If this were possible specific Ch5 lesions could be made. If the suggested organisation of PPTg were correct then lesions of these neurons would produce deficits in tasks requiring attention without producing the more executive deficits like orofacial stereotopies.

To summarise briefly, the study of the PPTg and its anatomical and functional position within the circuitry of the brain is an area that is at present still in need of further research. Recent experiments have shown that the mesopontine tegmental neurons have a much more important role than simply controlling behavioural state and motor output from the striatum. While these simple behavioural processes are undoubtedly one facet of the functional aspects of the PPTg is becoming ever clearer that researchers will have to look beyond these functions to fully understand the role of the PPTg in neural systems.

References

Ainge, J. A., T. A. Jenkins, et al. (1999). "Rewarded behaviour following excitotoxic lesions of the pedunculopontine tegmental nucleus II: Performance on a runway task." Society for Neuroscience Abstract **25**(533.4).

Ainge, J. A., S. C. Walker, et al. (2000). "The effects of excitotoxic lesions of the cuneiform nucleus and the pedunculopontine tegmental area on performance in the elevated plus maze." Society for Neuroscience Abstract **26**.

Alderson, H. L., T. A. Jenkins, et al. (2001). "The effects of excitotoxic lesions of the pedunculopontine tegmental nucleus on conditioned place preference to 4%, 12% and 20% sucrose solutions." Brain Research Bulletin **56**(6): 599-605.

Alderson, H. L., M. Latimer, et al. (2001). "The effect of training condition on D-amphetamine self-administration by pedunculopontine tegmental nucleus lesioned rats." Society for Neuroscience Abstract **27**(956.4).

Alderson, H. L., V. J. Brown, et al. (in press). "The effect of excitotoxic lesions of the pedunculopontine tegmental nucleus on performance of a progressive ratio schedule for reinforcement." Neuroscience.

Alexander, G. E., M. R. DeLong, et al. (1986). "Parallel organization of functionally segregated circuits linking basal ganglia and cortex." Annual Review of Neuroscience **9**: 357-81.

Allen, L. F. and P. Winn (1995). "Excitotoxic lesions of the pedunculopontine tegmental nucleus disinhibit orofacial behaviours stimulated by microinjections of d-amphetamine into rat ventrolateral caudate-putamen." Experimental Brain Research **104**(2): 262-74.

Allen, L. F. (1995). Defining a role for the pedunculopontine tegmental nucleus in striatal

outflow. School of Psychology, University of St Andrews.

Armstrong, D. M., C. B. Saper, et al. (1983). "Distribution of cholinergic neurons in rat brain: demonstrated by the immunocytochemical localization of choline acetyltransferase." Journal of Comparative Neurology **216**(1): 53-68.

Arvidsson, U., M. Riedl, et al. (1997). "Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems." Journal of Comparative Neurology **378**(4): 454-67.

Aston-Jones, G., M. T. Shipley, et al. (1995). The Locus Coeruleus, A5 and A7 Noradrenergic Cell Groups. The Rat Nervous System. G. Paxinos, Academic Press.

Bechara, A. and D. van_der_Kooy (1989). "The tegmental pedunculopontine nucleus: a brain-stem output of the limbic system critical for the conditioned place preferences produced by morphine and amphetamine." The Journal of Neuroscience : the Official Journal of the Society For Neuroscience **9**(10): 3400-9.

Bechara, A. and D. van_der_Kooy (1992). "A single brain stem substrate mediates the motivational effects of both opiates and food in nondeprived rats but not in deprived rats." Behavioral Neuroscience **106**(2): 351-63.

Beninato, M. and R. F. Spencer (1987). "A cholinergic projection to the rat substantia nigra from the pedunculopontine tegmental nucleus." Brain Research **412**(1): 169-74.

Berridge, K. C. (1996). "Food reward: brain substrates of wanting and liking." Neuroscience and Biobehavioral Reviews **20**(1): 1-25.

Bevan, M. D. and J. P. Bolam (1995). "Cholinergic, GABAergic, and glutamate-enriched inputs from the mesopontine tegmentum to the subthalamic nucleus in the rat." Journal of Neuroscience **15**(11): 7105-20.

Blaha, C. D. and P. Winn (1993). "Modulation of dopamine efflux in the striatum following cholinergic stimulation of the substantia nigra in intact and pedunculopontine tegmental nucleus-lesioned rats." Journal of Neuroscience **13**(3): 1035-44.

Blaha, C. D., L. F. Allen, et al. (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." Journal of Neuroscience **16**(2): 714-22.

Bolam, J. P., C. M. Francis, et al. (1991). "Cholinergic input to dopaminergic neurons in the substantia nigra: a double immunocytochemical study." Neuroscience **41**(2-3): 483-94.

Brantley, R. K. and A. H. Bass (1988). "Cholinergic Neurons in the Brain of a Teleost Fish (Porichthys- Notatus) Located with a Monoclonal-Antibody to Choline-Acetyltransferase." Journal of Comparative Neurology **275**(1): 87-105.

Bridges, R. J., M. S. Stanley, et al. (1991). "Conformationally defined neurotransmitter analogues. Selective inhibition of glutamate uptake by one pyrrolidine-2,4-dicarboxylate diastereomer." Journal of Medicinal Chemistry **34**(2): 717-25.

Brudzynski, S. M. and G. J. Mogenson (1985). "Association of the mesencephalic locomotor region with locomotor activity induced by injections of amphetamine into the nucleus accumbens." Brain Research **334**(1): 77-84.

Brudzynski, S. M. and D. Wang (1996). "C-Fos immunohistochemical localization of neurons in the mesencephalic locomotor region in the rat brain." Neuroscience **75**(3): 793-803.

Buscher, W., M. Schugens, et al. (1989). "Interhemispheric Relationship between Lateral

Hypothalamic Self-Stimulation and the Region of the Nucleus Tegmenti Pedunculo-Pontinus." Brain Research **487**(2): 321-334.

Carlson, J. D., R. D. Pearlstein, et al. (1999). "Regional metabolic changes in the pedunculopontine nucleus of unilateral 6-hydroxydopamine Parkinson's model rats." Brain Research **828**(1-2): 12-9.

Chapman, C. A., J. S. Yeomans, et al. (1997). "Increased striatal dopamine efflux follows scopolamine administered systemically or to the tegmental pedunculopontine nucleus." Neuroscience **76**(1): 177-86.

Charara, A. and A. Parent (1994). "Brainstem dopaminergic, cholinergic and serotoninergic afferents to the pallidum in the squirrel monkey." Brain Research **640**(1-2): 155-70.

Charara, A., Y. Smith, et al. (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." Journal of Comparative Neurology **364**(2): 254-66.

Clark, A. J., J. M. Clark, et al. (1990). "NMDA lesions of rat lateral hypothalamus: effects of dietary and physiological challenges." Neuroreport **1**(3-4): 263-6.

Clarke, P. B., D. W. Hommer, et al. (1987). "Innervation of substantia nigra neurons by cholinergic afferents from pedunculopontine nucleus in the rat: neuroanatomical and electrophysiological evidence." Neuroscience **23**(3): 1011-9.

Clarke, N. P., J. P. Bolam, et al. (1996). "Glutamate-enriched inputs from the mesopontine tegmentum to the entopeduncular nucleus in the rat." European Journal of Neuroscience **8**(7): 1363-76.

Clarke, N. P., M. D. Bevan, et al. (1997). "Glutamate-enriched cholinergic synaptic terminals in the entopeduncular nucleus and subthalamic nucleus of the rat." Neuroscience **81**(2): 371-85.

Clements, J. R. and S. Grant (1990). "Glutamate-like immunoreactivity in neurons of the laterodorsal tegmental and pedunculopontine nuclei in the rat." Neuroscience Letters **120**(1): 70-3.

Datta, S. (1995). "Neuronal activity in the peribrachial area: relationship to behavioral state control." Neuroscience and Biobehavioral Reviews **19**(1): 67-84.

Datta, S., E. H. Patterson, et al. (1997). "Endogenous and exogenous nitric oxide in the pedunculopontine tegmentum induces sleep." Synapse **27**(1): 69-78.

Datta, S. and D. F. Siwek (1997). "Excitation of the brain stem pedunculopontine tegmentum cholinergic cells induces wakefulness and REM sleep." Journal of Neurophysiology **77**(6): 2975-88.

Datta, S., E. H. Patterson, et al. (2001). "Excitation of the pedunculopontine tegmental NMDA receptors induces wakefulness and cortical activation in the rat." Journal of Neuroscience Research **66**(1): 109-16.

Delfs, J. M. and A. E. Kelley (1990). "The role of D1 and D2 dopamine receptors in oral stereotypy induced by dopaminergic stimulation of the ventrolateral striatum." Neuroscience **39**(1): 59-67.

Dellu, F., W. Mayo, et al. (1991). "Learning disturbances following excitotoxic lesion of cholinergic pedunculo-pontine nucleus in the rat." Brain Research **544**(1): 126-32.

Deurveilher, S. and E. Hennevin (2001). "Lesions of the pedunculopontine tegmental nucleus reduce paradoxical sleep (PS) propensity: evidence from a short-term PS

deprivation study in rats." European Journal of Neuroscience **13**(10): 1963-76.

Dormont, J. F., H. Conde, et al. (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." Experimental Brain Research **121**(4): 401-10.

Dragunow, M. and H. A. Robertson (1988). "Brain injury induces c-fos protein(s) in nerve and glial-like cells in adult mammalian brain." Brain Research **455**(2): 295-9.

Eagle, D. M., T. Humby, et al. (1999). "Differential effects of ventral and regional dorsal striatal lesions on sucrose drinking and positive and negative contrast in rats." Psychobiology **27**(2): 267-276.

Edley, S. M. and A. M. Graybiel (1983). "The afferent and efferent connections of the feline nucleus tegmenti pedunculopontinus, pars compacta." Journal of Comparative Neurology **217**(2): 187-215.

Erro, E., J. L. Lanciego, et al. (1999). "Relationships between thalamostriatal neurons and pedunculopontine projections to the thalamus: a neuroanatomical tract-tracing study in the rat." Experimental Brain Research **127**(2): 162-70.

Floresco, S. B., J. K. Seamans, et al. (1996). "Differential effects of lidocaine infusions into the ventral CA1/subiculum or the nucleus accumbens on the acquisition and retention of spatial information." Behavioural Brain Research **81**(1-2): 163-71.

Floresco, S. B., J. K. Seamans, et al. (1997). "Selective roles for hippocampal, prefrontal cortical, and ventral striatal circuits in radial-arm maze tasks with or without a delay." Journal of Neuroscience **17**(5): 1880-90.

Florio, T., A. Capozzo, et al. (1999). "The function of the pedunculopontine nucleus in the preparation and execution of an externally-cued bar pressing task in the rat."

Behavioural Brain Research **104**(1-2): 95-104.

Ford, B., C. J. Holmes, et al. (1995). "GABAergic neurons in the rat pontomesencephalic tegmentum: codistribution with cholinergic and other tegmental neurons projecting to the posterior lateral hypothalamus." Journal of Comparative Neurology **363**(2): 177-96.

Fujimoto, K., K. Ikeguchi, et al. (1992). "Impaired acquisition, preserved retention and retrieval of avoidance behavior after destruction of pedunculopontine nucleus areas in the rat." Neuroscience Research **13**(1): 43-51.

Futami, T., K. Takakusaki, et al. (1995). "Glutamatergic and cholinergic inputs from the pedunculopontine tegmental nucleus to dopamine neurons in the substantia nigra pars compacta." Neuroscience Research **21**(4): 331-42.

Gai, W. P., P. C. Blumbergs, et al. (1993). "Galanin-containing fibers innervate substance P-containing neurons in the pedunculopontine tegmental nucleus in humans." Brain Research **618**(1): 135-41.

Garcia-Rill, E. and R. D. Skinner (1987). "The mesencephalic locomotor region. I. Activation of a medullary projection site." Brain Research **411**(1): 1-12.

Garcia-Rill, E. and R. D. Skinner (1987). "The mesencephalic locomotor region. II. Projections to reticulospinal neurons." Brain Research **411**(1): 13-20.

Garcia-Rill, E., N. Kinjo, et al. (1990). "Posterior midbrain-induced locomotion." Brain Research Bulletin **24**(3): 499-508.

Garcia-Rill, E. (1991). "The pedunculopontine nucleus." Progress in Neurobiology **36**(5): 363-89.

Gegelashvili, G. and A. Schousboe (1998). "Cellular distribution and kinetic properties of

high-affinity glutamate transporters." Brain Research Bulletin **45**(3): 233-8.

Gould, E., N. J. Woolf, et al. (1989). "Cholinergic projections to the substantia nigra from the pedunculopontine and laterodorsal tegmental nuclei." Neuroscience **28**(3): 611-23.

Groenewegen, H. J., H. W. Berendse, et al. (1993). "Organization of the output of the ventral striatopallidal system in the rat: ventral pallidal efferents." Neuroscience **57**(1): 113-42.

Grofova, I. and M. Zhou (1998). "Nigral innervation of cholinergic and glutamatergic cells in the rat mesopontine tegmentum: light and electron microscopic anterograde tracing and immunohistochemical studies." Journal of Comparative Neurology **395**(3): 359-79.

Hallanger, A. E., A. I. Levey, et al. (1987). "The origins of cholinergic and other subcortical afferents to the thalamus in the rat." Journal of Comparative Neurology **262**(1): 105-24.

Hallanger, A. E. and B. H. Wainer (1988). "Ascending projections from the pedunculopontine tegmental nucleus and the adjacent mesopontine tegmentum in the rat." Journal of Comparative Neurology **274**(4): 483-515.

Halliday, G. M., Y. W. Li, et al. (1990). "Neuropathology of immunohistochemically identified brainstem neurons in Parkinson's disease." Annals of Neurology **27**(4): 373-85.

Hammond, C., B. Rouzaire_Dubois, et al. (1983). "Anatomical and electrophysiological studies on the reciprocal projections between the subthalamic nucleus and nucleus tegmenti pedunculopontinus in the rat." Neuroscience **9**(1): 41-52.

Hars, B. (1999). "Endogenous nitric oxide in the rat pons promotes sleep." Brain Research **816**(1): 209-19.

Heimer, L., D. S. Zahm, et al. (1991). "Specificity in the projection patterns of accumbal core and shell in the rat." Neuroscience **41**(1): 89-125.

Henderson, J. M., K. Carpenter, et al. (2000). "Loss of thalamic intralaminar nuclei in progressive supranuclear palsy and Parkinson's disease: clinical and therapeutic implications." Brain **123 (Pt 7)**: 1410-21.

Hernandez_Lopez, S., J. L. Gongora_Alfaro, et al. (1992). "A cholinergic input to the substantia nigra pars compacta increases striatal dopamine metabolism measured by in vivo voltammetry." Brain Research **598**(1-2): 114-20.

Hirsch, E. C., A. M. Graybiel, et al. (1987). "Neuronal loss in the pedunculopontine tegmental nucleus in Parkinson disease and in progressive supranuclear palsy." Proceedings of the National Academy of Sciences of the United States of America **84**(16): 5976-80.

Honda, T. and K. Semba (1994). "Serotonergic synaptic input to cholinergic neurons in the rat mesopontine tegmentum." Brain Research **647**(2): 299-306.

Honda, T. and K. Semba (1995). "An ultrastructural study of cholinergic and non-cholinergic neurons in the laterodorsal and pedunculopontine tegmental nuclei in the rat." Neuroscience **68**(3): 837-53.

Hoshino, K., Y. Y. Katoh, et al. (2000). "Distribution of terminals from pedunculopontine tegmental nucleus and synaptic organization in lateralis medialis-suprageniculate nucleus of cat's thalamus: anterograde tracing, immunohistochemical studies, and quantitative analysis." Visual Neuroscience **17**(6): 893-904.

Howell, D. C. (1996). Statistical methods for psychology, Wadsworth.

Ichinohe, N., B. Teng, et al. (2000). "Morphological study of the tegmental pedunculopontine nucleus, substantia nigra and subthalamic nucleus, and their interconnections in rat organotypic culture." **201**(6): 435-53.

Inglis, W. L., L. F. Allen, et al. (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**(4): 817-33.

Inglis, W. L., J. S. Dunbar, et al. (1994). "Outflow from the nucleus accumbens to the pedunculopontine tegmental nucleus: a dissociation between locomotor activity and the acquisition of responding for conditioned reinforcement stimulated by d-amphetamine." Neuroscience **62**(1): 51-64.

Inglis, W. L. and P. Winn (1995). "The pedunculopontine tegmental nucleus: where the striatum meets the reticular formation." Progress in Neurobiology **47**(1): 1-29.

Inglis, W. L., M. M. Thakkar, et al. (1995). "Ibotenic acid lesions of the rat pedunculopontine or laterodorsal tegmental nucleus: Effects on behavioural state control." Sleep Research **24A**: 217.

Inglis, W. L. and K. Semba (1996). "Colocalization of ionotropic glutamate receptor subunits with NADPH-diaphorase-containing neurons in the rat mesopontine tegmentum." Journal of Comparative Neurology **368**(1): 17-32.

Inglis, W. L., M. C. Olmstead, et al. (2000). "Pedunculopontine tegmental nucleus lesions impair stimulus--reward learning in autoshaping and conditioned reinforcement paradigms." Behavioral Neuroscience **114**(2): 285-94.

Inglis, W. L., M. C. Olmstead, et al. (2001). "Selective deficits in attentional performance on the 5-choice serial reaction time task following pedunculopontine tegmental nucleus

lesions." Behavioural Brain Research **123**(2): 117-131.

Jackson, A. and A. R. Crossman (1983). "Nucleus tegmenti pedunculopontinus: efferent connections with special reference to the basal ganglia, studied in the rat by anterograde and retrograde transport of horseradish peroxidase." Neuroscience **10**(3): 725-65.

Jellinger, K. (1988). "The pedunculopontine nucleus in Parkinson's disease, progressive supranuclear palsy and Alzheimer's disease." Journal of Neurology, Neurosurgery and Psychiatry **51**(4): 540-3.

Jenkins, T. A., M. P. Latimer, et al. (2002). "Determination of acetylcholine and dopamine content in thalamus and striatum after excitotoxic lesions of the pedunculopontine tegmental nucleus in rats." Neuroscience Letters **322**(1): 45-8.

Jones, B. E. and A. C. Cuello (1989). "Afferents to the basal forebrain cholinergic cell area from pontomesencephalic--catecholamine, serotonin, and acetylcholine--neurons." Neuroscience **31**(1): 37-61.

Jones, B. E. (1991). "Noradrenergic locus coeruleus neurons: their distant connections and their relationship to neighboring (including cholinergic and GABAergic) neurons of the central gray and reticular formation." Progress in Brain Research **88**: 15-30.

Jones, B. E. (1991). "Paradoxical sleep and its chemical/structural substrates in the brain." Neuroscience **40**(3): 637-56.

Jones, B. E. (1993). "The Organization of Central Cholinergic Systems and Their Functional Importance in Sleep-Waking States." Progress in Brain Research **98**: 61-71.

Jouvet, M. (1962). "Recherches sur les structures nervouses et les mechanismes responsables des differentes phases du sommeil physiologique." Archives Italien Biologique **100**: 125-206.

Kang, Y. and S. T. Kitai (1990). "Electrophysiological properties of pedunculopontine neurons and their postsynaptic responses following stimulation of substantia nigra reticulata." Brain Research **535**(1): 79-95.

Kasashima, S., Y. Muroishi, et al. (1998). "In situ mRNA hybridization study of the distribution of choline acetyltransferase in the human brain." Brain Research **806**(1): 8-15.

Keating, G. L. (1998). Examination of the role of the pedunculopontine tegmental nucleus in the control of behavioural processes. School of Psychology, University of St Andrews.

Keating, G. L. and P. Winn (2002). "Examination of the role of the pedunculopontine tegmental nucleus in the radial arm maze with or without a delay." Neuroscience **112**(3): 687-696.

Keating, G. L., S. C. Walker, et al. (in press). "An examination of the effects of bilateral excitotoxic lesions of the pedunculopontine tegmental nucleus on responding to sucrose reward." Behavioural Brain Research.

Kelland, M. D., A. S. Freeman, et al. (1993). "Ascending afferent regulation of rat midbrain dopamine neurons." Brain Research Bulletin **31**(5): 539-46.

Kim, J. H. and P. Vezina (1999). "Blockade of glutamate reuptake in the rat nucleus accumbens increases locomotor activity." Brain Research **819**(1-2): 165-9.

Kimura, H., P. L. McGeer, et al. (1981). "The central cholinergic system studied by choline acetyltransferase immunohistochemistry in the cat." Journal of Comparative Neurology **200**(2): 151-201.

Kimura, H., P. L. McGeer, et al. (1981). "The Central Cholinergic System Studied by Choline- Acetyltransferase Immunohistochemistry in the Cat." Journal of Comparative Neurology **200**(2): 151-201.

Koch, M., M. Kungel, et al. (1993). "Cholinergic Neurons in the Pedunculopontine Tegmental Nucleus Are Involved in the Mediation of Prepulse Inhibition of the Acoustic Startle Response in the Rat." Experimental Brain Research **97**(1): 71-82.

Kojima, J., Y. Yamaji, et al. (1997). "Excitotoxic lesions of the pedunculopontine tegmental nucleus produce contralateral hemiparkinsonism in the monkey." Neuroscience Letters **226**(2): 111-4.

Kolmac, C. I. and J. Mitrofanis (1998). "Patterns of brainstem projection to the thalamic reticular nucleus." Journal of Comparative Neurology **396**(4): 531-43.

Kolmac, C. I., B. D. Power, et al. (1998). "Patterns of connections between zona incerta and brainstem in rats." Journal of Comparative Neurology **396**(4): 544-55.

Kolmac, C. and J. Mitrofanis (2000). "Organization of brain stem afferents to the ventral lateral geniculate nucleus of rats." Visual Neuroscience **17**(2): 313-8.

Kozak, R., E. M. Bowman, et al. (2001). "The impact of bilateral excitotoxic lesions of the pedunculopontine tegmental nucleus on sustained attention in rats." Society for Neuroscience Abstract **28**(313.15).

Krauthamer, G. M., B. S. Grunwerg, et al. (1995). "Putative cholinergic neurons of the pedunculopontine tegmental nucleus projecting to the superior colliculus consist of sensory responsive and unresponsive populations which are functionally distinct from other mesopontine neurons." Neuroscience **69**(2): 507-17.

Lai, Y. Y., J. R. Clements, et al. (1999). "Brainstem projections to the ventromedial

medulla in cat: retrograde transport horseradish peroxidase and immunohistochemical studies." Journal of Comparative Neurology **408**(3): 419-36.

Lavoie, B. and A. Parent (1994). "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." Journal of Comparative Neurology **344**(2): 190-209.

Lavoie, B. and A. Parent (1994). "Pedunculopontine nucleus in the squirrel monkey: cholinergic and glutamatergic projections to the substantia nigra." Journal of Comparative Neurology **344**(2): 232-41.

Lee, H. J., D. B. Rye, et al. (1988). "Cholinergic vs. noncholinergic efferents from the mesopontine tegmentum to the extrapyramidal motor system nuclei." Journal of Comparative Neurology **275**(4): 469-92.

Lee, S. L., J. O. Rinne, et al. (2000). "The Pedunculopontine Nucleus: Its Role in the Genesis of Movement Disorders." Yonsei Medical Journal **41**(2): 167-184.

Leergaard, T. B., K. D. Alloway, et al. (2000). "Three-dimensional topography of corticopontine projections from rat barrel cortex: correlations with corticostriatal organization." Journal of Neuroscience **20**(22): 8474-84.

Lenz, F. A., I. M. Garonzik, et al. (1998). "Neuronal activity in the region of the thalamic principal sensory nucleus (ventralis caudalis) in patients with pain following amputations." Neuroscience **86**(4): 1065-81.

Leonard, C. S. and L. L. Llinas (1990). Electrophysiology of mammalian pedunculopontine and laterodorsal tegmental neurons *in vitro*: Implications for the control of REM sleep. Brain Cholinergic Systems. M. Steriade and D. Biesold. Oxford, Oxford University Press**:** 205-223.

Leonard, C. S. and R. Llinas (1994). "Serotonergic and cholinergic inhibition of mesopontine cholinergic neurons controlling REM sleep: an in vitro electrophysiological study." Neuroscience **59**(2): 309-30.

Leonard, T. O. and R. Lydic (1997). "Pontine nitric oxide modulates acetylcholine release, rapid eye movement sleep generation, and respiratory rate." Journal of Neuroscience **17**(2): 774-85.

Lepore, M. and K. B. J. Franklin (1996). "N-methyl-D-aspartate lesions of the pedunculopontine nucleus block acquisition and impair maintenance of responding reinforced with brain stimulation." Neuroscience **71**(1): 147-155.

Leri, F. and K. B. J. Franklin (1998). "Learning impairments caused by lesions to the pedunculopontine tegmental nucleus: an artifact of anxiety?" Brain Research **807**(1-2): 187-192.

Lokwan, S. J., P. G. Overton, et al. (1999). "Stimulation of the pedunculopontine tegmental nucleus in the rat produces burst firing in A9 dopaminergic neurons." Neuroscience **92**(1): 245-54.

Losier, B. J. and K. Semba (1993). "Dual projections of single cholinergic and aminergic brainstem neurons to the thalamus and basal forebrain in the rat." Brain Research **604**(1-2): 41-52.

Manaye, K. F., R. Zweig, et al. (1999). "Quantification of cholinergic and select non-cholinergic mesopontine neuronal populations in the human brain." Neuroscience **89**(3): 759-70.

Marin, O., W. Smeets, et al. (1997). "Distribution of choline acetyltransferase immunoreactivity in the brain of anuran (Rana perezi, Xenopus laevis) and urodele

(Pleurodeles waltl) amphibians." Journal of Comparative Neurology **382**(4): 499-534.

Matsumura, M., K. Watanabe, et al. (1997). "Single-unit activity in the primate nucleus tegmenti pedunculopontinus related to voluntary arm movement." Neuroscience Research **28**(2): 155-65.

Matsumura, M., A. Nambu, et al. (2000). "Organization of somatic motor inputs from the frontal lobe to the pedunculopontine tegmental nucleus in the macaque monkey." Neuroscience **98**(1): 97-110.

McCormick, D. A. (1989). "Cholinergic and noradrenergic modulation of thalamocortical processing." Trends in Neurosciences **12**(6): 215-21.

McCormick, D. A. and H. R. Feeser (1990). "Functional implications of burst firing and single spike activity in lateral geniculate relay neurons." Neuroscience **39**(1): 103-13.

McCormick, D. A. (1992). "Neurotransmitter actions in the thalamus and cerebral cortex and their role in neuromodulation of thalamocortical activity." Progress in Neurobiology **39**(4): 337-88.

Mediavilla, C., F. Molina, et al. (2000). "Electrolytic lesions of the pedunculopontine nucleus disrupt concurrent learned aversion induced by NaCl." Neurobiology of Learning and Memory **74**(2): 105-18.

Medina, L., W. Smeets, et al. (1993). "Distribution of Choline-Acetyltransferase Immunoreactivity in the Brain of the Lizard Gallotia-Galloti." Journal of Comparative Neurology **331**(2): 261-285.

Medina, L. and A. Reiner (1994). "Distribution of Choline-Acetyltransferase Immunoreactivity in the Pigeon Brain." Journal of Comparative Neurology **342**(4): 497-537.

Meltzer, L. T., C. L. Christoffersen, et al. (1997). "Modulation of dopamine neuronal activity by glutamate receptor subtypes." Neuroscience and Biobehavioral Reviews **21**(4): 511-8.

Mesulam, M. M., E. J. Mufson, et al. (1983). "Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1-Ch6)." Neuroscience **10**(4): 1185-201.

Mesulam, M. M., C. Geula, et al. (1989). "Human reticular formation: cholinergic neurons of the pedunculopontine and laterodorsal tegmental nuclei and some cytochemical comparisons to forebrain cholinergic neurons." Journal of Comparative Neurology **283**(4): 611-33.

Milner, K. L. and G. J. Mogenson (1988). "Electrical and chemical activation of the mesencephalic and subthalamic locomotor regions in freely moving rats." Brain Research **452**(1-2): 273-85.

Mineff, E. M., A. Popratiloff, et al. (1998). "Evidence for a possible glycinergic inhibitory neurotransmission in the midbrain and rostral pons of the rat studied by gephyrin." Archives of Physiology and Biochemistry **106**(3): 210-20.

Mitani, A., K. Ito, et al. (1988). "Cholinergic projections from the laterodorsal and pedunculopontine tegmental nuclei to the pontine gigantocellular tegmental field in the cat." Brain Research **451**(1-2): 397-402.

Mitchell, I. J., C. E. Clarke, et al. (1989). "Neural mechanisms underlying parkinsonian symptoms based upon regional uptake of 2-deoxyglucose in monkeys exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine." Neuroscience **32**(1): 213-26.

Mogenson, G. J. (1987). "Limbic Motor Integration." Progress in Psychobiology and

Physiological Psychology **12**: 117-170.

Moriizumi, T. and T. Hattori (1992). "Separate neuronal populations of the rat globus pallidus projecting to the subthalamic nucleus, auditory cortex and pedunculopontine tegmental area." Neuroscience **46**(3): 701-10.

Munro-Davies, L., J. Winter, et al. (2001). "Kainate acid lesions of the pedunculopontine region in the normal behaving primate." Movement Disorders **16**(1): 150-1.

Nadler, J. V., D. A. Evenson, et al. (1981). "Comparative toxicity of kainic acid and other acidic amino acids toward rat hippocampal neurons." Neuroscience **6**(12): 2505-17.

Nauta, H. J. (1979). "Projections of the pallidal complex: an autoradiographic study in the cat." Neuroscience **4**(12): 1853-73.

Nicolelis, M. A., L. A. Baccala, et al. (1995). "Sensorimotor encoding by synchronous neural ensemble activity at multiple levels of the somatosensory system." Science **268**(5215): 1353-8.

Oakman, S. A., P. L. Faris, et al. (1995). "Distribution of pontomesencephalic cholinergic neurons projecting to substantia nigra differs significantly from those projecting to ventral tegmental area." Journal of Neuroscience **15**(9): 5859-69.

Oakman, S. A., P. L. Faris, et al. (1999). "Characterization of the extent of pontomesencephalic cholinergic neurons' projections to the thalamus: comparison with projections to midbrain dopaminergic groups." Neuroscience **94**(2): 529-47.

Oda, Y. and I. Nakanishi (2000). "The distribution of cholinergic neurons in the human central nervous system." Histology and Histopathology **15**(3): 825-834.

Okazaki, S., Y. Nishida, et al. (1996). "Acute neurotoxicity of L-glutamate induced by

impairment of the glutamate uptake system." Neurochemical Research **21**(10): 1201-7.

Olmstead, M. C. and K. B. Franklin (1994). "Lesions of the pedunculopontine tegmental nucleus abolish catalepsy and locomotor depression induced by morphine." Brain Research **662**(1-2): 134-40.

Olmstead, M. C. and K. B. Franklin (1994). "Lesions of the pedunculopontine tegmental nucleus block drug-induced reinforcement but not amphetamine-induced locomotion." Brain Research **638**(1-2): 29-35.

Olmstead, M. C., E. M. Munn, et al. (1998). "Effects of pedunculopontine tegmental nucleus lesions on responding for intravenous heroin under different schedules of reinforcement." Journal of Neuroscience **18**(13): 5035-5044.

Olmstead, M. C., W. L. Inglis, et al. (1999). "Lesions of the pedunculopontine tegmental nucleus increase sucrose consumption but do not affect discrimination or contrast effects." Behavioral Neuroscience **113**(4): 732-43.

Owen, A. M., A. C. Roberts, et al. (1993). "Contrasting mechanisms of impaired attentional set-shifting in patients with frontal lobe damage or Parkinson's disease." Brain **116 (Pt 5)**: 1159-75.

Papa, M., D. F. Berger, et al. (1998). "A quantitative cytochrome oxidase mapping study, cross-regional and neurobehavioural correlations in the anterior forebrain of an animal model of Attention Deficit Hyperactivity Disorder." Behavioural Brain Research **94**(1): 197-211.

Parent, A. and L. N. Hazrati (1995). "Functional anatomy of the basal ganglia. I. The cortico-basal ganglia-thalamo-cortical loop." Brain Research. Brain Research Reviews **20**(1): 91-127.

Parent, A., M. Parent, et al. (1999). "Glutamatergic inputs to midbrain dopaminergic neurons in primates." Parkinsonism & Related Disorders **5**(4): 193-201.

Parker, J. L. and D. Vanderkooy (1995). "Tegmental Pedunculopontine Nucleus Lesions Do Not Block Cocaine Reward." Pharmacology Biochemistry and Behavior **52**(1): 77-83.

Podhorna, J. and K. B. J. Franklin (1998). "Lesions ef the pedunclopontine tegmental nucleus increase anxiety in rats." Neuroreport **9**(8): 1783-1786.

Ramcharan, E. J., J. W. Gnadt, et al. "Burst and tonic firing in thalamic cells of unanesthetized, behaving monkeys." Visual Neuroscience **17**(1): 55-62.

Redgrave, P., I. J. Mitchell, et al. (1987). "Descending projections from the superior colliculus in rat: a study using orthograde transport of wheatgerm-agglutinin conjugated horseradish peroxidase." Experimental Brain Research. Experimentelle Hirnforschung. Experimentation Cerebrale **68**(1): 147-67.

Reinagel, P., D. Godwin, et al. (1999). "Encoding of visual information by LGN bursts." Journal of Neurophysiology **81**(5): 2558-69.

Robertson, H. A. (1994). "The effect of pedunculopontine tegmental nuclesu and lateral hypothalamic excitotoxic lesions on motivational strength as measured by the progressive ratio paradigm." Unpublished M.Phil thesis: University of St Andrews.

Roghani, A., A. Shirzadi, et al. (1998). "Distribution of the vesicular transporter for acetylcholine in the rat central nervous system." Neuroscience **82**(4): 1195-212.

Rugg, E. L., J. S. Dunbar, et al. (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 Research **589**(2): 181-93.

Ruggiero, D. A., M. Anwar, et al. (1997). "The pedunculopontine tegmental nucleus issues collaterals to the fastigial nucleus and rostral ventrolateral reticular nucleus in the rat." Brain Research **760**(1-2): 272-6.

Rye, D. B., C. B. Saper, et al. (1987). "Pedunculopontine tegmental nucleus of the rat: cytoarchitecture, cytochemistry, and some extrapyramidal connections of the mesopontine tegmentum." Journal of Comparative Neurology **259**(4): 483-528.

Rye, D. B., H. J. Lee, et al. (1988). "Medullary and spinal efferents of the pedunculopontine tegmental nucleus and adjacent mesopontine tegmentum in the rat." Journal of Comparative Neurology **269**(3): 315-41.

Sahakian, B. J., P. Winn, et al. (1983). "Changes in body weight and food-related behaviour induced by destruction of the ventral or dorsal noradrenergic bundle in the rat." Neuroscience **10**(4): 1405-20.

Seamans, J. K., S. B. Floresco, et al. (1995). "Functional differences between the prelimbic and anterior cingulate regions of the rat prefrontal cortex." Behavioral Neuroscience **109**(6): 1063-73.

Semba, K., P. B. Reiner, et al. (1990). "Single cholinergic mesopontine tegmental neurons project to both the pontine reticular formation and the thalamus in the rat." Neuroscience **38**(3): 643-54.

Semba, K. and H. C. Fibiger (1992). "Afferent connections of the laterodorsal and the pedunculopontine tegmental nuclei in the rat: a retro- and antero-grade transport and immunohistochemical study." Journal of Comparative Neurology **323**(3): 387-410.

Semba, K. (1993). "Aminergic and cholinergic afferents to REM sleep induction regions of the pontine reticular formation in the rat." Journal of Comparative Neurology **330**(4):

543-56.

Shammah_Lagnado, S. J., G. F. Alheid, et al. (1996). "Efferent connections of the caudal part of the globus pallidus in the rat." Journal of Comparative Neurology **376**(3): 489-507.

Sherman, M. S. and R. W. Guillery (2001). Exploring the thalamus, Academic Press.

Shik, M. L., F. V. Severin, et al. (1966). "Control of walking and running by means of electric stimulation of the midbrain." Biofizika **11**(4): 659-66.

Shink, E., M. Sidibe, et al. (1997). "Efferent connections of the internal globus pallidus in the squirrel monkey: II. Topography and synaptic organization of pallidal efferents to the pedunculopontine nucleus." Journal of Comparative Neurology **382**(3): 348-63.

Shiromani, P. J., C. Floyd, et al. (1990). "Pontine cholinergic neurons simultaneously innervate two thalamic targets." Brain Research **532**(1-2): 317-22.

Shouse, M. N. and J. M. Siegel (1992). "Pontine regulation of REM sleep components in cats: integrity of the pedunculopontine tegmentum (PPT) is important for phasic events but unnecessary for atonia during REM sleep." Brain Research **571**(1): 50-63.

Sidibe, M., M. D. Bevan, et al. (1997). "Efferent connections of the internal globus pallidus in the squirrel monkey: I. Topography and synaptic organization of the pallidothalamic projection." Journal of Comparative Neurology **382**(3): 323-47.

Skinner, R. D. and E. Garcia-Rill (1984). "The mesencephalic locomotor region (MLR) in the rat." Brain Research **323**(2): 385-9.

Smith, Y., A. Charara, et al. (1996). "Synaptic innervation of midbrain dopaminergic neurons by glutamate-enriched terminals in the squirrel monkey." Journal of Comparative

Neurology **364**(2): 231-53.

Sofroniew, M. V., J. V. Priestley, et al. (1985). "Cholinergic projections from the midbrain and pons to the thalamus in the rat, identified by combined retrograde tracing and choline acetyltransferase immunohistochemistry." Brain Research **329**(1-2): 213-23.

Spann, B. M. and I. Grofova (1991). "Nigropedunculopontine projection in the rat: an anterograde tracing study with phaseolus vulgaris-leucoagglutinin (PHA-L)." Journal of Comparative Neurology **311**(3): 375-88.

Spann, B. M. and I. Grofova (1992). "Cholinergic and non-cholinergic neurons in the rat pedunculopontine tegmental nucleus." **186**(3): 215-27.

Spreafico, R., A. Amadeo, et al. (1993). "Branching projections from mesopontine nuclei to the nucleus reticularis and related thalamic nuclei: a double labelling study in the rat." Journal of Comparative Neurology **336**(4): 481-92.

Stefurak, T. L. and D. Vanderkooy (1994). "Tegmental Pedunculopontine Lesions in Rats Decrease Saccharins Rewarding Effects but Not Its Memory-Improving Effect." Behavioral Neuroscience **108**(5): 972-980.

Steininger, T. L., D. B. Rye, et al. (1992). "Afferent projections to the cholinergic pedunculopontine tegmental nucleus and adjacent midbrain extrapyramidal area in the albino rat. I. Retrograde tracing studies." Journal of Comparative Neurology **321**(4): 515-43.

Steininger, T. L., B. H. Wainer, et al. (1997). "Serotonergic dorsal raphe nucleus projections to the cholinergic and noncholinergic neurons of the pedunculopontine tegmental region: a light and electron microscopic anterograde tracing and immunohistochemical study." Journal of Comparative Neurology **382**(3): 302-22.

Steininger, T. L., B. H. Wainer, et al. (1997). "Ultrastructural study of cholinergic and noncholinergic neurons in the pars compacta of the rat pedunculopontine tegmental nucleus." Journal of Comparative Neurology **382**(3): 285-301.

Steriade, M., S. Datta, et al. (1990). "Neuronal activities in brain-stem cholinergic nuclei related to tonic activation processes in thalamocortical systems." Journal of Neuroscience **10**(8): 2541-59.

Steriade, M. (1992). "Basic mechanisms of sleep generation." Neurology **42**(7 Suppl 6): 9-17; discussion 18.

Sturm, W., A. de Simone, et al. (1999). "Functional anatomy of intrinsic alertness: evidence for a fronto-parietal-thalamic-brainstem network in the right hemisphere." Neuropsychologia **37**(7): 797-805.

Swerdlow, N. R. and G. F. Koob (1987). "Lesions of the dorsomedial nucleus of the thalamus, medial prefrontal cortex and pedunculopontine nucleus: effects on locomotor activity mediated by nucleus accumbens-ventral pallidal circuitry." Brain Research **412**(2): 233-43.

Swerdlow, N. R. and M. A. Geyer (1993). "Prepulse Inhibition of Acoustic Startle in Rats after Lesions of the Pedunculopontine Tegmental Nucleus." Behavioral Neuroscience **107**(1): 104-117.

Takakusaki, K., T. Shiroyama, et al. (1996). "Cholinergic and noncholinergic tegmental pedunculopontine projection neurons in rats revealed by intracellular labeling." Journal of Comparative Neurology **371**(3): 345-61.

Takakusaki, K., T. Shiroyama, et al. (1997). "Two types of cholinergic neurons in the rat tegmental pedunculopontine nucleus: electrophysiological and morphological characterization." Neuroscience **79**(4): 1089-109.

Takayama, K., T. Suzuki, et al. (1994). "The comparison of effects of various anesthetics on expression of Fos protein in the rat brain." Neuroscience Letters **176**(1): 59-62.

Thakkar, M. M., R. E. Strecker, et al. (1998). "Behavioral state control through differential serotonergic inhibition in the mesopontine cholinergic nuclei: a simultaneous unit recording and microdialysis study." Journal of Neuroscience **18**(14): 5490-7.

Tolman, E. C. and C. H. Honzik (1930). "Degrees of hunger, reward and non-reward, and maze learning in rats." University of California Publications in Psychology **4**(17): 241-256.

Tolman, E. C. and C. H. Honzik (1930). "Introduction and removal of reward, and maze learning in rats." University of California Publications in Psychology **4**(17): 257-275.

Torterolo, P., J. Yamuy, et al. (2001). "GABAergic neurons of the laterodorsal and pedunculopontine tegmental nuclei of the cat express c-fos during carbachol-induced active sleep." Brain Research **892**(2): 309-19.

Usunoff, K. G., V. N. Kharazia, et al. (1999). "Nitric oxide synthase-containing projections to the ventrobasal thalamus in the rat." **200**(3): 265-81.

Vincent, S. R., K. Satoh, et al. (1986). "Neuropeptides and NADPH-diaphorase activity in the ascending cholinergic reticular system of the rat." Neuroscience **17**(1): 167-82.

Vincent, S. R. and H. Kimura (1992). "Histochemical mapping of nitric oxide synthase in the rat brain." Neuroscience **46**(4): 755-84.

Vincent, S. R. (2000). "The ascending reticular activating system--from aminergic neurons to nitric oxide." Journal of Chemical Neuroanatomy **18**(1-2): 23-30.

Webster, H. H. and B. E. Jones (1988). "Neurotoxic lesions of the dorsolateral pontomesencephalic tegmentum-cholinergic cell area in the cat. II. Effects upon sleep-waking states." Brain Research **458**(2): 285-302.

Williams, J. A. and P. B. Reiner (1993). "Noradrenaline hyperpolarizes identified rat mesopontine cholinergic neurons in vitro." Journal of Neuroscience **13**(9): 3878-83.

Winn, P., M. P. Latimer, et al. (1995). "The pedunculopontine tegmental nucleus as a striatal output station. II. Effects of PPTg lesions on reaction time measures in a conditioned visual discrimination task." Society for Neuroscience Abstract **21**(2079).

Winn, P., V. J. Brown, et al. (1997). "On the relationships between the striatum and the pedunculopontine tegmental nucleus." Critical Reviews in Neurobiology **11**(4): 241-61.

Winn, P. (1998). "Frontal syndrome as a consequence of lesions in the pedunculopontine tegmental nucleus: a short theoretical review." Brain Research Bulletin **47**(6): 551-63.

Wong-Riley, M. T. (1989). "Cytochrome oxidase: an endogenous metabolic marker for neuronal activity." Trends in Neurosciences **12**(3): 94-101.

Woolf, N. J. and L. L. Butcher (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**(5): 603-37.

Woolf, N. J. and L. L. Butcher (1989). "Cholinergic systems in the rat brain: IV. Descending projections of the pontomesencephalic tegmentum." Brain Research Bulletin **23**(6): 519-40.

Wyvell, C. L. and K. C. Berridge (2000). "Intra-accumbens amphetamine increases the conditioned incentive salience of sucrose reward: Enhancement of reward "wanting" without enhanced "liking" or response reinforcement." Journal of Neuroscience **20**(21):

8122-8130.

Yamamoto, K., A. N. Mamelak, et al. (1990). "A cholinoceptive desynchronized sleep induction zone in the anterodorsal pontine tegmentum: locus of the sensitive region." Neuroscience **39**(2): 279-93.

Yasui, Y., D. F. Cechetto, et al. (1990). "Evidence for a cholinergic projection from the pedunculopontine tegmental nucleus to the rostral ventrolateral medulla in the rat." Brain Research **517**(1-2): 19-24.

Yeomans, J. S., A. Mathur, et al. (1993). "Rewarding brain stimulation: role of tegmental cholinergic neurons that activate dopamine neurons." Behavioral Neuroscience **107**(6): 1077-87.

Yeomans, J. S., A. Mathur, et al. (1993). "Rewarding Brain-Stimulation - Role of Tegmental Cholinergic Neurons That Activate Dopamine Neurons." Behavioral Neuroscience **107**(6): 1077-1087.

Zahm, D. S., E. A. Williams, et al. (2001). "Ventral mesopontine projections of the caudomedial shell of the nucleus accumbens and extended amygdala in the rat: double dissociation by organization and development." Journal of Comparative Neurology **436**(1): 111-25.

Zweig, R. M., P. J. Whitehouse, et al. (1987). "Loss of pedunculopontine neurons in progressive supranuclear palsy." Annals of Neurology **22**(1): 18-25.

Appendix

CRESYL FAST VIOLET STAIN FOR NISSL SUBSTANCE.

MATERIALS

Cresyl Fast Violet Acetate[aqueous]	0.5g.
glacial Acetic acid	25ml
distilled Water	475ml.

Dissolve the cresyl violet powder in the water and acid use of the ultrasonic bath is invaluable this probably takes around 60mins without ultrasound perhaps 2-3 days. Please check to see no animals are close before you use the ultrasonic bath. check pH and adjust to 3.5 with Sodium acetate solution
The pH is critical to this stain check before use.

METHOD

1. Mount the sections onto pretreated slides allow to dry for 12hours at least
2 Fix sections in formalin gas bath for at least 30mins
3 Defat the sections in Xylene--2mins
4 Rehydrate the sections in graded alcohols 100%,50%. then to tap water
5 Stain in Cresyl fast violet solution --2mins[do a test slide first]
6 Rinse in running tap water --5mins
7. Differentiate in 50%alcohol then in absolute [test slide for time]
8 Clear in Xylene and mount in DPX or other Xylene based mountant

RESULTS Nissl substance --Purple-Blue
Nuclei and some cytoplasmic processes of neurons --Paler blue

NADPH DIAPHORASE

MATERIALS

1. b-NADPH[tetrasodium salt type 1]		Sigma N1630
2. Nitro Blue Tetrazolium	Sigma	N6876 [10x10mg]
3. Glycerol gelatin		Sigma GG1

SOLUTIONS

1. 10% Triton X in distilled water
2. 0.3% Triton X Use the solution above take 1.5ml make up to 50ml with 0.1m Phosphate buffer
3. Nitro Blue Tetrazolium concentrate. Take 10mg vial add 1ml 0.3% triton Make sure this is in solution. Keeps in the fridge for upto 3days
4. Weigh out NADPH in disposable vial 10mg add to this 9.9ml 0.3% Triton and then add 0.1ml NBT concentrate. Use within 15mins.

METHOD

1. Perfuse using normal technique with 4%paraformaldehyde in 0.1m phosphate buffer. DO NOT USE GLUTERALDEHYDE.
2 Remove the brain and put into 20% sucrose in 0.1m phosphate buffer.
3. Keep in fridge till you are ready to cut the sections . Reaction should be done within 14 days
4. Cut sections at 50 microns on the freezing microtome into phosphate buffer
5. Pretreat the sections in 20%sucrose in 0.1m phosphate buffer for 30mins
6. Treat with the solution of NBT and NADPH for 20-30 mins at 37oC
7. Wash the sections in 0.1m phosphate buffer

8. Mount the sections from water onto gelatin coated slides.
9. Dry overnight at room temperature
10. Coverslip using glycerol gelatin mountant.

COSSH CONSIDERATIONS

1 NBT is considered to be dangerous wear gloves and eye protection when handling solutions containing it.

2. All waste stain solution should be kept in the bottle it is made up in and kept for disposal by the safety office it should be clearly labeled ,as to its contents..

this note may not cover all the risks associated with this proceedure a risk assessment form for this proceedure is on file