Mesopontine Mechanisms in Anxiety

A thesis submitted for the degree of PhD

by

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Abbreviations

Anatomical & Biological

ACTH	adreno corticotropic hormone
ß-CCE	ethyl ß-carboline-3-carboxylate
DAB	diaminobenzidene
DNA	deoxyribonucleic acid
GABA	gamma amino butyric acid
HCRT	hypocretin
IgG	immunoglobulin G
LDTg	laterodorsal tegmental nucleus
MRNA	messenger RNA
PAG	periaquductal grey
PPTg	pedunculopontine tegmental nucleus
NADPH	nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-d-aspartate
REM	rapid eye movement sleep
SAP	saporin
VTA	ventral tegmental area

Quantitative

cm	centimetre
g	gram
kg	kilogram

kJ	kilo joule
M	molar
ml	millilitre
mm	millimetre
ng	nanogram

Methodological

CS	conditioned stimulus
DV	dorsal-ventral
EEG	electroencephalogram
FMRI	functional magnetic resonance imaging
IAL	inter aural line
ip	intraperitoneal
IVSA	intravenous self-administration
ML	midline
PPI	prepulse inhibition
rCBF	regional cerebral blood flow
SAS	supervisory attentional system

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Abstract

The PPTg has been suggested to be important in a wide range of behavioural functions from locomotion to attention. Recently, several behavioural studies have reported that bilateral PPTg lesions produce and increase in anxiety compared to sham lesioned controls.

Yet, in the studies reported here no evidence of heightened anxiety was found in rats with discrete excitotoxic lesions of PPTg, in any of the behavioural tests used. However, lesion damage to the adjacent cuneiform nucleus reliably produced elevations in anxiety as measured by elevated plusmaze, food neophobia and openfield tests.

In an anatomical mapping study complementary results were produced, with rats exposed to the elevated plusmaze on two consecutive days showing heightened Fos immunoreactivity in the cuneiform nucleus but not the PPTg.

A number of studies have indicated that lesions of the PPTg disrupt responses to positively reinforcing stimuli in a variety of paradigms. Consistent with this, in a study measuring food, water and sucrose intake, over an extended period, rats with ibotenate lesions of the PPTg over-consumed 20% sucrose solution compared to normal rats. However, they were able to successfully modulate their energy intake by reducing consumption of other food sources.

Unexpectedly, behavioural control was also disrupted in response to aversive stimuli, both weak quinine solution and plusmaze exposure produced disinhibited behaviours in PPTg lesioned rats.

Chapter 1

General Introduction

1.1 The Pedunculopontine tegmental nucleus

Structure

The PPTg is one of the mesopontine nuclei found at the junction of the pons and the midbrain. It is bordered dorsally by the cuneiform nucleus, posteriorly by the parabrachial nuclei, and anteriorly by the substantia nigra. In the area of the PPTg there are, in the broadest terms, two groups of neurones: one group is cholinergic and the other non-chloinergic. In the past these two groups have been treated as individual structures, the cholinergic Ch5 neurones (Mesulam et al. 1983) being used to define the PPTg, with the adjacent non-cholinergic neurones termed the midbrain extrapyramidal area (Rye et al. 1987). However, more recent studies have indicated that these two groups of neurones are not separate at all but are interspersed (Honda & Semba 1995). The non-cholinergic cells of the PPTg include both glutamatergic and GABAergic neurones (Steiniger et al. 1997). However it is difficult to distinguish cholinergic from non cholinergic neurones in morphological terms given their similar size. Additionally they are not chemically distinct since both contain the same peptides, including substance P and corticotropin releasing hormone (Vincent et al. 1986) also, neurotransmitters GABA and glutamate are co-localised in both cholinergic and non cholinergic neurones (Manaye et al. 1999 and Bevan & Bolam 1995). There are reports too that the PPTg's cholinergic projections are comprised of

at least two distinct neural subtypes as measured by their electrical membrane properties, with one group firing short and the other longer duration action potentials, thus indicating they may be functionally distinct (Takakusaki et al. 1997). The cholinergic neurones of the mesopontine tegmentum are distinct from many other cholinergic groups in the brain as virtually all contain NADPH-diaphorase (Inglis & Semba 1996).

Clues to the function of any brain area are likely to be through its connections, and in the case of the PPTg these are extensive. Not only is it the lowest point in the brain to receive direct input from the whole of the basal ganglia (Winn et al. 1997) it was also cited by Alexander et al. (1986) as a key part of one of several subsidiary pathways which are proposed to have a modulatory influence on information transmitted via circuits, which they named the cortico-striato-pallido thalamic loops. With it's ascending projections to the thalamus, basal ganglia and hence, indirectly, to cortex, as well as descending connections to the pons, medulla and spinal cord the PPTg is in a prime position to influence a number of sensory and motor functions. The PPTg also receives extensive inputs from both dorsal and ventral striatum as well as motor cortex, with additional innervation from the ascending reticular activating system and superior colliculus. Therefore it appears that the PPTg is an important component of a complex distributed neural system as indicated by Figures 1.1 and 1.2 below.

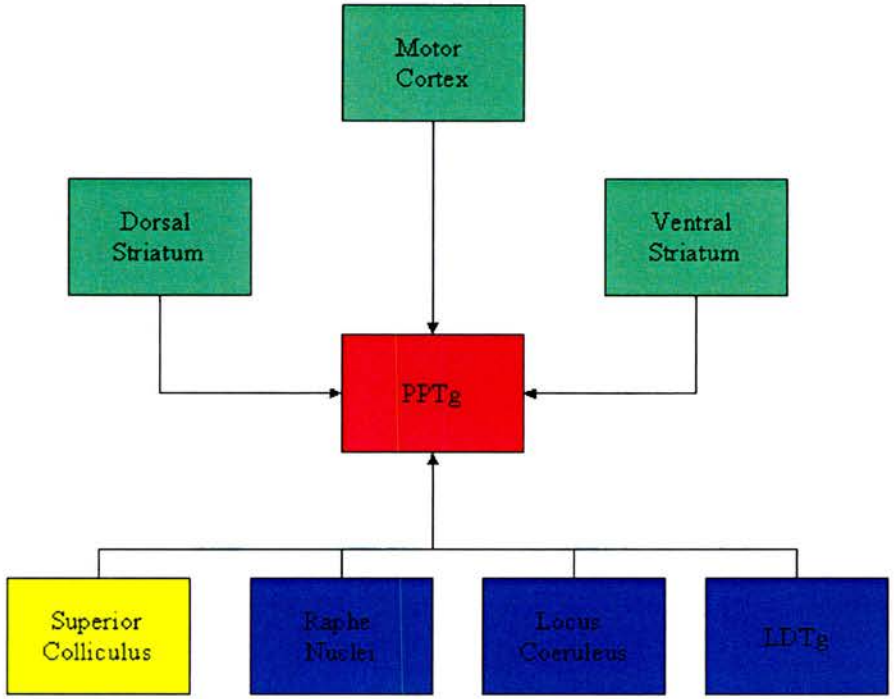


Figure 1.1 summarises the PPTg's main afferent connections. It receives innervation from the nuclei of the ascending reticular activating system as well as from superior colliculus. It has inputs from both dorsal and ventral striatum and their associated outputs including nucleus accumbens & ventral pallidum (ventral striatum) and substantia nigra, globus pallidus and subthalamic nucleus (dorsal striatum). Additionally the PPTg receives input from the region of motor cortex known as Broadman's area 4.

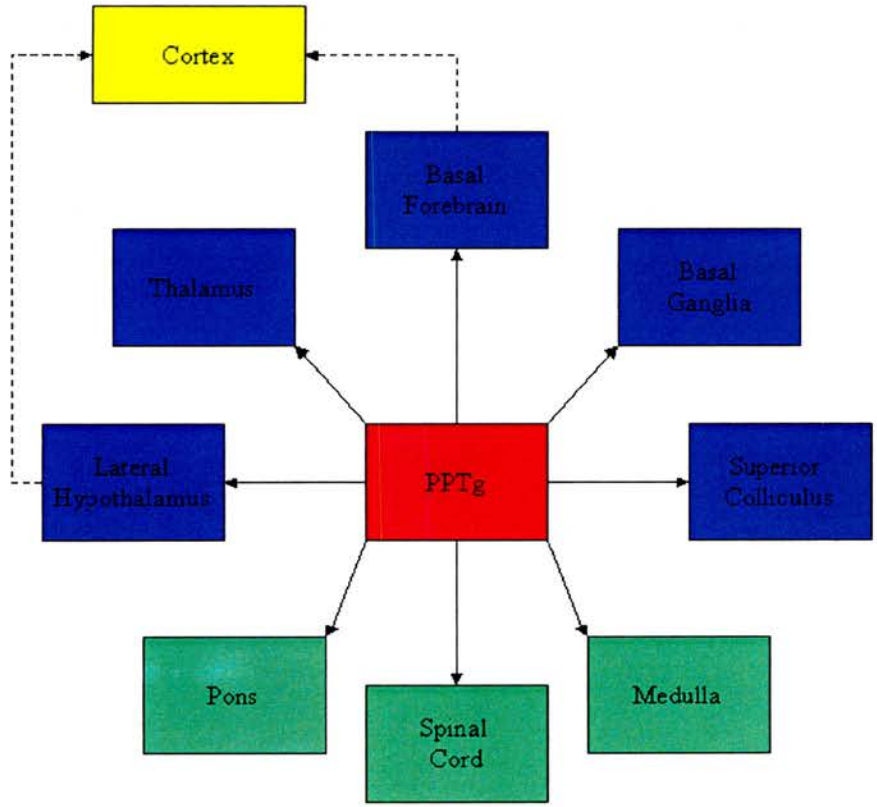


Figure 1.2 summarises the PPTg's efferent connections. It has descending connections to the pons, medulla and spinal cord. Additionally it makes extensive innervation of the basal ganglia and related structures including, substantia nigra, subthalamic nucleus, globus pallidus and VTA. Via its' connections with lateral hypothalamus and the basal forebrain the PPTg makes indirect input to cortex. Finally the PPTg has connections to virtually all thalamic nuclei, mainly those associated with sensorimotor functions

Functions

Extensive studies of behaviour following lesions of the PPTg have shown that, rather than a disruption of basic behaviours which might be expected if the PPTg merely passed on pre-processed information to low level sites of initiation and action, what we actually see are much more complex deficits in responses in a variety of experimental paradigms. While rats with lesions of the PPTg eat, drink, and move normally their responses in a variety of tasks reveal more subtle, possibly cognitive, abnormalities in processes such as learning and attention. Different categories of function are discussed below.

Motor Functions

Joel & Weiner (1994) proposed that some of the basal ganglia-thalamo cortical circuitry, first described by Alexander et al. (1986), are best viewed as open loops. They found that input from cortex to basal ganglia often projects back, via thalamus to an entirely different area of cortex, thus suggesting that the loops are not physically, and therefore functionally, segregated, and indicating that basal ganglia and cortex are extensively interconnected. One of the functions associated with both frontal and striatal regions is the initiation and selection of action and the connections of PPTg suggest it has a role in the output of motor programmes to lower level structures. The motor system is traditionally viewed as consisting of cerebellum, basal ganglia and cerebral cortex, as well as lower level brain stem and spinal cord areas (Mogenson et al.1980). The system is viewed as a hierarchical structure, with higher level areas involved in the initiation and planning of actions

exerting control over lower level brain stem and spinal cord regions which produce the behaviours. However, it is likely that in certain situations information is not processed as high up as cortex and basal ganglia performs the role of action selection, (Redgrave et al. 1999) thus initiating motor ouput more rapidly than if processing took place at a cortical level.

Several researchers have cited the PPTg as a major component of the mesencephalic locomotor region (Mogenson et al. 1980 & Skinner et al. 1990a,b.). Although, with several other sites in the midbrain, electrical stimulation of the PPTg in decerebrate animals does elicit stereotyped stepping (Garcia-Rill 1991) it has been proved on numerous occasions that bilateral electrolytic and excitotoxic lesions of the PPTg fail to disrupt drug induced locomotor activity (Swerdlow & Koob 1987, Inglis et al. 1994 a & b and Olmstead & Franklin 1994). Additionally Allen et al. (1996) found that excitotoxic lesions of the PPTg had no effect on spontaneous locomotion in an open arena as measured over three, three hour test sessions. Indeed neither Alderson et al. (2001) nor Keating et al. (2002) found any locomotor deficits in PPTg lesioned rats as measured in conditioned place preference studies.

Prepulse inhibition of the startle reflex (PPI) occurs on a single trial in response to a stimulus of any modality, presented for too brief a time to elicit conscious inhibition (Swerdlow & Geyer 1993). It is thought that several brain regions are vital for mediating PPI, and the PPTg is one (Fendt et al. 1996). Inhibition of PPTg neurones using scopolamine, a muscarinic receptor agonist, blocks PPI (Fendt et al. 2001). Lesions of the PPTg reduce PPI by around 65% while increasing baseline startle. Disruption of the startle reflex is also known to occur

when there is excess dopamine in the nucleus accumbens (Swerdlow et al. 1992). Therefore, reduced pre pulse inhibition seen following lesions of the PPTg is possibly the result of disruption of its' ventral striatal connections.

Several studies have also indicated that the reciprocal connections between dorsal striatum and PPTg are of functional significance. Dorsal striatum is known to be of critical importance in reaction time performance (Robbins & Brown 1991). Rats with lesions of the PPTg were found to be significantly slower than control rats to release an operant lever on presentation of a light, which signalled food reward (Winn et al 1995). Additionally, injection of amphetamine into the ventrolateral caudate putamen of PPTg lesioned rats was found to produce oral motor responses, such as gnawing and biting which were significantly disinhibited compared to control animals (Allen & Winn 1995). While Inglis et al. (1994a) found no impairment in either spontaneous or d-amphetamine stimulated locomotion in PPTg lesioned rats, they did observe abnormal orofacial stereotypies. Amphetamine generally does not produce such orofacial stereotypies unless administered directly to caudate putamen. It therefore appears likely that the abnormalities observed resulted from a disruption of PPTg caudate putamen connections.

Learning

Lesions of the PPTg impaired active avoidance acquisition but not general locomotor activity (Satorra-Marin 2001) and performance in the 8 arm radial maze was impaired following both pre and post training lesions of the PPTg while no motor impairment was found. In fact, lesioned rats were actually quicker around the

maze than sham lesioned controls, thus suggesting that the deficits observed are not due to motor or motivational abnormalities (Keating & Winn 2002).

Pavlovian conditioning paradigms have frequently been used to investigate the role of striatal systems in control of behaviour. Indeed, injections of amphetamine into the nucleus accumbens have been shown greatly to increase responses on the reinforced lever for food reward (Taylor & Robbins 1984). Rats with lesions of the PPTg also show several abnormalities in this paradigm. Though they do display a drug induced increase in lever pressing, just like normal rats, they continue pressing the non-reinforced as well as the reinforced lever (Inglis et al. 1994a). Additionally they fail to respond for food reward, following presentation of the conditioned reinforcer on more occasions than normal rats. It seems therefore, that the lesions of the PPTg in some way disrupt the rat's ability to establish an association between conditioned and unconditioned stimuli.

Inglis et al. (2000) found that rats with lesions of the PPTg were abnormal in their performance in both a visual autoshaping and a conditioned reinforcement paradigm. In the visual autoshaping, pavlovian conditioning experiment, rats had to discriminate between a visual stimulus being presented on one side of a screen, signalling reward (CS+), and the same stimulus on the other side of the screen which never signalled reward (CS-). Inglis et al. found that control rats made more approaches to the food hopper than those in the lesion group and approached more on presentation of the CS+ than CS- stimulus, improving across trials. Rats with lesions of the PPTg, on the other hand, did not discriminate between CS+ and CS- stimuli. Approach latencies also differed between groups, with control rats being

quicker to approach the food hopper on presentation of the CS+ stimulus than the lesioned group. They also showed a decrease in response latencies to CS+ across trials. Lesioned rats were slower overall to approach the hopper on presentation of a stimulus and did not improve across trials.

In the conditioned reinforcement paradigm used by Inglis et al. (2000) rats were trained to recognise that a visual stimulus signalled food reward. In the test phase, rats had a choice of two levers: one always elicited the conditioned stimulus (CS) and the other had no effect. They found PPTg lesioned rats made fewer magazine entries than sham-operated controls and, that control, but not lesioned rats, increased the number of CS paired magazine entries over time. Finally, they found that control, but not lesioned rats, showed increased reinforced but not non reinforced lever pressing over time. These results suggest that PPTg lesions interfere with learning in some way.

Reward & Motivation

There are several distinct motivational processes, primary and incentive motivation for example, it is likely therefore that distributed brain systems process different aspects of motivated behaviours (Robbins & Everitt 1996). The PPTg has inputs to both lateral hypothalamus and substantia nigra pars compacta. Both of these areas are associated with motivated behaviour and reward. Dopamine release from substantia nigra pars compacta neurones is thought to be vital in production of goal directed motor actions. Additionally the PPTg receives inputs from lateral hypothalamus, as well as the ventral tegmental area, ventral pallidum and therefore,

indirectly from nucleus accumbens. All of these areas have been identified as important in reward processes (Winn et al 1997).

In the autoshaping and conditioned reinforcement paradigms, described previously, PPTg lesioned rats showed longer approach latencies than control rats. Since PPTg lesions are known not to impair basic locomotor activity, it is possible that rats bearing bilateral lesions of the PPTg had motivational abnormalities which could account for the learning deficits seen in a number of reward related paradigms. Though, Dunbar et al. (1992) found no observable regulatory defects as measured by chow and water consumption as well as food spillage following lesions of the PPTg.

However, the literature does show mixed results (Bechara & van der Kooy 1989, 1992; Stefurak & van der Kooy 1994), for example several studies have produced evidence that PPTg lesions prevent development of conditioned place preference in sated and drug naïve rats. Bechara and van der Kooy (1989) found that ibotenate lesions of the PPTg disrupted formation of conditioned place preference to morphine and amphetamine in drug naïve rats. Bechara and van der Kooy (1992) found a similar effect with food in non deprived rats. However in both studies lesions of the PPTg did not block conditioned place preference in drug dependent and food deprived rats. Bechara and van der Kooy concluded that separate brain substrates must underlie reward mechanisms in deprived and non deprived states, and that PPTg lesions differentially disrupt the positively reinforcing effects in non deprived states. Stefurak and van der Kooy (1994) also found that PPTg lesions decreased the positively rewarding effects of saccharin in a conditioned place preference paradigm, but had no impact on its' memory improving effects. Abnormalities in responding

have also been reported in intra-cranial self stimulation of the hypothalamus and in a progressive ratio schedule for intra-venous heroin, following PPTg lesions (Olmstead et al. 1998).

On the other hand, several studies do not support this pattern of data. Waraczynski & Perkins (1998) found no disruption to medial forebrain bundle stimulation following large lesions of the PPTg. Also, Parker and van der Kooy (1995) found PPTg lesioned rats developed conditioned place preference for cocaine even when drug naïve, and rats with discrete bilateral lesions of the whole of PPTg are able to form normal conditioned place preference to sucrose reward (Alderson et al. 2001, Keating et al. 2002). Alderson el al (2001) found that both PPTg and sham lesioned, food deprived rats formed place preference to sucrose solutions of 12 and 20% concentration, but neither group did so for 4% solutions. This suggests that lesions of the PPTg do not alter perception of reward, at least as measured by conditioned place preference to sucrose. Keating et al. (2002) found that PPTg lesioned rats formed normal place preference to 20% sucrose solution both when in deprived and non deprived states. In a basic measure of incentive motivation, they also found that PPTg lesioned rats overconsumed sucrose, delivered in the home cage, compared to controls, at concentrations of 12% and greater, again indicating that PPTg lesions do not affect perception of reward per se. Olmstead et al. (1999) provide further support for this argument in their measurements of sucrose consumption. This study too found that rats with lesions of the PPTg overconsumed sucrose solution compared to sham lesioned controls, but just like normal rats, their consumption increased with increasing sucrose concentration. They also showed

preference for high concentration over low when given free choice and showed normal within session contrast effects. Together these data suggest that lesions of the PPTg do not impair perception of natural rewards and do not affect primary motivation.

Frontal Like Disruption

Alexander et al. (1986) point out that sites receiving projections from frontal systems are likely to share frontal functions. The fronto-striatal loops process information which shapes motor actions. Therefore, it seems reasonable to assume that because both cortical and striatal motor outputs target pontine, and other lower level structures such as the medulla, control of action must in some way be processed at these areas low in the neuraxis.

Deficits seen following lesions of the PPTg have been examined in terms of their similarity to frontal disruption. It appears that many of the behavioural abnormalities seen in PPTg lesioned rats can be categorised as one of three different behavioural types, perseverative, inappropriate and disinhibited responses (Winn 1998). Indeed, these classifications can be applied to many of the abnormalities already discussed in relation to striatal connections. For example, in an eight arm radial maze task, designed to test working memory, rats with lesions of the PPTg perseverate, continually re-entering already explored arms (Dellu et al. 1991). Reduced inhibition of the startle reflex, by pre pulse, as reported by Koch et al. (1993) and Swerdlow and Geyer (1993) can be described as disinhibited behaviour because rats were unable to inhibit the startle response. Inappropriate behaviours are

clearly seen by PPTg lesioned rat's responses on a conditioned reinforcement paradigm. While rats were able to acquire this task, they continually responded inappropriately on the non-reinforced lever as well as the reinforced one (Inglis et al. 1994). Alderson et al. (2002) found that PPTg lesioned rats trained pre operatively on a progressive ratio schedule for food reward showed significantly lower breaking points, longer post reinforcement pauses and increased pressing on the non reinforced lever compared to control rats. These deficits became more pronounced as the schedule increased. Since these rats appeared to be able to perceive reward normally and were just as quick as controls to respond when reward was delivered, it appears necessary to search for an alternative explanation for the observed abnormalities. Many of the deficits reported in behavioural testing following lesions of the PPTg could be explained in terms of deficits in inhibiting inappropriate responding. From increased levels of drug induced responding to inappropriate lever pressing and unrewarded approach behaviours, all could be the result of impaired behavioural inhibition as a result of disruption to cortico-striatal connections.

Without higher structures movement can still be initiated but its execution is more restricted and stereotyped in nature, as seen in decerebrate animals. Also, in vertebrates, such as reptiles and fish, where the basal ganglia probably forms the highest level of motor processing, movements are reactive behavioural responses to sensory stimuli (Korn & Faber 1996). This suggests that prefrontal cortex is key in the production of novel, willed motor actions seen in mammals. Indeed, evidence from human frontal lobe dysfunction and experimentally induced lesions in animals repeatedly indicates a disruption in the organisation and sequencing of behavioural

responses (Robbins 1995). This helps to explain why, lesions of the PPTg in rats produce deficits generally associated with both frontal and striatal disorders. Stereotypy, perseveration, disinhibition and inappropriate actions, as seen following lesions of the PPTg, are also often seen in association with neurological or experimental damage to frontal and striatal areas.

Attention

Though there is a lot of evidence for learning disturbances following lesions of the PPTg that cannot be accounted for in terms of either motor or motivational deficits, it is hard to determine whether impairments observed reflect a generalised learning deficiency, memory deficits or attentional abnormalities (Dellu et al. 1991). Dellu et al. (1991) found that rats bearing bilateral quisqualic acid lesions of the PPTg made more errors in an 8 arm radial maze task purported to test working memory, where with all arms baited on each trial rats are allowed to explore until every food pellet has been obtained. In the Morris water maze test of reference memory PPTg lesioned rats took longer to find the submerged platform, located in the same place across trials, than sham lesioned controls. However the same animals were not impaired in a cross maze task where 4 pellets had to be collected in every trial, one from each arm. The authors conclude that the deficits seen did not relate to memory but attentional abnormalities, and that the important difference between these tests was task difficulty, they suggest PPTg lesioned rats failed in the more demanding tasks because of an inability to sustain attention.

Attention is not a unitary process and as such is unlikely to the function of a single brain region. Generally attention is broken down to 3 or 4 different subtypes. Selective attention refers to the ability to analyse specific information while ignoring all other input. Divided attention is the ability to process more than one sensory input at a given time. Sustained attention and vigilance are closely related concepts: while sustained attention is required to perform novel tasks not under automatic control, vigilance is the process of detecting unpredictable events (Winn 2001).

Since the PPTg has connections to thalamus, basal ganglia and basal forebrain, all of which are associated with modulation of attention, it is possible that the PPTg too may be important in attentional processes (Steckler 1994). For example, Inglis et al. (1994a) found that lesions of the PPTg disrupt responding in a conditioned reinforcement paradigm stimulated be d-amphetamine. More recent studies have shown the same patterns of disruption in the absence of psychomotor stimulation. Inglis et al. (2000) suggest that the learning deficits seen in conditioned reinforcement and autoshaping paradigms are the result of disruption of attentional processes mediated by PPTg inputs to the thalamus. In their conditioned reinforcement task Inglis et al. explain the deficits seen in PPTg lesioned rats as a deficit in attentional or sensory gating. They suggest that the PPTg, via connections with thalamus, may be important in the integration of sensory, motor and motivational information that elicit behavioural responses to external stimuli. Additionally, disinhibited responses to high concentration sucrose reward and inappropriate lever pressing in a progressive ratio schedule could also be interpreted

as disruption of attentional processes resulting from damage to PPTg's cholinergic thalamic connections.

The basal forebrain cholinergic neurones are known to be vital for the accurate performance of tasks requiring sustained attention. Voytoko et al. (1994) found that lesions of the cholinergic basal forebrain, to which the PPTg has significant connections, impaired attention but not memory of monkeys in several behavioural tests. Indeed, Kozak et al. (2001) found that bilateral lesions of the PPTg impaired performance in a task requiring sustained attention, resulting in a reduction of correct and an increase in missed responses to a light signalling food reward. Inglis et al. (2001) found that bilateral lesions of PPTg impaired performance, on a variety of measures, in a 5 choice serial reaction time task, thus suggesting that deficits were global rather than reflecting specific attentional processes.

Interestingly, Inglis et al. also reported that the deficits seen were more severe when lesion damage was centred on cholinergic cells in posterior PPTg. Indeed, there is good evidence for the importance of cholinergic pathways in the modulation of attention. Cholinergic nicotinic receptor agonists that increase cholinergic activity have been shown to increase speed and accuracy of both healthy subjects and Alzheimer's patients in sustained attention but not memory tasks. Scopolamine, a cholinergic muscarinic antagonist, decreases cholinergic activity and consequently accuracy in sustained attention tasks (Broks et al. 1988).

Arousal

The term arousal refers to a general state of alertness that can be increased by sensory input, motivational incentives or stimulants, and decreased by both sensory and sleep deprivation. The traditional view of arousal was as a continuum of behavioural state from sleep to consciousness (Coul 1998). However, the arousal system is in fact a complex one consisting of several autonomic and pharmacologically identified systems that are implicated in a variety of behavioural functions. The thalamus plays a central role in modulating arousal state, relaying low level inputs, of different sensory modalities, from peripheral and brainstem systems up to cerebral cortex (Steriade 1996).

Four different patterns of neural activity relate to different neural states, reflecting different points on the traditional arousal continuum from deep sleep to alertness. Arousal is measured physiologically by the electrical activity of cortex, different stages of sleep and waking produce different patterns of neural activity. Low amplitude, high frequency beta waves are correlated with subjective reports and behavioural measures of alertness. Additionally external stimuli can have specific phasic effects (Robbins 1997). In a state of high arousal a fast desynchronised EEG is recorded, which is also a feature of REM sleep (Winn 2001). Exposure to arousing stimuli and REM sleep are both accompanied by an increase in cortical acetylcholine release. Muscarinic receptor stimulation of the medial pontine reticular formation is thought to be sufficient and necessary for the induction of REM sleep. The PPTg Ch5 neurones, along with the LDTg Ch6 neurones, supply substantial cholinergic innervation of the thalamus and consequently are thought to be critically important in

the sleep wake cycle. (Inglis et al. 1995). Additionally, cholinergic cells in both PPTg and LDTg, that show increased firing during REM sleep cause release of acetylcholine into the medial pontine reticular formation and therefore appear to be important for production and maintenance of REM sleep. However, while both LDTg and PPTg neurons are active in REM sleep, (Reese et al. 1995), and REM sleep is disrupted for up to four days following lesion of the PPTg, after this time rats appear to have no difficulty entering and maintaining this state (Inglis et al. 1995).

The basal forebrain is very closely connected to the brainstem cholinergic centres and is thought to be a functional extension of this region. Like PPTg and LDTg, basal forebrain activity correlates with EEG desynchrony and shows flutuations related to the sleep wake cycle. Cortical activation by the basal forebrain is thought to be important in modulation of attentional states including sustained and selective attention as well as maintaining vigilance in preparation for expected stimuli. Rather than modulating arousal per se, it seems that the basal forebrain cholinergic neurones are important in modulating attention and processes closely related to and dependent upon alertness and arousal. These neurones also show heightened metabolic activity during REM sleep, and this region too receives extensive projections from PPTg and LDTg cholinergic neurones. Indeed electrical stimulation of the PPTg produces an increase in cortical acetylcholine release indirectly via projections to the basal forebrain. (Sarter & Bruno 2000).

Arousal & Attention

Traditionally arousal encompasses all forms of cognitive and spatial arousal as well as non specific arousal of the sympathetic nervous system, such as in sates of fear or anxiety. Given the multiple neural correlates and transmitter systems involved, arousal is in all likelihood not a unitary process, and can be confused with other closely related concepts such as attention, alertness and vigilance (Brown & Bowman 2002). Though arousal and attention are functionally and anatomically distinct processes they are also interrelated behavioural functions. According to the Yerkes & Dodson law (1908) an animal will not perform many tasks optimally if either over or under aroused. The law predicts that, increasing arousal will improve task performance to a task specific optimum which is inversely proportional to task difficulty, after this point further increases in arousal are likely to impair performance. Indeed, while under some circumstances physiological activation can impede task performance cognitive arousal may enhance it (Brown & Bowman 2002). This phenomenon is true of the relationship between arousal and attention, where performance in tasks increases with moderate arousal but decreases if stress or excitement elevates arousal too much (Coul 1998).

Sherman & Guillery (1996) suggested that the functional organisation of thalamocortical connections implies far more complex processing takes place in thalamus than the modulation of arousal states, from sleep to wakefulness. In their view, the thalamus does not simply relay sensory information to cortex, but actively alters the nature of information transmitted. They suggest that thalamus monitors information and switches processing to a more detailed level of analysis when, for

example, novel or potentially dangerous stimuli are identified. Therefore the thalamus seems well placed to mediate the interaction between arousal and attentional processes (Portas et al. 1998). Indeed, Portas et al. found that human subjects performing an attentional task under different levels of arousal showed alterations of ventral lateral thalamic activity as a function of arousal state, as measured by fMRI. The highest levels of thalamic activity were observed under conditions of low arousal, following sleep deprivation. There was no significant change in performance of subjects across different arousal states, proving that changes in thalamic activity did not relate directly to task performance. The increase in thalamic activity was accompanied by participants' subjective reports of the need for more mental effort to perform the task in the low arousal, sleep deprivation, condition. Price et al. (1997) found that in a 60 minute test of auditory vigilance regression analysis showed a clear distinction between cortical and subcortical activity as measured by EEG and rCBF. Subcortical changes were seen in thalamus and a negative correlation was observed between thalamic activity and reaction time (Coull1998). The problem with such subjective questionnaires and performance measures is that it is impossible to be certain that it is arousal which is being described and not some other related but distinct process.

As well as REM sleep, cholinergic neurones of the PPTg are also active in the waking state, implying that the PPTg plays a role in maintaining arousal in the waking state too. It is therefore possible that the apparent attentional deficits seen following lesions of the PPTg could reflect disruption of arousal processes. Sustained attention, which is known to be disrupted following PPTg lesions (Kozak

et al. 2001, Inglis et al. 2001), is thought to rely on arousal to a greater extent than other types of attention (Sarter & Bruno 2000).

It is difficult to measure arousal behaviourally without the influence of other factors, but in the same respect, levels of arousal are something researchers must be very aware of when attempting to test a variety of behaviours. For example, Dellu et al.'s results (1991) that seem to fit an explanation of attentional deficits so readily could equally be interpreted as a consequence of elevated arousal levels. Indeed deficits in PPTg lesioned rats performance is often most readily seen in tasks when levels of motivation, and therefore arousal, are high (Keating & Winn 1996 and Ainge et al. 1999).

Neuropsychology

The PPTg is also altered in several human neurodegenerative disorders. This is not solely of interest in investigating cause and treatment of these disorders specifically, but also implies a role for the PPTg in cognitive processes (Steckler et al. 1994). Degeneration and damage to the PPTg is known to take place in Parkinsons disease where there are reports of from 43-57% of cholinergic neurones in caudal PPTg being lost. In dementia of both the Alzheimers and Lewy Body type around 25-34% of PPTg cholinergic neurones are lost. Additionally in progressive supranuclear palsy, a parkinsonian syndrome, there is almost total cholinergic neuronal death (75-80%) in the PPTg while cortex remains unaffected. Finally, there has been a suggestion of an increase in NADPH diaphorase positive neurones in the PPTg in schizophrenic patients, suggesting over activity of the PPTg in this disorder

(Scarnati & Florio 1997). Common symptoms of all of these disorders are attentional deficits, control of which has been suggested as a function of the PPTg. In the case of Parkinsons disease it is not clear whether degeneration of cholinergic cells in the PPTg is caused by altered outflow from the pallidum as a result of dopamine loss in the striatum (Mitchell et al 1989) or whether glutamate, co-localised on the cholinergic and dopaminergic cells of the PPTg - substantia nigra pars compacta pathway, mediates excitotoxic neural degeneration in both structures (Scarnati & Florio 1997).

1.2 The Cuneiform Nucleus

The cuneiform nucleus lies immediately ventral to the caudal parts of inferior colliculus, within the midbrain. Functionally the cuneiform nucleus has been implicated in mediation of behavioural changes in response to threatening stimuli. More specifically, it is thought to be vital in the modulation of cardiovascular responses to stress and physiological reactions behind defence responses (Lam & Verberne 1997). Electrical stimulation of the cuneiform nucleus produces physiological changes such as, increases in arterial blood pressure. The cuneiform nucleus has connections to a variety of brain stem nuclei known to play a role in autonomic regulation (Gayata & Pasaro 1998), including the locus coeruleus, part of the ascending reticular activating system which controls behavioural arousal, the nucleus of the solitary tract, important for monitoring signals from the vagus nerve about gustatory, visceral and cardiovascular changes, as well as the ventrolateral medulla which controls functions associated with the cardiovascular and respiratory system (Lam et al. 1997). Other cuneiform connections include inputs from laminal cells in the dorsal horn of the spinal cord, suggesting a role in processing painful stimuli, and reciprocal connections to the central gray as well as several forebrain structures associated with the modulation of fear and anxiety (Redgrave et al. 1988). These connections too support a role for the cuneiform nucleus in mediation of defensive behaviours to painful or threatening stimuli.

The cuneiform nucleus is one of the main targets of the ipsilateral descending pathway from superior colliculus (Redgrave et al. 1987b). Stimulation of superior colliculus, electrically or chemically produces a wide range of responses, which can

be divided into two categories, defensive behaviours and orienting/approach movements. Stimulation of the cuneiform nucleus with glutamate produces a subset of these, defence like behaviours, suggesting that the connection between the two structures is an important part of the brain's defence mechanism (Mitchell et al. 1998b). The vast majority of behaviours produced by glutamate stimulation of the cuneiform nucleus were defensive rather than orienting. These included, freezing, darting, rapid running and circling in an openfield. It was also noted that behaviours elicited by cuneiform stimulation changed with repeated microinjections, for example from freezing to fast running. Mitchell et al. inferred that the cuneiform nucleus could therefore be important to learning. An animal has to be adaptive in order to survive: it is known that defence behaviours can change with type and proximity of threat and it is therefore possible that the cuneiform nucleus plays some role in modulating behavioural adaptability to threat.

Forebrain structures known to have connections to cuneiform include various hypothalamic and thalamic nuclei (Lam et al. 1997). Analysis of c-fos and nerve growth factor-1 presence following intermittent low intensity electrical stimulation of the cuneiform nucleus revealed significant immediate early gene presence in various hypothalamic nuclei, most strongly in ventromedial hypothalamus, as well as piriform cortex, and ipsilaterally in the medial amygdaloid nucleus. The amygdala is a central part of the brain's fear circuitry and is important in mediating responses to potentially threatening stimuli (LeDoux 1997) and the hypothalamus plays an important part in mediating the sympathetic part of the fight or flight response (Carlson 1994).

The cuneiform nucleus has also been suggested as a possible site for the mesencephalic locomotor region. Electrical stimulation of the hypothesised location of this region produces a locomotor response, the speed of which is positively correlated with intensity of stimulation. The exact location of the mesencephalic locomotor region is unclear, low threshold electrical stimulation of the cuneiform, as well as several other midbrain structures including the PPTg, can elicit a stepping response in decerebrate animals (Coles et al. 1989). However, Allen et al. (1996) found no effect of bilateral excitotoxic cuneiform lesions on either spontaneous or nucleus accumbens induced locomotion. It seems likely that any motor response modulated by the cuneiform nucleus is associated with escape from aversive stimuli.

The cuneiform nucleus reliably shows significant anatomical labelling following exposure of animals to behavioural tests that induce anxiety and stress. Subordinate hamsters showed significant c-fos activity in the cuneiform nucleus following social interaction with a dominant unfamiliar conspecific (Kollack-Walker et al. 1997). Also, rats exposed to cat odour showed significantly greater Fos immunoreactivity in cuneiform, as well as a variety of other brain areas, compared to controls (Dielenberg et al. 2001). Similar findings were reported following plusmaze exposure (Silveira et al. 1993) and conditioned fear (Campeau et al. 1997).

1.3 Emotion

History of Emotion Study

The study of emotion, in modern times, began with psychologist William James who, with Danish physiologist Lange, proposed that emotions are the result of physiological reaction to external stimuli. Another physiologist, Walter Cannon (1927) was the main opponent of the James-Lange theory (Lang 1994). While he agreed that emotions were primarily a physiological phenomenon, and like Lange proposed that the main structures involved in emotional response were likely to be sub-cortical, he disagreed that emotions were the direct result of physiological changes occurring in the body in readiness for fight or flight. He claimed that, if this were the case patients with spinal cord injury would be impaired in feeling emotions (Hohmann 1966). Social psychologist Stanley Schachter (1959) agreed with Cannon that emotional arousal was generalised and not emotion specific. However, Schachter proposed that attributions about physiological changes lead directly to emotions, thus supporting James' hypothesis that emotional experience comes directly from a state of physiological arousal (Schachter & Singer 1962) According to Antonio Damasio's somatic marker hypothesis, human decision making processes depend upon emotions. He claims that there are biological processes influencing behavioural responses to emotional stimuli that can be processed both overtly and covertly (Damasio 1996). Indeed, in his gambling task experiments he has found that subjects begin following an advantageous strategy before they were overtly aware what that strategy is (Bechara et al. 1997).

In summary, emotions are mental states that manifest themselves in three different ways. Firstly they produce changes in peripheral nervous system activity such as hormone release and autonomic responses. Secondly, emotions each have their own characteristic behavioural expressions. Finally, in humans at least they produce subjective feelings (Winn 2001). It is unlikely that rodents experience emotions in the same subjective way humans do, and even if they did it would be impossible to measure, therefore all reference to emotions in the work reported here refers purely to measured behaviours and does not infer consciousness.

1.4 Anxiety

The term anxiety can refer both to an appropriate biological response to perceived threat and a pathological state in humans, which inappropriately activates the body's defence systems. Anxiety in response to perceived threat has both physiological and behavioural manifestations in animals. Physiological features include changes in autonomic reactions such as increases in heart rate, respiration and blood pressure. Psychological changes include a heightened state of vigilance in readiness for potential threats (Winn 2001). Fear on the other hand is an emotional description of the physiological and psychological state experienced in the face of threat. Since it would be wrong to infer emotional experience in rodents fear and anxiety are used interchangeably here, relating solely to physiological and behavioural responses, with no implication of subjective, conscious experience.

Behavioural Tests

There are a number of stimuli that automatically produce fear like responses such as, loud unexpected noises, heights, and species specific sounds and odours (Carlson 1994). These stimuli are utilised by researchers when investigating mechanisms of fear and anxiety in the laboratory. The elevated plusmaze, openfield and pre-pulse inhibition all use natural unlearned behaviours to measure changes in baseline anxiety. In the case of pre-pulse inhibition a weak pre stimulus inhibits the startle reflex to a strong startling stimulus. This is a homologus animal model as the behaviour produced in a rodent is the same as that seen in a human (Menard & Treit 1999). In the elevated plusmaze and openfield rodents face an approach-avoidance conflict. The fear of an unknown exposed area coupled with the desire to explore a novel environment (Montgomery 1958). Both of these tests are reliable correlational models of anxiety used to test the efficacy of novel drug treatments for human anxiety disorders (Menard & Treit 1999). As well as using natural fears to elicit unlearned reactions it is also possible to condition an emotional response by teaching an animal to recognise a given situation as dangerous. Following conditioning this learned situation will produce the same physiological and behavioural reactions such as increased heart rate, blood pressure and freezing, associated with reactions to innately frightening stimuli. Examples of this sort of behavioural test include conditioned fear and fear potentiated startle. In conditioned fear, a previously neutral stimulus repeatedly paired with an aversive event comes to produce the same response as the aversive stimuli (Wilkinson et al. 1998). Reactions seen include freezing and suppression of ongoing behaviour. Fear potentiated startle paradigms

combine the natural startle reflex with classical conditioning, repeated pairing leads to the unconditioned stimulus eliciting the startle reflex (Menard & Treit 1999). It is important for an animal to be flexible in its response to aversive stimuli. Learning that an unpleasant event can be avoided in the future is highly advantageous for survival. Several tests have been developed to help identify brain regions important in emotional learning. In a two-way active avoidance test animals learn to change compartment on presentation of a light in order to avoid footshock. These behavioural and operant tests have been very useful in the identification of neuroanatomical and neurochemical systems involved in anxiety and ultimately in the testing of new drug reliability for the treatment of the various human anxiety disorders.

Neuroanatomy

Any circuitry that modulates behavioural responses to stimuli eliciting anxiety or fear must include regions that perform a variety of different functions which work together to determine behavioural response. In humans, dysfunction of the brain circuitry which modulates reaction to aversive events, can result in anxiety disorder (Deakin 1998).

LeDoux (1984) found that lesions of sensory and motor cortex have no effect on fear conditioning but that damage to specific subcortical areas completely blocked it. This is most likely because when an animal's survival is under threat responses must be as quick as possible, meaning that brain signals never reach cortical regions. It therefore seems sensible to study anxiety in regions further down the neuraxis.

Figures 1.3 summarises some of the major subcortical structures and connections important in the modulation of anxiety and "fear like" behaviours. It is likely that behavioural reactions are sometimes elicited without sensory input ever reaching forebrain or even midbrain structures. Figure 1.4 schematically represents different levels of neural processing that could potentially be activated depending on the imminence of the perceived threat and the speed of behavioural output required.

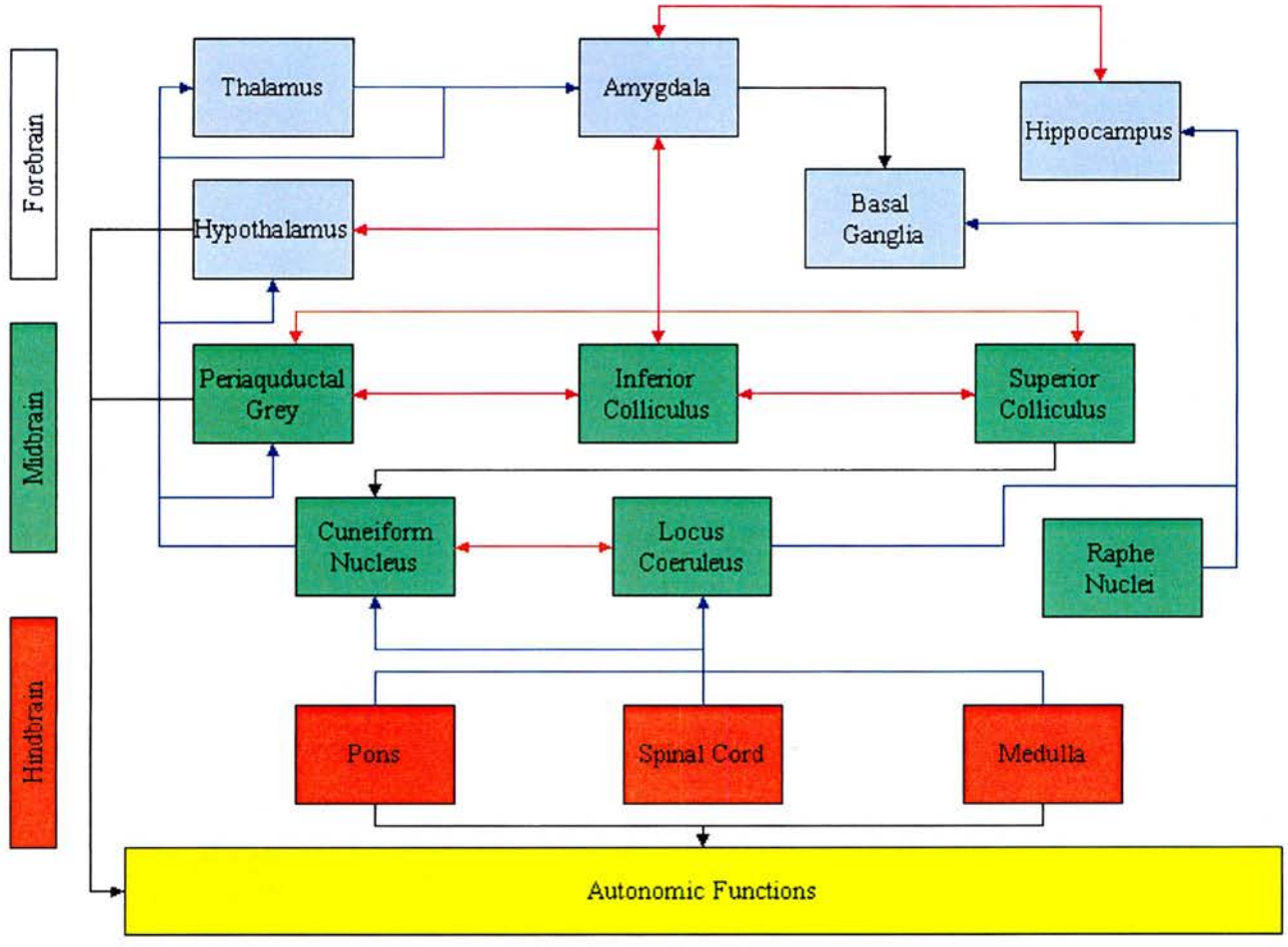


Figure 1.3 summarises the connections between some of the main neural structures involved in processing anxiogenic stimuli. Though it is a hierarchical network, anxiety type responses can be elicited solely by midbrain and hindbrain regions.

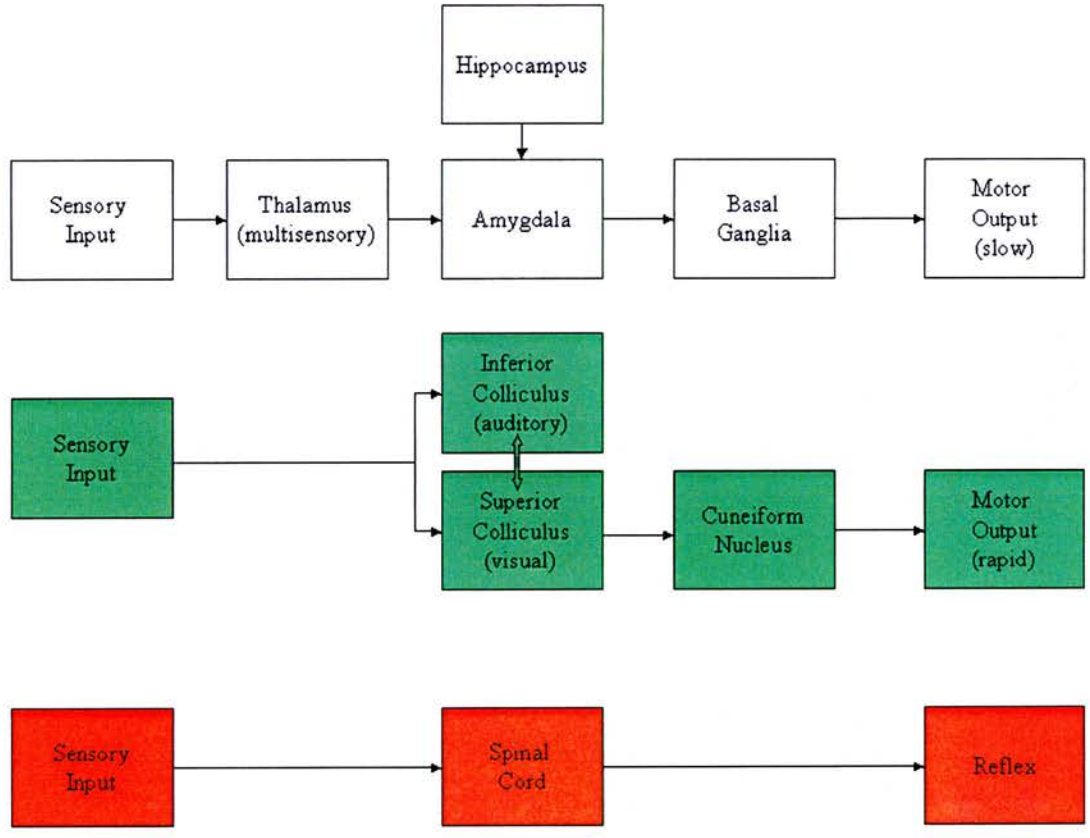


Figure 1.4 summarises three different levels of processing in response to perceived threat. Low down sensory input can be relayed, for example, directly from the cochlear to the motor neurones on the spinal cord, producing a rapid reflex reaction. Higher up in the midbrain rapid processing of sensory input by inferior or superior colliculus will produce a motor response to perceived threat, either directly or via the cuneiform nucleus. In the forebrain processing is likely to be more detailed and therefore slower, with information from the amygdala and hippocampus about putast experience influencing eventual behavioural output.

Forebrain

Amygdala and Hippocampal Formation

The amygdala is thought to be at the heart of this "fear circuitry" and is vital in identifying and acting upon emotionally significant stimuli. Lesions of the amygdala entirely block fear conditioning (LeDoux 1998). It appears that different nuclei within the amygdala are important for different types of fear conditioned behaviours. Kilcross et al. (1997) found that lesions of the basolateral nuclei impaired rats ability to avoid conditioned punishment but did not affect conditioned suppression, yet lesions of the central nucleus impaired suppression of response to an aversively conditioned stimulus but did not affect avoidance directed behaviour. Damage to the amygdala also has severe effects on real life behaviours in dangerous situations. Monkeys with lesions of the amygdala have shown a tendency to approach objects they are usually fearful of, such as snakes. Humans with bilateral amygdala lesion damage are, for the most part, unable to live independently without endangering themselves and others through poor judgement or decision making (Bechara et al. 1999). The amygdala receives sensory specific inputs from the various thalamic nuclei as well as context specific inputs from the hippocampal formation. It uses this information to assess the emotional relevance of stimuli and outputs to various brain stem nuclei, via extensive connections from the central nucleus, which elicits the relevant behavioural and autonomic responses (Kapp et al. 1982).

In terms of fear, memory can be declarative, that is a conscious recollection of a frightening event, or implicit, an unconscious memory activated by emotionally

salient stimuli. The hippocampus has long been known to be involved in memory function. Indeed, the much studied amnesiac, HM had considerable temporal lobe damage (LeDoux 1998). It has been proposed that the amygdala and hippocampus are involved in two separate aspects of memory function activated in parallel in response to emotional stimuli (LeDoux 1998). The amygdala activates implicit emotional memories that elicit bodily sensations and the hippocampus activates explicit emotional memories about time and place. Indeed imaging studies have revealed abnormalities in the hippocampus of humans suffering from post-traumatic stress disorder (Bremner et al. 1995).

Hypothalamus

The hypothalamus is known to be important in controlling autonomic functions and behaviours associated with survival such as eating, mating and fight or flight responses (Carlson 1994). For example in a recent study Shekha et al. (2002) reported that blockade of $GABA_A$ receptors in dorsomedial hypothalamus produced autonomic changes associated with a panic response, including increased heart rate, blood pressure and respiration rate. It also appears that the role of the dorsomedial hypothalamus in modulation of anxiety and fear is sensitive to behavioural tests used. Sajdyk et al. (1997) found an increase in noradrenaline and dopamine levels in dorsomedial hypothalamus in rats exposed to fear potentiated startle but not elevated plusmaze or social interaction tests.

Basal Ganglia & Thalamus

To recognise a stimulus as anxiety or fear inducing the brain's sensory systems must be active (Charney et al. 1998). The thalamus is an important relay station for sensory information of all modalities, and is therefore an important part of any fear circuit. The amygdala receives sensory information from a variety of sensory specific thalamic nuclei and is thought to act as a filter identifying emotionally relevant stimuli (Danion 1993).

One of the principle responses to fear inducing stimuli is escape therefore fear circuitry must also include a motor component. The basal ganglia are a major component of the motor system and have been proposed to be a centralised "selection device" important in the selection of motor actions particularly in the face of competing demands (Redgrave et al. 1999). Indeed the striatum receives extensive innervation from the amygdala suggesting the importance of these regions in the production of motor responses to anxiety inducing stimuli (Mogenson et al. 1980).

Brainstem

Several midbrain structures within the mesencephalon are known to be important in defensive behaviours. Electrical stimulation of the dorsal periaquductal grey (PAG), inferior and superior colliculus produces fear like behaviours including escape, defence responses and autonomic reactions such as elevated blood pressure, heart rate and respiration. Evidence of the role of these structures in responses to aversive stimuli comes from a number of experimental studies. For example, stimulation of the deep layers of superior colliculus produces head and visual

orienting movements as well as cardiovascular changes (Dean et al. 1988a). Lesions of this area lead to a decrease in defensive responses to threatening visual stimuli, such as overhead threats. The inferior colliculus is primarily an acoustic structure its role in defence is perhaps associated with filtering defence relevant sounds. Lesions of this region have been found to reduce startle suppression to rapidly presented intense acoustic stimuli (Jordan & Leaton 1983). Lesions of basolateral nucleus of the amygdala increase, and lesions of the central nucleus of the amygdala decrease, aversive reactions usually seen following stimulation of inferior colliculus, which is a further indication of the dissociation between these two nuclei in mediating fear responses. Stimulation of the PAG produces a large number of behavioural responses, from vocalization to freezing, as well as various autonomic changes. The PAG can be subdivided into several distinct regions; the dorsomedial and dorsolateral PAG are thought to be vital in escape behaviours, and electrical stimulation here produces autonomic changes like increased blood flow to muscles and reduced supply to skin ready for flight. Stimulation of lateral and ventromedial PAG produces hypotension and freezing (Brandao et al. 1999). PAG, superior colliculus and inferior colliculus have direct reciprocal connections between themselves as well as both direct and indirect links to the amygdala and medial hypothalamus. Together these structures are thought to be vital in the integration of aversive states such as fear and anxiety. In c-Fos studies following electrical or chemical stimulation of dorsal PAG or experimental exposure to aversive stimuli, extensive labelling has been seen in the amygdala, medial hypothalamus, dorsal PAG, deep layers of superior colliculus and inferior colliculus (Brandao et al. 1999).

In humans, stimulation of the medial hypothalamus, amygdala and PAG produces autonomic activation, analgesia, fear and distress, as well as reports of a desire to flee (Deakin 1998). These responses are very similar to those seen during spontaneous panic attacks.

Several hindbrain structures have also been implicated in modulating fear and anxiety states. The raphe nuclei lying directly ventral to the ventral PAG matter of the midbrain make extensive serotonergic innervation of the forebrain including the striatum, frontal cortex and hippocampus. While the main role of the PAG is thought to be in mediating reflexive flight or flight responses to proximal unconditioned events, the dorsal raphe is thought to be important in mediating inhibition of behaviour including anticipatory anxiety responses (Brandao et al. 1999). Abnormalities in anticipatory anxiety, in humans, are thought to underlie the symptoms of generalised anxiety disorder (Deakin 1998). Lesions of this region have anxiolytic effects. The median raphe nucleus, on the other hand is thought to play a role when behavioural adaptation has failed to avert danger and anxiety becomes chronic (Deakin 1998). The locus coeruleus, with direct and indirect noradrenergic connections to cortex, among other sites, and sympathetic input from the medulla and spinal cord, is in a good position to influence a number of emotional behaviours (Bernstan et al. 1998). Neurones in locus coeruleus show increased activity in the face of stress and anxiety as well as to rewarding stimuli. It therefore seems that this brain region responds to emotionally significant stimuli in general and not fear inducing ones in particular (Aston-Jones 1996). Stimulation of this structure produces autonomic arousal and other symptoms associated with anxiety. The locus

coeruleus contains one of the major groups of noradrenergic neurones in the brain (Cooper et al.1991), elevated levels of noradrenaline result in a rapid heart rate and increased blood pressure. Drugs that inhibit levels of noradrenaline cell firing successfully relieve symptoms of anxiety (Ninan 1999). Activity of locus coeruleus neurones is modified by arousing and stressful stimuli, pain and cardiovascular changes (Singewald & Philippu 1998). In a cat exposed to either a dog or an aggressive cat, noradrenaline cells in locus coeruleus increased their firing rate to at least twice that of controls exposed to a non aversive novel stimuli (Levine et al. 1990).

Autonomic Nervous System

Several of the areas important in an anxiety response have connections to the autonomic nervous system, which controls such physiological mechanisms as heart rate, blood pressure and skin conductance. Evidence for low level processing of aversive stimuli comes from studies in decerebrate animals which, with only an intact brain stem remaining, show normal cardiovascular responses to emotionally salient sensory stimuli. Additionally, humans suffering from anxiety disorders often have abnormally high autonomic responses (Bernstan et al. 1998). The hypothalamus is vital in controlling the autonomic nervous system (Carlson 1994) A main area of importance in controlling the sympathetic response to anxiety inducing stimuli is the lateral hypothalamus. Stimulation of this area causes release of corticotropin releasing hormone which sets of a chain of reactions, stimulating release of adrenocorticotropic hormone (ACTH) from the pituitary gland and ultimately

adrenaline form the adrenal gland into the bloodstream, helping to mobilise the bodies energy resources ready for fight or flight. Adrenaline is the hormone most associated with physiological arousal in response to fear, its release produces increases in blood pressure, sweating and heart rate in humans The release of adrenaline into the blood stream stimulates the vagus nerve that terminates in the nucleus of the solitary tract. The locus coeruleus, part of the ascending reticular activating system, receives input from the nucleus of the solitary tract in the pons and releases noradrenaline into the forebrain (Charney et al. 1998a).

1.5 Aims and objectives

The broad aim of this research project is to investigate further functions of the pedunculopontine tegmental nucleus (PPTg), using excitotoxic lesions and behavioural tests. More specifically it will examine the effect of bilateral lesions of the PPTg on behaviour in tests sensitive to anxiety.

Recent studies have reported that ablation of the PPTg produces an elevation in anxiety as measured by several behavioural paradigms (Podhorna & Franklin 1999). Podhorna & Franklin found increased anxiety in PPTg lesioned rats exposed to the elevated plusmaze. They made fewer open arm entries and spent less time in the open arms of the maze than sham operated controls, throughout several weeks of testing, indicating that these rats also failed to habituate to the maze. In a social interaction test increased anxiety was reported as measured by decreased social investigation such as sniffing and following, and increased freezing and defensive behaviour. Additionally, in a conditioned fear paradigm though PPTg lesioned rats did not show higher levels of fear than controls, freezing behaviour did persist for longer than control rats during extinction trials.

These results do not fit well with the behavioural data already reviewed. Indeed, preliminary studies in our own laboratory have failed to find an increase in anxiety in PPTg lesioned rats exposed to the elevated plusmaze, in comparison to sham lesioned controls (Alderson et al. 2000). Therefore I propose to investigate an alternative explanation for Franklin et al.'s results, that it is lesion damage to an adjacent brain region, namely the cuneiform nucleus, which causes the elevations in anxiety reported. If this is the case then it highlights the importance of accurate

surgical technique and careful interpretation of histological data when conducting lesion studies.

In recent years there has been an increase in the number of anatomical mapping studies, particularly using immediate early genes such as c-fos. I propose to compare the results of lesion studies using the elevated plusmaze test of anxiety with Fos activity observed following plusmaze exposure, to see if alternative methedological techniques produce complementary results.

Finally, an important direction for future research is to elucidate specific functions of the PPTg's extensive efferent and afferent connections. It seems likely that different connections have different functional roles, and only by selectively lesioning particular neural types will it be possible to determine what these may be. Therefore the final study reported here is a pilot of a new antibody-toxin conjugate that may produce small selective lesions of specific PPTg cell groups, and in the future could be used to investigate functions of specific pontine connections.

Chapter 2

General Methods

2.1 Animals

Male hooded Lister rats (Charles River chapters 3-6, Harlan-Olac chapters 7 & 8) were used in all experiments. Housed individually (except chapter 7) on a 12 hour light dark cycle, they were fed ad libitum (except for 3 days prior to food neophobia testing in chapters 5 & 6) on Special Diet Services (SDS) maintenance diet 1 and tap water. Animals were kept in accordance with national (Animals [Scientific Procedures] Act, 1986) and international (European Communities Council Directive of 24 November 1986 [86/609/EEC]) legislation governing the maintenance of laboratory animals and their use in scientific experiments.

2.2 Surgery

Rats were anaesthetised with an intraperitoneal injection of Sagatal (Rhône Mérieux; sodium pentobarbitone,60mg/ml 1ml/kg ip.) mixed with an equal volume of sterile water. Rimadyl analgesia was given prior to surgery (Pfizer; carprofen 0.01ml/kg sc.). They were placed in a stereotaxic frame (Kopf) with the skull level. Injections of excitotoxin were made using a 0.5 μl syringe (Scientific Glass Engineering) mounted on the stereotaxic frame.

Pedunculopontine Tegmental Nucleus:

To ensure maximal rates of survival separate unilateral lesions were carried out 1 week apart.

a) Ibotenic Acid.

Two injections were made in each hemisphere, each of 0.2µl 0.12 M ibotenic acid (Tocris Cookson) dissolved in phosphate buffer (Sorenson's buffer pH7.4). The stereotaxic co-ordinates used in chapters 3 and 4 were 0.8mm anterior to the interaural line, +/- 1.6mm from the midline, and 7.0mm below skull surface (posterior PPTg) and 1.5mm anterior to the interaural line +/- 1.7mm from the midline and 7.8mm below skull surface (anterior PPTg). In chapters 5 and 6 the co-ordinates used were 0.7mm anterior to the interaural line from, +/- 1.5mm from the midline, and 7.0mm below skull surface (posterior PPTg), and 1.4mm anterior to the interaural line, +/- 1.6mm from the midline and 7.7mm below skull surface. In all cases the toxin was delivered at a rate of 0.01μl at 10 second intervals, using a step down procedure, and was left in situ for 5 minutes to allow the liquid to diffuse.

b) NMDA.

The procedure used was exactly the same as that described in Leri & Franklin (1998). One injection was made per hemisphere using 0.5μl 0.1M NMDA (Sigma) at a rate of 0.02 μl at 10 second intervals, using a step down procedure. The co-ordinates were, 7.8mm posterior to bregma +/-1.8mm

from the midline and 2.8mm below the interaural line. The syringe was left in situ for 10 minutes to allow diffusion.

Cuneiform Nucleus:

Bilateral lesions were carried out in one surgical procedure. The anaesthetic regime was the same as for the PPTg group. Each hemisphere received 2x 0.14μl of 0.12M ibotenic acid. This was administered using a step down procedure of 0.02μl every 15 seconds. The syringe was left in place for 5 minutes to allow liquid to diffuse. The co-ordinates were, 0.2mm anterior to the interaural line, +/- 1.8mm from the midline and 6.0mm below skull surface.

Sham-lesioned controls:

Control rats underwent the same surgical procedure as lesion groups but were infused with an equal volume of sterile phosphate buffer instead of excitotoxin.

Orexin-SAP Micropipette Pilot Lesions:

These lesions were made using a glass micropipette with tip broken to 30 microns. 0.2 μl of toxin in Dulbecco's saline buffer (HCRT-2-SAP; Advanced Targeting Systems) was delivered unilaterally to the PPTg using co-ordinates, 0.7mm anterior to the interaural line +/- 1.5mm from the midline and either 6.0mm or 6.2mm below dura (except when otherwise stated).

2.3 Behavioural Testing

Behavioural testing began two weeks after recovery from surgery, except in chapter 5 when it was only one week. Rats were handled and weighed daily during recovery.

Elevated Plusmaze: apparatus

The maze was made of wood, consisting of two open arms each measuring 50cm in length. The two closed arms; again 50 cm long also had side walls, which were 40 cm deep. There was a central area that measured 10 cm^2. The maze was elevated on a stand, half a metre from the floor. See Figure 2.1 below.

Figure 2.1 is a photograph of an elevated plusmaze similar to the one used in chapters 3-6.

Elevated Plusmaze: procedure

Testing was carried out under dimmed white light. Rats were placed in the central platform facing one of the open arms and allowed to explore freely for 10

minutes. The maze was wiped down with distilled water after each rat. Behaviour was scored by an observer at the time of testing using a specially designed report sheet. Observations were made at 10 second intervals over the 10 minutes of testing. Measures were taken of time spent in open and closed arms and number of arm entries as well as "risk assessment" behaviours (Podhorna & Franklin 1999); latency to enter open arms, latency to enter closed arms, head dips over the side of the maze, freezing (complete cessation of movement for more than 3 seconds), closed arm returns (exiting a closed arm with forepaws only and returning to the same arm.) and stretch attend postures (forward elongation of head and shoulders followed by retraction to original position). Increased anxiety was measured as an increase in closed arm and a decrease in open arm activity, as well as increased latency to enter open arms. Rats were exposed to the apparatus for 1 session on each of 10 consecutive days.

Food Neophobia: apparatus

The open arena used was a square of approximately 50 cm^2, with a covering of sawdust on the floor. In the centre one bowl of cheese (Tesco mature chedder {flavour strength 2}), and one of lab chow (SDS maintenance diet 1) were placed equidistant from the start corner.

Food Neophobia: procedure

Testing was carried out in a different room than the plusmaze, under fluorescent white light. Rats were placed in the corner facing the centre of the arena

and allowed to explore freely for 10 minutes. Measures were taken by an observer at the time of testing using a specially designed data collection proforma. Observations were made, at 10 second intervals, of rats' position in the arena, as well as measures of their latency to contact and to eat cheese and chow, and time spent eating. Testing took place on 1 day only.

In the studies reported in chapters 5 & 6 rats were placed on food restriction 3 days before food neophobia testing. They received 10g of lab chow per day for 3 days, and full food was returned after completion of neophobia testing.

Openfield: apparatus

The square open arena used was $1m^2$ and had a covering of sawdust on the floor.

Openfield: procedure

Openfield testing was carried out the day after, and in the same room, under the same lighting conditions as the food neophobia test. Rats were placed in a corner facing the centre of the field and spontaneous locomotor activity was recorded for 10 minutes. The arena was divided into several different regions. Each side was given a corresponding letter A,B,C and D and the corners designated AB, BC, CD & AD. The area closest to the edge was labelled region 1, the next third known as region 2 and finally the centre, as summarised in Figure 2.2 below. Observations of the rat's position in the field were made at 10 second intervals.

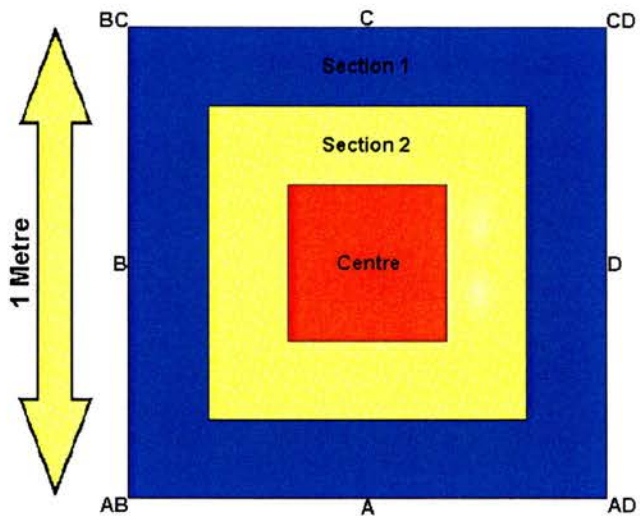


Figure 2.2 is a schematic diagram of the open field, showing the virtual regions used for scoring behaviour.

Sucrose Consumption

The rats were housed in individual plastic cages (41cm long x 25cm wide x 20.5 cm deep) with a wire grid lid and floor and separate spillage tray below lined with absorbent paper. On this tray a square of tin foil was placed beneath the food hopper to catch food spillage. These cages replaced the normal home cages, which were identical except for the absence of grid flooring and spill tray. Measurements were made to the nearest 0.1g over a 3 day period. Daily measures were taken of, weight of food in the hopper, weight of spillage and weight of water bottle

From this information daily food and water intake was calculated. Additionally body weight was recorded every day. On the second day all rats were

given access to 20% sucrose solution for 24 hours along with lab chow and water. On the final day rats had access to only lab chow and water.

Chow and sucrose intake was converted to kilo Joules of energy consumed per gram of body weight. The food pelllets contain14.8 kJ/g (data provided by the manufacturer, Special Dietry Services) and sucrose 16.8 kJ/g (Paul & Southgate 1978). 20 % sucrose solution contained 3.17kJ/g.

Quinine Consumption

The procedure was the same as for the sucrose experiment except on the second day water was replaced with 0.01% quinine (Sigma) solution.

c-Fos: procedure

Behavioural testing took place over 2 days. Rats were kept in pairs in their home cage. This pairing was used in testing so each animal exposed to the plusmaze had it's own control. On day one each pair received a 10 minute exposure to the experimental room. The control rat remained in the home cage while it's partner was placed on the maze and behaviour was recorded using the protocol already described. Following exposure the experimental rat was replaced in the home cage and the pair left in a darkened room for 1 hour 15 minutes. The following day the pair received a second 10 minute exposure, the control rat remaining in the home cage and the experimental partner on the elevated plusmaze. The pair were then put, in their home cage, in the darkened room for 1hour 15, after which time, both were anaesthetised with an overdose of sodium pentobarbitone and perfused.

Counting & Analysis

50μm coronal sections were cut and 1 in 4 processed for Fos immunoreactivity. Fos positive cells were visualised using brown Diaminobenzidene (DAB) (Sigma). Cells displaying Fos immunoreactivity were counted, by an experimenter blind to condition, on a colour monitor under a 16x objective lens. In the cuneiform cells were counted in one field of view per hemisphere on 3 50μm sections, the borders of the PPTg were identified by double staining sections with NADPH-diaphorase. Fos positive cells were counted on 4 sections representing rostral and 4 representing caudal PPTg.

2.4 Histology

Following completion of testing animals were anaesthetised with an intra peritoneal injection of 0.8 ml Dolethal (Univet; sodium pentobabitone, 200mg/ml) and perfused transcardially with 0.1M phosphate buffered saline followed by around 300ml of 4% paraformaldehyde in 0.1M phosphate buffer. After 1 hour, brains were removed and placed in 20% sucrose solution in the fridge overnight. Sections were cut coronally 50μm thick on a freezing microtome.

In all studies sections were stained for Nissl substance using cresyl violet in order to identify location and extent of damage. Lesioned tissue was identified by the presence of gliosis and degenerating neuronal somata. Immunostaining for neuron-specific nuclear protein (NeuN) was used to visualise neurones. This produces immunohistochemical staining in most cell types throughout the brain, mainly in the cell nuclei, with lighter staining in the cytoplasm (Chemicon International). In later

studies double staining NeuN and cresyl violet was found to facilitate identification of lesion area. Damage to the PPTg was also assessed using NADPH-diaphorase histochemistry. The enzyme NADPH-diaphorase is found throughout the nervous system but the cholinergic cells of the LDTg (CH6) and PPTg (CH5) stain particularly intensely, thus providing a useful marker for these structures (Leigh et al. 1990). All sections were viewed using a Leitz "Diaplan" microscope fitted with a Sony DXC-300P video camera to visualise sections on a colour monitor.

2.5 Statistical Analysis

All data was analysed parametrically using a repeated measures ANOVA, one-way ANOVA or t-test and post hoc analysis of group differences was carried out using Tukey's method for multiple comparisons on SPSS (version 10). Planned comparisons were carried out, where necessary using Statistica. Data were graphed using Sigma Plot 2000.

Chapter 3

The effects of excitotoxic lesions of the PPTg and cuneiform nucleus on performance in two behavioural tests of anxiety.

3.1 Introduction

Recently, several studies have indicated that the PPTg may play a role in the modulation of anxiety and fear (Leri & Franklin 1998; Podhorna & Franklin 1998; Podhorna & Franklin 2000). Leri & Franklin (1998) reported that N-methyl-D aspartate (NMDA) lesions of the PPTg blocked acquisition of a delayed non-matching to position T-maze task, and that these deficits were reversed by pre testing injections of diazepam. They also reported that pre-training injections of diazepam facilitated acquisition of the task in the PPTg lesioned rats while blocking learning in the controls. In a subsequent study, Podhorna and Franklin (1999) reported that electrolytic lesions of the caudal half of PPTg resulted in a "long lasting increase in anxiety" as measured in a number of behavioural tests, including the elevated plusmaze. Thirdly, in a study comparing the effects of PPTg lesions with the anxiogenic behaviour produced by ethyl β-carboline-3-carboxylate (β-CCE), a benzodiazepine partial inverse agonist. Podhorna and Franklin (2000) concluded that electrolytic lesions of caudal PPTg produce an increase in anxious behaviour, as measured by the elevated plusmaze, which is "quantitatively and qualitatively similar to that produced by...β–CCE." (p.267). If accurate, these observations would have significant implications for previous findings from PPTg lesion studies. Elevated

levels of stress or anxiety impair learning and memory function as has previously been noted in stressed rats performing the Morris water maze task (LeDoux 1998) and could account for the learning deficits seen following loss of PPTg function.

It is true that the position of the PPTg suggests the possibility of influence in the modulation of behaviours associated with the fight or flight response. The PPTg has reciprocal connections to several areas of the limbic system implicated in the modulation of anxiety states, including the central nucleus of the amygdala and the lateral hypothalamus. Additionally input from several areas of the ascending reticular activating system including, locus coeruleus and the raphe nuclei, both linked with anxiety induced behaviours (Winn et al. 1997) could suggest a role for the PPTg in control of anxiety.

However, the deficits seen following lesions of the PPTg point to attentional and behavioural control abnormalities rather than an increase in baseline anxiety. It seemed necessary therefore to look for an alternative explanation for Franklins' findings. The cuneiform nucleus lies dorsal to PPTg and is involved in behaviours related to the fight or flight response (Mitchell et al. 1988). For example, stimulation of this area with microinjections of glutamate is known to produce responses such as freezing, darting and fast running all associated with fear behaviours. The cuneiform nucleus receives ipsilateral projections from superior colliculus, which plays a role in mediating defensive behaviours. Hence, the role of the cuneiform nucleus appears to be in the production of behaviours in response to negatively reinforcing stimuli (Allen et al. 1996). It therefore seems possible that a lesion of the caudal half of

PPTg could destroy part, or all, of the cuneiform nucleus above it, thus accounting for the anxious behaviour reported by Franklin and colleagues.

The aim of the present study was to compare the behaviour of rats with lesions of the cuneiform nucleus with that of rats bearing lesions of the PPTg at the coordinates used in this laboratory, which produce no or minimal cuneiform damage, in an elevated plusmaze and a food neophobia paradigm.

The elevated plusmaze, like most animal models of anxiety, was developed to test the anxiolytic effects of drugs, used primarily in the treatment of human anxiety disorder (Pellow et al. 1985). Measures taken are of unlearned reactions to natural fears and therefore rely only on spontaneous activity. Montgomery (1958), who developed the first version of the maze, described the behaviour seen as an approach avoidance conflict, the conflict being a desire to explore a novel environment coupled with the fear of an unknown area. The plusmaze is comprised of two open and two enclosed arms, and anxiety is defined as an increase in time spent on the closed arms and a decrease in open arm activity.

The open field food neophobia test allows animals, placed in a novel environment, the choice between a familiar food (chow) and a novel preferred food (cheese). Measures are taken of both general locomotor activity as well as specific measures of response to the novel food. Suppression of eating the novel, desirable food, in the open field setting is taken as the measure of environmental neophobia (Burns et al. 1996). More evidence of environmental neophobia is expected in those rats with the highest levels of anxiety.

Hypothesis

It is predicted that cuneiform but not PPTg lesioned rats will show higher levels of anxiety, as measured by the elevated plusmaze, and heightened food neophobia compared to sham lesioned controls.

3.2 Methods

Animals

27 rats were used in this experiment; 9 with bilateral ibotenic acid lesions of the cuneiform nucleus, 9 with bilateral ibotenic acid lesions of the PPTg along with 9 sham lesioned control rats. Body weights at the time of initial surgery varied between 298–360g.

Behavioural Testing

Rats were not placed on food restriction prior to testing in the either behavioural paradigm.

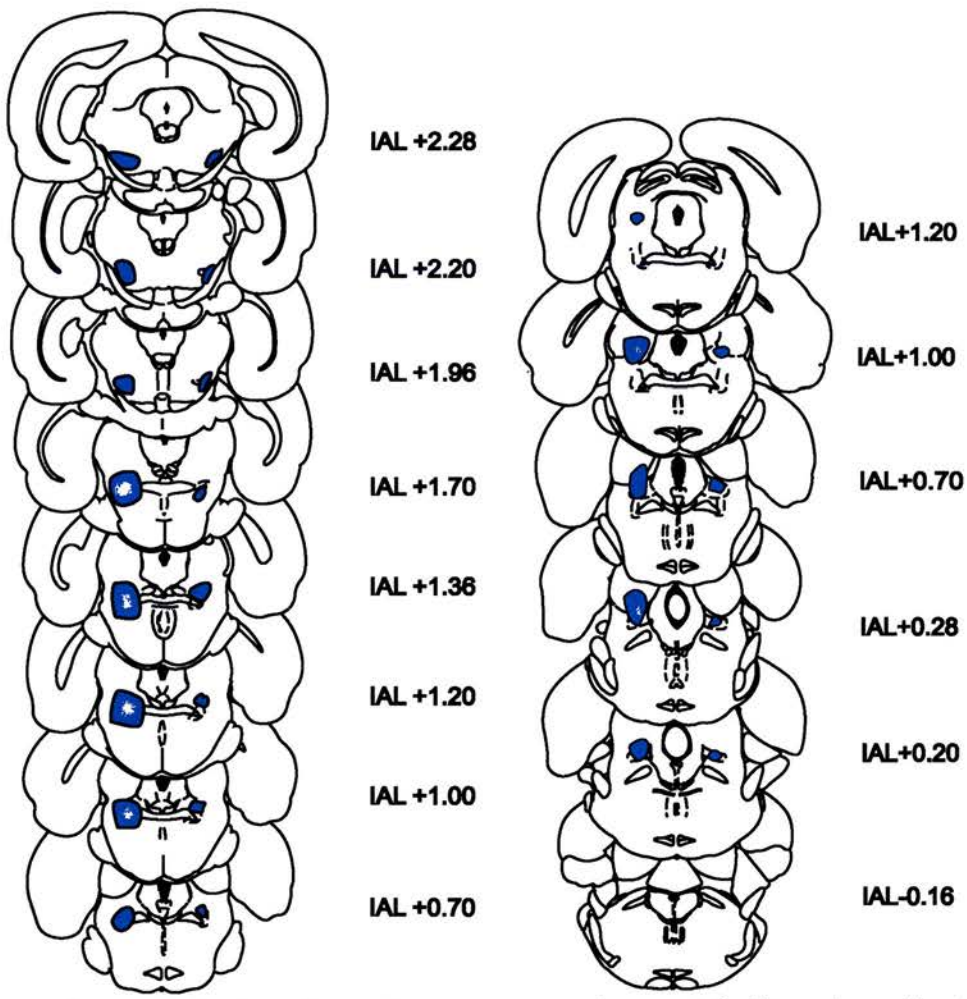
3.3 Results

Histology

Staining for Nissl substance showed that the majority of the cuneiform group had discrete bilateral lesions of the cuneiform nucleus. In two rats the lesions were small, with partial bilateral damage. In another two there was additional damage to the inferior colliculus. The majority of rats in the PPTg group had significant bilateral loss of PPTg cells as revealed by staining with diaphorase. One animal had an extremely large lesion with significant damage to LDTg and cuneiform as well as other neighbouring structures. The damage to areas out with the PPTg was so severe that the data collected from this animal is not reported.

Figure 3.1 is a schematic showing one half of the largest and smallest bilateral lesion of each type as assessed by visual analysis of the lesioned area.

Figure 3.1: Schematic showing the extent of damage produced by the largest and smallest lesion of each type

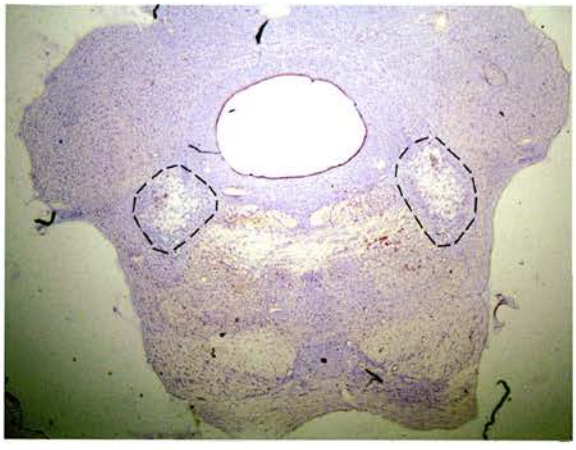


Largest (left) and smallest (right) PPTg lesions

Largest (left) and smallest (right) cuneiform lesions

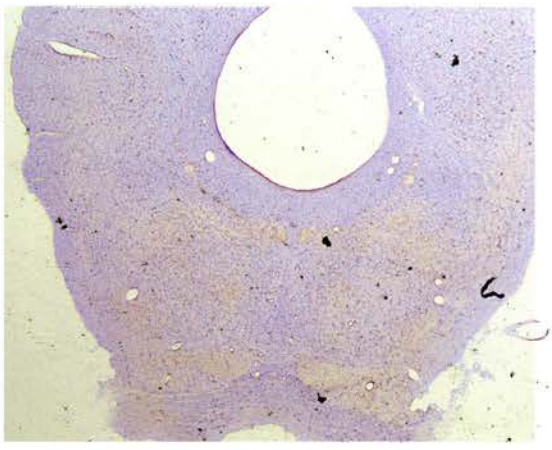
Photograph 3A shows part of an average PPTg lesion in comparison to photograph 3B, a sham lesioned control. Photograph 3C indicates loss of diaphorase positive cells in the PPTg (presumed ch5) in comparison to photograph 3D a sham lesioned control. The NeuN stained section in photograph 3E indicates the extent of cuneiform damage in comparison to a sham lesioned control in photograph 3F.

A



PPTg lesion, cresyl violet.
Dotted lines mark areas of gliosis following lesion damage.

B

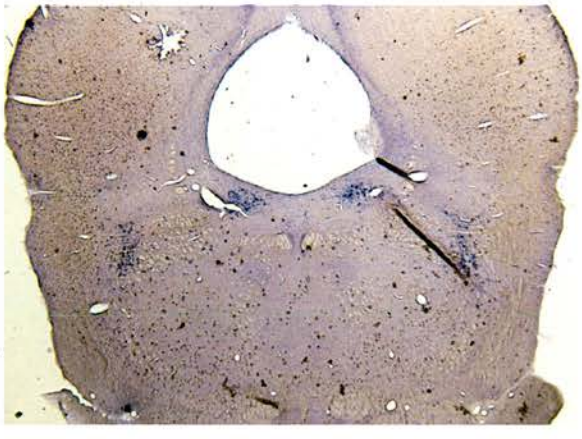


Control, cresyl violet.

C

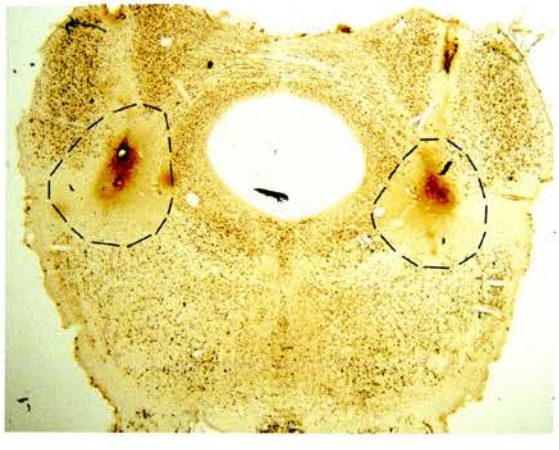
PPTg lesion, diaphorase.
There is almost total loss of diaphorase positive Ch5 cells while the Ch6 group remain intact.

D



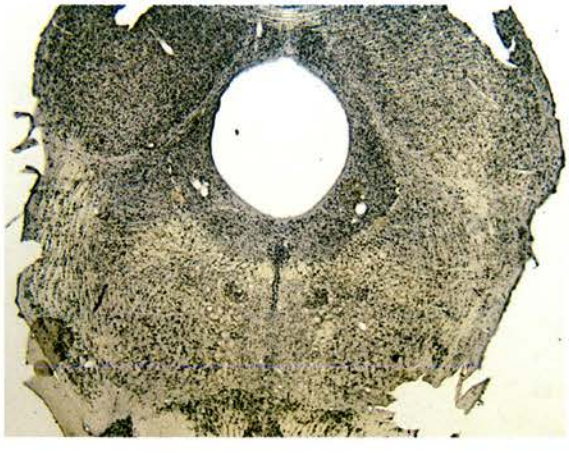
Control, diaphorase.

E



Cuneiform lesion, NeuN.
Dotted lines indicate the extent of bilateral cuneiform damage.

F



Control, NeuN.

Elevated Plusmaze

In the elevated plusmaze anxiety is generally defined as, a decrease in open arm activity (Pellow et al. 1995). In this study statistical analysis showed there was a significant effect of group $F(2,23)=3.89$, $p<0.05$. There was also a significant effect of day $F(9,15)=6.79$, $p<0.01$ but no day by group interaction $F(18,32)=1.64$, NS. Post hoc tests indicated that PPTg lesioned rats spent significantly longer in the open arms of the maze than the cuneiform group. These findings are summarised in Figure 3.2.

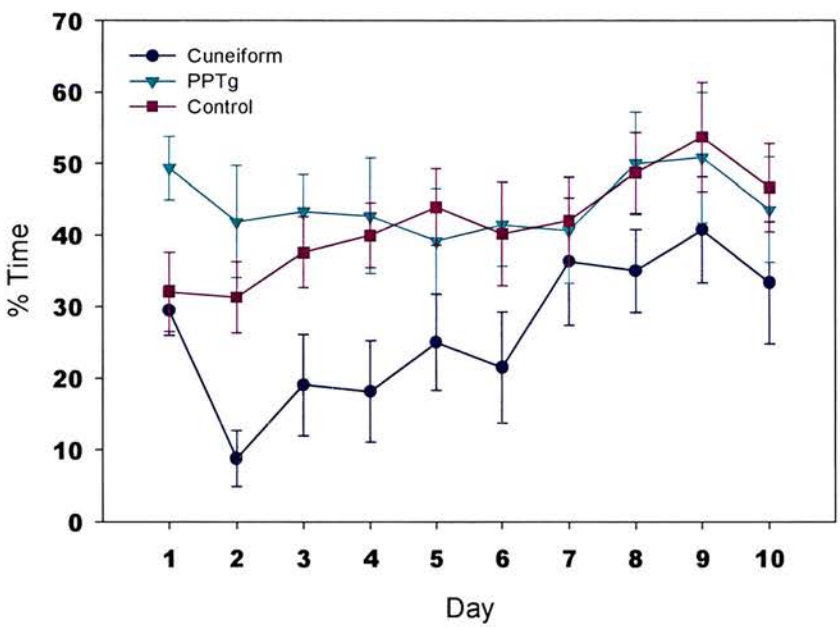


Figure 3.2: Mean percentage of test time spent on the open arms of the plusmaze over the 10 days of testing (+/- SE). There is a significant effect of day p<0.01 and of group p<0.05, but no day x group interaction.

Podhorna and Franklin (1998) measured anxiety in the opposite way, as an increase in time spent in the closed arms. On this measure a repeated measures ANOVA showed a significant effect of day, $F(9,15)=5.80$, $p<0.01$, and a significant

day x group interaction, $F(18,32)=2.49$, $p<0.01$. There was also a significant effect of group, $F(2,23)=5.07$, $p<0.05$. Planned comparisons were carried out to compare goup differences on individual days, on days 1-4 & 6 cuneiform lesioned rats spent significantly longer in the closed arms of the maze than PPTg lesioned rats ($p<0.05$).

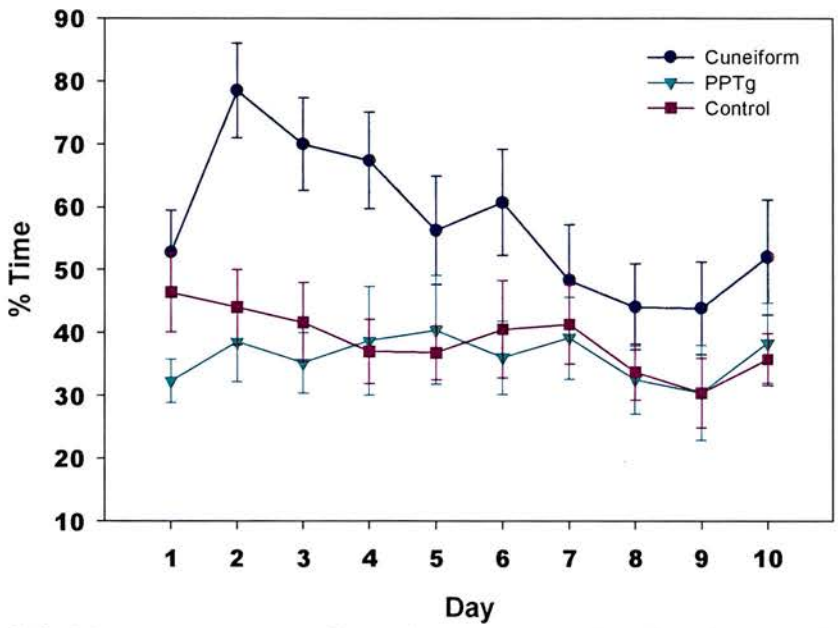


Figure 3.3: Mean percentage of test time spent on the closed arms of the plusmaze over the 10 days of testing (+/- SE). There was a significant effect of group, $p<0.05$, and of day, $p<0.01$, and a significant day x group interaction, $p<0.05$.

On days 2-4 & 6 they also spent significantly longer in the closed arms than the control group ($p<0.05$). PPTg and control rats did not differ significantly from each other on any day. Figure 3.3 indicates that while the cuneiform lesioned rats clearly habituated to the maze over successive test sessions the PPTg and control rats did not appear to change much in their level of closed arm activity.

Podhorna and Franklin (1999) also made measures of "risk assessment behaviours." These included open and closed arm entry latencies, closed arm returns,

freezing, self grooming and stretch attend postures. Figure 3.4 shows the log of the time taken by rats to initially enter one of the open arms of the maze. This graph indicates that the PPTg lesioned rats were faster to enter the open arm than either of the two other groups, while the cuneiform lesioned group took the longest over the majority of the 10 test days. A repeated measures ANOVA showed there was neither a significant effect of day $F(9,15)=2.06$ NS nor a significant day x group interaction $F(18,32)=0.84$ NS. However, there was a significant effect of group, $F(2,23)=5.23$, $p<0.05$. Post hoc tests revealed that the difference was significant between the PPTg and the cuneiform groups, PPTg lesioned rats were significantly faster to enter the open arms than those with lesions of the cuneiform nucleus.

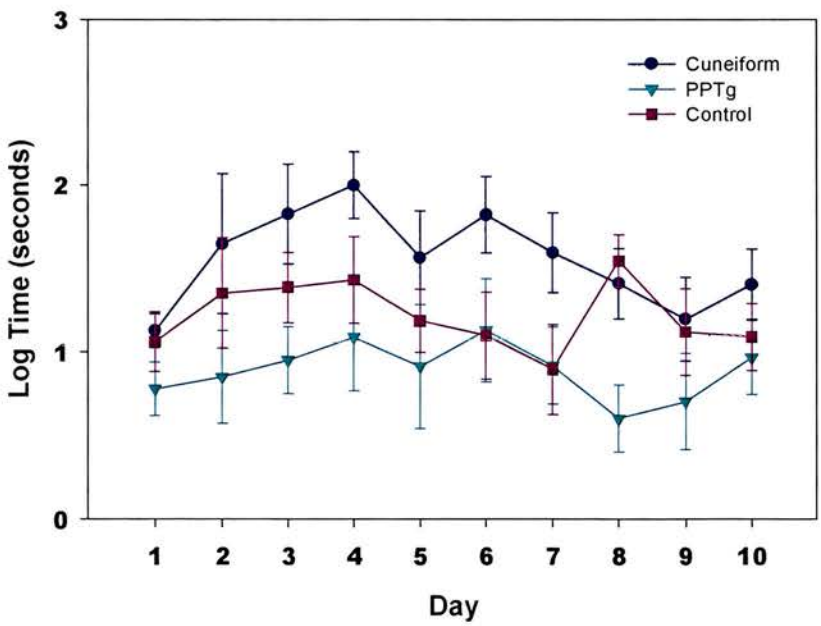


Figure 3.4: Mean latency to enter an open arm from the start of each of the 10 test sessions (+/-SE). There was a significant effect of group, $p<0.05$, but no significant effect of day or a day x group interaction.

Measures were also taken of grooming, freezing, closed arm returns and head dipping behaviours. Figure 3.5 shows the number of times grooming behaviour was seen over the ten test sessions. A repeated measures ANOVA showed no significant effect of day $F(9,15)=0.83$ NS and no day x group interaction $F(18,32)=1.83$ NS. There was however a significant effect of group, $F(2,23)=4.68$, $p<0.05$. Post hoc tests showed that the control group performed significantly more self-grooming than the cuneiform lesioned rats.

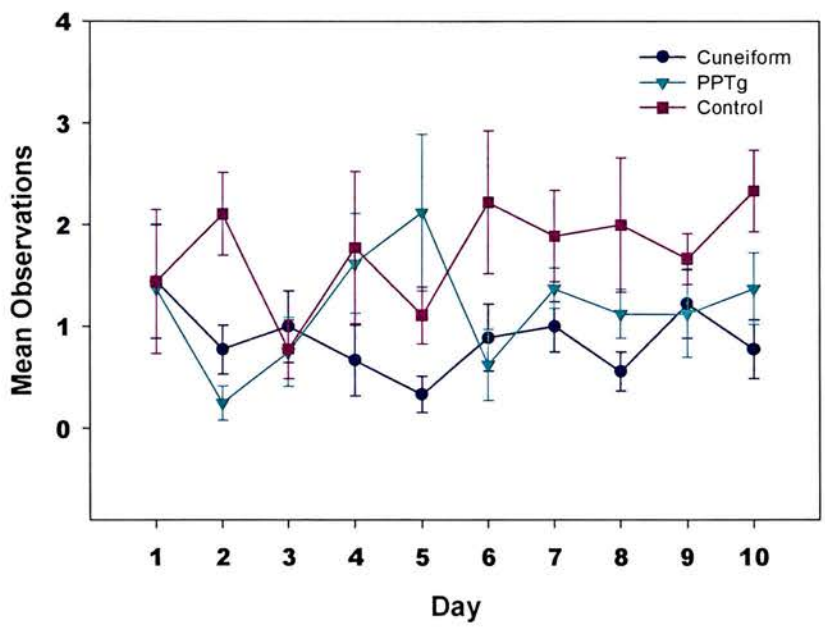


Figure 3.5: Mean observations of self grooming behaviour performed by each of the 3 groups on each of the 10 test sessions (+/-SE). There was a significant effect of group, $p<0.05$, but no significant effect of day or a day x group interaction.

Figure 3.6 shows the number of closed arm returns observed in the three groups. No such behaviours were seen after the third day of testing which would suggest all groups were habituated to the plusmaze. Eight of the nine cuneiform lesioned rats showed at least one example of this behaviour in the first three days of

testing. 6 of the 9 sham lesioned control rats performed closed arm returns, none of the PPTg lesioned group did so. A repeated measures ANOVA revealed a significant effect of day, $F(3,21)=8.91$, $p<0.01$, and a significant day x group interaction, $F(6,44)=2.942$, $p<0.05$. There was also a significant effect of group, $F(2,23)=5.42$, $p<0.05$. Planned comparisons were used to compare groups on individual days. On day 2 cuneiform lesioned rats made significantly more closed arm returns than either of the other 2 groups ($p<0.01$). There were no significant differences on any of the other days.

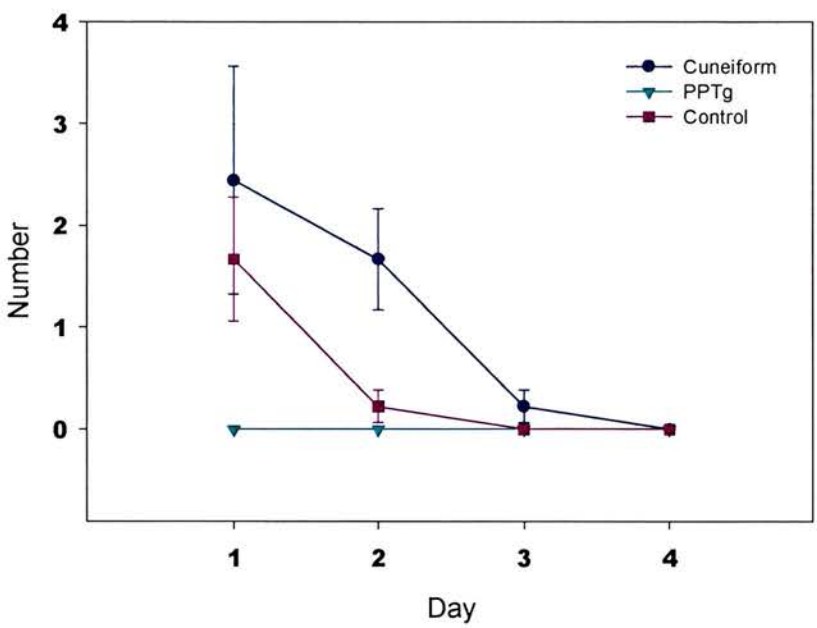


Figure 3.6: Total number of closed arm returns performed by each of the 4 groups during the first 4 test days (+/- SE). There was a significant effect of group $p<0.05$, and of day, $p<0.01$, there was also a significant day x group interaction, $p<0.05$.

Measures of freezing behaviours and stretch attend posturing produced so few observations in any group that they have not been analysed. These behaviours were seen only in the first 2 days of testing.

Food Neophobia

A number of measures were taken of time spent in a variety of areas of the open field throughout the 10 minute test session. Figure 3.7 shows the percentage of test time rats spent in the centre of the openfield. There was a significant effect of group, $F(2,23)=12.87$, $p<0.01$. The cuneiform lesioned rats spent the least time in the centre of the field and the PPTg lesioned rats spent the most time here. Post hoc tests showed that the differences between all three groups were significant.

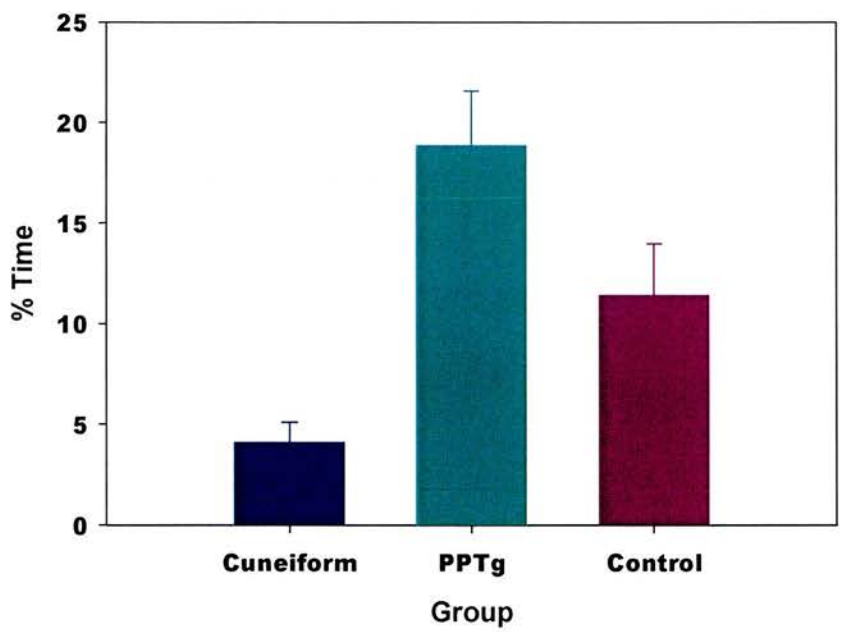


Figure 3.7: Mean percentage of test time each of the 3 groups spent in the centre portion of the open field (+SE). There was a significant effect of group, $p<0.01$. Post hoc tests showed that the difference between all 3 groups was significant

Figure 3.8 shows the percentage of the ten minute test time that rats were physically making contact with the outer walls of the openfield thigmotaxic behaviour. There was a significant effect of group, $F(2,23)=6.19$, $p<0.01$. The

cuneiform lesioned rats displayed the most thigmotaxis and PPTg lesioned rats the least; post hoc tests revealed that the difference between these two groups was significant.

Measures were also taken of time to contact the cheese and chow in the centre, as well as eating latencies and time spent eating. There was no significant difference between the three groups in the time elapsed before initial contact was made with either chow, $F(2,23)=0.42$ NS, or cheese, $F(2,23)=0.96$, NS. These findings are summarised in Figure 3.9.

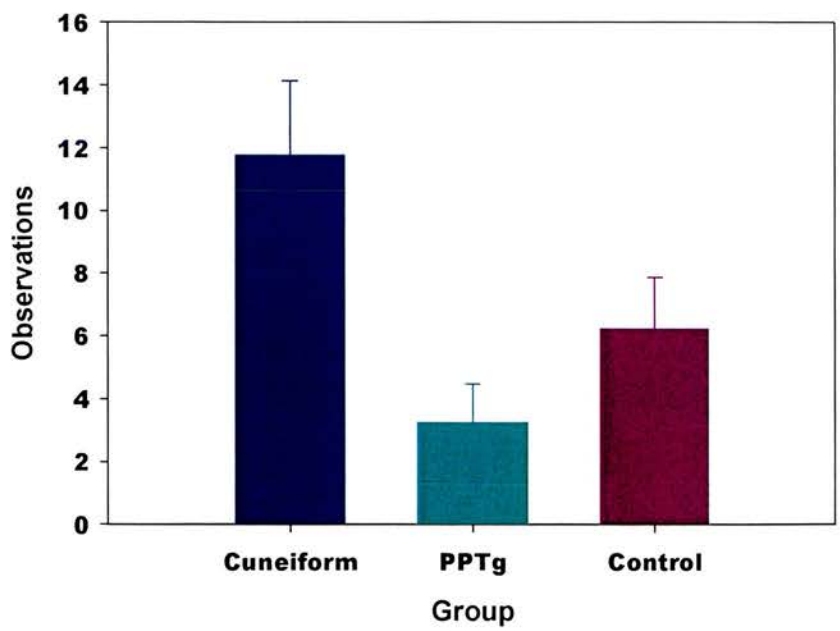


Figure 3.8: Mean number of displays of thigmotaxic behaviour observed in each of the 3 groups (+SE). There was a significant effect of group, $p<0.01$. Post hoc tests revealed that the differencde between cuneiform and PPTg lesioned rats was significant.

It was not possible to take mean eating latencies because, as Table 3.1 shows not all the rats in any group actually consumed the food. There were however, very obvious differences in the behaviours of the different groups. While all of the eight

PPTg lesioned and seven out of nine sham lesioned control rats ate some cheese, less than half the cuneiform group, four out of nine, ate any cheese at all. There were also clear differences between the groups when it came to eating the chow. None of the cuneiform and only one of the sham lesioned control rats ate any chow. However seven of the eight PPTg lesioned rats ate some chow. In summary the pattern of these results show, the cuneiform lesioned rats were less likely to eat either food, and spent less time doing so than either of the other groups. The PPTg lesioned rats, while reaching the food and spending a similar amount of time eating cheese as the sham lesioned control group were more likely to eat the chow too, and spent longer doing this.

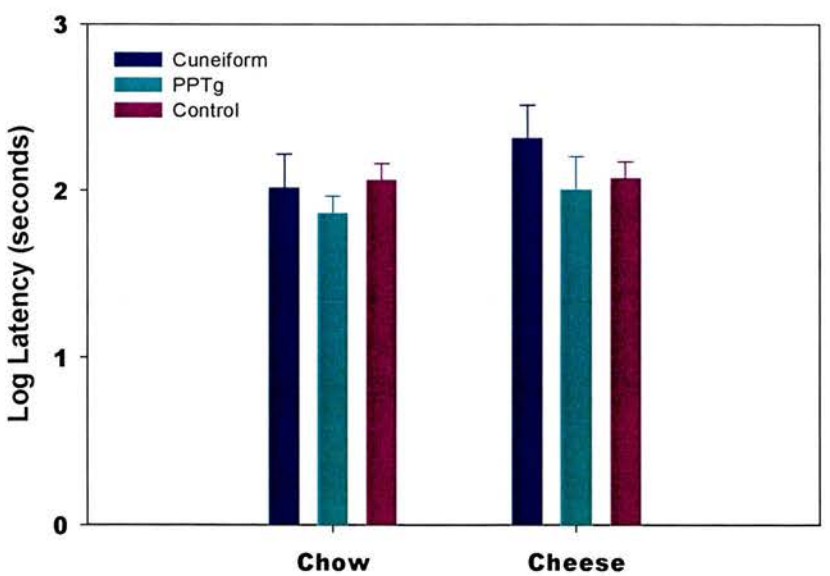


Figure 3.9: Mean latency to contact cheese and chow from the start of the test session (+SE). There were no significant differences between any of the groups on either measure.

Table 3.1 summarises data collected from the food neophobia experiment. It shows numbers of animals from each group eating cheese and chow.

	Cuneiform N=9	PPTg N=8	Control N=9
No. Eating Cheese	4	8	7
No. Eating Chow	0	7	1

3.4 Discussion

The results of this elevated plusmaze study, in contrast to Franklin et al.'s work, show no evidence of increased anxiety in PPTg lesioned rats. Rats with lesions of the PPTg did not differ from the control group in terms of the time spent in the closed arms of the maze, or in latency to enter the open arms. In contrast, those rats with discrete lesions of the cuneiform nucleus did show evidence of heightened anxiety on the maze. They spent longer than both PPTg and control groups in the closed arms, the traditional measure of anxiety on the plusmaze. Additionally, the cuneiform lesioned rats also showed evidence of heightened anxiety using Franklin's novel measure of arm entry latency.

The food neophobia test also revealed a degree of anxiety in the cuneiform lesioned rats that was not present in the PPTg lesioned or control groups. When placed in a novel open environment a rat will naturally remain close to the edges of the arena in contact with the side, a behaviour known as thigmotaxis. However, prolonged measures of this behaviour are evidence of elevated anxiety levels. Once more there was no evidence of increased anxiety in the PPTg lesioned rats. Increased time in the centre of the field and less evidence of thigmotaxis in fact suggests reduced anxiety in the PPTg and control rats in comparison to the cuneiform group. Though in terms of time spent in contact with the walls the sham lesioned control group did not differ significantly from either lesioned group, their behaviour was, in general, more like the PPTg lesioned rats.

There could be several reasons for the pattern of findings seen here. It could be that the cuneiform lesioned rats were indeed more anxious than those in either of

the other two groups, but high variance prevented there being a significant difference from the control group. Alternatively the PPTg lesioned group could show lower than normal levels of anxiety, thus suggesting that both experimental groups are abnormal in terms of their anxiety but in opposite ways. Several of the other measures taken in these studies indicate that this might be the case, and possibly limitations of apparatus and or group number prevented them from being apparent in all instances. For example, while 8 out of 9 cuneiform and 6 out of 9 sham rats performed closed arm returns during the first day of plusmaze testing, none of the PPTg lesioned rats did so at any point. This indicates that, on this measure at least, the PPTg lesioned rats are not the same as controls. The abnormality however does not appear to be an increase in anxiety but if anything, a decrease.

The PPTg lesioned rats spent significantly more time in the centre of the open field than the control rats, suggesting that, while the cuneiform group had heightened levels of anxiety, levels in the PPTg lesioned group were lower than normal rats. Although initial time to make contact did not differ significantly, the PPTg lesioned group were more likely to eat both cheese and chow than the cuneiform lesioned group, indicating heightened food neophobia in the cuneiform group. Although in terms of cheese consumed there was little difference between the PPTg lesioned rats and the control group, suggesting no difference in neophobic reaction, there was a difference in numbers eating familiar chow. Only one of the control rats ate any chow at all despite the fact that 7 out of 9 ate the cheese. The PPTg lesioned rats were almost as likely to eat the chow as the novel cheese suggesting an abnormality in their behaviour. Despite spending longer in the centre than either of the other two

groups the PPTg lesioned rats did not maximise potential reward since they split their time eating the novel desirable cheese, with eating familiar lab chow, a pattern of behaviour not seen in the other groups.

Therefore it appears that, while the cuneiform lesioned rats show elevated levels of anxiety the PPTg lesioned rats did not behave as normal in these two behavioural tests. This disinhibition seen in rats with lesions of the PPTg has been noted before in other behavioural paradigms. For example, on a progressive ratio schedule for food reinforcement rats with lesions of the PPTg made more responses on the non reinforced control lever than sham lesioned control rats (Alderson et al. 2002). Similarly, in a place preference paradigm, PPTg lesioned rats, while spending no longer than controls in the sucrose half, consumed more sucrose than control rats (Alderson et al. 2001).

Franklin et al. report that, in addition to their own results, there is increasing evidence for the role of the PPTg in regulation of defensive behaviour and anxiety. Primarily, they cite the evidence that rats with lesions of the PPTg show abnormal prepulse inhibition of the startle reflex (Swerdlow & Geyer 1993). However, rather than being a model of anxiety per se, prepulse inhibition of the startle reflex provides a model for the inhibition of behaviour in response to information, in this case sensory input. Rats with lesions of the PPTg show reduced prepulse inhibition. Rather than being the result of elevated anxiety levels, it is more likely to reflect disinhibited behaviour, seen frequently in rats with bilateral lesions of this area resulting in an inability to inhibit responses possibly due to disruption in attentional or arousal processes (Steckler et al. 1994). Also, PPTg lesioned rats show decreased

levels of freezing in response to overhead threat (Allen 1995). This disinhibited behaviour is very much at odds with the view that lesions of the PPTg produce an elevation in anxiety levels. Again, any suggestion of abnormality is, if anything, in the opposite direction to that proposed by Franklin et al. (1997,98, 99).

Other studies of anxiety concur with our view that lesions of the cuneiform nucleus and not the PPTg disrupt brain regions and or circuits involved in the modulation of anxiety states. Stimulation of the cuneiform nucleus produces freezing, darting and fast running behaviours all associated with the fear response (Mitchell et al. 1988). Also two experiments using c-Fos have shown evidence of the role of the cuneiform nucleus in anxiety. Silveira et al. (1993) induced Fos immunoreactivity following 15 minutes exposure to an elevated plusmaze. Along with the amygdala, locus coeruleus, inferior and superior colliculus, extensive labelling was also found in the cuneiform nucleus. While Fos is known to be neither a complete or exclusive marker of neuronal activity, a complementary study by Sandner et al. (1993) found a similar pattern of c-Fos staining following stimulation of GABA receptors using a benzodiazepine inverse agonist as were seen following environmentally induced anxiety.

In conclusion, using Franklin et al.'s methodology no evidence was found for increased anxiety in rats with bilateral ibotenate lesions of the PPTg. Evidence for anxiety, very similar to that reported by Franklin et al., was found in rats bearing excitotoxic lesions of the cuneiform nucleus. The behaviour of the PPTg lesioned rats was fundamentally similar to that of the sham lesioned control rats in both the plusmaze and the food neophobia paradigm. Differences, where they were detected,

were explicable as a disinhibition of behaviour particularly in the presence of a positive reinforcer, an abnormality previously described in rats bearing PPTg lesions.

Chapter 4

A study of sucrose consumption and energy modulation following bilateral excitotoxic lesions of the PPTg.

4.1 Introduction

Several studies of PPTg function have indicated that, along with other abnormalities, rats with excitotoxic lesions of the PPTg respond abnormally to positive reinforcement. For example in a runway task, rats with lesions of the PPTg, though no faster to reach the sucrose reward at the end, which suggests normal levels of motivation, consumed more 20% sucrose solution than control rats did (Ainge et al. 1999). This increased intake in reward had been noted previously, particularly under conditions of high motivational demand. In a place preference paradigm, rats with bilateral lesions of the PPTg, while showing no deficits in place conditioning, did show higher sucrose intake than normal rats in the positively paired environment (Alderson et al. 2001, Keating et al. 2002). However, these abnormalities are not specific to sucrose. Comparable patterns of behaviour have been seen using both drug and food rewards. In a conditioned reinforcement paradigm PPTg lesioned, like sham rats, increased lever pressing to gain food reward following pre test injections of amphetamine. The abnormality was their continued pressing on the non-reinforced lever (Inglis et al. 1994a). Also, when working under a progressive ratio schedule for intravenous heroin rats with PPTg lesions have elevated breaking points compared to controls (Olmstead et al. 1998).

With respect to reward related behavioural deficits seen following lesions of the PPTg, a study was conducted of the food and water intake of a group of PPTg lesioned rats in comparison to a group of sham lesioned control and additionally a group of cuneiform lesioned rats over a three day period. Previous studies have shown that, when given access to 20% sucrose solution, rats with lesions of the PPTg consume significantly more than control rats. The aim of this study was to determine whether this over consumption would be apparent during an extended period of sucrose availability, and whether a compensatory change in lab chow intake would take place to maintain normal energy balance.

Hypothesis

It is predicted that, as previous studies have shown, PPTg lesioned rats will consume significantly more sucrose solution than either cuneiform or sham lesioned control rats, however if this does not reflect a basic regulatory or motivational deficit, as is hypothesised, they will compensate for this increase in calorie intake by reducing consumption of lab chow.

4.2 Methods

Animals

The 27 rats used in this experiment had previously taken part in the study reported in chapter 3.

Experiment

Rats were placed in cages with spill trays for eight days, however only data from the 3^{rd}, 4^{th} and 5^{th} days is reported.

4.3 Results

In this study data were adjusted for bodyweight. This is because the PPTg lesioned rats, which have undergone two separate lesion surgeries tend to be lighter than both the control and cuneiform lesioned rats, which undergo only one. Indeed a one way ANOVA showed that while there was no significant difference between pre surgery body weights, $F(2,23)=0.34$ N.S., as shown in Figure 4.1, there was a significant pre testing difference, $F(2,23)=4.06$ $p<0.05$, as summarised in Figure 4.2.

Figure 4.3 shows the sucrose consumption for each of the three groups as measured by kilo Joules of energy intake, per gram of body weight. The PPTg lesioned rats consumed significantly more than either of the other two groups. $F(2,23)= 17.31$, $p<0.01$.

Figure 4.4 however, indicates that the PPTg lesioned rats did not differ significantly from the other two groups in their total combined energy intake for the

sucrose day, F(2,23)=1.94, N.S., or indeed either of the other test days. Day 1, F(2,23)=2.32, NS and Day 3, F(2,23)=1.77, NS.

Measures were also taken of water consumed by the different groups, which were also adjusted for body weight. There was a significant drop in the amount of water consumed by all groups over the 24 hours sucrose was available. Although there was a significant effect of day, F(2,22)= 30.08, $p<0.01$, there was not a significant between group difference F(2,23)=2.90 NS. These data are summarised in Figure 4.5.

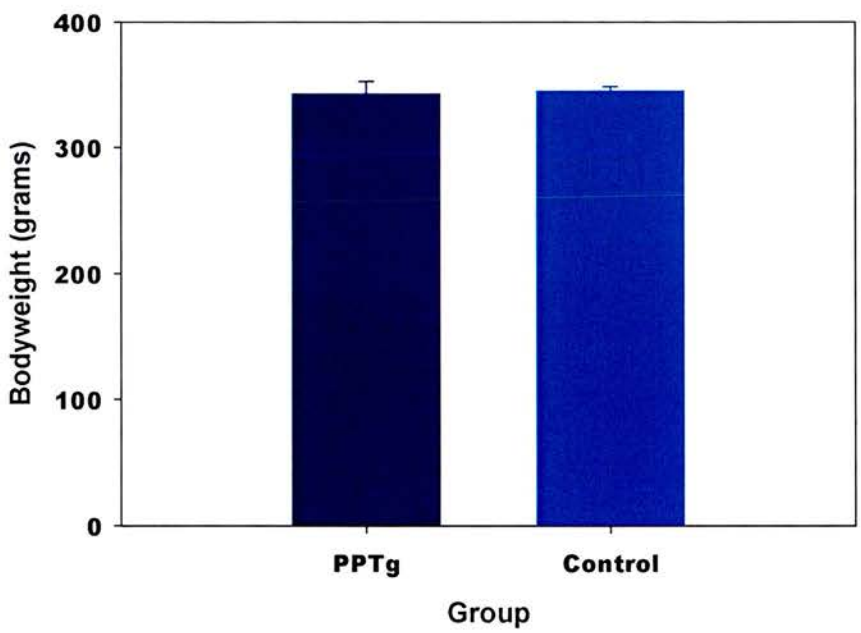


Figure 4.1: Mean body weights (g) of PPTg and control rats before surgery (+SE). There was no significant difference between groups.

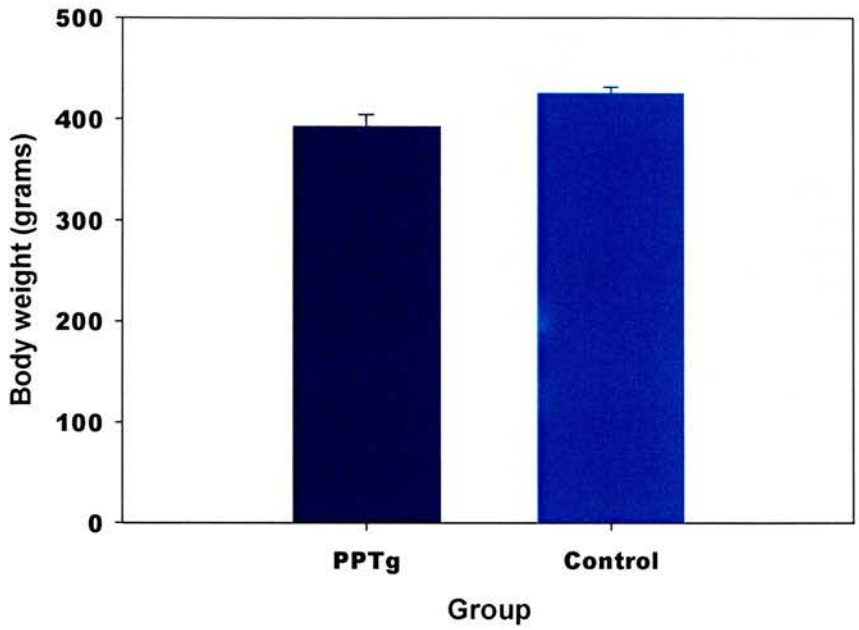


Figure 4.2: Mean body weight (g) of PPTg lesioned and control rats before testing, 4 weeks post surgery (+SE). There was a significant difference between groups, $p<0.05$.

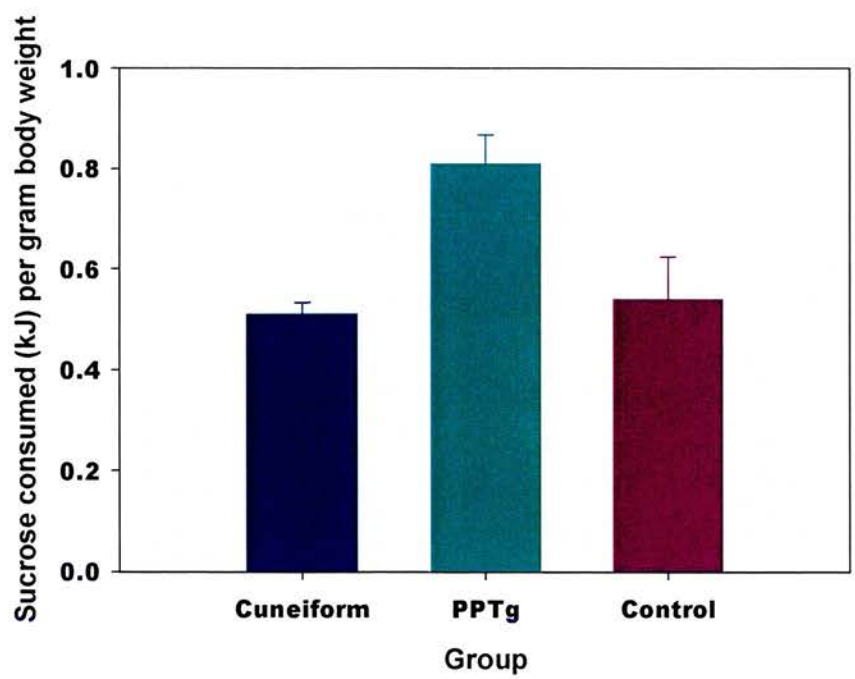


Figure 4.3: Mean sucrose consumption (kJ) per gram of body weight of each of the 3 groups over 24 hours (+SE). PPTg lesioned rats consumed significantly more sucrose than either of the other groups, $p<0.01$.

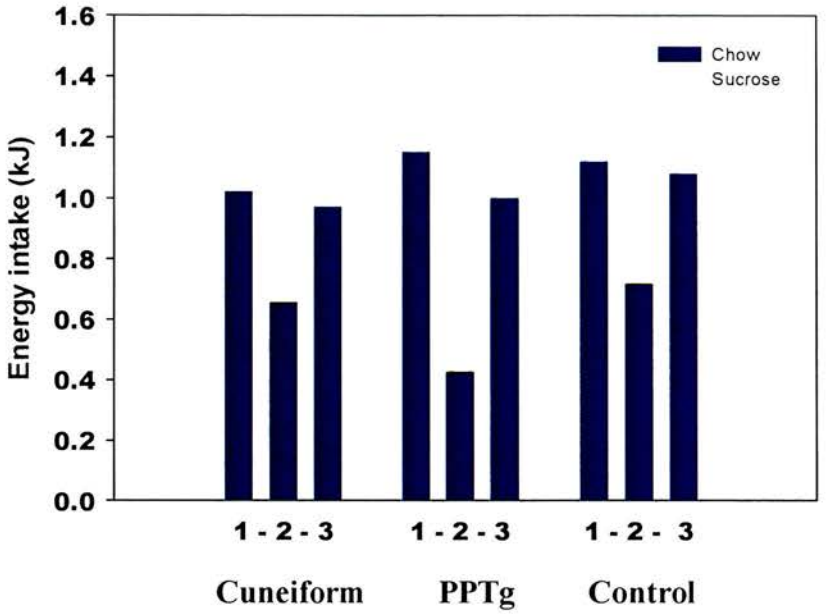


Figure 4.4: Average energy intake (separated into sucrose and lab chow consumption on day 2) for each of the 3 groups on each of the 3 test days. There was no significant difference between any of the groups in their total energy intake on any of the 3 test days.

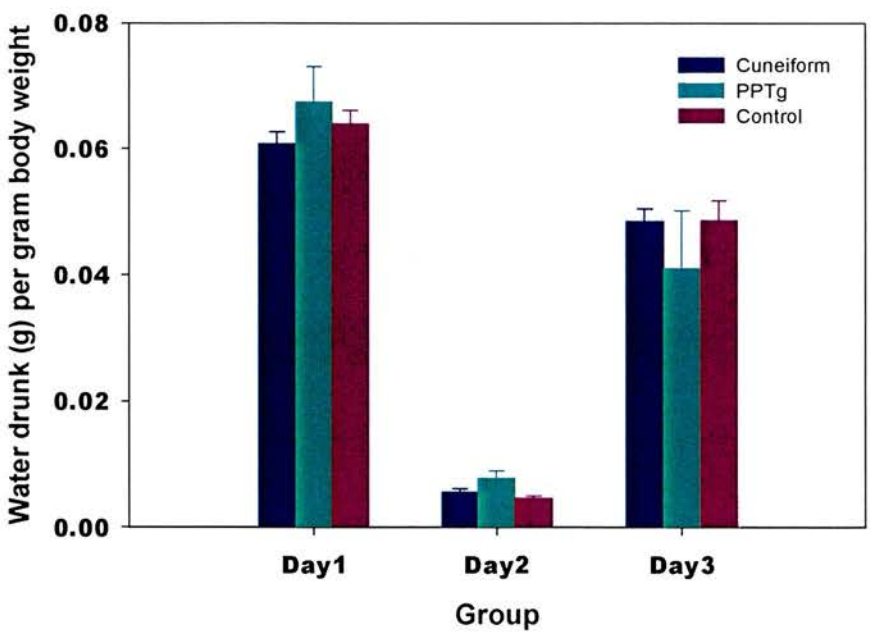


Figure 4.5: Mean water consumption (g) per gram of body weight of the 3 groups over each of the 3 test days (+SE). There was a significant effect of day, $p<0.01$, but no significant effect of group.

4.4 Discussion

The results from this study tie in well with previous findings that, when given free access to sucrose, PPTg lesioned rats consumed significantly more than either the control or cuneiform lesioned rats. Yet this study has also revealed that rats with lesions of the PPTg were able successfully to modulate their energy intake. Thus they can compensate for an increase in calorie intake by adjusting consumption of other energy sources, just like normal rats.

Rats with bilateral lesions of the PPTg are generally of lower body weight than sham lesioned control rats. This appears to be solely an effect of surgery. In the initial days following surgery PPTg lesioned rats fail to eat and drink, but once this post surgery trauma has come to an end they gain weight at the same rate as other rats (Inglis et al. 1994b). The results from the current study provide further evidence that, while basic behaviours remain intact following lesions of the PPTg, responses to positively reinforcing stimuli are disrupted. However the precise nature of this disruption is not entirely clear.

These findings are, for the most part, compatible with the results of a study conducted by Olmstead et al. (1999) that investigated how PPTg lesions affect responses to alterations in reward magnitude and ability to differentiate different reward strengths. Their findings were somewhat contradictory to their original hypothesis that either PPTg lesioned animals are unable to perceive differences in reward strength, or at the very least they fail to alter behaviour in response to these changes. What they actually found was that lesions of the PPTg did not reduce the rewarding properties of the sucrose solution in either food deprived or free fed

animals. These rats, just like the sham lesioned controls increased their consumption as concentration increased and were able to discriminate the reward strength of two simultaneously presented solutions. Where the PPTg lesioned group did differ was in the overall volume of sucrose consumed. They found that PPTg lesioned rats consumed more sucrose than controls when they were food deprived but not when free fed. This finding is partially at odds with our own given that the animals we tested were not food restricted. This suggests that the amount of time the sucrose is available is an important factor in the results. Olmstead et al. found that the PPTg lesioned rats were slower to approach the sucrose after initial presentation, and in the first half of the test consumed similar volumes to the control group. However, the PPTg lesioned rats maintained this level of consumption throughout the 30 minute test. In our study sucrose was available for 24 hours, and over this time period the difference between lesioned and control groups was obvious in free fed rats.

Olmstead et al. (1999) found that latencies to approach and lick a burette containing 10% sucrose solution decreased across trials in all groups, free fed or food restricted, lesion or control. However the free fed groups consistently took longer to approach the burette irrespective of lesion. These findings help to explain why differences between PPTg lesioned and control rats are sometimes easier to achieve in food deprived than non deprived groups where general levels of motivation are lower and therefore approach latencies longer. Keating et al. (2002) also found that increasing levels of food deprivation decreased approach latencies and that PPTg lesioned rats, in general, had longer approach latencies. PPTg lesioned, non deprived rats did not show disinhibited sucrose drinking until

concentration increased to 24%, where as it was apparent in food deprived rats at 12%, again showing that, only when motivation is high are abnormalities seen

Further evidence for disruption in response to positively reinforcing stimuli comes from an intravenous self-administration study (IVSA). Olmstead et al. (1998) found that NMDA lesions of the PPTg disrupted, and in some cases completely blocked acquisition of IVSA of heroin on a fixed ratio schedule. Lesioned animals also had lower breaking points than normal rats. While these data support previous findings that the PPTg is important in the rewarding effects of drugs, there was no effect of the lesion on extinction and reacquisition, in rats that were trained pre surgery. Olmstead et al. suggest therefore that the difference between drug naïve and drug dependent animals following PPTg lesions may be related to levels of drug exposure rather than the lesions per se. In their study animals that would not previously self-administer heroin did so after experimenter administration. It is therefore possible that repeated drug administration alters the neural substrates mediating reward.

Given the extensive connections of the PPTg to basal ganglia circuitry, it is not surprising that reward related behaviours are disrupted. Mogenson's (1980) seminal paper discusses, specifically the role of the nucleus accumbens in translating motivation into action. A great deal of experimental evidence suggests that the PPTg itself may have a fundamental role as a striatal outflow station (Inglis & Winn 1995). The abnormalities seen in the current study cannot be described as either basic locomotor or motivational deficits. What we do see is a disinhibited response to positively reinforcing stimuli given as primary reinforcement, particularly under

conditions of heightened motivation. These disruptions however do not appear to affect perception of reward.

Chapter 5

The effects of different PPTg lesion methodologies on, performance in behavioural tests sensitive to anxiety, and sucrose consumption, a comparison with lesions of the cuneiform nucleus.

5.1 Introduction (a)

The broad aim of this research is to further define the role of the PPTg in behavioural control. Studies so far have indicated the importance of its connections with the cortico-striato-pallido-thalamic loops (Alexander et al. 1986), with deficits observed similar to those seen following damage to frontal and striatal areas. While these connections strongly suggest a role for the PPTg in cognitive functioning, this can only be determined absolutely through the careful design and application of experiments which separate possible cognitive influences from non cognitive ones, like incentive motivation, motor control and anxiety.

Lesion studies entail destroying part of the brain and evaluating the subsequent effect on behaviour. It is important that an effective technique is used to ensure maximal damage to the target area while minimising effects on surrounding regions. Electolytic lesions involve placing an electrode in the target region and passing electrical current through, with the consequence of destroying cell bodies and axons in the surrounding area. This can be done in two different ways, either by

using a direct current device to create a chemical reaction, the products of which destroy neurones near by, or alternatively using very high frequency current which does not stimulate surrounding tissue but destroys cells with the heat generated by the resistance of the tissue to the passing current. These lesions are non selective and destroy all tissue around the tip including fibres of passage. Therefore any conclusions drawn about the specific functions of the target area must be cautious, as functions of other connected regions could also be dramatically altered (Carlson 1994).

Study of the excitatory effects of several amino acids in the brain has lead to the discovery of a number of naturally occurring analogs which act both specifically and potently to depolarise cells. Ibotenic acid, which was isolated from mushrooms, and N-mehtyl-d-aspartate (NMDA) are both analogs of glutamate and produce highly selective lesions by binding to specific receptor subtypes resulting in cell depolarisation. At high concentrations they are toxic either by causing overstimulation of the target neurone or by blocking cell depolarisation, allowing uncontrolled Ca^{2+} influx, both ultimately resulting in cell death (Cooper et al. 1991). If the correct volume and concentration of these excitotoxins is used, fibres of passage should remain intact. However, excitotoxins differ in level of toxicity within a given brain region depending on density and type of receptor present (Brace et al. 1997). Additionally level and type of anaesthesia can alter the size of lesions produced (Inglis et al. 1993): in general terms lighter anaesthesia tends to produce large lesions (Brace et al. 1997)

One key difference between the work described here, and the Franklin studies, is lesion technique. While ibotenic acid was used here to make fibre sparing lesions of the PPTg, Franklin et al. have used both electrolytic and NMDA lesions. NMDA, like ibotenic acid is effective in lesioning PPTg, making similar sized lesions, for equivalent volumes (Rugg et al. 1992). For the studies reported here rats received two injections of 0.2 μl of 0.12M ibotenic acid per hemisphere, one into rostral and one into caudal PPTg. This maximises damage to the long thin structure of PPTg itself while minimising damage to surrounding areas. Leri & Franklin (1998) report giving 0.5μl of 0.1M NMDA per hemisphere and so much larger, different shaped lesions are likely, with possible damage to fibres of passage at the injection site as well as to surrounding regions.

It was therefore decided to rerun the plusmaze, food neophobia, and sucrose experiments, with an openfield test, using the PPTg and cuneiform lesioned groups along with an additional PPTg group bearing NMDA lesions, using the stereotaxic co-ordinates, excitotoxin volumes and concentrations reported by Leri & Franlin (1998). This will, hopefully, confirm the hypothesis, that these lesions of caudal PPTg also cause damage to surrounding areas including the cuneiform nucleus. Hence, we could conclude using the evidence from our own discrete lesion studies that it is the damage to cuneiform nucleus and not the PPTg that produces the elevation in anxiety described.

In this study the co-ordinates of our own ibotenic acid lesions of the PPTg were changed slightly. They were made more caudal to ensure that successful lesioning of the same area of PPTg as Franklin et al.. Therefore it is safe to conclude

that any differences in behaviour seen between the lesion groups are not due to differences in damage to the PPTg itself.

Hypothesis

It is predicted that the NMDA lesions of the PPTg, made using the same methodology as Leri & Franlkin (1998), will consistently produce significant neural loss within the boundaries of the cuneiform nucleus. As a consequence these rats will behave like the cuneiform lesioned group displaying higher levels of anxiety and food neophobia than either sham lesioned control or PPTg lesioned rats.

5.2 Methods (a)

Animals

29 Lister hooded rats were used in this study. Body weights at time of surgery were between 298g and 360g. 5 rats received ibotenic acid lesions of the PPTg, 7 with NMDA lesions of the PPTg, 7 with ibotenic acid lesions of the cuneiform nucleus and 9 with sham lesions.

Behavioural Testing

Behavioural testing began 1 week after completion of surgery.

5.3 Results (a)

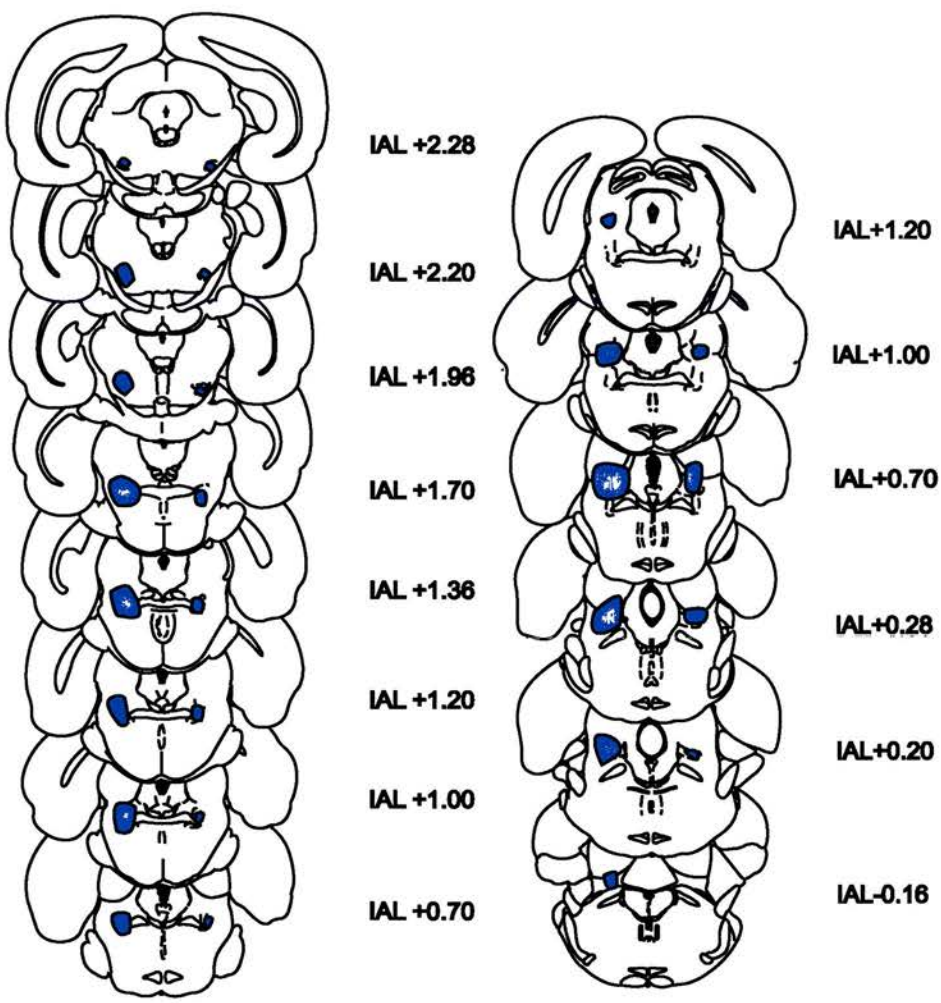
Histology

Figures 5.1 and 5.2 show the damage caused by the largest and smallest lesion of each type. Slides showed successful bilateral lesions of the PPTg in all rats in this group. All the cuneiform lesioned animals also had successful discrete bilateral lesions. The NMDA lesions were, as expected very large, spreading from areas near the front of PPTg to back beyond the cuneiform nucleus.

The extent of PPTg damage was assessed by counting diaphorase positive cells in the PPTg of all groups. Statistical analysis showed there were significantly fewer cells in the PPTg of both the ibotenic acid and NMDA lesioned than either of the other groups. The cuneiform and control groups did not differ significantly from each other showing that lesions of the cuneiform nucleus did not significantly

Figure 5.1 shows the extent of damage produced by the largest and smallest cuneiform and PPTg lesions.

Figure 5.1: Schematic showing the extent of damage produced by the largest and smallest cuneiform and PPTg lesions

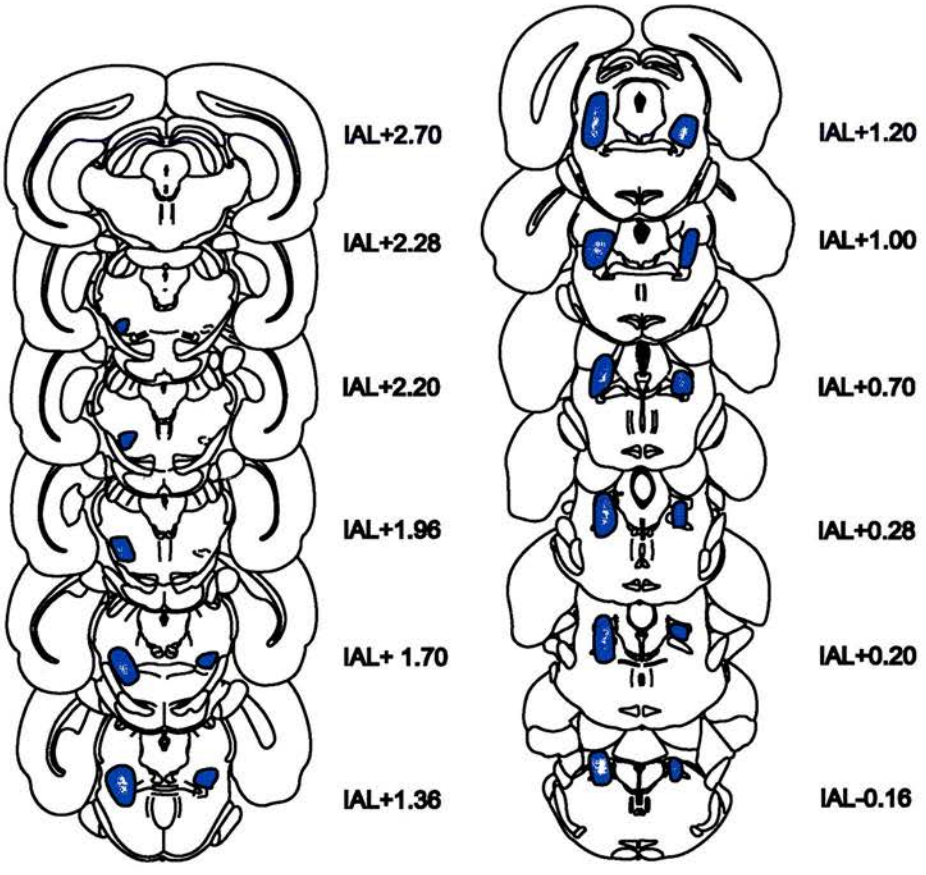


Largest (left) and smallest (right) PPTg lesions

Largest (left) and smallest (right) cuneiform lesions

Figure 5.2 shows the extent of damage produced by the largest and smallest NMDA lesions.

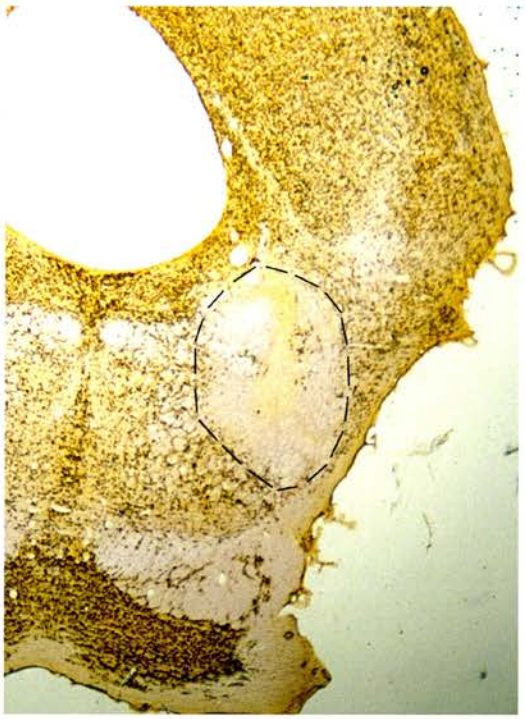
Figure 5.2: Schematic showing the extent of damage produced by the largest and smallest NMDA lesions



The left hemisphere shows the largest and the right hemisphere the smallest NMDA lesion.

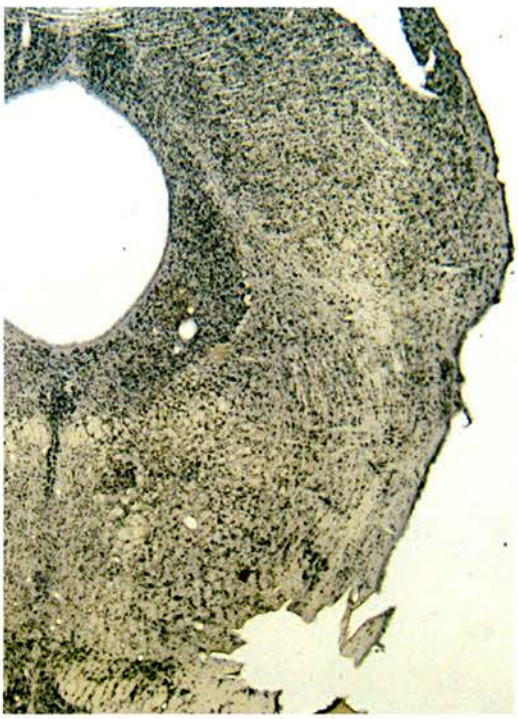
Photograph 5A shows lesion damage produced by an ibotenic acid lesion of PPTg in comparison to 5B a sham lesioned control. Photograph 5C shows a significant loss of diaphorase positive neurones in the PPTg in comparison to 5D a sham lesioned control. Ch6 neurones of LDTg remain intact in all cases.

A



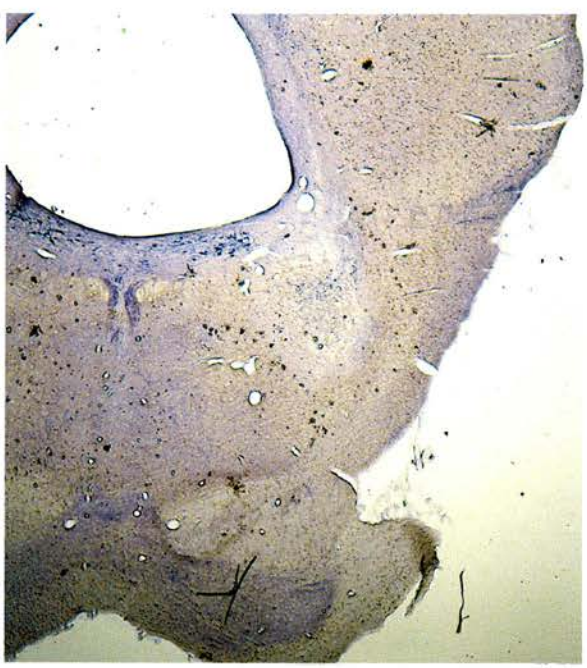
PPTg lesion, NeuN.
Dotted line indicates area of lesion damage.

B



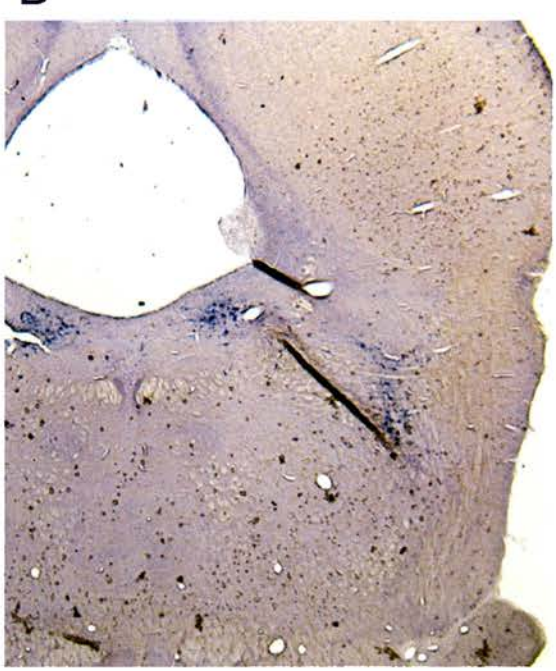
Control, NeuN.

C



PPTg lesion, diaphorase.
There is clear loss of diaphorase positive Ch5 neurones while the Ch6 group remain intact.

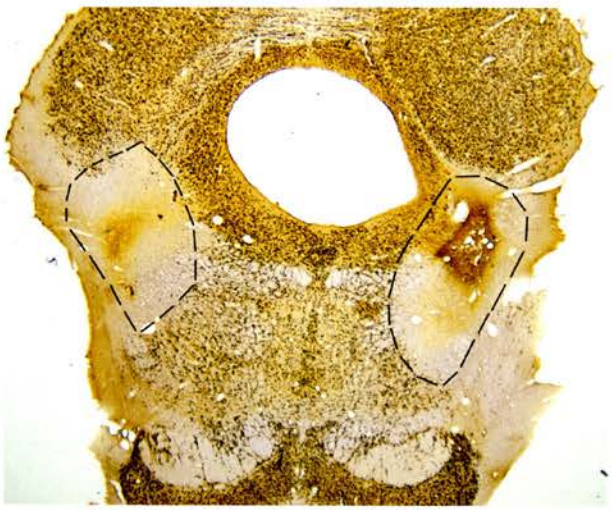
D



Control, diaphorase.

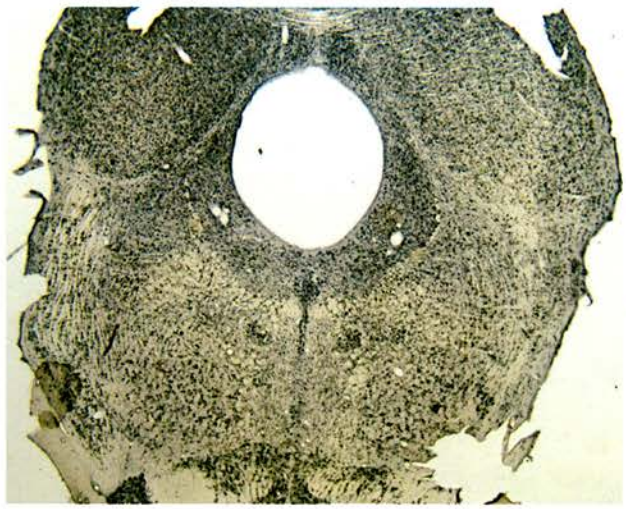
Photograph 5E indicates the extent of lesion damage produced by NMDA lesions of caudal PPTg. The lesion area clearly extends into cuneiform bilaterally. Photograph 5G indicates that this lesion also produced significant destruction of diaphorase positive neurones in PPTg when compared to 5H, a sham lesioned control.

E



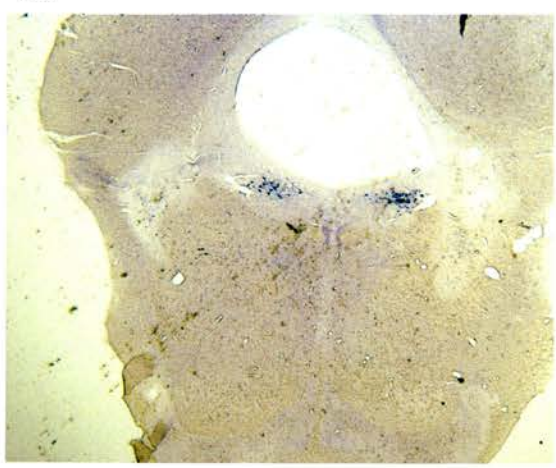
NMDA lesion, NeuN.
Dotted lines indicate extent of lesion area, encompassing the cuneiform nucleus and PPTg.

F



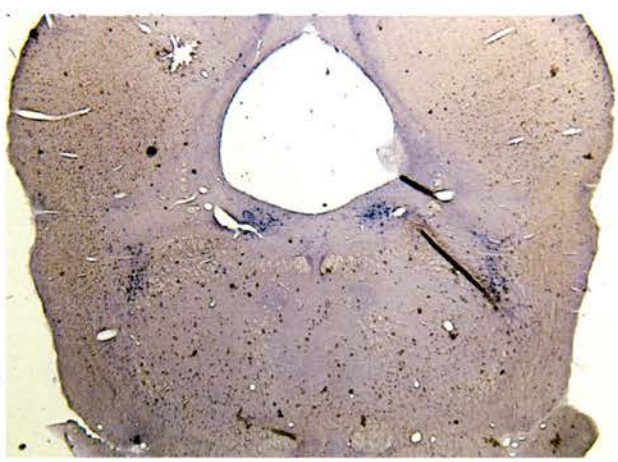
Control, NeuN

G



NMDA lesion, diaphorase.
There is significant bilateral loss of diaphorase positive Ch5 neurones in the PPTg.

H



Control, Diaphorase.

impinge into caudal PPTg. $F(3,23)=51.08$, $p<0.01$. These results are summarised in Figure 5.3. Ibotenic acid lesions produced, on average, 80% destruction of diaphorase positive cells in the PPTg and 77% in the caudal half specifically. The NMDA lesions caused, on average, 67% destruction of diaphorase cells throughout the extent of PPTg and 70% destruction in the caudal half as summarised in Figure 5.4 below.

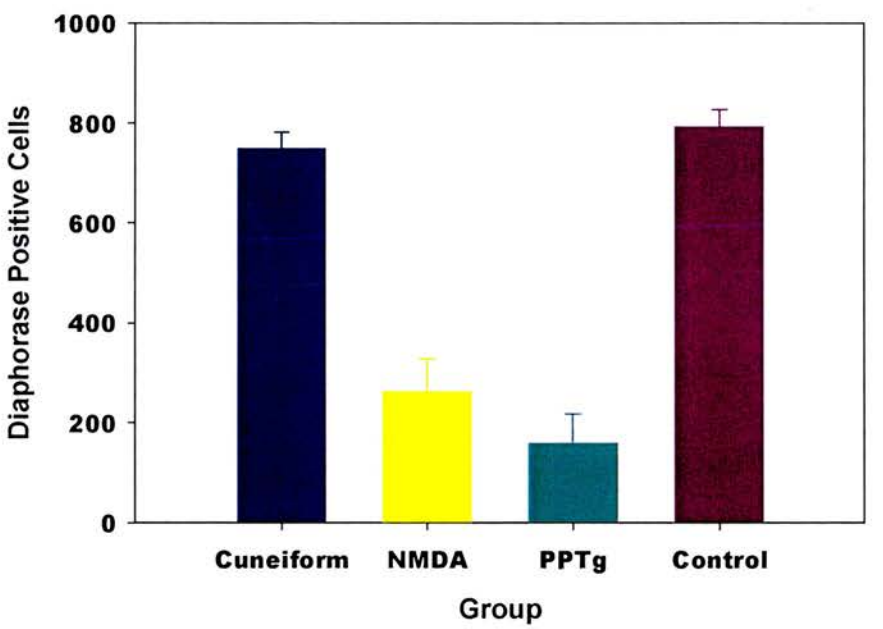


Figure 5.3: Mean number of diaphorase positive cells counted bilaterally in the PPTg of each of the 4 groups (+SE). There were significantly fewer cells in the PPTg of Ibotenic acid and NMDA lesioned groups than either cuneiform or sham lesioned control groups, $p<0.01$.

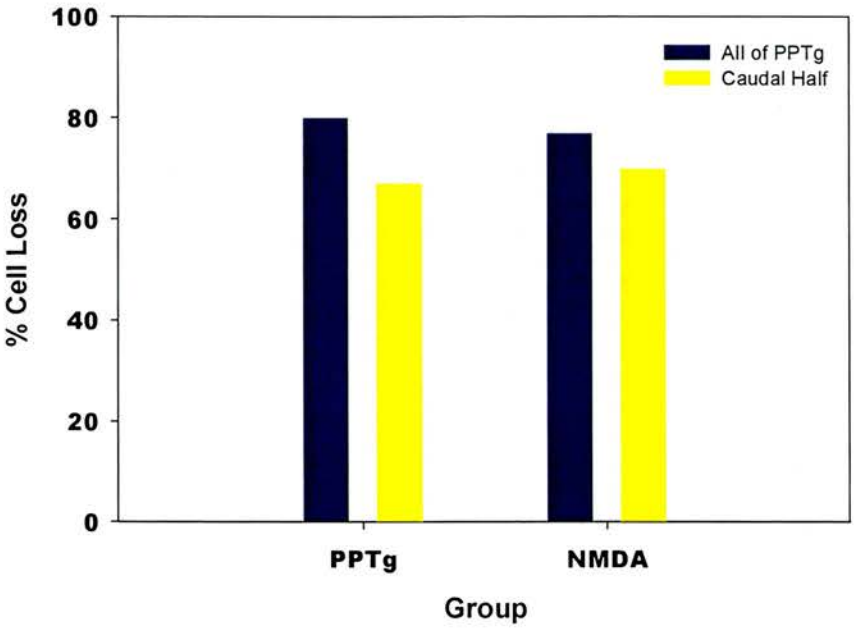


Figure 5.4: Mean percentage of bilateral diaphorase positive cell loss in the PPTg following the two different types of lesion.

Elevated Plusmaze

Anxiety in the elevated plusmaze was measured as per Podhorna and Franklin (1998) by the amount of time spent in the closed arms. The percentage of test time each group spent in the closed arms of the maze is shown in Figure 5.5. There were no significant differences between any of the groups in the time spent in the closed arms, $F(3,25)=0.71$, NS. Nor was there a significant effect of day $F(9,17)=1.55$, NS.

Since the traditional measure of anxiety in the elevated plusmaze is as a decrease in time spent in the open arms, this too was measured. Again there was neither a significant effect of group, $F(3,25)=0.44$, NS, nor a significant effect of day, $F(9,17)=1.25$, NS. These data are shown in Figure 5.6.

A measure was also made of the time taken to enter an open arm at the beginning of a test session. The results of these measurements are shown in Figure 5.7. Again no significant differences were found between any of the groups, $F(3,25)=1.18$, NS. However, the graph does indicate a trend for the cuneiform lesioned rats to show longer open arm latencies, especially after the first three days of testing. The PPTg lesioned animals appear to be quicker than any other group to enter the open arm on the first 4 test days. There was a significant effect of day, $F(9,17)=3.06$, $p<0.05$. The total number of open and closed arm entries made by the rats during each test session was also recorded. Figure 5.8 shows the combined total arm entries made by each of the groups over the test days. There was a significant effect of group, $F(3,25)=3.98$, $p<0.05$, and post hoc tests showed that the PPTg lesioned rats made significantly more arm entries than either the cuneiform or the control groups. None of the other three groups differed significantly from each other. There was no significant effect of day, $F(9,17)=0.85$, NS nor was there a significant day x group interaction, $F=0.99$, d.f.=27, NS.

Again unprotected head dips were examined as per Leri & Franklin (1998). There was no significant effect of group, $F(3,25)=0.55$, NS, but there was a significant effect of day, $F(9,17)=6.83$, $p<0.01$. This is summarised in Figure 5.9 below.

Rearing and grooming are both traditional measures of general activity, rather than anxiety, on the elevated plusmaze. There was no significant effect of group, $F(3,25)=0.36$ NS, and $F(3,25)=2.72$, NS, on either measure as summarised in Figures 5.10 and 5.11.

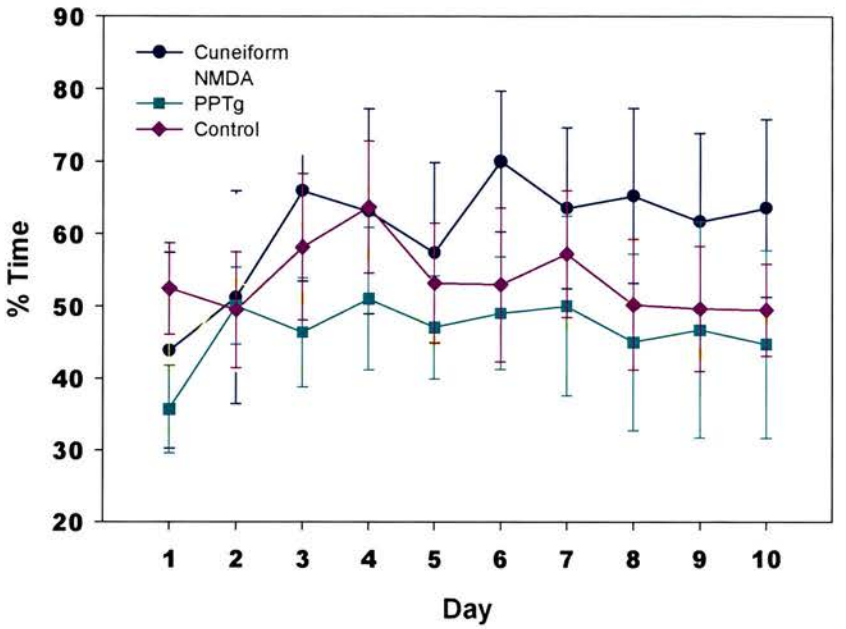


Figure 5.5: Mean percentage of test time the four groups spent on the closed arms of the plusmaze on each of the 10 test days (+/-SE). There was neither a significant effect of group nor day.

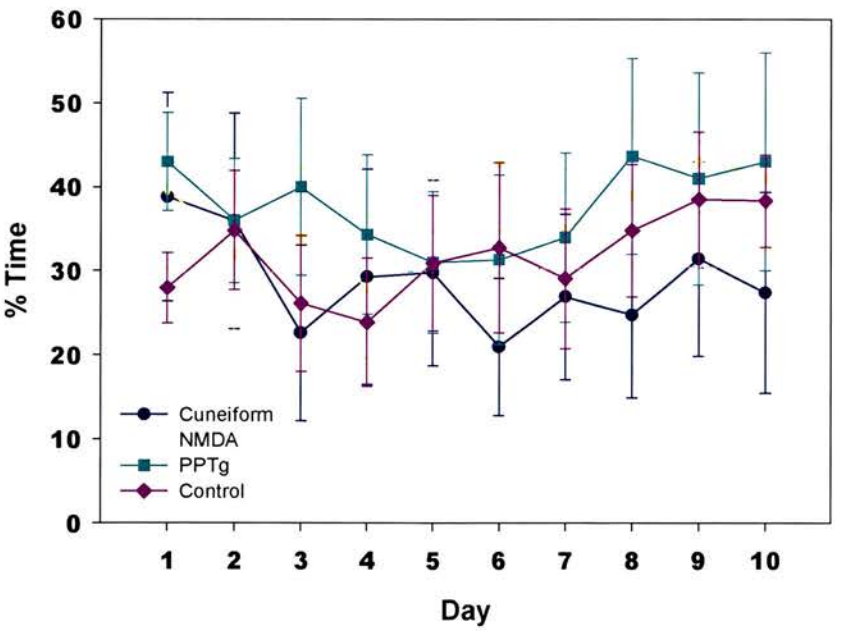


Figure 5.6: Mean percentage of test time the 4 groups spent on the open arms of the maze during each of the 10 test sessions (+/-SE). There was neither a significant effect of group nor of day.

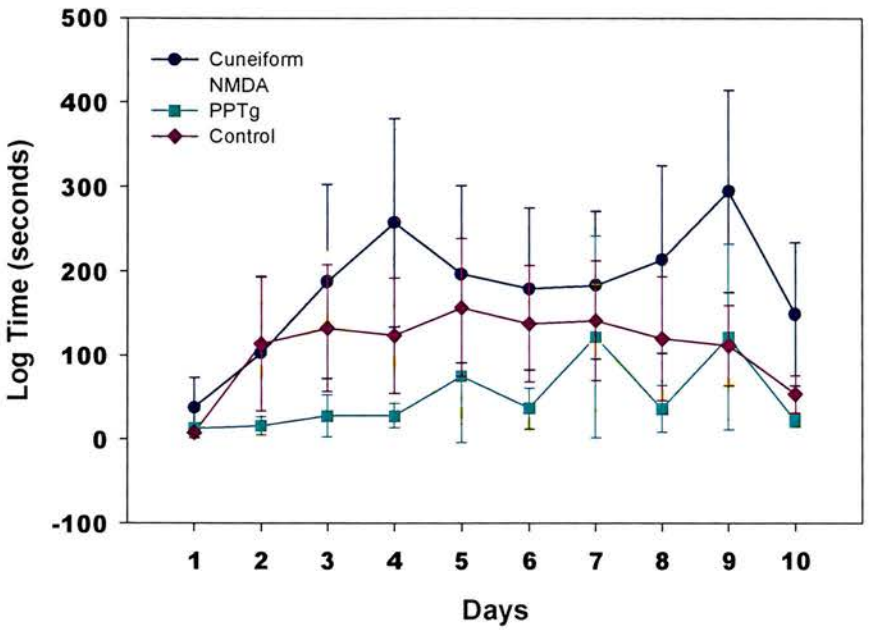


Figure 5.7: Mean latency to enter one of the open arms of the plusmaze at the start of each of the 10 test sessions (+/-SE). There was a significant effect of day, $p<0.05$, but not of group.

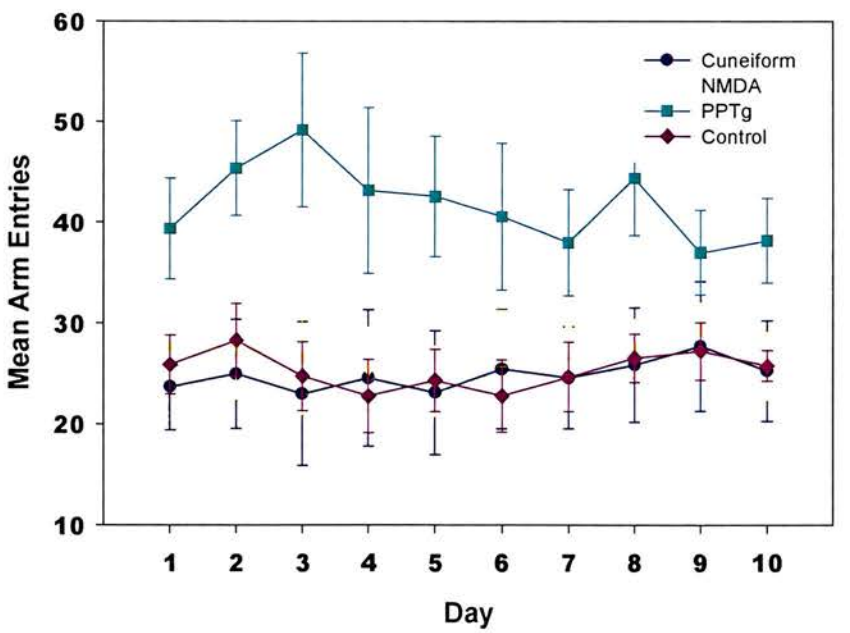


Figure 5.8: Total number of arm entries made on average by the 4 groups on each of the 10 test sessions (+/-SE). There was a significant effect of group, $p<0.05$ but not a significant effect of day.

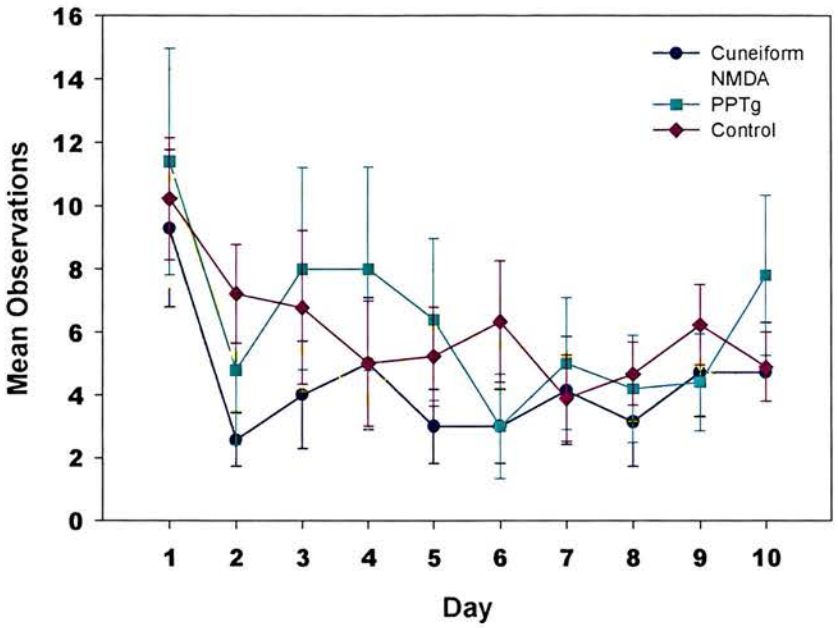


Figure 5.9: Mean number of head dips over the sides of the open arms of the plusmaze observed during each of the 10 test sessions (+/-SE). There was no significant effect of group, but there was a significant effect of day, $p<0.01$.

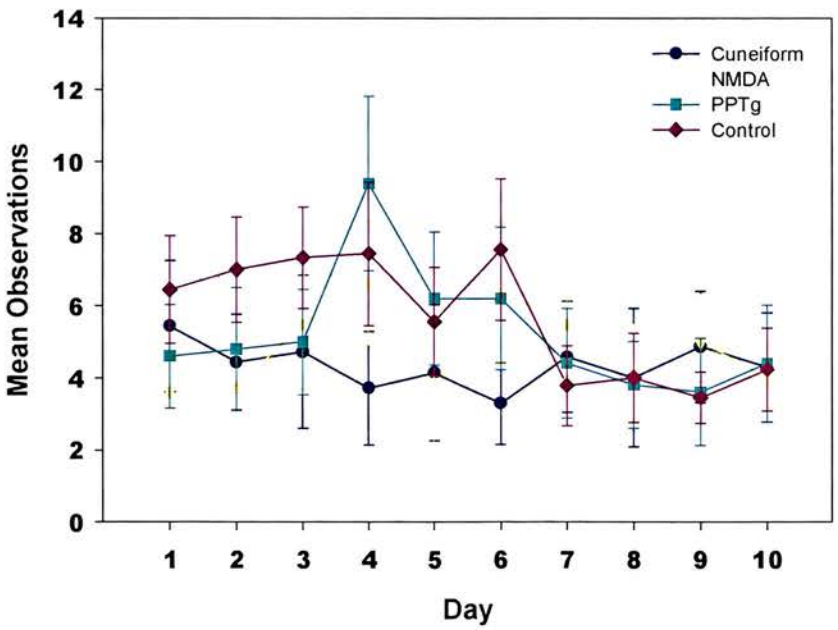


Figure 5.10: Mean number of displays of rearing behaviour observed in each of the four groups over the 10 test sessions (+/-SE). There was no significant effect of group or day.

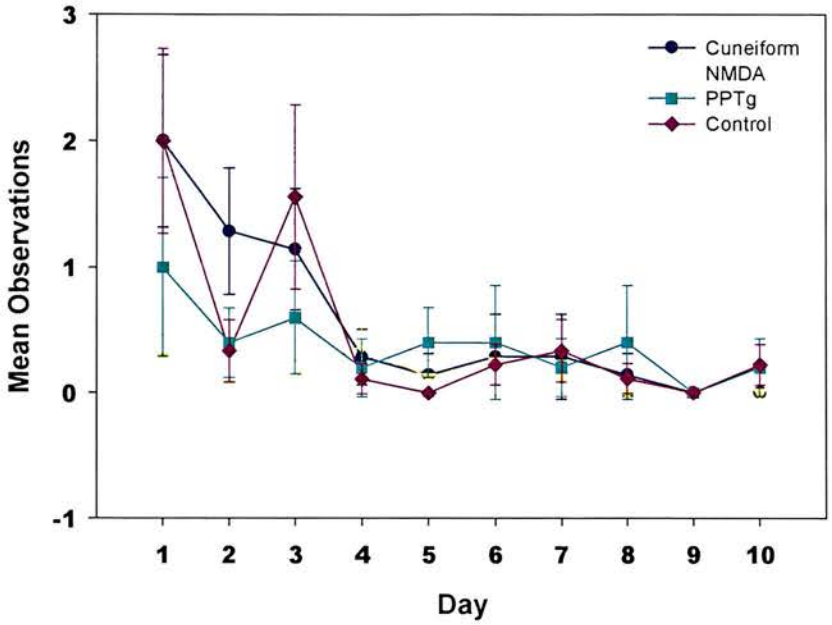


Figure 5.11: Mean number of displays of self grooming behaviour observed in each of the four groups over the 10 test sessions (+/-SE). There was no significant effect of group or day.

Food Neophobia

The food neophobia paradigm tests the rats reactions to a novel food presented in the centre of a novel open environment. Rats showing higher levels of anxiety take longer to make contact with and are less likely to eat the novel reward.

Figure 5.12 shows the percentage of the 10 minute test time the rats spent in the centre of the arena. Analysis showed that no group differed significantly from another, $F(3,24)=1.42$ NS.

Measures were also taken of time spent in contact with the outer wall of the test arena, a behaviour that indicates anxiety. Again there were no significant differences between groups, $F(3,24)=2.24$ NS, although the cuneiform lesioned

animals tended to spend more time in contact with the outer wall than any of the other rats, as is seen in Figure 5.13.

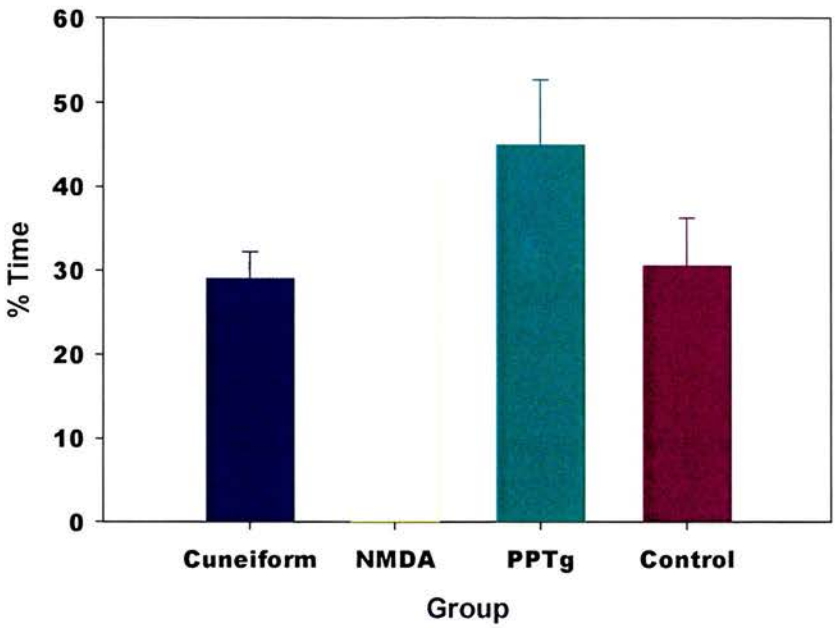


Figure 5.12: Mean percentage of total test time each of the 4 groups spent in the centre of the open arena (+SE). There were no significant between group differences.

As well as position in the arena, measures were taken of the rat's reaction to food. Figure 5.14 shows the percentage of total test time the animals spent eating. While the PPTg lesioned rats spent more time eating than the other groups, and the cuneiform lesioned rats the least, the difference was not significant, $F(3,24)=1.23$, NS. In this study cheese was the novel desirable food placed in the middle of the arena along with familiar lab chow.

Figure 5.15 shows there were clear differences between the groups in weight of cheese eaten. This difference was significant, $F(3,24)=3.46$ $p<0.05$. This suggests that the PPTg and NMDA lesioned rats were significantly less neophobic than

control or cuneiform lesioned rats. However post hoc tests failed to find and significant group differences.

Additionally, in comparison to the previous food neophobia experiment there was a reduction in the disinhibited eating response observed in PPTg lesioned rats. The PPTg lesioned rats are no more likely to consume chow than either of the other two lesion groups. Indeed it was the control rats that were most likely to eat chow.

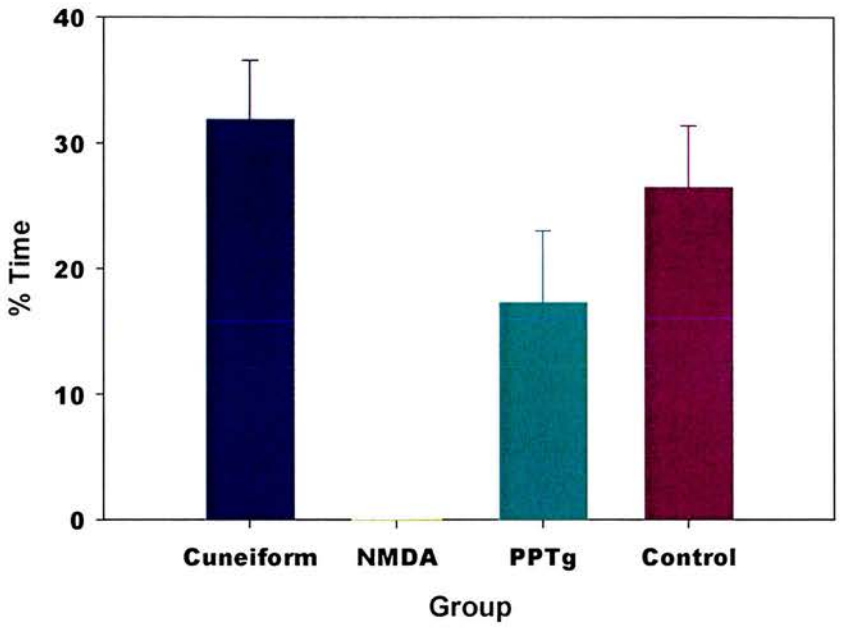


Figure 5.13: Mean percentage of the total test time groups spent in contact with the sides of the open arena (+SE). There were no significant differences between groups.

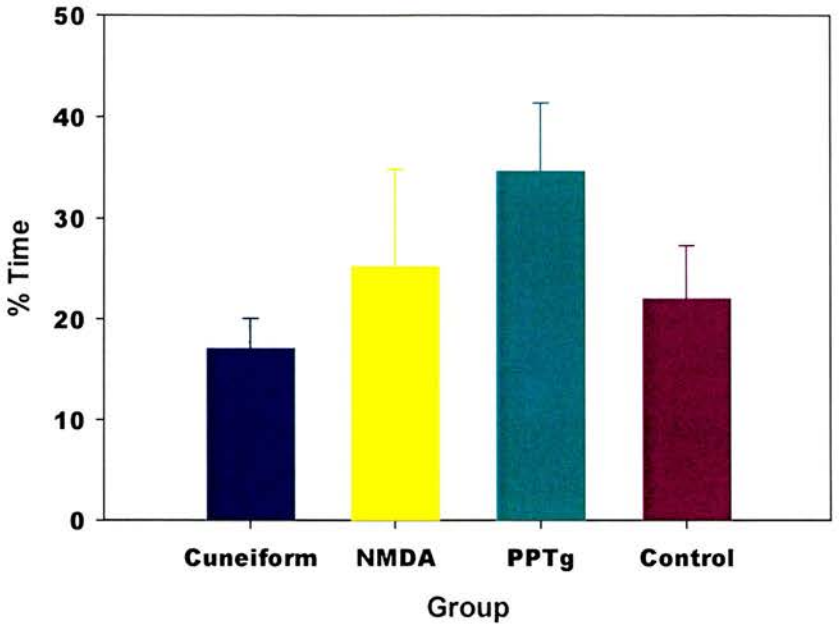


Figure 5.14: Mean percentage of the total test time each of the 4 groups spent eating cheese and chow (+SE). There were no significant differences between groups.

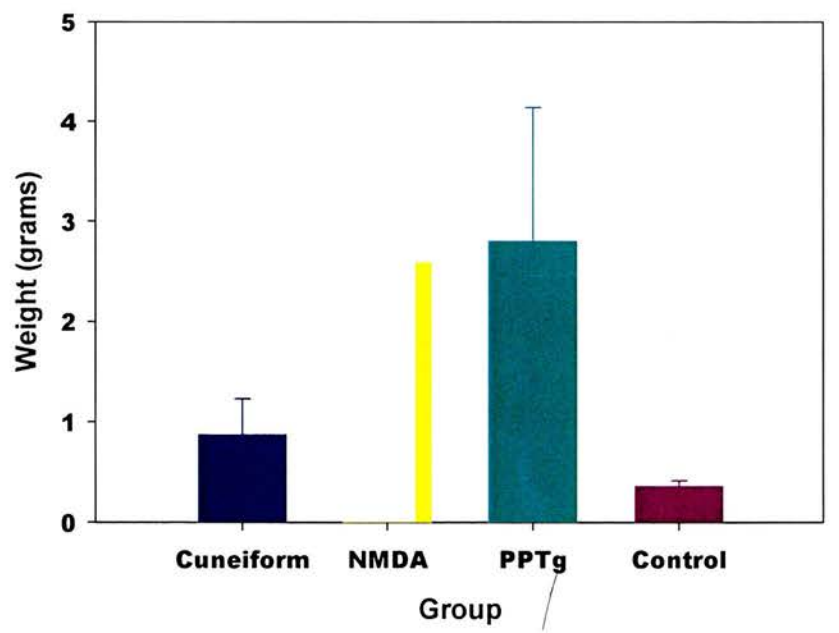


Figure 5.15: Mean weight of cheese (g) consumed by each of the four groups during the test session (+SE). There was a significant effect of group, $p<0.05$.

Table 5.1: Shows the percentage of rats from each group, making contact with and eating both cheese and chow.

	Contacting (%)		Eating (%)	
	Cheese	Chow	Cheese	Chow
PPTg	100	100	80	40
NMDA	71	100	71	43
Cuneiform	100	100	100	43
Control	100	100	89	67

Openfield

Behaviour was also measured in an open field, comparing the groups' tendency to venture into the centre or stay near the edge of the large open arena. Figure 5.16 shows the percentage of the 10 minute test time rats spent in the centre of the field. The PPTg lesioned rats spent more time here than either the NMDA or the cuneiform lesioned groups. This difference was significant at $F(3,24)=3.23$ $p<0.05$. The measure of thigmotaxic behaviour backs up these findings and is summarised in Figure 5.17. The NMDA and cuneiform lesioned groups spent significantly more time in contact with the outer walls of the field than either PPTg or control rats. $F(3,24)=3.26$ $p<0.05$. However, again post hoc tests failed to find differences between any of the groups.

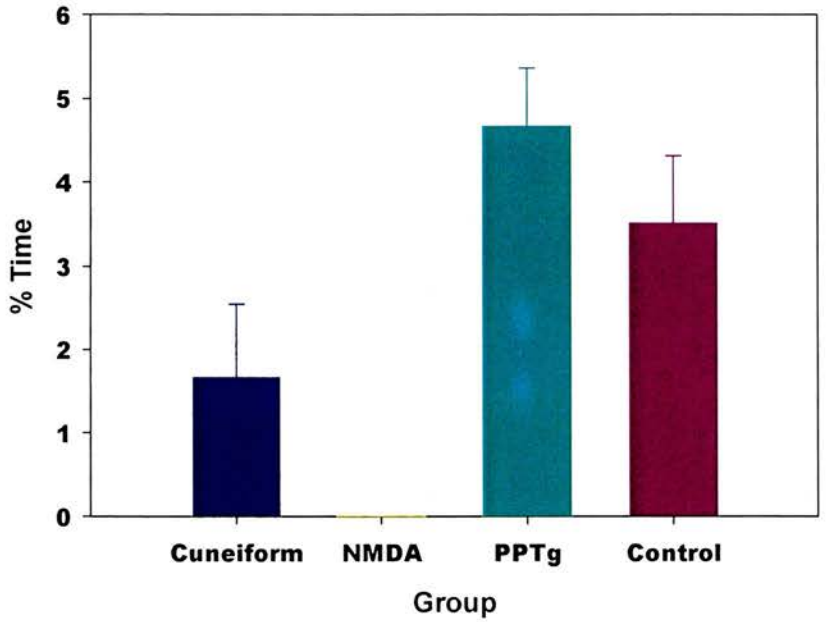


Figure 5.16: Mean percentage of total test time each of the 4 groups spent in the centre portion of the open field (+SE). There was a significant effect of group, $p<0.05$.

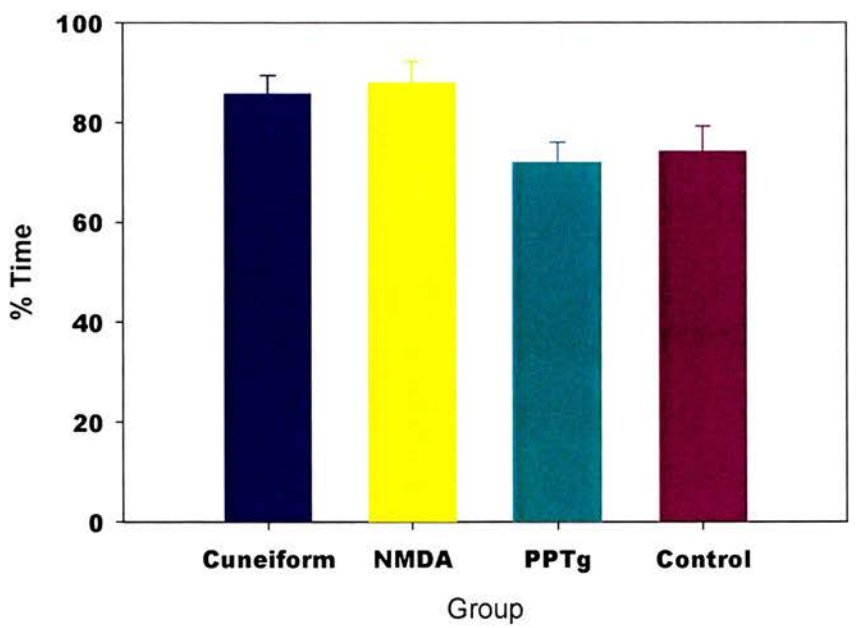


Figure 5.17: Mean percentage of total test time each of the 4 groups sepnt in contact of the walls of the open field (+SE). There was a significant effect of group $p<0.05$.

Chapter 5b

A comparison of 20% sucrose consumption over 24 hours free access.

5.4 Introduction (b)

It has already been shown, in chapter 4, that while rats with lesions of the PPTg overconsume 20% sucrose solution they are still able to modulate energy intake, and compensate for this over consumption by reducing consumption of other energy sources. This study aimed to compare the effects of the NMDA lesion given that earlier studies showed that, damage to the cuneiform nucleus does not disrupt sucrose consumption.

5.5 Methods (b)

Animals

24 rats were used in this study, 5 with ibotenic acid lesions of the PPTg, 5 with ibotenic acid lesions of the cuneiform nucleus, 7 with NMDA lesions producing damage to caudal PPTg and cuneiform and 7 sham lesioned control rats. All had previously been used in the tests described earlier in this chapter.

Behaviour

Rats were given 24 hours free access to 20% sucrose solution in their home cage. Grams consumed were recorded.

5.6 Results (b)

One way analysis of variance revealed a significant effect of group, $F(3,20)=3.43$ $p<0.05$. Data in Figure 5.18 suggests that NMDA and PPTg lesioned rats consumed more sucrose than control and cuneiform groups. However post hoc tests showed that only the difference between NMDA and controls was significant.

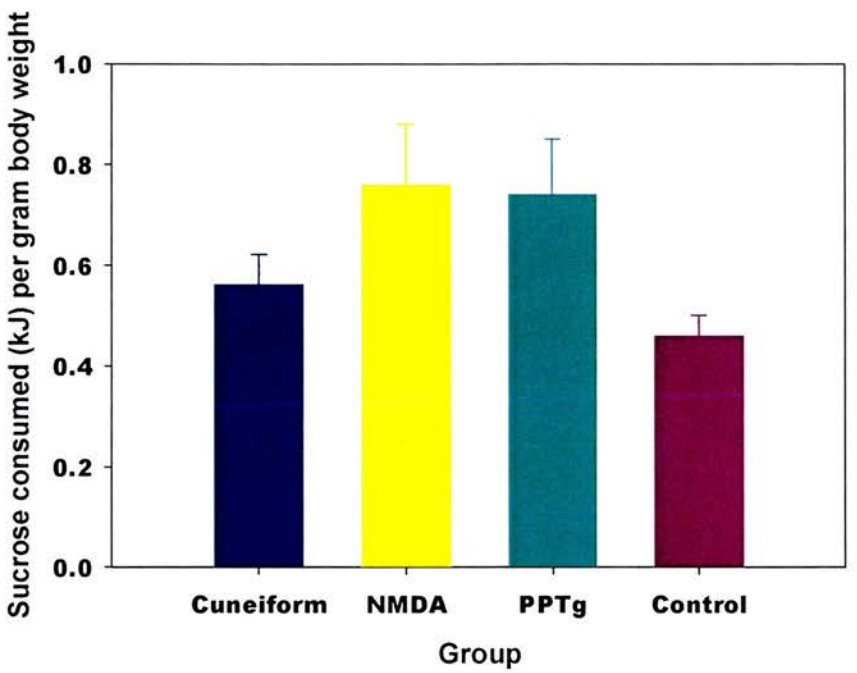


Figure 5.18: Average amount of sucrose solution consumed (kJ per gram of body weight) by each of the 4 groups over a 24 hour period (+SE). There was a significant effect of group. Post hoc tests revealed that the difference between NMDA and sham lesioned control rats was significant.

5.7 Discussion

The results of this study do not replicate Franklin's findings that NMDA lesions of caudal PPTg produce increased anxiety, as measured in the elevated plus maze. Nor do they replicate our previous findings, which found that lesions of the cuneiform nucleus decrease open arm activity, while ibotenate lesions of the PPTg increase time spent on the open arms.

There were no significant effect of group on measures of open and closed arm time or open arm entry latency in the elevated plusmaze. Nor were there any differences in the risk assessment activities including unprotected head dips, rearing and self grooming.

The only significant effect of group that was apparent on the elevated plusmaze was in total number of arm entries. Ibotenic acid lesioned PPTg rats made significantly more arm entries than any other group, which is in contrast to Podhorna & Franklin's (1998) findings that PPTg lesioned rats made significantly fewer arm entries than control rats.

In the food neophobia test too, analysis did not reveal any differences between the four groups. There were no changes in thigmotaxic behaviour or time spent eating. There was a significant group difference in weight of cheese consumed and Figure 5.15 indicates that NMDA and PPTg lesioned rats consumed more than cuneiform or control groups, however high variance prevented post hoc tests being significant.

There was a notable difference in the behaviour of PPTg lesioned rats in this test compared to those in the previous study. There was no disinhibited consumption

of chow along with cheese, which possibly reflects a change in incentive motivation brought about by food deprivation.

In the openfield there were significant effects of group both in terms of thigmotaxic behaviour and time spent in the centre of the field. Looking at Figures 5.16 & 5.17 it seems clear that PPTg and control rats were displaying fewer anxiety type behaviours than the other two groups. However, the difference was not significant in post hoc tests.

When we measured sucrose consumption we again found a significant effect of group and Figure 5.18 indicates that PPTg and NMDA lesioned rats consumed more sucrose than cuneiforms or controls. However in post hoc tests only the difference between NMDA and control rats is significant.

It is possible that these unexpected results are produced due to elevated baseline anxiety in all groups since they had only one, as opposed to two weeks recovery post surgery. This hypothesis is supported when data from this and the previous study are compared. The control rats from the previous study spent less time in the closed arm of the maze than did the control rats and the NMDA lesioned rats in the current study. Also the PPTg lesioned rats in the previous study spent less time in the closed arms than did either the current PPTg or the NMDA lesioned rats. In the cuneiform lesioned rats there was not a difference between behaviour in this and the previous study. This too supports the theory of recovery time being an influencing factor, while both the NMDA and PPTg lesioned rats go through two surgeries one week apart, the cuneiform group receive bilateral lesions in one surgery and so had longer to recover before testing began.

Another factor, which could have prevented some trends from being significant, is the high levels of within group variance. This is the result of both, small group sizes and the nature of the tests used. Since none of the tasks involve training and measures are of unconditioned reactions individual differences are likely to be large.

Significant differences in anxiety levels, between groups, started to become apparent during the latter stages of testing, though group size and individual variance prevented these from being significant in post hoc tests. In the openfield PPTg and control rats showed less thigmotaxic behaviour than NMDA and cuneiform groups. The results of this test support our hypothesis that it is the damage to the cuneiform nucleus produced by the NMDA lesions and not damage to the PPTg that results in heightened anxiety. These results also indicate that baseline anxiety, heightened by the trauma of surgery, dissipates over time.

Another interesting finding came from the sucrose study. While limitations of group size and therefore high levels of variance prevented all differences from being significant it is clear that those rats with Franklin's NMDA lesions look more like the other PPTg group than cuneiform or control rats when presented with free access to highly rewarding sucrose solution.

Chapter 6

A comparison of the effects of excitotoxic damage to the PPTg and cuneiform nuclei on, performance in behavioural tests sensitive to anxiety and consumption of sucrose and quinine solutions.

6.1 Introduction

In the previous study there was no evidence of the predicted increase in anxiety, as measured by the elevated plusmaze and food neophobia paradigms, though rats with damage to the cuneiform nucleus did show heightened anxiety in the openfield. It was suggested that this unexpected result may have been due to the short recovery time between surgery and testing. The previous experiment was therefore repeated with 4 new groups of rats, bearing the same lesions described in the previous study, but leaving two weeks recovery time between completion of surgery and the beginning of testing.

It also appeared in the last study, that although differences between groups became more apparent in later stages of testing these were not significant because of high levels of variance which occurs due to the spontaneous nature of behaviour measured in the test. Therefore, in this study group sizes were increased to counteract variance produced by individual differences in control group behaviour.

Additionally to complement the sucrose consumption data it was decided also to compare the different groups reactions to a weakly aversive quinine solution. It was predicted that, given the heightened levels of anxiety and food neophobia seen previously in rats bearing cuneiform lesions, this group may be more sensitive than other rats to this mildly aversive drink and therefore consume less.

Hypothesis

It is predicted that rats with NMDA lesions of the PPTg, known to produce significant neural loss within the boundaries of the cuneiform nucleus, will behave like cuneiform lesioned rats on the elevated plusmaze, in the food neophobia and openfield tests. Displaying higher levels of anxiety and food neophobia than either PPTg lesioned or sham lesioned control rats.

It is also hypothesised, given the heightened levels of anxiety and food neophobia observed previously in cuneiform lesioned rats, that this group will be more sensitive to, and therefore consume less of, a weakly aversive quinine solution than PPTg lesioned or sham lesioned control rats.

6.2 Methods (a)

Animals

68 Lister hooded rats were used in this experiment; 19 had bilateral ibotenic acid lesions of the cuneiform nucleus, 16 bilateral ibotenic acid lesions of the PPTg, 15 with bilateral NMDA lesions causing damage to caudal PPTg and cuneiform and 18 were sham lesioned controls. Weights at time of initial surgery varied between 280 – 400g.

Behavioural Testing

Testing began 2 weeks after completion of surgery

6.3 Results (a)

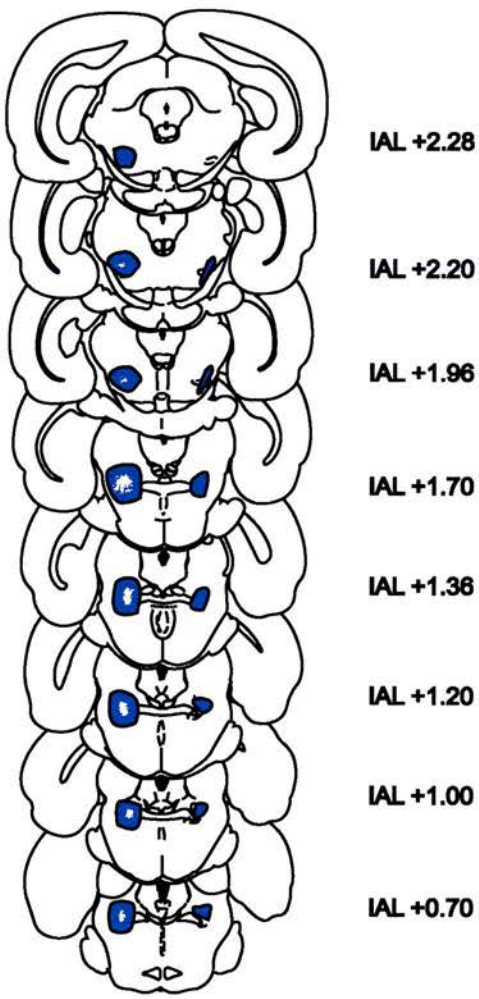
Histology

Following histological examination data from several rats was excluded from further analysis. 3 PPTg and 5 cuneiform lesioned rats were excluded because lesions extended significantly beyond the bounds of the target structure. In the case of the PPTg lesions this included damage to the cuneiform nucleus and LDTg. In the cuneiform lesioned group this included damage to caudal PPTg and or inferior colliculus. One further rat from the cuneiform lesioned group was excluded due to problems with histological processing. One rat with an NMDA lesion was excluded because there was no lesion damage to caudal PPTg.

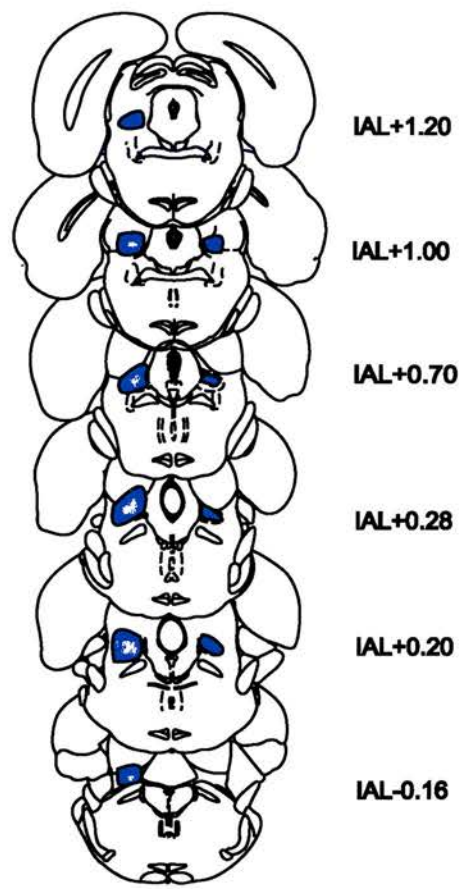
Therefore data from 58 rats is reported in this study, 13 with lesions of the cuneiform nucleus, 13 with ibotenic acid lesions of the PPTg, 14 with NMDA lesions of caudal PPTg and 18 sham lesioned control rats.

Figure 6.1 shows the extent of damage produced by the largest and smallest PPTg and cuneiform lesions.

Figure 6.1: Schematic showing the extent of damage produced by the largest and smallest PPTg and cuneiform lesions.



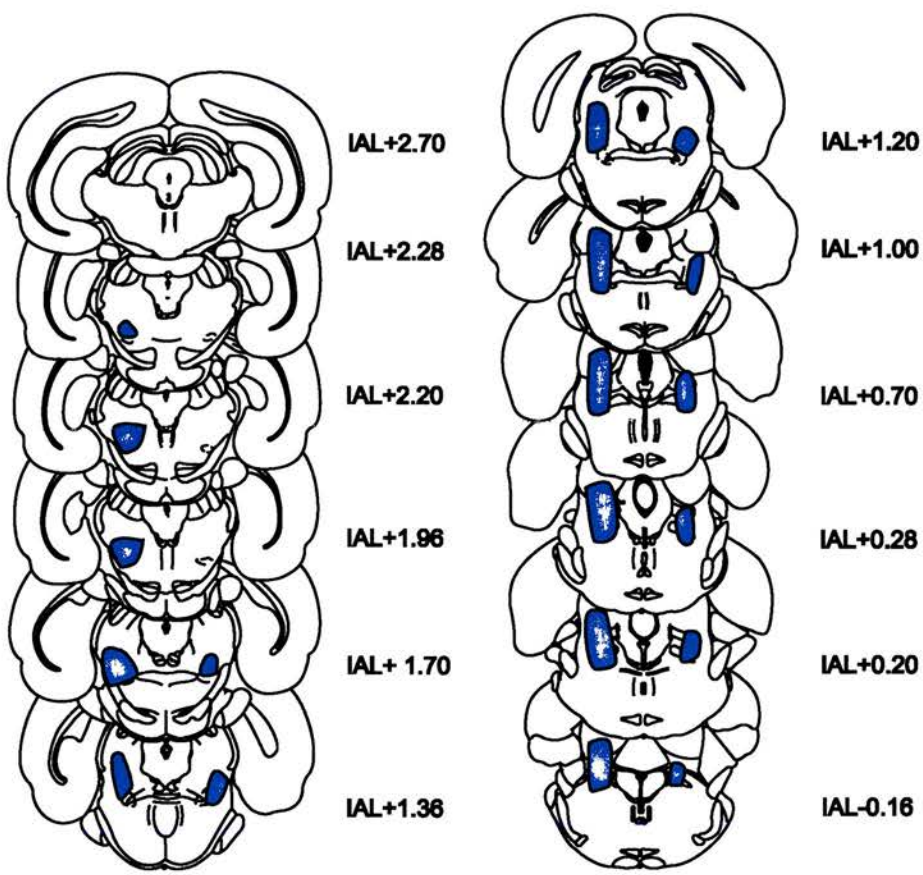
Largest (left) and smallest (right) PPTg lesions



Largest (left) and smallest (right) cuneiform lesions

Figure 6.2 shows the extent of the damage produced by the largets and smallest NMDA lesions.

Figure 6.2: Schematic showing the extent of damage produced by the largest and smallest NMDA lesions



The left hemisphere shows the largest and the right hemisphere the smallest NMDA lesion.

Figures 6.1 & 6.2 are schematics showing the largest and smallest lesion of each type.

Elevated Plusmaze

According to several validation studies, the most accurate measure of anxiety in the elevated plusmaze is percentage time spent in the open arms. In this experiment there was a significant effect of group, $F(3,54)=9.62$ $p<0.01$ and a significant effect of day, $F(9,46)=8.291$ $p<0.01$ and a significant day x group interaction, $F(27,144)=1.61$ $p<0.05$. Planned comparisons were carried out to compare groups on each individual day. There were no significant differences between groups on day 1. On day 2 PPTg lesioned rats spent more time on the open arms than any of the other groups ($p<0.05$). On day 3 PPTg and control groups spent more time on the open arm than the cuneiform lesioned rats ($p<0.05$), The PPTg lesioned rats also spent significantly more time on the open arms than the NMDA group ($p<0.01$). On days 4-7 & 9-10 PPTg and control rats spent significantly longer on the open arms than either NMDA or cuneiform lesioned groups ($p<0.05$). On day 8 Cuneiform and NMDA lesioned groups again spent less time on the open arms than PPTg lesioned groups ($p<0.01$). NMDA lesioned rats also spent significantly less time there than controls ($p<0.01$). These findings are summarised in Figure 6.3 below.

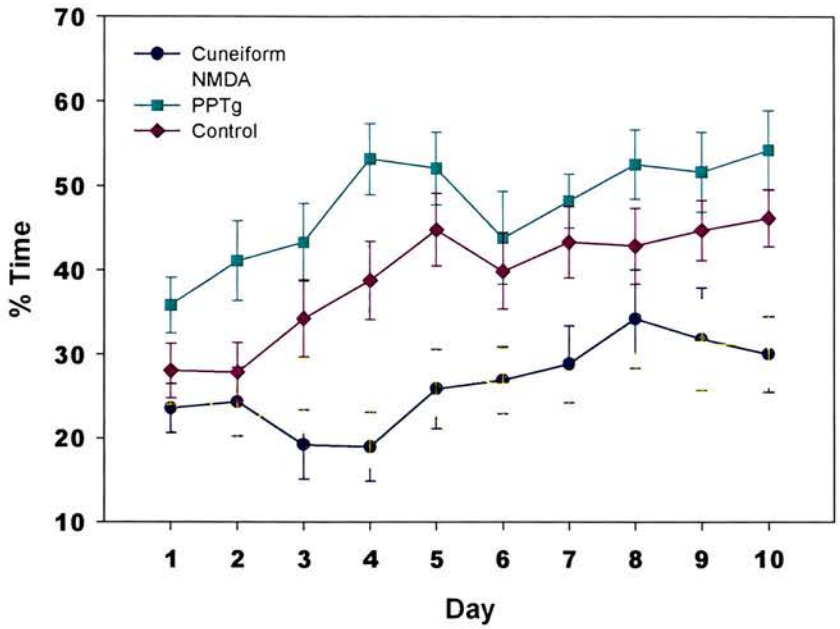


Figure 6.3: Mean percentage of test time each of the 4 groups spent on the open arms of the maze on each of the 10 test days (+/-SE). There was a significant effect of group, $p<0.01$, a significant effect of day, $p<0.01$ and a significant day x group interaction, $p<0.05$.

Figure 6.4 shows the mean percentage of test time each group spent in the closed arm of the maze over the 10 days of testing. Statistical analysis revealed a significant effect of group; $F(3,54)=9.18$ $p<0.01$, and a significant effect of day; $F(9,46)=5.21$ $p<0.01$. There was also a significant day x group interaction, $F(27,144)=2.03$ $p<0.05$. Planned comparisons were used to compare groups on individual days. On day one there were no significant differences between groups. On day 2 PPTg lesioned rats spent significantly less time in the closed arms than any of the other groups ($p<0.05$). On days 3 and 4 cuneiform and NMDA lesioned rats spent significantly more time in the closed arms than the PPTg lesioned group ($p<0.05$), cuneiform lesioned rats also spent more time in the closed arms than

controls ($p<0.05$). On days 5-7, 9 and 10 cuneiform and NMDA lesioned rats spent significantly longer in the closed arm than PPTg and control groups ($p<0.05$). On day 8 PPTg lesioned rats spent less time in the closed arms than NMDA or cuneiform groups ($p<0.05$), control rats spent less time in the closed arm than NMDA lesioned rats ($p<0.01$).

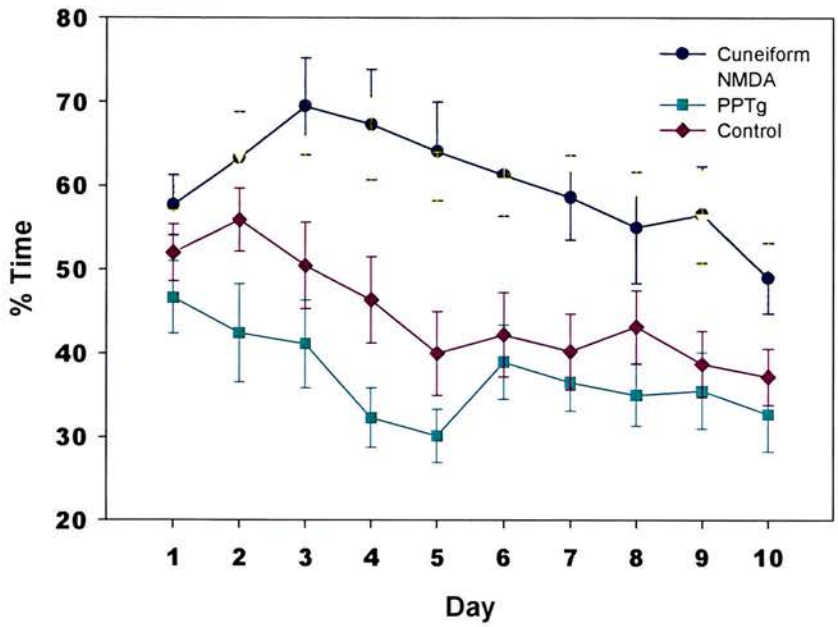


Figure 6.4: Mean percentage of test time the 4 groups spent on the closed arms of the maze during each of the 10 test sessions (+/-SE). There was a significant effect of group, $p<0.01$ and a significant effect of day, $p<0.01$, as well as a significant day x group interaction, $p<0.05$.

Figure 6.5 shows the latency of rats to enter one of the open arms of the maze after the start of a test session. The graph indicates a trend for the NMDA and cuneiform lesioned groups towards longer arm entry latencies than the PPTg or control groups. A repeated measures ANOVA revealed there was a significant effect of group, $F(3,54)=4.34$ $p<0.01$, but no significant effect of day, $F(9,46)=1.57$ NS.

Post hoc tests revealed however that only the NMDA and control groups differed significantly with NMDA lesioned rats taking significantly longer to make an initial open arm entry than controls.

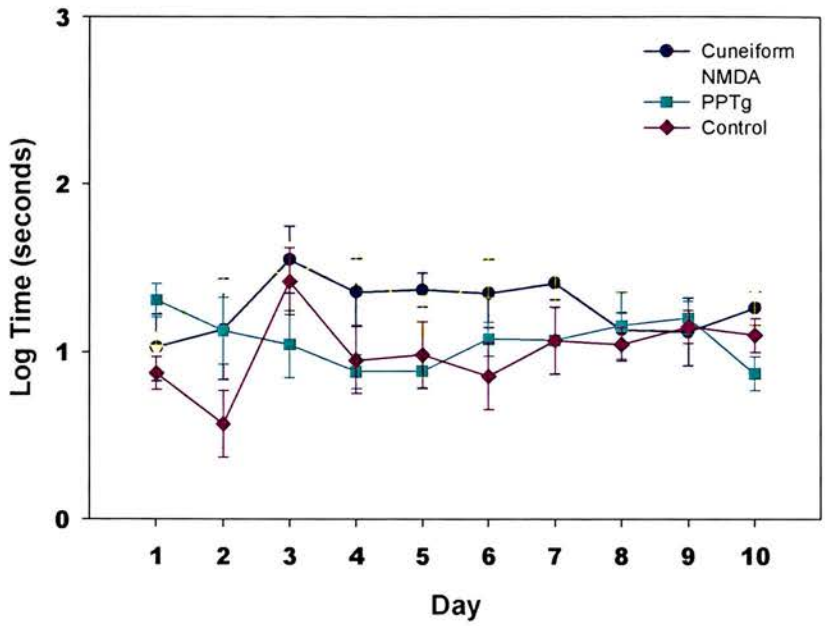


Figure 6.5: Mean latency to enter an open arm at the start of each of the 10 test sessions (+/-SE). There was a significant effect of group, p<0.01, but no significant effect of day and no day by group interaction.

Examination of closed arm entry latency, as shown in Figure 6.6, there was a significant effect of group, $F(3,54)=4.64$ $p<0.01$ and a significant effect of day, $F(9,46)=17.22$ $p<0.01$, but no interaction, $F(27,144)=1.21$, NS. Post hoc tests showed that both NMDA and cuneiform groups were quicker to enter the closed arms of the maze than sham lesioned controls at the start of a test session.

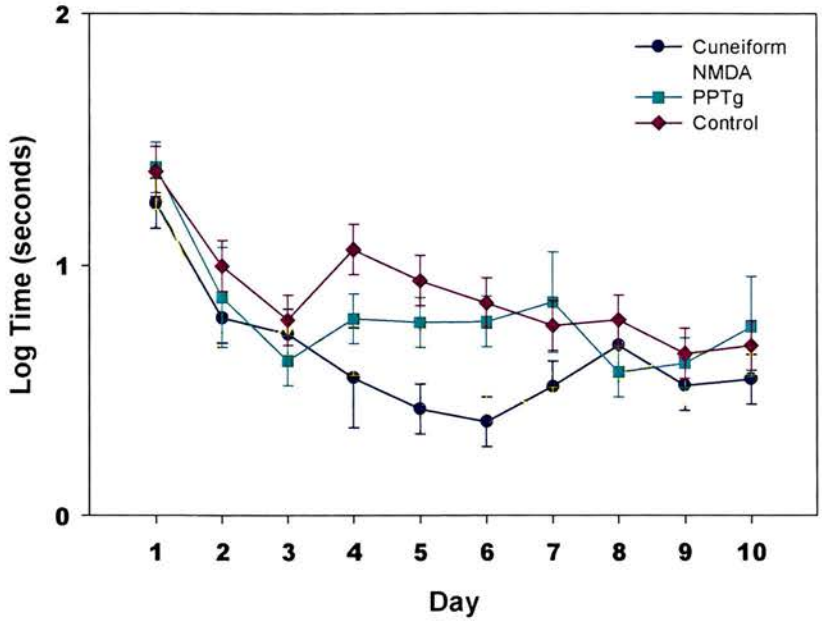


Figure 6.6: Mean latency to enter a closed arm of the maze at the start of each of the 10 test sessions (+/-SE). There was a significant effect of group, p<0.01 and day p<0.01 but no significant day x group interaction.

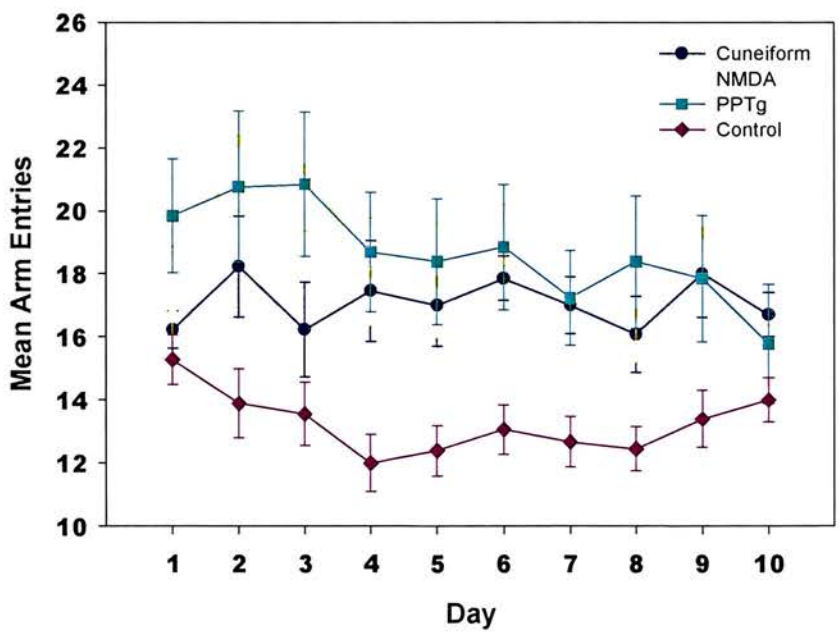


Figure 6.7: Mean number of entries into the closed arms of the plusmaze made by the 4 groups on each of the 10 test days (+/-SE). There was a significant effect of group, p<0.01 and day, p<0.05, but no significant day x group interaction.

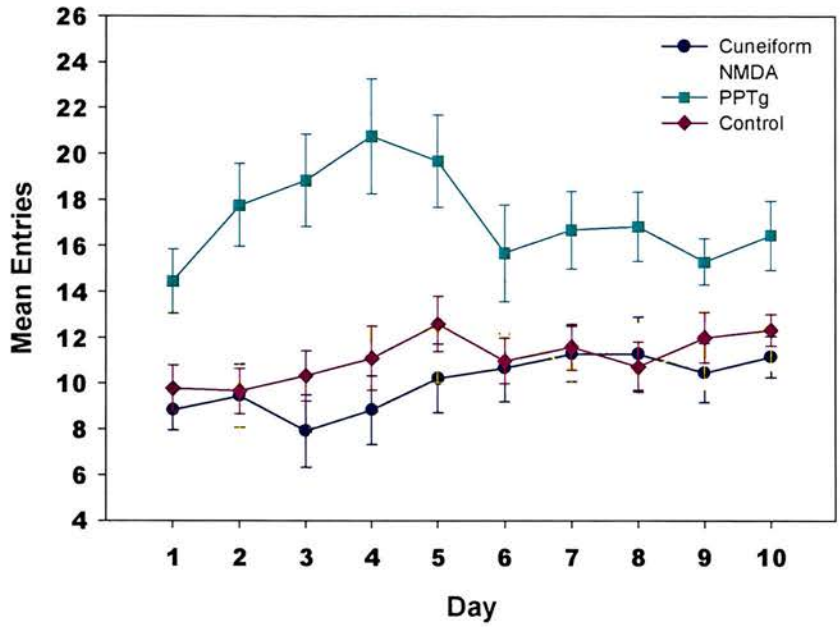


Figure 6.8 Mean number of entries made into the open arms of the plus maze by the 4 groups on each of the 10 test days (+/-SE). There was a significant effect of group, p<0.01, but no significant effect of day. Post hoc tests revealed that PPTg lesioned rats made significantly more open arm entries than any of the other groups.

Figures 6.7, 6.8 and 6.9 show the mean number of arm entries made by the different groups over the ten day test period. Figure 6.7 shows the number of entries made into the closed arms during the ten, ten minute test sessions. Analysis using a repeated measures ANOVA showed a significant effect of group, $F(3,54)=6.21$, $p<0.01$, and a significant effect of day $F(9,46)=3.41$, $p<0.05$. There was no day x group interaction, $F(27,144)=1.43$, NS. Post hoc comparisons revealed that PPTg and NMDA lesioned rats made significantly more closed arm entries than did cuneiform rats. NMDA rats also made significantly more closed arm entries than controls. Figure 6.8 shows the total number of open arm entries made. Again a repeated measures ANOVA indicated a significant effect of group, $F(3,54)=7.25$ $p<0.01$, but no significant effect of day, $F(9,46)=2.01$, NS. Post hoc comparisons revealed that

the PPTg lesioned rats made significantly more open arm entries than any of the other groups.

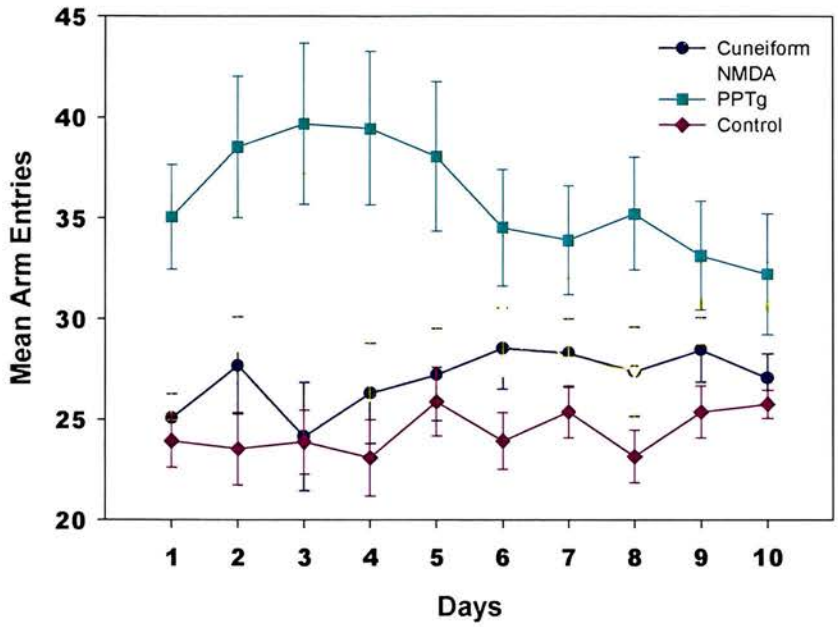


Figure 6.9: Total number of arm entries made on average by the 4 groups over each of the 10 test sessions (+/-SE). There was a significant effect of group, p<0.01 but no significant effect of day. Post hoc tests revealed that PPtg lesioned rats made significantly more arm entries than cuneiform or sham lesioned control rats.

Food Neophobia

In the food neophobia test of anxiety measures were taken of the rats position in the field, in the centre or at the side, as well as latency to make contact with the food and time spent eating. Figure 6.10 shows the average amount of test time rats spent in the centre portion of the field. Analysis using a one-way ANOVA showed a significant main effect of group, $F(3,54)=19.95$ $p<0.01$. Post hoc comparisons indicated that PPTg and NMDA lesioned rats spent significantly longer in the centre of the field than either cuneiform or control rats. Figure 6.10 also shows the levels of thigmotaxic behaviour displayed by each of the four groups. Analysis again showed

a significant main effect of group, $F(3,54)=12.16$ $p<0.01$. Post hoc tests revealed that the cuneiform lesioned group spent significantly more time at the edges of the field than the other three groups, which did not differ significantly from each other.

Finally Figure 6.10 shows a comparison of the percentage of test time each of the groups spent eating. A one-way ANOVA revealed a significant main effect of group $F(3,54)=12.84$ $p<0.01$. Post hoc comparisons showed that the PPTg lesioned group spent significantly more time eating than any of the other groups, which did not differ significantly from each other.

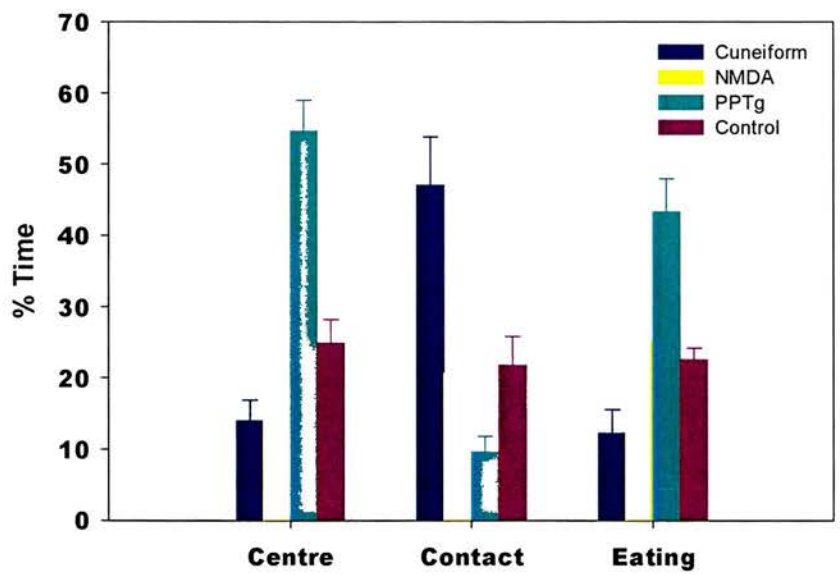


Figure 6.10: Mean percentage of test time each of the 4 groups spent in the centre of the field, in contact with the sides and eating (+SE). PPTg and NMDA lesion groups spent significantly longer in the centre of the field than either cuneiform lesioned or sham lesioned control groups, $p<0.01$. Cuneiform lesioned rat displayed significantly more thigmotaxic behaviour than any other group, $p<0.01$. PPTg lesioned rats spent ssignificantly more time eating than any other group, $p<0.01$.

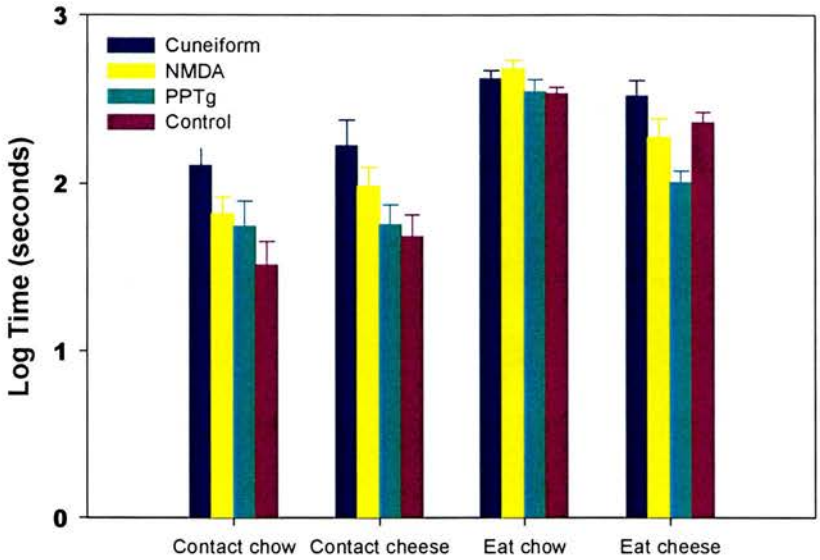


Figure 6.11: Mean latencies to contact and eat both cheese and chow (+SE). Cuneiform lesioned rats were significantly slower than sham lesioned control rats to contact both cheese, $p<0.05$, and chow, $p<0.05$. There were no significant differences in latency to eat chow, however PPTg lesioed rats were significantly quicker to eat cheese than either cuneiform lesioned or sham lesioned control rats, $p<0.01$.

Measures were made of latency to contact and eat both cheese and chow from the start of the test session; Figure 6.11 summarises these results. There were significant main effects of group revealed by analysis of both chow, $F(3,54)=3.54$ $p<0.05$, and cheese, $F(3,54)=3.79$ $p<0.05$, contact latencies. Post hoc comparisons showed that cuneiform lesioned rats were significantly slower to contact both chow and cheese than the control group. No differences were found in latency to eat chow, $F(3,54)=1.97$ NS. However there was a significant group effect of cheese, $F(3,54)=6.48$ $p<0.01$. Planned comparisons revealed that the PPTg lesioned rats were significantly quicker to eat cheese than either cuneiform or control groups.

Openfield

In the openfield the arena is divided into sections close to the wall, away from the wall and in the centre. Recordings are made at 10 second intervals of rat's position in the field. Figure 6.12 shows the percentage of the 10 minute test session spent in the centre of the field. Analysis with a one way ANOVA revealed a significant effect of group, $F(3,54)=5.52$ $p<0.01$. Posthoc tests revealed that the PPTg lesioned rats spent significantly more time in the centre than either cuneiform or NMDA lesion groups. Thigmotaxic behaviour refers to a rats tendency to remain close to walls or high sided structures when anxious. A one way ANOVA indicated a significant effect of group, $F(3,54)=6.48$, $p<0.01$, and post hoc tests again showed that NMDA and cuneiform lesion groups displayed more thigmotaxic type behaviour than PPTg lesioned rats.

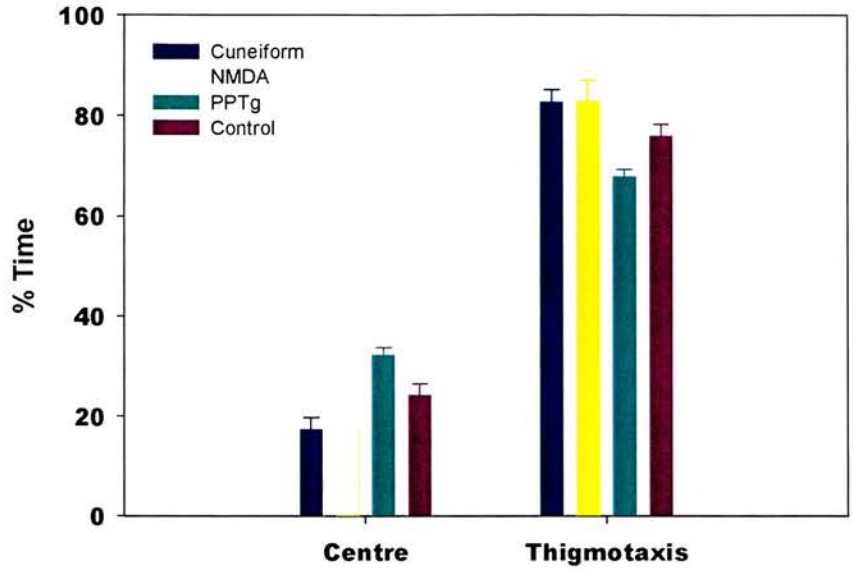


Figure 6.12: Mean percentage of test time groups spent in the centre and in contact with the sides of the open field (+SE). PPTg lesioned rats spent significantly more time in the centre than either cuneiform of NMDA lesion groups, p<0.01. Cuneiform and NMDA lesion groups displayed significantly more thigmotaxic behaviour than PPTg lesioned rats, p<0.01.

Sucrose Consumption

6.4 Methods (b)

Animals

The rats used in this experiment had previously been used in the study reported in the first part of this chapter. There were 13 with lesions of the cuneiform nucleus, 12 with ibotenic acid lesions of PPTg, 14 with NMDA lesions of caudal PPTg and 17 sham lesioned controls.

Behavioural Testing

Rats were allowed 48 hours habituation to the grid bottom cages before any measures were taken. Testing took place over three consecutive days.

6.5 Results (b)

Figure 6.13 summarises the pattern of water intake of the 4 groups over the 3 days of monitoring. A repeated measures ANOVA confirmed there was no significant effect of group, $F(3,52)=1.41$, NS but there was a significant effect of day, $F(2,51)=321.31$, p,0.01. Figure 6.14 shows a comparison of the sucrose consumed by each of the 4 groups. One way analysis revealed a significant effect of group, $F(3,52)=6.58$ $p<0.01$; post hoc comparisons showed that the PPTg lesioned rats consumed significantly more sucrose than cuneiform or control rats.

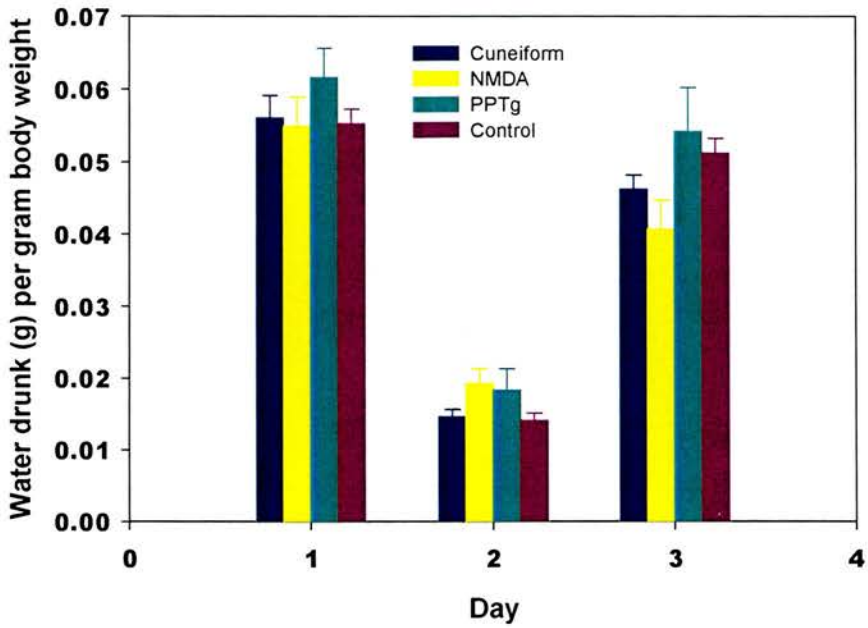


Figure 6.13: Mean amount of water consumed (grams per gram of body weight) by the 4 groups on each of the 3 test days (+SE). There was no significant effect of group, but there was a significant effect of day, p<0.01.

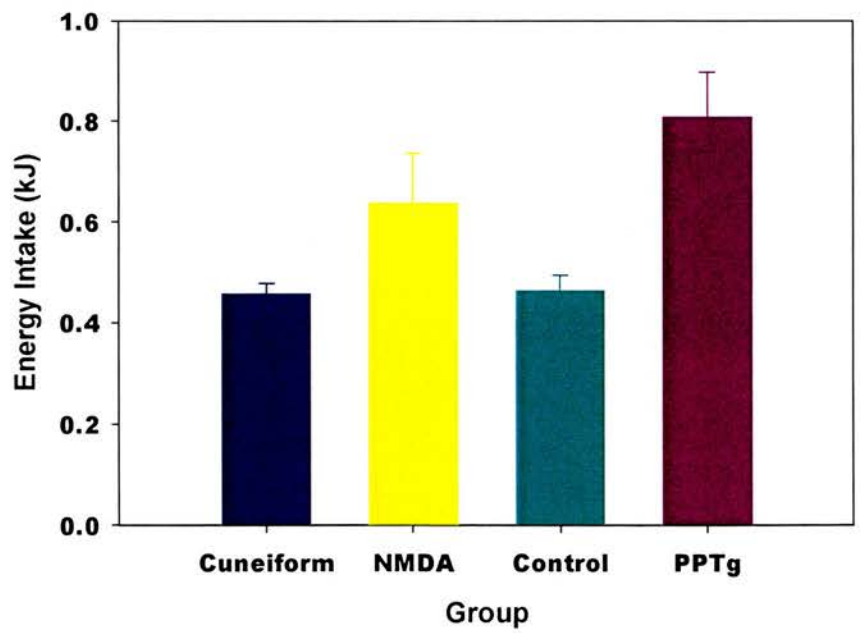


Figure 6.14: Mean sucrose consumed (kJ per gram of body weight) by each of the 4 groups (+SE). PPTg lesioned rats consumed significantly more sucrose than cuneiform lesioned or sham lesioned control rats, p<0.01.

Figure 6.15 summarises the average, total energy intake of each of the 4 groups over the three test days. A repeated measures ANOVA confirmed there was no significant effect of group, $F(3,52)=2.25$ NS; there was a significant effect of day, $F(2,51)=51.78$, $p,0.01$, but there was no day x group interaction $F(6,104)=0.66$ NS.

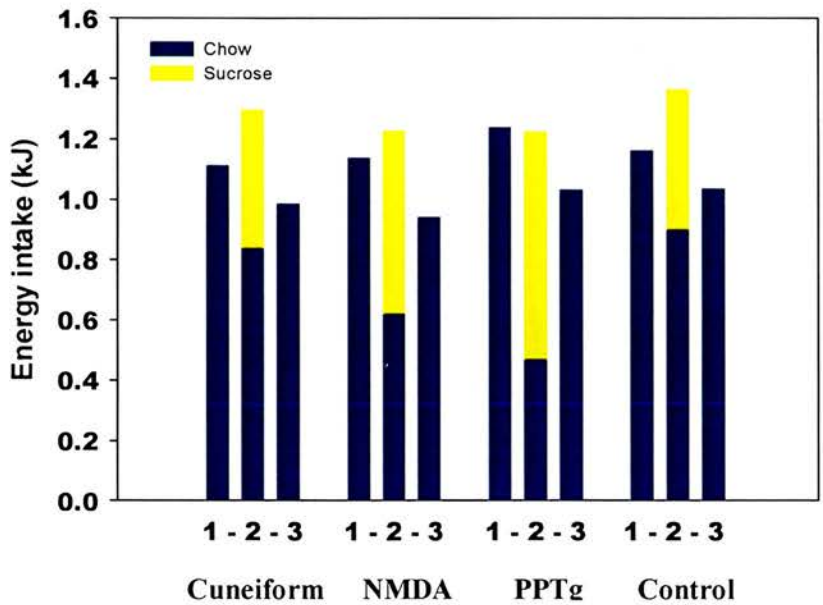


Figure 6.15: Mean energy intake (separated into lab chow and sucrose on day 2) for each of the 4 groups. There was no significant effect of group, but there was a significant effect of day, $p<0.01$.

Quinine Consumption

6.6 Methods (c)

Animals

The rats used in this experiment were some of those used in studies already described in this chapter. 6 had ibotenic acid lesions of the PPTg, 6 had NMDA lesions of the PPTg, 7 had bilateral cuneiform lesions and 7 were control rats.

Behavioural Testing

Following 24 hours habituation to the grid bottom cages measures were taken over 3 consecutive days of body weight, food consumed and water drunk. On day 2 of testing rats were given 0.01% quinine solution instead of water.

6.7 Results (c)

Figure 6.16 shows a comparison of amounts of quinine consumed per gram of body weight. A one way ANOVA showed a significant effect of group, $F(3,22)=8.99$, $p<0.01$. Post hoc analysis indicated that both PPTg and NMDA groups drank significantly more quinine than control rats. PPTg lesioned rats also consumed significantly more quinine solution than the cuneiform group.

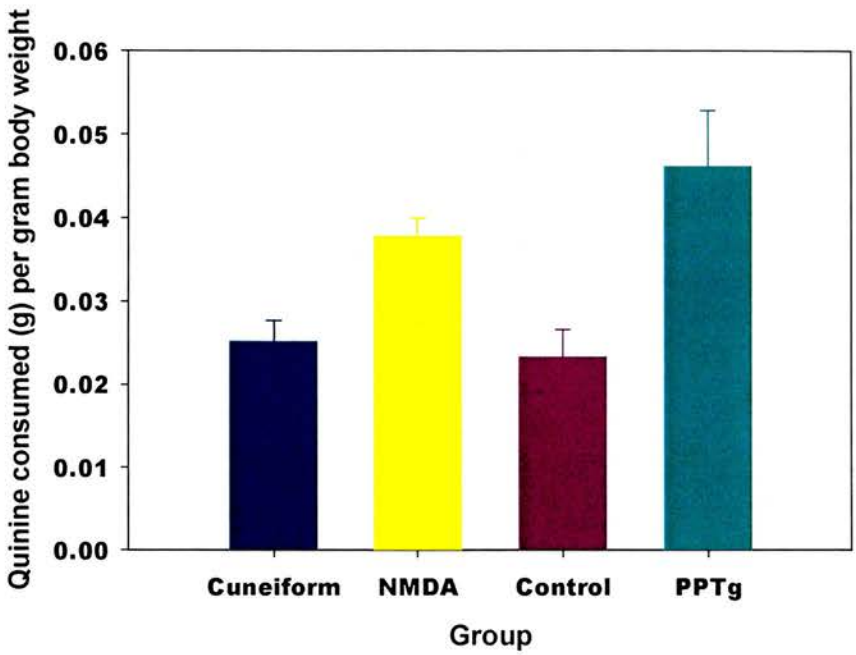


Figure 6.16: Mean quinine consumed (grams per gram of bodyweight) by each of the 4 groups (+SE). There was a significant effect of group, $p<0.01$. Both PPTg and NMDA lesioned rats drank significantly more than sham lesioned control rats. PPTg lesioned rats also consumed more than the cuneiform lesioned group.

6.8 Discussion

The results from this study reconfirm our findings that PPTg lesioned rats do not show heightened levels of anxiety as measured by the elevated plusmaze. They also indicate that those rats bearing Franklin's NMDA lesions of the PPTg behave more like cuneiform than our own PPTg lesioned group on the elevated plusmaze.

Measures of percentage arm entries confirmed that both the cuneiform and NMDA group spent significantly more time in the closed arms and less in the open arms of the maze than either PPTg or control groups, which did not differ significantly from each other.

In terms of arm entry latencies NMDA and cuneiform groups took longer to enter the open arms of the maze than controls at the start of a test session, again suggesting an elevation in normal anxiety levels in these two groups.

The other traditional measure of anxiety in the elevated plusmaze is the number of open arm entries. Anxiolytic effects are measured as an increase in open arm entries without any change in total arm entries (Andretoni & Bacellar 2000). While PPTg lesioned rats made significantly more open arm entries than any other group, they also showed a higher level of total arm entries than either control or cuneiform groups. These findings suggest a behavioural abnormality in the PPTg lesioned group, that cannot be explained purely as a decrease in general anxiety, but seems more like an increase motor activity.

While on the elevated plusmaze the NMDA lesioned rats looked more like the cuneiform lesioned group than ibotenate lesioned PPTg's, but this distinction was not so clear cut in the food neophobia test. PPTg and NMDA lesioned rats showed

less thigmotaxic behaviour than cuneiform or control groups, spending more time in the centre of the field. Again in the cuneiform group there was evidence of elevated anxiety, they spent more time in contact with the edges of the field than any other group.

The PPTg lesioned rats were less neophobic than any of the other groups as they spent a higher percentage of the test time eating. They were also significantly quicker to begin eating cheese than either the cuneiform or control groups. In contrast eating latencies to the familiar lab chow did not differ significantly between groups.

In the openfield, when food was no longer a factor, there was a return to the pattern of results seen in the elevated plusmaze. NMDA and cuneiform groups displayed higher levels of anxiety in comparison to PPTg rats, which spent more time in the centre and showed less thigmotaxic behaviour than the other lesion groups.

In summary, the data from these three behavioural tests indicate a degree of anxiety in the cuneiform and NMDA lesion groups that is not present in PPTg or control rats as measured by percentage closed arm time in the plusmaze and thigmotaxic behaviour in the openfield. Yet in the food neophobia experiment, where there was food available, the NMDA rats looked generally more like PPTg than cuneiform lesions.

In the sucrose drinking experiment too the behaviour of the NMDA group was not easily definable. While this study successfully replicated previous finding that PPTg lesioned rats consume significantly more sucrose when given free access

than either cuneiform or controls, the NMDA group did not differ significantly from any other. However, looking at Figure 6.14 it seems the same tendency for over consumption is apparent in this group as in our own PPTg group and that high levels of variance prevents the differences being significant.

A possible reason the NMDA group show higher levels of variance and we fail to find significant differences between them and other groups is because there has not been the same subject exclusion procedures applied to this as the other lesion groups. While generally data is excluded from any rats when lesions spread too far to surrounding areas this was not done with the NMDA lesioned rats as it was not clear what an accurate lesion should look like, and exclusion could bias results towards those with most severe cuneiform damage. Thus only one NMDA rat has been excluded from analysis in this study due to an absence of lesion damage in caudal PPTg.

The data from the quinine drinking experiment also provides some interesting findings. Previously disinhibited consummatory behaviour reported following lesions of the PPTg has been explained as an abnormality in control of response to positive reward. However, in this study we saw the same disinhibited drinking response to a weakly aversive quinine solution. Franklin's NMDA group also showed higher levels of consumption than controls though they did not differ significantly from cuneiform lesioned rats.

These findings suggest that behavioural abnormalities in PPTg lesioned rats do not purely reflect a disinhibited response when levels of motivation are elevated by reward but also under aversive conditions. In the behavioural tests of anxiety

these rats are not normal. The behaviour seen is not due to an anxiogenic effect of the lesion, according to traditional measures, nor is there a clear cut anxiolytic effect. What we see instead is a general disinhibition of outwardly directed behaviour, coupled with a reduction in neophobic reaction.

Disinhibited behaviours have been reported in PPTg lesioned rats in a variety of different behavioural tests. Over consumption of sucrose has been reported in a number of studies, from measures of free home cage access to conditioned place preference paradigms (Keating et al. 2002). In all cases while basic regulatory and motivational behaviours, such as energy modulation, reward strength perception and place preference formation, remain intact, the same increase in high concentration sucrose consumption is seen. Other types of reward also produce disinhibited consummatory responses in PPTg lesioned rats. In an IVSA study for amphetamine, PPTg lesioned rats showed higher injection rates and breaking points than controls (Keating et al. 1997). Also on a conditioned ratio schedule for food reward PPTg lesioned rats show normal levels of responding on the reinforced lever but also respond equally on the non reinforced lever meaning they fail to balance effort with reward and hence have lower than normal breaking points (Alderson et al. 2002). The fact that these rats adjust behaviour when output outweighs reward is another indication that PPTg lesions are not disrupting basic motivational processes.

Basic motor responses also appear disinhibited in PPTg lesioned rats fail to inhibit the startle reflex following an acoustic pre pulse warning, and additionally show reduced freezing behaviour to overhead threats. These abnormalities sit well with the locomotor responses observed in the elevated plusmaze. A wide range of

studies have failed to find a disruption in basic locomotor activity following lesions of the PPTg so an alternative explanation is required. While a decrease in general anxiety would account for an increase in open arm entries, the global increase in arm entries suggests either a disinhibition of exploratory response or perseverative arm re-entry as seen in rats with similar lesions the radial arm maze (Dellu et al. 1991). In either case the behaviours seen suggest an abnormality in action selection.

It appears therefore that irrespective of response type, be it to reward or under conditions of heightened anxiety, there is a loss of behavioural control in PPTg lesioned rats that produces the disinhibited behaviours reported. Shallice (1988) proposed a model for action selection which involves two separate psychological processes. He names the first contention scheduling, which refers to a decentralised low level selection of a particular action over several competing possibilities. Shallice refers to the second process as the supervisory attentional system (SAS). This higher level system is not thought to control behaviour overtly but has a modulatory effect on lower level contention scheduling by activating or inhibiting particular action schemata. Shallice claims that the SAS is important in situations involving, novelty, danger and decision making when routine stimulus driven action selection is not sufficient. He also postulates that damage to or absence of the supervisory system would result in behavioural deficits like perseveration, distractability, impulsivity and an inability to co-ordinate complex actions, all deficits seen following damage to the frontal lobes.

Frontal and striatal systems are intimately connected both structurally and functionally (Alexander et al. 1986). Damage to striatal systems also produces

deficits in action selection. For example, ventral striatum, and nucleus accumbens in particular is important in co-ordinating reward driven actions (Brown & Bowman 1995). Eagle et al (1999) found that ventral striatal lesions increased consumption of weak sucrose solution without altering contrast effects. Dorsal striatum is important in motor control, lesions produce deficits in reaction time performance (Robbins & Brown 1991) and perseverative behaviours (Eagle et al. 1999). Since the PPTg has such strong connections to fronto-striatal regions and potentially forms the lowest part of fronto-striatal circuitry as part of a subsidiary loop (Alexander et al. 1986) it is possible that deficits in action selection reported following lesions of the PPTg could reflect disruption to fronto-striatal connections and particularly to cognitive processes controlled by, what Shallice's supervisory attentional system.

In terms of the lesion study the behavioural data suggest much the same as the histological data that Franklin's NMDA lesions do successfully destroy the caudal half of PPTg and this damage accounts for some of the behaviour seen. However there is also significant damage to the cuneiform nucleus and it is this which produces the anxiogenic effects seen on traditional anxiety measures on the elevated plusmaze.

Differences in lesions provide some interesting indications of the importance of caudal PPTg in a number of behavioural functions associated with the PPTg. They also suggest that lesion differences may be at the core of contradictions in the literature on PPTg function. For example several studies have produced evidence that PPTg lesions prevent the development of conditioned place preference in sated and drug naïve animals. Bechara and van der Kooy (1989) found that ibotenate

lesions of the PPTg disrupted formation of conditioned place preference to morphine in drug naive rats. Bechara and van der Kooy (1992) found a similar effect with food in non deprived rats. However in both studies lesions of the PPTg did not block conditioned place preference in morphine dependent and food-deprived rats. Bechara and van der Kooy concluded that separate brain substrates must underlie reward mechanisms in deprived and non-deprived states, and that PPTg lesions differentially disrupt the positively reinforcing effects in non deprived states. However, a number of studies do not support the pattern of data described above. Parker & van der Kooy (1995) found PPTg lesioned rats developed conditioned place preference for cocaine even when drug naïve. Alderson et al. (2001) & Keating et al. (2002) found no deprivation state dependent effects in a conditioned place preference paradigm maintained by sucrose.

While these findings could potentially be explained by differences in reward used, it is difficult to see an obvious distinction along these lines. For example, there is not a clear difference in response to natural and artificial rewards. An alternative explanation is that differences in methodology, and in particular lesion technique, produce the behavioural differences reported. As previously described, in this laboratory we make two small injections of ibotenic acid one into caudal and one into rostral PPTg. In contrast van der Kooy et al. use a stronger solution of ibotenic acid and a one placement strategy, suggesting a larger more diffuse lesion would result. Additionally differences in co-ordinates used mean damage is centred on more rostral and medial portions of PPTg than our own lesion.

Together these differences could account for some of the literature anomalies in PPTg studies, and while it does not invalidate any findings, it makes generalising across studies more difficult. We know from anatomical studies of PPTg that inflow and outflow connections are specific to different neural groups within PPTg, and while ibotenic acid itself is not selective for cell type, differing injection sites could bias damage to particular cells with specific input output connections.

Is it possible to account for all the deficits seen following removal of the PPTg under a unitary behavioural description? In general terms disruption in behavioural control, as described by Shallice (1988), does account for what Winn (1998) described as frontal type deficits seen in PPTg lesioned rats in a number of behavioural tests. However, the term does not account so well for the attentional type abnormalities that have been reported in recent years following PPTg lesions (Inglis et al. 2000, Kozak et al. 2001). However perhaps this is not surprising given what we know about the structure of PPTg its different neurone groups with separate inflow and outflow connections. Possibly the abnormalities seen are better divided along anatomical lines, with the frontal type behavioural control abnormalities reflecting a disruption in striatal outflow, and the attentional type deficits reflecting a disruption in thalamic inputs from PPTg and other low levels sites of behavioural activation and arousal. This suggests an important future direction for PPTg research is in separating these anatomical connections in functional terms.

In methodological terms this again brings up the question of lesion technique and the importance of using an accurate strategy to ensure that any findings can be accurately interpreted. In current research whether a single or dual placement

strategy is used using excitotoxins means unselective lesions of all cell groups in the PPTg.

Chapter 7

A comparison of Fos immunoreactivity in the PPTg and cuneiform nucleus following exposure to the elevated plusmaze.

7.1 Introduction

C-fos is one of a family of genes known as immediate early genes and was first isolated in 1982. Produced in response to neuronal stimulation, this second messenger gene codes for the protein Fos. The immediate early gene proteins are transcription factors: on entering the nucleus of a cell they bind to a particular region of the DNA located there, and can then initiate and regulate the transcription of mRNA (Herdegen & Leah 1998). In recent years immediate early genes and their protein products have been widely used as metabolic mapping tools, and Fos in particular has been utilised to identify cells and circuits activated by various stimuli (Kovacs 1998).

Immunohistochemical techniques allow identification of specific neurones that have been activated by experimental stimulation. By incubating fixed tissue sections in Fos-antibody and tagging the resulting antigen-antibody complex, cells that contain the Fos protein can be visualised (Winn 2001).

C-fos is a particularly good mapping tool for a number of reasons. First, both Fos protein and *fos* mRNA are present in only very low levels under normal conditions even in brain areas with a generally high level of metabolic activity, like

visual cortex (Kovacs 1998). Second, a wide range of different types of stimulation induce reactivity, from exploration of a novel environment, which produces relatively weak activation (Wirtshafter et al. 1998), to direct electrical stimulation of a particular brain region (Lam & Verberne 1997). Finally, the time scale of *c-fos* presence within a cell is very useful, mRNA *c-fos* is induced within 1-2 minutes of stimulus presentation with levels peaking after around 30-60 minutes, Fos protein is present 1-3 hours after stimulation, and disappears completely 4-6 hours later (Kovacs 1998). Therefore presence of *c-fos* or the Fos protein indicates recent cellular activation.

There are certain limitations to the use of *c-fos* and its protein product in anatomical mapping tools. Fos is neither a complete nor an exclusive marker of neuronal activity, meaning that its absence in given region does not prove lack of activation (Sandner et al. 1993), and brain regions differ in threshold of Fos activation (Kovacs 1998). Additionally it must be borne in mind that Fos and other inducible transcription factors are sometimes expressed due to the absence of an expected stimulus. However, stress and anxiety related brain pathways have been particularly widely studied using Fos mapping techniques. Studies have successfully identified sites of neuroendocrine, autonomic and behavioural response to stress (Herdegen & Leah 1998).

Behavioural models of anxiety reliably induce Fos immunoreactivity in various limbic and cortical brain areas, particularly prefrontal cortex, amygdala, and the hypothalamus, all areas that have previously been implicated in the modulation of fear and anxiety states. Brainstem activation is also reliably seen following

various behavioural tests associated with fight or flight responses including colliculus, central grey and locus coeruleus (Duncan et al.1996). Silveira et al. (1993) found that a 15 minute exposure to an elevated plusmaze induced Fos immunoreactivity in several forebrain and cortical areas as well as brain stem regions which included the periaquductal grey, colliculus, dorsal raphe, locus coeruleus and the cuneiform nucleus.

Results from earlier chapters clearly show that lesions of the cuneiform nucleus, and not the PPTg, produce anxiogenic effects as measured by the elevated plusmaze. Indeed results from some behavioural tests suggest a reduction in anxiety in rats with lesions of the PPTg compared to control rats. The literature also shows that exposure to the elevated plusmaze is an intense enough stressor to induce Fos immunoreactivity (Duncan et al. 1996, Silveria et al. 1993). Therefore, the aim of the current study was to replicate previous results using intact animals. To do this, levels of activation of Fos protein in the PPTg and cuneiform nucleus of plusmaze exposed and control rats will be compared. Previous plusmaze studies have reported an increase in open arm avoidance following a second exposure to the elevated plusmaze, suggesting that the type and extent of anxiety changes from fear of a novel exposed area on trial 1 to a stronger and specific fear of heights on trial 2 (Treit et al. 1993 & Bertiligo & Carobrez 2000). For this reason, levels of Fos were measured after 10 minutes plusmaze exposure on 2 consecutive days.

Hypothesis

It is hypothesised that there will be a significant increase in Fos activation in the cuneiform nucleus following the exposure to the elevated plusmaze in comparison to homecage controls that will not be seen in the PPTg. Additionally it is predicted there will be a positive correlation between level of Fos immunoreactivity in the cuneiform nucleus of plusmaze exposed rats, and time spent in the closed arms of the maze.

7.2 Methods

Animals

18 animals were used in this experiment. They were housed from arrival in 9 home cage pairs. Each of the pairs was randomly assigned, one to plusmaze exposure with the other as homecage control.

7.3 Results

Histology

Following histological analysis two of the nine pairs were eliminated due to pale staining and unevenly sliced sections in one case, and poor section quality making accurate counting in the cuneiform nucleus impossible in another.

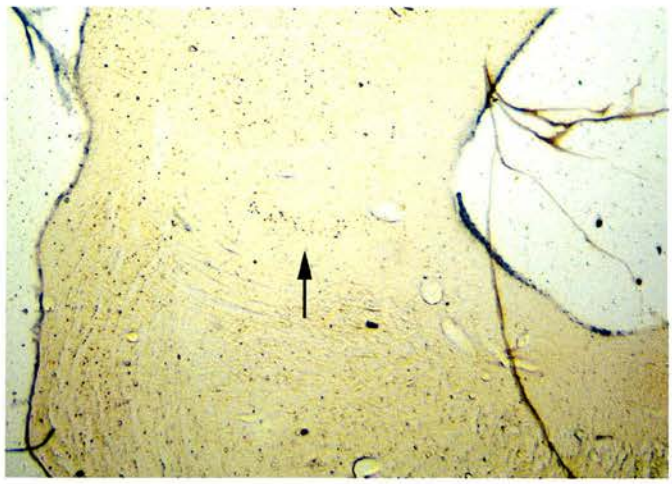
Photograph 7A shows significant Fos like immunoreactivity in both the cuneiform nucleus and inferior colliculus of a plusmaze exposed rat in comparison to photograph 7B which shows much weaker Fos immunoreactivity in the same region of a control. Photographs 7C & D show the PPTg of a plusmaze exposed and control rat respectively. Only weak diffuse Fos like immunoreactivity can be seen in both photographs.

Behaviour

Figure 7.1 shows the average number of Fos positive cells counted in the cuneiform of the plusmaze exposed and control groups. T-test analysis showed that a significantly higher number of Fos positive cells were counted in those rats exposed to the elevated plusmaze, $t=4.29$, $df=12$ $p<0.001$. Figure 7.2 shows a

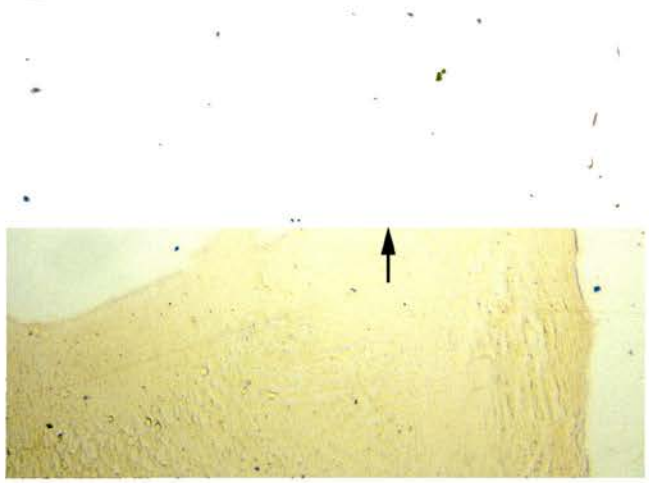
Photograph 7A shows significant Fos like immunoreactivity in both the cuneiform nucleus and inferior colliculus of a plusmaze exposed rat in comparison to photograph 7B which shows much weaker Fos immunoreactivity in the same region of a control. Photographs 7C & D show the PPTg of a plusmaze exposed and control rat respectively. Only weak diffuse Fos like immunoreactivity can be seen in both photographs

A



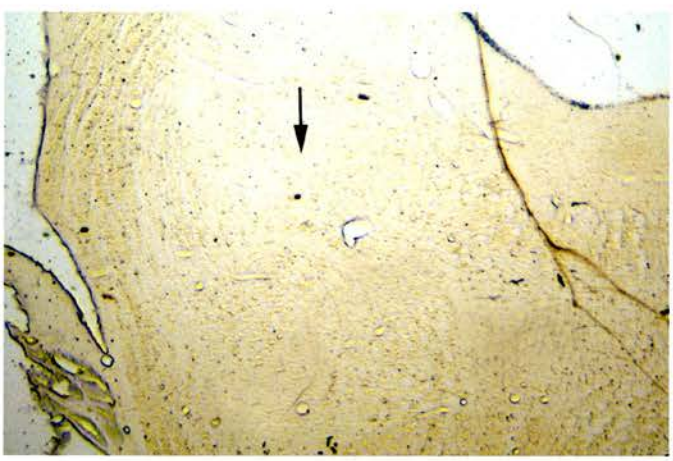
Cuneiform of plusmaze exposed rat.

B



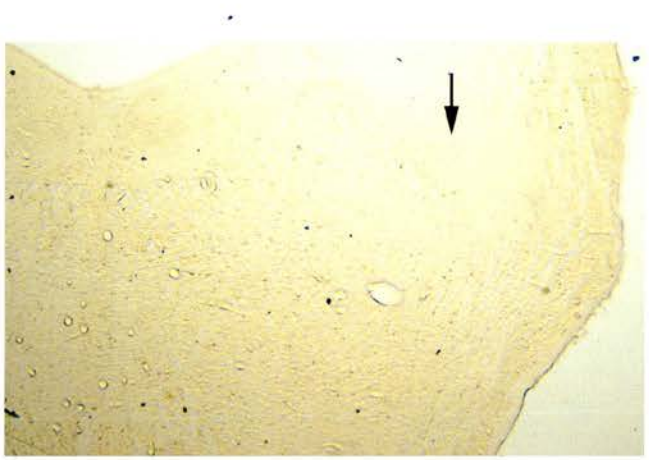
Cuneiform of control.

C



PPTg of plusmaze exposed rat.

D



PPTg of control.

comparison of the number of Fos positive cells found in the PPTg of plusmaze exposed and control animals. A t-test analysis indicated no significant difference between the two groups, t=0.887 df=12 NS.

A count of Fos positive cells was also made at different anatomical levels of the PPTg. Separate counts were made of caudal and rostral PPTg, and the findings are summarised in Figures 7.3 and 7.4. T-tests revealed that there was no significant difference between plusmaze exposed and control rats in terms of Fos activation in either caudal, t=0.615, df=12 p<0.55, or rostral PPTg, t=0.412, df=12 p<0.687.

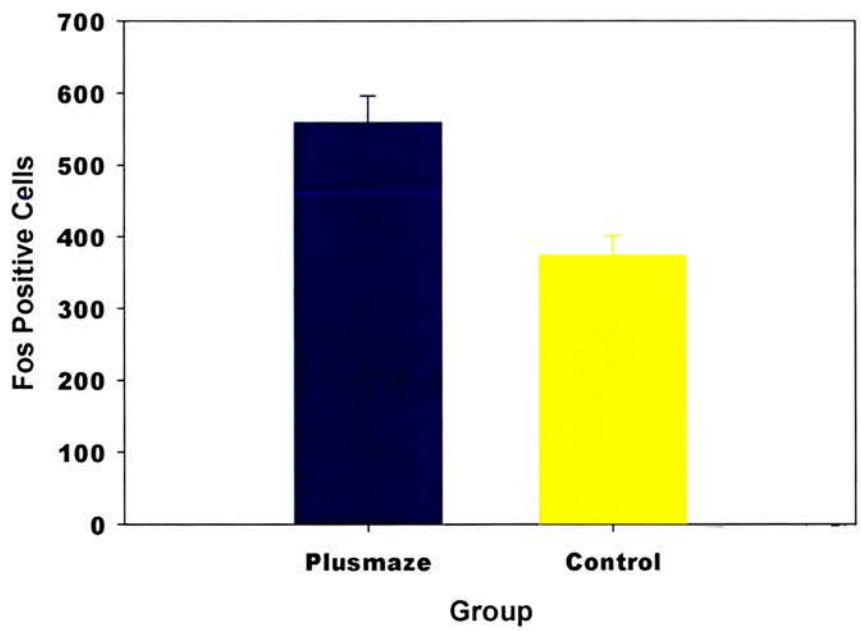


Figure 7.1: Mean number of Fos positive cells counted in the cuneiform nucleus of plusmaze exposed and control rats (+SE). Significantly more Fos positive cells were counted in those rats exposed to the elevated plusmaze, p<0.001.

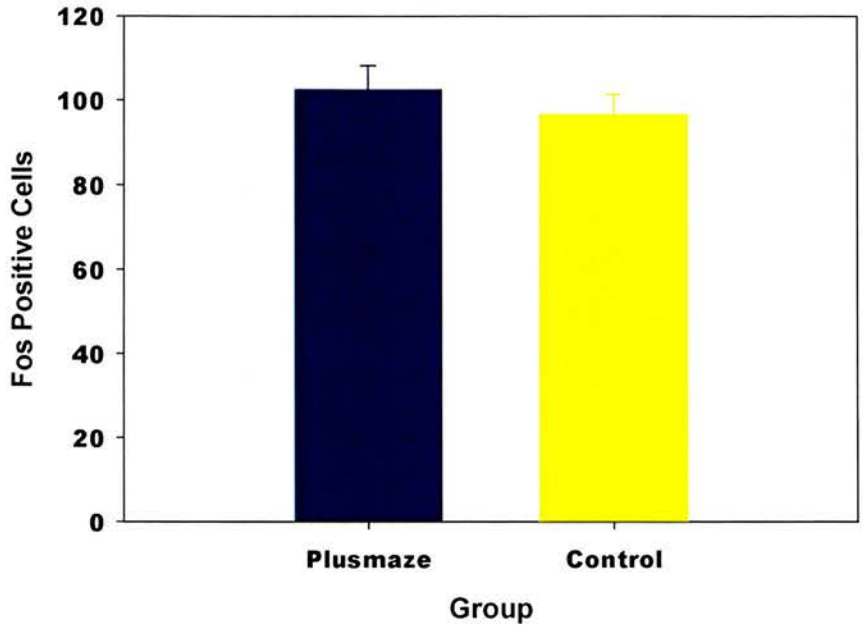


Figure 7.2: Mean number of Fos positive cells counted in the PPTg of plusmaze exposed and control rats (+SE). There was no significant difference between the two groups.

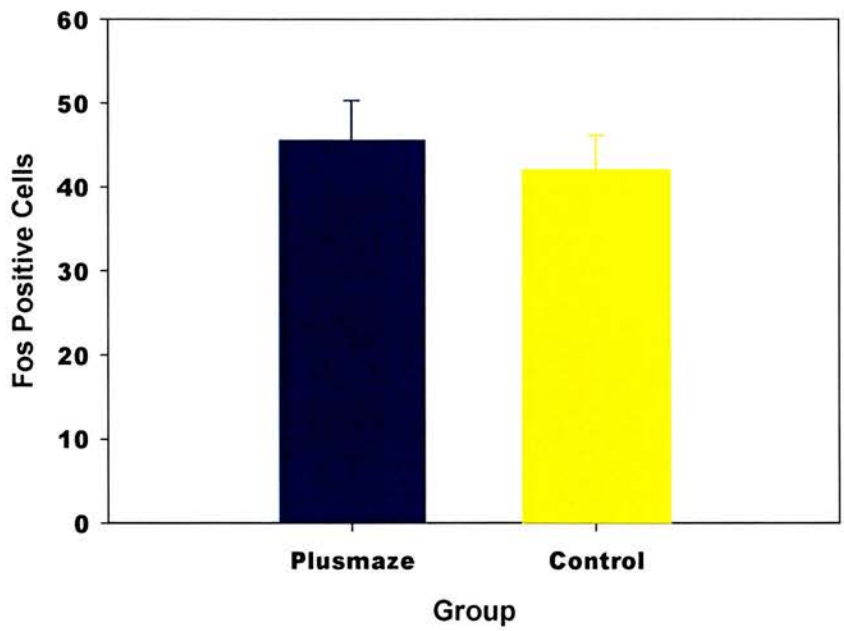


Figure 7.3: Mean number of Fos positive cells counted in the caudal half of the PPTg of plusmaze exposed and control rats (+SE). There was no significant difference between groups.

Correlations were calculated between degree of Fos immunoreactivity in the two nuclei and percentage time spent in the open and closed arms of the maze. No significant correlations were found on any of the 4 measures; Fos in the cuneiform and percentage time on the closed arms, r = -0.262 (2 tailed) NS, Fos in the PPTg and percentage time on the closed arms, r = 0.32 (2 tailed) NS, Fos in the cuneiform and time on the open arms, r = 0.062 (2 tailed) and Fos in the PPTg and time on the open arms, r = -0.567 (2 tailed) NS.

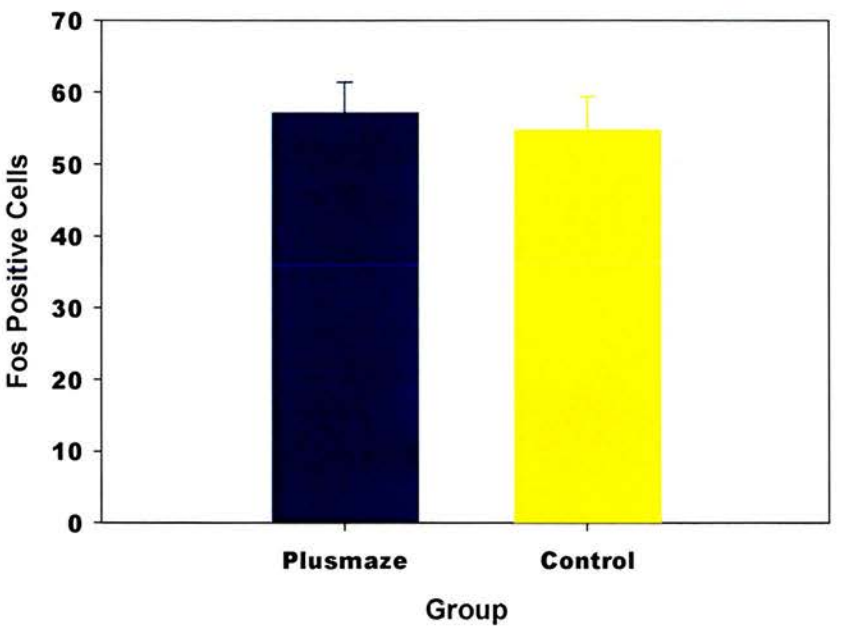


Figure 7.4: Mean number of Fos positive cells counted in the rostral half of the PPTg of plusmaze exposed and control rats (+SE). There was no significant difference between groups.

7.4 Discussion

The findings from this anatomical mapping study confirm previous results from lesion studies that the cuneiform nucleus and not the PPTg is important for behaviour stimulated by exposure to the elevated plusmaze. While rats exposed on two consecutive days to the plusmaze showed significantly more Fos immunoreactivity in the area of the cuneiform nucleus than did controls, there was no difference between the two groups in PPTg activation. This suggests that the cuneiform nucleus is important in the modulation of anxiety states, at least as measured by plusmaze exposure.

Measures were also made of plusmaze activity, and it was predicted that increased time in the closed arm of the maze, indicating heightened anxiety would be positively correlated with Fos expression in the cuneiform nucleus. However the rat with the highest level of Fos immunoreactivity in the cuneiform spent less time than average in the closed arm of the maze, and the opposite pattern was observed in the rat with the lowest levels of immunoreactivity thus indicating any correlation would be in the opposite direction. In fact analysis showed there to be no significant correlation between either open of closed arm time and levels of Fos immunoreactivity in the cuneiform or PPTg nuclei. Results from other Fos mapping studies support this finding as in many cases intensity and or duration of a stimulus shows no relation to the extent of Fos expression observed, possibly because the expression of proteins such as Fos in vivo represents a more complex process than neural response to a specific stimulus (Herdegen & Leah 1998). This is likely to be

amplified in a study such as this, where the stimulus used is relatively weak and diffuse.

It is important to emphasise that the cuneiform nucleus was far from the only structure to show significant levels of Fos. In the brain stem alone, high levels of immunoreactivity were observed in the central grey and inferior colliculus. Both of these structures have previously been implicated in the modulation of anxiety states (Fendt 1998 & Pandossio et al. 2000). Indeed, Silveira et al. (1993) report significant Fos immunoreactivity in these along with several other brainstem, forebrain and cortical regions following 15 minutes exposure to the elevated plusmaze.

These observations tie in well with other literature that shows weakly aversive behavioural tests successfully stimulate Fos production in the brain. For example, Dielenberg et al. (2001) found that exposure to cat odour produced Fos immunoreactivity in several brain nuclei including ventromedial hypothalamus and PAG, which have known roles in defensive behaviour, as well as "strong and selective" Fos induction in the cuneiform nucleus. Kollack-Walker (1997) found significant expression of c-fos mRNA in several cortical and midbrain areas as well as the dorsal PAG, dorsal raphe, locus coeruleus and the cuneiform nucleus of subordinate hamsters following a 30 minute interaction with an unknown dominant male.

It is possible that the Fos activation observed in the region of the cuneiform nucleus was not due to anxiety at all but to exploratory locomotor activity. However, there are a number of reasons that this is unlikely to be the case. Firstly, Allen et al. (1996) found that rats with bilateral lesions of the cuneiform nucleus were no

different to control rats in measures of either spontaneous or amphetamine induced locomotion. Additionally Silveira et al. (1993) report that rats placed for 15 minutes in a circular open arena, to which they had previously been habituated, showed only very weak Fos immunoreactivity, confined to the dorsal midbrain and medial hypothalamus. Similarly, Wirtshafter et al. (1998) found that one hour exposure to a novel open field produced significant Fos like immunoreactivity in the supramammilary regions of the hypothalamus and areas that receive input from it.

In conclusion the results from this study show that on a second 10 minute exposure to the elevated plusmaze the cuneiform and not the PPTg shows significant immunoreactivty, indicating that comparable results can be successfully achieved using lesion and mapping studies.

Chapter 8

A pilot study using hypocretin-saporin to lesion the PPTg.

8.1 Introduction

The main focus of the research in this thesis has been an investigation of anxiety related functions of the mesopontine nuclei. Studies have clearly shown an anxiogenic effect following lesions of the cuneiform nucleus not seen following lesions of the PPTg. Additionally it has highlighted the importance of using an accurate and consistent lesion technique, in order to ensure that behavioural effects recorded are the result of damage to the target region. The limitations of electrolytic lesions and the problem of toxin spread have already been highlighted. There are other, often undiscussed, effects of commonly used toxins. For example studies have indicated demyelination following NMDA lesions, which recovers with time but means disruption of connections between as well as cells within the target region for a period following surgery (Brace et al. 1997).

In recent years much work has gone into developing more targeted toxins which produce selective lesions of specific neural groups within a given brain area. Axonally transported toxins are based on anatomical tracing dyes, producing lesions retrograde to the original injection site. More recently a number of toxic plant proteins have been utilised. When conjugated with an antibody that will target specific cell receptors, the complex binds to the target cell. Once inside, the cells' transport system delivers the toxin to the endoplasmic reticulum, where it attacks the

ribosomes, thus arresting protein synthesis and ultimately resulting in cell death (Wiley & Kline 2000). One of the most successful immunolesioning tools has been 192 IgG saporin. The antibody specifically targets neurons that express a neurotrophin receptor, $p75^{NTR}$, found in rat. Once the antibody has bound to the cell surface the complex is taken up by the axons and into the cell by retrograde transport, where the saporin, a cytotoxic plant protein, acts to prevent protein synthesis, causing cell death. This antibody-toxin conjugate has been highly successful in producing specific lesions of cholinergic cells within the basal forebrain (Wiley et al. 1991). Even more recently several neuropeptide toxin conjugates have been developed such as substance P- saporin, which targets neurokinin 1 receptors, and demorphin – saporin that targets μ opiate receptors (Wiley & Kline 2000).

Hypocretin / Orexin is a peptide produced exclusively in the lateral hypothalamus. It was discovered in the late 1990s simultaneously by two independent research groups, one naming it hypocretin due to its apparent similarity to secretin (de Lecea et al. 1998) and the other orexin because of its hypothesised role in feeding (Sakurai et al. 1998). Though localised in lateral hypothalamus, these neurones have extensive projections throughout the brain and spinal cord and are thought to be important in both feeding and sleep (Kilduff & Peyron 2000).

The original development of a targeting toxin utilising this neuropeptide was as a tool in sleep research. Chemelli et al. (1999) found that orexin gene knockout mice displayed narcoleptic symptoms. To aid further investigation a toxin was required that would destroy neurones that express the hypocretin / orexin receptor,

allowing exploration of the functions of these connections. The cytotoxin which was developed; hypocretin–2 / orexin–B–sap was found to produce narcoleptic symptoms when injected into the lateral hypothalamus (Gerashchenko et al. 2001a). Additionally the same group found that injections of this toxin into the medial septum of the basal forebrain, a target of hypocretin neurones, destroyed both Parv and ChAT positive cells in this region. This pattern of neural destruction was different to that produced by IgG saporin, and binding was specific to the hypocretin / orexin receptors no cross reactivity with other peptides was found (Gerashchenko et al. 2001b).

Hypocretin recepter proteins and mRNA have been identified in many pontine nuclei including the dorsal raphe, locus coureleus, LDTg and PPTg (Greco & Shiromani 2001). Thus suggesting that this peptide may play a role in more than just sleep (Kilduff & Peyron 2000). Given that hypocretin receptors have been located within the PPTg it was thought there was potential to develop a lesion technique that would be effective at target cells but, given the nature of its functional mechanism, would minimise or eliminate damage to surrounding areas.

The aim of the current study was to investigate the efficacy of targeted toxin hypocretin-2-sap in producing small selective lesions of PPTg without damage to surrounding regions.

Hypothesis

It is hypothesised, given its role in modulating of arousal via cholinergic connections to thalamus, that hypocretin / orexin receptors are located on the cholinergic cells of PPTg and that the hypocretin-sap conjugate toxin will produce selective lesions of these neurones.

8.2 Methods

Surgery

Surgery was carried out as per the methodology already described.

Stereotaxic Co-ordinates:

Co-ordinates were varied to find the optimal combination for delivery of toxin directly to the target region.

Unlike in previous studies where two injections are made per hemisphere into PPTg, in this study only the caudal co-ordinates were used. Initially injections were made 0.8mm anterior to the interaural line, +/- 1.6mm from the midline and 6mm below dura. Subsequent injections were made more caudally, 0.7mm anterior to the interaural line +/-1.5mm from the midline and either 6.0mm or 6.2mm below dura.

Toxin Concentrations:

High doses of saporin conjugate have been reported to cause non specific damage of neurones and myelinated axons (Wiley et al. 1991). Therefore part of this pilot study was assessing the optimal concentration of toxin.

Hypocretin-Sap was dissolved in Dulbecco's saline buffer; dilutions for use were made with sterile phosphate buffer. Unilateral injections of 0.2µl were made of 40, 50, 60 and 200*ng*/µl solution.

Histology

Unlike excitotoxins, there is a significant time lag between injection of an axonally transported toxin and lesion development. According to Wiley & Lappi (1994) cell death occurs 2 to 3 days after inhibition of protein synthesis, and full neuronal disintegration takes several days more. Therefore all rats were kept alive for at least 7 days post surgery.

Rats were perfused and brains processed according to the methodology already described. Sections were stained with NADPH-diaphorase to identify the cholinergic cells of PPTg and NeuN was used to visualise lesion area

8.3 Results

Table 8.1 summarises the results from this study. It indicates the degree of lesion damage produced by various toxin concentrations and differing stereotaxic co-ordinates. Though lesions varied in the extent to which they centred on the target region, it is clear from counting diaphorase positive cells on in the lesioned hemisphere, compared with the contralateral control, that the hypocretin-saporin conjugate toxin successfully destroyed the cholinergic neurones of PPTg. However, it is apparent from the NeuN/cresyl stain that non-specific neural destruction also occurred both within and out with the boundaries of PPTg. Figures 8.1 & 8.2 show the extent of each lesion.

Table 8.1 show toxin concentrations, stereotaxic co-ordinates and percentage loss of diaphorase cells on the lesion vs. contralateral control side. Reference numbers for the corresponding lesion schematic and photographs are also shown.

Lesion Schematic	Photographs	Toxin Concentration (*ng/μl*)	Stereotaxic Co-ordinates			% Loss of Diaphorase Positive Cells	Comments
			I.A.L.	M.L.	D.V.		
1	A & B	200	+0.8	+1.6	-6	78.9	Too large
2	C & D	50	+0.8	+1.5	-6	23.79	Too rostral
3	E & F	50	+0.7	+1.5	-6	37.99	Small & discrete
4	G & H	50	+0.7	+1.5	-6	0	Too dorsal
5	I & J	60	+0.7	+1.5	-6	30.75	Too medial
6	K & L	60	+0.7	+1.5	-6.2	29.7	Missed rostrally
7	M & N	50	+0.7	+1.5	-6.2	35.9	Small & discrete

Schematic 8.1 summarises the extent of damage produced by each lesion and concentration of toxin used.

Figure 8.1 shows the extent of damage produced by each lesion and concentration of toxin used

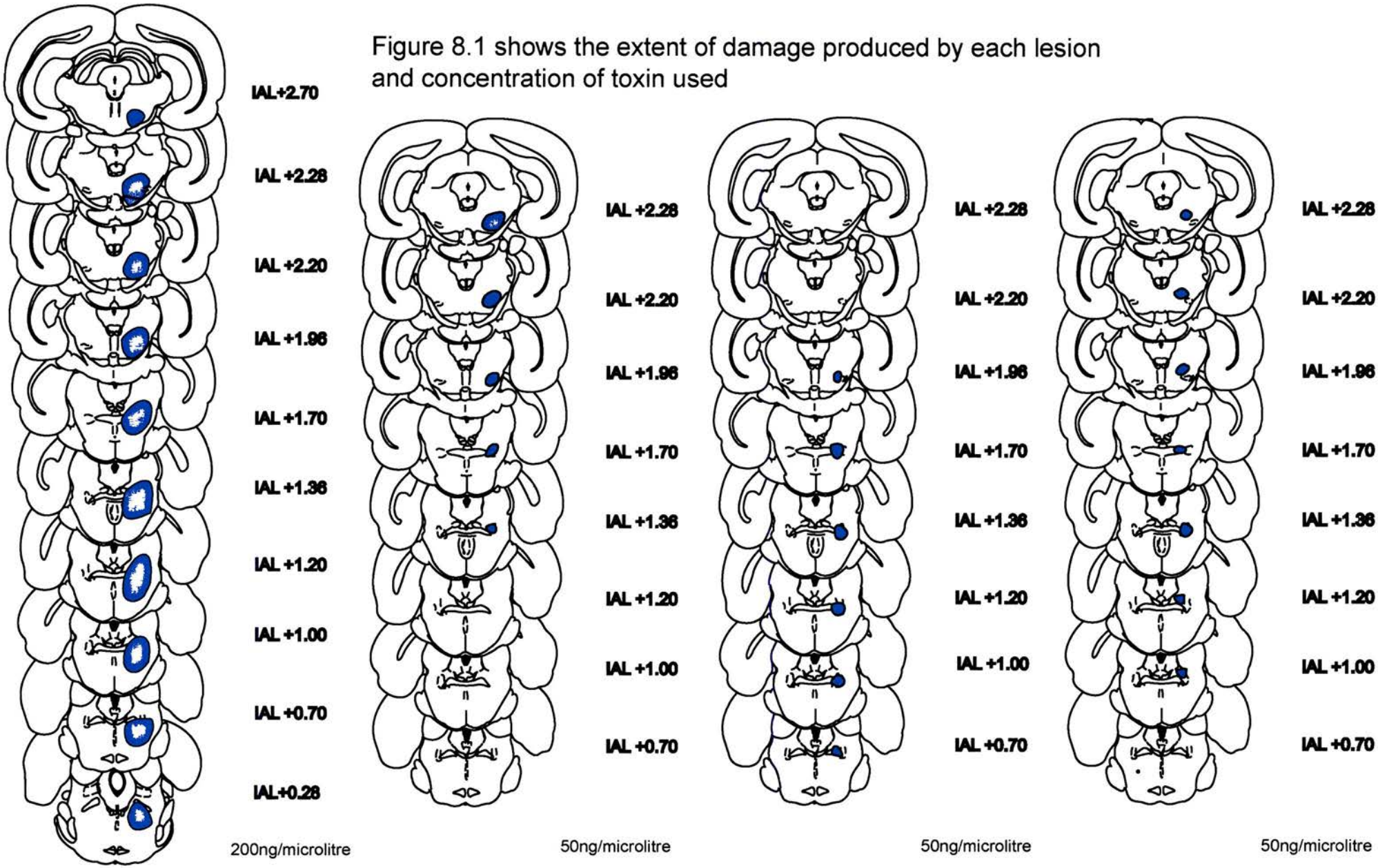
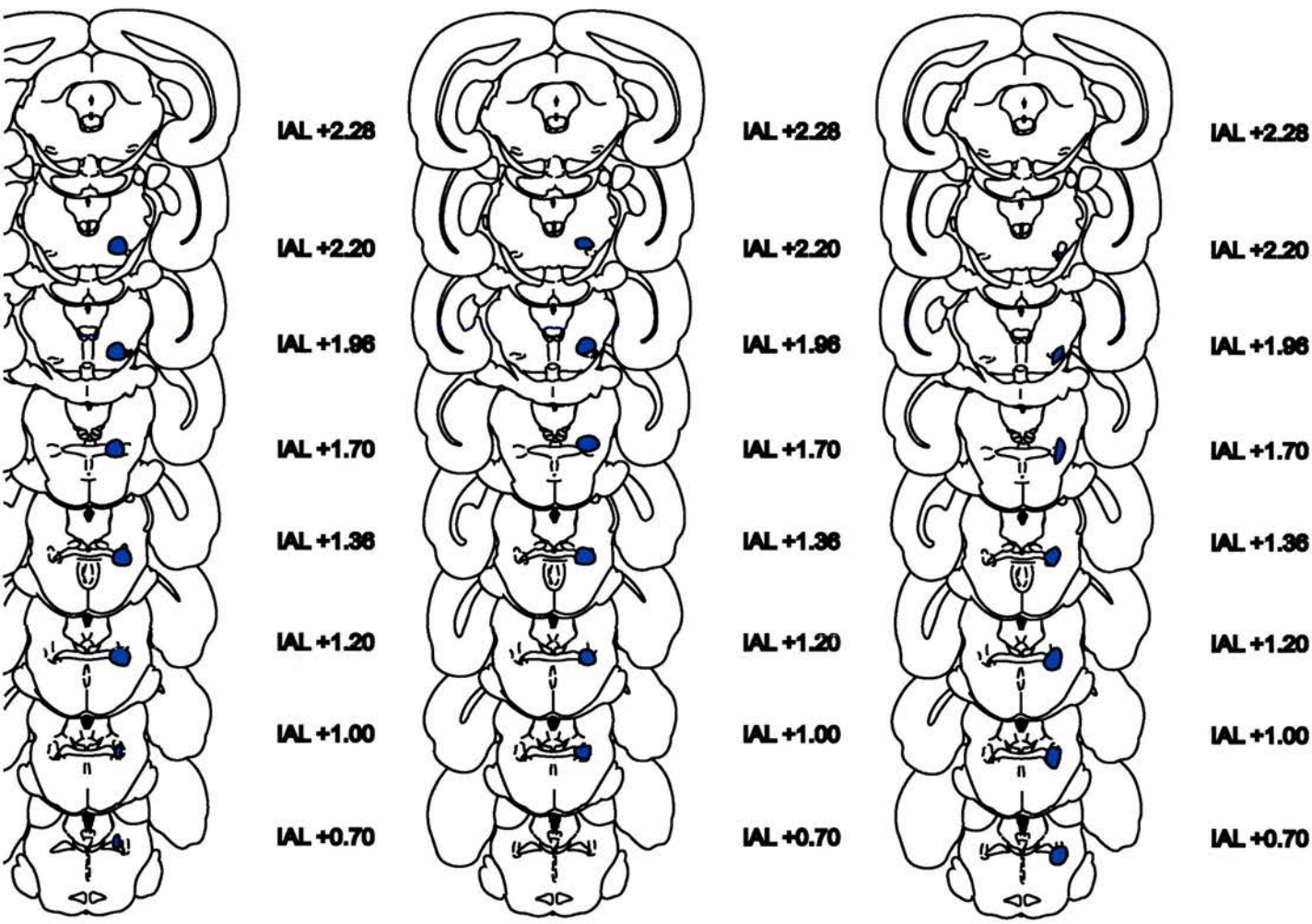


Figure 8.2 summarises the extent of damage produced by each lesion and concentration of toxin used

Figure 8.2 shows the extent of damage produced by each lesion and concentration of toxin used



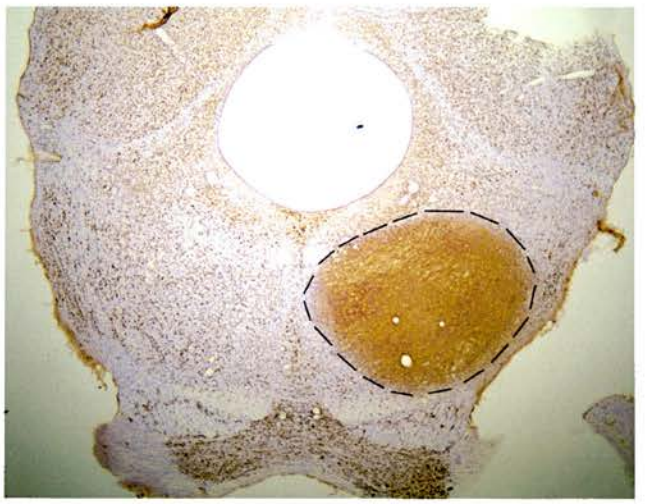
0ng/microlitre

60ng/microlitre

50ng/microlitre

Photographs A and B indicate the large area of damage produced by a toxin concentration of 200ng/µl. The area of damage clearly extends well outside the boundaries of PPTg. Photographs C and D show the damage produced by the toxin at a concentration of 50ng/µl. The damaged area is rostral of the target region. Photographs E and F show descrete lesion damage to caudal PPTg, with significant unilateral diaphorase cell loss.

A



200ng/µl, NeuN.
Dotted line indicates area of lesion damage.

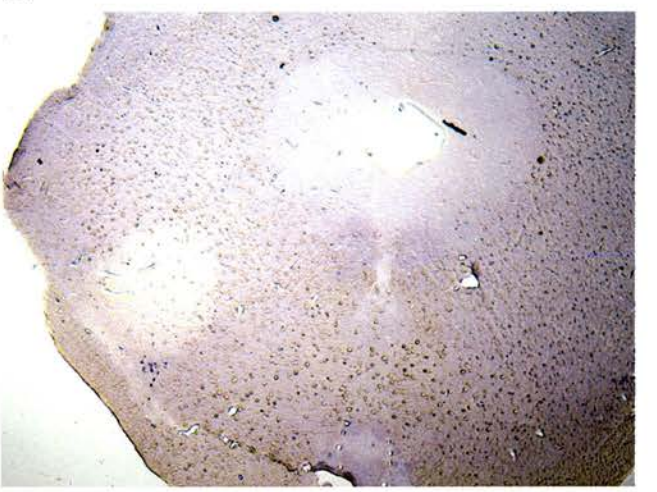
B

200ng/µl, Diaphorase.
Significant unilateral loss of diaphorase positive cells from the PPTg.

C

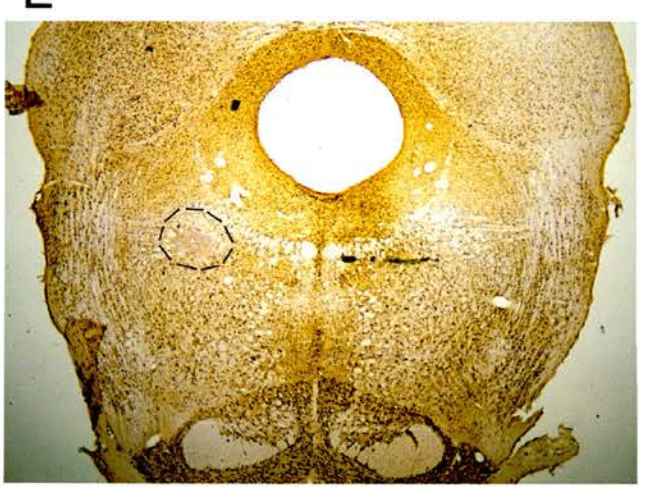
50ng/µl, NeuN.
Dotted line indicates area of lesion damage.

D



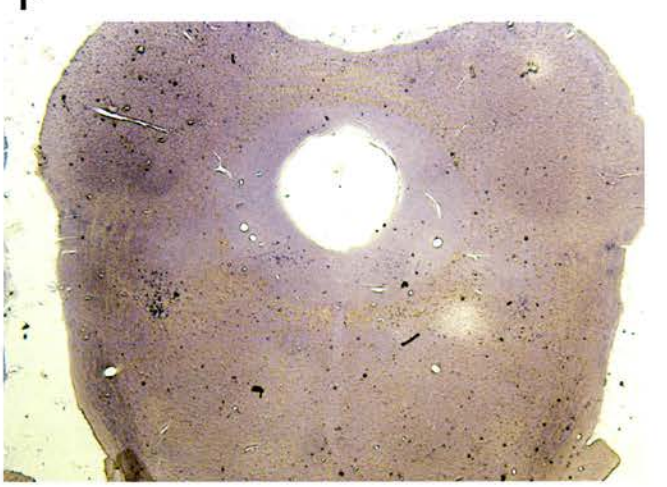
50ng/µl, Diaphorase.
Significant unilateral loss of diaphorase positive cells from the PPTg.

E



50ng/µl, NeuN.
Dotted line indicates area of lesion damage.

F



50ng/µl, Diaphorase.
Significant unilateral loss of diaphorase positive cells from the PPTg.

Photographs G and H show that the toxin damage is centered on an area dorsal of the PPTg. Photographs I and J show a lesion which has successfully destroyed diaphorase positive cells in PPTg but which is slightly too medial.

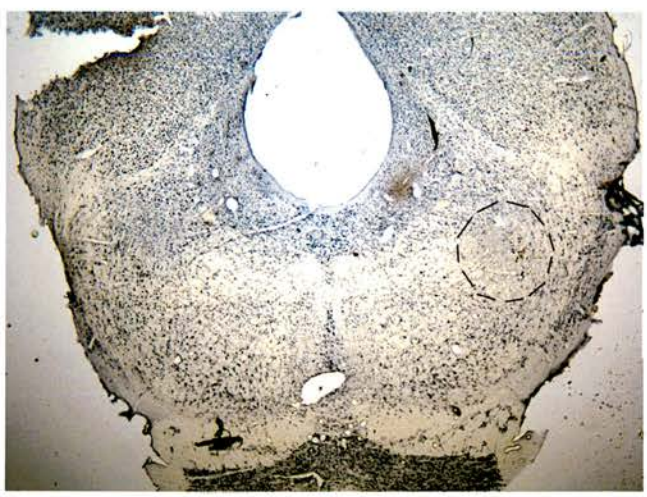
G

50ng/µl, NeuN.
Dotted line indicates area of lesion damage.

H

50ng/µl, Diaphorase.
No significant loss of diaphorase positive cells from the PPTg.

I



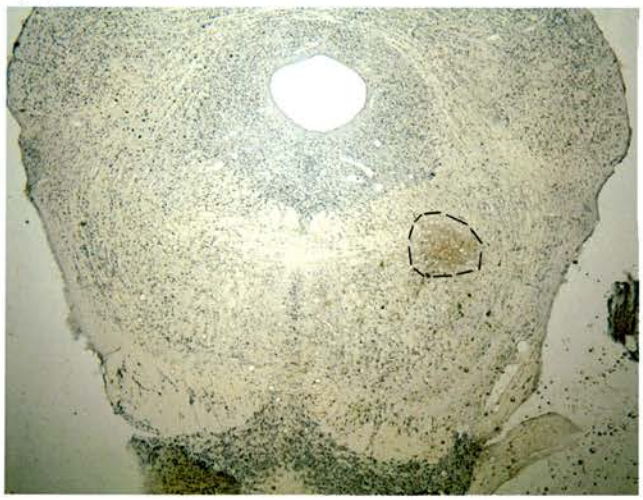
60ng/µl, NeuN.
Dotted line indicates area of lesion damage.

J

60ng/µl, Diaphorase.
Significant unilateral loss of diaphorase positive cells from the PPTg.

Photographs K and L show partial damage to the PPTg at a toxin concentration of 60ng/µl. Photographs M and N show successful unilateral damage to PPTg, with significant loss of diaphorase positive cells.

K

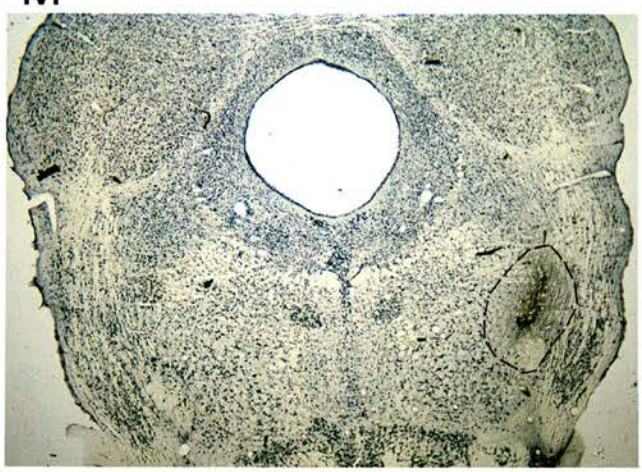


60ng/µl, NeuN.
Dotted line indicates area of lesion damage.

L

60ng/µl, Diaphorase.
Significant unilateral loss of diaphorase positive cells from the PPTg.

M



50ng/µl, NeuN.
Dotted line indicates area of lesion damage.

N

50ng/µl, Diaphorase.
Significant unilateral loss of diaphorase positive cells from the PPTg.

8.4 Discussion

The findings from this pilot lesion study suggest that while the hypocretin-sap conjugate toxin does successfully lesion the cholinergic cells of PPTg it also destroys non-cholinergic neurones within and outwith the boundaries of PPTg. This suggests that orexin / hypocretin receptors are present on several different cell types within PPTg and are also on the neurones in surrounding structures.

Although previous studies have reported that saporin produces non-specific cell damage at high concentrations, (Wiley et al. 1991) it is unlikely that this is the cause for the cell death observed here. While for the initial pilot a high concentration, 200ng/µl, was used and as photographs A & B show substantial lesion damage was produced subsequent lesions were made at the lower end of the toxin's effective range. Indeed at 40ng/µl no visible cell loss was observed.

Counting of diaphorase positive neurones within PPTg showed that, while a significant proportion of these cells were destroyed by the toxin, the percentages were always less than 50% of the hemisphere total, which is low considering the degree of toxin spread throughout the structure. However, it may be that in the PPTg, as has been found in the LDTg, HCRT- receptors exist on only a subset of the cholinergic cells (Greco & Shromani 2001).

Two different types of hypocretin / orexin have been isolated, hypocretin 1 / orexin A and hypocretin 2 / orexin B, each with it's own receptor subtype. Hypocretin (orexin) 1 receptors bind orexin A preferentially over orexin B whereas the hypocretin (orexin) 2 receptor binds both to high affinity. Some studies report that hypocretin – R1 receptors are found in significantly higher density in the PPTg

and LDTg than hypocretin – R2. This could be significant since the peptide used in the toxin is hypocretin 2 because it is easier to attach the saporin to than hypocretin 1, which does not have a free binding site (Sakurai et al. 1998). However Kilduff & Peyron (2000) claim that hypocretin 2/ B receptors are 2 to 3 times more common than 1/A receptors in all brainstem nuclei.

Since the study of hypocretin / orexin, it's projections and receptor distribution is at an early stage, it is not surprising there are some conflicts in the literature. Indeed, the level of projecting hypocretin fibre density is also disputed. While Peyron et al. (1998) report dense fibre innervation of the PPTg and other mesopontine nuclei, Cutler et al. (1999) report finding very few hypocretin-1 fibres in any of the pontine nuclei, yet Nambu et al. (1999) found moderately dense orexin fibres in PPTg and densely packed fibres within LDTg.

What is clear from the present data is that hypocretin-saporin is not an ideal tool for selectively lesioning cholinergic PPTg, not only may it not effectively target all cholinergic cells within the structure, it appears to destroy non-cholinergic neurones as well. Therefore, an important direction for future PPTg research is finding a suitable toxin that will produce specific lesions of PPTg neural subtypes. It is not possible to use a nerve growth factor, saporin conjugate, like 192 IgG-saporin that has been so successful in producing cholinergic lesions of the basal forebrain since such receptors are not found on PPTg neurones.

This study has been successful in developing a lesion methodology that produces smaller more discrete lesions of PPTg than the traditional syringe method used in previous studies. Micropippette lesions using traditional excitotoxins such as

ibotenic acid and NMDA should help to significantly reduce unintentional damage to brain structures surrounding the target region.

A particular problem of developing selective agents to lesion specific cell groups within PPTg is that that the neurotransmitters found there, acetylcholine and glutamate for example, are widely distributed throughout the brian, and neuropeptides are often found in several neural groups (Vincent et al 1986, Manaye et al 1999). Urotensin II is a peptide found both in peripheral tissue and rodent brain, the receptor for this peptide co-localises with acetylcholine transferase in the mesopontine tegmentum, including PPTg and LDTg (Clark et al 2001). This discovery may provide an opportunity to selectively lesion the cholinergic cells of PPTg using, a urotensin II-toxin conjugate.

Chapter9

General Discussion

The broad aim of this research was to further define the role of the PPTg in behavioural control. Studies so far have indicated the importance of its connections with the cortico-striato-pallido-thalamic loops (Alexander et al. 1986), with deficits observed similar to those seen following damage to frontal and striatal areas. While these connections strongly suggest a role for the PPTg in cognitive functioning, this can only be determined absolutely through the careful design and application of experiments which separate possible cognitive influences from non cognitive ones, like incentive motivation, motor control and anxiety.

The PPTg, with extensive ascending and descending, afferent and efferent connections has been implicated in a wide range of functions from motor control to attention. Recently it has been suggested that a fundamental property of PPTg lesions is to induce an anxiety like state. Podhorna & Franklin found increased anxiety in PPTg lesioned rats exposed to the elevated plusmaze. They made fewer open arm entries and spent less time in the open arms of the maze than sham operated controls, throughout several weeks of testing, indicating that these rats also failed to habituate to the maze. In a social interaction test increased anxiety was reported as measured by decreased social investigation such as sniffing and following, and increased freezing and defensive behaviour. Additionally, in a conditioned fear paradigm though PPTg lesioned rats did not show higher levels of fear than controls, freezing behaviour did persist for longer than control rats during extinction trials.

However, in the first experiment reported here no evidence of heightened anxiety was found following PPTg lesions, in either elevated plusmaze or food neophobia tests. Yet, rats with lesions of the adjacent cuneiform nucleus did show abnormally high levels of anxiety in both these paradigms. Findings from the studies reported here confirm what a literature review would suggest about cuneiform function. The cuneiform nucleus is clearly important in mediating behavioural changes in response to threatening stimuli. Given its position as a major target of the ipsilateral descending projection from the superior colliculus as well as its role in mediating physiological responses to stress it seems that the cuneiform nucleus is probably part of an anatomically low level, rapid response system vital for dealing with unexpected threatening events.

In a complementary study measuring, food, water and sucrose intake rats with ibotenate lesions of the PPTg over consumed 20% sucrose solution compared to normal rats, studies conducted here confirmed for the first time that these rats can successfully modulate their energy intake by reducing consumption of other energy sources. We know that the deficit is not motivational as PPTg lesioned rats successfully discriminate between solutions of different concentrations and increase their consumption with increasing concentration, unlike rats with ventral striatal lesions, the only abnormality is in total volume consumed. These results, along with those of several other studies, suggest that, while basic motivational behaviours remain intact following excitotoxic lesions of the PPTg, disinhibited consummatory responses to positively rewarding stimuli are observed.

However, this disruption of behavioural control is not seen solely in response to reward. It was hypothesised that, given the increase in anxiety like behaviour exhibited by cuneiform lesioned rats, these animals may be more sensitive than normal to a weakly aversive quinine solution. However surprisingly, while cuneiform lesioned rats did not differ from sham lesioned controls in their consumption, PPTg lesioned rats overconsumed quinine contaminated water compared to the other groups. This is the first example of disinhibited consummatory behaviours towards an aversive stimuli following this lesion. This suggests that either PPTg lesioned rats are less sensitive than normal to this noxious tasting solution specifically or, taken with findings from other studies reported here, they are abnormal in response to potentially dangerous stimuli. For example, while there was no apparent increase in anxiety, PPTg lesioned rats did display disinhibited exploratory behaviours on the elevated plusmaze. It is likely that the disinhibited responses seen following lesions of PPTg reflect a lack of behavioural control possibly as a result of disruption to fronto-striatal connections.

Another aspect of the work reported here was methodological. The hypothesis that it is damage to the cuneiform nucleus and not the PPTg which produces elevations in anxiety reported in a number of studies, was confirmed. The NMDA lesions of caudal PPTg produced significant damage to the cuneiform nucleus, and behavioural changes seen reflected both PPTg and cuneiform damage with disinhibited consummatory and exploratory behaviour accompanied by increased anxiety, as measured by the elevated plusmaze and openfield paradigms. These results suggest that differences in lesion technique and consequently patterns

of tissue damage could account for inconsistencies seen in the experimental literature. For example the fact that van der Kooy and Bechara report a state dependent abnormality in response to food and drug rewards in PPTg lesioned rats, concluding that the PPTg modulates the postitively reinforcing effects of these rewards only in non deprived, free fed and drug naïve states. Yet these results have not been replicated using a slightly different lesion technique, Alderson et al (2001) & Keating et al (2002) found no deprivation state dependent effects of PPTg lesions in a conditioned place preference paradigm.

Lesion studies entail destroying part of the brain and evaluating the subsequent effect on behaviour. It is important that an effective technique is used to ensure maximal damage to the target area while minimising effects on surrounding regions. This work has highlighted the importance of using an accurate and consistent lesion technique, in order to ensure that behavioural effects recorded are the result of damage to the target region. Electrolytic lesions are non selective and destroy all tissue around the tip including fibres of passage. Therefore any conclusions drawn about the specific functions of the target area must be cautious, as functions of other connected regions could also be dramatically altered (Carlson 1994). There are other, often undiscussed, effects of commonly used toxins. For example studies have indicated demyelination following NMDA lesions, which recovers with time but means disruption of connections between as well as cells within the target region for a period following surgery (Brace et al. 1997).

To help define more accurately the functional importance of given brain regions an alternative technique to lesion studies was used. Anatomical mapping

using the Fos protein produced complementary results to earlier lesion experiments. The cuneiform nucleus, but not the PPTg was significantly activated by exposure to the elevated plusmaze. The fact that lesioning the cuneiform nucleus increases anxiety, and exposure to anxiogenic stimuli activates it, further suggests that this structure in important in modulating anxiety states possibly integrating autonomic inputs with low level motor output.

In recent years much work has gone into developing more targeted toxins which produce selective lesions of specific neural groups within a given brain area. An important direction for future research into PPTg function is to elucidate the specific role of neural subtypes within the structure. It was hoped that a neuropeptide-toxin conjugate hypocretin-saporin would facilitate this work by selectively lesioning the cholinergic neurones of PPTg. However this was not successful since the neuropeptide appeared to bind to several neural types both within and out with the PPTg. On the other hand, the more refined surgical technique used to pilot this toxin produces smaller more accurate lesions, even with traditional excitotoxins, thus minimising toxin spread and reducing the chances of damage to surrounding structures confounding results.

In summary, the work reported here has shown that the PPTg is not vital for modulating anxiety type behaviour, however it has confirmed the importance of this structure in behavioural control, not only in response to rewarding but also aversive stimuli. Taken together findings from the present studies and results from previous studies do suggest and abnormality in action selection which is neither anxiety nor reward specific. Shallice's model of action selection proposes that the supervisory

attentional system is probably located in the frontal lobes of the brain since damage to this area produces deficits such as perseveration, disihibition and impaired decision making. Given the PPTg's anatomical position as an important outflow station for information from cortex and striatum it is possible that lesions of this area disrupt outputs to low level sites of initiation and action resulting in the disinhibited, perseverative and inappropriate behaviours observed in a variety of experimental paradigms.

However, disruption to behavioural control processes does not account so well for the attentional abnormalities frequently reported following removal of this structure. It is likely that such attentional functions of the PPTg are modulated via connections with thalamic and basal forebrain nuclei. Future research into PPTg function must dissect these two apparent roles by isolating the functions of its' many neural connections. One way of doing this is selectively lesioning neural groups within PPTg and investigating the subsequent behavioural changes while remaining neurones continue to function. Not only would such studies help define the role of the PPTg specifically it may also provide clues about the brains neural architecture and how such brainstem nuclei influence and are influenced by higher level forebrain and cortical structures. A particular difficulty in selecting an appropriate agent to selectively lesion PPTg is the extensive co-localisation of neuropeptides and neurotransmitters GABA and glutamate in the cholinergic and non cholinergic neurones of the structure, as well as their wide distribution throughout the entire brain. One potential solution is a toxin conjugate of the peptide urotensin II found in

rodents in both peripheral and CNS tissue, receptors for which appear to co-localise with acetylcholine transferase in the PPTg and LDTg (Clark et al 2001).

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