

MIDBRAIN CONTROL OF MICTURITION IN THE RAT

by

ELLA STONE

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Neuronal Networks Group
Neuroscience and Neurophysiology Section
School of Clinical and Experimental Medicine
College of Medical and Dental Sciences
University of Birmingham

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ABSTRACT

The role of the periaqueductal grey (PAG) in the central control of micturition was investigated in urethane-anaesthetised rats, with the aims of furthering understanding of the central control of micturition and identifying novel therapeutic targets for urinary incontinence. Experiments using microinjection of GABA_A agonists and antagonists into the midbrain showed that transmission through a localised region of the caudal ventrolateral PAG (cvlatPAG) is critical for reflex voiding and the micturition pathway is normally subject to tonic inhibitory GABAergic control. Experiments to determine the role of dopamine in controlling micturition by selectively lesioning dopamine-containing neurons in the cvlatPAG and microinjection of dopamine agonists and antagonists were inconclusive and require further work. Micturition could however be suppressed completely by trains of electrical stimulation applied throughout the midbrain. Microinjection of an excitatory amino acid over the same area reduced the frequency of micturition without disrupting the pattern of voiding. Though further work is required to determine the mechanism by which electrical stimulation inhibits reflex micturition, collaboration with clinical colleagues has indicated the exciting translational potential of electrical stimulation of the midbrain in human patients to treat urinary disturbances that have proven refractory to pharmacotherapy.

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PUBLICATIONS

FULL PAPERS

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Stone E, Coote JH, Allard J & Lovick TA. (2011). GABAergic control of micturition within the periaqueductal grey matter of the male rat. *J Physiol* **589**, 2065-2078.

ORAL COMMUNICATIONS

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Stone E, Coote JH, Allard J & Lovick TA (2009). Midbrain control of micturition in the rat. *Proc Physiol Soc* **15**, C100.

POSTER COMMUNICATIONS

Stone E, Janardhanan PSJ, Coote JH & Lovick TA (2011). A micturition switch in the periaqueductal grey matter in rats. *Proc Physiol Soc* **23**, PC42.

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LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ABC	Avidin-biotin complex
AC	Anterior commissure
APO	Apomorphine
Arb. U	Arbitrary units
ATP	Adenosine triphosphate
BIC	Bicuculline (GABA _A antagonist)
BP	Blood pressure
BPM	Beats per minute
Br.PM	Breaths per minute
CoCl₂	Cobalt chloride
cvlat	Caudal ventrolateral
D1	Dopamine 1 subtype receptor
DAB	Di-aminobenzoate
DBH	Dopamine β-hydroxylase
DBS	Deep brain stimulation
DLH	D,L-homocysteic acid
dPAG	Dorsal periaqueductal grey
EMG	Electromyograph / electromyography
EUS	External urethral sphincter
Fig.	Figure
fMRI	Functional magnetic resonance imaging
GABA	γ-aminobutyric acid

HR	Heart rate
ID	Inner diameter
IP	Intraperitoneal
IUS	Internal urethral sphincter
L-DOPA	L-3,4-dihydroxyphenylalanine
L-region	Lateral region / pontine storage centre
MABP	Mean arterial blood pressure
MCC	Maximal cystometric capacity
ME5	Mesencephalic trigeminal nucleus
mmHg	Millimetres of mercury
Musc	Muscimol (GABA _A agonist)
MRI	Magnetic resonance imaging
n	Number in group
NHS	Normal horse serum
NO	Nitric oxide
OD	Outer diameter
PAG	Periaqueductal grey
PBS	Phosphate buffered saline
PC	Posterior commissure
PFA	Paraformaldehyde
PMC	Pontine micturition centre
PSB	Pontamine sky blue dye
PVG	Periventricular grey
RF	Respiratory flow
RN	Red nucleus
RR	Respiratory rate
SC	Superior colliculi

SNM	Sacral nerve modulation
STIM	Stimulation
Teg	Ventral tegmentum
TH	Tyrosine hydroxylase
TH+	Tyrosine hydroxylase positive
UI	Urinary incontinence
vPAG	Ventral periaqueductal grey
VPL	Ventral posterolateral nucleus of the hypothalamus
W/V	Weight / volume

CHAPTER 1: GENERAL INTRODUCTION

Medical advances, improved sanitation and increased quality of life have led to an increased life expectancy and an aging population. United Kingdom (UK) Government statistics suggest that for the first time in England and Wales since records began, the percentage of the population made up by people above 65 years old exceeds that made up by under 16 year olds. Between 1981 and 2007 the growth in the population above retirement age was around 1%, however between 2006 and 2007 this growth doubled to 2% (UK National statistics, 2008). The increase in life expectancy inevitably means an increase in diseases associated with aging. One such disease with high prevalence amongst the elderly, though certainly not exclusively associated with age, is urinary incontinence.

1.1 URINARY INCONTINENCE

The term 'urinary incontinence' (UI) refers to the inability to control voiding adequately, or a leaking of urine in-between voiding. There are numerous types of incontinence covered by the term: stress urinary incontinence (urine loss associated with coughing, laughing etc.); urge urinary incontinence (where the urge to void comes on suddenly and cannot always be suppressed until an appropriate time); mixed urinary incontinence (combined symptoms of stress and urge incontinence); and enuresis (the involuntary loss of urine, which can occur in the daytime or at night). Accurate assessment of the prevalence of urinary incontinence throughout the community and institutional settings is often complicated by sample bias, a reliance on self-reporting of past events and

inconsistencies in the definition of incontinence between different studies (Lagace *et al.*, 1993; Cheater & Castleden, 2000).

O'Brien *et al.* (1991) estimate incontinence prevalence at 1 million women and 100,000 men in the UK suffering from regular incontinent episodes. Their prediction is based on an initial postal questionnaire, followed up with interviews of those who replied. In their initial survey, they found that 16.1% of women (aged 35 and above) and 7.4% of men (of the same age category) regularly suffer from incontinence ('regular' is defined as two or more episodes a month) (O'Brien *et al.*, 1991). More recently, Milsom (2009) suggests that 10% of women of all ages suffer from incontinence, and this value increases to 20% of women over the age of 70, though he points out that this may be a modest prediction as not all sufferers will approach the medical profession. A review of studies which estimate the prevalence of urinary incontinence in the community and institutions has been undertaken by Cheater and Castleden (2000), who summarise studies which estimate a range from 3 – 58% prevalence in the community and 6 – 72% prevalence in institutional settings.

The cost of UI to the UK National Health Service is substantial, with estimates of up to £536 million per year, and on top of this there is substantial monetary cost for the individual in terms of prescription charges, over-the-counter drugs, pads and travel expenses for medical appointments (Turner *et al.*, 2004). As well as the financial demands, there are significant social costs associated with UI. Some of these include social isolation (for fear of embarrassment with uncontrollable bladder emptying),

depression and a decreased quality of life (Temml *et al.*, 2000; Margalith *et al.*, 2004; Ko *et al.*, 2005).

1.2 THEORIES OF INCONTINENCE

Incontinence occurs where there is a failure to store or release urine adequately. The pathologies underlying incontinence are varied. The slightly higher prevalence in females may be explained by incontinence as a result of the trauma of giving birth, but the inequality in incidence becomes less significant after menopause and is equal between men and women in age groups of 75 years and above (McGrother *et al.*, 1987).

There are a number of theories that explain incontinence where an obvious nerve or tissue injury is absent, which focus on neurological changes and physiological alterations in the bladder wall and external urethral sphincter.

1.2.1 Non-neurologic causes

A common cause for incontinence in males presenting with an inability to empty the bladder is benign prostatic hyperplasia. This can be treated effectively with surgery in 66% of cases. There is a similar prognosis for females suffering urinary retention as a result of a prolapsed vagina which causes bending and consequent blockade of the urethra (Keane & O'Sullivan, 2000). Obstruction of the urethra in guinea pigs caused a decrease in the passage of electrical signals between neighbouring myocytes of the detrusor, which did not fully recover after the obstruction was removed (Seki *et al.*,

1992a, b). This could explain why success rate in humans suffering benign prostatic hyperplasia, treated with surgery lies at 66%, not 100%, as one would expect if the problem was a simple obstruction.

Another theory of incontinence suggests that bladder over-activity leading to urge incontinence has a myogenic basis. Brading (1997) suggests that increased activity of the detrusor muscle could be explained by increased tonic activity in myocytes within the detrusor, and a lower threshold for the spread of excitability between neighbouring cells (Brading, 1997), which could lead to the urgency that so many sufferers report. Other research has proposed that the disease could have a neural basis.

1.2.2 Neurologic causes

In a comprehensive review of literature on the neural control of micturition, de Groat (1997) puts forward the argument that changes in both peripheral and central neural pathways can lead to bladder over-activity. He suggests that neuronal changes could mean a reduction in central inhibition of the micturition pathway, a reduction in sensitivity of the bladder and EUS to descending inhibition, an increase in excitatory input to the micturition reflex pathway, or an increase in afferent activity from the bladder in to the micturition circuitry (de Groat, 1997). The suggestion of a neural origin for incontinence is supported by neuro-imaging studies in humans which have shown decreased activity (measured by functional magnetic resonance imaging) in the orbitofrontal cortex in incontinent compared to continent subjects (Griffiths *et al.*, 2005), and the high incidence of urge incontinence in patients with Parkinson's disease – a disorder known to be

caused by a loss of central dopaminergic neurons (Singer, 1998; Sakakibara *et al.*, 2010).

As Brading and de Groat show in their 1997 reviews, there are likely to be a number of underlying pathologies that result in incontinence. To be able to treat the problem pharmacologically, it is important to be able to understand the underlying cause, otherwise treatments simply mask the symptoms, as opposed to targeting the cause of the disorder. This is likely to lead to less efficacious results. However, in reality, often the cause of incontinence is unknown and available treatments ineffective.

1.3 THERAPIES FOR INCONTINENCE

Current treatments for urinary incontinence include both behavioural and pharmacological approaches. Behavioural methods include monitoring of fluid intake and avoidance of drinks in the evening with the aim of reducing nocturnal enuresis. Pharmacological approaches primarily target the bladder, and often aim to reduce the activity of the detrusor muscle.

No existing therapeutic approach is completely successful in 'curing' incontinence, which is most likely a combination of mixed underlying pathologies and also as a result of targeting the symptoms as opposed to the cause of the problem. Most success is achieved in treating urge incontinence (Andersson, 2000). Some of the available

therapies have been included in this introduction, but for a more comprehensive review see Andersson (2000).

1.3.1 Antimuscarinic drugs

The first line of treatment in those who present themselves to a medical professional with an over-active bladder is likely to be antimuscarinic drugs. These act via antagonism of M-type muscarinic receptors in the bladder wall to prevent over-activity (Andersson, 2000). These systemic drugs have undesirable side effects such as xerostomia, constipation, blurred vision, dyspepsia and nausea (Lazzeri & Spinelli, 2006). However the benefits of reduced urinary frequency, reduced sensation of urgency, increased voiding volume and an increase in quality of life are presumed sufficient to out-weigh the potential side effects (Chapple *et al.*, 2008).

1.3.2 Botulinum toxin

It was initially hypothesised that injection of botulinum toxin A into the detrusor could prevent detrusor activity solely through preventing pre-synaptic acetylcholine release, thus reducing the power of the parasympathetically-mediated detrusor contraction (Schurch *et al.*, 2000). It has since been realised that the effects of botulinum A are more complex. A review by Apostolidis *et al.* (2006) proposes that the effects of botulinum toxin A (increasing bladder capacity, compliance and increasing the volume voided at the first reflex detrusor void) are brought about via inhibition of vesicular release of adenosine triphosphate (ATP) and substance P as well as acetylcholine. They also suggest that the botulinum toxin A has direct effects on myofibroblasts within

the lamina propria causing changes in the expression of receptors within the plasma membrane (Apostolidis *et al.*, 2006).

1.3.3 Antidepressants

Imipramine is an agent used to treat depression, and has a spectrum of effects via action on a number of neurotransmitter systems, including systemic antimuscarinic action and serotonin and noradrenaline re-uptake inhibition (Andersson, 2000). It has shown some success in treating incontinence as a result of bladder instability, though it is suggested that these success rates with imipramine may be more attributable to the change in habits alongside the administration of the drug, as opposed to an actual effect of the drug itself. There are also undesirable side effects such as xerostomia and constipation (Castleden *et al.*, 1986)

1.3.4 Sacral nerve modulation

Electrical stimulation of the sacral spinal cord via electrodes connected to an implanted pulse generator, (sacral nerve modulation (SNM)) has proven effective in reducing urinary disturbance caused by urge urinary incontinence and non-obstructive urinary retention that is refractory to other therapies (Tanagho *et al.*, 1989; Van Kerrebroeck & Marcelissen, 2011; Monga *et al.*, 2012). The mechanism of action of SNM is unclear, but clinical studies suggest that this treatment is effective in up to 70% of patients (Monga *et al.*, 2012). Implantation of the neuromodulatory system is considered to be minimally invasive, however some patients report pain and discomfort during stimulation, but this can be limited by the patient since the implantable pulse generator can be switched off

when not required, and in serious cases the electrodes can be repositioned (Van Kerrebroeck & Marcelissen, 2011).

The available therapies have limited efficacy amongst incontinence sufferers, which is likely to be explained by a varied spectrum of the pathology of incontinence. To be able to tackle incontinence more successfully, it is essential to further the understanding of the normal control of continence, to be able to target areas of the control system that may be inactive or damaged in incontinent subjects.

1.4 CURRENT UNDERSTANDING OF CONTINENCE

The bladder acts a reservoir, and collects urine until a socially acceptable time for emptying. The organ consists of a smooth muscle wall (the detrusor), which is lined with transitional epithelium. This specialised epithelium can accommodate a large degree of stretch as the bladder fills with urine. The bladder receives urine, containing waste products and metabolites from the kidneys via the ureters, receiving between 0.5 and 5 ml per minute in the human (Keane & O'Sullivan, 2000). Because of the distensible properties of the detrusor and the transitional epithelium, the pressure rise in response to filling is minimal, allowing the storage of large volumes of urine. The external urethral sphincter (EUS) is a striated muscle structure, which surrounds the urethra and is responsible for preventing bladder emptying until it is safe and socially acceptable to do so. During micturition, coordinated contraction of the detrusor and relaxation of the

external urethral sphincter allow the bladder to empty. This coordination is mediated both spinally and supra-spinally.

Figure 1.1 shows a basic schematic of the micturition pathway linking the bladder and EUS, the spinal cord and the brain. Sensory information from the bladder and proximal urethra is conveyed to higher centres within the midbrain and pons via the spinal cord, which integrate this information and if deemed socially acceptable, signal to the bladder and EUS and allow bladder emptying. The following section will describe the current understanding of the role of each level (the bladder, spinal cord, and brain stem), paying attention to the tissue types and structures, innervation, and neurotransmitters involved in maintaining continence.

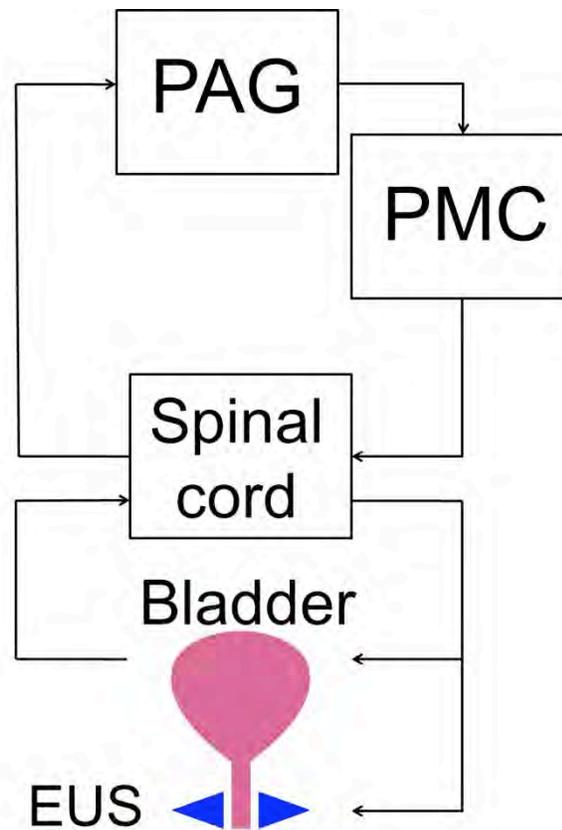


Figure 1.1. Schematic diagram of the micturition pathway. Intravesicular volume is detected by stretch receptors in the bladder wall, which signal to the sacral spinal cord. Projections from the sacral cord reach the periaqueductal grey (PAG), which also receives projections from the frontal cortex that may be involved in the conscious control of the micturition reflex. The PAG sends projections to the pontine micturition centre (PMC), which is essential for the coordination of the detrusor muscle and external urethral sphincter (EUS). The PMC signals via the sacral spinal cord and causes contraction of the detrusor in synchrony with relaxation of the EUS, allowing the bladder to empty.

1.4.1 Bladder and external urethral sphincter

The bladder and external urethral sphincter (EUS) act in concert to maintain continence. In humans and some other species, as the bladder fills, the activity of the EUS increases to counteract the pressure build-up of the fluid accumulating in the bladder. This is known as the guarding reflex, and is a spinal mechanism that helps to maintain continence (Garry *et al.*, 1959). The guarding reflex can be seen experimentally in both humans and some animals through measurement of intravesicular pressure and electromyography (EMG) of the EUS. Since there is little increase in the level of the EMG activity in the EUS during the period between voids when the bladder is being filled (Conte *et al.*, 1991; Streng *et al.*, 2004; D'Amico *et al.*, 2011), it is generally suggested that rats do not show a guarding reflex (McMurray *et al.*, 2006), and the pattern of EMG activity during voiding shows significant difference from that seen in humans. The EUS of the rat contracts intermittently during flow through the urethra, with EMG bursts interspersed between EMG silences (Conte *et al.*, 1991; Streng *et al.*, 2002; Streng *et al.*, 2004; Peng *et al.*, 2006; Sadananda *et al.*, 2011), whereas EMG of the EUS is silent during flow in humans (de Groat, 2011). It is thought that these phasic contractions of the EUS in rats facilitate the flow of urine and are important in scent marking behaviours (Van Asselt *et al.*, 1995; Park *et al.*, 1997).

1.4.2 Neuronal control of the bladder and EUS

In neonates and also following spinal injury above the lumbosacral level, the control of bladder emptying is a spinal reflex. As the intravesicular pressure builds up with the

inflow of urine from the ureters, there is an increase in firing in afferent fibres which project to the lumbosacral spinal cord. This activity is conveyed via spinal interneurons to efferent fibres projecting to the EUS. The immediate response to this increased efferent firing is an increase in the tone of the EUS, to prevent the bladder from emptying. Eventually the pressure inside the bladder overcomes the internal urethral sphincter (IUS), and there is flow of urine into the urethra. This is detected by flow receptors in the urethral wall, and their activation results in the relaxation of the EUS, allowing urine to empty from the bladder (de Groat, 2006). Without the control of supra-spinal mechanisms, this cycle could happen at any time. The input of higher centres allows bladder emptying to be deferred until an individual is in a safe and socially acceptable environment, even when the bladder is at near full capacity (Janig & Morrison, 1986; Fowler *et al.*, 2008).

1.4.2.1 Sympathetic control

During bladder filling, the detrusor is relaxed and the smooth muscle IUS is contracted. This is mediated by tonic activity in the sympathetic nervous system. Postganglionic sympathetic fibres originate in the thoracolumbar portion of the spinal cord and reach the bladder and EUS via the hypogastric and pelvic nerves (Fowler *et al.*, 2008). Their effect is to relax the detrusor (via action of noradrenaline on β -adrenoceptors) and cause contraction of the IUS (via action of noradrenaline on α -adrenoceptors), (de Groat, 2006; Michel & Vrydag, 2006).

1.4.2.2 Parasympathetic control

During voiding, the detrusor contracts and the EUS relaxes, allowing urine to be expelled. This is mediated by parasympathetic projections from the sacral spinal cord (Fowler *et al.*, 2008) and also the lower lumbar cord in rats (Morrison 1997). These parasympathetic fibres reach the bladder in the pelvic nerves, and cause contraction of the detrusor in response to the release of acetylcholine (ACh), which acts principally on M3 receptors in the detrusor, and also via M2 receptors (Hegde, 2006). ACh also facilitates contraction via action on presynaptic M1 receptors, causing the release of more ACh during activity, and can also inhibit neurotransmitter release via M4 receptors (Somogyi *et al.*, 1998; Fowler *et al.*, 2008). As well as ACh, adenosine triphosphate (ATP) and nitric oxide (NO) released from nerve terminals contribute to the parasympathetic control of the bladder. ATP elicits contractile activity, predominantly via the P2X₁ receptor in humans, and it has been shown that expression of other subtypes of the P2X receptors are up-regulated in some pathological conditions (O'Reilly *et al.*, 2001). The role of nitric oxide in the expulsion phase is contentious, with some suggesting it functions only under pathological conditions (Andersson & Arner, 2004). However, it has also been suggested that NO released from parasympathetic nerve terminals relaxes the smooth muscle of the internal urethral sphincter to allow flow through (Bennett *et al.*, 1995; Fowler *et al.*, 2008).

1.4.2.3 Somatic efferent control

Somatic cholinergic fibres originate in Onuf's nucleus within the sacral spinal cord and project to the EUS via the pudendal nerve (Morrison 1997). These fibres play an important role in maintaining continence when it is not socially inappropriate to void by increasing activity of the external urethral sphincter, preventing urine outflow. It is suggested that Onuf's nucleus receives projections from a lateral region of the pons, which promotes continence. This is discussed in more detail below.

1.4.2.4 Sensory afferents of the bladder and EUS

As well as carrying efferent signals to the bladder, A δ and C fibres in the hypogastric, pelvic and pudendal nerves transmit sensory information from the bladder and EUS. In the cat myelinated A δ fibres are activated by both bladder distension and contraction (Janig & Morrison, 1986; Habler *et al.*, 1993). The unmyelinated C-fibres are not activated in response to bladder filling, or in response to isovolumetric contractions of the bladder (Bahns *et al.*, 1987), but they do respond under conditions such as inflammation and mechanical stimulation, suggesting a role in transmitting sensory information on painful and pathological bladder conditions (Habler *et al.*, 1990). In the rat, the electrophysiological activity in the A δ fibres is indistinguishable from that in the C fibre afferents (Sengupta & Gebhart, 1994).

1.4.3 Central control of micturition

Transneuronal retrograde tracing techniques have identified areas of the brain likely to be involved in the central control of micturition. Immunotracing studies using the pseudorabies virus, have shown immunoreactivity in the sacral parasympathetic nucleus, other areas of the spinal cord, the PMC and also the periaqueductal grey, locus coeruleus, the subcoeruleus alpha and the red nucleus following microinjection of the virus into the detrusor and external urethral sphincter (Nadelhaft *et al.*, 1992; Vizzard *et al.*, 1995; Sugaya *et al.*, 1997; Grill *et al.*, 1999). Marson's (1997) work supported these findings, and also found pseudorabies immunoreactivity in forebrain regions when the survival times were longer. These areas were the lateral hypothalamus, the parvocellular region of the paraventricular nucleus and the medial preoptic area (Marson, 1997). These regions may play a part in the conscious control of micturition, in which decisions are made as to whether voiding is safe and socially acceptable.

1.4.3.1 The periaqueductal grey

The periaqueductal grey (PAG) is well known for its functions in pain and analgesia, the fight-fright-flight response (also known as the defence response), anxiety, cardiovascular control and lordosis behaviour (for a review see (Behbehani, 1995). However, there are indications that the PAG also plays an important role in the control of micturition. In an interesting case study by Yaguchi *et al.* (2004), a 31 year old male presented with acute urinary retention. Upon examination and after extensive tests, the only abnormality was shown up by an MRI scan: a lesion in the PAG. Treatment with

steroid therapy reduced the size of the lesion, and the patient's continence returned (Yaguchi *et al.*, 2004). This case provides a small but valuable insight into the critical involvement of the PAG in the control of micturition in humans and shows how an underlying pathology in the structure can have profound effects on continence. Other studies in humans using a number of neuroimaging techniques have suggested a role for the PAG in the control of micturition (Blok *et al.*, 1997a; Blok *et al.*, 1997b; Blok *et al.*, 1998; Griffiths & Tadic, 2008; Fowler & Griffiths, 2010).

Transneuronal retrograde tracing techniques in animals have highlighted the PAG as an area which has neural connections with the bladder and EUS (Nadelhaft *et al.*, 1992; Vizzard *et al.*, 1995; Marson, 1997). An increase in neuronal activity in the PAG evoked by an intravesicular infusion of saline has also been shown in the urethane anaesthetised rat by functional magnetic resonance imaging (fMRI) (Tai *et al.*, 2009). Micturition-related neuronal firing has also been recorded in the PAG under isovolumetric bladder conditions in decerebrate cats (Liu, Sakakibara *et al.* 2004). The responsive cells were located predominantly in the ventrolateral PAG. Interestingly, when Matsuura *et al.* (2000) inhibited synaptic transmission in the ventrolateral PAG of the rat with cobalt chloride, they found that this blocked reflex micturition evoked by continuous infusion of saline into the bladder. They also found that under isovolumetric conditions, microinjection of the excitatory amino acid L-glutamate at the same sites at which cobalt chloride blocked neurotransmission, caused contraction of the bladder (as reflected by an increase in intravesicular pressure) and evoked corresponding activity in the EUS. Taniguchi *et al.* followed this work in 2002, and showed in cats that electrical

stimulation or microinjection of the excitatory amino acid D,L-homocysteic acid (DLH) into a similar region of PAG could initiate bladder contraction (coupled with appropriate activation of the EUS). These studies suggest that the ventrolateral part of the PAG contains synaptic relays that are important for normal micturition.

The descending pathway from the PAG relays in the pontine micturition centre (PMC). With the use of neuronal tracers, the ventrolateral PAG has been shown to send projections to the PMC in the cat and the PMC receives a projection from the dorsomedial region of the PAG too (Blok & Holstege, 1994; Ding *et al.*, 1998). The projection from the PAG to the PMC is bilateral since unilateral microinjection of the retrograde neuronal tracer fluorogold into the PMC, resulted in labelling on both the right and left hand side of the PAG (Taniguchi *et al.*, 2002). Matsuura (2000) showed that the excitatory effects of L-glutamate in the PAG could be blocked with cobalt chloride microinjected into the PMC (Matsuura *et al.*, 2000) thus providing unequivocal evidence that the micturition reflex pathway relays via the PMC.

1.4.3.2 The pontine micturition centre (PMC) and storage centre (L-region)

The importance of the pontine micturition centre (PMC), in the control of micturition has been recognised for over 80 years. Barrington was the first to investigate the effects of lesioning the brainstem on micturition in cats (Barrington, 1925). He found that pontine lesions either caused an increase in residual volume (despite maintenance of the desire to void as judged by persistence of the behavioural traits normally preceding voiding in

the cat), loss of consciousness of the desire to void without any increase in residual volume, or a combination of the above. Post-mortem histological analysis of the brains of the cats identified a common region in the pons, which is now known as 'pontine micturition centre' or 'Barrington's nucleus'.

As Barrington concluded, the integrity of the PMC is essential for normal micturition to take place, and it is now known that projections from the PMC, via the sacral spinal cord (Holstege *et al.*, 1979) are responsible for activating the synchronous contraction of the smooth muscle of the detrusor and relaxation of the striated EUS that allows bladder emptying to take place (Holstege *et al.*, 1979; Holstege *et al.*, 1986; Blok *et al.*, 1995; Sasaki, 2005).

As well as a pontine micturition centre, which allows the coordinated contraction of the bladder detrusor and relaxation of the EUS, it has been suggested that more laterally in the pons, a 'pontine storage centre' or 'lateral region' (L-region), becomes active during the storage phase. This region was identified following the discovery of projections from the region to Onuf's nucleus in the lumbosacral spinal cord in the cat (Holstege *et al.*, 1979). Electrical stimulation in this region led to contraction of the musculature of the pelvic floor and the external urethral sphincter (and anal sphincter) (Holstege *et al.*, 1986). It was later found that bilateral lesion of the region caused a decrease in bladder capacity and an increase in detrusor activity, which resulted in incontinence in cats (Griffiths, Holstege *et al.* 1990). Neuroimaging in humans has also suggested that there may be an equivalent region in the pons, which is active during the withholding of urine against a 'full' bladder (Blok *et al.*, 1997b). Though this evidence strongly suggests a

role for the L-region in the control of continence in some species (no such equivalent region has been identified in the rat (Morrison *et al.*, 2005)), no direct neural projections between the L-region and the PMC can be identified (Blok & Holstege, 1999). This suggests that unless they are linked indirectly, rather than there being some reciprocal coordination between the areas, they appear to act independently in controlling the storage and voiding phases. It is more likely that activation of the L-region continuously during bladder filling plays a role in initiating contraction of the EUS, and then during micturition, this excitatory control over the EUS is overcome by activation of the pathways via the PMC, which allow the bladder to empty.

1.5 AIMS AND HYPOTHESIS

The primary aim of the present experiments was to seek further understanding of the control of the micturition reflex at the level of the PAG using a rat model, with a secondary, more long-term aim of identifying new targets for therapeutic intervention in the treatment of urinary incontinence in human patients. Though it is suggested that the PAG may play a role in integrating and processing afferent input from the distended bladder, the specific role of this midbrain structure in the control of micturition is not fully understood. The fact that micturition can be suppressed in humans and socialised animals until an appropriate time infers that there is a higher level of control over the micturition reflex circuitry. We hypothesised that the PAG is involved in the inhibitory control over the micturition reflex that allows voiding to be deferred at will, and modulation of neuronal activity in this region might be a useful clinical tool for the treatment of urinary disturbances in human patients in the future.

CHAPTER 2: GENERAL METHODS

2.1 ANIMALS

Male Sprague Dawley rats (Charles River, Kent, UK) weighing between 206 and 410 g were housed under a 12 hour light/dark cycle, with lights on at 7 am and off at 7 pm. Temperature was maintained at 21°C with 50% humidity, and food and water were available in home cages ad libitum. All procedures were in accordance with agreed local ethical guidelines of the University of Birmingham and complied with the UK Animals (Scientific Procedures) Act 1986. Every care was taken to minimise suffering of subjects and to reduce the number of animals used.

2.2 ACUTE SURGICAL PREPARATION

2.2.1 Anaesthesia

For terminal procedures, animals were anaesthetised with 1.4 g kg⁻¹ urethane intraperitoneally (20% weight/volume (W/V) solution, 0.7 ml 100g⁻¹ administered. Vehicle: dH₂O. Sigma, Poole, Dorset). Depth of anaesthesia was assessed as sufficient when the lower limb flexor reflex was absent. Experiments were terminated with an intravenous overdose of pentobarbitone (Sigma, Poole, Dorset), followed by cervical dislocation to confirm death.

2.2.2 Tracheal cannulation

A tracheal cannula was inserted to allow recording of respiratory flow via a spirometer and to maintain a clear airway throughout the procedure. A 15 to 20 mm longitudinal midline incision of the skin over the trachea was made to expose the underlying salivary gland tissue, which was then separated from the underlying longus colli muscle. The

vertebral portion of the sternohyoid was bluntly dissected longitudinally to expose the underlying trachea. The trachea was separated from the surrounding connective tissue and a loose ligature placed around it distally. Curved forceps were placed under the trachea to raise it clear from the surrounding tissue and a small transverse cut was made between two cartilaginous rings using a scalpel. The first 2 to 3 mm of a bevelled 30 mm polythene cannula (1.67 mm inner diameter (ID), 2.42 mm outer diameter (OD) Portex tubing, SIMS Portex Ltd, Kent, UK) was inserted pointing towards the lungs and secured with the previously placed suture.

2.2.3 Cannulation of femoral artery and vein

Cannulation of the femoral artery and vein allowed monitoring of blood pressure and provided a route for administering fluids and drugs respectively. A longitudinal incision of the skin of the inner right thigh was made to expose the underlying inguinal fat pad, which was ligated, divided and reflected with clamps to allow access to the underlying vessels. Blunt dissection was used to separate the femoral artery and the femoral vein from surrounding tissue and from each other, and obvious side branches from the vessels were tied to prevent blood loss during the cannula insertion. The artery was tied off peripherally and a loose ligature placed centrally and a small artery clip was used to temporarily occlude the vessel while the cannula was inserted. A small incision was made in the vessel between the clip and the tight ligature and a polythene cannula (1.14 mm ID, 1.57 mm OD Portex tubing, SIMS Portex Ltd, Kent, UK) filled with heparinised saline (10 units ml⁻¹ Multiparin™ CP Pharmaceuticals Ltd, Wrexham, UK) was advanced

into the vessel in a retrograde direction, and secured in place with ligatures on either side of the site of entry into the vessel, and the clamp removed. A second cannula (1.14 mm ID, 1.57 mm OD Portex tubing, SIMS Portex Ltd, Kent, UK) containing 0.9% physiological saline was inserted into the femoral vein in an identical fashion, but in an orthograde direction for administration of fluids (Gelofusine Ecobag, plasma substitute, B Braun Medical Ltd., Sheffield, UK) and supplementary anaesthesia if necessary.

2.2.4 Laparotomy and cannulation of the bladder

Rats were placed prone in a stereotaxic instrument (Kopf Instruments, California, USA) in the attitude described by Paxinos and Watson (1986). A temperature sensing probe attached to a homeothermic blanket system (Harvard Apparatus, Kent, UK) was inserted into the rectum and secured in place by taping to the tail. The lower body was twisted and the left leg was raised to give access to the lower abdomen. Rat tooth forceps were used to lift the abdominal skin away from the underlying abdominal muscle. A midline abdominal incision extending 20 mm from the point at which the penis folds allowed exposure of the underlying linea alba muscle. This muscle was lifted with rat tooth forceps to separate it from the abdominal contents, and gently cut until perforated. This hole was extended 20 mm along the midline from the fold of the penis, taking care not to puncture the abdominal contents. The laparotomy was extended 10 mm bilaterally at the distal end so that the muscle and skin could be reflected.

A cotton bud was used to gently reflect the abdominal contents and locate the bladder with minimal tissue damage. Where the bladder contained residual urine, the bladder

pressure and hence the tension in the wall was increased by gently squeezing the bladder between the index and middle finger, placed close to the bladder neck. For cannulating the bladder, a 23 gauge hypodermic needle was cut 10 mm from the tip and the blunt end inserted into a length of polythene tubing (0.58 mm ID 0.96 mm OD Portex Tubing, SIMS Portex Ltd, Kent, UK). The assembly was filled with saline and connected to a T-piece with one arm connected to a pressure transducer (AD Instruments, Oxfordshire, UK) and the other to an infusion pump (Harvard Apparatus, Kent, UK). Cannulation was achieved by piercing the bladder through the dome. Insertion into an empty bladder meant the bladder dome was grasped gently with blunt forceps so that sufficient tension could be applied to the wall to allow insertion of the needle. Once the cannulation had been performed the tubing was secured to a supportive bar with Bluetack[®], and was not moved for the remainder of the experiment. Cling film was placed over the wound to prevent drying.

2.2.5 Electromyographic recordings from the external urethral sphincter (EUS)

A cotton bud was used to gently reflect the bladder and forceps were used to separate connective tissue overlying the urethra. Excess abdominal fluid was absorbed with cotton wool. Two stainless steel needle electrodes fashioned by soldering the 10 mm ends from 26G hypodermic needles (insulated except for the tips) to a length of insulated flexible wire were inserted bilaterally into the external urethral sphincter proximal to the symphysis pubis which remained intact (Fig. 2.1). For connections see

Fig. 2.2. Correct placement of the electrodes in the EUS was verified by the appearance of EMG activity in response to gentle application of pressure on to the bladder.

2.2.6 Craniotomy

A longitudinal midline incision was made to expose the dorsal surface of the skull. Skin was reflected using clamps, and the periosteum was scraped back. Areas of the skull seeping blood were sealed by “spot” drilling the bone over the area with a dental drill (W&H, Austria). A limited craniotomy 3 mm caudal to lambda (approximately 5 mm x 5 mm) was made to expose the cortex overlying the midbrain. The skull was thinned using the drill until the remaining bone could be removed with fine forceps. The tip of a hypodermic needle was used to pierce the dura and this hole was extended using Vannas microscissors.

2.2.7 Electrode placement

An injection cannula, made from either stainless steel or glass, depending on the experiment (see individual chapter methods for details) was inserted into the midbrain using a microdrive (Harvard Apparatus, Kent, UK) attached to a stereotaxic frame (Kopf Instruments, California, USA). Stereotaxic placement of the microcannula into the PAG was originally guided by coordinates from the Paxinos and Watson Rat Brain Atlas (1986). Often the optimal coordinates lay directly below the confluence of the sagittal and transverse sinuses so the cannula was inclined caudally at a 10° angle to be able to reach the more caudal regions of the PAG. With experience, cannula placement was guided by the location of cerebral vessels, in particular the sagittal and transverse

sinuses. The pattern of smaller surface vessels varied between experiments, and these vessels were carefully avoided to prevent blood loss. Depth measurements were also guided by the atlas of Paxinos and Watson (1986) and ranged from -4.0 mm to -10.00 mm below the surface of the cortex, in order to reach the most dorsal and most ventral midbrain respectively (Paxinos & Watson, 1986).

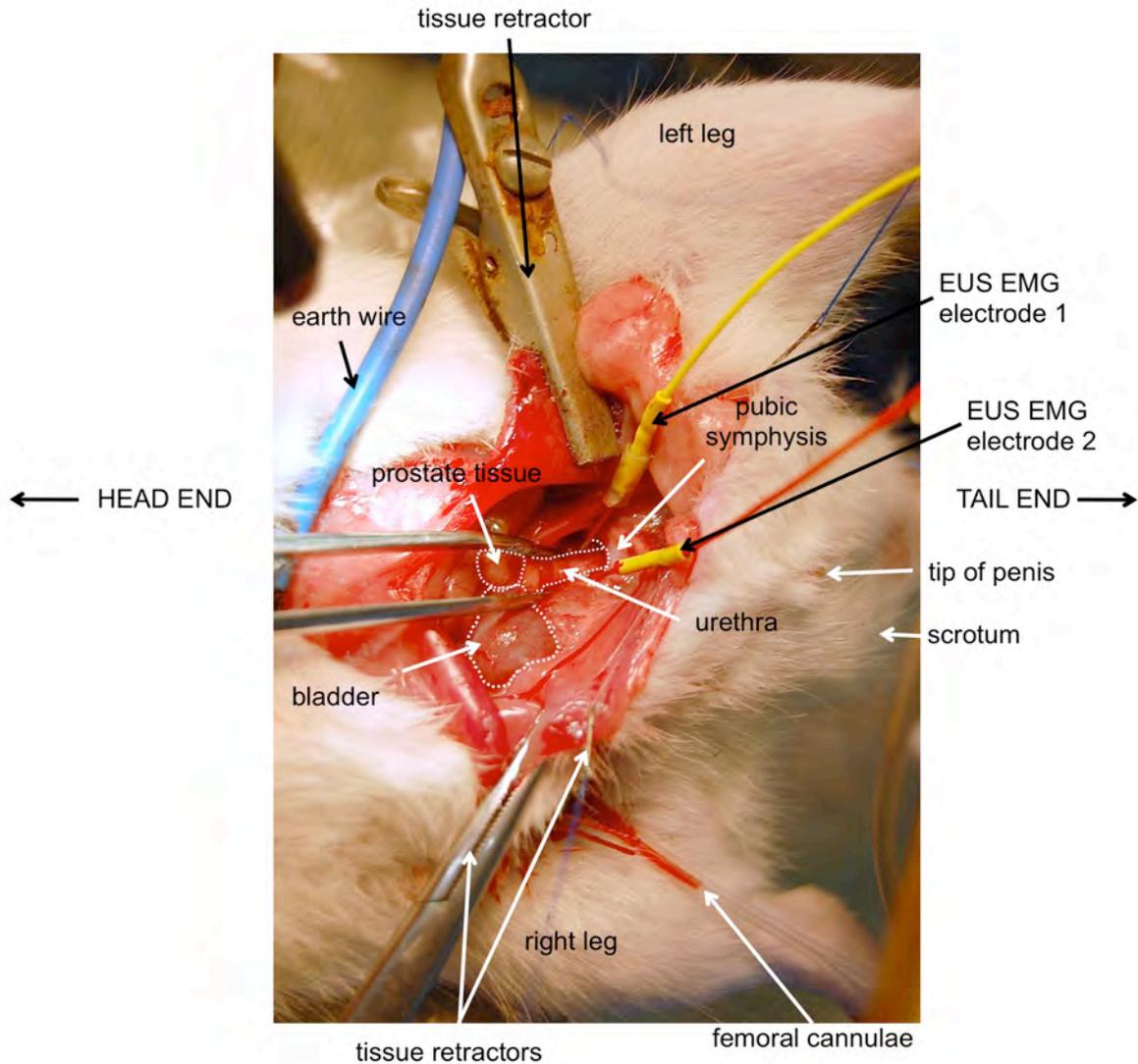


Figure 2.1. Labelled photograph showing location of electrodes placed to record EMG activity of the external urethral sphincter (EUS). Two electrodes, insulated except for the tip were inserted into the EUS on either side of the urethra and proximal to the pubic symphysis to record bipolar EMG activity of the skeletal musculature.

2.2.8 Cystometry

In the majority of acute experiments, saline was infused continuously into the bladder at a rate of 6ml h^{-1} via polyethylene tubing connected to an infusion pump (Harvard Apparatus Syringe Pump 11, Model: 555-1111, Kent, UK). Intravesicular pressure was recorded via a pressure transducer connected to a t-arm from the infusion tubing. The urethral outlet remained open in all experiments. Drop output during cystometry was recorded by an automatic infra-red drop counter (AD Instruments, Oxford, UK), which was positioned below the channel that drops naturally exited the urethral opening. A manual counter was always available and was used in the initial stages of setting up the drop counter in the correct position, and also at times when the trajectory of the drop channel changed and the automatic counter did not detect all drops.

2.3 DATA COLLECTION

All data inputs in acute experiments fed into a PowerLab data acquisition system running Chart 4.2.3 software module. Signals from the pressure transducers (AD Instruments, Oxford, UK), the spirometer, and infra-red drop counter all fed in via bridge amplifiers (AD Instruments, Oxford, UK). EMG electrodes were connected to a headstage, and the signal was amplified ($\times 5000$) and filtered (low freq. 15 – 500 Hz, high freq. 50 – 5000 Hz) using NeuroLog apparatus (Digitimer, Welwyn Garden City, UK), before feeding into the PowerLab. The manual drop counter connected directly into PowerLab that sampled at 100 Hz (AD Instruments, Oxford, UK). The thermostatic heat blanket displayed the temperature of the preparation; this was monitored regularly to ensure it remained within a physiological range.

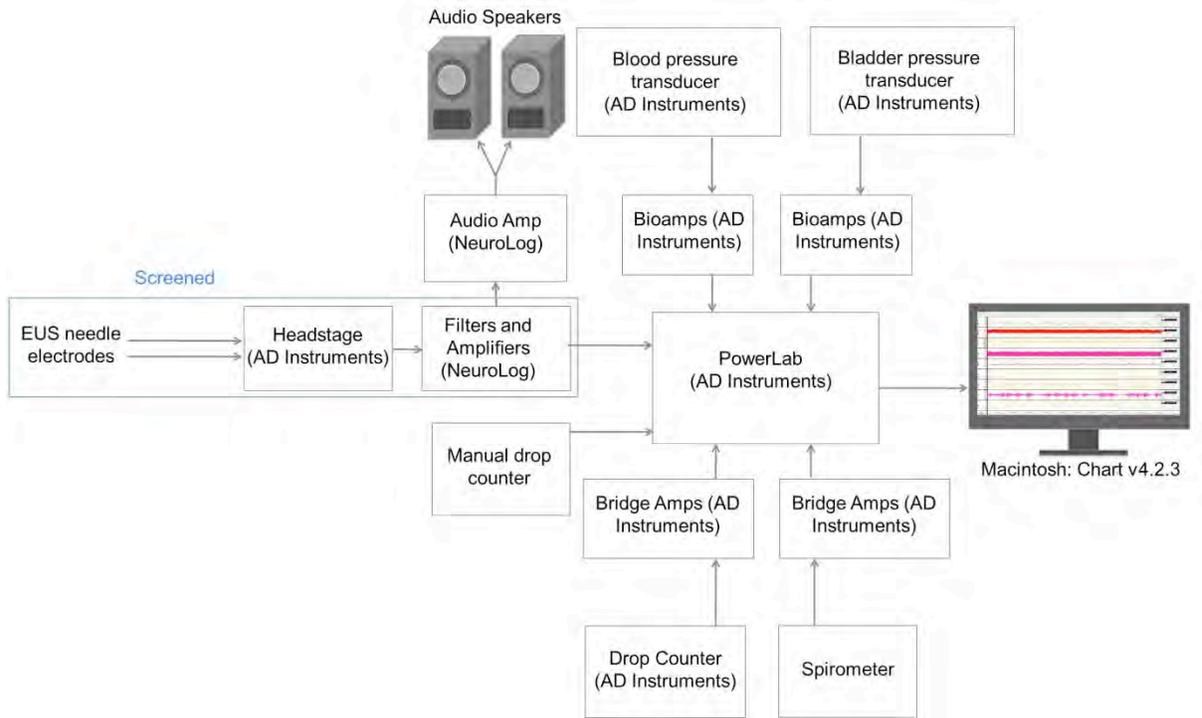


Figure 2.2. Diagram representing inputs and outputs to NeuroLog and PowerLab apparatus that allowed recording of intravesicular pressure, blood pressure, heart rate, respiratory flow, urine output, EMG of the external urethral sphincter (EUS) during cystometry in the urethane anaesthetised rat.

2.4 PREPARATION OF TISSUE FOR HISTOLOGICAL ANALYSIS

In terminal experiments that involved the microinjection of drugs and electrical stimulation of the midbrain, rats were killed by an overdose of pentobarbitone whilst under urethane anaesthesia and cervically dislocated (see Section 2.2.1: Anaesthesia). The whole brain was then removed and placed into 10% formol saline solution for fixation. Once fixed, the midbrain was blocked for sectioning (see Section 2.5: Sectioning brain tissue for histology). In terminal experiments that required immunohistochemical analysis of the midbrain, rats were terminally anaesthetised and perfused with fixative retrogradely via the descending aorta with the jugular veins cut bilaterally to allow the perfusate to drain from the circulation (see Chapter 5, Section 5.3.4). Brains were removed and stored in paraformaldehyde before being transferred to 30% sucrose in phosphate buffer (0.1M, pH 7.4) to afford cryoprotection (see Chapter 5, Section 5.3.5 for immunohistochemistry protocol).

2.5 SECTIONING BRAIN TISSUE FOR HISTOLOGY

Blocks of brain containing the PAG were frozen on a microtome (Measuring and Scientific Equipment Ltd., London, UK) and cut in coronal sections either 40 or 60µm thick. Sections that were required for locating microinjection tracks only were cut to a 60µm thickness and were mounted on to gelatinised slides, dried at 37°C and then stained as outlined in the individual chapter methods. Where fluorescent dyes were used for identification of microinjection sites, alternate sections were nissl stained to

identify brain structures, and the remaining sections were left unstained and viewed under the fluorescence microscope.

2.6 ANALYSIS

All data are represented as mean \pm standard error of the mean (SEM) unless stated otherwise. Normality of the distributions was assessed with a D'Agostino-Pearson Omnibus test in Prism 4 (Omnibus K2 version, GraphPad Software, Inc, California, USA). Paired data sets displaying a normal distribution were compared with a Student's Paired *t* test unless otherwise stated. Data failing to follow the normal distribution were analysed with the Mann-Whitney U Test. Data from 3 or more groups, were compared using a one-way ANOVA with Bonferroni post-hoc analysis. Significance was assumed when $P \leq 0.05$. Where administration of a drug or stimulation produced a response, the maximal effect during the response was compared to the mean response before administration.

2.6.1 Definition of parameters of a contraction

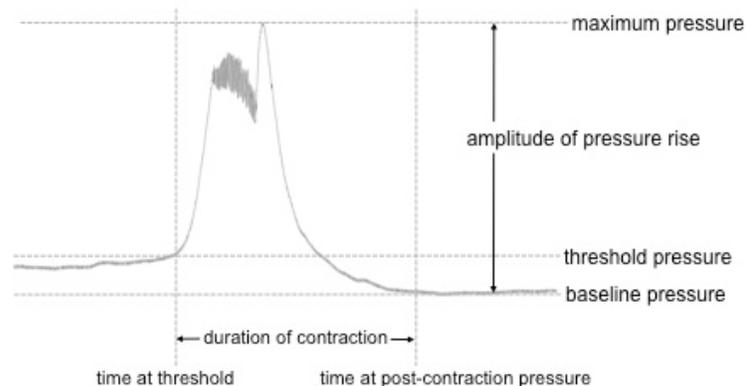


Figure 2.3. Sample contraction. Broken lines define parameters measured for analysis of the periodic increases in bladder pressure associated with voiding during continuous infusion of saline into the bladder of the urethane anaesthetised rat.

**CHAPTER 3: THE EFFECTS OF GABA_A AGONISM AND ANTAGONISM IN THE
PERIAQUEDUCTAL GREY ON THE MICTURITION REFLEX EVOKED BY
CONTINUOUS INTRAVESICULAR INFUSION OF SALINE IN THE URETHANE
ANAESTHETISED MALE RAT.**

3.1 INTRODUCTION

Micturition is a spino-midbrain-spinal event that requires coordinated contraction of the detrusor and relaxation of the external urethral sphincter (EUS) to allow the bladder to empty. Humans and socialised animals are able to defer bladder emptying until a socially appropriate moment, which suggests that there is inhibitory control over the micturition reflex. However, the level of the micturition circuitry at which this inhibitory control is imposed is presently unknown.

As the bladder fills with urine, afferent signals from stretch receptors in the bladder wall are transmitted to the sacral spinal cord. After synapsing in the sacral spinal cord, these fibres project to the periaqueductal grey (PAG) (Vizzard *et al.*, 1995; Marson, 1997). The ventral PAG sends dense bilateral projections to the pontine micturition centre (Blok & Holstege, 1994; Ding *et al.*, 1998) which is known to be integral to the coordination of contraction of the bladder and relaxation of the EUS that allows voiding, via projections to the sacral spinal cord (Barrington, 1925; Holstege, 2005; Fowler *et al.*, 2008; Drake *et al.*, 2010; Holstege, 2010).

Recent evidence suggests that inhibitory control of the micturition reflex may be exerted at the level of the PAG. The PAG contains a dense population of inhibitory γ -aminobutyric acid-containing (GABAergic) interneurons (Reichling & Basbaum, 1990; Lovick & Paul, 1999; Griffiths & Lovick, 2005) which exert tonic inhibitory control over

output neurons that coordinate behavioural responses such as flight and anti-nociception (Bandler *et al.*, 1985; Carrive *et al.*, 1986; Morgan & Clayton, 2005). Furthermore, there is evidence that the extracellular levels of GABA in the ventrolateral PAG are reduced during periods of intravesical distension (Kitta *et al.*, 2008), suggesting that GABAergic inhibition of the micturition circuitry is withdrawn to permit voiding to occur.

3.2 AIMS AND HYPOTHESIS

We hypothesised that GABAergic neurotransmission is involved in the inhibitory control of the micturition circuitry as it passes through the periaqueductal grey. Our initial objective was to determine the location at which the micturition reflex pathway passes through the PAG using microinjection of the GABA_A agonist, muscimol to inhibit local neurotransmission at the site of injection in the urethane-anaesthetised rat during continuous cystometry. We then investigated whether GABA tonically inhibits the micturition reflex at the level of the PAG by microinjecting the GABA_A antagonist, bicuculline into the region.

3.3 METHODS

Male Sprague Dawley rats ($n = 79$, $197 - 410$ g, Charles River, Kent, UK) were anaesthetised with urethane (20% W/V, 0.7 ml 100g^{-1}) and prepared for cystometry along with recording of cardiovascular and respiratory parameters as described in Chapter 2: General methods, Section 2.2). A dorsal craniotomy exposed the surface of the brain, which allowed insertion of a stainless steel cannula (500 μm diameter) into the periaqueductal grey (PAG) and surrounding midbrain for the administration of drugs.

3.3.1 Microinjection of drugs

The effects of microinjection of the GABA_A agonist, muscimol (5×10^{-3} M, pH adjusted to 7.4 using NaOH, vehicle: 0.9% saline, Sigma-Aldrich, Gillingham, UK), GABA_A antagonist, bicuculline methiodide (2×10^{-2} M, pH 6.8, vehicle: dH₂O, Sigma-Aldrich, Gillingham UK) and saline (0.9%, pH 7.4) into the PAG on the micturition reflex evoked by continuous infusion of saline into the bladder (6 ml h^{-1}) were investigated in the majority of experiments ($n = 73$). Doses were based on those reported to have been used in the literature and in preliminary experiments, and were administered in 50 nl volumes. To allow post mortem histological location of microinjection sites, approximately 2 mg ml^{-1} of pontamine sky blue (PSB, Searle Diagnostics, High Wycombe, UK) was added to the muscimol and saline solutions. The bicuculline solution contained 0.2% yellow-green fluorescent microbeads (FluoSphere, Invitrogen Molecular Probes, USA) since PSB caused precipitation of bicuculline from solution.

3.3.2 Stepped volume infusion experiments

In 6 experiments, the bladder was emptied manually using a 1 ml syringe connected via a 3-way tap to the infusion tubing. Saline was then introduced into the bladder in stepped increases in volume and the effects on EMG activity of the EUS were recorded. This protocol was repeated following microinjection of bicuculline into the PAG in order to determine whether the changes in EUS activity seen following microinjection represented a primary response or a secondary effect related to changes in intravesicular pressure.

3.3.3 Histology

Experiments were terminated by an intravenous overdose of pentobarbitone anaesthetic followed by cervical dislocation. Brains were removed, stored in 10% formol saline and sectioned (60 μm , see Chapter 2: General methods, sections 2.4 and 2.5 for details). Sections from experiments in which pontamine sky blue dye was used to mark injection sites were all stained with Neutral Red (1% in water, Sigma-Aldrich, Gillingham, UK). Only alternate sections were stained with Neutral Red where fluorescent beads were used for identification of brain structures, and unstained sections were viewed under a fluorescence microscope exciting at 450 – 500 nm and detecting at 475 – 700 nm (Olympus BH-2 Microscope, filtered with Omega Optical XF100-2, Olympus, Southend-on-Sea, UK). Images of whole sections were enlarged x17.5 using a Carl Zeiss documenter (Carl Zeiss, Welwyn Garden City, UK). The location of the centre of the microinjection site as judged by the most densely stained region with PSB dye or

fluospheres, was marked on to a map of four representative levels of the PAG (P -5.6, P -6.2, P -8.0 and P -8.8) taken from the stereotaxic atlas of Paxinos and Watson (1986).

3.3.4 Analysis

All values are represented as mean \pm SEM, and unless otherwise stated, paired data sets have been compared with a Student's paired t test, with significance assumed where $P \leq 0.05$. See Chapter 2: General methods, section 2.6 for details).

3.3.4.1 Bladder and EMG of the EUS

A bladder 'contraction' was defined as a change in the intravesicular pressure of more than 7 mmHg, and the parameters of contractions measured have been defined in Chapter 2: General methods, Section 2.6.1). For analysis of the frequency of contractions, voiding and non-voiding contractions were grouped together. A text file from a 60 s period of raw EMG signal from the EUS in between bladder contractions recorded in LabChart (AD Instruments, Oxford, UK) was exported in to Microsoft Excel (Microsoft, Reading, UK), where values were rectified. The area under the curve was calculated by summing the amplitude of the rectified activity in individual bins in Microsoft Excel (Microsoft, Reading, UK), see appendix A for detailed instructions.

3.3.4.2 Cardiorespiratory parameters

Mean values for arterial blood pressure, heart rate and respiratory rate over 1 min during the pre-drug control period were compared to the maximum values reached in the post-drug response period.

3.4 Results

3.4.1 Response to infusion of saline into the bladder

Continuous infusion of saline (room temperature, 0.9%, 6 ml h⁻¹) into the bladder of the urethane anaesthetised male rat elicited a micturition reflex that was characterised by (1) a sharp rise in intravesicular pressure of 7 mmHg or greater, (2) an increase in the level of tonic EMG activity in the EUS, which adopted a bursting pattern with EMG bursts followed by EMG silence and (3) drop output from the urethral meatus (Fig. 3.1).

The micturition reflex occurred at a frequency of 0.7 ± 0.04 contractions min⁻¹ (n = 68), though there was a considerable degree of inter-rat variability, with frequencies ranging from 0.1 to 1.9 contractions min⁻¹. The amplitude of the periodic increase in intravesicular pressure was 21.5 ± 0.6 mmHg (range 10.7 to 35.1) and lasted for 32.3 ± 2.4 s (range from 14 to 124 s). The number of drops per void was also highly variable, with a mean of 5.6 ± 0.4 drops void⁻¹, and range of 1 to 35 drops void⁻¹. With the exception of frequency, the mean values were the same in all treatment groups (Table 3.1). Despite this, there was no relationship between the control voiding frequency and the effect of microinjection of the GABA agonist and antagonist on the voiding frequency. In 25 rats that had EMG electrodes in the EUS, there was an increase in the level of tonic EMG activity as the pressure in the bladder increased at the onset of a contraction. At the peak of the contraction, this EMG activity adopted a bursting pattern (6.8 ± 0.1 Hz), with EMG silences interspersed between bursts. During this time, drop output from the urethra occurred. This rhythmic bursting was reflected as small oscillations in the intravesicular pressure (Fig. 3.1 A ii). Following voiding, EMG of the

EUS returned to a sustained tonic activity and the levels gradually decreased to baseline. This reflex remained stable for up to 9 hours or until the experiment was terminated.

In 11 rats (13.9%), this reflex failed to develop and as saline was infused into the bladder, the intravesicular pressure rose to 31.0 ± 1.2 mmHg. As the infusion continued, fluid began to exit the urethral meatus in a dropwise fashion (2.2 ± 0.1 drops min^{-1}) as opposed to in clusters of drops as was normally seen during micturition. There was no coordinated bursting activity in the EMG recorded from the EUS, and periodic increases in intravesicular pressure were absent. These experiments were terminated after a minimum of 2 hours and no data was obtained.

Table 3.1. Mean frequency of contractions for each group before microinjection of drug or control solutions into the PAG.

	Muscimol	Bicuculline	Control
Mean frequency \pm SEM (contractions min^{-1})	0.8 0.04	\pm 0.7 \pm 0.04	0.8 \pm 0.04
Minimum frequency (contractions min^{-1})	0.3	0.1	0.6
Maximum frequency (contractions min^{-1})	1.9	1.7	1.1

Although there was a significantly lower frequency of contractions in rats that went on to receive microinjections of bicuculline ($F(2, 142) = 5.2, P = 0.007$), there was no relationship between the control voiding frequency and the effect of microinjection of the GABA agonist, antagonist or saline on the voiding frequency.

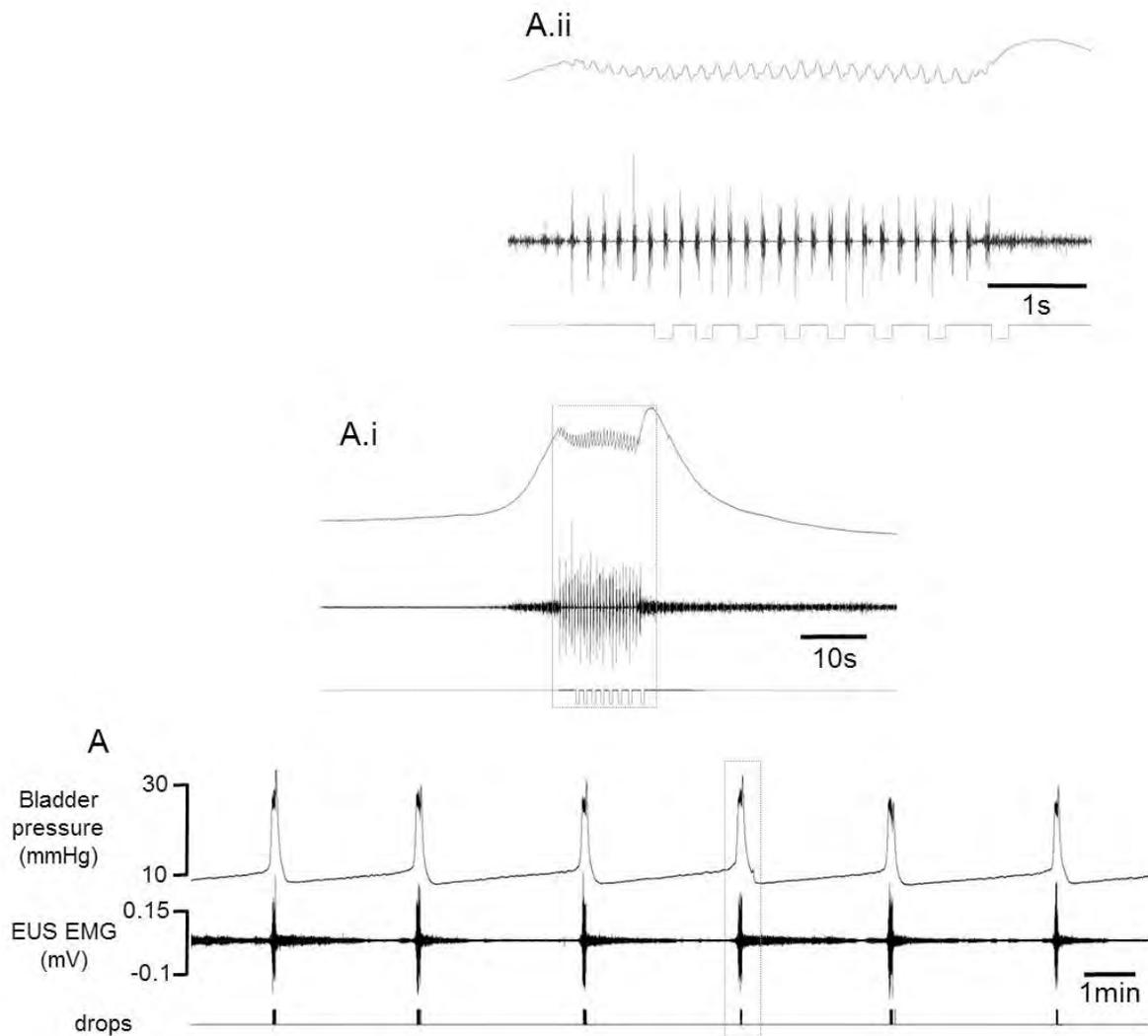


Figure 3.1. Sample sequence of voiding evoked by continuous infusion of saline into the bladder of the urethane anaesthetized rat. (A) Periodic increases in intravesicular pressure were accompanied by an increase in EMG activity and drop output from the urethral meatus. Rectangle highlights void shown on a faster time base in Ai. Ai, EMG activity of the EUS initially showed a tonic increase, and adopted a bursting pattern which was associated with drop output via the urethra. Bursting stopped shortly before the last drop was expelled and EUS EMG activity gradually declined after the void. Aii, EMG bursts were followed by EMG silence and occurred at a frequency of approximately 7Hz.

3.4.2 Effect of microinjection of a GABA_A agonist into the PAG on reflex contractions of the bladder

Microinjections of the GABA_A agonist, muscimol (250 pmol, 50 nl) were made at 61 sites distributed over a wide area of the PAG and adjacent tegmentum, on either side of the midline. At most injection sites (n = 50) there was no change in the micturition reflex evoked by continuous infusion of saline into the bladder (Fig. 3.2 A, open circles). There was no change in the frequency (0.9 ± 0.1 v. 0.8 ± 0.1 contractions min⁻¹), amplitude (19.4 ± 0.4 v. 19.6 ± 0.4 mmHg) or duration of contractions (22 ± 1 v. 26 ± 2 s before and after microinjection of muscimol respectively). There was also no significant change in the number of drops of fluid expelled per void (7.2 ± 0.6 v. 7.2 ± 0.6 drops per void).

At 11 sites (Fig. 3.2 A, filled circles, 7 on the left hand side and 4 on the right hand side), microinjection of muscimol evoked an inhibitory effect on the micturition reflex induced by continuous infusion of saline into the bladder. At these inhibitory sites, the effects of microinjection of muscimol into the left hand side of the PAG were indistinguishable from those evoked by microinjection on the right hand side (Table 3.2). Therefore data from both sides of the PAG have been pooled.

Table 3.2. Comparison of left and right PAG microinjections of muscimol on the frequency of voiding evoked by continuous cystometry.

	Left hand side of PAG	Right hand side of PAG	P =
n =	7	4	
Pre-muscimol frequency (contractions min ⁻¹)	0.9 ± 0.1	1.2 ± 0.1	0.79
Post-muscimol frequency (contractions min ⁻¹)	0.3 ± 0.1	0.2 ± 0.1	0.23

The inhibitory effects of microinjection of muscimol into the left hand side of the PAG were indistinguishable from those evoked by microinjection on the right hand side of the PAG. Therefore data from the left and right hand side have been pooled. Analysed with non-parametric Mann Whitney U test.

At 7 of the inhibitory sites, the frequency of voids was reduced from 0.9 ± 0.1 to 0.4 ± 0.1 contractions min⁻¹ (Fig. 3.3 A i, $P < 0.001$). There was no change in the amplitude of the pressure rise (23.0 ± 1.7 v. 21.0 ± 1.7 mmHg, $P = 0.22$), duration of contraction (27.0 ± 2.9 v. 39.3 ± 9.6 s, $P = 0.21$), or number of drops per void (5.5 ± 0.9 v. 6.5 ± 0.7 drops void⁻¹ $P = 0.2$) at these sites (Fig. 3.3 B, C, D consecutively). The inhibitory effect on the frequency of contractions lasted for 49.0 ± 26.2 min (range 3.3 to 236.9 min). At the other 4 sites, the micturition reflex was completely abolished (Fig. 3.4 B). Within 14.8 min (mean: 7.1 ± 3.9 min) of microinjection of muscimol into the midbrain, cyclic voiding ceased. As the infusion of saline continued, the intravesicular pressure began to rise until reaching a plateau at 20.3 ± 5.0 mmHg. Urine output resumed, but it was not in

association with any sharp rise in intravesicular pressure, or the development of bursting activity in the EUS which was normally associated with a void. Indeed, rather than the clusters of drops normally seen during a void, drops of fluid were expelled from the penis in a regular fashion at a rate of $2.7 \pm 0.3 \text{ min}^{-1}$. In 6 of the experiments the EMG from the EUS was also recorded alongside the intravesicular pressure. Following microinjection of muscimol, there was a significant decrease in the amplitude of tonic EMG activity present in between voids in comparison to control values (Fig. 3.4 C, $0.3 \pm 0.1 \text{ v. } 0.2 \pm 0.1 \text{ } \mu\text{V.s}$, $P = 0.03$, $n = 6$). At sites where there was a reduction in the frequency of the micturition reflex, the relationship between the intravesicular pressure, bursting activity in the EMG of the EUS and the drop output was maintained despite the reduction in the tonic EMG of the EUS during the inter-void period (Fig. 3.4 A).

Sites at which muscimol produced inhibitory effects were clustered together in the caudal ventrolateral (cvlat) region of the PAG at P levels -8.0 and -8.8mm (Paxinos and Watson, 1986) (Fig. 3.2 A). Within this region, injection sites at which muscimol evoked the two types of inhibitory effect ('partial': grey circles and 'complete': black circles) were intermingled.

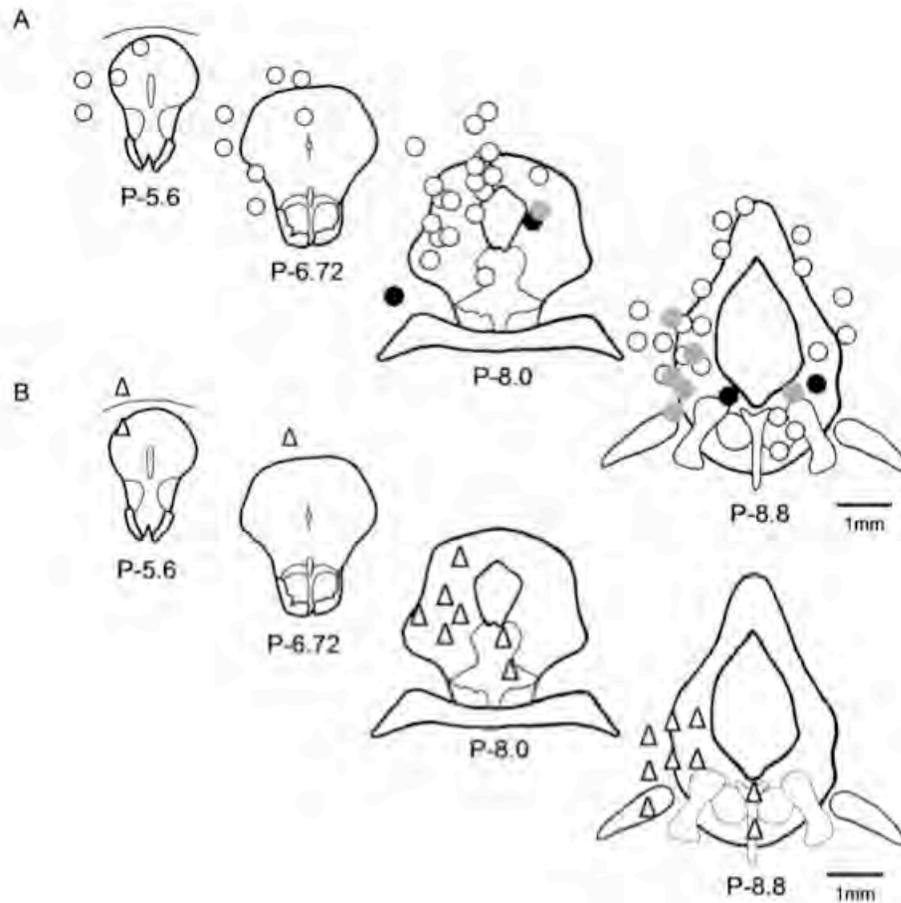


Figure 3.2. Location of sites where muscimol was microinjected in and around the PAG plotted on to outlines of the PAG taken from the Atlas of Paxinos and Watson (1986). A, Black filled circles represent the centre of sites at which microinjection of muscimol completely inhibited the micturition reflex in response to continuous intravesicular filling (6 ml h^{-1}). Grey filled circles represent the centre of sites at which microinjection of muscimol reduced the frequency of the micturition reflex in response to continuous infusion of saline into the bladder. Open circles represent the centre of sites at which microinjection of muscimol produced no change in the frequency of the micturition reflex. B, open triangles represent the centre of sites where saline was microinjected into the PAG. Numbers indicate distance (mm) caudal to Bregma.

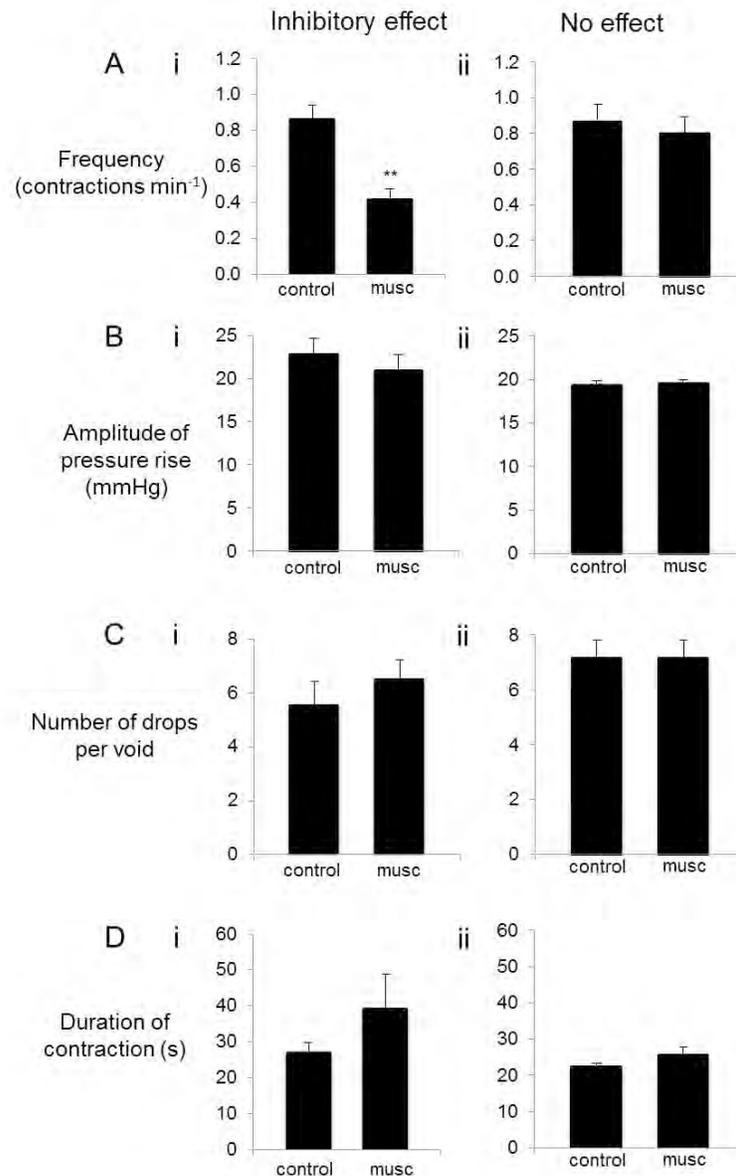


Figure 3.3. Quantification of the effect of microinjection of muscimol into and around the PAG on the micturition reflex evoked by continuous cystometry. A i, At 11 sites located in the caudal ventrolateral PAG, microinjection of muscimol significantly reduced the frequency of contractions. These sites are marked on fig. 3.2 A as grey and black filled circles. A ii, At the remaining 50 sites microinjection of muscimol did not affect the frequency of contractions (marked as open circles in the map of microinjection sites, fig. 3.2 A). Microinjection of muscimol did not affect B i and ii, the amplitude of the pressure rise associated with a void, C i and ii, the number of drops per void, or D i and ii, the duration of contraction at any sites in the midbrain during continuous cystometry. < 0.001.

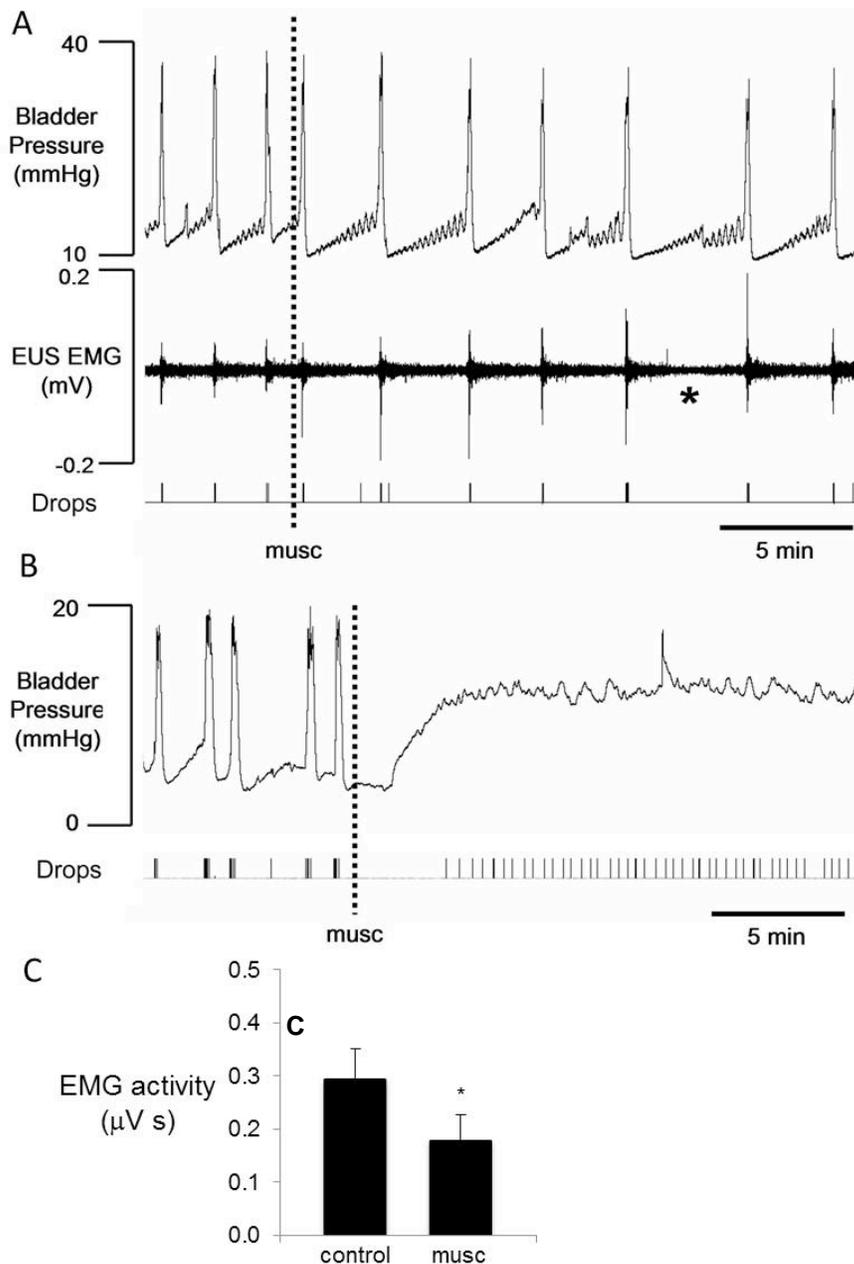


Figure 3.4. Effect of microinjection of muscimol (250 pmol in 50 nl) into the caudal ventrolateral PAG on the micturition reflex evoked by intravesicular filling: Sample traces. A, At most effective sites ($n = 7$), microinjection of muscimol reduced the frequency of reflex voiding which was accompanied by a decrease in the level of EMG activity in the EUS in the inter-void period (asterisk). B, at 4 sites, muscimol completely suppressed the micturition reflex. As the infusion of saline continued, drop output returned but was not in association with the sharp change in the intravesicular pressure that normally indicates a void. C, at the 6 inhibitory sites where EMG of the EUS was recorded, microinjection of muscimol produced a significant decrease in the EUS EMG activity during the inter-void period. * $P \leq 0.05$.

3.4.3 Effect of microinjection of muscimol into the PAG on cardiorespiratory function

Microinjection of muscimol at sites in and around the PAG did not produce changes in any of the cardiovascular and respiratory parameters recorded. This was true both at sites where muscimol reduced the frequency or abolished cyclic voiding in response to infusion of saline into the bladder (Fig. 3.5 A) and at sites where muscimol evoked no effect on micturition (Fig. 3.5 B).

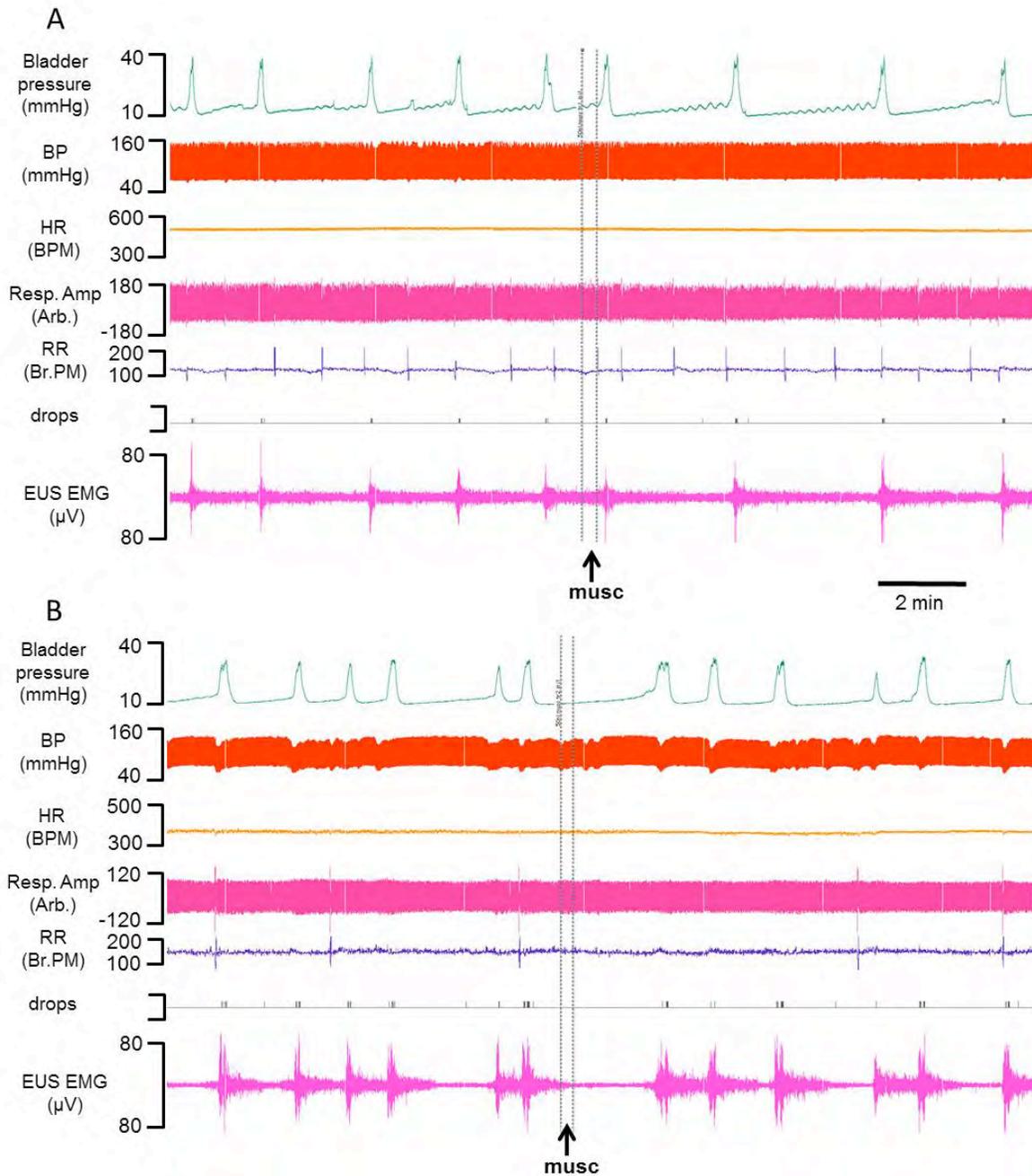


Figure 3.5. Microinjection of muscimol (250 pmol in 50 nl) evoked no changes in the cardiovascular and respiratory parameters recorded at both inhibitory (A) and 'no effect' (B) sites. Abbreviations: BP: Blood pressure, HR: heart rate, BPM: beats min^{-1} , RF: respiratory flow, Arb: arbitrary units, RR: respiratory rate, Br.PM: breaths min^{-1} , MABP: mean arterial blood pressure.

3.4.4 Control experiments

Microinjection of saline at 19 sites throughout the midbrain and PAG (Fig. 3.2 B) did not affect the micturition reflex evoked by continuous infusion of saline into the bladder (Fig. 3.6 A). There was no significant change in the frequency of contractions (Fig. 3.6 B, 0.8 ± 0.04 v. 0.8 ± 0.03 contractions min^{-1}) or any other parameters recorded. There was also no change in the mean arterial blood pressure (Fig. 3.6 C, 77.6 ± 4.3 v. 84.9 ± 5.7 mmHg), heart rate (Fig. 3.6 D, 481.1 ± 20.6 v. 492.7 ± 20.6 beats min^{-1}) or respiratory rate (Fig. 3.6 E, 164.9 ± 9.5 v. 189.4 ± 13.6).

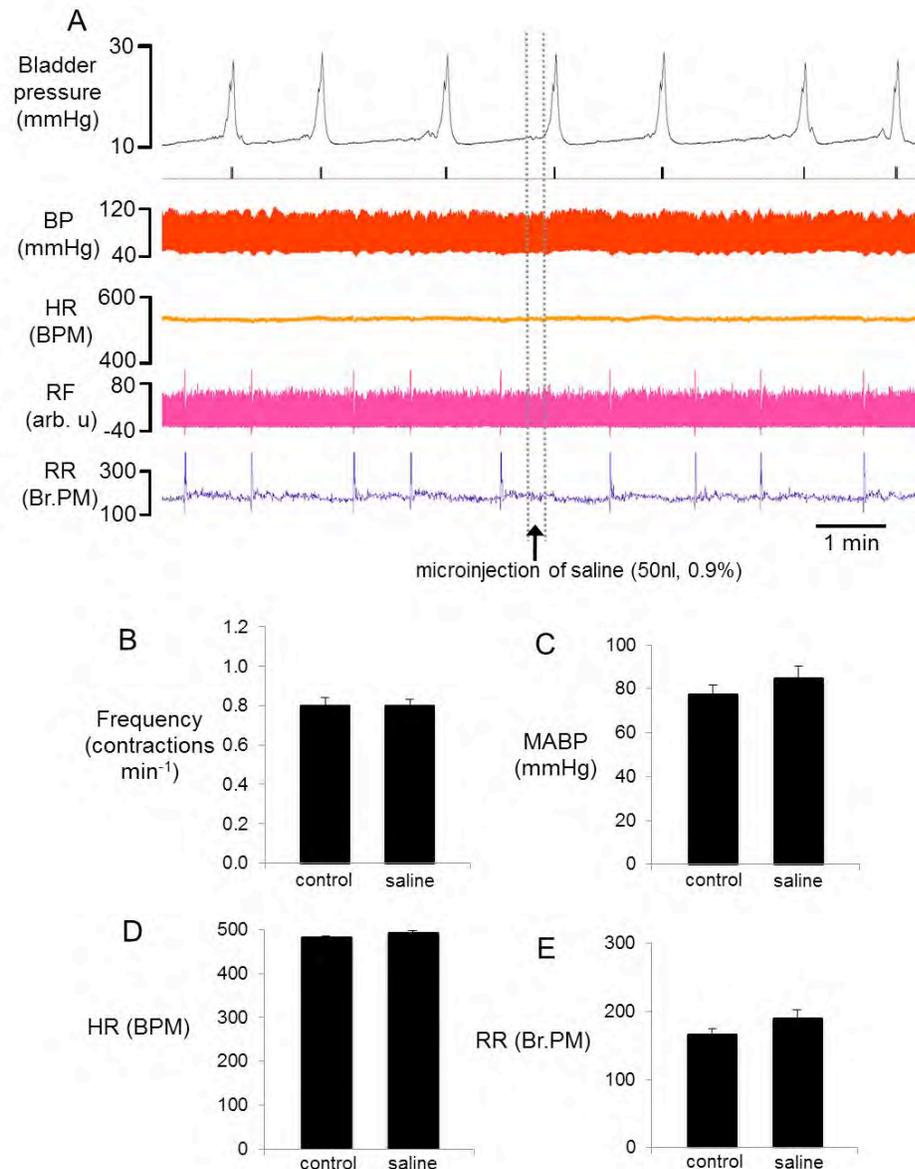


Figure 3.6. Effect of microinjection of saline (0.9%, 50 nl) into the PAG on the micturition reflex induced by continuous infusion of saline into the bladder and on the cardiorespiratory system. A, sample trace demonstrating that microinjection of saline into the PAG (between horizontal broken lines) evoked no change in the micturition reflex or cardiovascular and respiratory parameters recorded. B, microinjection of saline evoked no change in the frequency of the micturition reflex during continuous cystometry ($n = 11$). C, D, E, microinjection of saline into the PAG did not affect MABP (mmHg), HR (BPM) or RR (Br.PM) respectively. Abbreviations: BP: Blood pressure, HR: heart rate, BPM: beats min^{-1} , RF: respiratory flow, Arb. U: arbitrary units, RR: respiratory rate, Br.PM: breaths min^{-1} , MABP: mean arterial blood pressure.

3.4.5 Effect of microinjection of a GABA_A antagonist into the PAG on reflex contractions of the bladder

3.4.5.1 Microinjection of bicuculline in the ventral PAG

The inhibitory effects evoked by microinjection of muscimol into the cvlat PAG on the micturition reflex induced by continuous cystometry suggested that synapses in this part of the PAG could be subject to tonic GABAergic inhibition. To investigate this further, the GABA_A antagonist, bicuculline was microinjected into the region in the cvlat PAG where microinjection of muscimol inhibited micturition.

Microinjection of bicuculline (1 nmol in 50 nl) into the cvlat PAG facilitated the micturition reflex evoked by continuous infusion of saline into the bladder (Fig. 3.7, black filled circles and Fig. 3.8). At 16/26 sites, this effect was characterised by an increase in the frequency of the micturition reflex (from 0.6 ± 0.1 to 3.5 ± 0.5 contractions min^{-1} , $P < 0.0001$, Fig. 3.9 A). In contrast to the control period, when each contraction of the detrusor was associated with a void, following microinjection of bicuculline, non-voiding contractions developed. These were classified as periodic increases in intravesicular pressure of 7 mmHg or greater without associated expulsion of drops from the urethra (Fig. 3.8, Ai). There was also a significant reduction in the amplitude of contractions (pressure change from baseline to maximum) from 23.2 ± 1.1 to 16.3 ± 2.4 mmHg, (Fig. 3.9 B, $P = 0.02$). Following microinjection of bicuculline there was a significant increase in the amplitude of tonic EMG activity of the EUS (Fig. 3.8 A ii, from 0.2 ± 0.1 to 0.4 ± 0.1 $\mu\text{V} \cdot \text{s}$, $P = 0.02$, $n = 7$) along with a disruption of the bursting activity normally seen in the

EMG of the EUS during voiding. Although some bursts were evident these were superimposed on a tonic level of EMG activity rather than being interspersed with silences in the EMG as seen in the control period (Fig. 3.8 A i). Normal voiding returned within 21.4 ± 5.5 min of the initial microinjection of bicuculline (range 6.1 to 70.2 min).

At the remaining 10 sites, microinjection of bicuculline into the cvlat PAG during continuous cystometry inhibited the cyclic pattern of voiding completely (Fig. 3.8 B). In 6/10 cases this followed a short period (9.4 ± 2.2 min) of contractions of increased frequency (from 0.7 ± 0.2 to 1.7 ± 0.4 contractions min^{-1}). Intravesicular pressure rose and was maintained at 27.6 ± 1.1 mmHg. Low amplitude oscillations in bladder pressure were superimposed on top of the raised baseline (Fig. 3.8 B). In 3 cases where EMG activity of the EUS was also recorded there was an increase in the level of tonic EMG activity (Fig. 3.8 Bi, from 0.1 ± 0.02 to 0.3 ± 0.1 $\mu\text{V}\cdot\text{s}$). As the infusion of saline into the bladder continued, drops of fluid were expelled from the penis in an irregular manner, rather than in clusters of drops that characterised a void during the control period. Moreover, drops were not associated with the sharp rise in bladder pressure that was normally associated with a void.

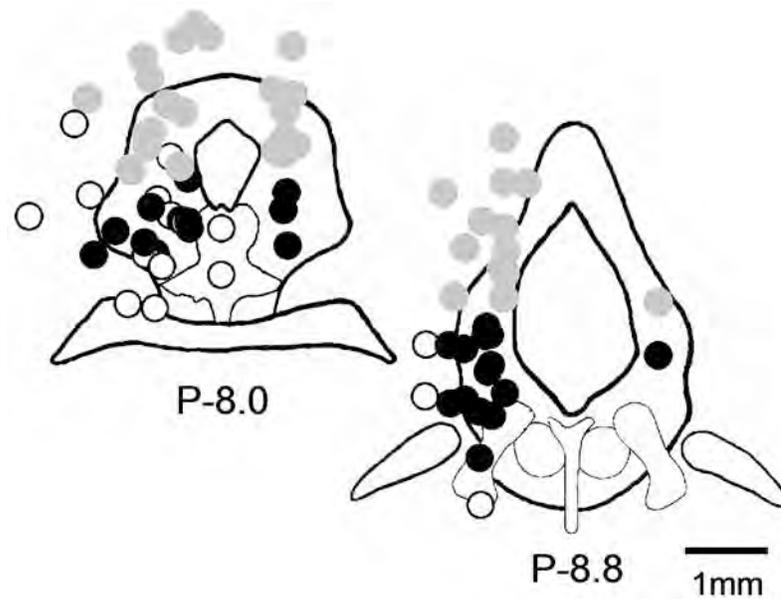


Figure 3.7. Location of sites where bicuculline was microinjected into the PAG plotted onto outlines of the PAG taken from the atlas of Paxinos and Watson (1986). Black circles represent the centre of sites at which microinjection of bicuculline facilitated micturition evoked by continuous cystometry, accompanied by only moderate cardiorespiratory changes. Grey circles represent the centre of sites where microinjection of bicuculline facilitated micturition evoked by continuous cystometry, and this was accompanied by intense autonomic activation. Open circles represent the centre of sites at which microinjection of bicuculline evoked no effect on the micturition reflex. Numbers indicate distance (mm) caudal to Bregma.

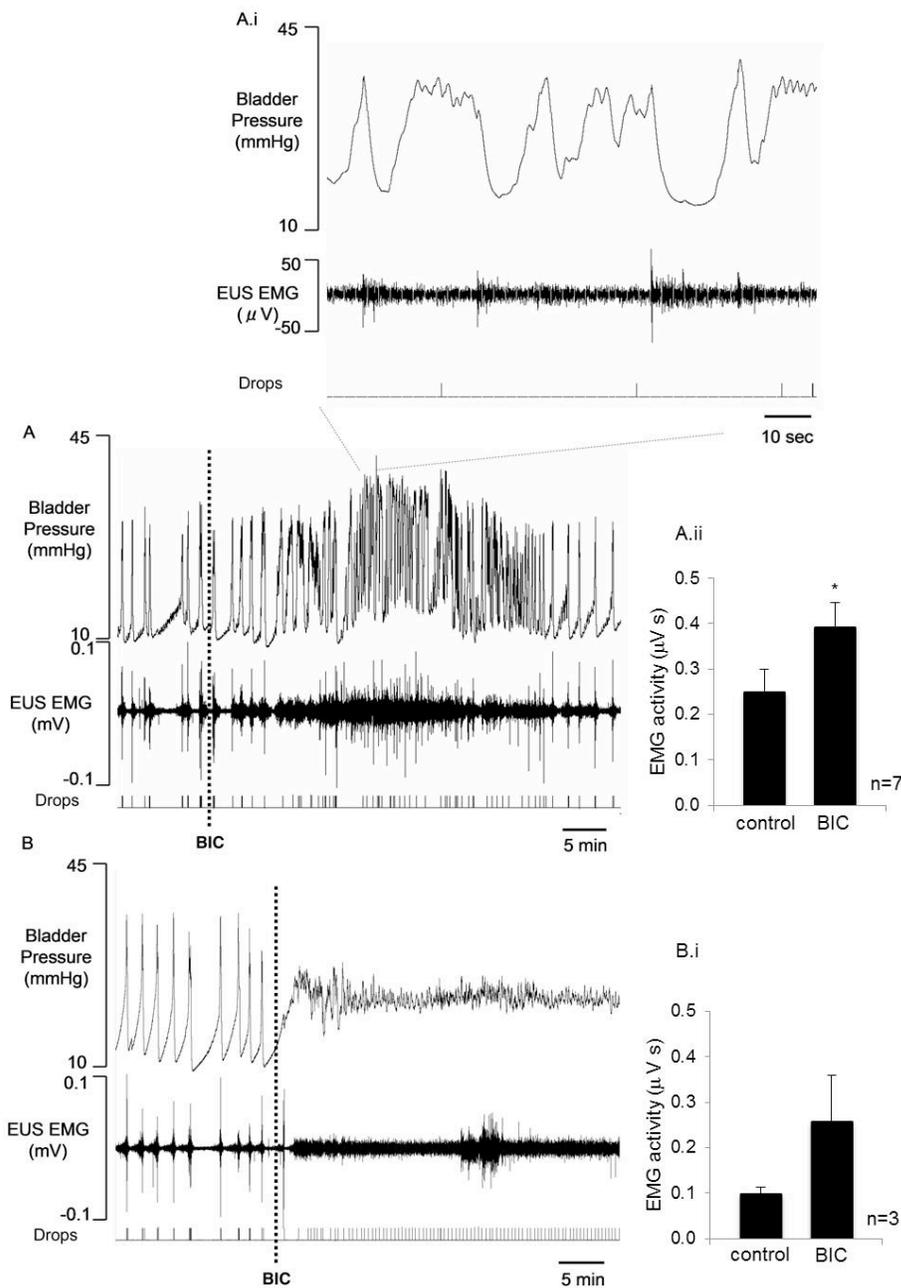


Figure 3.8. Effect of microinjection of bicuculline (BIC, 1 nmol in 50 nl) into the caudal ventrolateral PAG on the micturition reflex evoked by continuous infusion of saline into the bladder. A, at most sites ($n = 16$), microinjection of bicuculline caused an increase in the frequency of the micturition reflex, the appearance of non-voiding contractions (A.i), and a significant increase in the level of tonic EMG activity in the external urethral sphincter (EUS, A.ii) paired with desynchronisation of the EMG bursting activity (A.i). B, at 10 sites, following microinjection of bicuculline, micturition ceased. Despite an increase in EMG activity of the EUS (B.i), overflow incontinence developed as the infusion of saline into the bladder continued. * $P < 0.05$.

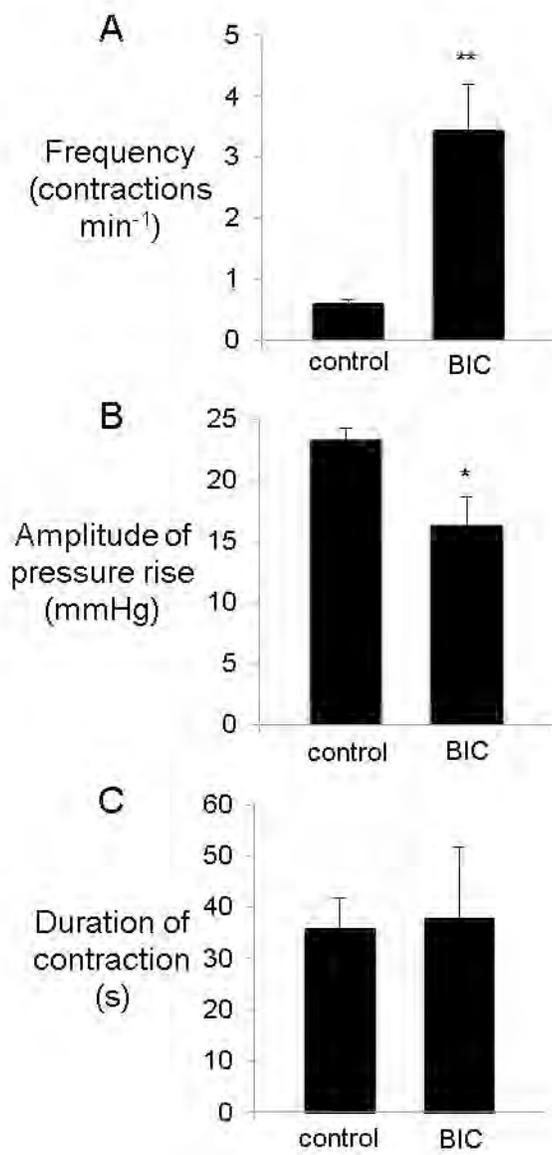


Figure 3.9. Quantification of the effect of microinjection of bicuculline (1 pmol in 50 nl) into the caudal ventrolateral PAG on the micturition reflex evoked by continuous infusion of saline into the bladder at sites where the micturition reflex persisted (16/26 sites). A, microinjection of bicuculline caused a significant increase in the frequency of the cyclic voids induced by continuous cystometry. B, in these cases there was also a significant decrease in the amplitude of the pressure rise normally associated with a void. C, there was no change in the duration of each contraction following microinjection of bicuculline (BIC) into the PAG. *P < 0.05, **P < 0.001.

We were concerned that the increase in the level of tonic EMG activity following microinjection of bicuculline into the PAG was secondary to increased bladder pressure, rather than due to direct activation of afferent pathways projecting through the PAG. In six experiments, the bladder was emptied by withdrawing fluid through the injection port and the relationship between the intravesicular volume and level of tonic EMG activity in the EUS was investigated. There was a direct relationship between the level of tonic EMG activity and bladder volume when increasing volumes of saline were injected into the bladder (Fig. 3.10 A). Microinjection of bicuculline into the PAG when the bladder was empty caused an increase in the tonic EMG activity of the EUS (from 0.1 ± 0.00 to $0.3 \pm 0.1 \mu\text{V s}$, $n = 4$, $P < 0.05$, Mann Whitney U test). This was accompanied by a small, transient rise in intravesicular pressure (Fig. 3.10 B, $+8.7 \pm 2.6 \text{ mmHg}$). When incremental volumes of saline were infused into the bladder following microinjection of bicuculline into the PAG, this relationship was maintained but the curve was shifted upwards, reaching significance at 0.4 ml intravesicular volume (Fig. 3.10 A).

At 7 other sites (Fig. 3.10 C, open circles), at which microinjection of bicuculline did not produce a change the level of tonic EMG activity, there was no shift in the curve when saline was infused into the bladder in incrementally increasing volumes (Fig. 3.10 C, open circles).

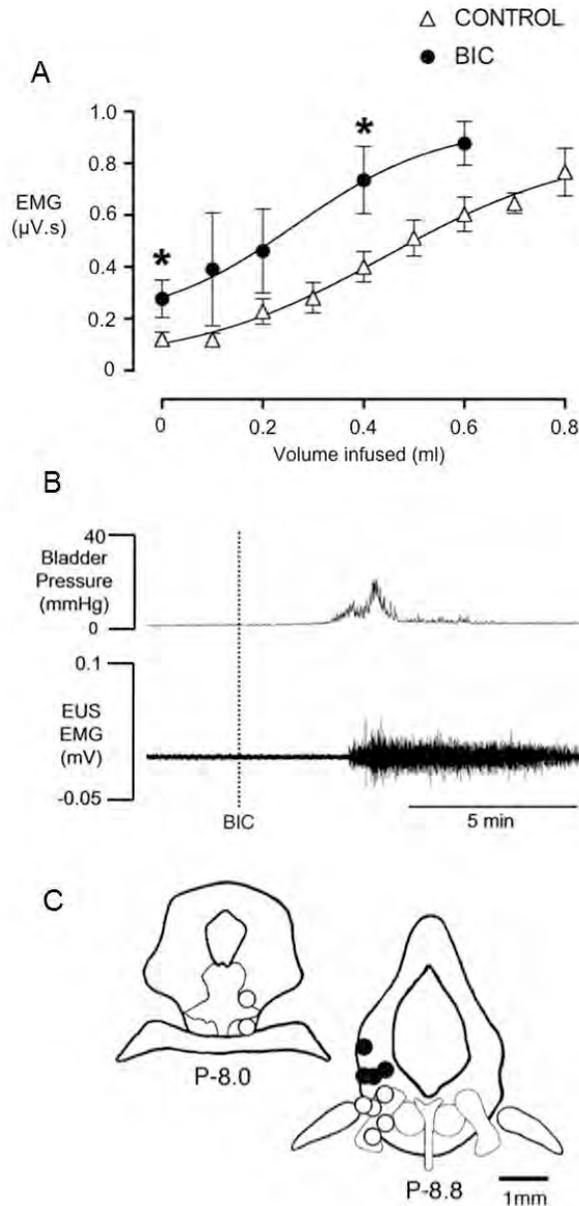


Figure 3.10. Effect of microinjection of bicuculline into the caudal ventrolateral PAG on the response of the external urethral sphincter (EUS) to stepped infusion of saline into the bladder. A, effect of microinjection of bicuculline (BIC) into the caudal ventrolateral PAG on the response of the EUS to stepped infusion of saline into the bladder before (open triangles) and after (filled circles) the microinjection ($n = 8$ in control group and $n = 4$ in BIC group). Note that due to the necessity of limiting the experiment to within the period that BIC was active after microinjection, it was not possible to test the response of the EUS to the complete range of intravesicular volumes tried in the control experiments. * $P < 0.05$ significant increase in the tonic EMG activity following microinjection of BIC into the PAG without distension of the bladder (Multiple

Mann Whitney U tests. Given the problems with multiple measurements, in future a greater n and more sophisticated statistical analysis may be warranted). B, example of EUS EMG activity and increase in intravesicular pressure evoked after microinjection of BIC into the cvlat PAG in a rat in which the bladder had previously been emptied. C, location of microinjection sites in the PAG mapped on to two representative levels taken from the atlas of Paxinos and Watson (1986). Black circles represent the centre of sites at which microinjection of BIC increased the level of tonic EMG activity in the EUS whilst the bladder was empty. Open circles show centre of sites where microinjection of BIC had no effect on tonic EMG activity of the EUS.

Alongside the changes in the micturition reflex in response to infusion of saline into the bladder, microinjection of bicuculline into the cvlat PAG produced a pressor effect ($+32.7 \pm 4.9$ mmHg), tachycardia ($+59.9 \pm 9.6$ beats min^{-1}) and tachypnoea ($+124.8 \pm 16.1$ breaths min^{-1}) (Fig. 3.11 A, B, C ventral PAG graphs). This finding was unexpected since previous studies have reported depressor effects upon activation of the cvlat PAG (Lovick, 1992; Behbehani, 1995). To determine whether the preparatory surgery or distension of the bladder contributed to these cardiovascular effects, a series of experiments was carried out in which bicuculline was microinjected into the midbrain when the bladder was cannulated but not distended ($n = 4$). Microinjection of bicuculline into the cvlat PAG when the intravesicular pressure was low, evoked a transient depressor response (Fig. 3.12 A, C, -22.1 ± 5.9 mmHg). The microinjection of bicuculline was then repeated at the same site after distension of the bladder by infusion of saline. Under these conditions, as well as a facilitation of the micturition reflex in response to infusion of saline into the bladder (see above), microinjection of bicuculline evoked a pressor effect ($+27.0 \pm 12.2$ mmHg), tachycardia ($+74.8 \pm 42.5$ beats min^{-1}) and tachypnoea ($+83.7 \pm 26.2$ breaths min^{-1}) (Fig. 3.12 B, C).

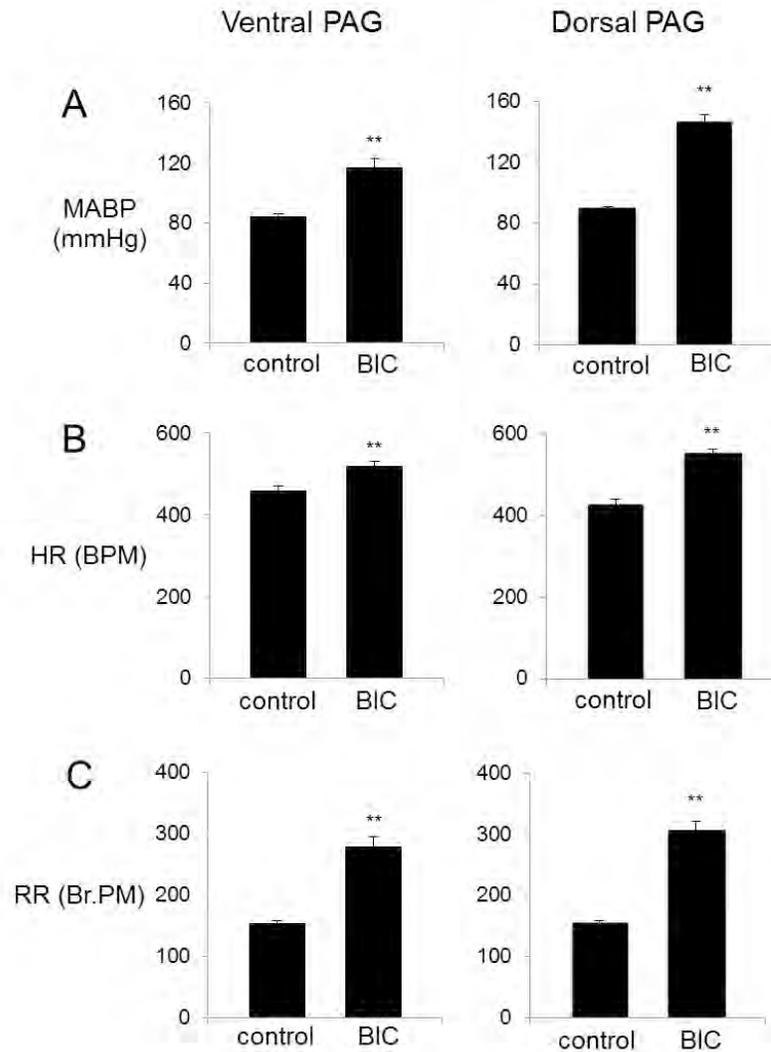


Figure 3.11. Quantification of the effect of microinjection of bicuculline into the ventral PAG (left column, n = 26) and dorsal PAG (right column, n = 34) on cardiorespiratory parameters. A, effect on mean arterial blood pressure (MABP, mmHg), B, heart rate (HR, BPM: beats min⁻¹). C, respiratory rate (RR, Br.PM: breaths min⁻¹). All values show mean for the control period before microinjection compared to the maximum reached during the response. **P < 0.001.

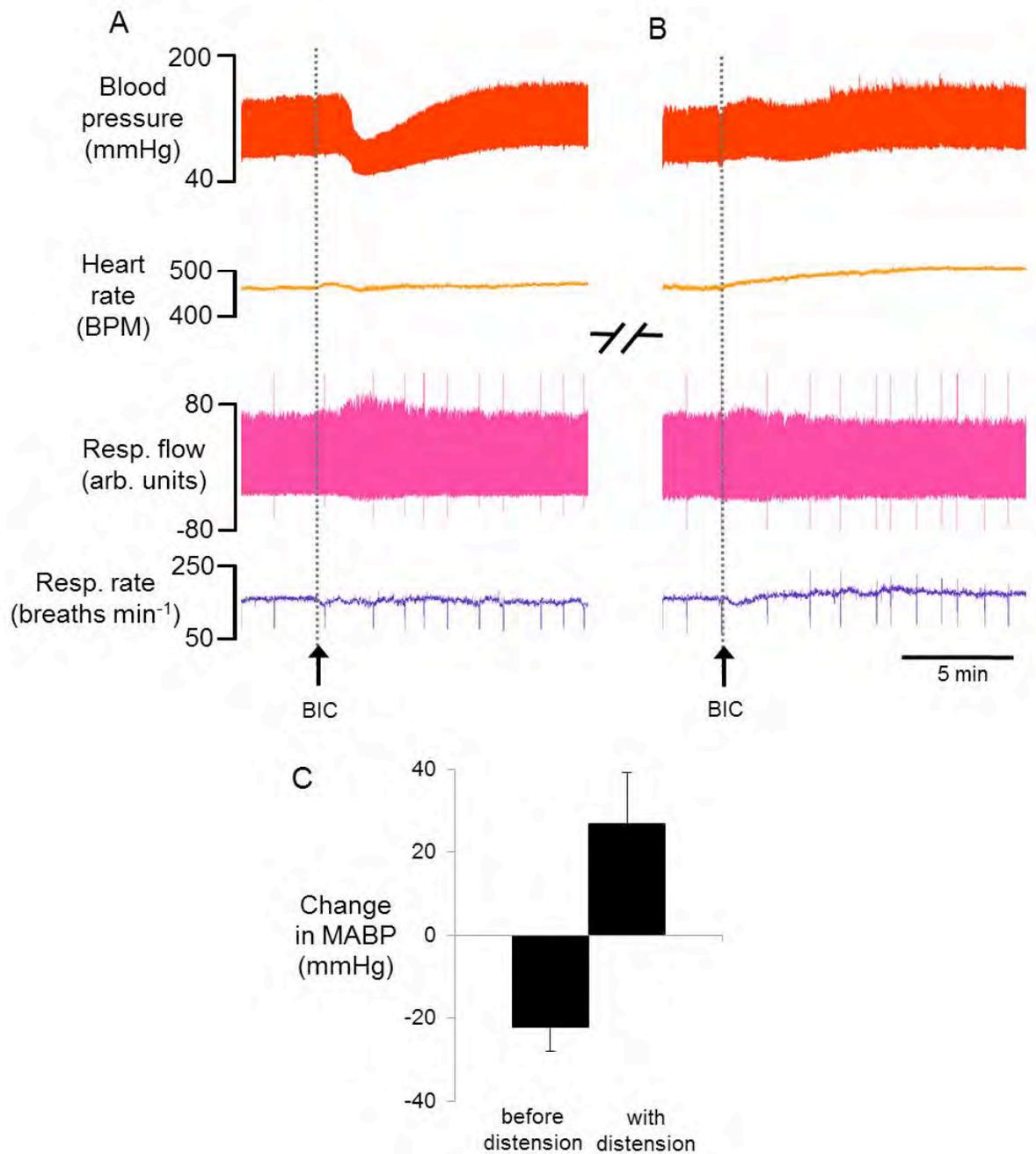


Figure 3.12. Effect of microinjection of bicuculline into the caudal ventrolateral (cvlat) PAG on the cardiorespiratory system before and after distension of the bladder. A, sample trace: microinjection of bicuculline into the cvlat PAG with the bladder exposed and cannulated but before distension caused a marked depressor effect. B, microinjection of bicuculline at the same midbrain site when the bladder was distended caused a pressor response ($n = 4$). C, Change in mean arterial blood pressure (MABP, mmHg) with microinjection of bicuculline into the cvlat PAG before distension of the bladder and then 'with distension'.

3.4.5.2 Microinjection of bicuculline into the dorsal PAG

In 18 experiments, microinjections of bicuculline were also made into the dorsal PAG ($n = 34$), an area in which microinjection of muscimol evoked no effect on the micturition reflex (Fig. 3.7, compare to Fig. 3.2 A). At most sites in this region ($n = 21$), bicuculline caused an increase in the frequency of the micturition reflex during continuous infusion of saline into the bladder, with the frequency of contractions increasing from 0.7 ± 0.1 to $2.5 \pm 0.4 \text{ min}^{-1}$ (Fig. 3.13 A). There was no change in the amplitude of the pressure rise associated with a void (Fig. 3.13 B, 20.8 ± 1.1 v. $19.7 \pm 1.2 \text{ mmHg}$, $P = 0.41$) or the duration of the contraction (Fig. 3.13 C, 30.2 ± 4.1 v. $25.2 \pm 1.6 \text{ s}$, $P = 0.26$). At these dorsal sites bicuculline also evoked marked signs of sympatho-activation, including a pressor effect (Fig. 3.11 A dorsal PAG, $+57.2 \pm 5.6 \text{ mmHg}$), tachycardia (Fig. 3.11 B dorsal PAG, $+122.6 \pm 15.4 \text{ beats min}^{-1}$), tachypnoea (Fig. 3.11 C, dorsal PAG, $+152.3 \pm 15.7 \text{ breaths min}^{-1}$), exophthalmos, pupillary dilatation and bilateral vibrissae twitching. At the remaining 13 sites, following an initial increase in the frequency of the micturition reflex, coordinated voiding ceased. As the infusion of saline into the bladder continued, the bladder pressure rose and urine output returned in an irregular dropwise manner rather than a clustering of drops seen during normal voiding. In addition, expulsion of drops of fluid from the penis was not associated with the sharp rise in bladder pressure normally associated with a void. Tonic EMG activity in the EUS was increased (from 0.1 ± 0.02 to $0.7 \pm 0.23 \text{ } \mu\text{V s}$, Fig. 3.13 D ii, $n = 3$). The coordinated micturition reflex showed no sign of recovery within 2 hours, when the experiment was terminated.

The dorsal PAG was exceptionally sensitive to the effects of bicuculline. At 23 sites, moving the cannula into the dorsal PAG was enough alone to evoke sympatho-activation and facilitate the micturition reflex, presumably evoked by passive diffusion of bicuculline from the cannula tip. These effects could be reversed by withdrawing the cannula from the brain.

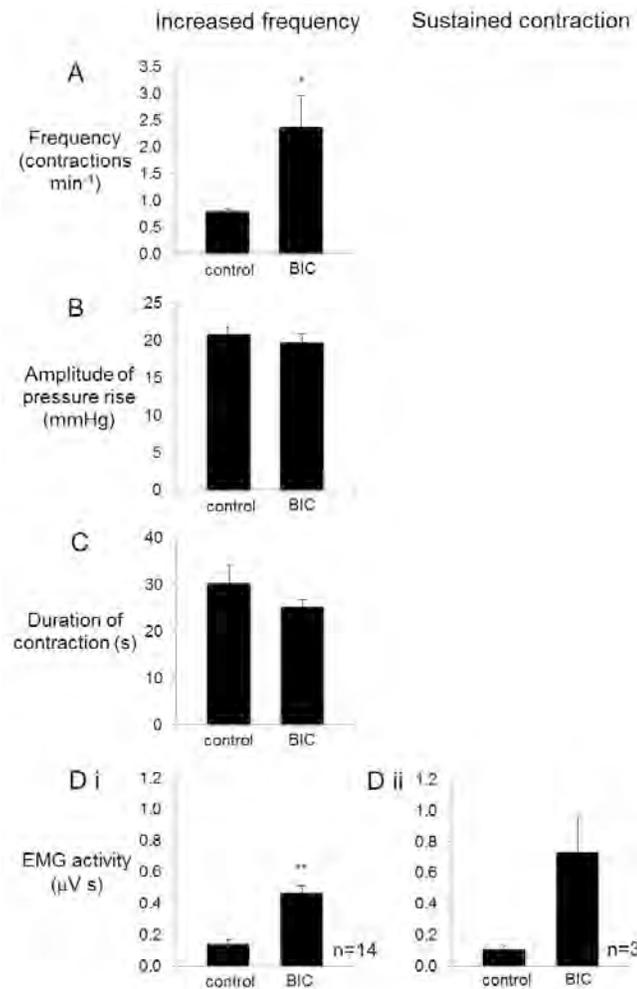


Figure 3.13. Quantification of the effect of microinjection of bicuculline (BIC) into the dorsal PAG on the micturition reflex evoked by continuous infusion of saline into the bladder. Effect of microinjection of bicuculline into the dorsal PAG on A, frequency (contractions min⁻¹), B, amplitude of the pressure rise associated with a void (mmHg), C, duration of contraction (s) and D, level of tonic EMG activity in the EUS. D, Right hand column shows increase in EMG activity at sites where coordinated micturition ceased. *P < 0.05, **P < 0.001.3.5

DISCUSSION

Continuous infusion of saline into the bladder elicited periodic voids characterised by sharp increases in intravesicular pressure accompanied by bursting activity in the EMG recorded from the EUS. In between bursts EMG activity was silenced. This sequence of events was associated with expulsion of several drops of fluid from the urethral meatus. Similar responses have been reported previously using a comparable experimental protocol (Maggi *et al.*, 1986; Kruse *et al.*, 1993; Van Asselt *et al.*, 1995; Matsuura *et al.*, 1998; Matsuura *et al.*, 2000).

3.5.1 Microinjection of muscimol into the PAG

Microinjection of the GABA agonist, muscimol inhibited the micturition reflex. At effective sites, there was either a significant reduction in the frequency of phasic bladder contractions, accompanied by a reduction in the level of tonic EMG activity of the EUS during the lengthened inter-void interval. Alternatively, at some sites contractions ceased completely following microinjections of muscimol and as the saline infusion continued, a type of 'overflow incontinence' developed as fluid dripped regularly from the penis. These inhibitory effects were only seen when the microinjection of muscimol was made into the caudal ventrolateral periaqueductal grey (cvlat PAG), not in other areas of the PAG and midbrain. These negative sites were not due to insufficient dosing because two and three times the standard doses administered in the rostral and dorsal PAG in preliminary experiments also evoked no effect on the micturition reflex. Interestingly a similar reduction in voiding frequency has also been reported following microinjection of

the μ -opioid agonist DAMGO or the synaptic inhibitor CoCl_2 into the ventrolateral PAG (Matsuura *et al.*, 1998; Matsumoto *et al.*, 2004) suggesting that the integrity of synaptic transmission through this region is essential for the development of reflex voiding patterns. Though muscimol is widely thought to primarily act as an agonist at GABA_A receptors, there is also suggestion that the inhibitory effects may be mediated via activation of pre-synaptic GABA_B receptors (Yamauchi *et al.*, 2000). The concentration of muscimol microinjected in the present experiments (5 mM) would have been sufficient, according to Yamauchi and colleagues work in the calyx of Held, to activate any pre-synaptic GABA_B receptors present as well as post-synaptic GABA_A receptors, thus the inhibitory effects on micturition seen in the present experiments might have been evoked by both a pre- and post-synaptic effect on GABA_A and GABA_B receptors.

Although microinjection of muscimol reduced the frequency of voiding, each void remained a co-ordinated event and there was no significant change in the threshold, amplitude, duration of contraction, in the number of drops of fluid expelled per void or in the pattern of bursting in the EMG activity of the EUS. This suggests that GABAergic neurotransmission in the region gates the circuitry in an on/off fashion, rather than being involved in the coordination of a void, which is likely to be integrated in the pontine micturition centre (Holstege *et al.*, 1979; Holstege *et al.*, 1986; Blok *et al.*, 1995; Sasaki, 2005).

The cvlat PAG receives bilateral projections from the bladder and lumbosacral spinal cord (Blok & Holstege, 1994; Vizzard *et al.*, 1995; Marson, 1997; Ding *et al.*, 1998).

Interestingly, both the dorsal and the ventrolateral PAG project to the pontine micturition centre (PMC) (Blok & Holstege, 1994; Ding *et al.*, 1998). Barrington showed in 1925 that the integrity of the PMC is essential for normal micturition to take place (Barrington, 1925), and projections from the PMC, via the sacral spinal cord cause synchronous contraction of the detrusor and relaxation of the EUS (Holstege *et al.*, 1979; Holstege *et al.*, 1986; Blok *et al.*, 1995; Sasaki, 2005). In the present study, microinjection of muscimol into the dorsolateral PAG evoked no effect on the micturition reflex induced by continuous infusion of saline into the bladder. Thus the pathway from the dorsal PAG to the PMC is unlikely to play a role in the reflexive control of micturition, at least during cystometry in the urethane-anaesthetised rat. It is likely that muscimol inhibited transmission in the reflex circuitry that controls micturition at the level of the cvlat PAG, and the functional inhibition was translated to the bladder and EUS via these projections from the cvlat PAG to the PMC.

Projections from the bladder and urethra to the cvlat PAG and from the cvlat PAG to the PMC are bilateral (Blok & Holstege, 1994; Blok *et al.*, 1995; Vizzard *et al.*, 1995; Marson, 1997; Ding *et al.*, 1998; Taniguchi *et al.*, 2002). In the present study, all microinjections were made unilaterally thus it is possible that afferent signals from the bladder were still integrated on the contralateral side of the PAG. This explains the incomplete inhibition of the micturition reflex seen when microinjections of muscimol were made in the cvlat PAG. However, occasionally, microinjection of muscimol did produce a complete inhibition, as also reported by Matsuura (1998) following unilateral microinjection of CoCl_2 in the ventrolateral PAG. Thus it is possible that in these cases

the GABA agonism was sufficient to inhibit activity in both the ipsi and contralateral PMC (Fig. 3.14). It seems possible that these complete inhibitory effects might have been evoked at sites that were in the centre of an 'effective' region. However, sites at which microinjection of muscimol produced transient and complete inhibitory effects on the micturition reflex were intermingled in the caudal ventrolateral PAG. Therefore it cannot be concluded from their anatomical location that the transient responses are simply weaker effects due to injection of muscimol at the periphery of the effective region.

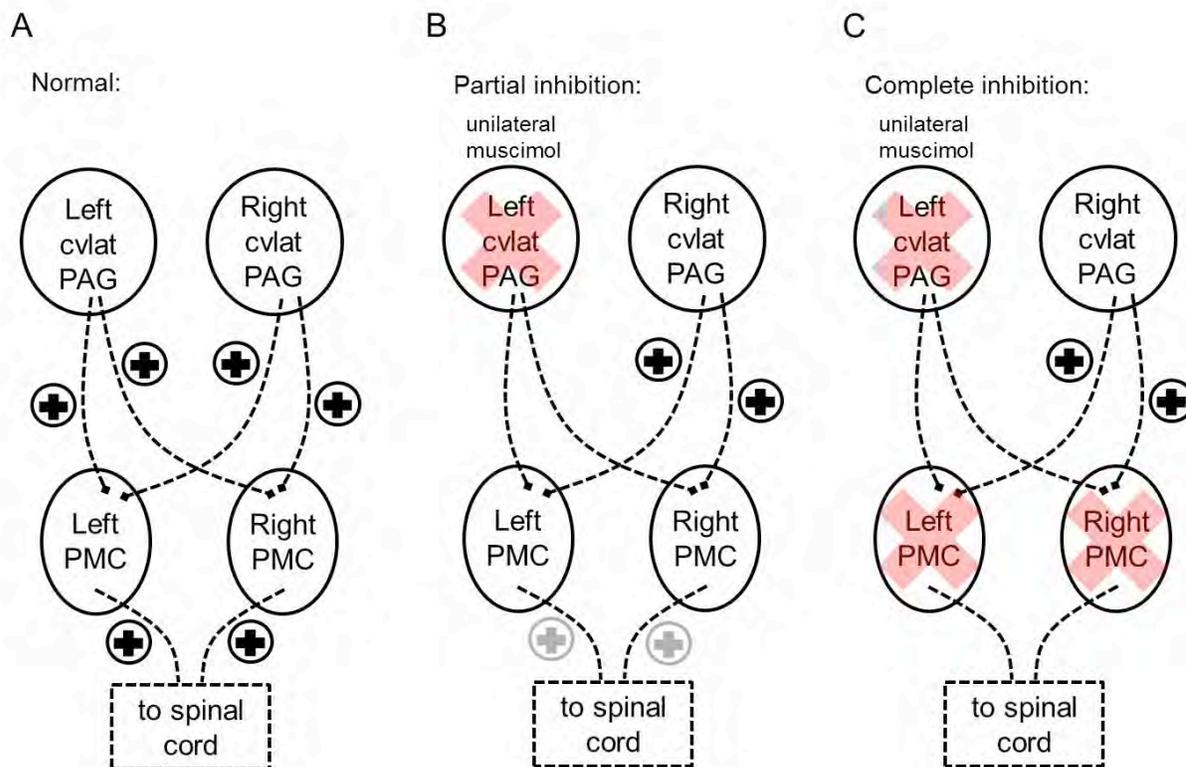


Figure 3.14. Schematic diagram demonstrating how unilateral microinjection of muscimol might produce partial and complete inhibitory effects on the micturition reflex evoked by continuous infusion of saline into the bladder of the urethane anaesthetised rat. A, normally, bilateral projections from the PAG to the left and right PMC are active during micturition. B, Microinjection of muscimol into the left hand side of the PAG could prevent activity in projections from this area to both the left and right hand sides of the PMC. However the projections from the right hand side of the PAG to the PMC would still be active. Therefore the level of neuronal output from the PMC might be lower, but not completely inhibited, producing a reduction in the frequency of the micturition reflex, without complete prevention of voiding. C, in cases where the micturition reflex was completely inhibited by unilateral microinjection of muscimol, the drug could have been sufficient to block activity in both sides of the PMC, thus preventing the output of the micturition circuitry that would normally coordinate a void.

As well as the projections from the lumbosacral spinal cord to the PAG (Vizzard *et al.*, 1995; Marson, 1997; Peng *et al.*, 2006; Peng *et al.*, 2008), direct projections from the bladder and urethra to the PMC have been identified in the rat (Ding *et al.*, 1998). The functional importance of these connections in the rat is unclear. It is unlikely in our experimental model, that these projections are functional and short-circuit the projections via the cvlat PAG; otherwise the micturition reflex evoked by continuous cystometry could not have been inhibited (both partially and completely) by microinjection of muscimol into the cvlat PAG, which lies more rostral in the micturition circuitry than the PMC.

3.5.2 Microinjection of bicuculline into the caudal ventrolateral PAG

Microinjection of the GABA_A antagonist, bicuculline, into cvlat PAG increased the frequency of the micturition reflex evoked by continuous cystometry. This was associated with the appearance of non-voiding contractions and a centrally mediated increase in the level of tonic EMG activity in the EUS. The bursting activity in the EMG of the EUS normally seen during a void became dyssynergic and EMG bursts were not always followed by EMG silence. It is during this EMG silence that urine output normally occurs (Maggi *et al.*, 1986; Peng *et al.*, 2006; Peng *et al.*, 2008) so this dyssynergia probably contributed to the appearance of non-voiding contractions (Matsuura & Downie, 2000) .

These findings indicate that micturition-related synapses in the cvlat PAG are normally subject to a tonic GABAergic influence, and the lifting of this tone may be a prerequisite for voiding to occur. In support of this theory, Kitta and colleagues (2008) found that extracellular levels of GABA in the cvlat PAG were reduced during periods of intravesicular distension in the conscious rat. The source of the inhibitory GABAergic tone is unknown; however a wealth of evidence suggests that it originates in frontal regions, and GABAergic interneurons seem strategically placed in the PAG to act as a local source of GABA (Reichling & Basbaum, 1990; An *et al.*, 1998; Adolphs, 1999; Floyd *et al.*, 2000; Fowler *et al.*, 2008; Griffiths & Tadic, 2008; Fowler & Griffiths, 2010). In the urethane anaesthetised preparation it is likely that the activity in the frontal cortices is reduced, however there is suggestion that some activity in the motor cortex and higher centres may be preserved during urethane anaesthesia (Tai *et al.*, 2009).

Occasionally, following microinjection of bicuculline into the cvlat PAG the coordinated micturition reflex ceased despite continued infusion of saline into the bladder. In these cases small oscillations in intravesicular pressure were transposed on top of the tonically raised bladder pressure, urine drops were not uniformly expelled and tonic EMG activity in the EUS was increased. This suggested that the bladder was in an active 'tonic contraction' as opposed to the passive overflow seen when muscimol was microinjected at the same site. Recording the efferent activity in the pelvic nerve might resolve this question, though ideally, one would develop a reliable technique to record the EMG activity of the smooth muscle of the detrusor itself, which in the past, has proven difficult (Ballaro *et al.*, 2001; Ballaro, 2008).

In the present study, microinjection of bicuculline into the cvlat PAG induced a pressor response. This is in contrast to a previous reports in which the drug evoked a fall in blood pressure in urethane anaesthetised rats (Lovick, 1991). However, Matsuura et al. (2000) also saw a pressor effect when they activated neurons in the ventrolateral PAG by microinjection of glutamate in a similar surgical preparation to the one used in the present study. Pressor responses were also evoked following microinjection into the dorsal PAG, raising the possibility that the pressor responses produced by bicuculline were a result of the drug leaking up the cannula track to act at more dorsal sites. However, this seems unlikely as there was no difference in the latency to onset of the responses evoked from the dorsal and ventral PAG. A major difference between the preparation used in the present studies compared to previous work that showed a depressor region in the ventrolateral PAG was the laparotomy carried out to gain access to the bladder for cannulation and also distension of the bladder. Interestingly, in the absence of intravesicular distension, microinjection of bicuculline into the cvlat PAG evoked depressor effects, in agreement with previous studies. Distension of the bladder has been shown to produce a sympathetically mediated redistribution of splanchnic blood flow (Guttmann & Whitteridge, 1947; Cunningham *et al.*, 1953; Mukherjee, 1957; Weaver, 1985; Ward *et al.*, 1995) which might have masked any depressor effect that would normally be produced by activation of neurons in the ventrolateral PAG. Also, a high level of visceral nociceptor activation of the ventrolateral PAG might be expected to be evoked by bladder pressures produced by the cystometry protocol (Su *et al.*, 2008). Thus, it is possible that in the present study, the high level of visceral input to the

ventrolateral PAG, which could have been nociceptive (Keay & Bandler, 1993; Lumb, 2002), may have caused some change in local neurotransmitter activity in the region. Therefore, the effects of antagonising GABA in the cvlat PAG were not the same as they may have been in the absence of this afferent input. This theory is in accord with that of Behbehani, who suggests in his 1995 review that the efferent output of the PAG is dependent on the level of afferent input.

3.5.3 Microinjection of bicuculline in the dorsal PAG

Though it is clear that the functional integrity of the cvlat PAG is essential for reflex micturition to occur, the role of the dorsal PAG is much less clear. Microinjection of muscimol into the dorsal PAG failed to affect reflex micturition, suggesting that the area is not integral to the micturition reflex in response to continuous cystometry. However, activation of the dorsal PAG is undoubtedly able to influence micturition because microinjection of bicuculline into the area in the present series of experiments increased the frequency of reflexive voiding. Also, electrical stimulation and microinjection of glutamate in the dorsal PAG can modulate micturition (Vargas *et al.*, 2000; Bittencourt *et al.*, 2004; Schenberg *et al.*, 2005), and strategically placed projections from the dorsal PAG to the PMC have been shown (Blok & Holstege, 1994; Ding *et al.*, 1998).

The dorsal PAG is known to integrate and initiate the cardiovascular defence response, which primes the cardiovascular system for motor behaviour in response to imminent threat (Behbehani, 1995). Micturition (and defaecation) are recognised responses to extreme fright in humans and animals (Gray, 1987; Goetz *et al.*, 1994; Schenberg *et al.*,

2001). Thus it is possible that these are components of the same behavioural response of the whole animal to imminent threat, and the dorsal PAG may initiate descending activity in the micturition circuitry required to initiate a void in the absence of an efferent drive from distension of the bladder. The dorsal PAG could also play a role in the coordination of volitional voiding for scent marking in animals and pre-emptive bladder emptying in humans.

The dorsal PAG was extremely sensitive to the effects of bicuculline. In some cases, lowering the cannula containing the bicuculline solution into the dorsal PAG was enough to evoke a full bladder, cardiovascular and respiratory response which could not be intensified by microinjection of 1 pmol of bicuculline. Since microinjection of 1 pmol of bicuculline was always required to initiate a response when the cannula was in the region of the ventrolateral PAG, it is likely that a difference in the ongoing level of GABAergic tone in the dorsal PAG in comparison to the ventral exists. This difference may, in part, explain the absence of an effect when muscimol was microinjected into the dorsal PAG as the level of GABA tone may have already been sufficient to inhibit any on-going GABAergic activity in the region. The sensitivity to changes in the levels of GABAergic neurotransmission in the dorsal PAG may reflect the need for rapid activation of the region to initiate behavioural responses in reaction to imminent threat. The dose of bicuculline (1 pmol) used was lower than that which others have used in investigating GABA function in the PAG (McMullan & Lumb, 2006; Numata *et al.*, 2008), but much higher than the dose used in some experiments into the bicuculline-evoked defence response in the dorsal PAG (Morgan & Clayton, 2005). This suggests that

future investigation may warrant a lower dose of bicuculline in the dorsal PAG; however the dose appears to be sufficient to bring about reversible effects on the bladder and cardiorespiratory system in the ventrolateral PAG.

3.6 SUMMARY AND CONCLUSIONS

In summary, the present experiments have demonstrated that the functional integrity of synapses in the caudal, ventrolateral PAG is essential for the micturition reflex in response to bladder filling to take place. The use of a GABA_A antagonist has shown that the micturition pathway that passes through the caudal ventrolateral PAG is tonically inhibited by GABAergic neurotransmission. Modulation of GABAergic neurotransmission at this level of the micturition circuitry might be the mechanism by which voiding is allowed or deferred, depending on the social situation. The role of the dorsal PAG in micturition is less clear. It is evidently not involved in the control of reflex voiding in response to bladder filling, but may play a role in 'active voiding' by activating descending pathways controlling micturition in the absence of an afferent input from the bladder. This could be important for controlling volitional voiding (or scent marking in animals) and the bladder emptying associated with the defence response integrated and initiated in the dorsal PAG.

3.7 Key Findings

3.7.1 Microinjection of GABA_A agonist (muscimol) into the PAG

1. Reduced the frequency of the micturition reflex only when applied in the caudal ventrolateral region, suggesting that synapses involved in the control of reflex micturition are present in this region.
2. Did not affect reflex micturition when applied in the dorsal and rostral PAG, suggesting that these regions are not involved in the micturition reflex, at least in the urethane anaesthetised rat.
3. Did not affect the cardiovascular and respiratory parameters recorded.

3.7.2 Microinjection of GABA_A antagonist into the ventral PAG

1. Increased the frequency of reflex micturition, suggesting that GABA tonically inhibits micturition in this region, since removing its effects facilitated micturition.
2. Caused a pressor effect, tachycardia and tachypnoea alongside the effects on micturition. However, we evoked a depressor effect when the bladder was not distended, suggesting that the degree of afferent input to the PAG determines the effect on the blood pressure.

3.7.3 Microinjection of GABA_A antagonist into the dorsal PAG

1. Increased the frequency of micturition evoked by continuous cystometry, despite the fact that microinjection of muscimol in the same region evoked no changes. This suggests that though the dorsal PAG may not be involved in the control of reflex micturition, it may play a role in volitional voiding or voiding that is associated with extreme fright.
2. Evoked intense sympatho-activation, characterised by a pressor effect, tachycardia, tachypnoea, exophthalmos, pupillary dilatation, and vibrissae twitching.

**CHAPTER 4: THE EFFECT OF MONOPOLAR ELECTRICAL STIMULATION OF
THE PERIAQUEDUCTAL GREY ON REFLEX MICTURITION IN THE URETHANE
ANAESTHETISED MALE RAT**

4.1 INTRODUCTION

The experiments reported in Chapter 3 showed that the micturition reflex pathway synapses in a restricted area within the caudal ventrolateral periaqueductal grey (PAG) and that transmission through this region is normally tonically inhibited by a GABAergic influence (Stone *et al.*, 2011, Chapter 3). Interestingly, in the conscious rat, during periods of intravesicular distension the extracellular concentration of GABA in the caudal ventrolateral PAG is reduced (Kitta *et al.*, 2008), implying that this GABAergic inhibition might be transiently lifted to permit micturition to take place. This critical synapse in the ventrolateral PAG presents a target to modulate or modify transmission through the micturition reflex pathway.

Electrical stimulation has been used for decades by Physiologists as a tool to activate neuronal pathways, typically by delivering single shocks or short trains of pulses. More recently longer or even continuous trains of pulses have been used in the clinical setting to modulate or normalise pathophysiological activity underlying certain disease states. The technique of deep brain stimulation (DBS) is currently used clinically for the treatment of a number of disorders, including Parkinson's disease and intractable pain (for reviews see Perlmutter and Mink, 2006, Kringelbach *et al.* 2007, Deniau *et al.* 2010). It is an attractive therapeutic option where pharmacotherapy has failed because stimulation can be applied directly to the area of interest, and the effects are of rapid onset and are reversible.

It is possible some forms of urge incontinence may be due to a lack of inhibitory control of the micturition circuitry. Therefore DBS could be a valuable therapeutic tool if it could be used to manipulate the activity of the voiding circuitry.

4.2 AIMS AND HYPOTHESIS

We hypothesised that electrical stimulation of the PAG could be used to manipulate the activity of the micturition reflex circuitry in the urethane-anaesthetised rat. Previous studies have shown contrasting effects of electrical stimulation of the midbrain on micturition in the rat, cat and dog (Nishizawa *et al.*, 1987; Kruse *et al.*, 1990; Matsuura *et al.*, 2000; Liu *et al.*, 2004; Ito *et al.*, 2006; Numata *et al.*, 2008). The objective of the present experiments was to investigate the effects of monopolar electrical stimulation of the caudal PAG with varying pulse parameters, on voiding evoked by continuous cystometry in the urethane-anaesthetised rat. The excitatory amino acid D,L-homocysteic acid (DLH), a non-specific agonist of glutamate receptors, was microinjected at sites where the effects of electrical stimulation on micturition were investigated to determine whether the electrical stimulation primarily activated axons of passage or cell bodies. The results have provided an important insight into the potential for using electrical stimulation of the midbrain (or DBS) for the treatment of drug-refractory urinary disturbances in the future.

4.3 METHODS

39 male Sprague Dawley rats (201 – 341 g, Charles River, Kent, UK) were anaesthetised with urethane (IP, 1.4 g kg⁻¹) and prepared for cystometry and monitoring of vital signs as described in the general methods (Chapter 2, Section 2.2). A dorsal craniotomy allowed access to the midbrain with two types of electrode (Fig. 4.1). Experiments 1 – 13 were conducted using the two-channel electrode design. This was modified to the one-channel design for experiments 13 – 39.

4.3.1 Electrode design

Both electrodes were used to apply monopolar electrical stimulation at midbrain sites. The differential electrode connected — via a wire soldered to a crocodile clip — to the open wound on the head from the craniotomy to the positive pole of the stimulator (SD9, Grass Telefactor, W. Warwick, RI, USA). The preparation was earthed via a wire — connected to the abdominal wall by a crocodile clip — which fed into a common ground with the headstage used to record the EMG activity of the external urethral sphincter.

4.3.1.1 Electrode 1

Electrode 1 (Fig. 4.1 A) was pulled from two tubes of borosilicate glass capillary tubing (1.5 mm OD, 1.17 mm ID, Harvard Apparatus, Kent, UK), fused together with heat-shrink tubing and Araldite[®] at either end (Fig. 4.2 A). The centre point of the fused tubes was heated in an open flame, twisted — to maintain the dual structure and ensure the tips remained together (Fig.4.2 B) — and pulled to approximately 250 µm tip diameter (Fig. 4.2 C). When snapped apart, this created two dual, heat-pulled glass pipettes for

future use. The overall electrode tip was approximately 500 x 250 μm . To allow connection to polyethylene tubing (1.67 mm ID, 2.42 mm OD, Portex Tubing, SIMS Portex Ltd, Kent, UK) and to the stimulator, both barrels were gently heated (distal to the Araldite/heat shrink tubing support) in an open flame and bent through 90° (as in Fig. 4.1 A). One channel (250 μm) was filled with Wood's metal and was connected — via wire soldered into the Wood's metal — to the negative pole of an electrical stimulator (SD9, Grass Telefactor, W. Warwick, RI, USA). The other channel (250 μm) contained 0.2 M D,L-homocysteic acid (DLH, pH 7.4, Sigma Aldrich, Gillingham, UK) with pontamine sky blue dye (PSB, 2% W/V, Searle Diagnostics, High Wycombe, UK) and was connected via heat pulled polyethylene tubing (1.67 mm ID, 2.42 mm OD, Portex Tubing, SIMS Portex Ltd, Kent, UK) to a microsyringe for application of discrete volumes of DLH.

4.3.1.2 Electrode 2

Electrode 2 (Fig. 4.1 B) was designed following concerns that DLH might leak dorsally up the channel between the two glass tubes. It was pulled from one tube of borosilicate glass capillary tubing (1.5 mm OD, 1.17 mm ID, Harvard Apparatus, Kent, UK) using a Narishige weighted electrode puller (Narishige, London, UK). The tip of the electrode was broken off after pulling so that the diameter was approximately 250 μm . Polyethylene tubing (1.67 mm ID, 2.42 mm OD, Portex Tubing, SIMS Portex Ltd, Kent, UK) was attached to the distal end of the barrel and was heat pulled (without twisting) so that it could be push-fitted to a microsyringe for application of DLH (Fig. 4.1 B). The external junction at which the polyethylene tubing met the glass tubing was sealed with

Araldite[®] to prevent leakage of the DLH solution to be contained within (Fig. 4.1 B). A heated 26 G needle tip was used to puncture a hole in the polyethylene tubing distal to its junction with the glass tubing, and a fine tungsten wire, insulated except for the tip (50 μm bare, 100 μm coated, AM Systems INC, USA) was fed down into the electrode until it reached the tip (Fig. 4.1 B). The wire was secured and sealed with Araldite[®] at its external junction with the polyethylene tubing (Fig. 4.1 B). The distal end of the tungsten wire was stripped of its insulation and connected, via a wire soldered to a crocodile clip, to the stimulator (SD9, Grass Telefactor, W. Warwick, RI, USA).

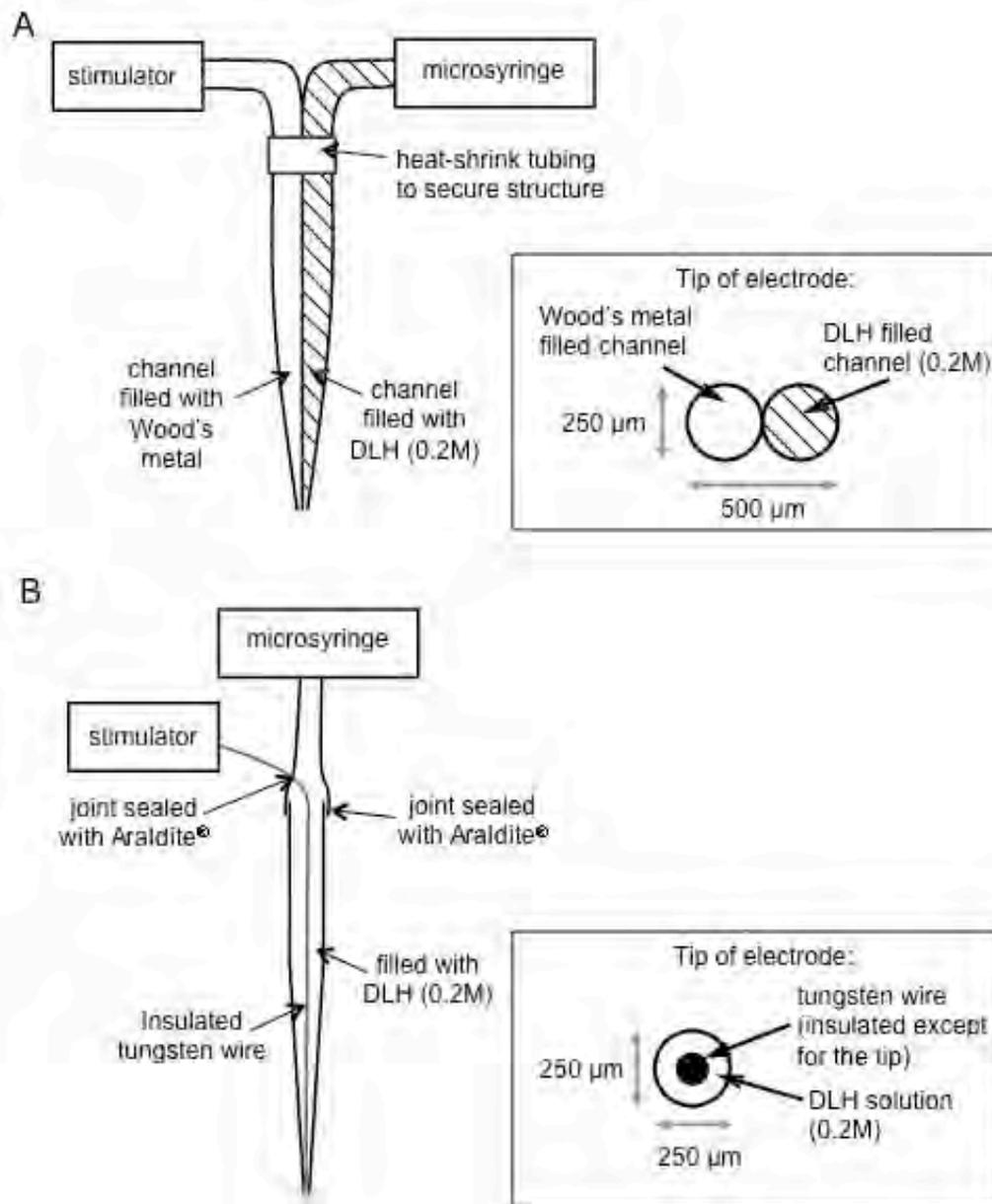


Figure 4.1. Diagram of the two types of electrodes used to apply electrical stimulation and D,L-homocysteic acid (DLH, 50 nl, 0.2 M) in the midbrain. A, electrode 1 was heat-pulled from two tubes of borosilicate glass. One channel (250 μm) contained Wood's metal for electrical stimulation and DLH was contained in a separate glass channel (250 μm). B, electrode 2 was designed following concerns that DLH may leak dorsally between the two channels. It consisted of one tube of heat-pulled borosilicate glass (250 μm), containing a fine tungsten wire (insulated except for the tip, 50 μm bare, 100 μm coated) for electrical stimulation. DLH filled the electrode around the wire and was injected following recovery from electrical stimulation using a microsyringe via polyethylene tubing.

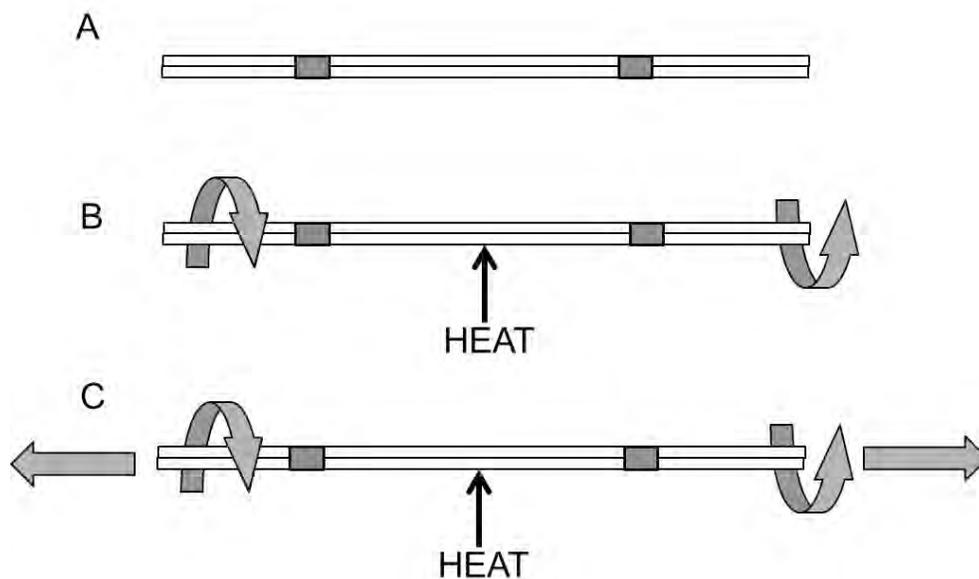


Figure 4.2. Instructions for manual heat-pulling of double barrelled electrode 1 from two borosilicate glass capillary tubes. A, two tubes are sealed together using heat-shrink tubing, which is secured with Araldite[®] glue for stability. B, The centre of the tubes was heated in an open flame, and they were gently twisted when the glass reached melting point. C, the ends of the tubing were pulled apart whilst still gently twisting. Once snapped apart in the middle, this could be used to produce two dual lumen electrodes.

4.3.2 Electrical stimulation

The electrode was advanced into the brain at a 10° angle to a site within the caudal midbrain. Thereafter the electrode was advanced through the brain in 0.5 – 1 mm steps. At each step electrical stimulation was applied using square wave pulses of 60 Hz, 0.5 ms and with an amplitude of between 0.5 and 2.5 V. Stimulus parameters were based on preliminary experiments and those reported to be effective in the literature (Noto *et al.*, 1989, 1991; Liu *et al.*, 2004). For voltage threshold mapping experiments the frequency and duration of pulses (60 Hz, 0.5 ms, 60 s) were kept constant. Recovery of micturition reflexes and the cardiovascular and respiratory parameters recorded was allowed before repeated stimulations were applied, and no less than 10 minutes elapsed between stimulations.

4.3.2.1 Continuous cystometry

Stimulation was started following a minimum of 3 control contractions, at the onset of a void signified by a sharp increase in the intravesicular pressure, and lasted for 60 s. At each stimulation site the minimum voltage required to inhibit urine output during continuous infusion of saline into the bladder (6 ml h⁻¹) for the duration of the 60 s stimulation period was sought. A similar protocol was used to determine the optimum frequency and pulse duration. The effect of stimulation between voids and longer periods of stimulation (> 60 s) were investigated at some of these sites (n = 13 and n = 12 respectively).

4.3.2.2 Discontinuous cystometry

The effect of electrical stimulation on the threshold volume for initiation of a void was investigated during discontinuous cystometry. The bladder was emptied manually via a syringe connected to the infusion tubing through a 3-way tap and was then filled with saline ($0.1 - 0.2 \text{ ml min}^{-1}$) until a void was initiated whereupon the infusion was stopped. The voided volume was collected in a beaker placed under the rat, and this was measured by collection in a 1 ml syringe. The manual drop counter was used to record the number of drops expelled. The residual intravesicular volume was recorded as the volume of fluid that was removed from the bladder following the void using a 1 ml syringe connected to the infusion tubing via a 3-way tap. The discontinuous cystometry protocol was repeated a minimum of three times at any one stimulation site to ensure reproducibility. The protocol was then repeated while electrical stimulation was applied to the midbrain (60 Hz, 0.5 ms, amplitude set at threshold voltage for complete inhibition of urine output during continuous cystometry) and the same parameters were recorded. The pre-stimulation control value (i.e. stimulator off) was compared to the 'stimulator on' condition.

4.3.3 Microinjection of D,L-homocysteic acid (DLH)

In 20 experiments, D,L-homocysteic acid DLH (50 nl, 0.2 M, in saline vehicle, pH 7.4, containing 2% PSB dye. Sigma-Aldrich, Gillingham, UK) was microinjected at sites where the effect of electrical stimulation in the midbrain on micturition had been investigated, during continuous cystometry. The microinjections were applied during the

inter-void period, and the 50nl volumes were injected slowly over 30 s using a 1 μ l Hamilton syringe (Hamilton Bonaduz AG, Switzerland). The effect of microinjection of DLH on the micturition reflex and the cardiovascular and respiratory parameters recorded was measured.

Experiments were terminated with an overdose of pentobarbital, followed by cervical dislocation. Brains were removed and fixed in 10% formol saline for at least 48 hours before they were sectioned to determine the location of electrode placement. Stimulation sites were identifiable following sectioning as blue spots from the pontamine sky blue dye added to the DLH (for details see Chapter 2: General methods, Sections 2.4 and 2.5).

4.3.4 Analysis

All data have been reported as mean \pm SEM, and analysed with a Student's paired t test unless otherwise stated (for further details see Chapter 2: General methods, Section 2.6).

4.3.4.1 Electrical stimulation

An 'inhibitory effect' where electrical stimulation was applied at the onset of a void was defined as the complete prevention of drop output during the 60 s period of stimulation that was started at the onset of a void as judged by the sharp increase in intravesicular pressure normally associated with voiding. A facilitatory effect was defined as an increase in the drop output during stimulation that was more than double that of the

control mean (derived from 3 control contractions), and was also accompanied by a doubling (or greater) of the duration of bursting activity associated with urine output in comparison to the mean of 3 control contractions. All responses falling between these two definitions are described as 'no effect'.

4.3.4.2 DLH

Due to the inherent variability in the interval between voids in any one rat, the short duration of action of DLH, and the difficulty in identifying when a response started, an 'effect' was defined as any inter-void interval between the first 5 contractions following microinjection of DLH that lay more than 3 standard deviations away from any of the 5 control inter-void intervals immediately prior to the injection starting. Any inter-void intervals that were less than 3 standard deviations away from any of the control inter-void intervals were deemed 'no effect'.

4.4 RESULTS

4.4.1 Effect of electrical stimulation on reflex micturition when applied at the onset of a void

An initial series of experiments were carried out to determine whether electrical stimulation in the periaqueductal grey (PAG) could influence the cycles of filling and voiding induced by continuous infusion of saline into the bladder. These preliminary experiments revealed that at many sites in the PAG and surrounding tegmentum it was possible to suppress urine output completely during stimulation. When the train of pulses was initiated within 5 s of an imminent void, signalled by a sharp rise in bladder pressure, the void was aborted (Fig. 4.3). Urine output was suppressed completely but voiding resumed once the stimulation ceased. Based on the preliminary data, in subsequent experiments we chose to use a “standard” 60 s stimulus train of 0.5 ms pulses at 60 Hz and up to 2.5 V intensity to identify effective stimulation sites.

Although electrical stimulation at many midbrain sites was able to inhibit voiding completely, the suppression of urine output was accompanied by two distinct patterns of change in bladder pressure and activity in the external urethral sphincter (EUS). In the first type of response (Type 1, n = 53) the sharp rise in intravesicular pressure was aborted once the electrical stimulation started and the pressure dropped rapidly back towards the baseline, resulting in a significant reduction in the amplitude of the pressure

rise and duration of contraction (Fig. 4.4, from 22.3 ± 0.6 to 8.4 ± 0.9 mmHg, $P < 0.0001$, and from 34.6 ± 1.3 to 15.2 ± 1.2 s, $P < 0.0001$ respectively). No bursting activity developed in the EMG of the EUS but tonic activity was maintained throughout the stimulation period. No urine escaped from the penis for the duration of the stimulus, despite intravesicular pressure and bladder volume rising further as the infusion of saline into the bladder continued (Fig. 4.3 A). On other occasions (Type 2 responses, $n = 13$), electrical stimulation still reduced the amplitude of the pressure rise normally associated with a void from 23.3 ± 0.7 to 15.8 ± 2.3 mmHg (Fig. 4.4, $P = 0.001$), but the duration of contraction was increased from 36.4 ± 5.3 to 71.2 ± 5.6 s (Fig. 4.4, $P < 0.0001$). In these cases, despite the contraction being maintained for the duration of the stimulation period, no urine was expelled and in all cases bursting activity in the EUS normally associated with drop output failed to develop (Fig. 4.3).

At a small minority of sites (4/70 effective sites) in 3 rats, electrical stimulation in the midbrain produced facilitatory effects on the micturition reflex (Fig. 4.5, grey filled stars). These responses were characterised by an increase in the number of urine drops produced (Fig. 4.6 A, $+9.9 \pm 1.4$ drops void⁻¹), an increase in the duration of contraction (Fig. 4.6 B from 37.3 ± 3.8 to 85.5 ± 17.6 s) and an increase in the duration of the bursting activity in the EUS that is associated with urine output (Fig. 4.6 C, from 4.1 ± 0.8 to 32.5 ± 9.8 s). However, there was no obvious change in the amplitude of the pressure rise associated with each contraction (Fig. 4.6 D, 26.5 ± 0.5 v. 26.9 ± 2.1 mmHg).

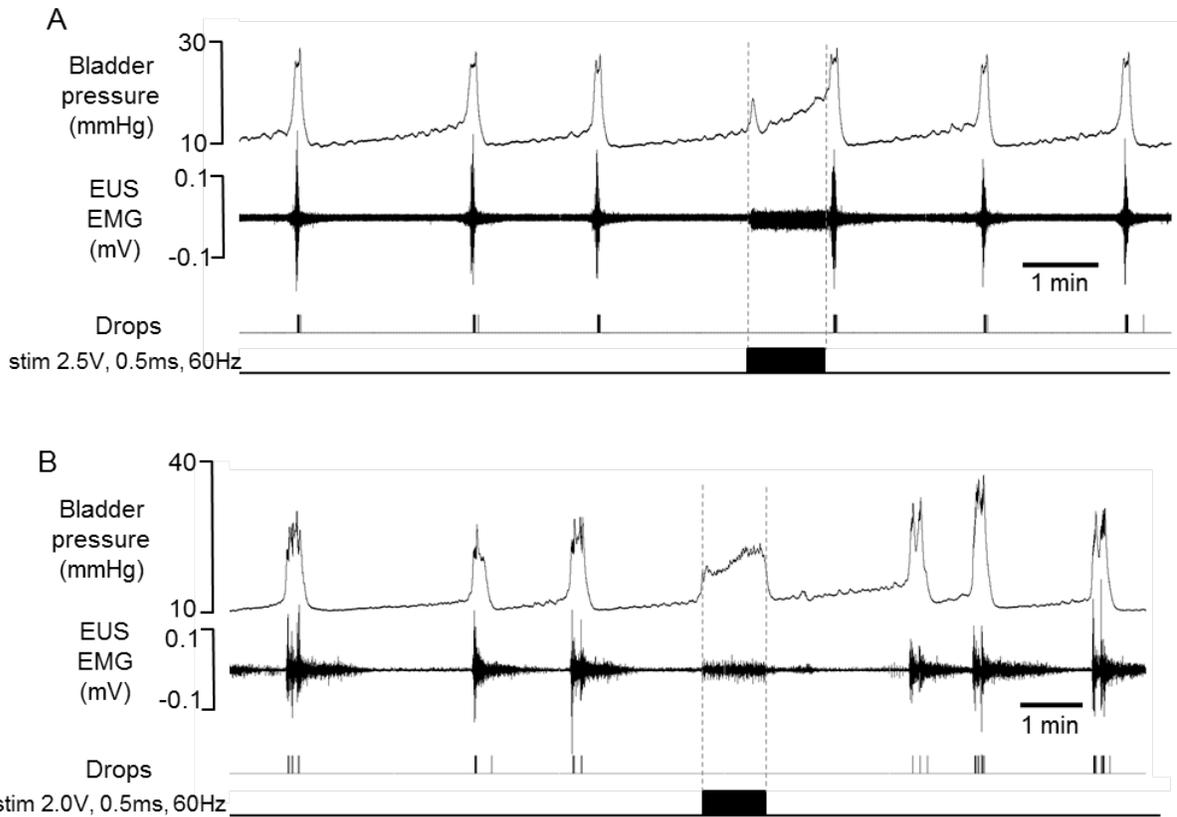


Figure 4.3. Electrical stimulation of the midbrain suppressed cyclical voiding responses evoked by continuous infusion of saline (6 ml h^{-1}) into the bladder. A: Type 1 pattern of response was associated with a reduction in the amplitude of the pressure rise and duration of voiding alongside inhibition of drop output during electrical stimulation at midbrain sites. **B:** Type 2 pattern of response was associated with inhibition of voiding during electrical stimulation and a reduction in the amplitude of the pressure rise, but an increase in the duration of contraction. Thick black line in between vertical broken lines indicates stimulation period.

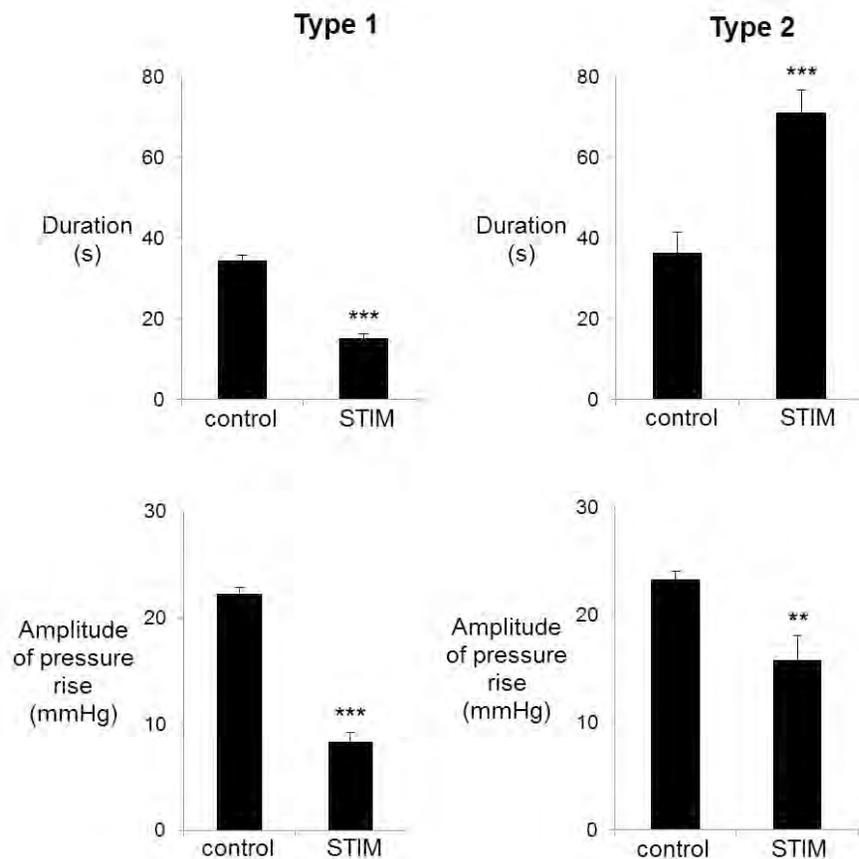


Figure 4.4. Electrical stimulation of the midbrain inhibited urine output and this suppression was accompanied by two distinct patterns of change in bladder pressure during stimulation: quantification. In the first type of response, ‘type 1’ (left hand side, $n = 53$) the duration and amplitude of the pressure rise normally associated with a contraction was significantly reduced. In ‘type 2’ responses (right hand side, $n = 13$), the duration of contraction was significantly increased during stimulation, along with a significant reduction in the amplitude of the contraction. *** $P < 0.0001$, ** $P = 0.001$.

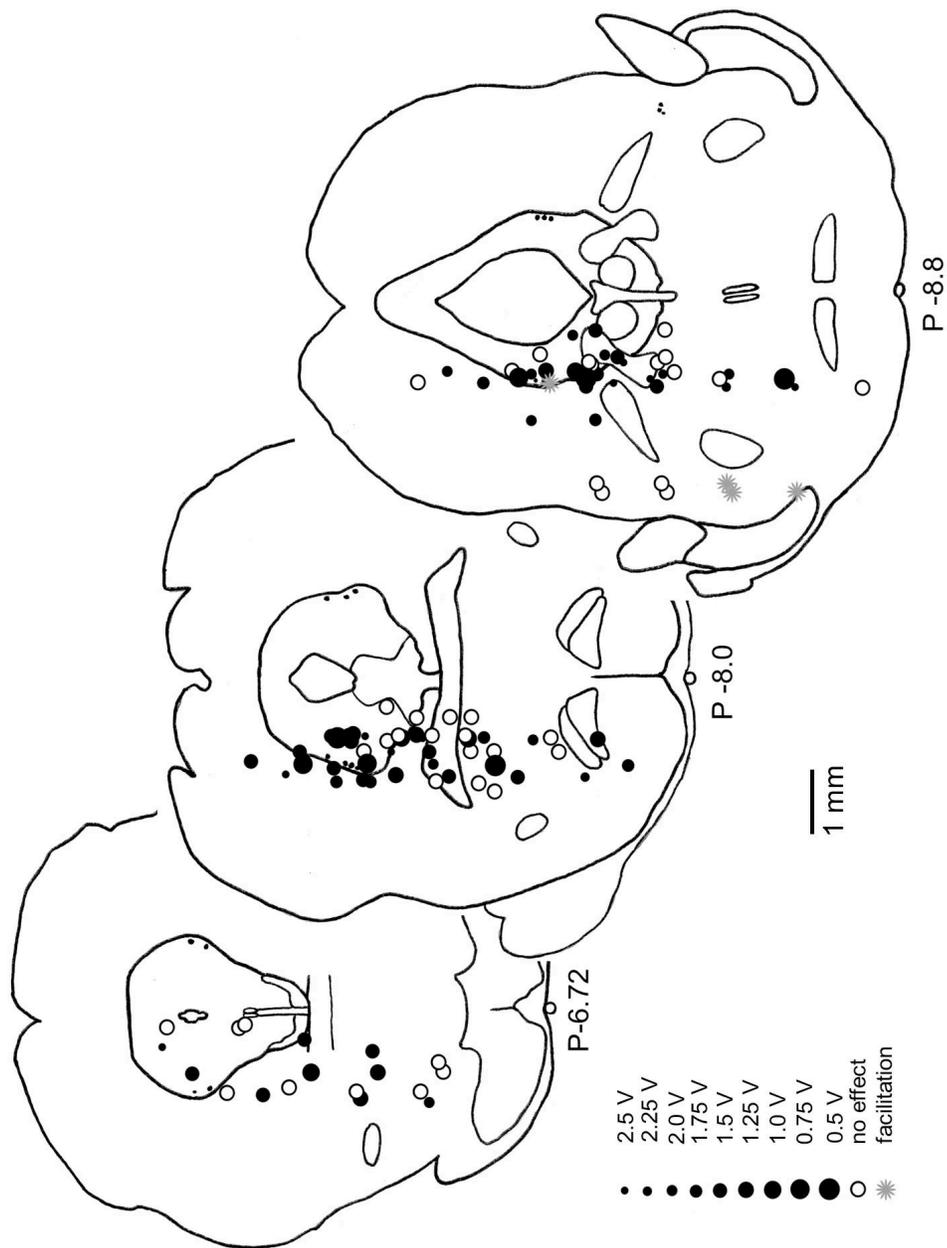


Figure 4.5. Location of sites where electrical stimulation was applied in the midbrain. Plotted onto outlines of the midbrain taken from the Atlas of Paxinos and Watson (1986). Filled circles: sites at which electrical stimulation inhibited micturition. Diameter of circles inversely proportional to the minimum stimulus intensity needed to inhibit micturition at that site. Grey stars represent centre of sites at which electrical stimulation facilitated micturition. Open circles: no effect of electrical stimulation. Numbers below section outlines represent mm caudal to Bregma.

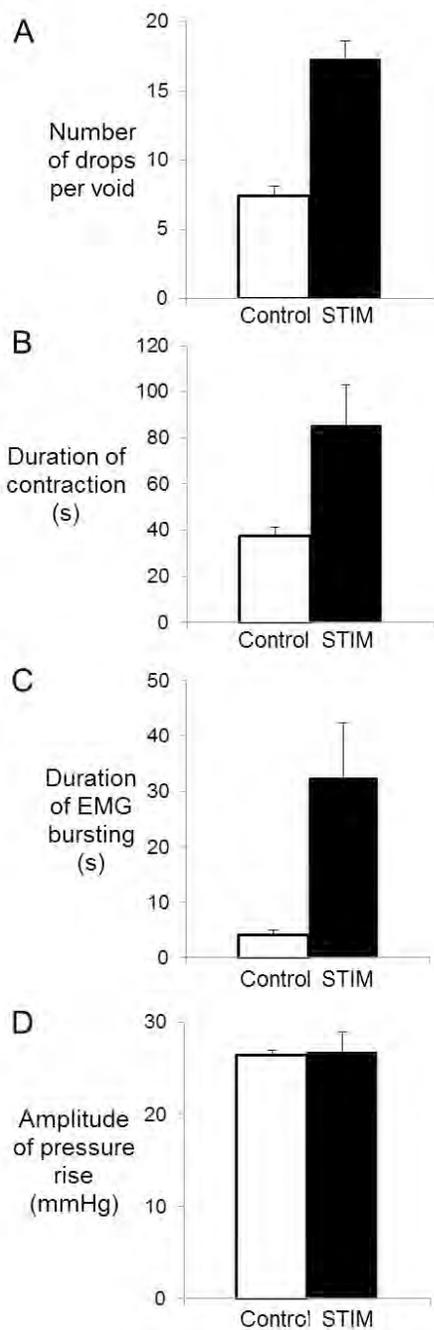


Figure 4.6. Electrical stimulation facilitated micturition when applied at the onset of a void signalled by a sharp increase in intravesicular pressure at 4 midbrain sites. This facilitation was characterised by A, an increase in the number of drops associated with a void in comparison to the control period, B, an increase in the duration of the contraction, C, an increase in the duration of EMG bursts followed by EMG silence, and D, no obvious change in the amplitude of the pressure rise associated with voiding.

4.4.2 Effect of stimulation between voids on reflex micturition

At 13 sites where application of electrical stimulation at the onset of a void could inhibit micturition, stimulation was applied during the filling phase between contractions. There was no change in the inter-void interval following stimulation and there was no change in the volume of the post-stimulation void in comparison to the mean of the three pre-stimulation control contractions (Fig. 4.7, and Table 4.1). However there was an increase in mean arterial blood pressure (MABP, $+21.4 \pm 7.4$ mmHg), heart rate ($+22.6 \pm 5.3$ beats min^{-1}) and respiratory rate ($+76.2 \pm 39.2$ breaths min^{-1}), table 4.2.

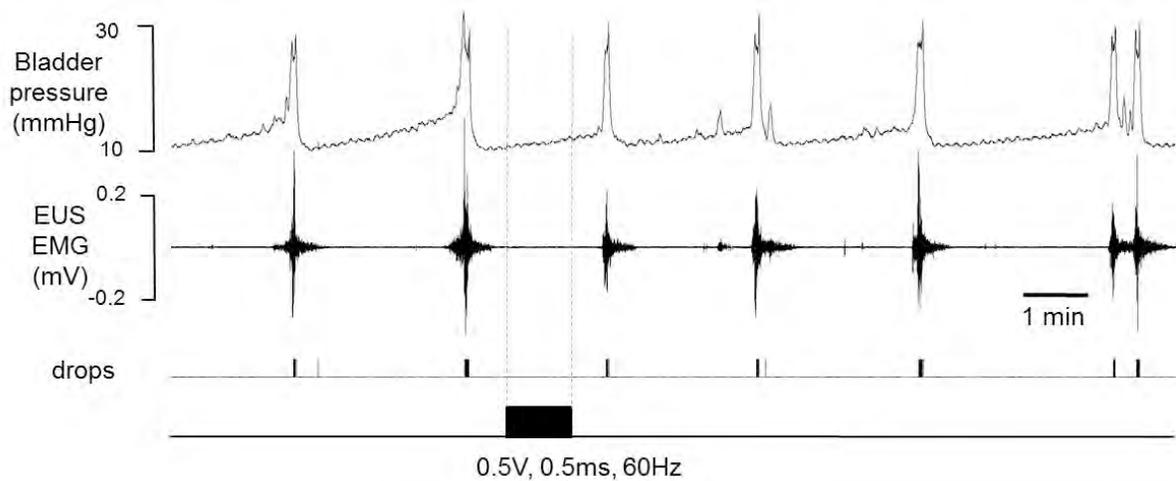


Figure 4.7. Effect of stimulation between voids at a site where stimulation applied at the onset of a void was able to inhibit urine output for the duration of the stimulus. Abbreviations: EUS; external urethral sphincter, EMG; electromyograph.

Table 4.1. Effect of stimulation between voids at inhibitory sites in the midbrain.

	Control	STIM	P =
Number of drops per void	7.2 ± 0.7	7.6 ± 1.7	0.9
Inter-void interval (s)	113.6 ± 18.5	163.0 ± 25.0	0.4

Mean ± SEM, Student's paired *t* test, n = 13.

Table 4.2. Effect of electrical stimulation of the midbrain between voids on cardiorespiratory parameters.

	Control	STIM	P =
MABP (mmHg)	105.8 ± 2.3	127.2 ± 7.5	0.01
Heart Rate (BPM)	434.3 ± 12.1	456.9 ± 11.6	0.001
Respiratory Rate (Br.PM)	170.0 ± 5.4	246.2 ± 40.8	0.11

Abbreviations: mean arterial blood pressure (MABP, mmHg), heart rate (beats min⁻¹ (BPM)) and respiratory rate (breaths min⁻¹ (Br.PM)). Mean ± SEM, Student's paired *t* test, n = 13.

4.4.3 Effect of longer periods of stimulation on reflex micturition

At 12 sites where stimulation using standard parameters (0.5 ms, 60 Hz, 0.5 – 2.5 V) could inhibit urine output for at least 60 s, the effects of longer periods of stimulation were investigated. Stimulation was started at the onset of a void (as judged by a sudden increase in the bladder pressure) and was continued until drop output returned (Fig. 4.8). Stimulation increased the inter-void interval by up to 438 s (Fig. 4.8, range +49.3 s to +438.0 s, n = 12). At two of these sites urine output was not accompanied by a transient increase in bladder pressure and bursting in the EMG of the EUS (as in Fig. 4.8 A), but was accompanied by a sustained, high bladder pressure and tonic EMG activity (Fig. 4.8 B), however there was still a significant increase in the inter-void interval during application of the stimulus (Fig. 4.8 C). This suppression was accompanied by a pressor effect ($+29.3 \pm 3.2$ mmHg, range +10.3 to +45.6 mmHg), tachycardia ($+36.4 \pm 7.0$ beats min^{-1} , range +5.0 to +73.0 beats min^{-1}) and tachypnoea ($+47.6 \pm 7.8$ breaths min^{-1} , range +19.0 to +98.0 breaths min^{-1}).

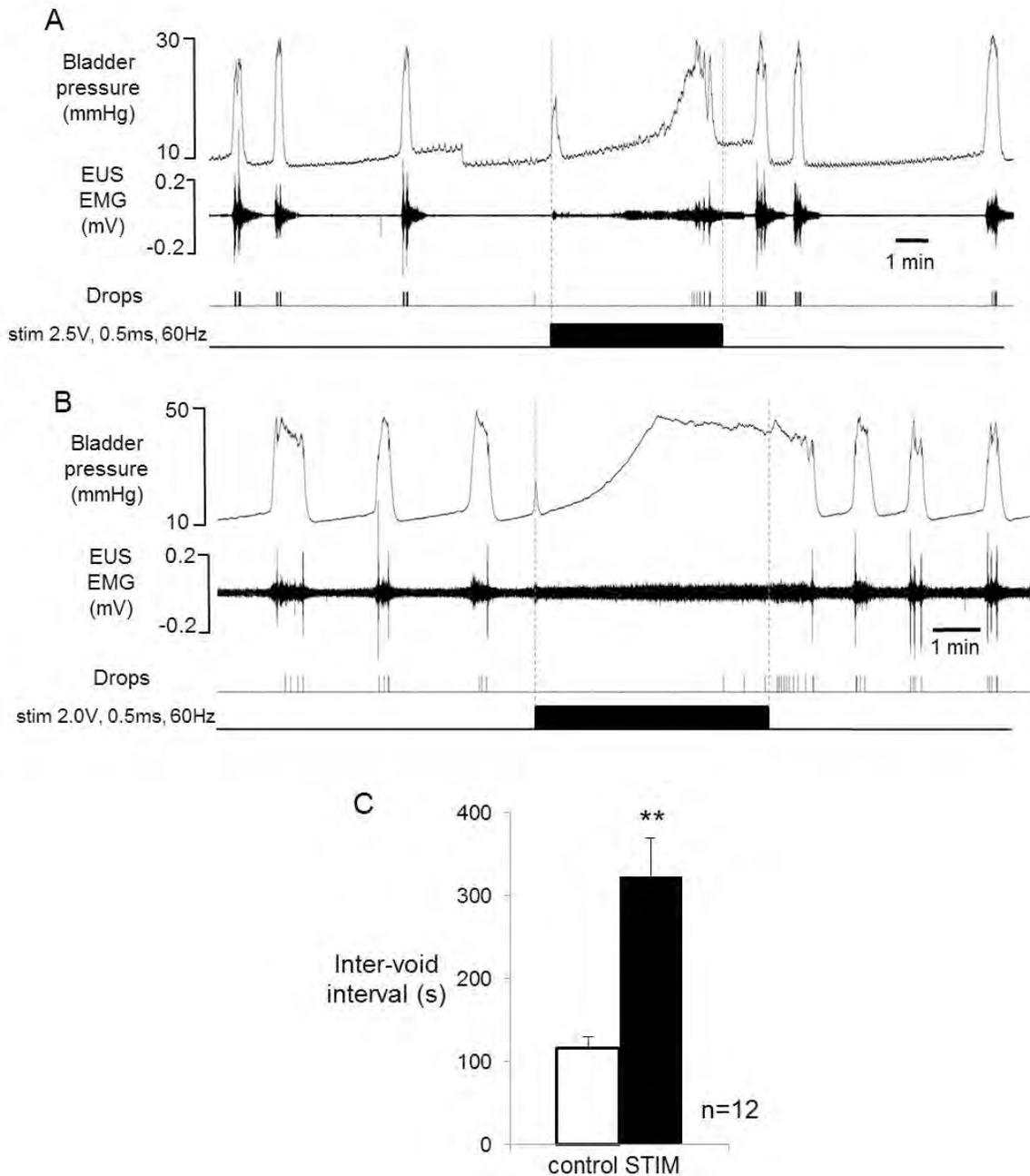


Figure 4.8. Effect of longer periods of electrical stimulation in the midbrain on reflex micturition. A: In 10 cases, the coordinated relationship between the bladder and EUS was maintained, but the inter-void interval was increased. B: In 2 cases, the intravesicular pressure reached a sustained level and urine output was not associated with bursting activity in the EUS. C: Long periods of electrical stimulation (> 60 s) significantly increased the inter-void interval. Stimulation period (STIM) was compared to control period with stimulation turned off.; Abbreviations: EUS; external urethral sphincter, stim; stimulation. **P < 0.001

4.4.4 Localisation of effective stimulation sites

4.4.4.1 Inhibitory sites

A threshold mapping protocol was adopted to determine whether there was any localisation of the effect of electrical stimulation in the midbrain. Once an inhibitory site had been identified, the minimum intensity at which urine drop output was inhibited for the duration of the 60 s period of stimulation was determined. The maximum intensity applied was 2.5 V and the frequency and pulse duration was kept constant (60 Hz, 0.5 ms) throughout these experiments. The effect of stimulation at 113 midbrain sites in 34 rats was tested (Fig. 4.5). At 66 sites, urine output was completely inhibited for the duration of the stimulation period (Fig. 4.5, black filled circles). Inhibitory sites that required the lowest voltage for complete inhibition of drop output (0.5 – 1.5 V) were primarily located within and close to the PAG at the caudal levels (Fig. 4.5, P -8.0 and P -8.8 mm) though some were located in the underlying tegmentum at all levels of the PAG studied.

4.4.4.2 'No effect' sites

At 43 sites, urine output could not be prevented for the duration of the stimulation period, even with the amplitude set at 2.5 V (Fig. 4.5, open circles). There was no significant change in the number of drops per void or the amplitude of contractions at these sites (Fig. 4.9 A and B: 6.6 ± 0.3 v. 7.7 ± 0.8 drops void⁻¹, $P > 0.05$, 23.1 ± 0.5 v. 22.2 ± 0.7 mmHg, $P > 0.05$). There was however, an overall increase in the duration of bladder

contraction during the stimulation period (Fig. 4.9 C: $+11.0 \pm 4.0$ s, $P < 0.01$). These ineffective sites were located mainly within the PAG at rostral levels (Fig. 4.5, P -5.6 mm) and in the tegmentum ventral to the PAG at caudal levels (Fig. 4.5, P -8.0 and P -8.8 mm).

4.4.4.3 Facilitatory sites

As described earlier, at 4 sites in 3 rats, electrical stimulation in the midbrain produced facilitatory effects on the micturition reflex (Fig. 4.5 grey filled stars). Three of the facilitatory sites were clustered in the ventral midbrain within the region of the motor nucleus of the trigeminal nerve (Fig. 4.5, P -8.8 mm grey stars). During stimulation at these three sites, rhythmic movement of the lower jaw was noted and the ipsilateral vibrissae vibrated. The remaining facilitatory site was located on the boundary of the caudal ventrolateral PAG near to the mesencephalic trigeminal nucleus as outlined in the atlas of Paxinos and Watson (1986), ME5 (Fig. 4.5, P-8.8 mm, grey star).

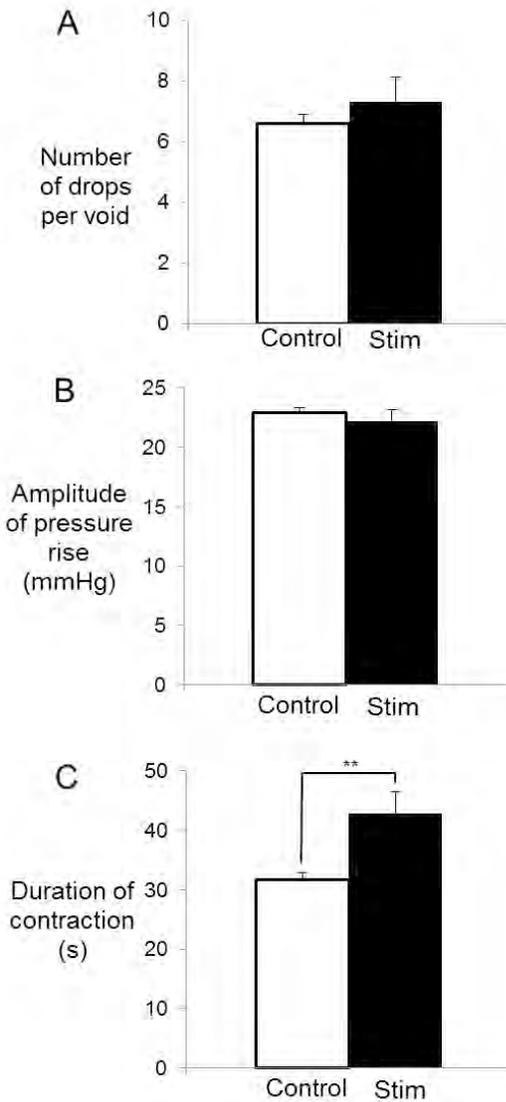


Figure 4.9. Electrical stimulation did not inhibit reflex micturition at 43 midbrain sites. A, Drop output could not be prevented for the 60 s duration of the stimulation period with pulse amplitudes ≤ 2.5 V. B, At these sites there was no change in the amplitude of the pressure rise normally associated with a void, however there was, C, a significant increase in the duration of contraction. ****P<0.001.**

4.4.5 Determining the optimal stimulus parameters

At 12 midbrain sites in 6 rats, the effects of electrical stimulation whilst varying either the frequency or duration of pulses at a constant amplitude was investigated to determine whether a) there was any difference in the effect on the micturition reflex, and b) whether changing the pulse parameters either increased or decreased the intensity of the associated cardiovascular and respiratory changes.

4.4.5.1 Effect of changing the frequency of pulses on reflex micturition

Increasing the frequency of square wave electrical pulses (of constant pulse duration; 0.5 ms) reduced the number of drops per void (Fig. 4.10 A). At frequencies of 40 Hz and above, drop output could be completely suppressed at times. Trains of stimulation with a frequency of less than 40 Hz did not completely prevent drop output for the duration of the 60 s stimulation at any time (Fig. 4.10 A).

The amplitude of the pressure rise normally associated with urine output was reduced by the greatest percentage when stimulation was applied at 80 Hz and above (Fig. 4.10 B). There was no relationship between the 'type' of response (i.e. 'type 1', shorter duration of contraction during stimulation and 'type 2', longer duration of contraction during stimulation) and the frequency of pulses (Fig. 4.10 C), with individual rats showing a mixture of these responses (Fig. 4.11).

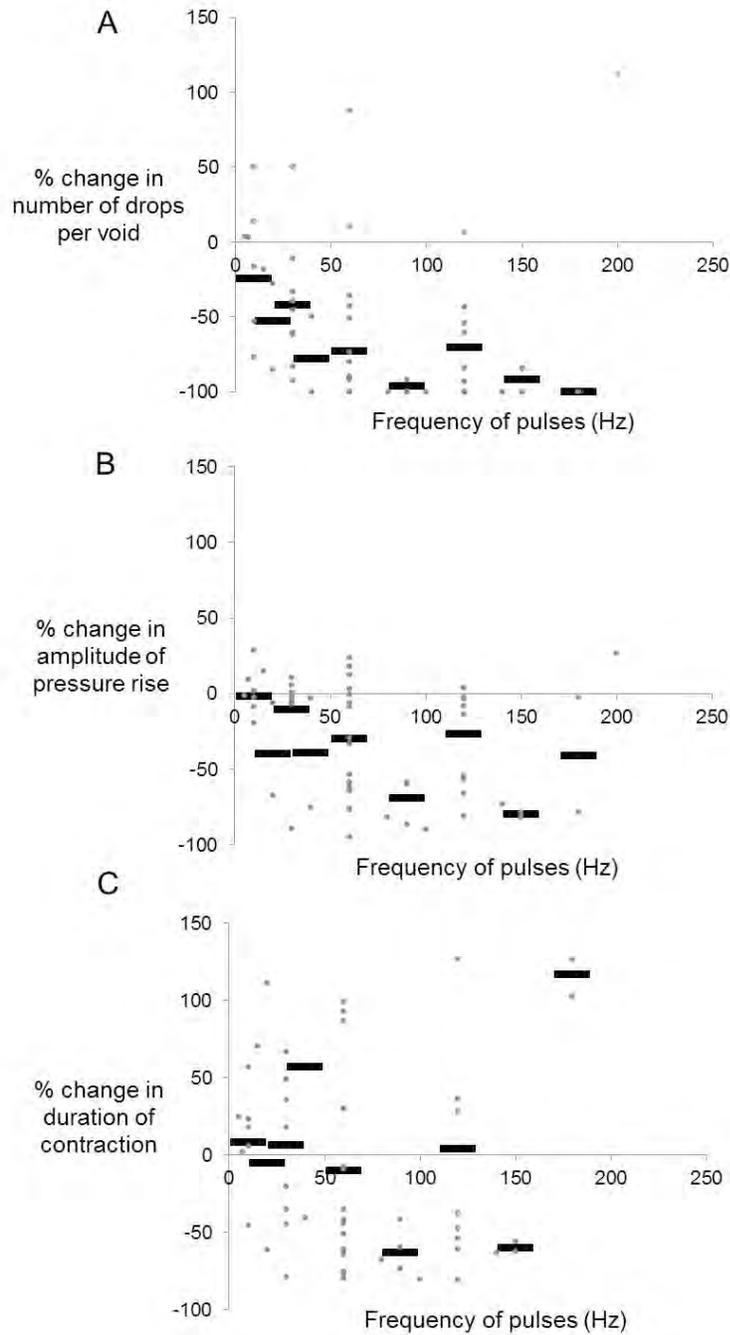


Figure 4.10. Effect of changing the frequency of electrical pulses applied at midbrain sites on reflex micturition (59 stimulations at 8 different sites in the midbrain). Data represented as % change in A; number of drops, B; duration of contraction and C; amplitude of the pressure rise normally associated with drop output. Grey spots show raw data for each stimulation. Horizontal black lines: Data average for each frequency. Nb. groups without any repeats have no average.

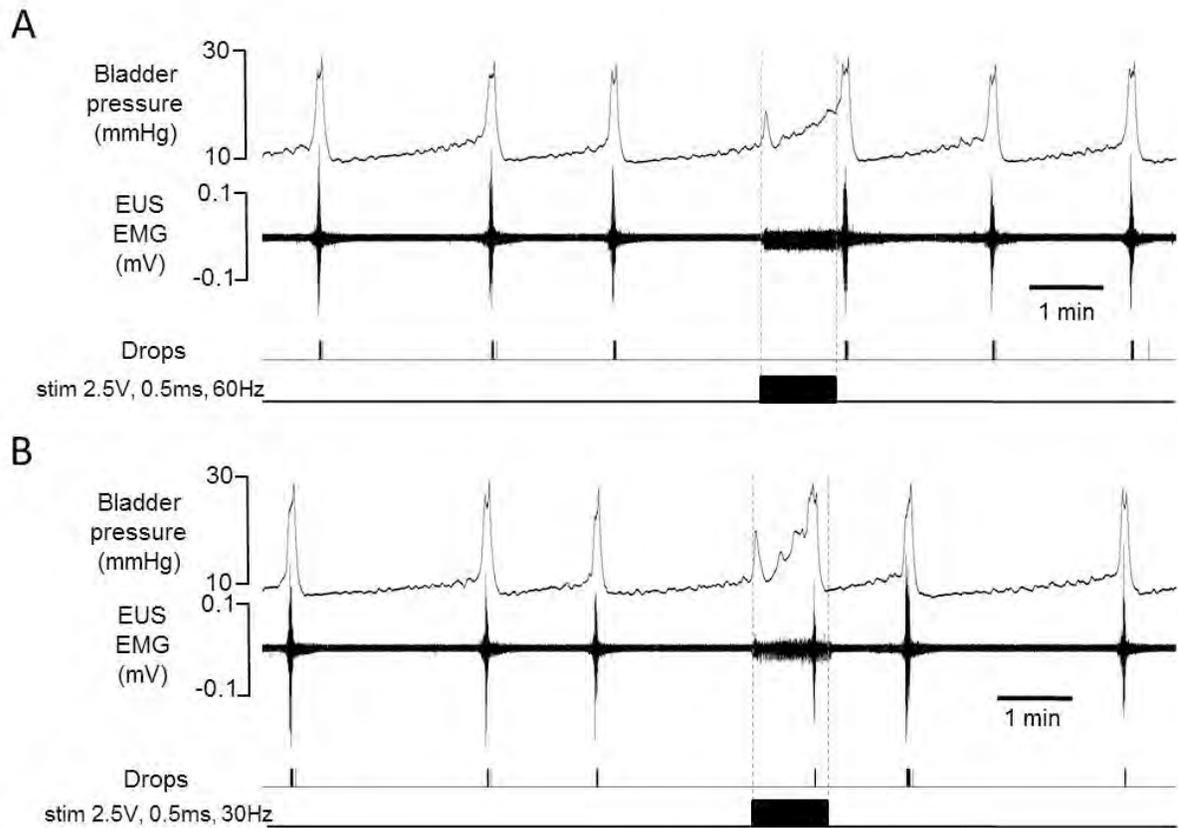


Figure 4.11. Effect of changing the frequency of electrical pulses applied at one site in the midbrain on the micturition reflex evoked by continuous infusion of saline into the bladder (6 ml h^{-1}). A, Typical 'type 1' response. The duration of contraction and the amplitude of the intravesicular pressure rise associated with a contraction were both reduced and urine output was completely inhibited. B, When the frequency of stimulation was reduced to 30 Hz, the duration of contraction was increased. There was no change in the amplitude of the pressure rise associated with a void and though there was a reduction in the number of drops expelled, output was not completely inhibited as it was at 60 Hz. Abbreviations: EUS; external urethral sphincter, stim: stimulation.

4.4.5.2 Effect of changing the duration of pulses on reflex micturition

Increasing the duration of square wave electrical pulses applied at 7 sites in 7 rats in the midbrain with a constant pulse frequency (60 Hz), reduced the number of drops per void (Fig. 4.12 A, Fig. 4.13 A, B, C). At pulse durations of 0.5 ms and above, drop output could be completely suppressed at times (Fig. 4.12 A). Pulses of less than 0.5 ms did not completely prevent drop output at any time. Increasing the pulse duration increased the % reduction in the amplitude of the intravesicular pressure rise normally associated with a void (Fig. 4.12 B). There was no causal relationship between the pulse duration and the 'type' of response (i.e. 'type 1', a reduction in the duration of contraction during stimulation, or 'type 2' an increase in the duration of contraction during stimulation, Fig. 4.12 C).

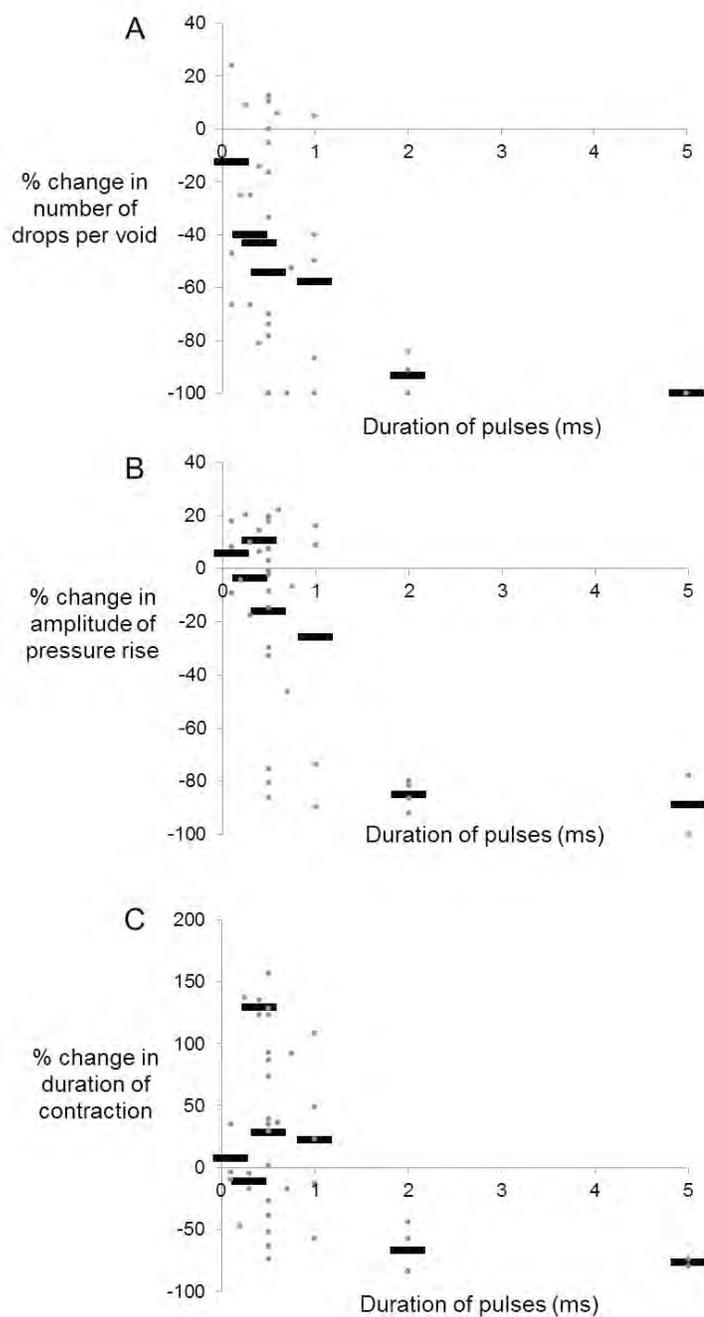


Figure 4.12. Effect of changing the duration of electrical pulses applied at midbrain sites on reflex micturition (41 stimulations at 7 different sites in the midbrain). Data represented as % change in A; number of drops, B; duration of contraction and C; amplitude of the pressure rise normally associated with drop output. Grey spots show raw data for each stimulation. Horizontal black lines: Data average for each frequency. Nb. groups with no repeats have no average.

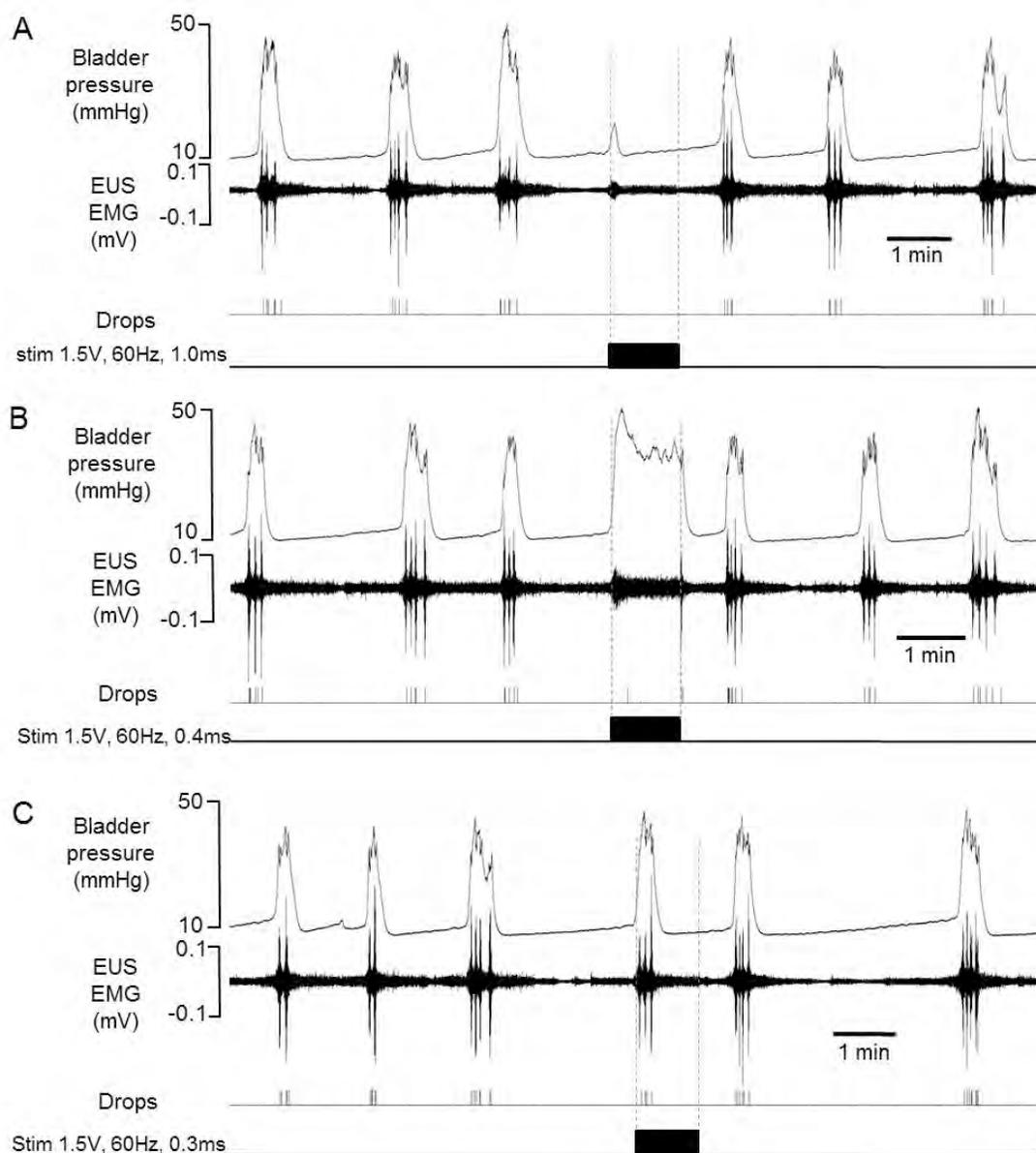


Figure 4.13. Effect of changing the duration of electrical pulses applied at one site in the midbrain on the micturition reflex evoked by continuous infusion of saline into the bladder (6 ml h^{-1}). Sample traces are all from the same stimulation site. A: Response typical of 'type 1' effect, duration and amplitude of contraction were reduced and voiding was aborted. B: When pulse duration was reduced to 0.4 ms, the duration of contraction was increased. There was no change in the amplitude of the pressure rise and there was a reduction in the number of drops expelled with as part of the 'void'. C: When pulse duration was reduced further to 0.3 ms, electrical stimulation evoked no obvious change in the duration or amplitude of contraction, and there was no reduction in the number of drops voided⁻¹. Abbreviations: EUS; external urethral sphincter, stim: stimulation.

4.4.6 Effect of electrical stimulation of the midbrain on the cardiovascular and respiratory system

Electrical stimulation at midbrain sites also evoked cardiovascular and respiratory changes, typically a pressor response, tachycardia and tachypnoea. These responses were intensified when the duration of the square-wave pulses was increased (Fig. 4.14 A, B, C). The effects of increasing the frequency of pulses on the mean arterial blood pressure (MABP) and heart rate reached a maximal at 60 Hz. Increasing the frequency above this level did not further intensify the effects (Fig. 4.15 A, B, C). Analysis of the cardiovascular and respiratory effects at 10 sites which lay in and around the PAG showed that pressor effects could be evoked at sites where micturition was not suppressed by the highest intensity stimulation ($+33.2 \pm 4.4$ mmHg, 2.5 V, 0.5 ms, 60 Hz).

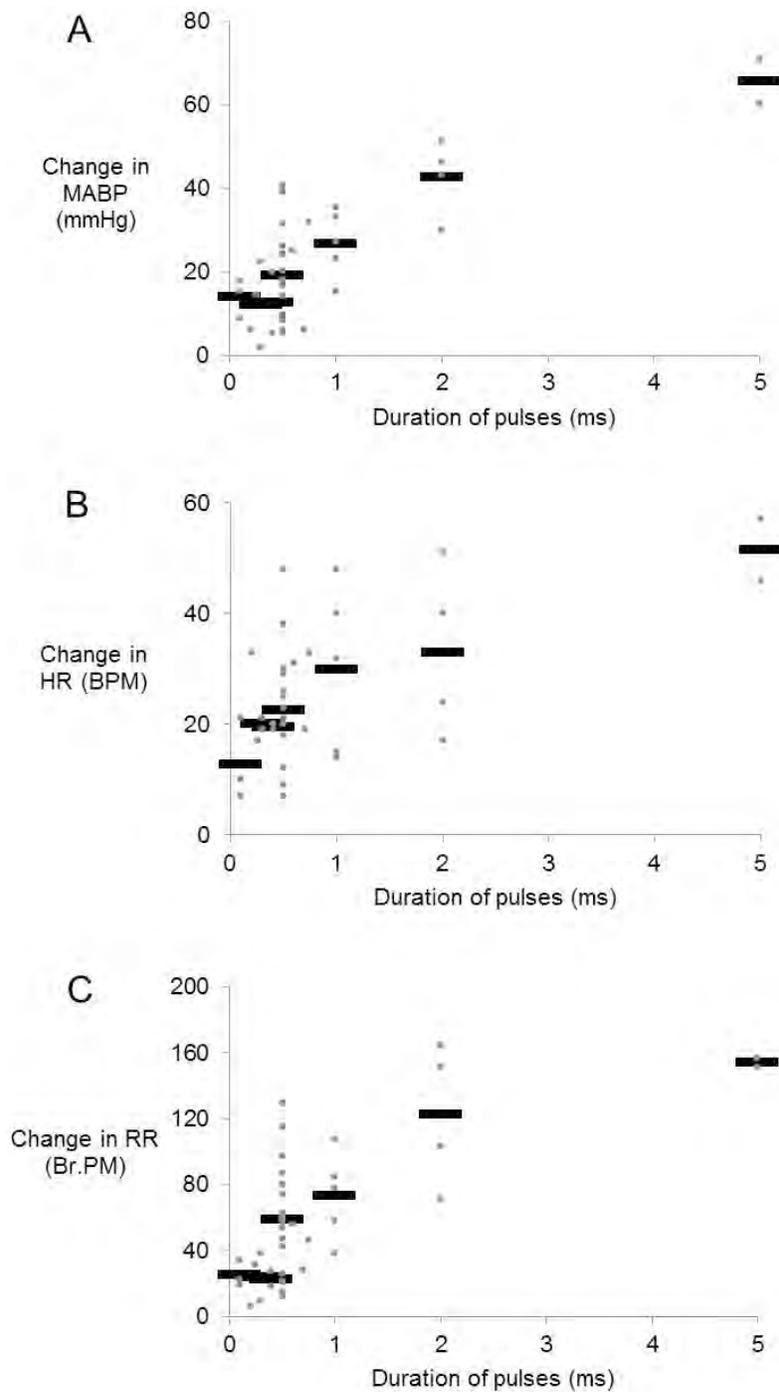


Figure 4.14. Effect of changing the duration of square wave pulses applied in the midbrain on the cardiorespiratory parameters recorded. A: Mean arterial blood pressure, B; heart rate, and C; respiratory rate. Grey spots show raw data for each stimulation. Horizontal black lines: Data average for each frequency. Nb. groups with only one repeat have not been averaged. Abbreviations: MABP: mean arterial blood pressure, HR: heart rate BPM: beats min^{-1} , RR: respiratory rate, Br.PM: breaths min^{-1} .

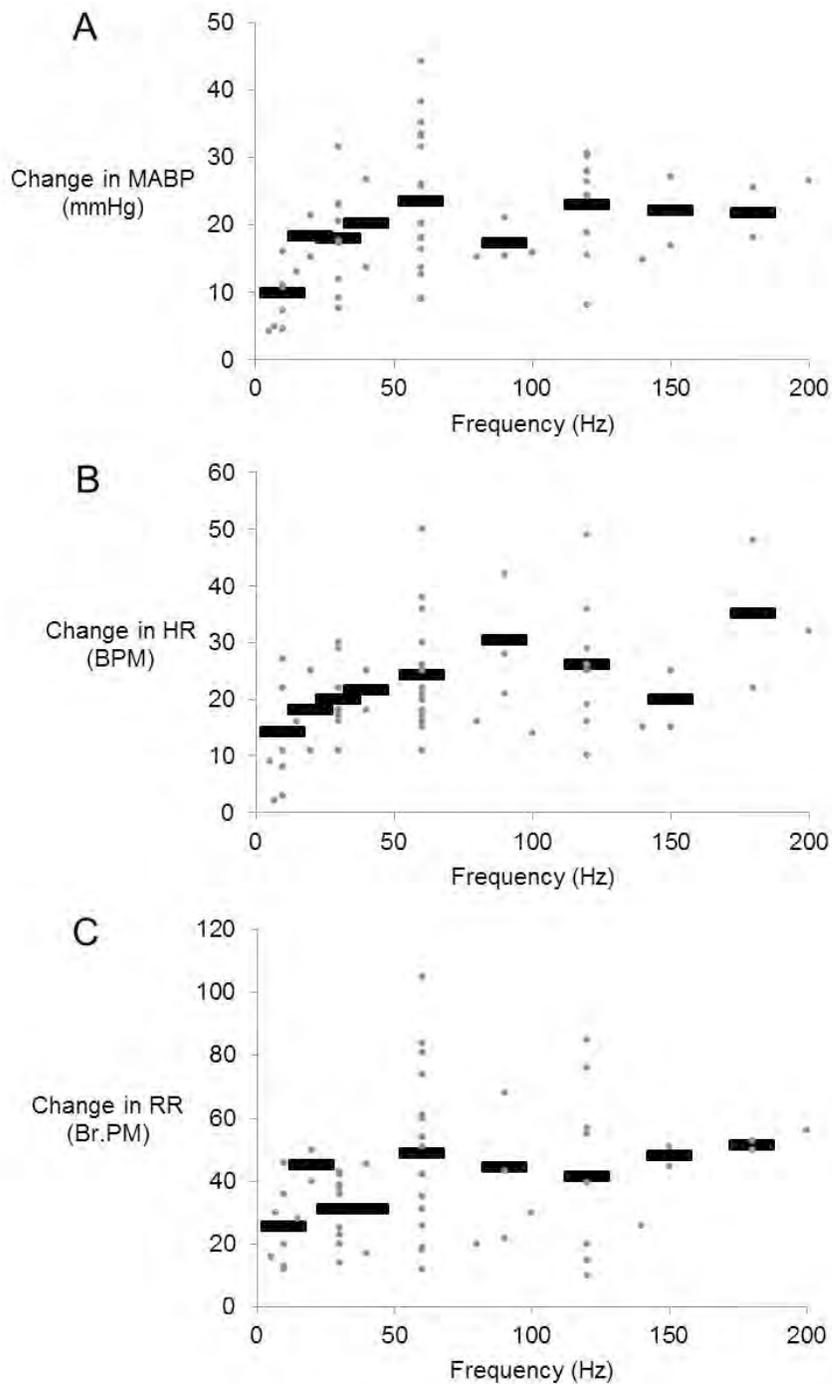


Figure 4.15. Effect of changing the frequency of square wave pulses applied in the midbrain on the cardiorespiratory parameters recorded. A: Mean arterial blood pressure, B; heart rate, and C; respiratory rate. Grey spots show raw data for each stimulation. Horizontal black lines: Data average for each frequency. Nb. groups with only one repeat have not been averaged. Abbreviations: MABP: mean arterial blood pressure, HR: heart rate BPM: beats min^{-1} , RR: respiratory rate, Br.PM: breaths min^{-1} .

4.4.7 Effect of electrical stimulation on the volume threshold for reflex micturition using discontinuous cystometry

In order to investigate whether the threshold intravesicular volume for evoking a coordinated void changed during stimulation a different protocol was used. At 10 sites in 5 rats, once an inhibitory site (i.e. a site where 60 s stimulation ≤ 2.5 V, 60 Hz, 0.5 ms could inhibit drop output) had been identified using electrical stimulation in the midbrain during continuous cystometry, the infusion into the bladder was stopped and the bladder was emptied manually by withdrawal of fluid via the injection port. Saline was then infused into the bladder ($0.1 - 0.2$ ml min^{-1}) until a void occurred. The latency to drop output was increased when stimulation was applied in the midbrain (Fig. 4.16 Ai, ii). The intravesicular volume at which a void was triggered was increased from 0.5 ± 0.1 ml to 0.8 ± 0.4 ml when the stimulation was applied (Fig. 4.16 B, $P < 0.001$, $n = 10$). At 8 sites, the bladder entered a state where urine output recommenced during the stimulation period but was not in association with bursting activity in the EUS. At this point, the stimulation was stopped and coordinated voiding returned within 5.4 ± 3.7 s (range 1 – 20 s). At the remaining 2 sites, coordinated voiding activity (with EUS EMG bursting) remained during stimulation, but the void was delayed in comparison to the control. There was a large degree of inter-rat variability in cystometric capacity (i.e. the total volume that each bladder held before drops of fluid were expelled from the penis, range 0.5 – 1.5 ml with stimulation on). However in every case, the threshold volume for urine output was increased during stimulation (Fig. 4. 16 B).

There was no difference in bladder compliance — i.e. the pressure-volume relationship — on or off stimulation (Fig. 4.16 C).

The intravesicular infusion of saline during discontinuous cystometry without electrical stimulation did not produce any changes in the MABP in comparison to the pre-infusion control period (Fig. 4.17 A, 86.8 ± 2.6 v. 87.1 ± 2.8 mmHg, $n = 10$, $P > 0.05$). However there was a significant increase in the MABP during stimulation in comparison to both pre-stimulation controls (Fig. 4.17 A, $+31.0 \pm 2.8$ mmHg, $P < 0.0001$). There was no correlation between the MABP and the latency to drop output (Fig. 4.17 B).

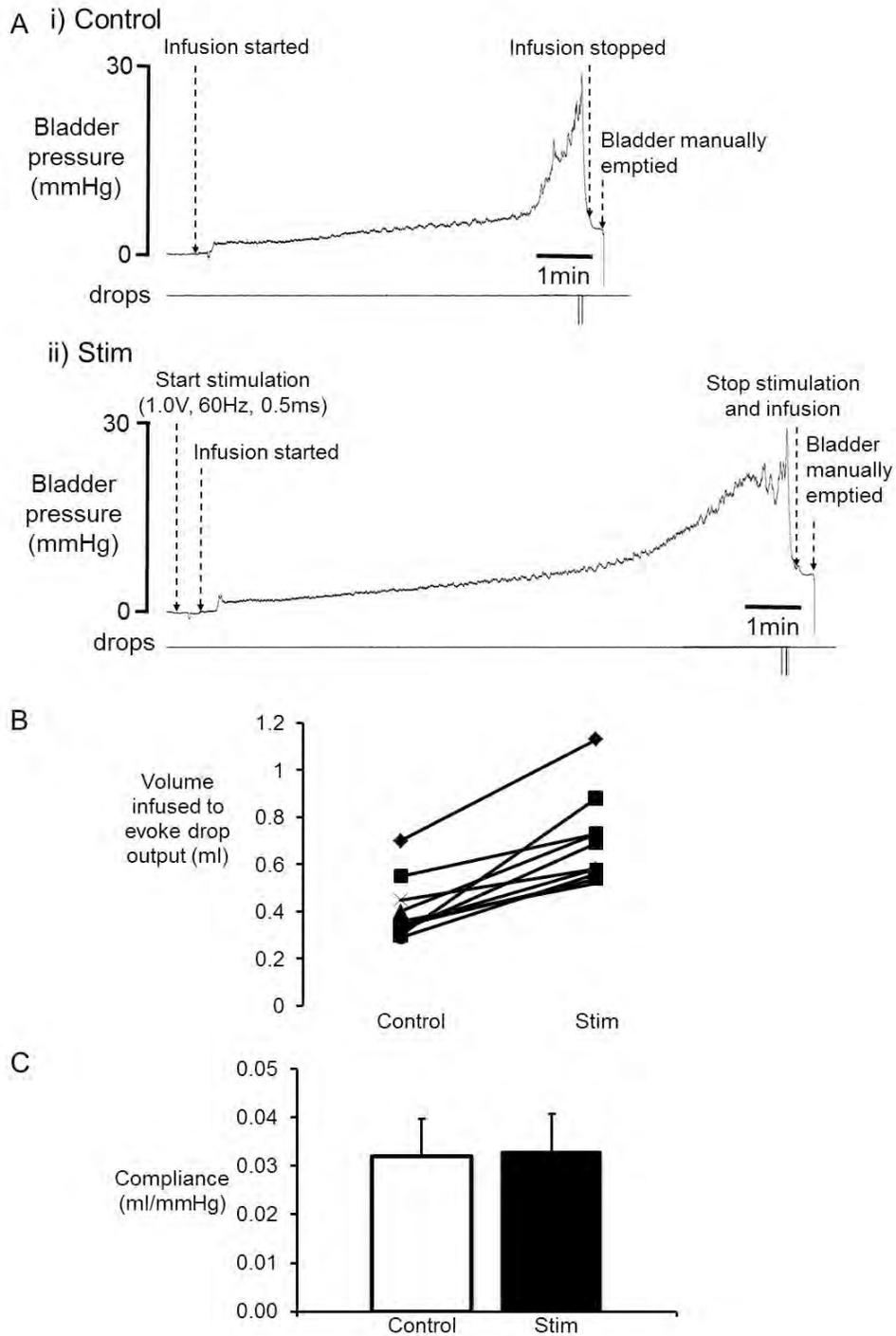


Figure 4.16. Alteration of the threshold volume for micturition during electrical stimulation of the midbrain at 10 sites in 5 rats. A, i, Control: Saline was infused into

an empty bladder ($0.1 - 0.2 \text{ ml min}^{-1}$). The latency from the start of the infusion to first drop output was measured with the stimulator turned OFF, and the threshold volume for urine output could be calculated from this latency and the flow rate. A, ii, Stim: This protocol was repeated while stimulation was applied ($\leq 2.5 \text{ V}$, 60 Hz , 0.5 ms). B: At every site there was an increase in the intravesicular volume at which a void occurred in the 'stimulator on' condition compared to the control 'stimulator off' condition. C: There was no change in the pressure-volume relationship (i.e. compliance) when the bladder was filled from empty during stimulation of the midbrain in comparison to the control 'stimulation off; condition.

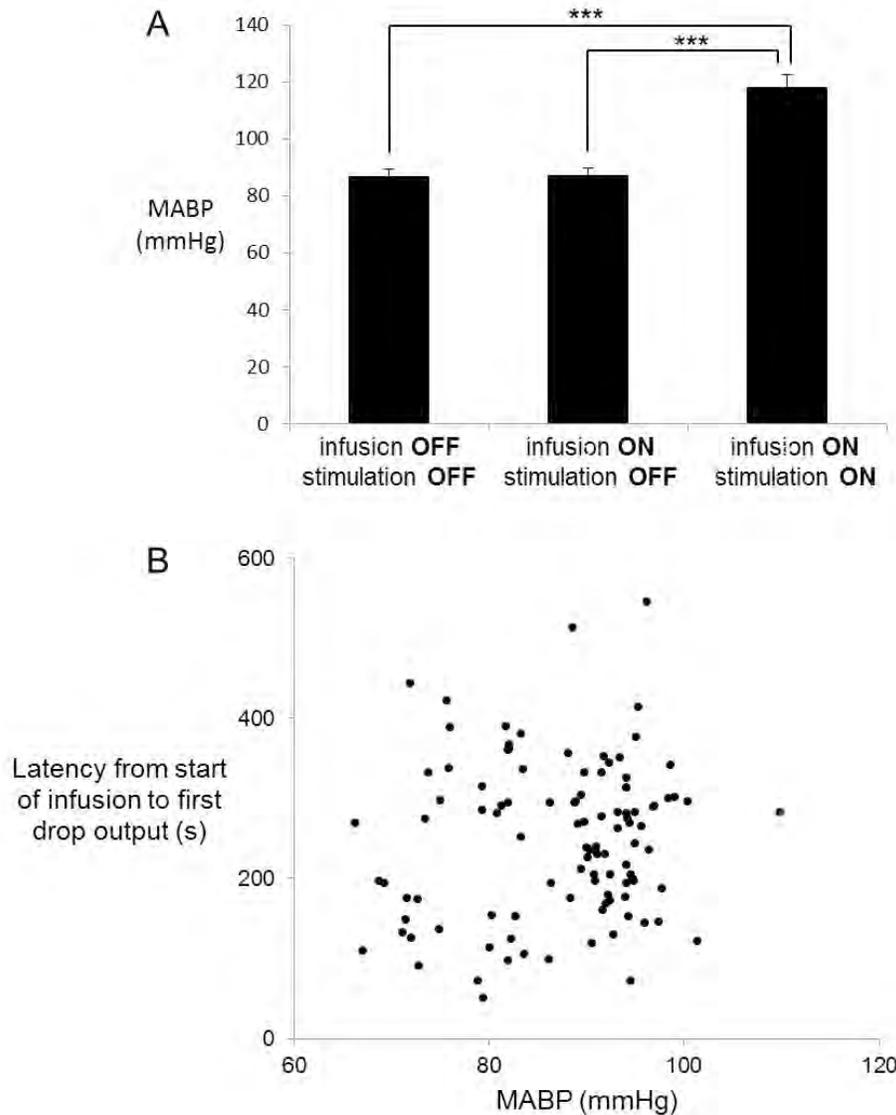


Figure 4.17. Comparison of effect of intravesicular distension and electrical stimulation at midbrain sites on blood pressure. A, intravesicular infusion of saline alone (“infusion ON, stimulator OFF”) did not affect the resting mean arterial blood pressure (MABP, “infusion OFF, stimulation OFF”). There was however, a significant increase in the MABP when electrical stimulation (≤ 2.5 V, 60 Hz, 0.5 ms) was applied at midbrain sites during infusion of saline into the bladder (“infusion ON, stimulation ON”, $n = 10$). B, there was no relationship between the mean arterial blood pressure and the latency to urine drop output, suggesting that the delayed drop output during electrical stimulation was not due to the pressor effect evoked by electrical stimulation. *** $P < 0.0001$.

4.4.8 Effect of microinjection of D,L-homocysteic acid (DLH) into the midbrain on reflex micturition

In 20 experiments, DLH (50 nl, 0.2 M, pH 7.4 containing pontamine sky blue dye) was microinjected at sites where the effect of electrical stimulation on micturition during continuous cystometry had been investigated. At the majority of sites ($n = 50$), microinjection of DLH evoked an increase in the inter-void interval from 104.4 ± 4.1 to 236.0 ± 9.8 s, (Fig. 4.18 B, C I, $P < 0.0001$). At 28 of these sites, electrical stimulation had been shown to inhibit voiding. Conversely, at 3 sites where microinjection of DLH produced inhibitory effects, electrical stimulation facilitated micturition (Fig. 4.19, compare with Fig. 4.5). At 4 sites, microinjection of DLH produced a facilitatory effect on the micturition reflex, with the inter-void interval being reduced from 183.2 ± 30.6 to 98.9 ± 13.3 s (Fig. 4.18 A, C ii).

Fig. 4.19 shows the location of injection sites. Inhibitory effects on micturition were seen following injections made in the midbrain, including in the periaqueductal grey and the underlying tegmentum. Facilitatory sites were predominantly clustered in the caudal end of the PAG in the lateral portion (Fig. 4.19, P -8.0 mm, grey stars, $n = 3$), an area at which electrical stimulation inhibited micturition, usually with low voltages (refer to Fig. 4.5). One facilitatory site was located more rostrally and ventral to the PAG (Fig. 4.19, P -6.72 mm, $n = 1$). At the remaining sites, microinjection of DLH had no effect on the micturition reflex (Fig. 4.19, open circles, $n = 30$).

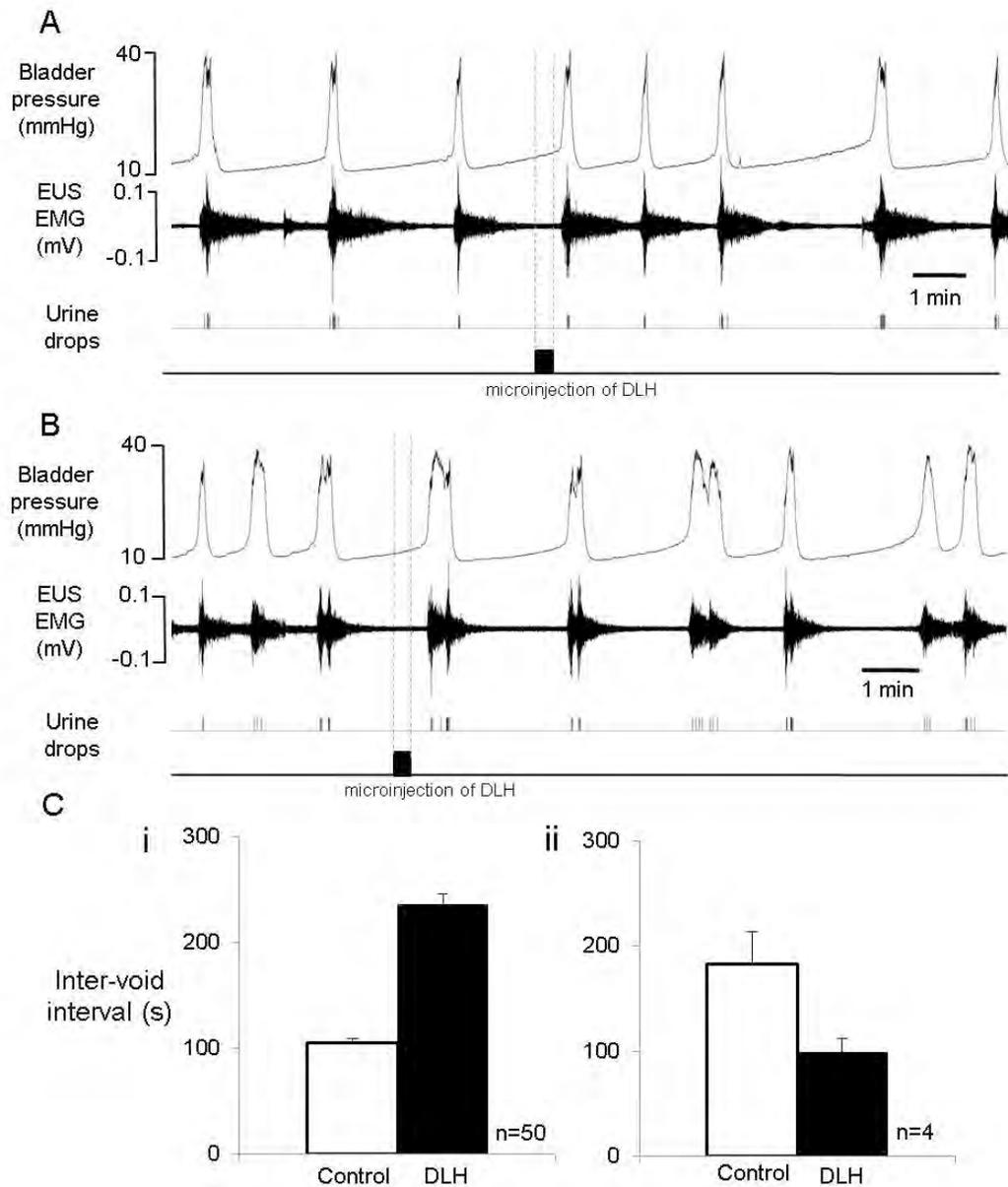


Figure 4.18. Effect of microinjection of D,L-homocysteic acid (DLH, 50 nl, 0.2 M) into the midbrain on micturition evoked by continuous intravesicular filling (6 ml h^{-1}). A: Sample trace shows facilitatory effect of microinjection of DLH into the midbrain during continuous cystometry ($n = 4$). B, Sample trace showing the inhibitory effects of microinjection of DLH into other areas of the midbrain. C, i: At 50 sites, microinjection of DLH increased the inter-void interval, and ii) at four sites, the inter-void interval was reduced following microinjection of DLH. $P < 0.001$.**

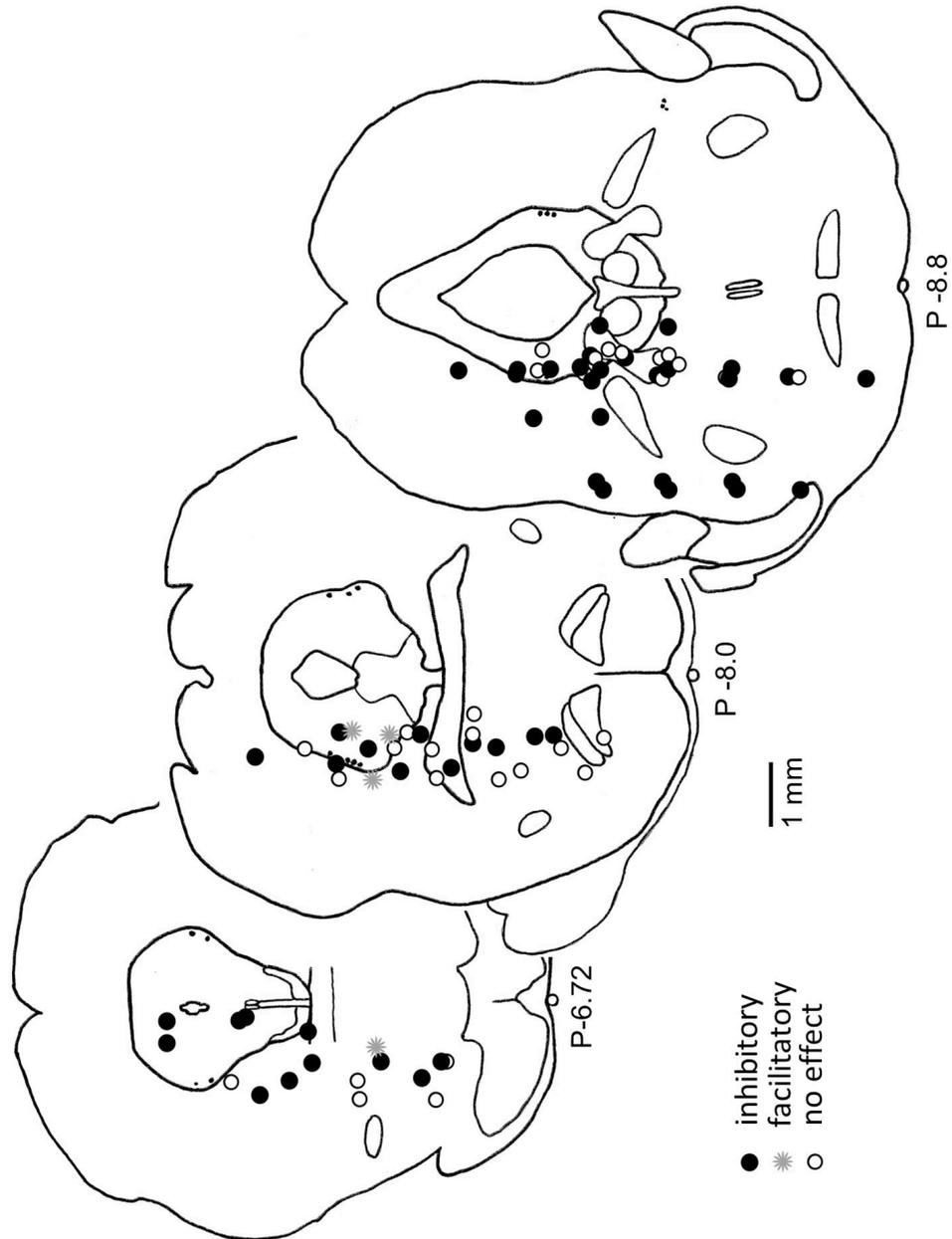


Figure 4.19. Location of sites at which 50 nl 0.2 M D,L-Homocysteic acid (DLH) with pontamine sky blue was microinjected into the midbrain. Plotted onto outlines of the midbrain taken from the Atlas of Paxinos and Watson (1986). Filled black circles represent the centre of sites where microinjection increased the inter-void interval during cystometry. Open circles represent the centre of microinjection sites where DLH had no effect on the inter-void interval. Grey filled stars represent the centre of sites at which microinjection of DLH reduced the inter-void interval between contractions during continuous cystometry.

4.5 DISCUSSION

In the present study electrical stimulation over an extensive area of the midbrain inhibited voiding evoked by continuous infusion of saline into the bladder. This finding is in line with previous studies in the rat, cat and dog which also showed that the micturition reflex could be modulated by electrical stimulation of the midbrain (Nishizawa *et al.*, 1987; Kruse *et al.*, 1990; Fukuda & Koga, 1991; Matsuura *et al.*, 2000; Liu *et al.*, 2004; Ito *et al.*, 2006; Numata *et al.*, 2008) .

In most cases, when applied at the onset of a void, electrical stimulation aborted the coordinated contraction of the bladder and bursting activity in the EUS normally associated with a void, and bladder pressure returned to baseline levels until stimulation was stopped. This was termed a 'type 1' response. Occasionally, electrical stimulation increased the duration of contraction whilst still preventing drop output, coordinated bursting in the EMG of the EUS and reducing the amplitude of the pressure rise that would normally be associated with drop output. This was termed a 'type 2' response. Noto and colleagues (1989) describe similar effects of electrical stimulation in the midbrain to the 'type 1' response shown in the present experiments, but did not report evoking 'type 2' responses. Since Noto and colleagues applied much shorter trains of stimulation, (between 0.3 and 3 s, compared to our 60 s train of stimulus pulses) it is unsurprising that they did not see sustained effects on the bladder and EUS (Noto *et al.*, 1989).

Interestingly, when electrical stimulation was applied during the interval in between voids, it had no effect on bladder pressure or on the level of on-going tonic activity in the EUS, despite the same stimulation inhibiting micturition when applied at the onset of a void. Moreover, the first void after cessation of stimulation was neither inhibited nor delayed, suggesting that the electrical stimulation disrupts the circuitry that coordinates voiding only when that circuitry is already activated by the afferent drive from a distended bladder, and not when that coordinating circuitry is inactive during the inter-void period. In contrast, Noto *et al.* (1989) found that stimulation in between voids produced a small drop in intravesicular pressure. However this was using an isovolumetric system, with the intravesicular pressure held supra-threshold for activation of the micturition circuitry, thus the afferent input from the distended bladder to the PAG would have been consistently high. This 'inter-void stimulation' condition would be represented in our model as stimulation at the onset of a void, where the intravesicular volume was sufficient to trigger a void, and afferent input to the PAG was at its highest, rather than an inter-void stimulation in our preparation when afferent input would have been at its lowest. Since it has been suggested that the efferent output of the PAG is dependent on the afferent input (Behbehani, 1995), this is an important clarification to make, and perhaps a criticism of the isovolumetric cystometry technique for investigation of the role of the midbrain in micturition.

It is unlikely that the inhibitory effects of electrical stimulation of the midbrain on micturition were simply due to activation of the inhibitory sympathetic drive to the bladder causing detrusor relaxation for two reasons (Skehan *et al.*, 1993; Khadra *et al.*,

1995; Smith *et al.*, 2011). Firstly, although there were clear signs of systemic sympatho-activation (including a pressor effect and tachycardia) when electrical stimulation inhibited micturition at the onset of a void, these sympathetically-mediated effects were also present when stimulation was applied during the inter-void period and evoked no changes in the micturition reflex. This suggests that the effects of electrical stimulation on micturition and the cardiovascular and respiratory systems are not evoked by a shared mechanism. Secondly, electrical stimulation of the midbrain during discontinuous cystometry evoked no change in the compliance of the bladder when the stimulation was applied. If the effects on micturition of electrical stimulation were due to activation of the inhibitory sympathetic pathway, then one would expect the bladder to become more relaxed and compliant when the stimulation was applied, and therefore should accommodate greater volumes of fluid at lower intravesicular pressures. In fact, there was no change in the compliance of the bladder during electrical stimulation of the midbrain, suggesting that the inhibitory effects on micturition were via a different mechanism.

Existing evidence suggests that application of electrical stimulation to the region in and around the PAG can have both inhibitory and excitatory effects on the bladder and EUS (Nishizawa *et al.*, 1987; Noto *et al.*, 1989; Kruse *et al.*, 1990; Noto *et al.*, 1991; Ito *et al.*, 2006). In the present experiments, electrical stimulation facilitated micturition when applied at one site in the PAG, and three sites within the region of the motor nucleus of the trigeminal nerve. Similarly, microinjection of the excitatory amino acid, DLH facilitated micturition when applied at a limited number of sites in the caudal

ventrolateral PAG. The localisation of the sites at which DLH facilitated micturition in the caudal ventrolateral PAG is unsurprising, since synapses in this region are known to play a role in the micturition reflex circuitry (Stone *et al.*, 2011) and Chapter 3). Microinjection of DLH is thought to initially depolarise neurons (Curtis *et al.*, 1959, 1960), thus it is possible that DLH increased the frequency of micturition either by a) directly activating excitatory neurons in the micturition circuitry in this region, b) by lowering the threshold for excitation of efferent outputs of the micturition circuitry which would allow micturition to be evoked by a lower level of afferent input from the bladder or c) via a depolarisation block of inhibitory neurons in the micturition circuitry. The location of the sites in the ventral midbrain at which electrical stimulation facilitated micturition is more surprising, since this region is not reported in the literature to be involved in the midbrain control of micturition. It is possible that this region may contain neurons involved in the afferent or indeed efferent pathways of the micturition reflex circuitry and that electrical stimulation activated these projections, or alternatively there may have been a spread of electrical current dorsally via a low resistance route up the electrode track. Further experiments, such as ortho- and anterograde neuronal tracing from the region, electrophysiological recording during periods of intravesicular distension, or use of a bipolar stimulating electrode to focus the area of influence of the applied current would be required for conclusions on the importance of the area in the control of micturition to be drawn and to reduce the likelihood of the current spreading to dorsal regions.

At the majority of midbrain sites, electrical stimulation and microinjection of DLH evoked inhibitory effects on reflex micturition as a result of intravesicular filling. These

functionally inhibitory effects seem counter-intuitive, since electrical stimulation and DLH both activate neurons (Curtis *et al.*, 1959, 1960; Kringelbach *et al.*, 2010). However these experiments are not the first reports suggesting electrical stimulation can evoke an inhibitory effect on micturition (Nishizawa *et al.*, 1987; Kruse *et al.*, 1990; Fukuda & Koga, 1991; Matsuura *et al.*, 2000; Liu *et al.*, 2004; Ito *et al.*, 2006; Numata *et al.*, 2008).

It is thought that DLH primarily activates the cell body of a neuron, due to the relative absence of glutamate receptors on the axonal membrane in comparison to the somatic. Thus DLH has been used in the past as a tool to identify whether the effects of electrical stimulation are evoked by somatic or axonal activation (Shaikh *et al.*, 1987; al Maskati & Zbrozyna, 1989; Zhang *et al.*, 1997), and this was the purpose of microinjection in the present experiments. Therefore the widespread locations in the midbrain at which DLH evoked inhibitory effects on micturition was unexpected, since we have shown that critical synapses involved in controlling the micturition reflex circuitry are located in a discrete region of the caudal ventrolateral PAG (Chapter 3, Stone *et al.*, 2011). However, recent evidence has suggested the existence of some subtypes of glutamate receptors on myelinated axons (Ouardouz *et al.*, 2009a; Ouardouz *et al.*, 2009b), therefore the effects of DLH on micturition in the present experiments may not have solely been via activation of the cell body. Nonetheless, it is still a possibility that DLH activated inhibitory interneuron pools (Reichling & Basbaum, 1990) via projections which course to the PAG, thus increasing their inhibitory tone over the micturition circuitry as it passes through the PAG. It must also be considered that these widespread effects might have been due to diffusion of the DLH back up the cannula track to the PAG, though

there was not a greater delay to the onset of effects as the cannula was advanced ventrally. Furthermore, inhibitory effects on micturition were evoked by microinjection of DLH laterally, as well as ventral to the PAG. Using the present experimental protocol, we cannot be sure that DLH did not evoke some effects on micturition via glial cell activation, since these cells have been shown to possess glutamate receptors (Teichberg, 1991; Steinhauser & Gallo, 1996; Bergles *et al.*, 2010; Parpura *et al.*, 2012). Future experiments should use an alternative method to deduce the mechanism of action of electrical stimulation of the midbrain on micturition, such as recording the electrophysiological activity at other levels of the micturition reflex circuitry: in the pontine micturition centre, or in the region of Onuf's nucleus in the sacral spinal cord during electrical stimulation of the midbrain.

There are a number of theories suggesting how activation of neurons by electrical stimulation might produce inhibitory effects. Electrical stimulation can activate afferent or efferent axons at the site of stimulation (Nowak & Bullier, 1998), probably in both ortho- and antidromic directions (Perlmutter & Mink, 2006; Kringelbach *et al.*, 2007; Deniau *et al.*, 2010). Therefore the inhibitory effects on micturition that were evoked in the present experiments might have been via a central effect on the descending micturition circuitry, by a peripheral effect on the bladder and sensory afferent transmission, or a combination of the two. However, since neither the compliance of the bladder nor the subjective responses to intravesicular filling in human participants were changed during stimulation — despite a significant increase in the maximal cystometric capacity during electrical stimulation of the midbrain using a similar cystometry protocol in human

patients (Green *et al.*, 2012) — it is unlikely that the inhibitory effects on micturition were due to a decrease in the sensory responsiveness of the bladder during electrical stimulation of the midbrain.

It is possible that electrical stimulation of the midbrain evoked inhibitory effects on the micturition reflex by causing release of the inhibitory neurotransmitter GABA. GABA is known to tonically inhibit the micturition reflex at the level of the caudal ventrolateral PAG (Chapter 3, Stone *et al.*, 2011). A range of evidence gathered from *in vitro*, *in vivo* and clinical settings has shown that high frequency electrical stimulation selectively causes GABA release (Moser *et al.*, 2003; Li *et al.*, 2004; Ogura *et al.*, 2004; Li *et al.*, 2006; Mantovani *et al.*, 2006; Hiller *et al.*, 2007). Interestingly, it has also been suggested that in the thalamus, high frequency electrical stimulation can produce similar behavioural effects as microinjection of the GABA agonist muscimol (Pahapill *et al.*, 1999). In support of this, we found that microinjection of muscimol into the caudal ventrolateral PAG evoked inhibitory effects on micturition, seen as a reduction in the frequency or complete inhibition of voids evoked by a continuous intravesicular infusion of saline (Chapter 3, Stone *et al.*, 2011). However the profile of the micturition contractions (i.e. the amplitude and duration) was unchanged following microinjection of muscimol, whereas electrical stimulation completely aborted drop output and reduced the amplitude of the contraction. This might suggest that the two inhibitory responses are not evoked by the same mechanisms. However it is possible that the responses evoked by electrical stimulation could be ‘stronger’ versions of the inhibitory effects evoked by microinjection of muscimol, since electrical stimulation might cause the

release of more GABA than was introduced by unilateral microinjection of the GABA agonist. The inhibitory effects of electrical stimulation were evoked over a much wider area of the midbrain than those evoked by microinjection of muscimol, which were clustered in the caudal ventrolateral PAG. However, since the effects of muscimol were specifically via agonism at GABA_A receptors, whilst electrical stimulation could potentially activate any cell bodies or axons from a number of nuclei passing into the PAG, and cause the release of GABA either from the interneuron pool (Reichling & Basbaum, 1990), it might be expected that electrical stimulation should have a greater area of influence. Further experiments that simultaneously apply electrical stimulation alongside a GABA antagonist, or analysis of the perfusate from microdialysis in the caudal ventrolateral PAG during electrical stimulation could elucidate whether the electrical stimulation of the midbrain in the current preparation causes release of GABA in the region of the PAG.

Often overlooked glial cells might also be involved in producing the functional inhibition of the micturition reflex that is evoked by electrical stimulation of the midbrain. High frequency stimulation causes depolarization of glial cells and release of adenosine (Kang *et al.*, 1998; Bekar *et al.*, 2008; Tawfik *et al.*, 2010). The action of adenosine via A1 receptors is to inhibit synaptic transmission by activation of post-synaptic potassium channels, and action on pre-synaptic calcium channels prevents neurotransmitter release (Trussell & Jackson, 1985; MacDonald *et al.*, 1986; Dunwiddie & Masino, 2001; Vedam-Mai *et al.*, 2012). It has also been shown that the inhibitory effect of electrical stimulation was absent in the A1 deficient mouse and also following application of an A1

receptor antagonist (Bekar *et al.*, 2008). It is impossible to assess the contribution that glial cell activation during high frequency stimulation had under the present experimental conditions, though it would be interesting to apply an A1 antagonist within the PAG during electrical stimulation to find out whether adenosine plays a role in the inhibitory effects on micturition in this region in the urethane anaesthetised rat.

It must also be considered that activation of neurons by repeated pulses of electrical stimuli might have caused a block of depolarisation thus an overall inhibition of the neuronal activity. Beurrier and colleagues (2001) found that high frequency stimulation of the subthalamic nucleus *in vitro* produced a brief inhibition of voltage-gated currents in these neurons (Beurrier *et al.*, 2001). These findings however, have not been repeatable through *in vivo* experiments which have shown that in the same area of the brain, high frequency stimulation reduces but does not block neuronal activity (Tai *et al.*, 2003; Meissner *et al.*, 2005). Also, other research has shown that the neuronal activity in the target nucleus is increased during high frequency stimulation (Hashimoto *et al.*, 2003; Maurice *et al.*, 2003), not reduced as one might expect if the stimulated neurons were inhibited. In the present experiments, if depolarisation blockade were responsible for the functional inhibition of the micturition reflex, then one would hypothesise that when pulse trains were applied at low frequencies, the stimulation should either activate or facilitate the reflex by activation of neurons, and at high frequencies, neuronal activity would inhibit micturition due to depolarisation blockade. In fact, changing the frequency of the pulses applied at a number of midbrain sites did not reverse the inhibitory effect on micturition. Moreover, application of low frequency electrical stimulation during the

inter-void period never evoked voiding at sites where application of electrical stimulation at the onset of a void had inhibited micturition.

A more recent theory explaining the functionally inhibitory effects of electrical stimulation is 'neurophysiological jamming'. High frequency electrical stimulation can cause activated neurons to become 'time locked' into a state where they fire at a similar rate to the stimulus that is being applied (Garcia *et al.*, 2003; Hashimoto *et al.*, 2003; Maurice *et al.*, 2003; Li *et al.*, 2007; Hammond *et al.*, 2008; Deniau *et al.*, 2010). Similarly to findings in the thalamus (Garcia *et al.*, 2003; Hashimoto *et al.*, 2003; Maurice *et al.*, 2003; Li *et al.*, 2007; Hammond *et al.*, 2008), electrical stimulation in the midbrain may entrain neurons projecting through the micturition circuitry at the same frequency as the applied stimulus, which could cause the network activity reaching the peripheral voiding machinery from the spinal outflows to be 'jammed' into this frequency, preventing the physiological neuronal activity that would normally coordinate a void. This hypothesis would explain why electrical stimulation in the midbrain only had an inhibitory effect on micturition evoked by continuous cystometry when applied at the onset of a void, and not during the inter-void interval when the network that coordinates voiding was inactive. The neurophysiological jamming theory could also explain why electrical stimulation produced inhibitory effects when applied over such a large area of the midbrain. Tracing experiments have shown that inputs to Barrington's nucleus (the PMC) course not only through the PAG, but as lateral as the medial lemniscus and more ventral than the brachium conjunctivum (Blok & Holstege, 1994). Stimulation in the region of any of these afferent, efferent or descending frontal projections into the micturition circuitry

would have the same net effect: to drive the whole micturition circuitry at the prescribed frequency and prevent the translation of physiological network activity in to a coordinated void. To determine whether neuronal 'jamming' is the mechanism by which electrical stimulation inhibits micturition when applied at midbrain sites, one could record the electrophysiological activity in different levels of the micturition circuitry during electrical stimulation of the midbrain and PAG, including in the PMC, the sacral spinal cord and in both pelvic efferents and afferents to determine whether stimulation drives the micturition circuitry at the prescribed frequency.

If neurophysiological jamming is the mechanism by which normal neuronal network activity is prevented from coordinating a void then theoretically one should achieve the same inhibitory effects on micturition when stimulation is applied at any level of the micturition circuitry, not specifically within the midbrain. Indeed, electrical stimulation (known as sacral nerve modulation, SNM) is already used to treat urge incontinence and non-obstructive urinary retention in around 26,000 patients (Van Kerrebroeck & Marcelissen, 2011). This stimulation is applied via an electrode implanted at the level of the sacral roots (Tanagho *et al.*, 1989), and is reported to improve urinary disturbance by up to 70% (Monga *et al.*, 2012). Similarly to electrical stimulation of the brain used in the clinic, the mechanism of action of SNM is unclear. Though there are indications that the neural stimulation works via a spinal mechanism, much evidence points to afferent effects of SNM on structures in the brain (Dasgupta *et al.*, 2005; Blok *et al.*, 2006). Thus stimulation of neurons involved in the micturition circuitry at the level of the midbrain (as in the present experiments) or the sacral cord may evoke effects on micturition via a

similar mechanism. In which case, if electrical stimulation of the midbrain were to be considered in the future for human patients who demonstrate urinary disturbances that are refractory to pharmacotherapy, then the attendant risk of surgery associated with implanting stimulating electrodes in to the brain could be reduced by targeting the micturition circuitry peripherally rather than centrally. Investigation into the effects of applying electrical stimulation at the level of the sacral cord on reflex micturition in the urethane anaesthetised rat would allow comparison to the effects of electrical stimulation of the midbrain on reflex micturition. Furthermore, recording the neuronal activity at different levels of the micturition circuitry during SNM in the rat, including in pelvic afferents and efferents, at the level of Onuf's nucleus and centrally in the PAG and PMC would deduce more about the mechanism of action of SNM and whether the effects are evoked by a central or peripheral mechanism.

4.6 SUMMARY AND CONCLUSIONS

In summary, the present experiments have shown that stimulation of the midbrain both electrically and chemically predominantly inhibits micturition evoked by continuous infusion of saline into the bladder. The mechanism of action of this functional inhibition is unclear. Inhibitory effects on micturition were evoked by both electrical stimulation and DLH over a wide area of the midbrain, despite the critical synaptic relays in the micturition circuitry being situated in the caudal ventrolateral PAG (Chapter 3, Stone *et al.*, 2011). This may be due to electrical stimulation readily activating axons of passage, which could have been afferent or efferent inputs in to spinal relays controlling the bladder and EUS. The widespread action of DLH in the midbrain is more surprising, since it is generally thought to activate cell bodies rather than axons of passage, and synapses in the micturition circuitry have not been identified in the caudal midbrain ventral and lateral to the PAG. Further experiments are required to understand the mechanism of action and to deduce how electrical stimulation, which is thought to activate neurons, can evoke an inhibitory effect on micturition. Nevertheless, these findings have important implications and demonstrate the potential of using localised electrical stimulation of the midbrain as a tool for treating urinary incontinence involving uncontrolled bladder emptying in patients that have proven refractory to the currently available therapies.

4.7 CHAPTER 4: KEY FINDINGS

4.7.1 Electrical stimulation of the midbrain (60 Hz, 0.5 ms, 0.5 – 2.5 V)

1. When applied at the onset of a void signalled by a sharp rise in intravesicular pressure, inhibited voiding.
2. Did not evoke effects on the micturition reflex when applied during the inter-void period, however did evoke the same sympatho-activation of the cardiovascular and respiratory systems, suggesting that the inhibitory effects on micturition are not due to activation of the inhibitory sympathetic drive to the detrusor.
3. Stimulation was effective at sites ventral and lateral to the PAG, some distance away from the synapses involved in reflex micturition that we identified in chapter 3. This suggests that we may be activating axons of passage, which could be from afferent or efferent neurons in the micturition circuitry.
4. Stimulation was inhibitory at the majority of sites in the midbrain, except at 4 sites (1 in the PAG and 3 close to the motor nucleus of the trigeminal nerve). Since electrical stimulation primarily activates neurons, the predominantly inhibitory effects seem counter intuitive. Further experiments would be required to deduce the mechanism of action, which might be via release of inhibitory neurotransmitters, activation of glial cells or neurophysiological jamming.

4.7.2 Chemical stimulation of the midbrain with D,L-homocysteic acid

(DLH)

1. Microinjection evoked inhibitory effects on micturition at the majority of midbrain sites, seen as an increase in the inter-void interval. Since DLH depolarises neurons, this might be via activation of inhibitory pathways controlling the micturition circuitry.
2. At 3 sites in the caudal ventrolateral PAG, DLH facilitated micturition (reduced the inter-void interval). This may have been via synaptic activation of excitatory projections from the PAG.
3. The widespread inhibitory action of DLH in the midbrain on micturition was unexpected, since it is thought to primarily activate cell bodies as opposed to axons, and synapses in this area of the brain have been identified in a localised area of the PAG, some distance from the centre of effective sites. This may be due to spread of the drug, or activation of more recently identified axonal glutamate receptors and glial cells.

**CHAPTER 5: THE ROLE OF DOPAMINERGIC NEUROTRANSMISSION IN THE
PERIAQUEDUCTAL GREY IN THE CONTROL OF MICTURITION IN BOTH THE
CONSCIOUS AND THE URETHANE ANAESTHETISED MALE RAT**

5.1 INTRODUCTION

We have shown in Chapter 3 that the micturition reflex pathway synapses in the caudal ventrolateral periaqueductal grey (PAG), and that these synapses are subject to ongoing GABAergic inhibition (Stone et al., 2011, Chapter 3). In the conscious rat, the extracellular concentration of GABA in the caudal ventrolateral PAG is reduced during periods of intravesicular distension (Kitta *et al.*, 2008), suggesting that this inhibitory GABA tone is withdrawn to allow micturition to occur. Kitta and colleagues (2008) also showed that the extracellular concentration of dopamine in the same region of the PAG is increased during periods of intravesicular distension, and local application of D1 receptor antagonist, SCH23390 by microdialysis increased the amplitude of the intravesicular pressure rise associated with voiding. This suggests that dopaminergic neurotransmission in the ventrolateral PAG may play a role in the central control of the micturition circuitry.

Interestingly, a population of dopaminergic cells has been identified in a discrete region of the caudal ventrolateral PAG (Saper & Petit, 1982; Flores *et al.*, 2004; Lu *et al.*, 2006), raising the possibility that these dopamine cells could be the source of the increase in levels of dopamine in the ventrolateral PAG during intravesicular distension in the conscious rat (Kitta *et al.*, 2008).

Furthermore, in animal models of Parkinson's disease that have used selective neurotoxins to lesion dopaminergic cells of the nigro-striatal pathway, the lesions have been shown to extend as far as the PAG (Shaw *et al.*, 2010), and urinary disturbances as a result of this lesioning have been reported (Soler *et al.*, 2011). Thus it is possible that lesioning the population of dopaminergic cells in the caudal ventrolateral PAG could lead to urinary disturbances.

6-hydroxydopamine (6-OHDA) is a neurotoxin that selectively lesions dopaminergic cells and is used to produce animal models that demonstrate some of the key features of Parkinson's disease (for reviews see Blandini *et al.*, 2008; Duty & Jenner, 2011 and Blandini & Armentero, 2012). The chemical structure of 6-OHDA is very similar to that of dopamine (Fig. 5.1), so the neurotoxin is readily taken up into dopaminergic cells by dopamine transporters (Duty & Jenner, 2011). Indeed, the structure is also similar to noradrenaline, but specificity for dopaminergic terminals can be improved by systemic pre-treatment with the noradrenaline re-uptake inhibitor, desipramine (Luthman *et al.*, 1989; Duty & Jenner, 2011). Once inside the cell, 6-OHDA is oxidised (Heikkila & Cohen, 1972; Salonen *et al.*, 1996), causing the release of toxic free radicals that ultimately lead to cell death (Blum *et al.*, 2001). 6-OHDA is also taken up by mitochondria where it impairs the electron transport chain via inhibition of complex I (Glinka *et al.*, 1997; Duty & Jenner, 2011).

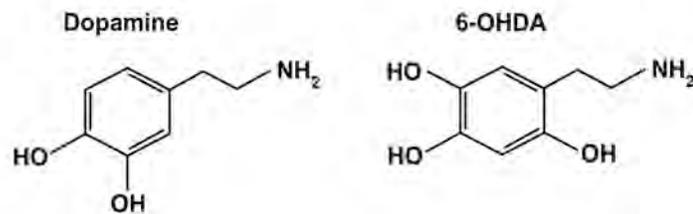


Figure 5.1. Diagram showing chemical structure of the neurotransmitter dopamine and the neurotoxin 6-hydroxydopamine (6-OHDA). Due to its structural similarities with dopamine (left), 6-OHDA (right) is readily taken up by the dopamine transporter into dopaminergic cells. Once inside the cell, 6-OHDA oxidises and ultimately causes cell death.

5.2 AIMS AND HYPOTHESIS

We hypothesised that the increase in extracellular dopamine in the ventrolateral PAG associated with periods of intravesicular distension (Kitta *et al.*, 2008) originates from the population of dopaminergic cells located in the caudal ventrolateral PAG (Saper & Petito, 1982; Flores *et al.*, 2004; Lu *et al.*, 2006), and that dopaminergic neurotransmission in this region could play a role in the control of the micturition circuitry. Therefore loss of these dopaminergic cells could lead to urinary disturbances. We used bilateral localised microinjections of the neurotoxin 6-OHDA to selectively lesion dopaminergic cells in the caudal ventrolateral PAG of male rats. The effects of this lesion on micturition were investigated by recording the 24 h urine output in the conscious rat, and by carrying out continuous cystometry in the urethane-anaesthetised rat. The effectiveness of the lesion was assessed by tyrosine hydroxylase immunohistochemistry of the PAG, following which the number of tyrosine hydroxylase — an enzyme involved in the production of dopamine — containing cells were counted.

5.3 METHODS

Male Sprague Dawley rats (n = 20, 220 – 377 g, Charles River, Kent, UK) were randomly assigned to the 6-OHDA and sham (saline) lesioning groups for chronic experiments. Acute experiments investigating the effects of microinjection of a dopamine agonist and antagonist were carried out on 8 urethane-anaesthetised rats (198 – 244 g, Charles River, Kent UK) that had not been subjected to any pre-treatment.

5.3.1 24 h urine output in the conscious, unrestrained rat

Each rat was placed into a metabolism cage (Nalgene, Harvard Apparatus, UK) to monitor urine output over 24 h before surgery, and then again 14 days after lesioning surgery. Rats were placed into the cages during the early part of the light cycle between 8 am and 10 am and were removed 24.5 h later. Recordings of urine output were started after a 30 min acclimatisation period in the metabolism cage. An automatic drop counter (AD Instruments, Oxford, UK), which was connected to a Powerlab acquisition system (AD Instruments, Oxford, UK) was positioned below the urine chute to record (in LabChart v 4.2.3, AD Instruments, Oxford, UK) each time a drop fell from the metabolism cage (Fig. 5.2). This data was collected continuously for the 24 h period and allowed measurement of the total voided volume, the inter-void interval, the number of drops per void and comparison of voiding activity during the light and dark periods. The protocol was identical for the second exposure to the metabolism cage carried out 14 days after lesioning surgery.

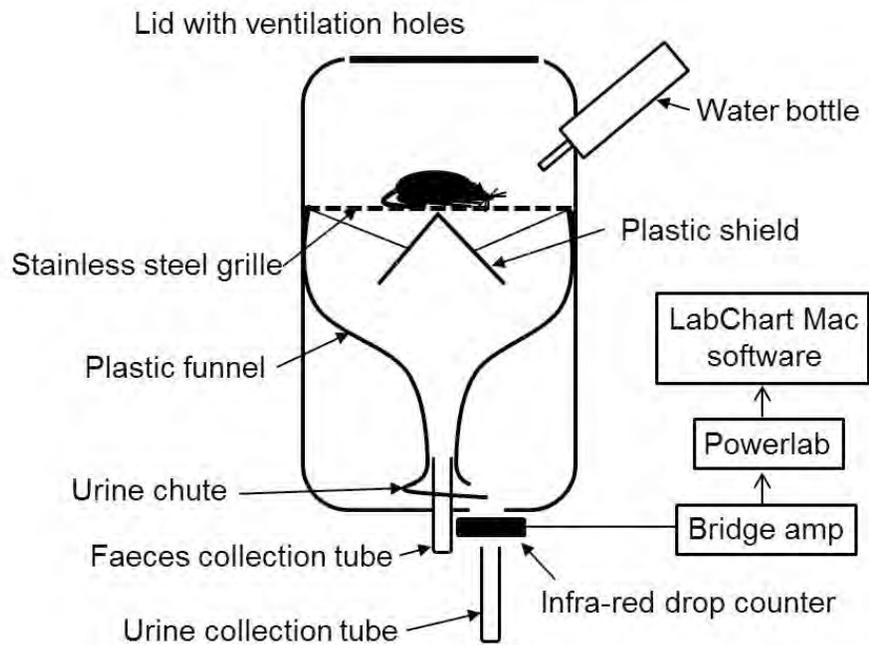


Figure 5.2. Diagram of Nalgene metabolism cage. Rats were placed into the cage with food and water available ad libitum for 24 h. During this time, urine output was monitored. The infra-red drop counter streamed data to the LabChart programme continuously for the 24 h and recorded when a drop fell from the collection chute into the urine collection tube. Faeces fell directly into the central collection tube, whereas urine trickled down the side and into the chute, then into the collection tube. The plastic shield prevented urine falling directly into the faeces tube. A shield below the water bottle prevented water drops from entering the metabolism cage (not shown in diagram).

5.3.2 6-OHDA lesions of the periaqueductal grey

These recovery procedures were carried out in the Biomedical Services Unit at the University of Birmingham and a sterile surgical technique was employed to reduce the chance of post-surgical infections. 30 minutes after intra-peritoneal injections of desipramine (25 mg kg⁻¹) and pargyline (30 mg kg⁻¹ both from Sigma-Aldrich, Gillingham, UK) were made to protect noradrenergic and serotonergic terminals, a combination of medetomidine and ketamine in dH₂O (0.04 mM, Tocris, Bristol, UK and 80 mM Pfizer, UK, respectively) was administered via an intraperitoneal injection in volumes of 3ml kg⁻¹ to induce general anaesthesia. Depth of anaesthesia was deemed sufficient when the pedal and corneal reflexes were absent. Rats were placed on to an electric heating blanket and mounted prone in a Kopf stereotaxic frame. A rectal temperature-sensing probe was inserted to monitor core body temperature and a heating lamp was used to warm the rat when core body temperature fell below 37.0°.

The dorsal surface of the skull was exposed by incision with a scalpel, and a portable, wireless drill (Draper Tools, Hampshire, UK) with a dental burr was used to create a limited dorsal craniotomy (4 x 4 mm). A sterile 26 G needle was used to perforate the dura and allowed insertion of a stainless steel cannula (500 µm diameter), at an inclination of 10° from vertical and under stereotaxic guidance, into the ventral periaqueductal grey (PAG). The cannula was left in place for 5 min before administration of drug or sham solution. Microinjections of 6-hydroxydopamine (6-OHDA, 1 µl, 5 mg ml⁻¹, with ascorbic acid for stabilisation, Sigma-Aldrich, Gillingham, UK) or saline (0.9%)

were administered over a 5 min period, and the cannula was left in place for a further 5 min before being withdrawn. This process was then repeated on the contralateral side of the PAG. The analgesic, buprenorphine (0.03 mg kg^{-1}) was administered subcutaneously at this point to afford analgesia during the initial phase of recovery. The cranial wound was closed using vicryl absorbable suture (Ethicon, Johnson and Johnson Medical Limited, West Lothian, UK) and 5 ml of glucosaline was administered subcutaneously to replace fluids lost under anaesthesia.

Anaesthesia was reversed with atipamezole (0.5 mg in 0.15 ml, Pfizer, UK) and rats were placed onto a heated table for approximately 1 h while the effects of the anaesthetic were reversed. Recovery for the first 24 h was in individual cages before being returned to shared cages for the remainder of the 14 day recovery period.

5.3.3 Acute cystometry

Either one or two days after the second 24 h exposure to the metabolism cage (either 15 or 16 days after cranial surgery and microinjection of 6-OHDA or saline – sham – into the PAG), an acute terminal experiment was carried out under urethane anaesthesia. Rats ($n = 15$, 291 – 466 g) were terminally anaesthetised (IP urethane, 1.4 g kg^{-1}), and prepared for cystometry and monitoring of vital signs as described in the Chapter 2: General methods, Section 2.2 (excluding the craniotomy and electrode placement). Once the preparation was completed a 30 min stabilisation period was allowed. Saline

was then continuously infused into the bladder (6 ml h^{-1}) for at least 2 h and the resulting effect on the intravesicular pressure and EMG of the EUS were recorded.

5.3.4 Preparation of tissues for immunohistochemistry

At the termination of the experiment, the brain tissue was fixed by perfusion of a paraformaldehyde solution via the vascular system. The abdominal aorta was dissected from the surrounding tissue below the origin of the renal blood supply, and the flow in the aorta was temporarily halted with a clip. The distal end of the dissected aorta was tied with a tight ligature, and a loose ligature was placed around the middle of the dissected portion to secure the cannula once in place. A polyethylene cannula (1.67 mm ID, 2.42 mm OD, heat pulled at the tip, Portex tubing, SIMS Portex Ltd, Kent, UK) attached to a 50 ml syringe containing heparinised saline (10 units ml^{-1} , Multiparin, CP Pharmaceutics Ltd., Wrexham, UK) was advanced into the aorta in a retrograde fashion and was secured in place with the aforementioned ligature around the vessel and the tubing. The temporary clip was removed, the right and left jugular veins were severed. Heparinised saline (10 units ml^{-1} , 100 ml Multiparin, CP Pharmaceutics Ltd., Wrexham, UK) was washed through the circulatory system — entering via the aorta and exiting via the jugular veins — to clear blood from the vascular system. The rats were then perfused with 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PFA, pH 7.4, see appendix B for recipe), again via the abdominal aorta. The brain was removed and post fixed in PFA for 2 h before being stored in 30% sucrose in 0.1 M phosphate buffer to afford cryoprotection.

5.3.5 Immunohistochemistry

Tyrosine hydroxylase (TH) immunohistochemistry was carried out on 30 serial sections, each 40 µm thick that extended 1200 µm rostrally from the caudalmost PAG, from approximately 9.0 mm caudal to Bregma, to -7.8 mm caudal to Bregma. Sections were incubated for 1h in block solution containing normal horse serum (NHS, Vector Labs, Peterborough, UK) to limit non-specific binding, hydrogen peroxide (H₂O₂, Sigma-Aldrich, Gillingham, UK) to reduce endogenous peroxidase activity and phosphate buffered saline (PBS, 0.1 M, pH 7.4, see appendix C for recipe), containing Triton-X-100 (0.01%, Fluka Biochemika, Sigma-Aldrich Gillingham, UK) to increase the permeability of cell membranes. Following this, sections were incubated for 2 days in 0.01% primary TH antibody (Mouse anti-tyrosine hydroxylase, Alexa Flour[®] Conjugated monoclonal antibody, Millipore, Watford, UK) made up in solution containing PBS, Triton-X-100 (0.01%) and NHS (1.5%), at 5 °C and were gently agitated using an orbital shaker (IKA-Vibrax-VXR-17, IKA Laboratories, Staufen, Germany). After three washes with PBS, sections were transferred into a secondary antibody solution containing biotinylated anti-mouse IgG (0.2%, Biotinylated anti-mouse IgG (H&L), affinity purified, made in horse, Vector Laboratories, Peterborough, UK) and NHS (2.5%) in PBS. Sections were again washed 3 times in PBS and then transferred into avidin-biotin complex (ABC, Vector Laboratories, Peterborough, UK) solution for 1 h to label the biotinylated secondary antibody. Reaction products were developed using di-aminobenzoate (DAB, Sigma-Aldrich, Gillingham, UK) and H₂O₂, and development was monitored for a visual colour change and quenched after approximately 60 s by flooding vials with ice-cold PBS.

Sections were then washed 3 times in PBS before being mounted on to gelatinised slides and allowed to dry overnight. Following dehydration with ascending concentrations of alcohol slides were cover slipped with Histomount® (National Diagnostics, Hull, UK) and viewed under a light microscope (Olympus BH2, Olympus, Southend-on-Sea, UK). TH positive cells from each section were mapped onto outline drawings of the PAG with the use of the drawing tube attachment on the microscope. Large ($> 30 \mu\text{m}$) cells were marked as circles and small cells ($\leq 30 \mu\text{m}$) crosses. From these pictures, large and small cells were counted to assess the effectiveness of lesions.

5.3.6 Acute microinjection of dopamine agonists and antagonists

In 8 urethane anaesthetised (1.4 g kg^{-1}) and previously untreated rats, (198 – 244 g, Charles River, Kent, UK) the acute effects of microinjection of the non-selective dopamine agonist apomorphine (94.0 mM, containing ascorbic acid to reduce oxidation, RBI, Sigma-Aldrich, Gillingham, UK) and the D1 receptor antagonist, SCH23390 (5.0 mM, Tocris Bioscience, Bristol, UK) on reflex micturition were investigated. Rats were anaesthetised and prepared for cystometry and monitoring of vital signs as described in Chapter 2: General methods, Section 2.2. Microinjections (50 nl) were administered at 0.5 – 1.0 mm intervals in the caudal midbrain and PAG and the effects on the micturition reflex evoked by continuous infusion of saline into the bladder (6 ml h^{-1}) were recorded. The doses of apomorphine and SCH23390 administered in the present experiments

were within the range of those reported in the literature in the PAG and other brain regions (Melo *et al.*, 2009; Meyer *et al.*, 2009; Dang *et al.*, 2010; Chen *et al.*, 2011).

These experiments were terminated by overdose of pentobarbitone via the intravenous catheter, and cervical dislocation. The brains were removed and stored in 10% formol saline before processing for histological analysis (See Chapter 2: General methods, Sections 2.4 and 2.5 for details).

5.3.7 Analysis

All means are represented \pm SEM. Unless otherwise stated, paired data have been compared with a Student's paired t test. For details of statistical testing see Chapter 2: General methods, Section 2.6. Analysis of data from the metabolism cage, urethane-anaesthetised cystometry and TH immunohistochemistry was carried out blind to reduce experimenter bias. The TH cell population was counted manually from maps of the 40 μ m sections made using a drawing arm attached to a light microscope (Olympus BH2, Olympus, Southend-on-Sea, UK). Occasionally, one large TH+ cell spanned two 40 μ m sections, as seen by the presence at identical positions in two neighbouring sections. These cells were only counted once.

5.4 RESULTS

5.4.1 Chronic experiments: 6-OHDA lesioning of the PAG

In the 24 h following reversal of anaesthesia and cranial surgery, 5 rats (2 from the 6-OHDA group, and 3 from sham group) lost weight and demonstrated signs of illness (hunched posture, porphyrin staining around the eyes, piloerection, absence of grooming behaviour). These rats were euthanased with an IP injection of pentobarbitone and data from the pre-surgery exposure to the metabolism cage for these rats were discarded.

5.4.1.1 Effect on 24 h urine output

5.4.1.1.1 24 h voiding pattern in the conscious rat

The 24 h pattern of urine output was measured in 15 rats (of which 8 went on to receive 6-OHDA injections into the PAG and the other 7 went on to receive injections of the saline vehicle). Recording of drop output failed for 3 rats (2 from 6-OHDA lesion group and 1 from sham lesioned group). Therefore these subjects were not included in the drop pattern analysis since paired observations could not be made. Urine collection also failed in one of these subjects from the 6-OHDA group, so this rat was also excluded from the urine volume analysis. Therefore the number of rats used for analysis of 24 h urine volume was 7 from the 6-OHDA group, and 7 from the sham lesioned group. Analysis of the pattern of urine output was carried out on data from 6 rats from the 6-OHDA group and 6 rats from the sham lesioned group.

There was no difference in the volume or pattern of urine output between the groups before surgery, therefore the data have been pooled. The rats voided 20.2 ± 2.0 times ($n = 12$), producing 2.7 ± 0.4 ml 100g^{-1} of urine in the 24 h ($n = 14$). The volume of urine produced has been reported as a function of the rat weight in order to account for any weight gain upon the second exposure 14 days after the lesioning surgery.

There was a diurnal pattern in urine output, with a significantly greater number of voids occurring during the dark period (7 pm to 7 am, 12.7 ± 1.1 voids) in comparison to the light period (7 am to 7 pm, 7.5 ± 1.0 voids, $P < 0.0001$, Student's paired t test, $n = 12$, Fig. 5.3 A). Consequently, the inter-void interval was shorter in the dark period (63.8 ± 6.8 min) in comparison to the light period (110.6 ± 15.3 min, Fig. 5.3 B, $P < 0.01$, $n = 12$).

Interestingly, voids made during the dark period were smaller than during the light period (Fig. 5.3 C, 10.4 ± 1.4 v. 14.5 ± 2.1 drops void $^{-1}$ respectively, $P < 0.05$, $n = 12$), so that the total number of urine drops counted during the dark and light periods was similar (Fig. 5.3 D, 106.0 ± 7.5 v. 92.4 ± 10.6 drops 24 h^{-1} respectively, $P = 0.13$, $n = 12$).

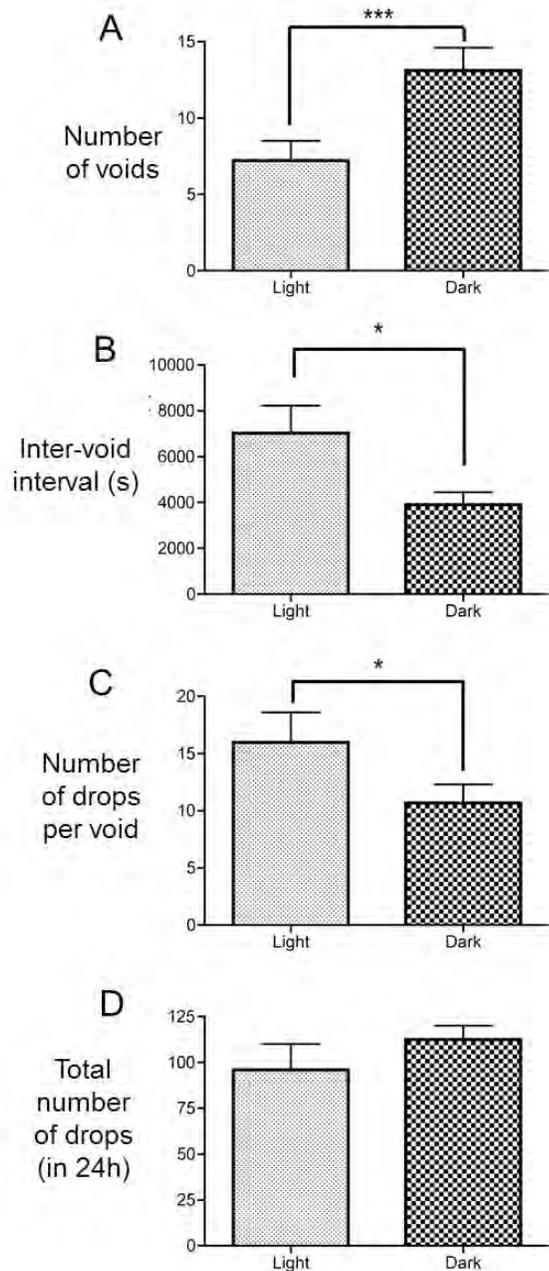


Figure 5.3. 24 h Urine output in the conscious unrestrained rat over 24 h period in metabolism cage before any treatment (n = 12). Lights on condition (7 am to 7 pm) compared to dark period (7 pm to 7 am). Data shown: A, total number of voids in 24 h, light v dark, B, inter-void interval, C, number of drops per void , D, total number of urine drops expelled in 24 h *** P < 0.001, * P < 0.05, Student's paired t test.

5.4.1.1.2 Voiding diaries in the 6-OHDA and sham lesioned rat

There appeared to be an increase in 24 h urine output in both the 6-OHDA lesioned and the sham lesioned groups upon their post-recovery exposure to the metabolism cage. This difference persisted even after the data had been corrected for the animals' weight gain over the 14 day period since the first 24 h collection was made, (Fig. 5.4 A). However, the difference did not reach significance. Interestingly, close examination of individual data sets revealed that of the 14 rats used, 4 (2 from the 6-OHDA lesioned group and 2 from the sham lesioned group) showed excessive urine production following surgery (> 100% increase in urine production). 24 h urine output increased by up to 298.4% (mean $246.9 \pm 25.7\%$, min 185.8%), compared to a $2.9 \pm 12.7\%$ increase (range -48.4 to 63.2%) in the other 10 rats, Fig. 5.4 B.

There was no difference in the total number of voids during the 24 h that 6-OHDA lesioned rats were exposed to the metabolism cage following recovery from surgery in comparison to their pre-surgery exposure (Fig. 5.5, 17.8 ± 2.1 v. 21.5 ± 4.5 voids, $P = 0.5$). The diurnal pattern of voiding remained post-surgery, with a significantly greater number of voids during the dark period in comparison to the light (Fig. 5.5, 14.5 ± 3.6 compared to 7.0 ± 1.1 voids, $P < 0.05$). Similarly, in the sham lesioned group, there was no difference in the total number of voids during the 24 h that subjects were exposed to the metabolism cage following recovery from surgery (Fig. 5.5, 19.3 ± 4.2 compared to 26.9 ± 3.2 voids, $P = 0.24$). The diurnal pattern in urine output was also maintained in the sham lesioned group (Fig. 5.5, 9.6 ± 1.2 v. 17.3 ± 2.7 , $P < 0.05$).

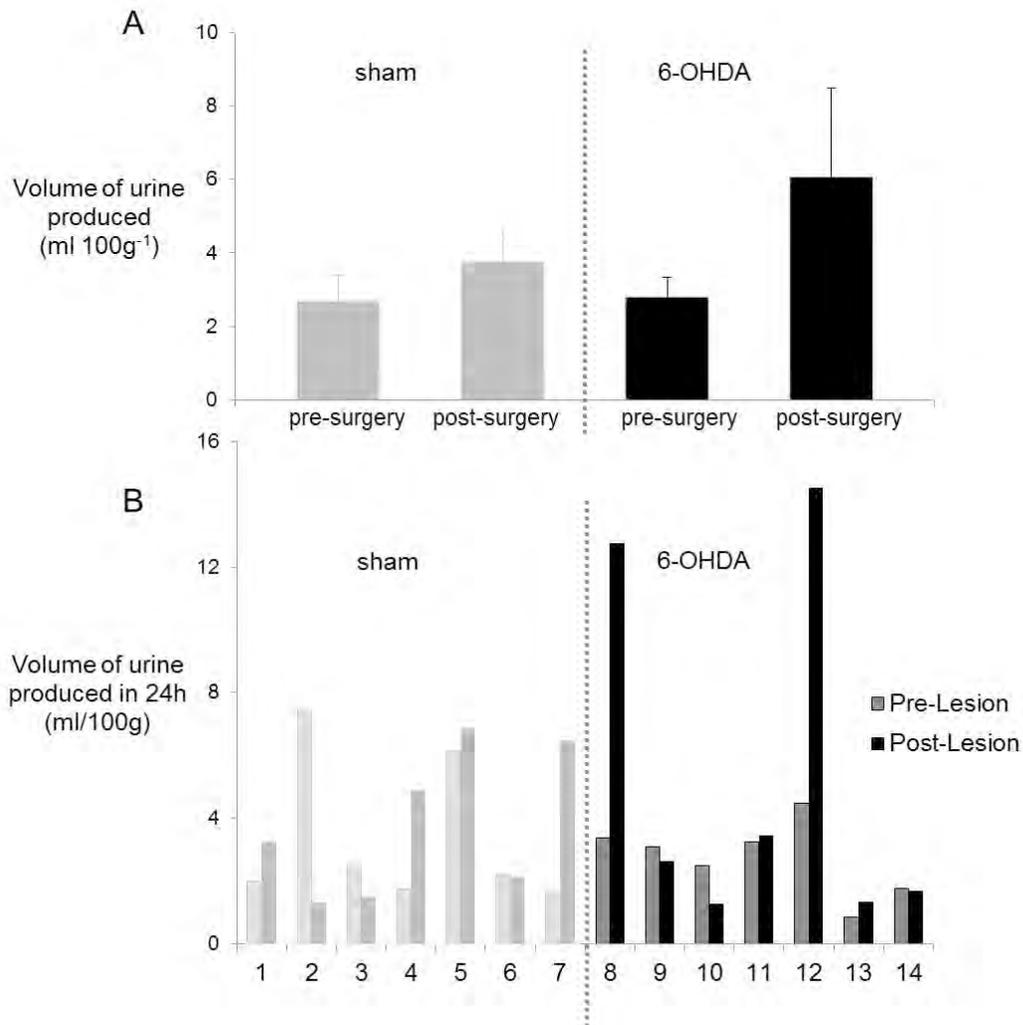


Figure 5.4. Volume of urine produced during 24 h exposure to the metabolism cage. A, Mean volume of urine produced in 24 h exposure to metabolism cage for the sham (n = 7) lesioned and 6-OHDA (n = 7) lesioned groups before and after lesioning surgery. B, Individual data sets showing 24 h urine output before and after injections of saline or 6-OHDA into the PAG.

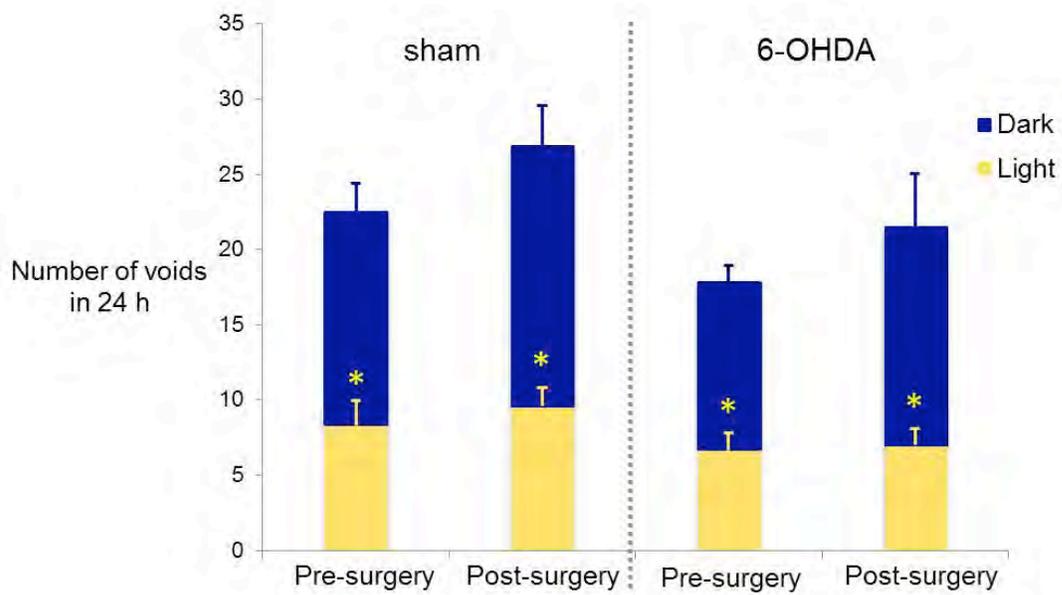


Figure 5.5. Number of voids in 24 h exposure to the metabolism cage during the light (yellow, 7 am to 7 pm) and dark (blue, 7 pm to 7 am) period in rats receiving microinjections of saline (sham n = 6) or 6-OHDA (n = 6) into the PAG. Asterisks indicate significant difference between dark and light periods for each group Student's paired *t* test, **P* < 0.05).

5.4.1.2 Effect of 6-OHDA lesioning on micturition reflex evoked by intravesicular filling in the urethane anaesthetised rat

One or two days after the second 24 h urine collection period (15 or 16 days after lesioning), every rat (sham lesioned $n = 7$, 6-OHDA lesioned $n = 8$) entered in to a terminal experiment using the same preparation and experimental protocol described previously in detail in the general methods (Chapter 2, Section 2.2). In both 6-OHDA and sham lesioned rats infusion of saline into the bladder (6 ml h^{-1}) elicited periodic increases in intravesicular pressure that were associated with development of bursting activity in EMG recorded from the EUS and the expulsion of drops of fluid from the urethral meatus. This reflex failed to develop within 2 h of the intravesicular infusion starting in one rat from the 6-OHDA group (12.5%), and two from the sham lesioned group (28.6%). There was no significant difference between any of the parameters of voiding measured during continuous cystometry in the sham and 6-OHDA lesioned groups (Table 5.1).

Table 5.1. Comparison of parameters of voiding induced by continuous infusion of saline into the bladder (6 ml h⁻¹) of urethane anaesthetised rats treated with 6-OHDA or sham microinjection into the midbrain

	6-OHDA treated		Sham treated	P =
Reflex developed (n)	7/8		5/7	n/a
Frequency of contractions (contractions min ⁻¹)	0.5 0.1	±	0.5 ± 0.1	0.7
Amplitude of contraction (mmHg)	21.4 1.7	±	21.6 ± 1.8	0.9
Baseline bladder pressure (mmHg)	8.8 0.5	±	10.2 ± 1.9	0.5
Duration of contraction (s)	31.0 2.0	±	27.0 ± 2.0	0.24
Number of drops per void	5.9 1.0	±	5.8 ± 1.2	0.95

Values shown as mean ± SEM. P values from Student's unpaired *t* tests.

5.4.1.3 Effect of 6-OHDA lesioning on tyrosine hydroxylase containing cell numbers in the caudal of the PAG

In sham lesioned rats, cells immunostained for tyrosine hydroxylase (TH) were present throughout the PAG. Two brains were spoilt during storage before immunohistochemistry, one from the 6-OHDA lesioned group and one from the sham saline group. Therefore the numbers are 7 and 6 respectively. At the most caudal levels of the PAG (P -8.8 mm), TH+ cells were clustered almost exclusively in the ventrolateral portion. At more rostral levels TH+ cells were spread more diffusely throughout the ventral PAG and ventral to the aqueduct in the dorsal raphe nucleus (though rarely dorsal to the central aqueduct), Fig. 5.6.

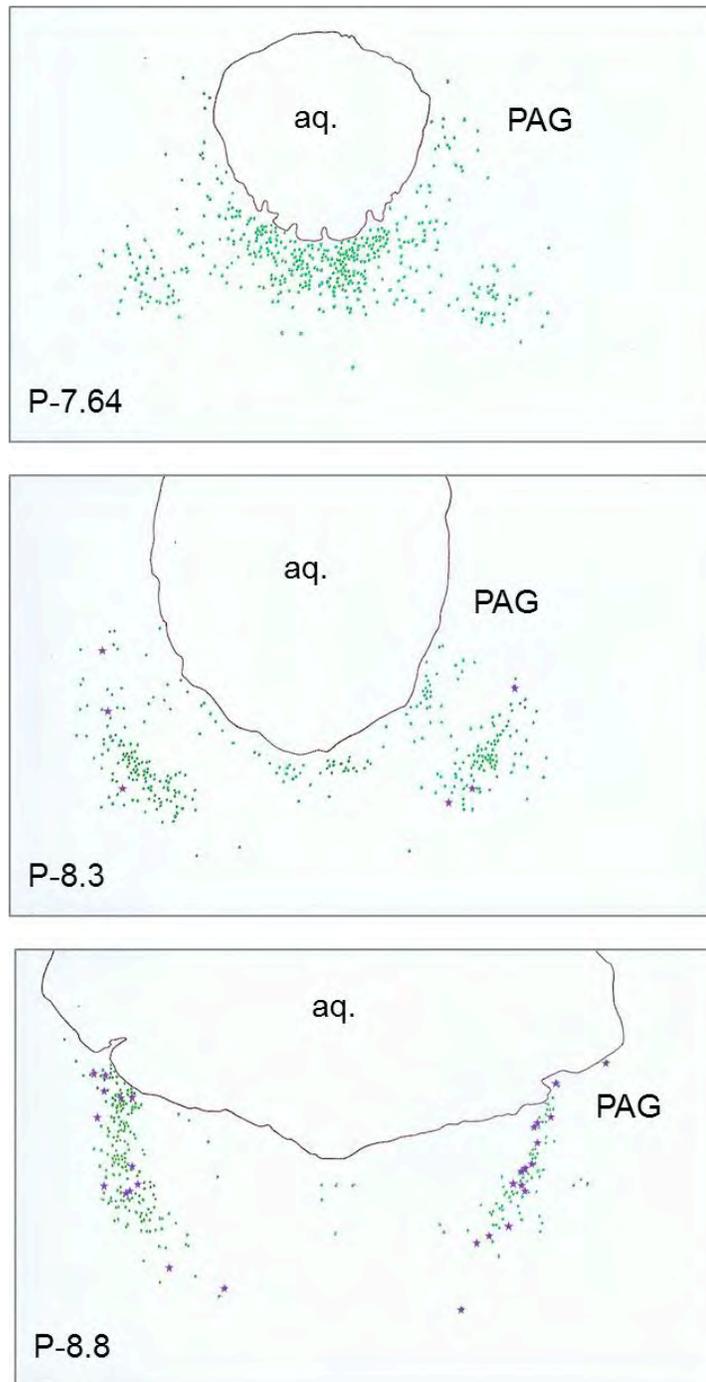


Figure 5.6. Map taken from camera lucida drawings of TH+ cell population in the caudal PAG of a rat that received bilateral microinjections of saline vehicle (sham). Cells are mapped on to three representative levels of the caudal PAG. Green spots represent small TH+ cells ($\leq 30\mu\text{m}$) and purple stars represent large TH+ cells ($> 30\mu\text{m}$) Abbreviations: Aq. aqueduct, PAG: periaqueductal grey, TH: tyrosine hydroxylase. Numbers represent mm caudal to Bregma.

5.4.1.3.1 TH+ cell population in sham rats.

There was a large degree of inter-rat variability in the total number of TH containing (TH+) cells in the caudal 1200 μm of the PAG (range 476 – 2211 cells, mean 1254.0 ± 260.3 , Fig. 5.7 A). Two types of TH+ cells could be distinguished in the PAG, defined as 'small' (diameter of cell body $\leq 30 \mu\text{m}$) and 'large' (diameter of cell body $> 30 \mu\text{m}$), Fig. 5.8. Population density of both the small and large cells was equal on both the left and right hand sides of the PAG in the sham group (large cells: 18.5 ± 9.1 v. 31.5 ± 8.0 , $P = 0.51$, small cells: 654.9 ± 131.1 v. 668.5 ± 129.1 , $P = 0.88$). Therefore cell numbers for both the left and right hand sides have been combined. There were significantly more small cells than large cells in the caudalmost 1200 μm of the PAG studied (Fig. 5.7 B, $P = 0.001$). Large diameter TH+ cells were more numerous in the more caudal 600 μm of the PAG studied in comparison to the more rostral 600 μm in the sham group (Fig. 5.7 C, Table 2).

5.4.1.3.2 Effect of 6-OHDA microinjections

Large and small TH+ cells were also present in the PAG of 6-OHDA lesioned rats, and followed a similar distribution to the sham lesioned group (Fig. 5.7 C, Table 5.2). However, there was no difference in the total number or distribution of TH+ cells when the 6-OHDA lesioned group was compared to the sham group (Fig. 5.7 A, Fig. 5.9, Table 5.2).

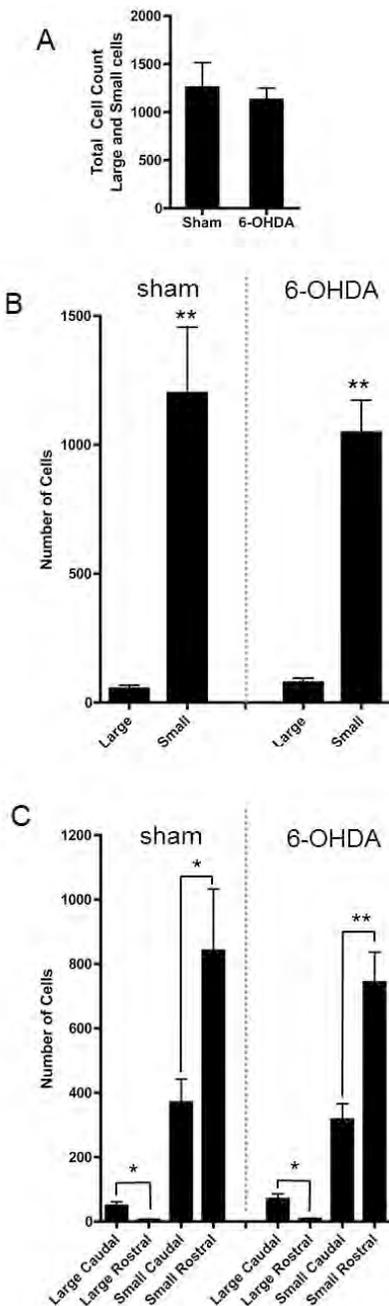


Figure 5.7. Effect of bilateral microinjection of 6-OHDA and saline (sham) into the caudal periaqueductal grey (PAG) on tyrosine hydroxylase containing (TH+) cell numbers. A, total number of TH+ cells in the caudal 1200 μm of the PAG, large and small TH+ cells combined for sham (n = 6) and 6-OHDA (n = 7). B, number of TH+ cells in the caudal 1200 μm of the PAG, showing small and large cells. C, number of TH+ cells in the caudal 1200 μm of the PAG, showing the number of cells in the most caudal portion (600 μm) separately to the most rostral 600 μm . ** $P \leq 0.001$, * $P < 0.05$.

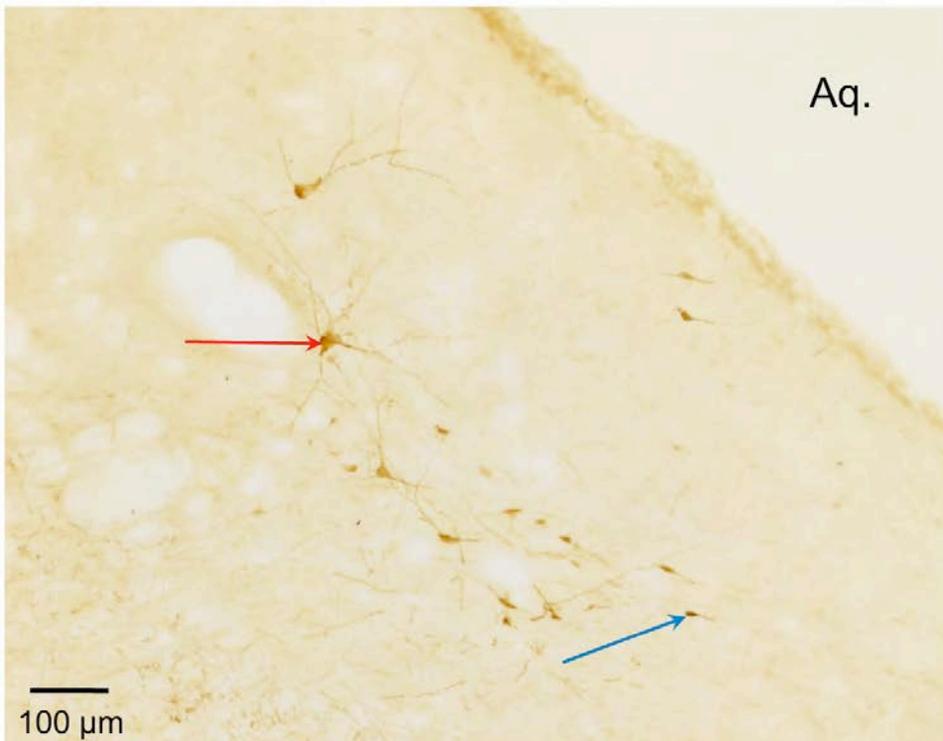
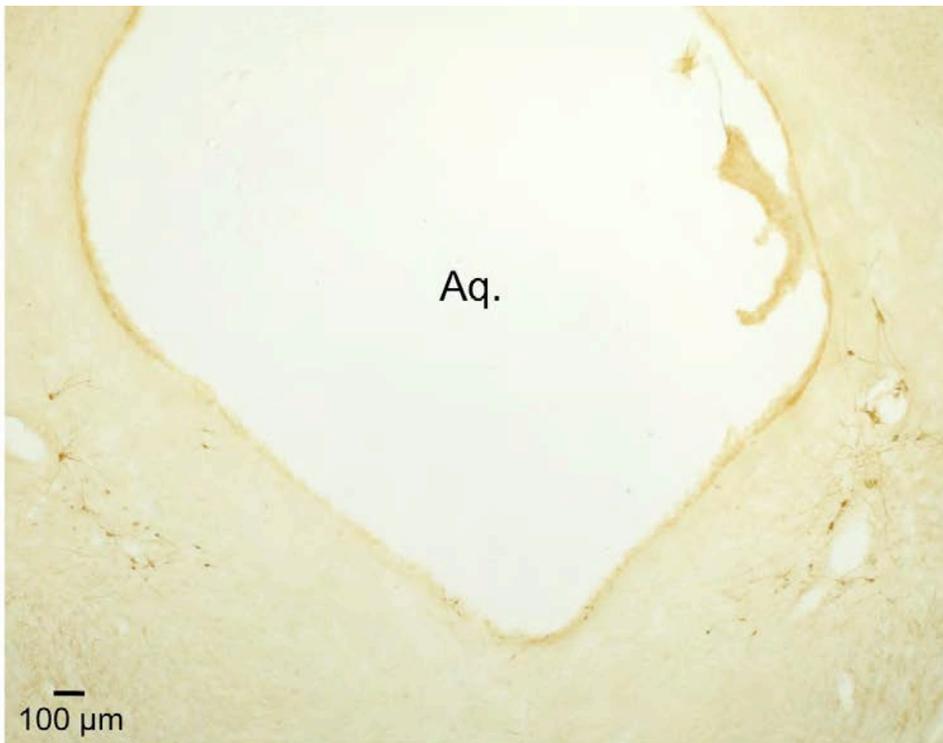


Figure 5.8. Photomicrograph of tyrosine hydroxylase positive (TH+) cells in the caudal ventrolateral PAG. Large and small diameter cells indicated by red and blue arrows respectively. Aq: central aqueduct.

Table 5.2. Effect of bilateral microinjection of 6-OHDA into the ventrolateral PAG on tyrosine hydroxylase positive (TH+) cells in the most caudal 1200 μ m of the PAG.

		6-OHDA treated	Sham treated
(n)		7	6
Total TH+ cell count 1200 μ m of PAG)	(caudal	1124.5 \pm 124.7	1373.4 \pm 261.1
Large cell count 600 μ m)	(caudal	69.9 \pm 15.9	48.6 \pm 12.7
Large cell count 600 μ m)	(rostral	8.0 \pm 2.4	5.6 \pm 2.3
Small cell count 600 μ m)	(caudal	316.9 \pm 48.7	369.6 \pm 73.1
Small cell count 600 μ m)	(rostral	742.9 \pm 94.1	841.2 \pm 191.5

Values shown as mean \pm SEM. 6-OHDA: 6-hydroxydopamine.

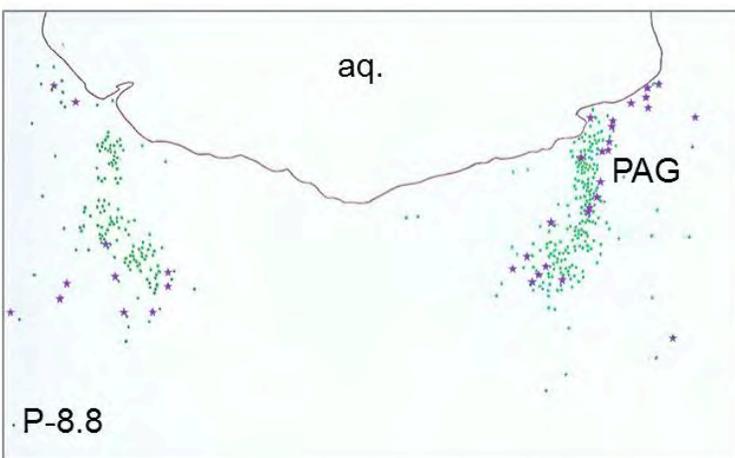
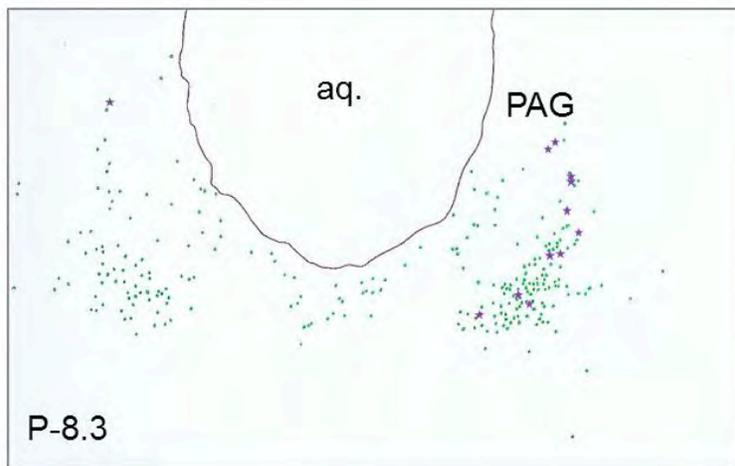
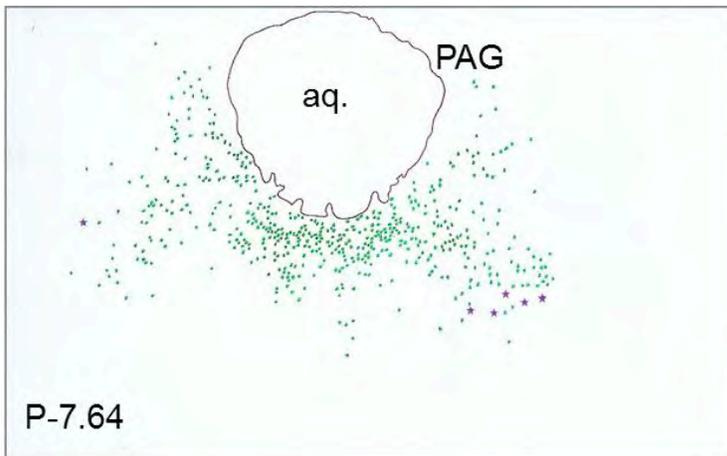


Figure 5.9. Map taken from camera lucida drawings of TH+ cell population in the caudal PAG of a rat that received bilateral microinjections of 6-OHDA. Cells are mapped on to three representative levels of the caudal PAG. Green spots represent small TH+ cells ($\leq 30\mu\text{m}$) and purple stars represent large TH+ cells ($> 30\mu\text{m}$) Abbreviations: Aq. aqueduct, PAG: periaqueductal grey, TH: tyrosine hydroxylase. Numbers represent mm caudal to Bregma.

5.4.2 Acute experiments: Effect of dopamine agonism and antagonism in the midbrain of the urethane anaesthetised rat

Infusion of saline into the bladder elicited periodic increases in bladder pressure (0.5 ± 0.03 contractions min^{-1} , 21.2 ± 0.3 mmHg) that were associated with an increase in the level of tonic EMG activity of the EUS that adopted a bursting pattern that coincided with urine output (6.6 ± 0.2 drops per void).

5.4.2.1 Microinjection of SCH23390 into the superior colliculi, dorsal PAG, ventral PAG and ventral tegmentum

Once periodic voiding had been established, microinjections of the D1 antagonist SCH23390 (5 mM, 50 nl) were made into the superior colliculi ($n = 3$), dorsal PAG ($n = 5$), ventral PAG ($n = 14$) and ventral tegmentum ($n = 7$, Fig. 5.10). SCH23390 did not affect any of the parameters of voiding including the threshold, maximum, baseline or amplitude of the pressure rise (Fig. 5.11). There was also no change in the frequency, the number of drops per void or the duration of contractions (Fig. 5.11). The bursting activity in the EMG of the EUS remained synchronous with the periodic increases in bladder pressure and expulsion of drops from the urethral meatus.

Following microinjection of SCH23390, at 2/29 sites (6.9%) one in the superior colliculus and the other in the ventral PAG, unusual burst activity in the EMG of the EUS developed within 12.3 min, which appeared to be unrelated to voiding (Fig. 5.12, sample

trace). EMG bursting (average frequency 0.4 and 0.7 Hz respectively) was accompanied by twitching of the scrotum and a very small rise in intravesicular pressure (< 5 mmHg). Bursting activity usually lasted around 60 s and was characterised by an initial high intensity high frequency activity (around 2.5 Hz) which gradually subsided and slowed down (slow \approx 0.4 Hz), Fig. 5.12. In one case, two drops of fluid were expelled from the urethral meatus during a burst of activity (Fig 5.12).

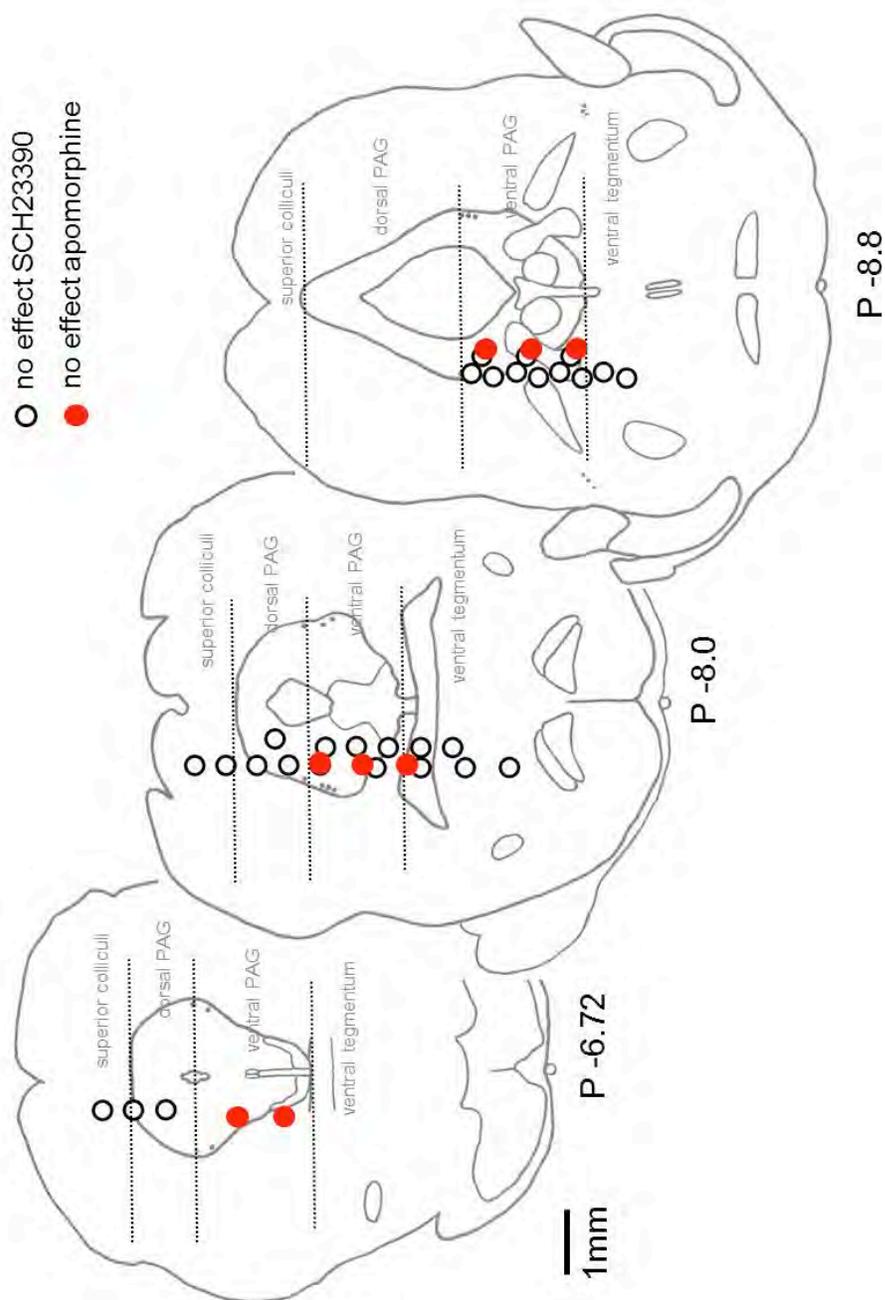


Figure 5.10. Location of sites where dopamine agonists and antagonists were microinjected into the superior colliculi, dorsal periaqueductal grey (PAG), ventral PAG and tegmentum, mapped on to outlines of the midbrain taken from the Atlas of Paxinos and Watson (1986). Black open circles represent the centre of sites at which SCH23390 was microinjected during continuous cystometry. Red filled circles represent the centre of sites where apomorphine was microinjected and had no effect on the micturition reflex evoked by continuous cystometry. Numbers indicate distance (mm) caudal to Bregma.

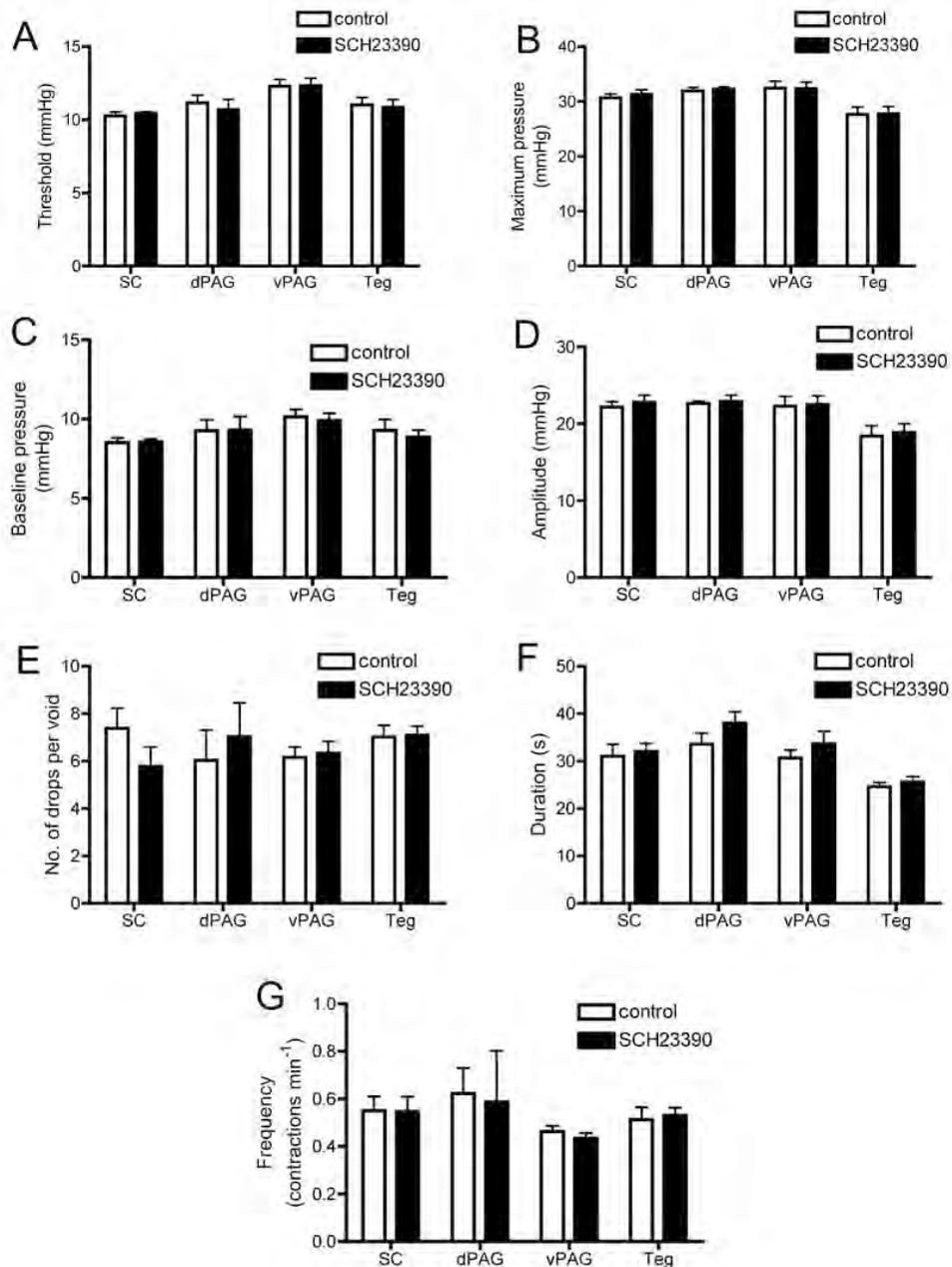


Figure 5.11. Effect of microinjection of SCH23390 into the superior colliculi (SC, n = 3), dorsal PAG (dPAG, n = 5), ventral PAG (vPAG, n = 14) and ventral tegmentum (Teg, n = 7) on A, threshold, B, maximum pressure, C, baseline pressure, D, amplitude of the pressure rise, E, number of drops per void, F, duration of contraction, G, frequency of contractions. Analysis: No statistical testing for small n in SC, Mann Whitney U test for paired observations in the dPAG, paired t test for vPAG and Teg. All P values where analysed > 0.05.

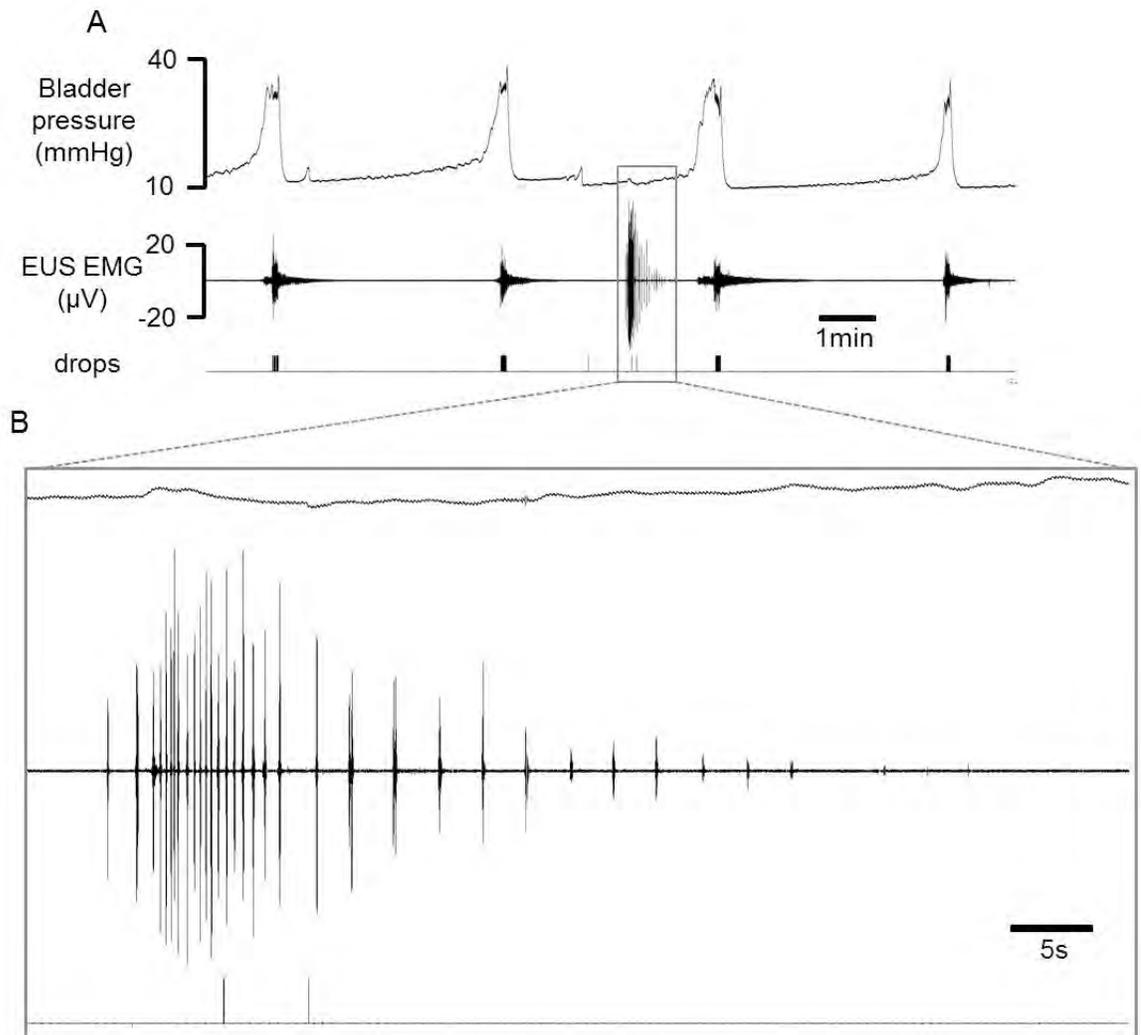


Figure 5.12. Sample trace showing atypical bursting in the EMG of the EUS following microinjection of SCH23390 into the PAG. A, periodic increases in bladder pressure are normally associated with increased activity in the EMG activity of the EUS and expulsion of drops from the urethral meatus. Independent bursting in EMG of the EUS in absence of a change in intravesicular pressure is highlighted on an increased timescale in B.

5.4.2.2 Microinjection of apomorphine into the ventral PAG

Microinjections of the D1 agonist, apomorphine (94 mM, 50 nl) were made at 8 sites in the ventral PAG (Fig. 5.10 map, red circles, n = 8) during continuous cystometry. There was no change in any of the parameters of voiding that were measured. Analysis was carried out into the effects on the threshold for initiation of contraction of the detrusor, maximum pressure reached during contraction, the baseline bladder pressure, the amplitude of the pressure rise, the number of drops per void, the duration of contraction and the frequency of contractions (Fig. 5.13). Paired *t* tests showed no significant differences between any of the above parameters before and after administration of apomorphine.

At 2/8 sites, following microinjection of apomorphine into the ventral PAG, bursting activity developed in the EMG of the EUS within 2.3 min at a mean frequency of 1.1 and 0.8 Hz in each case. Bursting started off at a slow frequency (\approx 0.4 Hz), then became fast for a short period (\approx 2.5 Hz) before slowing again. This bursting lasted for a maximum of 1.7 min and occurred in the absence of the sharp rise in intravesicular pressure that was normally associated with a void.

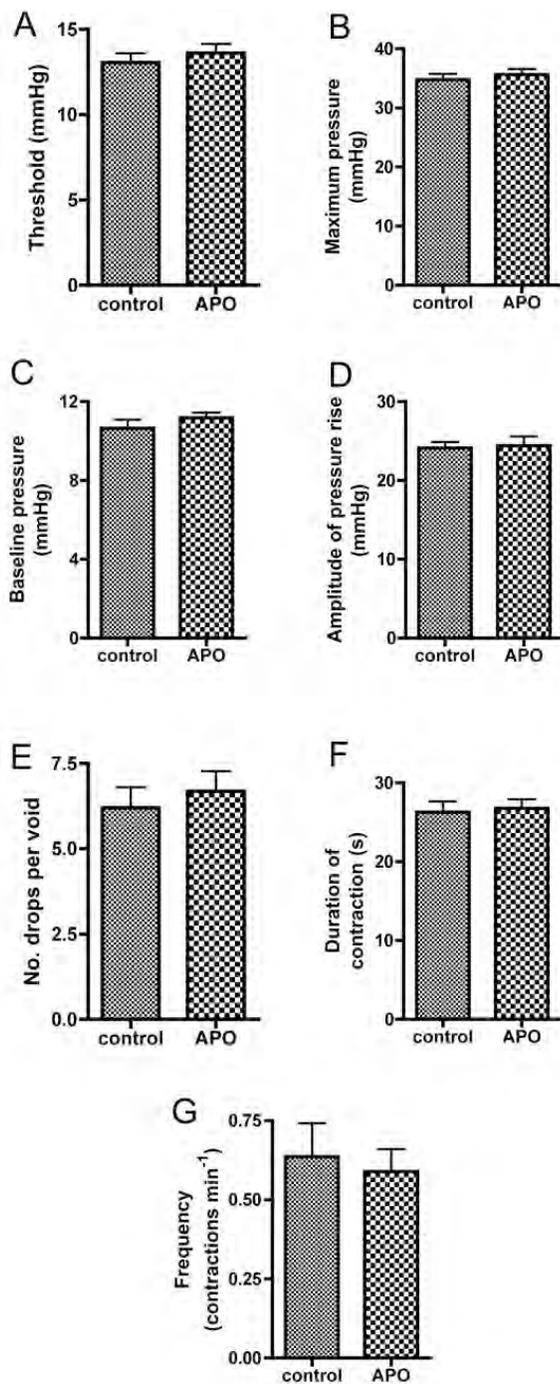


Figure 5.13. Microinjection of apomorphine (APO, n = 8): Effect on parameters of the micturition reflex evoked by continuous infusion of saline into the bladder of urethane anaesthetised rats. A, threshold pressure for contraction, B, maximum pressure reached during contraction, C, baseline bladder pressure, D, amplitude of pressure rise during contraction, E, number of drops per void, F, duration of contraction, G, frequency of contractions. All control means analysed with Student's paired t test to the APO values. All $P > 0.05$.

5.5 DISCUSSION

This study was designed to investigate the contribution of dopamine-containing cells in the PAG on micturition. 24 h urine output was measured before and after the 6-OHDA lesioning or sham procedure so that each animal could act as its own control.

All rats showed a diurnal pattern in urine output, with a greater number of voids during the dark period, when rats are awake and active, in comparison to the light period. This finding is in accord with previous studies, which reported more frequent and smaller volume voids during the dark period compared to the light (Longhurst *et al.*, 1992; Schmidt *et al.*, 2001; Herrera & Meredith, 2010). Surprisingly, the volume of urine produced during the 24 h period in the present experiments was almost half of that reported by Schmidt and colleagues (2001). It is possible that evaporation of fluid from the collection tube over the 24 h period led to an underestimation of the volume of urine produced. However, the rats used by Schmidt and colleagues (2001) had received 5 ml water by gavage in an attempt to standardise the baseline hydration status before urine collection was started. These animals would have been severely volume loaded which would have contributed to their high urine volume output over the subsequent 24 h.

Interestingly, in the present experiments, though the number of voids during the light, inactive period was lower than during the dark period, the volume of urine produced during this time was equal to the volume produced during the dark, active period.

Therefore during the light, inactive period, rats did fewer voids, but the volume of each void was greater than during the dark period. Usually, there is a diurnal pattern in the volume of urine produced, with production levels reduced in the inactive period compared to the active period (Mills, 1951; Schmidt *et al.*, 2001), which was not seen in the present experiments. It is possible that since this was a novel environment, and the first time alone in a cage for these rats, their diurnal patterns may have been disrupted. In the future, habituation to the metabolism cage, as carried out by Schmidt and colleagues (2001) would determine whether this was the cause.

When 24 h urine output was measured following the lesioning or sham-lesioning procedures, there was no difference in the volume of urine produced compared to the baseline pre-surgery values in either group. However, counts of tyrosine hydroxylase positive (TH+) cells in the periaqueductal grey (PAG) in the 6-OHDA-injected group were not significantly different to the sham-lesioned rats, suggesting that the lesioning procedure had not been effective.

In sham-lesioned rats the immunohistochemistry experiments revealed densely stained TH immunoreactive cells bilaterally in the ventrolateral PAG and dorsal raphe nucleus, in accordance with previous findings (Hokfelt *et al.*, 1976; Hasue & Shammah-Lagnado, 2002; Flores *et al.*, 2004). TH is an enzyme responsible for the hydroxylation of L-tyrosine to form L-3,4-dihydroxyphenylalanine (L-DOPA) which is then converted to dopamine in the dopamine synthesis pathway. Synthesis of noradrenaline shares a common pathway with dopamine being converted to noradrenaline by dopamine β

hydroxylase (DBH). Thus it is important to clarify that one is identifying dopaminergic neurons with TH immunohistochemistry as opposed to noradrenergic neurons that contain DBH. Flores and colleagues (2004) used a similar immunohistochemistry protocol to the present experiments, and as well as immunostaining for TH, they carried out DBH immunohistochemistry and found that there were no cells containing DBH in the PAG. This suggests that the TH cells stained in their sections, and indeed in the present experiments are likely to be dopaminergic neurons. Two morphologically distinct populations of TH+ cells (large and small) in the PAG with a morphology and distribution similar to those found in the present study have also been described previously (Flores et al 2004). However in contrast to the study of Flores et al (2004) where approximately one third of the immunostained population comprised large cells, only 5% of cells in the current study were classified as large. This discrepancy may be due to difference in the area sampled in the two studies. In the present experiments, TH+ cells in the dorsal raphe nucleus have been included in the total cell counts and it is not explicitly clear whether these cells were counted in Flores and colleagues' model used for estimation of cell numbers (Flores *et al.*, 2004).

In the present study there was a more than 4-fold range in the number of TH+ cells present in the caudalmost 1200 μ m of the PAG between individual sham-lesioned rats. Thus it was possible that the high degree of inter-individual variability might be masking an effect of 6-OHDA lesioning. Furthermore, the method employed to quantify the effect of the 6-OHDA lesion was a simple cell count that did not take into account the size of the remaining TH+ cells aside from identifying those which were less or greater than

30µm. Thus the possibility of cells being double-counted was not considered using a correction such as Abercrombie's which takes into account the size of cells and the thickness of the section. However one might expect the error caused by double counting to be of an equal magnitude in both the 6-OHDA and sham lesioned group since neither were subject to Abercrombie's correction, thus it is unlikely that this error masked any overall change in cell numbers. Moreover there was no correlation between the 24 h urine output and TH+ cell number in either the sham-lesioned or lesioned groups. Therefore we have to conclude that the lesioning procedure had been ineffective.

The reason for the ineffectiveness of 6-OHDA in the lesioning of the DA-containing cell population in the PAG is not clear. It is possible that the 6-OHDA became inactive due to oxidation. However, a number of precautions were taken to prevent this happening. On arrival, the drug was dissolved in aqueous solution containing ascorbate and was stored at -20°C. On the day of an experiment the aliquot was stored in darkness and on ice until the last possible moment before filling the microinjection cannula.

To carry out the lesioning procedure the rats were anaesthetised with an IP injection of ketamine and medetomidine. One possibility is that this anaesthetic interfered with the neurotoxic actions of the 6-OHDA. An interesting paper suggested that a similar anaesthetic mix of ketamine and xylazine protected dopaminergic cells from the lesioning effects of 6-OHDA (Ferro *et al.*, 2007). Ferro and colleagues (2007) found significantly fewer dopaminergic cells were lesioned when 6-OHDA was administered into the substantia nigra pars compacta under ketamine/xylazine anaesthesia in

comparison to under thiopental anaesthesia. However, Ferro et al (2007) did not find that this anaesthetic had a 100% neuroprotective effect. Moreover, others have also successfully used the 6-OHDA lesioning procedure in different brain regions using the same general anaesthesia as used in the present experiments (Yoshimura et al., 2003; Soler et al., 2011). This suggests that the success of 6-OHDA lesions in our surgeries carried out under ketamine/medetomidine anaesthesia may have been thwarted by the anaesthetic but this cannot explain the complete failure of the lesioning technique.

A further consideration is that the dose of 6-OHDA used in the present experiments may have been too low. Similar doses have been used to selectively lesion dopaminergic cells in the nigrostriatal pathway in animal models of Parkinson's disease (Ungerstedt, 1968; Sauer & Oertel, 1994; Yoshimura *et al.*, 2003; Blandini *et al.*, 2008; Soler *et al.*, 2011), but it is possible that different biophysical properties of dopaminergic cells in the PAG could leave them more resistant to 6-OHDA lesioning. However, an identical dose of 6-OHDA to that used in the present study administered into the PAG was found to lesion around 60% of large TH+ cells in the PAG without affecting the number of small cells (Flores et al 2004). In contrast, others used much higher doses of 6-OHDA in the PAG to achieve a similar reduction in the number of TH+ cells (Lu *et al.*, 2006). This study did not specify whether the neurotoxin produced differential effects on the large and small cell populations. This suggests that the ineffectiveness of 6-OHDA might have been due to insufficient dosing. Unfortunately, due to constraints imposed on the conditions of the Project Licence governing this work, we were unable to carry out further experiments to test this hypothesis.

An interesting and unexpected finding in the present study was that a significant minority of rats in both the sham-lesioned and 6-OHDA lesioned groups developed polyuria following the surgical procedure. Since the only common factor was the anaesthesia, it is possible that in these animals polyuria was a lasting effect of the ketamine/medetomidine anaesthetic. It is known that administration of this anaesthetic produces acute diuresis via α_2 activation (Cabral *et al.*, 1997; Cabral *et al.*, 1998). However there are no reports of long-lasting effects. We were unable to carry out control experiments to test this hypothesis due to restrictions on the Project Licence governing the work. To answer these questions fully, future experiments could repeatedly expose an age-matched control group to the metabolism cage, but without the intervening anaesthesia and lesioning surgery to see whether the polyuria was an effect of age. If there were no differences then another control group should be exposed to the metabolism cage, receive the anaesthetic and drugs associated with surgery for a similar length of time without any surgery being carried out.

Since lesioning experiments were stopped before methodological issues concerning optimising the procedure could be resolved, we moved to a pharmacological approach to investigate the role of dopaminergic transmission in the PAG on reflex micturition. The effects of microinjection of a dopamine agonist and an antagonist in the urethane anaesthetised rat in an acute preparation were investigated.

In our anaesthetised rat preparation, microinjection of neither the D1 antagonist SCH23390 nor the non-selective dopamine agonist apomorphine had any effect on any of the parameters of voiding. These findings are in contrast to previous studies in conscious rats in which application of SCH23390, into the ventrolateral PAG increased the maximal voiding pressure and reduced the inter-void interval during continuous cystometry (Kitta *et al.*, 2008) and apomorphine caused an increase in the maximal voiding pressure following systemic administration (Uchiyama *et al.*, 2009). The failure of these agonists to produce an effect in the present study was unexpected. Indeed, a previous study in conscious rats showed that during micturition, the levels of dopamine in the caudal ventrolateral PAG were increased whilst the levels of GABA decreased (Kitta *et al.*, 2008), suggesting that dopamine and GABA may work in concert in the ventrolateral PAG to control the micturition circuitry. In striatal tissue dopamine has been shown to inhibit the release of GABA (Harsing & Zigmond, 1996). It is possible that a similar relationship may exist in the caudal ventrolateral PAG, where dopamine could inhibit the release of GABA, thus allowing micturition to occur as the GABAergic inhibition is lifted. In the present study carried out under urethane anaesthesia, the levels of GABA in the whole of the brain, including the PAG, are likely to be high (Krnjević, 1986). Therefore if dopamine acts by inhibiting GABA release, but GABA levels are already high, then the effects of a dopamine agonist or antagonist on GABA release may be silenced. It is possible that the absence of an effect in these experiments was due to insufficient dosing of both the agonist and the antagonist, despite the doses being within the ranges reported in the literature (Melo *et al.*, 2009; Meyer *et al.*, 2009; Dang *et al.*, 2010; Chen *et al.*, 2011). Further experiments that

administer a higher dose of both SCH23390 and apomorphine would resolve this uncertainty but might lead to more non-specific activation of other receptor subtypes.

Although apomorphine was ineffective in influencing micturition, in 25% of cases it evoked atypical low frequency EMG bursting in the EUS in the absence of a change in the intravesicular pressure that would normally signify a void. Similar bursting patterns have been seen in investigations into the coital reflex in rats (Carro-Juarez & Rodriguez-Manzo, 2000). Moreover, administration of apomorphine systemically, and into other regions of the brain is known to induce ejaculation (Pehek *et al.*, 1988; Pehek *et al.*, 1989; Stafford & Coote, 2006; Yonezawa *et al.*, 2009). The PAG has been implicated in the control of sexual function in females, producing lordosis, but this role in males has not been described (Behbehani, 1995). Marson (2004) has shown that inhibition of the PAG can prevent the medial preoptic area induced coital reflex in male rats (Marson, 2004). Thus bursting activity in the EUS evoked by apomorphine may be related to sexual behaviour.

5.6 SUMMARY AND CONCLUSIONS

In summary, the present experiments have neither proven nor disproven the role that dopamine in the caudal ventrolateral PAG plays in the control of micturition. Optimisation of the lesioning technique is required to be able to draw any conclusions about whether degeneration of the dopaminergic cells in the caudal ventrolateral PAG might contribute to the urinary disturbances that have been reported in animal models of

Parkinson's disease (Soler *et al.*, 2011). Our experiments in the urethane anaesthetised rat indicate that this preparation is not an ideal experimental model for investigation of the role of dopamine in the midbrain in control of reflex micturition, possibly because the urethane anaesthetic interferes with central neurotransmission.

5.7 KEY FINDINGS: CHAPTER 5

5.7.1 Chronic experiments: Lesioning of dopaminergic cells in the

PAG with 6-OHDA

1. 24 h urine output can be recorded in the conscious, unrestrained rat by placement in a metabolism cage with an infra-red drop counter recording passage of drops into the urine collection tube via PowerLab and LabChart software. Future experiments should allow a longer acclimatisation period or habituation in case exposure to the novel metabolism cage environment affects voiding patterns.
2. Bilateral microinjection of a 5 mg ml⁻¹ solution of 6-OHDA did not reduce the number of TH+ cells in the ventral 1200 µm of the PAG in comparison to the sham lesioned controls. This may have been due to insufficient dosing or an interaction between the 6-OHDA and anaesthesia used for surgery. Future experiments should use a higher dose and an alternative anaesthetic.
3. There was a high degree of inter-rat variability in the number of TH+ cells in the caudal portion of the PAG. This should be considered when using the cell number to assess the extent of 6-OHDA lesioning.

5.7.2 Acute experiments: Microinjections of dopamine agonist and

antagonist into the PAG of the urethane anaesthetised rat

1. Microinjection of the D1 antagonist SCH23390 and the non-specific dopamine agonist apomorphine into the PAG did not affect the micturition reflex in the urethane anaesthetised rat; despite previous research suggesting that dopaminergic neurotransmission in the caudal ventrolateral PAG might play a role in the control of micturition in the conscious rat (Kitta *et al.*, 2008).
2. The urethane anaesthetised rat may not be a suitable preparation to use in these investigations because of the increased GABA tone that produces general anaesthesia.

CHAPTER 6: GENERAL DISCUSSION

These studies were designed with the short-term goal of furthering understanding of the normal control of micturition in the rat, and with a long-term goal of identifying novel therapeutic targets for human diseases that affect the urine storage and emptying function of the bladder.

6.1 KEY FINDINGS

Microinjection of the GABA agonist, muscimol into the periaqueductal grey (PAG) revealed that critical synapses involved in reflex micturition are located in a restricted area within the caudal ventrolateral region. Furthermore, these experiments have shown that synaptic transmission through this region is tonically suppressed by a GABAergic influence since microinjection of the GABA_A antagonist, bicuculline, into the same region of the PAG increased the frequency of voiding evoked by continuous infusion of saline into the bladder. The presence of on-going GABAergic tone is clearly an important factor for maintaining urinary continence. It is possible that disease states that lead to disruption of GABA levels in the PAG could cause a failure of this inhibitory mechanism leading either to urinary retention due to excess levels of GABA in the caudal ventrolateral PAG, or overactive bladder: a frequent desire to void, possibly caused by insufficient levels of GABAergic inhibition in the same area.

The PAG receives inputs from a number of brain regions, and integrates coordinated behavioural and emotional responses, including anxiety, sympathetic activation of the

cardiovascular and respiratory systems and nociception (Behbehani, 1995) (Behbehani, 1995). It is probable that the caudal ventrolateral PAG also acts as a site for integration in the control of micturition, receiving afferent signals from the distended bladder, and efferent projections from frontal regions (Marson, 1997) which might be involved in the conscious component of socialised voiding.

The ubiquitous nature of GABAergic transmission within the brain makes selective targeting of local GABAergic circuits for therapeutic gain by pharmacological means almost impossible. However, the presence of a discrete population of tyrosine hydroxylase containing (TH+) neurons localised in the ventrolateral PAG (Saper & Petito, 1982; Flores *et al.*, 2004; Lu *et al.*, 2006) corresponding to the area where critical synapses in the micturition circuitry had been identified (Stone *et al.* 2011, Chapter 3) raised the possibility that dopamine might be involved in the control of micturition. Moreover, Kitta and colleagues (2008) found that during periods of intravesicular distension, the extracellular levels of dopamine in the caudal ventrolateral PAG of the conscious rat were raised. We hypothesised that the population of TH+ cells in the ventrolateral PAG might be the source of this dopamine, and designed experiments to investigate the effects of lesioning these cells on micturition in the rat. The results of these studies were inconclusive. Technical problems in optimising a procedure for selectively lesioning this cell group did not allow us to establish whether they are a critical component of the micturition reflex pathway.

Pharmacological manipulation of dopaminergic transmission through the PAG using selective agonists and antagonists did not have any effect on reflex micturition in the urethane-anaesthetised rat. Since others have shown that similar manipulation, both systemically and locally evoked dose and time dependent effects on the frequency of voiding in the conscious rat (Kitta *et al.*, 2008; Uchiyama *et al.*, 2009), it is possible that the urethane anaesthesia in the present experiments interfered with the actions of the drugs. Refinement of experimental procedures would resolve many of the technical problems that arose in our investigations into the role of dopaminergic neurotransmission in the control of micturition and would provide conclusive evidence as to whether the population of dopaminergic cells in the PAG are involved in controlling micturition.

This study has however shown that powerful control of micturition can be produced by electrical stimulation within the PAG. These inhibitory effects were rapidly reversible, with voiding returning shortly after the stimulation was stopped. Electrical stimulation of discrete regions of the brain ('deep brain stimulation', DBS) has become increasingly popular as a clinical tool for the alleviation of symptoms of a number of disorders, including Parkinson's and intractable pain (for reviews see Perlmutter and Mink, 2006, Kringelbach *et al.* 2007, Deniau *et al.* 2010). In cases that are refractory to other treatments DBS is an attractive therapeutic option because although electrode implantation involves invasive neurosurgery, DBS has been shown to be effective, highly selective, of rapid onset and rapidly reversible. Thus the side effects are limited (and depend on the locus of stimulation).

6.2 TRANSLATIONAL POTENTIAL

Based on our findings in the rat, the translational potential of electrical stimulation in the PAG for controlling human continence has been explored by our clinical colleagues at the John Radcliffe and Churchill Hospitals in Oxford. The neurosurgical team at the John Radcliffe Hospital in Oxford and others use deep brain stimulation of the PAG to control pain in patients with chronic intractable pain (Nandi *et al.*, 2002; Bittar *et al.*, 2005; Hamani *et al.*, 2006; Owen *et al.*, 2006). We were therefore fortunate to have access to a cohort of 7 patients with electrodes already implanted into midbrain sites that consented to being participants in a study to investigate the effects of their DBS on bladder function. Six of these patients — with normal bladder function — had electrodes implanted into the PAG and/or ventral posterolateral nucleus of the hypothalamus (VPL) for the purpose of treating their chronic pain. A further patient had electrodes in the VPL only. A similar protocol was used to the ‘discontinuous cystometry’ experiments on rats (Chapter 4, Section 4.3.2.2). Maximum cystometric capacity, the volume at which patients could not tolerate further intravesicular filling, was increased when the PAG stimulator was turned on, compared to when the PAG stimulator was turned off (Fig. 6.1). Moreover, in two patients with electrodes implanted in the VPL, deep brain stimulation was ineffective (Fig. 6.1) (Green *et al.*, 2012). Importantly, the effects of DBS were not secondary to changes in pain.

These exciting results suggest that there may be therapeutic potential for DBS of the midbrain for the treatment of urinary incontinence in human patients. In addition, our

parallel study in animals validates the use of the anaesthetised rat model in basic scientific research into the central control of reflex micturition.

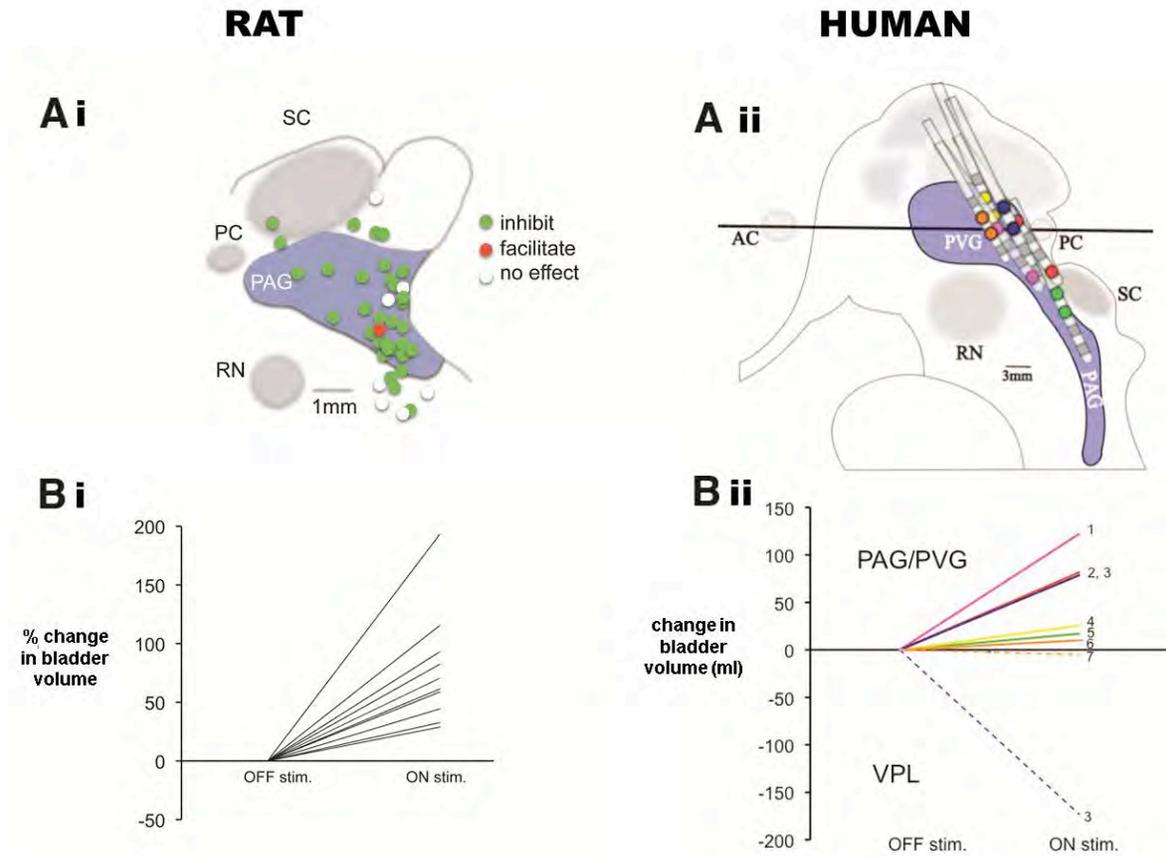


Figure 6.1. Effect of electrical stimulation of the midbrain of both anaesthetised rats and conscious humans on the maximal cystometric capacity. Figure adapted from collaborative work with Green et al. 2012. A, Parasagittal maps showing location of sites at which electrical stimulation was applied and the effects on bladder filling from empty were recorded in the rat (i) and human (ii). Centre of effective sites in the rat represented as green circles. Open circles represent 'no effect'. In human map (A ii), pairs of contacts are indicated by same coloured circles. Abbreviations: AC: anterior commissure; PAG: periaqueductal grey; PC: posterior commissure; PVG: periventricular grey; RN: red nucleus; SC: superior colliculus. B i, Effect of electrical stimulation on discontinuous cystometry in the rat: Change in bladder volume at which urine was first expelled ON v. OFF stimulation when the bladder was filled from empty (n = 10; P<0.001, Student's paired t test on raw data). B ii, Effect of electrical stimulation in human participants: PAG/PVG (unbroken lines, n = 6) and VPL (broken lines, n = 2)

showing an increase in maximum cystometric capacity (MCC) in participants. One participant (3, blue lines) had electrode placements in PAG where stimulation increased MCC and VPL (broken lines) where it decreased MCC. Corresponding electrode placements for each participant are shown by equivalent coloured circles in A ii. For PAG/VPL sites $P = 0.028$ ON v. OFF stimulation, Wilcoxon signed rank test carried out on raw data from human participants.

6.3 SUMMARY AND CONCLUSIONS

These experiments have shown that GABAergic neurotransmission in the caudal ventrolateral PAG tonically inhibits the micturition reflex evoked by continuous infusion of saline into the bladder of the urethane anaesthetised rat. Though we have been unable to draw conclusions on the effects of dopaminergic neurotransmission in the same area of the PAG, we have shown that electrical stimulation in the PAG and also over a wide area of the midbrain can inhibit reflex voiding in response to intravesicular distension. The translational potential of these findings is great, and indeed we have been in the rare position of being able to collaborate with clinical colleagues to translate our findings from laboratory animal into preliminary trials with human participants. The future for therapeutic intervention using DBS to alleviate urinary disturbances is exciting.

Nonetheless, there is still much scope for basic research to further understanding of the normal control of the micturition circuitry — particularly the pharmacology of conscious control of micturition and its integration with the reflexive autonomic control of the bladder and EUS — with a view to deducing the pathophysiology underlying the failure of the storage and emptying functions of the bladder and EUS that afflict so many. With this knowledge, novel, targeted therapies may be realised or existing therapies modified to improve success rates and the overall quality of life of individuals suffering from urinary disturbances.

LIST OF REFERENCES

- Adolphs R. (1999). Social cognition and the human brain. *Trends in cognitive sciences* **3**, 469-479.
- al Maskati HA & Zbrozyna AW. (1989). Cardiovascular and motor components of the defence reaction elicited in rats by electrical and chemical stimulation in amygdala. *Journal of the autonomic nervous system* **28**, 127-131.
- An X, Bandler R, Ongur D & Price JL. (1998). Prefrontal cortical projections to longitudinal columns in the midbrain periaqueductal gray in macaque monkeys. *J Comp Neurol* **401**, 455-479.
- Andersson KE. (2000). Drug therapy for urinary incontinence. *Baillieres Best Pract Res Clin Obstet Gynaecol* **14**, 291-313.
- Andersson KE & Arner A. (2004). Urinary bladder contraction and relaxation: physiology and pathophysiology. *Physiol Rev* **84**, 935-986.
- Apostolidis A, Dasgupta P & Fowler CJ. (2006). Proposed mechanism for the efficacy of injected botulinum toxin in the treatment of human detrusor overactivity. *Eur Urol* **49**, 644-650.
- Bahns E, Halsband U & Janig W. (1987). Responses of sacral visceral afferents from the lower urinary tract, colon and anus to mechanical stimulation. *Pflugers Arch* **410**, 296-303.
- Ballaro A. (2008). The elusive electromyogram in the overactive bladder: a spark of understanding. *Ann R Coll Surg Engl* **90**, 362-367.
- Ballaro A, Mundy AR, Fry CH & Craggs MD. (2001). A new approach to recording the electromyographic activity of detrusor smooth muscle. *J Urol* **166**, 1957-1961.
- Bandler R, Depaulis A & Vergnes M. (1985). Identification of midbrain neurones mediating defensive behaviour in the rat by microinjections of excitatory amino acids. *Behav Brain Res* **15**, 107-119.
- Barrington FJF. (1925). The effect of lesions of the hind- and mid-brain on micturition in the cat. *Experimental Physiology* **15**, 81-102.

- Behbehani MM. (1995). Functional characteristics of the midbrain periaqueductal gray. *Prog Neurobiol* **46**, 575-605.
- Bekar L, Libionka W, Tian GF, Xu Q, Torres A, Wang X, Lovatt D, Williams E, Takano T, Schnermann J, Bakos R & Nedergaard M. (2008). Adenosine is crucial for deep brain stimulation-mediated attenuation of tremor. *Nat Med* **14**, 75-80.
- Bennett BC, Kruse MN, Roppolo JR, Flood HD, Fraser M & de Groat WC. (1995). Neural control of urethral outlet activity in vivo: role of nitric oxide. *J Urol* **153**, 2004-2009.
- Bergles DE, Jabs R & Steinhauser C. (2010). Neuron-glia synapses in the brain. *Brain research reviews* **63**, 130-137.
- Beurrier C, Bioulac B, Audin J & Hammond C. (2001). High-frequency stimulation produces a transient blockade of voltage-gated currents in subthalamic neurons. *J Neurophysiol* **85**, 1351-1356.
- Bittar RG, Kar-Purkayastha I, Owen SL, Bear RE, Green A, Wang S & Aziz TZ. (2005). Deep brain stimulation for pain relief: a meta-analysis. *J Clin Neurosci* **12**, 515-519.
- Bittencourt AS, Carobrez AP, Zamprogno LP, Tufik S & Schenberg LC. (2004). Organization of single components of defensive behaviors within distinct columns of periaqueductal gray matter of the rat: role of N-methyl-D-aspartic acid glutamate receptors. *Neuroscience* **125**, 71-89.
- Blandini F, Armentero MT & Martignoni E. (2008). The 6-hydroxydopamine model: news from the past. *Parkinsonism Relat Disord* **14 Suppl 2**, S124-129.
- Blok BF, De Weerd H & Holstege G. (1995). Ultrastructural evidence for a paucity of projections from the lumbosacral cord to the pontine micturition center or M-region in the cat: a new concept for the organization of the micturition reflex with the periaqueductal gray as central relay. *J Comp Neurol* **359**, 300-309.
- Blok BF, Groen J, Bosch JL, Veltman DJ & Lammertsma AA. (2006). Different brain effects during chronic and acute sacral neuromodulation in urge incontinent patients with implanted neurostimulators. *BJU international* **98**, 1238-1243.
- Blok BF & Holstege G. (1994). Direct projections from the periaqueductal gray to the pontine micturition center (M-region). An anterograde and retrograde tracing study in the cat. *Neurosci Lett* **166**, 93-96.

- Blok BF & Holstege G. (1999). Two pontine micturition centers in the cat are not interconnected directly: implications for the central organization of micturition. *J Comp Neurol* **403**, 209-218.
- Blok BF, Sturms LM & Holstege G. (1997a). A PET study on cortical and subcortical control of pelvic floor musculature in women. *J Comp Neurol* **389**, 535-544.
- Blok BF, Sturms LM & Holstege G. (1998). Brain activation during micturition in women. *Brain* **121 (Pt 11)**, 2033-2042.
- Blok BF, Willemsen AT & Holstege G. (1997b). A PET study on brain control of micturition in humans. *Brain* **120 (Pt 1)**, 111-121.
- Blum D, Torch S, Lambeng N, Nissou M, Benabid AL, Sadoul R & Verna JM. (2001). Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Prog Neurobiol* **65**, 135-172.
- Brading AF. (1997). A myogenic basis for the overactive bladder. *Urology* **50**, 57-67; discussion 68-73.
- Cabral AD, Kapusta DR, Kenigs VA & Varner KJ. (1998). Central alpha2-receptor mechanisms contribute to enhanced renal responses during ketamine-xylazine anesthesia. *Am J Physiol* **275**, R1867-1874.
- Cabral AM, Varner KJ & Kapusta DR. (1997). Renal excretory responses produced by central administration of opioid agonists in ketamine and xylazine-anesthetized rats. *The Journal of pharmacology and experimental therapeutics* **282**, 609-616.
- Carrive P, Schmitt P & Karli P. (1986). Flight induced by microinjection of D-tubocurarine or alpha-bungarotoxin into medial hypothalamus or periaqueductal gray matter: cholinergic or GABAergic mediation? *Behav Brain Res* **22**, 233-248.
- Carro-Juarez M & Rodriguez-Manzo G. (2000). Sensory and motor aspects of the coital reflex in the spinal male rat. *Behav Brain Res* **108**, 97-103.
- Castleden CM, Duffin HM & Gulati RS. (1986). Double-blind study of imipramine and placebo for incontinence due to bladder instability. *Age Ageing* **15**, 299-303.
- Chapple CR, Khullar V, Gabriel Z, Muston D, Bitoun CE & Weinstein D. (2008). The effects of antimuscarinic treatments in overactive bladder: an update of a systematic review and meta-analysis. *Eur Urol* **54**, 543-562.
- Cheater FM & Castleden CM. (2000). Epidemiology and classification of urinary incontinence. *Baillieres Best Pract Res Clin Obstet Gynaecol* **14**, 183-205.

- Chen L, Qi R, Chen XY, Xue Y, Xu R & Wei HJ. (2011). Modulation of the activity of globus pallidus by dopamine D1-like receptors in parkinsonian rats. *Neuroscience* **194**, 181-188.
- Conte B, Maggi CA, Parlani M, Lopez G, Manzini S & Giachetti A. (1991). Simultaneous recording of vesical and urethral pressure in urethane-anesthetized rats: effect of neuromuscular blocking agents on the activity of the external urethral sphincter. *J Pharmacol Methods* **26**, 161-171.
- Cunningham DJ, Guttmann L, Whitteridge D & Wyndham CH. (1953). Cardiovascular responses to bladder distension in paraplegic patients. *J Physiol* **121**, 581-592.
- Curtis DR, Phillis JW & Watkins JC. (1959). Chemical excitation of spinal neurones. *Nature* **183**, 611-612.
- Curtis DR, Phillis JW & Watkins JC. (1960). The chemical excitation of spinal neurones by certain acidic amino acids. *J Physiol* **150**, 656-682.
- D'Amico SC, Schuster IP & Collins WF, 3rd. (2011). Quantification of external urethral sphincter and bladder activity during micturition in the intact and spinally transected adult rat. *Exp Neurol* **228**, 59-68.
- Dang YH, Zhao Y, Xing B, Zhao XJ, Huo FQ, Tang JS, Qu CL & Chen T. (2010). The role of dopamine receptors in ventrolateral orbital cortex-evoked anti-nociception in a rat model of neuropathic pain. *Neuroscience* **169**, 1872-1880.
- Dasgupta R, Critchley HD, Dolan RJ & Fowler CJ. (2005). Changes in brain activity following sacral neuromodulation for urinary retention. *J Urol* **174**, 2268-2272.
- de Groat W. (2011). Autonomic regulation of the urinary bladder and urethra. In *Central Regulation of Autonomic Functions*, 2 edn, ed. Llewellyn-Smith IJ & M VAJ, pp. 345 - 365. Oxford University Press, Inc., New York.
- de Groat WC. (1997). A neurologic basis for the overactive bladder. *Urology* **50**, 36-52; discussion 53-36.
- de Groat WC. (2006). Integrative control of the lower urinary tract: preclinical perspective. *Br J Pharmacol* **147 Suppl 2**, S25-40.
- Deniau JM, Degos B, Bosch C & Maurice N. (2010). Deep brain stimulation mechanisms: beyond the concept of local functional inhibition. *Eur J Neurosci* **32**, 1080-1091.

- Ding YQ, Wang D, Nie H, Guan ZL, Lu BZ & Li JS. (1998). Direct projections from the periaqueductal gray to pontine micturition center neurons projecting to the lumbosacral cord segments: an electron microscopic study in the rat. *Neurosci Lett* **242**, 97-100.
- Drake MJ, Fowler CJ, Griffiths D, Mayer E, Paton JF & Birder L. (2010). Neural control of the lower urinary and gastrointestinal tracts: supraspinal CNS mechanisms. *NeuroUrol Urodyn* **29**, 119-127.
- Dunwiddie TV & Masino SA. (2001). The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci* **24**, 31-55.
- Duty S & Jenner P. (2011). Animal models of Parkinson's disease: a source of novel treatments and clues to the cause of the disease. *Br J Pharmacol* **164**, 1357-1391.
- Ferro MM, Angelucci ME, Anselmo-Franci JA, Canteras NS & Da Cunha C. (2007). Neuroprotective effect of ketamine/xylazine on two rat models of Parkinson's disease. *Braz J Med Biol Res* **40**, 89-96.
- Flores JA, El Banoua F, Galan-Rodriguez B & Fernandez-Espejo E. (2004). Opiate antinociception is attenuated following lesion of large dopamine neurons of the periaqueductal grey: critical role for D1 (not D2) dopamine receptors. *Pain* **110**, 205-214.
- Floyd NS, Price JL, Ferry AT, Keay KA & Bandler R. (2000). Orbitomedial prefrontal cortical projections to distinct longitudinal columns of the periaqueductal gray in the rat. *J Comp Neurol* **422**, 556-578.
- Fowler CJ, Griffiths D & de Groat WC. (2008). The neural control of micturition. *Nat Rev Neurosci* **9**, 453-466.
- Fowler CJ & Griffiths DJ. (2010). A decade of functional brain imaging applied to bladder control. *NeuroUrol Urodyn* **29**, 49-55.
- Fukuda H & Koga T. (1991). Midbrain stimulation inhibits the micturition, defecation and rhythmic straining reflexes elicited by activation of sacral vesical and rectal afferents in the dog. *Exp Brain Res* **83**, 303-316.
- Garcia L, Audin J, D'Alessandro G, Bioulac B & Hammond C. (2003). Dual effect of high-frequency stimulation on subthalamic neuron activity. *J Neurosci* **23**, 8743-8751.
- Garry RC, Roberts TD & Todd JK. (1959). Reflexes involving the external urethral sphincter in the cat. *J Physiol* **149**, 653-665.

- Glinka Y, Gassen M & Youdim MB. (1997). Mechanism of 6-hydroxydopamine neurotoxicity. *Journal of neural transmission* **50**, 55-66.
- Goetz RR, Klein DF & Gorman JM. (1994). Consistencies between recalled panic and lactate-induced panic. *Anxiety* **1**, 31-36.
- Gray JA. (1987). *The psychology of fear and stress*. Cambridge University Press, Cambridge.
- Green A, Stone E, Sitsapesan H, Turney B, Coote J, Aziz T, Hyam J & Lovick T. (2012). Switching off micturition using deep brain stimulation at midbrain sites. *Ann Neurol* **In press**.
- Griffiths D, Derbyshire S, Stenger A & Resnick N. (2005). Brain control of normal and overactive bladder. *J Urol* **174**, 1862-1867.
- Griffiths D & Tadic SD. (2008). Bladder control, urgency, and urge incontinence: evidence from functional brain imaging. *Neurourol Urodyn* **27**, 466-474.
- Griffiths JL & Lovick TA. (2005). GABAergic neurones in the rat periaqueductal grey matter express alpha4, beta1 and delta GABAA receptor subunits: plasticity of expression during the estrous cycle. *Neuroscience* **136**, 457-466.
- Grill WM, Erokwu BO, Hadziefendic S & Haxhiu MA. (1999). Extended survival time following pseudorabies virus injection labels the suprapontine neural network controlling the bladder and urethra in the rat. *Neurosci Lett* **270**, 63-66.
- Guttmann L & Whitteridge D. (1947). Effects of bladder distension on autonomic mechanisms after spinal cord injuries. *Brain* **70**, 361-404.
- Habler HJ, Janig W & Koltzenburg M. (1990). Activation of unmyelinated afferent fibres by mechanical stimuli and inflammation of the urinary bladder in the cat. *J Physiol* **425**, 545-562.
- Habler HJ, Janig W & Koltzenburg M. (1993). Myelinated primary afferents of the sacral spinal cord responding to slow filling and distension of the cat urinary bladder. *J Physiol* **463**, 449-460.
- Hamani C, Schwalb JM, Rezai AR, Dostrovsky JO, Davis KD & Lozano AM. (2006). Deep brain stimulation for chronic neuropathic pain: long-term outcome and the incidence of insertional effect. *Pain* **125**, 188-196.
- Hammond C, Ammari R, Bioulac B & Garcia L. (2008). Latest view on the mechanism of action of deep brain stimulation. *Mov Disord* **23**, 2111-2121.

- Harsing LG, Jr. & Zigmond MJ. (1996). Dopaminergic inhibition of striatal GABA release after 6-hydroxydopamine. *Brain Res* **738**, 142-145.
- Hashimoto T, Elder CM, Okun MS, Patrick SK & Vitek JL. (2003). Stimulation of the subthalamic nucleus changes the firing pattern of pallidal neurons. *J Neurosci* **23**, 1916-1923.
- Hasue RH & Shammah-Lagnado SJ. (2002). Origin of the dopaminergic innervation of the central extended amygdala and accumbens shell: a combined retrograde tracing and immunohistochemical study in the rat. *J Comp Neurol* **454**, 15-33.
- Hegde SS. (2006). Muscarinic receptors in the bladder: from basic research to therapeutics. *Br J Pharmacol* **147 Suppl 2**, S80-87.
- Heikkila R & Cohen G. (1972). Further studies on the generation of hydrogen peroxide by 6-hydroxydopamine. Potentiation by ascorbic acid. *Molecular pharmacology* **8**, 241-248.
- Herrera GM & Meredith AL. (2010). Diurnal variation in urodynamics of rat. *PLoS One* **5**, e12298.
- Hiller A, Loeffler S, Haupt C, Litza M, Hofmann U & Moser A. (2007). Electrical high frequency stimulation of the caudate nucleus induces local GABA outflow in freely moving rats. *J Neurosci Methods* **159**, 286-290.
- Hokfelt T, Johansson O, Fuxe K, Goldstein M & Park D. (1976). Immunohistochemical studies on the localization and distribution of monoamine neuron systems in the rat brain. I. Tyrosine hydroxylase in the mes- and diencephalon. *Medical biology* **54**, 427-453.
- Holstege G. (2005). Micturition and the soul. *J Comp Neurol* **493**, 15-20.
- Holstege G. (2010). The emotional motor system and micturition control. *NeuroUrol Urodyn* **29**, 42-48.
- Holstege G, Griffiths D, de Wall H & Dalm E. (1986). Anatomical and physiological observations on supraspinal control of bladder and urethral sphincter muscles in the cat. *J Comp Neurol* **250**, 449-461.
- Holstege G, Kuypers HG & Boer RC. (1979). Anatomical evidence for direct brain stem projections to the somatic motoneuronal cell groups and autonomic preganglionic cell groups in cat spinal cord. *Brain Res* **171**, 329-333.

- Ito T, Sakakibara R, Nakazawa K, Uchiyama T, Yamamoto T, Liu Z, Shimizu E & Hattori T. (2006). Effects of electrical stimulation of the raphe area on the micturition reflex in cats. *Neuroscience* **142**, 1273-1280.
- Janig W & Morrison JF. (1986). Functional properties of spinal visceral afferents supplying abdominal and pelvic organs, with special emphasis on visceral nociception. *Progress in brain research* **67**, 87-114.
- Kang J, Jiang L, Goldman SA & Nedergaard M. (1998). Astrocyte-mediated potentiation of inhibitory synaptic transmission. *Nature neuroscience* **1**, 683-692.
- Keane DP & O'Sullivan S. (2000). Urinary incontinence: anatomy, physiology and pathophysiology. *Baillieres Best Pract Res Clin Obstet Gynaecol* **14**, 207-226.
- Keay KA & Bandler R. (1993). Deep and superficial noxious stimulation increases Fos-like immunoreactivity in different regions of the midbrain periaqueductal grey of the rat. *Neurosci Lett* **154**, 23-26.
- Khadra MH, Satchell PM & Vaughan CW. (1995). Sympathetic nervous system effects on feline bladder wall compliance throughout continence. *Acta Physiol Scand* **155**, 31-39.
- Kitta T, Matsumoto M, Tanaka H, Mitsui T, Yoshioka M & Nonomura K. (2008). GABAergic mechanism mediated via D receptors in the rat periaqueductal gray participates in the micturition reflex: an in vivo microdialysis study. *Eur J Neurosci* **27**, 3216-3225.
- Ko Y, Lin SJ, Salmon JW & Bron MS. (2005). The impact of urinary incontinence on quality of life of the elderly. *Am J Manag Care* **11**, S103-111.
- Kringelbach ML, Green AL, Owen SL, Schweder PM & Aziz TZ. (2010). Sing the mind electric - principles of deep brain stimulation. *Eur J Neurosci* **32**, 1070-1079.
- Kringelbach ML, Jenkinson N, Owen SL & Aziz TZ. (2007). Translational principles of deep brain stimulation. *Nat Rev Neurosci* **8**, 623-635.
- Krnjević K. (1986). Cellular and synaptic actions of general anesthetics. In *Molecular and cellular mechanisms of anaesthetics*, ed. Roth SH & Miller KW, pp. 3-16. Springer.
- Kruse MN, Belton AL & de Groat WC. (1993). Changes in bladder and external urethral sphincter function after spinal cord injury in the rat. *Am J Physiol* **264**, R1157-1163.

- Kruse MN, Noto H, Roppolo JR & de Groat WC. (1990). Pontine control of the urinary bladder and external urethral sphincter in the rat. *Brain Res* **532**, 182-190.
- Lagace EA, Hansen W & Hickner JM. (1993). Prevalence and severity of urinary incontinence in ambulatory adults: an UPRNet study. *J Fam Pract* **36**, 610-614.
- Lazzeri M & Spinelli M. (2006). The challenge of overactive bladder therapy: alternative to antimuscarinic agents. *Int Braz J Urol* **32**, 620-630.
- Li S, Arbuthnott GW, Jutras MJ, Goldberg JA & Jaeger D. (2007). Resonant antidromic cortical circuit activation as a consequence of high-frequency subthalamic deep-brain stimulation. *J Neurophysiol* **98**, 3525-3537.
- Li T, Qadri F & Moser A. (2004). Neuronal electrical high frequency stimulation modulates presynaptic GABAergic physiology. *Neurosci Lett* **371**, 117-121.
- Li T, Thumen A & Moser A. (2006). Modulation of a neuronal network by electrical high frequency stimulation in striatal slices of the rat in vitro. *Neurochemistry international* **48**, 83-86.
- Liu Z, Sakakibara R, Nakazawa K, Uchiyama T, Yamamoto T, Ito T & Hattori T. (2004). Micturition-related neuronal firing in the periaqueductal gray area in cats. *Neuroscience* **126**, 1075-1082.
- Longhurst PA, Eika B, Leggett RE & Levin RM. (1992). Comparison of urinary bladder function in 6 and 24 month male and female rats. *J Urol* **148**, 1615-1620.
- Lovick TA. (1991). Central nervous system integration of pain control and autonomic function. *News in Physiological Sciences* **6**, 82-86.
- Lovick TA. (1992). Inhibitory modulation of the cardiovascular defence response by the ventrolateral periaqueductal grey matter in rats. *Exp Brain Res* **89**, 133-139.
- Lovick TA & Paul NL. (1999). Co-localization of GABA with nicotinamide adenine dinucleotide phosphate-dependent diaphorase in neurones in the dorsolateral periaqueductal grey matter of the rat. *Neurosci Lett* **272**, 167-170.
- Lu J, Zhou TC & Saper CB. (2006). Identification of wake-active dopaminergic neurons in the ventral periaqueductal grey matter. *J Neurosci* **26**, 193-202.
- Lumb BM. (2002). Inescapable and escapable pain is represented in distinct hypothalamic-midbrain circuits: specific roles for Delta- and C-nociceptors. *Exp Physiol* **87**, 281-286.

- Luthman J, Fredriksson A, Lewander T, Jonsson G & Archer T. (1989). Effects of d-amphetamine and methylphenidate on hyperactivity produced by neonatal 6-hydroxydopamine treatment. *Psychopharmacology (Berl)* **99**, 550-557.
- MacDonald RL, Skerritt JH & Werz MA. (1986). Adenosine agonists reduce voltage-dependent calcium conductance of mouse sensory neurones in cell culture. *J Physiol* **370**, 75-90.
- Maggi CA, Giuliani S, Santicoli P & Meli A. (1986). Analysis of factors involved in determining urinary bladder voiding cycle in urethan-anesthetized rats. *Am J Physiol* **251**, R250-257.
- Mantovani M, Van Velthoven V, Fuellgraf H, Feuerstein TJ & Moser A. (2006). Neuronal electrical high frequency stimulation enhances GABA outflow from human neocortical slices. *Neurochemistry international* **49**, 347-350.
- Margalith I, Gillon G & Gordon D. (2004). Urinary incontinence in women under 65: quality of life, stress related to incontinence and patterns of seeking health care. *Qual Life Res* **13**, 1381-1390.
- Marson L. (1997). Identification of central nervous system neurons that innervate the bladder body, bladder base, or external urethral sphincter of female rats: a transneuronal tracing study using pseudorabies virus. *J Comp Neurol* **389**, 584-602.
- Marson L. (2004). Lesions of the periaqueductal gray block the medial preoptic area-induced activation of the urethro-genital reflex in male rats. *Neurosci Lett* **367**, 278-282.
- Matsumoto S, Levendusky MC, Longhurst PA, Levin RM & Millington WR. (2004). Activation of mu opioid receptors in the ventrolateral periaqueductal gray inhibits reflex micturition in anesthetized rats. *Neurosci Lett* **363**, 116-119.
- Matsuura S, Allen GV & Downie JW. (1998). Volume-evoked micturition reflex is mediated by the ventrolateral periaqueductal gray in anesthetized rats. *Am J Physiol* **275**, R2049-2055.
- Matsuura S & Downie JW. (2000). Effect of anesthetics on reflex micturition in the chronic cannula-implanted rat. *NeuroUrol Urodyn* **19**, 87-99.
- Matsuura S, Downie JW & Allen GV. (2000). Micturition evoked by glutamate microinjection in the ventrolateral periaqueductal gray is mediated through Barrington's nucleus in the rat. *Neuroscience* **101**, 1053-1061.

- Maurice N, Thierry AM, Glowinski J & Deniau JM. (2003). Spontaneous and evoked activity of substantia nigra pars reticulata neurons during high-frequency stimulation of the subthalamic nucleus. *J Neurosci* **23**, 9929-9936.
- McGrother CW, Castleden CM, Duffin H & Clarke M. (1987). A profile of disordered micturition in the elderly at home. *Age Ageing* **16**, 105-110.
- McMullan S & Lumb BM. (2006). Spinal dorsal horn neuronal responses to myelinated versus unmyelinated heat nociceptors and their modulation by activation of the periaqueductal grey in the rat. *J Physiol* **576**, 547-556.
- McMurray G, Casey JH & Naylor AM. (2006). Animal models in urological disease and sexual dysfunction. *Br J Pharmacol* **147 Suppl 2**, S62-79.
- Meissner W, Leblois A, Hansel D, Bioulac B, Gross CE, Benazzouz A & Boraud T. (2005). Subthalamic high frequency stimulation resets subthalamic firing and reduces abnormal oscillations. *Brain* **128**, 2372-2382.
- Melo LL, Pereira EC, Pagini CH, Coimbra NC, Brandao ML & Ferrari EA. (2009). Effects of microinjections of apomorphine and haloperidol into the inferior colliculus on the latent inhibition of the conditioned emotional response. *Exp Neurol* **216**, 16-21.
- Meyer PJ, Morgan MM, Kozell LB & Ingram SL. (2009). Contribution of dopamine receptors to periaqueductal gray-mediated antinociception. *Psychopharmacology (Berl)* **204**, 531-540.
- Michel MC & Vrydag W. (2006). Alpha1-, alpha2- and beta-adrenoceptors in the urinary bladder, urethra and prostate. *Br J Pharmacol* **147 Suppl 2**, S88-119.
- Mills JN. (1951). Diurnal rhythm in urine flow. *J Physiol* **113**, 528-536.
- Monga AK, Tracey MR & Subbaroyan J. (2012). A systematic review of clinical studies of electrical stimulation for treatment of lower urinary tract dysfunction. *International urogynecology journal*.
- Morgan MM & Clayton CC. (2005). Defensive behaviors evoked from the ventrolateral periaqueductal gray of the rat: comparison of opioid and GABA disinhibition. *Behav Brain Res* **164**, 61-66.
- Morrison J, Fowler C, Birder L, Craggs M, Groat Wd, Downie J, Drake M & Thor K. (2005). Neural control of the bladder. In *Incontinence*, ed. Abrams P, Cardozo L, Khoury S & Wein A, pp. 363-422. Health Publications Ltd, Paris.

- Moser A, Gieselberg A, Ro B, Keller C & Qadri F. (2003). Deep brain stimulation: response to neuronal high frequency stimulation is mediated through GABA(A) receptor activation in rats. *Neurosci Lett* **341**, 57-60.
- Mukherjee SR. (1957). Effect of bladder distension on arterial blood pressure and renal circulation: role of splanchnic and buffer nerves. *J Physiol* **138**, 307-325.
- Nadelhaft I, Vera PL, Card JP & Miselis RR. (1992). Central nervous system neurons labelled following the injection of pseudorabies virus into the rat urinary bladder. *Neurosci Lett* **143**, 271-274.
- Nandi D, Smith H, Owen S, Joint C, Stein J & Aziz T. (2002). Peri-ventricular grey stimulation versus motor cortex stimulation for post stroke neuropathic pain. *J Clin Neurosci* **9**, 557-561.
- Nishizawa O, Sugaya K, Noto H, Harada T & Tsuchida S. (1987). Pontine urine storage center in the dog. *The Tohoku journal of experimental medicine* **153**, 77-78.
- Noto H, Roppolo JR, Steers WD & de Groat WC. (1989). Excitatory and inhibitory influences on bladder activity elicited by electrical stimulation in the pontine micturition center in the rat. *Brain Res* **492**, 99-115.
- Noto H, Roppolo JR, Steers WD & de Groat WC. (1991). Electrophysiological analysis of the ascending and descending components of the micturition reflex pathway in the rat. *Brain Res* **549**, 95-105.
- Nowak LG & Bullier J. (1998). Axons, but not cell bodies, are activated by electrical stimulation in cortical gray matter. I. Evidence from chronaxie measurements. *Exp Brain Res* **118**, 477-488.
- Numata A, Iwata T, Iuchi H, Taniguchi N, Kita M, Wada N, Kato Y & Kakizaki H. (2008). Micturition-suppressing region in the periaqueductal gray of the mesencephalon of the cat. *Am J Physiol Regul Integr Comp Physiol* **294**, R1996-2000.
- O'Brien J, Austin M, Sethi P & O'Boyle P. (1991). Urinary incontinence: prevalence, need for treatment, and effectiveness of intervention by nurse. *BMJ (Clinical research ed)* **303**, 1308-1312.
- O'Reilly BA, Kosaka AH, Chang TK, Ford AP, Popert R & McMahon SB. (2001). A quantitative analysis of purinoceptor expression in the bladders of patients with symptomatic outlet obstruction. *BJU international* **87**, 617-622.
- Ogura M, Nakao N, Nakai E, Uematsu Y & Itakura T. (2004). The mechanism and effect of chronic electrical stimulation of the globus pallidus for treatment of Parkinson disease. *Journal of neurosurgery* **100**, 997-1001.

- Ouardouz M, Coderre E, Basak A, Chen A, Zamponi GW, Hameed S, Rehak R, Yin X, Trapp BD & Stys PK. (2009a). Glutamate receptors on myelinated spinal cord axons: I. GluR6 kainate receptors. *Ann Neurol* **65**, 151-159.
- Ouardouz M, Coderre E, Zamponi GW, Hameed S, Yin X, Trapp BD & Stys PK. (2009b). Glutamate receptors on myelinated spinal cord axons: II. AMPA and GluR5 receptors. *Ann Neurol* **65**, 160-166.
- Owen SL, Green AL, Stein JF & Aziz TZ. (2006). Deep brain stimulation for the alleviation of post-stroke neuropathic pain. *Pain* **120**, 202-206.
- Pahapill PA, Levy R, Dostrovsky JO, Davis KD, Rezai AR, Tasker RR & Lozano AM. (1999). Tremor arrest with thalamic microinjections of muscimol in patients with essential tremor. *Ann Neurol* **46**, 249-252.
- Park JM, Bloom DA & McGuire EJ. (1997). The guarding reflex revisited. *Br J Urol* **80**, 940-945.
- Parpura V, Heneka MT, Montana V, Oliet SH, Schousboe A, Haydon PG, Stout RF, Jr., Spray DC, Reichenbach A, Pannicke T, Pekny M, Pekna M, Zorec R & Verkhratsky A. (2012). Glial cells in (patho)physiology. *Journal of neurochemistry* **121**, 4-27.
- Paxinos G & Watson C. (1986). *The Rat Brain in Stereotaxic Coordinates*. Academic Press, Sydney.
- Pehek EA, Thompson JT, Eaton RC, Bazzett TJ & Hull EM. (1988). Apomorphine and haloperidol, but not domperidone, affect penile reflexes in rats. *Pharmacology, biochemistry, and behavior* **31**, 201-208.
- Pehek EA, Thompson JT & Hull EM. (1989). The effects of intracranial administration of the dopamine agonist apomorphine on penile reflexes and seminal emission in the rat. *Brain Res* **500**, 325-332.
- Peng CW, Chen JJ, Chang HY, de Groat WC & Cheng CL. (2006). External urethral sphincter activity in a rat model of pudendal nerve injury. *NeuroUrol Urodyn* **25**, 388-396.
- Peng CW, Chen JJ, Cheng CL & Grill WM. (2008). Role of pudendal afferents in voiding efficiency in the rat. *Am J Physiol Regul Integr Comp Physiol* **294**, R660-672.
- Perlmutter JS & Mink JW. (2006). Deep brain stimulation. *Annu Rev Neurosci* **29**, 229-257.

- Reichling DB & Basbaum AI. (1990). Contribution of brainstem GABAergic circuitry to descending antinociceptive controls: I. GABA-immunoreactive projection neurons in the periaqueductal gray and nucleus raphe magnus. *J Comp Neurol* **302**, 370-377.
- Sadananda P, Drake MJ, Paton JF & Pickering AE. (2011). An exploration of the control of micturition using a novel in situ arterially perfused rat preparation. *Front Neurosci* **5**, 62.
- Sakakibara R, Uchiyama T, Yamanishi T & Kishi M. (2010). Genitourinary dysfunction in Parkinson's disease. *Mov Disord* **25**, 2-12.
- Salonen T, Haapalinna A, Heinonen E, Suhonen J & Hervonen A. (1996). Monoamine oxidase B inhibitor selegiline protects young and aged rat peripheral sympathetic neurons against 6-hydroxydopamine-induced neurotoxicity. *Acta neuropathologica* **91**, 466-474.
- Saper CB & Petit CK. (1982). Correspondence of melanin-pigmented neurons in human brain with A1-A14 catecholamine cell groups. *Brain* **105**, 87-101.
- Sasaki M. (2005). Role of Barrington's nucleus in micturition. *J Comp Neurol* **493**, 21-26.
- Sauer H & Oertel WH. (1994). Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat. *Neuroscience* **59**, 401-415.
- Schenberg LC, Bittencourt AS, Sudre EC & Vargas LC. (2001). Modeling panic attacks. *Neurosci Biobehav Rev* **25**, 647-659.
- Schenberg LC, Pova RM, Costa AL, Caldellas AV, Tufik S & Bittencourt AS. (2005). Functional specializations within the tectum defense systems of the rat. *Neurosci Biobehav Rev* **29**, 1279-1298.
- Schmidt F, Yoshimura Y, Ni RX, Kneesel S & Constantinou CE. (2001). Influence of gender on the diurnal variation of urine production and micturition characteristics of the rat. *Neurourol Urodyn* **20**, 287-295.
- Schurch B, Schmid DM & Stohrer M. (2000). Treatment of neurogenic incontinence with botulinum toxin A. *N Engl J Med* **342**, 665.
- Seki N, Karim OM & Mostwin JL. (1992a). Changes in electrical properties of guinea pig smooth muscle membrane by experimental bladder outflow obstruction. *Am J Physiol* **262**, F885-891.

- Seki N, Karim OM & Mostwin JL. (1992b). The effect of experimental urethral obstruction and its reversal on changes in passive electrical properties of detrusor muscle. *J Urol* **148**, 1957-1961.
- Sengupta JN & Gebhart GF. (1994). Mechanosensitive properties of pelvic nerve afferent fibers innervating the urinary bladder of the rat. *J Neurophysiol* **72**, 2420-2430.
- Shaikh MB, Barrett JA & Siegel A. (1987). The pathways mediating affective defense and quiet biting attack behavior from the midbrain central gray of the cat: an autoradiographic study. *Brain Res* **437**, 9-25.
- Shaw VE, Keay KA, Ashkan K, Benabid AL & Mitrofanis J. (2010). Dopaminergic cells in the periaqueductal grey matter of MPTP-treated monkeys and mice; patterns of survival and effect of deep brain stimulation and lesion of the subthalamic nucleus. *Parkinsonism Relat Disord* **16**, 338-344.
- Singer C. (1998). Urinary dysfunction in Parkinson's disease. *Clinical neuroscience (New York, NY)* **5**, 78-86.
- Skehan AM, Downie JW & Awad SA. (1993). Control of detrusor stiffness in the chronic decentralized feline bladder. *J Urol* **149**, 1165-1173.
- Smith PP, Deangelis AM & Kuchel GA. (2011). Evidence of central modulation of bladder compliance during filling phase. *Neurourol Urodyn*.
- Soler R, Fullhase C, Santos C & Andersson KE. (2011). Development of bladder dysfunction in a rat model of dopaminergic brain lesion. *Neurourol Urodyn* **30**, 188-193.
- Somogyi GT, Zernova GV, Yoshiyama M, Yamamoto T & de Groat WC. (1998). Frequency dependence of muscarinic facilitation of transmitter release in urinary bladder strips from neurally intact or chronic spinal cord transected rats. *Br J Pharmacol* **125**, 241-246.
- Stafford SA & Coote JH. (2006). Activation of D2-like receptors induces sympathetic climactic-like responses in male and female anaesthetised rats. *Br J Pharmacol* **148**, 510-516.
- Steinhauser C & Gallo V. (1996). News on glutamate receptors in glial cells. *Trends in neurosciences* **19**, 339-345.
- Stone E, Coote JH, Allard J & Lovick TA. (2011). GABAergic control of micturition within the periaqueductal grey matter of the male rat. *J Physiol* **589**, 2065-2078.

- Streng T, Santti R, Andersson KE & Talo A. (2004). The role of the rhabdosphincter in female rat voiding. *BJU international* **94**, 138-142.
- Streng T, Santti R & Talo A. (2002). Similarities and differences in female and male rat voiding. *Neurourol Urodyn* **21**, 136-141.
- Su X, Riedel ES, Leon LA & Laping NJ. (2008). Pharmacologic evaluation of pressor and visceromotor reflex responses to bladder distension. *Neurourol Urodyn* **27**, 249-253.
- Sugaya K, Roppolo JR, Yoshimura N, Card JP & de Groat WC. (1997). The central neural pathways involved in micturition in the neonatal rat as revealed by the injection of pseudorabies virus into the urinary bladder. *Neurosci Lett* **223**, 197-200.
- Tai C, Wang J, Jin T, Wang P, Kim SG, Roppolo JR & de Groat WC. (2009). Brain switch for reflex micturition control detected by fMRI in rats. *J Neurophysiol* **102**, 2719-2730.
- Tai CH, Boraud T, Bezard E, Bioulac B, Gross C & Benazzouz A. (2003). Electrophysiological and metabolic evidence that high-frequency stimulation of the subthalamic nucleus bridges neuronal activity in the subthalamic nucleus and the substantia nigra reticulata. *Faseb J* **17**, 1820-1830.
- Tanagho EA, Schmidt RA & Orvis BR. (1989). Neural stimulation for control of voiding dysfunction: a preliminary report in 22 patients with serious neuropathic voiding disorders. *J Urol* **142**, 340-345.
- Taniguchi N, Miyata M, Yachiku S, Kaneko S, Yamaguchi S & Numata A. (2002). A study of micturition inducing sites in the periaqueductal gray of the mesencephalon. *J Urol* **168**, 1626-1631.
- Tawfik VL, Chang SY, Hitti FL, Roberts DW, Leiter JC, Jovanovic S & Lee KH. (2010). Deep brain stimulation results in local glutamate and adenosine release: investigation into the role of astrocytes. *Neurosurgery* **67**, 367-375.
- Teichberg VI. (1991). Glial glutamate receptors: likely actors in brain signaling. *Faseb J* **5**, 3086-3091.
- Temml C, Haidinger G, Schmidbauer J, Schatzl G & Madersbacher S. (2000). Urinary incontinence in both sexes: prevalence rates and impact on quality of life and sexual life. *Neurourol Urodyn* **19**, 259-271.

- Trussell LO & Jackson MB. (1985). Adenosine-activated potassium conductance in cultured striatal neurons. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 4857-4861.
- Turner DA, Shaw C, McGrother CW, Dallosso HM & Cooper NJ. (2004). The cost of clinically significant urinary storage symptoms for community dwelling adults in the UK. *BJU international* **93**, 1246-1252.
- Uchiyama T, Sakakibara R, Yoshiyama M, Yamamoto T, Ito T, Liu Z, Yamaguchi C, Awa Y, Yano HM, Yanagisawa M, Yamanishi T, Hattori T & Kuwabara S. (2009). Biphasic effect of apomorphine, an anti-parkinsonian drug, on bladder function in rats. *Neuroscience* **162**, 1333-1338.
- Ungerstedt U. (1968). 6-Hydroxy-dopamine induced degeneration of central monoamine neurons. *European journal of pharmacology* **5**, 107-110.
- Van Asselt E, Groen J & Van Mastrigt R. (1995). A comparative study of voiding in rat and guinea pig: simultaneous measurement of flow rate and pressure. *Am J Physiol* **269**, R98-103.
- Van Kerrebroeck PE & Marcelissen TA. (2011). Sacral neuromodulation for lower urinary tract dysfunction. *World J Urol.*
- Vargas LC, Marques TA & Schenberg LC. (2000). Micturition and defensive behaviors are controlled by distinct neural networks within the dorsal periaqueductal gray and deep gray layer of the superior colliculus of the rat. *Neurosci Lett* **280**, 45-48.
- Vedam-Mai V, van Battum EY, Kamphuis W, Feenstra MG, Denys D, Reynolds BA, Okun MS & Hol EM. (2012). Deep brain stimulation and the role of astrocytes. *Mol Psychiatry* **17**, 124-131, 115.
- Vizzard MA, Erickson VL, Card JP, Roppolo JR & de Groat WC. (1995). Transneuronal labeling of neurons in the adult rat brainstem and spinal cord after injection of pseudorabies virus into the urethra. *J Comp Neurol* **355**, 629-640.
- Ward J, Daly MB & Wood LM. (1995). Urinary bladder distension: its effects on carotid baroreceptor reflex left ventricular inotropic response in the dog. *J Physiol* **489 (Pt 3)**, 857-868.
- Weaver LC. (1985). Organization of sympathetic responses to distension of the urinary bladder. *American Journal of Physiology* **248**, R236-R240.
- Yaguchi H, Soma H, Miyazaki Y, Tashiro J, Yabe I, Kikuchi S, Sasaki H, Kakizaki H, Moriwaka F & Tashiro K. (2004). A case of acute urinary retention caused by

periaqueductal grey lesion. *Journal of neurology, neurosurgery, and psychiatry* **75**, 1202-1203.

Yamauchi T, Hori T & Takahashi T. (2000). Presynaptic inhibition by muscimol through GABA_B receptors. *Eur J Neurosci* **12**, 3433-3436.

Yonezawa A, Yoshizumi M, Ise SN, Watanabe C, Mizoguchi H, Furukawa K, Tsuru H, Kimura Y, Kawatani M & Sakurada S. (2009). Synergistic actions of apomorphine and m-chlorophenylpiperazine on ejaculation, but not penile erection in rats. *Biomed Res* **30**, 71-78.

Yoshimura N, Kuno S, Chancellor MB, De Groat WC & Seki S. (2003). Dopaminergic mechanisms underlying bladder hyperactivity in rats with a unilateral 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway. *Br J Pharmacol* **139**, 1425-1432.

Zhang YH, Hosono T, Yanase-Fujiwara M, Chen XM & Kanosue K. (1997). Effect of midbrain stimulations on thermoregulatory vasomotor responses in rats. *J Physiol* **503 (Pt 1)**, 177-186.

APPENDICES

APPENDIX A: CALCULATION OF TOTAL EMG ACTIVITY DURING THE INTER-VOID INTERVAL

Nb. The sampling frequency must remain constant during and between experiments in order to make mathematical comparisons of the EMG activity using the following method.

1. Sum the total rectified EMG signal over 60 s during the inter-void interval. To do this:
 - a. Highlight relevant section in Chart
 - b. Save as .txt file
 - c. Copy/paste values into Excel
 - d. Rectify the data by selecting any negative values (ctl+f) and replacing them with positive.
 - e. Sum the first 6000 values to calculate the total EMG activity in 60s (6000 because the sampling rate for these experiments was set at 100Hz, and want 60s period counted (100x60=6000))
2. Divide the total rectified EMG activity achieved by summing the first 6000 values by 5000. This is to take account for the x5000 gain on the NeuroLog amplifiers. This will give the total EMG activity recorded from the EUS in mV min.
3. Divide by 60 to convert units to seconds.
4. (If necessary multiply by 1000 and use $\mu\text{V s}$)

Example:

EXP ID: ES86 1.1 pre musc

1. Sum of EMG over 60s (6000 inter-void values) = 97.91 mV. min
2. Divide by 5000 to take into account gain on NeuroLog amplifier = 0.0196 mV. min
3. Divide by 60 to convert units to seconds = 0.00033 mV.s
4. Multiply by 1000 to convert to $\mu\text{V. s}$ = 0.33 $\mu\text{V. s}$

APPENDIX B: 0.1M PHOSPHATE BUFFER (PB) RECIPE

1. Dissolve 3.9 g sodium dihydrogen orthophosphate in 400 ml distilled water
2. Add 0.8 g sodium hydroxide and dissolve
3. Make up to 500 ml with distilled water
4. pH to 7.4 with sodium hydroxide

(All solid ingredients from Fisher Scientific, Loughborough, UK)

APPENDIX C: 0.1M PHOSPHATE BUFFERED SALINE (PBS) RECIPE

1. Dissolve 0.195g sodium dihydrogen orthophosphate in 400ml distilled water.
2. Add 0.535g disodium hydrogen orthophosphate and dissolve
3. Add 4.25g sodium chloride and dissolve
4. Make up to 500ml with distilled water
5. pH to 7.4 with sodium hydroxide

(All solid ingredients from Fisher Scientific, Loughborough, UK)

PUBLISHED PAPERS RELATED TO THIS THESIS

(Un-numbered pages)