An investigation into the effects of acute and chronic blockade of 5-HT_{1A} receptors involved in the control of micturition.

Thesis submitted for the

Degree of Doctor of Philosophy

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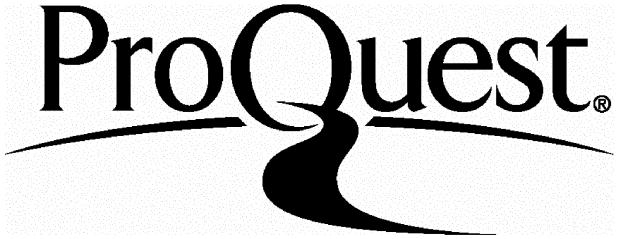

Abstract

The storage and periodic elimination of urine (micturition) is dependent upon a complex neural co-ordination of activities in the bladder and urethra. 5-HT_{1A} receptors have been suggested to be involved in the central control of micturition, and considered a potential therapeutic target, since 5-HT_{1A} receptor antagonists acutely inhibit micturition in anaesthetized rats. However, recent studies examining 5-HT-containing neuronal control in the field of affective disorders, have shown 5-HT_{1A} receptors to be highly labile and easily desensitized, suggesting that the long-term effects of chronic administration of 5-HT receptor ligands may differ from those observed after acute administration. Therefore, the present studies initially characterized the acute and chronic effects of 5-HT_{1A} receptor antagonists on micturition in urethane-anaesthetized and conscious female rats. Further characterisation of the effects of chronic administration of a 5-HT_{1A} receptor antagonist on supraspinal 5-HT control was obtained using radiotelemetry and quantitative autoradiography studies.

The 5-HT_{1A} receptor antagonist, WAY-100635, significantly suppressed micturition in a dose dependent manner after acute intracerebroventricular and intrathecal (L6/S1) administration in anaesthetized rats, thus confirming the involvement of 5-HT_{1A} receptors in the control of micturition, at both supraspinal and lumbosacral spinal levels. A similar suppression of micturition was observed in conscious rats after acute intravenous (i.v.) administration (3 h infusion) of WAY-100635 and robalzotan (NAD-299, a structurally distinct 5-HT_{1A} receptor antagonist). However, by the fourth day of chronic i.v. administration (via osmotic minipump) of WAY-100635 or robalzotan, neither 5-HT_{1A} receptor antagonist had any significant effect on conscious micturition measurements. This observation of a lack of effect of 5-HT_{1A} receptor antagonists on micturition after chronic treatment was confirmed using anaesthetized cystometry studies in chronically treated rats, thus suggesting that these animals had developed 'tolerance' to 5-HT_{1A} receptor antagonists after chronic administration. Further characterization of the onset of the 'tolerance' phenomenon was obtained in rats using radiotelemetry measurements of bladder activity and a subcutaneous injectable pump for i.v. administration of WAY-100635, which showed that 'tolerance' occurs within 24 hours of administration.

Since supraspinal 5-HT receptors, and 5-HT_{1A} receptors in particular, are highly labile, it is possible that the observed 'tolerance' phenomenon resulted from a change in the density of these receptors after chronic administration. This was investigated using quantitative autoradiography, which showed that chronic WAY-100635 administration caused almost a 50% increase in the level of [^{3}H]WAY-100635 binding in various brain regions including the raphé nuclei. 5-HT_{1A} receptor levels were still raised after a 6-day wash-out period, thus confirming this 'tolerance' phenomenon to be long lasting. Changes in the levels of 5-$HT_{1B/1D}$ receptors and the 5-HT transporter (5-HTT) were also observed, thus highlighting the complex level of interaction between serotonin receptors involved in supraspinal 5-HT-containing neuronal control.

These studies indicate that the chronic administration of 5-HT_{1A} receptor antagonists results in the onset of tolerance towards the ligands, which is characterized by an upregulation of 5-HT_{1A} receptors in various brain regions. Furthermore, additional changes in 5-$HT_{1B/1D}$ receptors and 5-HTT levels, suggest a high level of complexity in supraspinal 5-HT control. Thus, it appears that treating overactive bladder with long-term administration of 5-HT_{1A} receptor antagonists is unlikely to be clinically efficacious, and therefore has little therapeutic potential.

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Chapter 1

General Introduction

1.1 – Micturition: neurophysiology and control

The lower urinary tract (LUT) has two main functions, the storage and periodic elimination (micturition) of urine, which are dependent on the complex co-ordination of the activities of the bladder and urethra. The excretory function of these structures is regulated by a complex neural control system in the brain and lumbosacral spinal cord. This dependence on the central nervous system distinguishes the LUT from many physiological systems, such as the cardiovascular system, which are able to maintain a certain level of function even after neural input is eliminated (see de Groat, 1990). In the human, the level of voluntary control from higher centres in the brain enables the LUT to temporarily store urine until a socially convenient time to micturate, thus distinguishing the bladder and urethra, and other organs with similar sphincter control, such as the rectum and anus, as unique from other visceral structures (see de Groat *et al.*, 1993). In contrast to the tonic patterns of activity in most autonomic pathways, such as the cardiovascular organs, the neural circuits controlling micturition exhibit switch-like or phasic patterns of activity (see de Groat, 1990).

The functions of the LUT are dependent on the activity of smooth and striated muscles in the urinary bladder, urethra and external urethral sphincter (EUS) (see de Groat *et al.*, 2001), which are effected by a complex integration of parasympathetic, sympathetic, somatic and sensory systems (see de Groat *et al.*, 1993). Neural control of the bladder and urethra is further complicated by the presence of a substantial number of neurotransmitters including 5-hydroxytryptamine (5-HT; serotonin), γ-aminobutyric acid (GABA), acetylcholine (ACH), noradrenaline (NA), dopamine (DA), adenosine triphosphate (ATP), nitric oxide (NO) and neuropeptides. Disturbances at any level of

the complex relationship between the nervous system and the bladder and urethra results in disorders of normal LUT function.

1.1.1 – Functional changes in the bladder and urethra during voiding and urine storage

The storage and elimination of urine are dependent on the complex co-ordination of activities of the bladder and urethra. The bladder acts as a reservoir during storage and as a pump during voiding, whilst the urethra provides the resistance to maintain continence and also aids complete expulsion of urine from the bladder (see de Groat *et al.*, 1993). During urine storage, the bladder neck and proximal urethra are closed, and the bladder detrusor smooth muscle is quiescent, thus allowing intravesical pressure to remain low despite increasing volumes of urine (see Figure 1.1). Involuntary voiding occurs in animal species when the bladder capacity reaches its micturition threshold, however voiding can also be initiated voluntarily in the adult human. Micturition is characterized by an initial drop in urethral pressure, followed by a large detrusor contraction, which is accompanied by an opening of the bladder neck and urethra (see Figure 1.1). These functional changes raise intravesical pressure, induce urine flow and enable efficient voiding which, in rats and dogs, is also aided by high frequency bursting of the striated muscle of the urethra (Kakizaki *et al.*, 1997).

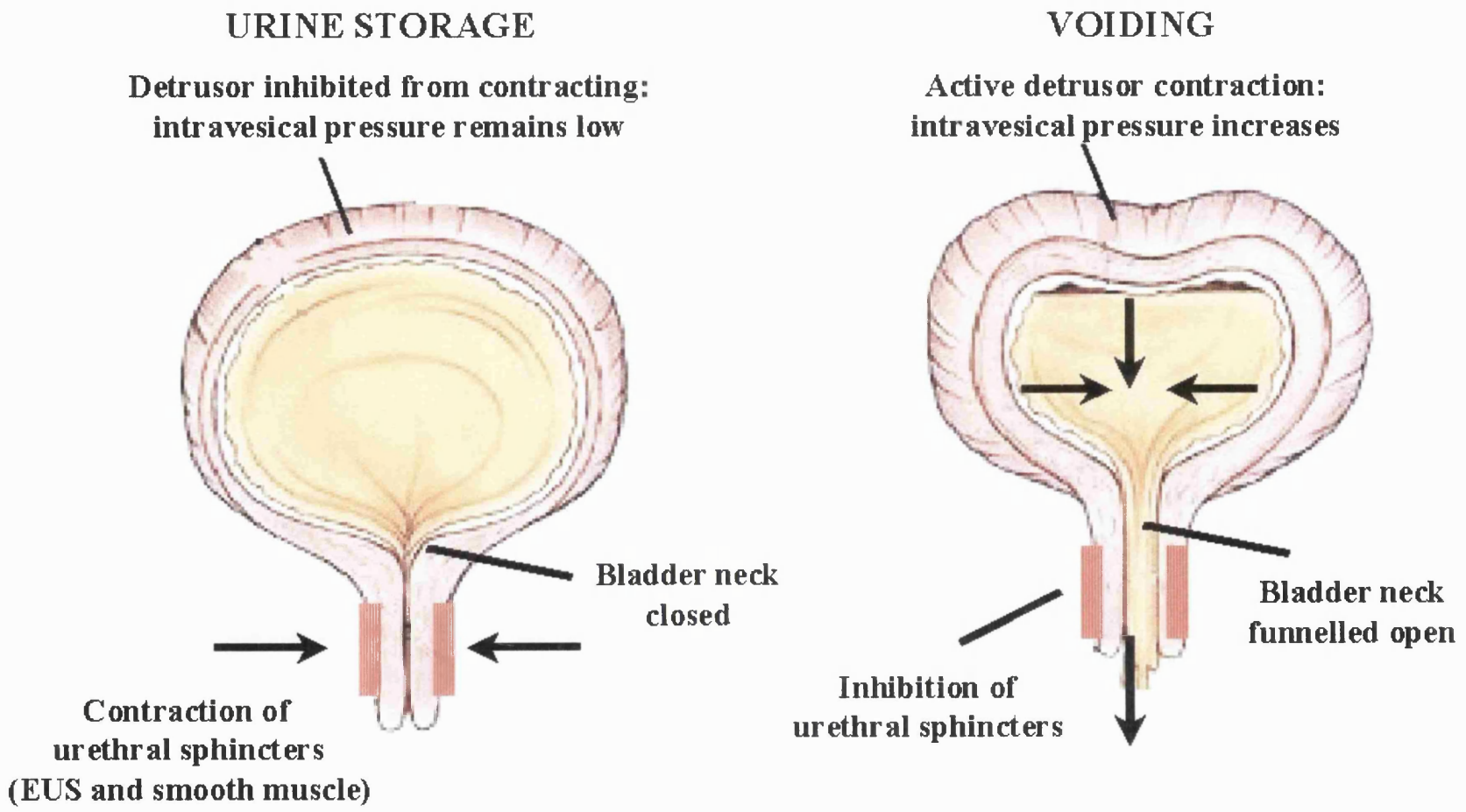


Figure 1.1 – Functional changes associtated with the bladder and urthra during urine storage and voiding. (Modified from Wibberley, 2000).

1.1.2 – Anatomy and innervation of the LUT

The two functional units of the LUT are the urinary bladder and the outlet consisting of the bladder neck and the smooth and striated muscles of the urethra. The bladder receives urine from the kidneys via the ureters, which enter the dorsal wall of the lower region of the bladder. The bladder narrows just below these vesico-ureteric junctions to form the bladder neck, from which the tubular urethra projects (see Blandy *et al.*, 1996). The bladder is divided into two distinct regions; the detrusor forms the smooth muscle of the fundus of the bladder and is highly distensible. In contrast, the trigone is a triangular area located at the bladder neck which does not change in size during bladder filling and is thought to prevent reflux of urine into the ureters when the pressure rises in the bladder during voiding (Shafik, 1998). In all mammalian species, the smooth muscle of the bladder consists of three layers, inner and outer longitudinal and middle circular which interlace so discrete layers are not discernible (Gosling *et al.*, 1975), therefore contraction of this muscle bundle leads to a reduction in all dimensions of the bladder lumen during micturition (Gosling, 1979). The bladder has the ability to maintain a remarkably low intravesical pressure during filling (Coolsaet, 1985), thus enabling the urethral pressure to remain higher than the bladder, and allowing the maintenance of continence until the micturition threshold is reached. This bladder compliance is thought to be due to intrinsic properties of the bladder wall and independent of neural input (Finkbeiner, 1999).

In the bladder neck, further circular smooth muscle extends distally to surround the proximal urethra completely. The urethra also consists of an inner longitudinal muscle layer, which is continuous with the inner longitudinal layer of the bladder. It has been

proposed that the circular smooth muscle of the bladder neck and the proximal urethra form an internal sphincter (Krane *et al.*, 1973). In all species, the urethra is surrounded by striated muscle (Thind, 1995) whose fibres have a circular orientation and is termed the external urethral sphincter (EUS; see Lincoln *et al.*, 1993). The EUS is more prominent in males than in females in all species and the EUS can maintain continence in males even when the internal sphincter has been damaged (Turner-Warwick, 1974). The striated muscle is thought to play a vital role in the maintenance of urethral tone at rest, and also during dynamic events such as physical activity and coughing (Thind, 1995).

The bladder and urethra are controlled by three sets of peripheral nerves (parasympathetic, sympathetic and somatic), which emerge from the sacral and thoracolumbar levels of the spinal cord (see de Groat *et al.*, 1993); (see de Groat, 1997). The sacral parasympathetic outflow (pelvic nerves) provides the major excitatory input (cholinergic and purinergic) to the bladder and also an inhibitory input (nitrergic) to the urethra (see de Groat *et al.*, 2001). Thoracolumbar sympathetic outflow (hypogastric nerves), which is mediated via noradrenaline, elicits a variety of responses in the LUT including an excitation of the bladder neck and urethra, modulation of cholinergic transmission in bladder parasympathetic ganglia (both facilitatory and inhibitory) and, in some species, inhibition of detrusor smooth muscle (de Groat, 1990). Lumbosacral efferent pathways in the pudendal nerves provide a cholinergic excitatory input to the EUS.

Afferent axons innervating the LUT are present in three sets of nerves (De Groat, 1986). The most important afferents for initiating micturition are those passing in the pelvic nerves to the sacral spinal cord (Kuru, 1965). These afferents consist of small myelinated

(Aδ) (45% in rats) and unmyelinated (C) fibres (55% in rats), which convey impulses from tension and volume receptors and nociceptors in the bladder wall (Sengupta *et al.*, 1994). Electrophysiological studies in cats have shown that Aδ bladder afferents respond to both passive distension and active contraction of the bladder in a graduated manner, and are therefore thought to trigger sensations of bladder filling (Morrison, 1999). C-fibre bladder afferents have very high thresholds and usually do not respond to even high levels of intravesical pressure (Habler *et al.*, 1990), although activity in these afferents is observed after chemical irritation of the bladder mucosa and cold temperatures (Fall *et al.*, 1990) therefore suggesting these afferents are involved in the signalling of noxious events in the LUT. Afferent nerves are also located in the serosal and muscle layers, as well as the epithelial lining (the urothelium) of the bladder, where they respond to changes in the composition of the urine (see de Groat, 1990). The bladder afferents carry information from 'in series' tension receptors (i.e. they increase their rate of discharge in response to bladder distension during filling) in the detrusor muscle to the sacral spinal cord neurones, and hence provide the sensory input which controls the activities of the bladder, urethra and EUS (Habler *et al.*, 1990; Habler *et al.*, 1993). Barrington (1931) showed that autonomic nerves (pudendal and pelvic) arising from the urethra also contain afferents responsible for reflexes which modify bladder filling and emptying (Barrington, 1931), and recently it has been demonstrated that saline flow through the urethra also increases the frequency of reflex-induced bladder contractions in the anaesthetized female rat (Jung *et al.*, 1999) and conscious ewe (Robain *et al.*, 2001), thus suggesting the presence of a urethrovesical reflex. This may have clinical relevance and induce bladder instability in patients with urinary incontinence (Hindmarsh *et al.*, 1983).

1.1.3 - Central influences and reflex mechanisms controlling the lower urinary tract.

The central pathways controlling the LUT are organized as simple on-off switching circuits, which maintain a reciprocal relationship between the urinary bladder and the urethral outlet. The main reflex components of these circuits are shown in Table 1.1. Urine storage mechanisms are mediated by sympathetic and somatic inputs (tonically active during bladder filling) to the urethral outlet, and are dependent on spinal reflex circuits initiated by the low level of bladder afferent activity during filling (see Edvardsen, 1968). Hence, this vesicosympathetic reflex acts as a negative feedback mechanism, whereby an increase in bladder pressure triggers an increase in inhibitory input to the bladder thus allowing the bladder to accommodate larger volumes of urine (see de Groat *et al.*, 1993). This spinal reflex circuit is also known as the 'guarding' reflex. During bladder filling, and hence storage, the EUS electromyogram (EMG) also increases due to an increase in efferent firing in the pudendal nerve. This causes an increase in outlet resistance and hence contributes to the maintenance of urinary continence.

The storage phase of the bladder can be switched to the voiding phase either involuntarily (reflexly) or voluntarily. Reflex voiding is observed in anaesthetized animals and the human infant, and occurs when the volume of urine in the bladder exceeds the micturition threshold. This causes increased afferent firing from the tension receptors in the bladder wall which reverse the pattern of efferent outflow, producing firing in the sacral parasympathetic efferent pathways (hence detrusor contraction) and inhibition of sympathetic and somatic pathways (hence urethral relaxation).

Afferent Pathway	*Efferent pathway*	*Central pathway*
Urine Storage Low level vesical afferent activity (pelvic nerve)	1. External sphincter contractions (somatic nerves) 2. Internal sphincter contraction (sympathetic nerves) 3. Detrusor inhibition (symapthetic nerves) 4. Ganglionic inhibition (sympathetic nerves) 5. Sacral parasympathetic outflow inactive	Spinal reflexes (also known as the "guarding" reflex)
Micturition High level vesical afferent activity (pelvic nerve)	1. Inhibition of EUS activity 2. Inhibition of sympathetic outflow 3. Activation of parasympathetic outflow	Spinobulbospinal reflexes

Table 1.1 - Reflexes involved in urine storage and micturition. (Modified from De Groat *et al.*, 1984).

The 'guarding' reflexes for urine storage are organized at the spinal level, whereas the parasympathetic outflow to the detrusor has a more complicated central control involving spinal and spinobulbospinal pathways (see de Groat, 1990). Thus, during micturition, inputs from higher centers in the brain inhibit spinal storage reflexes and activate the parasympathetic excitatory outflow to the bladder. This activation of bladder parasympathetic nerves is mediated by a spinobulbospinal pathway, which passes through a co-ordinating centre in the rostral brainstem known as the pontine micturition centre (PMC; also known as Barrington's nucleus; (Barrington, 1921; Barrington, 1925; Barrington, 1928). The PMC essentially acts as an 'on-off' switch that is activated by a critical level of afferent activity from the tension receptors in the bladder wall (De Groat, 1975), and modulates inhibitory and excitatory influences from areas of the brain rostral to the pons.

The PMC is located in the region of the dorsolateral pons in the cat (De Groat, 1975) and rat (Satoh *et al.*, 1978). Brain imaging studies in humans during micturition have also provided evidence of a comparable region in humans, with its location being similar to that of the cat (Blok *et al.*, 1997). The importance of the PMC in co-ordinating urine storage and release is shown by the observation that bilateral lesions of the PMC in both cats (Barrington, 1925; Griffiths *et al.*, 1990) and rats (Satoh *et al.*, 1978) abolishes micturition immediately followed by the slow development of involuntary, uncoordinated spinal voiding mechanisms. The PMC receives pelvic visceral information via projections from the lumbosacral spinal cord (Ding *et al.*, 1997), however, these projections are very weak, thus suggesting that another brainstem region may serve as a 'relay centre' for the transmission of afferent input to the pons. This 'relay' region is thought to be the mesencephalic periaqueductal grey (PAG) as it is the only caudal

brainstem structure known to project specifically to the PMC (Blok *et al.*, 1994). Further evidence for this possible role of the PAG is that stimulation of the PAG in the rat facilitates bladder reflexes and reduces bladder capacity (Kruse *et al.*, 1990), and interruption of some of the fibres between the PAG and PMC results in a low capacity bladder (Tang *et al.*, 1956). Ding *et al* (1997) have shown in the rat that the sensory neurones in the lumbosacral cord project directly onto neurones in the PMC, and it is therefore possible that both the direct and indirect (via the PAG) pathways for afferent information to reach the PMC are operational (see Blok *et al.*, 1999a).

Upon activation, the PMC sends excitatory projections to parasympathetic preganglionic motorneurones in the sacral spinal cord, which then promote detrusor contractions and urethral relaxation (Ding *et al.*, 1997; Blok *et al.*, 1999b). During micturition projections from the PMC to neurones in the sacral dorsal grey commissure (DGC) are also activated and these inhibit urethral sphincter motorneurones and produce a relaxation of the EUS (Blok *et al.*, 1998; see Figure 1.2).

The pontine storage center (PSC) is another group of neurones in the pons, which are located more ventrally and laterally in the pontine tegmentum than the PMC. The PSC sends excitatory projections to the motorneurones of the urethral sphincter in Onuf's nucleus in the sacral spinal cord (Holstege, 1996). Electrical stimulation of this region elicits EUS contractions and inhibits bladder activity (Kruse *et al.*, 1990) thus promoting urine storage. Furthermore, bilateral destruction of the PSC induces bladder hyperactivity and urinary incontinence (Griffiths *et al.*, 1990). It has been shown that the PSC has a continuous excitatory effect on Onuf's nucleus and the EUS, which must therefore be overrided during voiding by the excitatory projections from the PMC to

parasympathetic preganglionic motorneurones in the sacral spinal cord (see Blok *et al.*, 1999a). Recent studies have shown that the PMC and PSC are not interconnected directly, suggesting that these regions represent separate functional systems, which act independently (Blok *et al.*, 1999b).

Various other supraspinal sites, such as the cerebral cortex, diencephalon and medullary centres, also influence the activities of the bladder and urethra. The complex control of voluntary micturition is thought to be highly dependent on these sites. For example, the forebrain is not essential for the basic micturition reflex, but clinical observations suggest that it determines the start of micturition (Andrew *et al.*, 1996), and in the cat stimulation of forebrain structures such as the anterior cingulate gyrus, amygdala and the preoptic area of the hypothalamus elicit bladder contractions (see Blok *et al.*, 1999a). Lesion studies have revealed an inhibitory role for the cerebral cortex (see de Groat *et al.*, 1993), since lesions in this region cause an indirect increase in bladder activity due to the removal of the cortical inhibitory control over the anterior hypothalamic area which normally provides an excitatory input to the micturition centres in the pons. It appears that this anterior hypothalamic area sends direct projections to the PMC, with the overall hypothalamic control being facilitatory (Tang *et al.*, 1956). Medullary centres have been implicated in both inhibitory and facilitatory modulation of the micturition reflex pathway. For example, electrical stimulation of the nucleus raphé magnus (NRM)

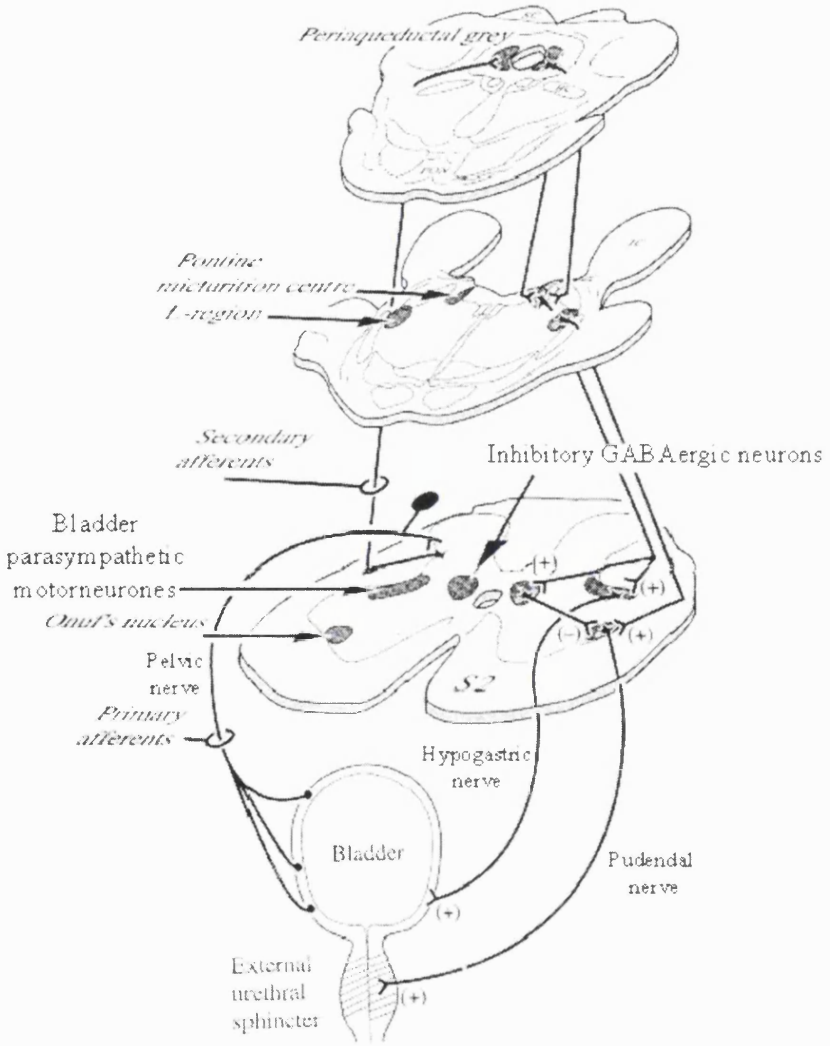


Figure 1. 2 – Schematic overview of the central pathways involved in the control of micturition. (Modified from Blok *et al.*, 2002)

in the cat inhibits reflex bladder contractions and reflex firing in the sacral efferent pathways to the bladder (McMahon *et al.*, 1982; Morrison *et al.*, 1986). Stimulation of the raphé also inhibits the firing of the spinal dorsal horn neurones activated by bladder afferents (Lumb, 1986a). This modulation of bladder sensory mechanisms in the spinal cord explains the inhibitory effect of raphé stimulation on bladder reflexes. In addition, physiological bladder distension in the rat and cat activates raphé neurones (Lumb *et al.*, 1984; Lumb, 1986b), therefore it is possible that this inhibitory raphé-spinal pathway is tonically active during bladder filling and suppresses micturition at the spinal level until afferent activity is sufficient to initiate micturition.

1.1.4 – Autonomic innervation of the bladder and urethra

1.1.4.1 – Parasympathetic nervous system: innervation and function

Parasympathetic innervation provides the main excitatory input to the bladder to evoke a contraction during micturition (Barrington, 1915; Barrington, 1931; Barrington, 1941; Elmer, 1975; Theobald, 1986), and the efferent pathways concerned with this response run in the pelvic nerves. The pelvic nerves originate from the sacral parasympathetic nucleus, which is located in the intermediolateral grey matter of the spinal cord. Parasympathetic preganglionic neurones leave the spinal cord and enter the pelvic plexus, from which postganglionics emerge to innervate the LUT. However, the pelvic plexus is not just considered to function merely as a relay station, but is thought to actually participate in the complex regulation of neural activities responsible for effecting co-ordinated activities in the bladder and urethra during urine storage and voiding (Dail, 1996; Keast, 1999). Species variation exists with regard to the lumbosacral segments from which preganglionic parasympathetic neurones arise (Table 1.2). In the rat, the

sacral parasympathetic nucleus is localised to spinal cord segments L6-S1 (Fletcher *et al.*, 1978; Hancock *et al.*, 1979). However, it has been suggested that differences exist amongst strains of rat with respect to the lumbosacral levels from which parasympathetic preganglionic neurones arise, originating in the S1 and S2 spinal segments of the Wistar rat, and the L6 and S1 segments in the Sprague Dawley rat (Pascual *et al.*, 1989), although more recently this has been disputed (Birder *et al.*, 1992).

The pelvic nerves also convey parasympathetic input to the urethra, where the parasympathetic nervous system has important roles in urethral function during micturition. Barrington was the first to demonstrate parasympathetic function in the urethra with his extensive work with cats, when he showed that bladder distension caused a relaxation of the urethra, and that this response involved a pelvic nerve efferent pathway (Barrington, 1931; Barrington, 1941). These findings have been corroborated by other investigators (McGuire *et al.*, 1978; Slack *et al.*, 1983; Jung *et al.*, 1999; Robain *et al.*, 2001; Shafik *et al.*, 2003), and stimulation of the parasympathetic spinal roots has also been shown to evoke urethral relaxations in the anaesthetized rat (Fraser *et al.*, 1995) and in man (Van Kerrebroeck *et al.*, 1991).

Species	*Afferent pathway*			*Efferent pathway*		
	Pelvic	***Hypogastric***	***Pudendal***	***Pelvic***	***Hypogastric***	***Pudendal***
Rat	L6-S1 [a]	T13-L3 [d]	L5-L6 [g]	L1-L6 [j]	L1-L2 [m]	L5-L6 [p]
Cat	S1-S3 [b]	L2-L5 [e]	S1-S2 [h]	S1-S3 [k]	L3-L5 [n]	S1-S2 [q]
Human	S2-S4 [c]	L3-S1 [f]	S2-S3 [i]	S2-S4 [l]	L1-L4 [o]	- *

Table 1.2 - The spinal cord segment origin of afferent and efferent neurones innervating the bladder and urethra in the cat, rat and human. (Taken from Wibberley, 2000); [a] (Fletcher *et al.*, 1978); [b] (Morrison, 1987); [c] (Juenemann *et al.*, 1988); [d] (Nadelhaft *et al.*, 1987); [e] (Morgan *et al.*, 1986); [f] (Steers, 1994); [g] (McKenna *et al.*, 1986); [h] (Thor *et al.*, 1989); [l] (Schroder, 1981); [j] (Birder *et al.*, 1991); [k] (Morgan *et al.*, 1981); [l] (White, 1943); [m] (Nadelhaft *et al.*, 1991); [n] (Applebaum *et al.*, 1980); [o] (Janig *et al.*, 1986); [p] (Hulsebosch *et al.*, 1982); [q] (Thor *et al.*, 1989). * To the author's knowledge, there are no studies regarding the spinal cord origin of pudendal afferents in man.

1.1.4.2 – Sympathetic nervous system: innervation and function

The sympathetic supply to the bladder and urethra arises from the intermediolateral nuclei of the lower thoracic and upper lumbar segments of the spinal cord in all species (Table 1.2). Preganglionic sympathetic fibres travel in the splanchnic nerves either to the inferior mesenteric ganglia, or pass caudally in the paravertebral chains to the level of the lumbosacral sympathetic chain ganglia. The hypogastric nerves arise from the inferior mesenteric ganglia, and carry a mixture of both preganglionic and postganglionic sympathetic nerve fibres to the bladder and urethra. From the sympathetic chain, sympathetic fibres join preganglionic parasympathetic axons in the pelvic nerve (De Groat *et al.*, 1980). Therefore, the pelvic nerve cannot be described as exclusively parasympathetic. Indeed, in the cat it has been estimated that there are at least twice as many sympathetic postganglionic fibres as parasympathetic preganglionic fibres in the pelvic nerve (Kuo *et al.*, 1984). In the rat, sympathetic axons represent approximately 25% of the efferent fibres in the pelvic nerve (Hulsebosch *et al.*, 1982). Therefore, sympathetic axons reach the pelvic plexus via both the hypogastric and pelvic nerves. Sympathetic neurones in the pelvic ganglia are in close proximity to their target organs, distinguishing these neurones from 'classic' sympathetic noradrenergic neurones, and are therefore often termed 'short adrenergic neurones' (see Keast, 1999).

The thorocolumbar sympathetic pathways elicit a variety of responses in the LUT including: (1) inhibition of detrusor muscle activity, (2) excitation of the bladder base and urethra and (3) modulation of cholinergic transmission in bladder parasympathetic ganglia (see de Groat *et al.*, 1993). While not essential for the storage or release of urine, in many species these sympathetic pathways make a significant contribution to the neural

control of bladder capacity and outlet resistance during the storage phase of bladder function. Studies have shown that sympathetic input to the LUT is tonically active during bladder filling (Edvardsen, 1968; De Groat *et al.*, 1972a; Floyd *et al.*, 1982; Satchell *et al.*, 1988; Boczek-Funcke *et al.*, 1990), and surgical or pharmacological blockade of the sympathetic pathways can reduce urethral resistance, reduce bladder capacity, reduce bladder wall compliance and increase the frequency and amplitude of bladder contractions (Edvardsen, 1968; Edvarsen, 1968a; Edvarsen, 1968b; Maggi *et al.*, 1983; Nishizawa *et al.*, 1985).

The role of the sympathetic nervous system in the control of the bladder was originally the subject of much debate since many contrasting observations have been obtained. For example, the observation that an intact sympathetic supply is not a requirement for micturition to take place (De Groat *et al.*, 1984), has questioned whether the sympathetic nervous system possesses any essential functions in the LUT. Similarly, sympathectomy and operations that affect sympathetic ganglia rarely cause a lasting effect on the micturition of an individual (Nordling *et al.*, 1980). Conversely, side effects such as urinary retention caused by sympathomimetics (e.g. ephedrine for asthma) or urinary incontinence following administration of sympatholytics (e.g. prazosin for hypertension) are frequently observed (Bradley *et al.*, 1982). Now it is generally accepted that the sympathetic nervous system has an inhibitory physiological role in the control of the bladder. These inhibitory effects are evoked following activation of bladder afferents in the pelvic nerve in response to bladder distension (De Groat *et al.*, 1972a; de Groat *et al.*, 1976), and are thought to represent an intersegmental spinal 'storage' reflex/negative feedback mechanism whereby elevations in intravesical pressure trigger sympathoinhibitory input to the bladder smooth muscle and pelvic ganglia, to allow

greater urine accommodation in the bladder (see de Groat *et al.*, 1993; Khadra *et al.*, 1995). This sympathetic outflow to the bladder may also be modulated by excitatory inputs from the brain, since it has been demonstrated that electrical stimulation of various supraspinal sites (e.g. ventromedial areas in the hypothalamus) elicits an inhibtion of reflex bladder activity (Gjone, 1966). The importance of the sympathetic nervous system in the control of the bladder in man remains to be established owing to a lack of conclusive experimental evidence. However, the functions of the sympathetic nervous system in healthy humans may differ from those in conditions of bladder dysfunction. After long-term parasympathetic decentralisation, an increase in sympathetic innervation and a change in sympathetic receptor function was observed in both the cat (Sundin *et al.*, 1973; Norlen, 1977) and human (Sundin *et al.*, 1977) bladder, suggesting that the physiology of the sympathetic nervous system may differ in various disease states.

The sympathetic nervous system also plays a role in the control of the bladder outlet. Sympathetic stimulation produces contractile responses of the bladder neck and urethra in the rat (Maggi *et al.*, 1989; Martinez-Pineiro *et al.*, 1992; Kontani *et al.*, 2000), cat (Mcguire *et al.*, 1979; Slack *et al.*, 1983; Kakizaki *et al.*, 1991) and dog (Creed, 1979). This response acts to close the bladder outlet, and is therefore thought to be involved with the maintenance of continence during urine storage in these species. These effects are closely co-ordinated with the functions of the somatic nervous system, which act to increase the activity of the EUS to augment this urethral closure mechanism (see section 1.1.4.3).

There is also evidence to suggest a level of sympathetic regulatory control exerted over the pelvic excitatory pathways to the bladder. For example, transection of the

sympathetic nerves to the bladder in the cat results in an increase of both spontaneous and reflex-evoked bladder contractions (Gjone, 1965; Edvardsen, 1968). Similarly, stimulation of the hypogastric nerve decreases both spontaneous and pelvic nerve-evoked bladder contractions in the anaesthetized cat (De Groat *et al.*, 1972b) and dog (Creed, 1979; Ohtsuka *et al.*, 1980). A site of action for this inhibition is thought to exist at the level of the pelvic ganglia since it has been demonstrated that stimulation of the hypogastric nerve possesses inhibitory effects on transmission in parasympathetic ganglia of the bladder in the cat (De Groat *et al.*, 1971). Moreover, a facilitatory effect of hypogastric nerve stimulation on transmission in these ganglia has also been described, and it has been shown that these contrasting inhibitory and facilitatory responses are mediated by specific adrenergic receptor subtypes (Keast *et al.*, 1990). These authors also suggested that these two actions of the sympathetic nervous system on parasympathetic transmission maybe important during different stages of urine storage or voiding (Keast *et al.*, 1990). Similar observations have also been found in the anaesthetized dog (Bosch *et al.*, 1990) and guinea pig (Dave *et al.*, 1976), and histochemical studies have confirmed the presence of noradrenaline-containing nerve terminals alongside cholinergic ganglion cells in the human pelvic ganglion (Gosling *et al.*, 1977). However, there is no evidence for modulation of pelvic ganglionic synaptic transmission in the rat (Tabatai *et al.*, 1986; Mallory *et al.*, 1989), suggesting that sympathetic regulatory control over pelvic excitatory pathways to the bladder may vary between species.

1.1.4.3 – Somatic nervous system: innervation and function

In all species, the pudendal nerves supply the somatic innervation to the striated muscle of the EUS, although some species variation exists with regard to the spinal cord segments from which somatic motorneurones arise (see Table 1.2). Anatomically, in the rat, somatic motorneurones are contained in dorsolateral and dorsomedial nuclei in spinal cord segments L5-L6 (McKenna *et al.*, 1986), whereas in the human, monkey and cat, these neurones are contained within a single well defined nucleus known as Onuf's nucleus (Onufrowicz, 1899).

The pudendal nerves have been ascribed a storage role, based on findings that pudendal motorneurone firing occurs upon bladder filling to increase urethral striated muscle activity, and therefore increase urethral resistance (Blaivas *et al.*, 1977; see de Groat *et al.*, 1993). This phenomenon, in combination with the previously mentioned sympatho-excitatory input to the urethra via the hypogastric nerve, constitutes the 'guarding' or 'continence' reflex, that is active during bladder filling to help promote continence (see Park *et al.*, 1997). During voiding, pudendal motorneurones in Onuf's nucleus are inhibited, thereby inhibiting the EUS to promote an opening of the bladder outlet. This inhibition is dependent in part on supraspinal mechanisms since in chronic spinal animals and paraplegic patients it is weak or absent (see de Groat, 1994), and stimulation of various supraspinal sites, such as the PSC (see section 1.1.3), elicits EUS contractions and inhibits bladder activity (Kruse *et al.*, 1990) thus promoting urine storage. Furthermore, bilateral destruction of the PSC induces bladder hyperactivity and urinary incontinence (Griffiths *et al.*, 1990).

In rats and dogs, but not humans nor guinea-pigs, the striated muscle of the urethra also exhibits high frequency bursting during voiding that is mediated by the somatic nervous system (Kakizaki *et al.*, 1997). The function of this phasic activity is unclear, although it is possible it may enable the urethra to function like a pump to enhance urine flow along the urethra (Kakizaki *et al.*, 1997), or induces intermittent isometric bladder contractions thus enhancing bladder emptying (Maggi *et al.*, 1986). It has also been suggested that this phasic activity maybe involved in territory marking (Van Asselt *et al.*, 1995) or more recently, that it is purely a side effect of relaxations of the urethra that occur during micturition (Le Feber *et al.*, 1999).

1.1.5 – Neurotransmitters involved in the micturition reflex pathways.

Various neurotransmitters have been implicated in the neural regulation of the LUT, including 5-HT, dopamine, acetylcholine, noradrenaline, excitatory and inhibitory amino acids, adenosine triphosphate (ATP), nitric oxide and neuropeptides (see de Groat *et al.*, 2001). Figure 1.3 shows the inhibitory or excitatory influences of the variety of neurotransmitters thought to regulate transmission in the PMC. In addition, ATP is co-released with ACH in the bladder and is largely responsible for the initiation of voiding in the rat (Igawa *et al.*, 1993), noradrenaline is released by the sympathetic pathways and contracts the bladder neck and urethra during continence (see Conley *et al.*, 2001) and nitric oxide is the main neurotransmitter involved in mediating relaxations of the urethral smooth muscle (see Andersson *et al.*, 1994). Since this thesis mainly details the role of 5-HT_{1A} receptors in the control of micturition, only the role of 5-HT as a neurotransmitter involved in micturition will be discussed in detail.

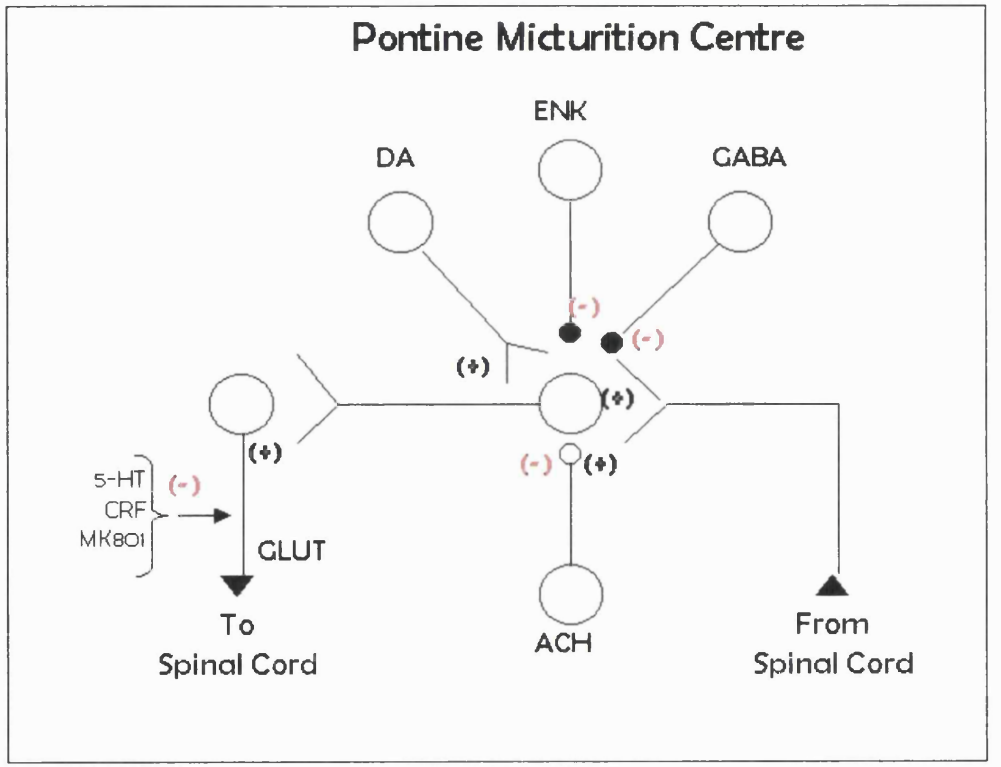


Figure 1.3 – A variety of neurotransmitters may regulate transmission in the PMC and in the descending limb of the micturition reflex pathway. DA, dopamine; ENK, enkephalins (depress micturition and sphincter reflexes); GABA, γ-aminobutyric acid (tonic GABAergic inhibitory control of supraspinal pathway regulates bladder capacity); GLUT, glutamic acid (principal excitatory neurotransmitter in descending pathways from the PMC to the spinal cord, also thought to be involved in the ascending limb of the spinobulbospinal pathway); ACH, acetylcholine (has both inhibitory and excitatory effects depending on the level of afferent activity reaching the PMC (synapse indicated by open circle)); 5-HT, 5-hydroxytryptamine; MK-801, a noncompetitive NMDA glutamate antagonist; CRF, corticotrophin releasing factor (descending limb of the reflex can be depressed by CRF, 5-HT agonists and MK-801). (+) excitatory, (-) inhibitory synapse (indicated by filled circles). (Modified from de Groat *et al.*, 1993).

1.1.5.1– 5-HT

5-HT exerts effects on the LUT at both peripheral and central sites. Peripheral administration of 5-HT induces contraction of the rat urinary bladder (Bom *et al.*, 1986) which is thought to be mediated via 5-HT_2 receptors (Saum *et al.*, 1973). The peripheral effect of 5-HT could be mediated at various levels: 5-HT exerts stimulatory activity on isolated bladder of many species (see Lecci *et al.*, 1992), it primarily inhibits, and occasionally facilitates, cholinergic ganglionic transmission in the pelvic ganglia (Saum *et al.*, 1973) and also induces complex neurotransmission in the sympathetic ganglia (Newberry *et al.*, 1989). Despite this pharmacological evidence for the existence of peripheral 5-HT receptors affecting micturition, there is no indication for physiological serotonergic control of micturition at a peripheral level (see Lecci *et al.*, 1992).

5-HT, along with various other neurotransmitters, has been implicated in the central neural regulation of the lower urinary tract (McMahon *et al.*, 1982; Lumb, 1986b; Lecci *et al.*, 1992; Espey *et al.*, 1995; Espey *et al.*, 1998; Testa *et al.*, 1999; see de Groat *et al.*, 2001; see de Groat, 2002). 5-HT receptors are widely distributed in the CNS including various areas concerned with the control of micturition. For example, 5-$HT_{1A/1B}$, 5-HT_2 and 5-HT_3 receptors are all present in rat and cat lumbosacral spinal cord (Monroe *et al.*, 1983; Glaum *et al.*, 1988; Murphy *et al.*, 1988; Thor *et al.*, 1993), and 5-HT also modifies the activity of sympathetic preganglionic neurones (Lewis *et al.*, 1990). In addition, 5-HT receptors are also present in various brain regions known to be important in the control of micturition, for example, 5-HT_{1A} receptors are found on 5-HT-containing neurones in the raphé nucleus (Aghajanian *et al.*, 1986).

1.1.5.1.1 – Role of 5-HT$_{1A}$ receptors in the control of micturition.

Several 5-HT receptor types, including 5-HT$_{1A}$, 5-HT$_2$, 5-HT$_3$, 5-HT$_4$ and 5-HT$_7$, have been studied with regard to their role in controlling micturition (see later, section 1.1.5.1.2), however, due to the early availability of various selective 5-HT$_{1A}$ ligands, the role of 5-HT$_{1A}$ receptors in the control of micturition is better understood than that of other 5-HT receptors. Previous studies in the rat have revealed that activation of spinal and supraspinal 5-HT$_{1A}$ receptors using the 5-HT$_{1A}$ receptor agonist, 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) facilitates the micturition reflex in the presence of intact C-fibre afferents (Lecci *et al.*, 1992), whereas administration of the 5-HT$_{1A}$ neutral receptor antagonists WAY-100635 (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)-cyclohexanecarboxamide trichloride) and NAD-299 ((*R*)-3-*N,N*-Dicyclobutylamino-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide hydrogen (*2R,3R*)-tartrate monohydrate; robalzotan) both intravenously (Testa *et al.*, 1999; Pehrson *et al.*, 2002) and intrathecally (Kakizaki *et al.*, 2001; Pehrson *et al.*, 2002) suppress the micturition reflex. 5-HT$_{1A}$ receptor antagonists that also show partial agonist activity do not inhibit the micturition reflex (Testa *et al.*, 1999).

Histochemical studies have confirmed that autonomic nuclei in the lumbosacral spinal cord, which are known to contain preganglionic neurones innervating the LUT, include a collection of 5-HT containing nerves that originate in the raphé nucleus in the caudal brain stem (Dahlstrom *et al.*, 1965). Transneuronal tracing studies with pseudorabies virus have also shown that raphé neurones in the brainstem of the rat are labelled after injection of the virus into either the bladder (Nadelhaft *et al.*, 1992), urethra (Vizzard *et al.*, 1995) or EUS (Marson, 1997) and both electrical and chemical stimulation of these

raphé neurones inhibits the micturition reflex in the rat and cat (McMahon *et al.*, 1982; Lumb, 1986a; Morrison *et al.*, 1986; Chen *et al.*, 1993). All the above evidence suggests the possible presence of an inhibitory descending raphé-spinal pathway that is activated by afferent input from the bladder, and inhibits the micturition reflex (Testa *et al.*, 1999). Since 5-HT_{1A} receptors are found on serotonergic neurones in the raphé nucleus (Aghajanian *et al.*, 1986), where they are thought to act as autoreceptors and play an important role in the control of raphé firing (Aghajanian *et al.*, 1986; see 1.3.1) it is not surprising that modulating 5-HT_{1A} activity can have significant effects on the micturition reflex. Studies using intrathecal administration of WAY-100635 in the urethrane anaesthetized rat has shown that 5-HT_{1A} receptors at the L6/S1 level of the spinal cord play an important physiological role in the tonic control of the descending limb of the micturition reflex pathway (Kakizaki *et al.*, 2001). The recent publication of a study investigating the effect of intracerebroventricular administration of WAY-100635 in the anaesthetized female rat (Yoshiyama *et al.*, 2003) has also demonstrated an actual physiological role for supraspinal 5-HT_{1A} receptors in the control of micturition. This effect may be mediated via blockade of raphé 5-HT_{1A} autoreceptors, which increases the raphé neuron firing rate, hence increasing the input from the decending inhibitory raphé-spinal pathway and resulting in the suppression of bladder activity. The inhibition of micturition by 5-HT_{1A} receptor antagonists can be antagonized by pre treatment with mesulergine, a 5-HT_2 receptor antagonist, indicating that 5-HT_2 receptors are also involved in descending raphé-spinal inhibitory mechanisms (Testa *et al.*, 1999). Figure 1.4 shows the possible serotonergic pathways controlling bladder function in the rat. Desending projections from the brainstem raphé neurones to the spinal cord activate interneurones via 5-HT_{2C} receptors, which provide an inhibitory input through as yet

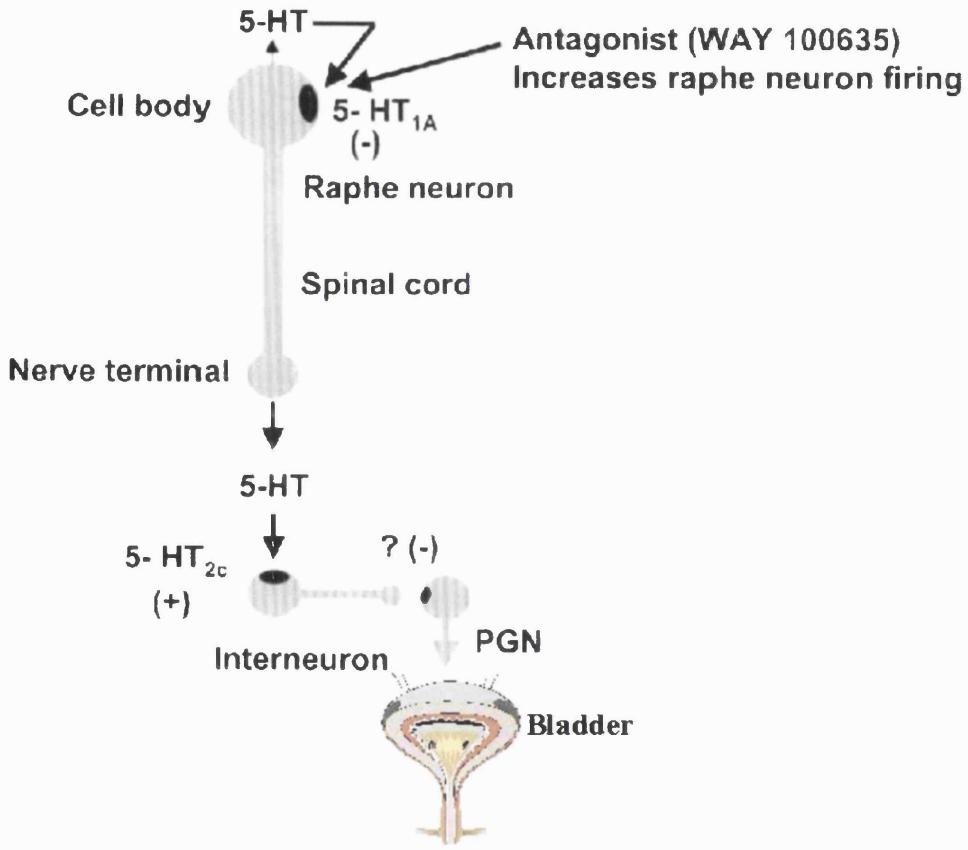


Figure 1.4 – Central serotonergic pathways controlling bladder function in the rat. Blockade of 5-HT_{1A} autoreceptors (by antagonists such as WAY-100635) in the raphé enhances raphé neuron firing, and therefore increases the release of 5-HT in the spinal cord. 5-HT activates excitatory 5-HT_{2C} receptors on inhibitory interneurones which provides an inhibitory input, via as yet unknown receptors, to the parasympathetic preganglionic neurones (PGN) and suppresses their firing. The site of action of 5-HT_{1A} receptor antagonists in the spinal cord is not shown. (Modified from de Groat, 2002).

unknown receptors, to the parasympathetic preganglionic neurones innervating the bladder.

1.1.5.1.2 – 5-HT$_1$ receptor subtypes, other than 5-HT$_{1A}$, involved in the control of micturition.

The role of 5-HT receptors, other than 5-HT$_{1A}$, in the control of micturition has been difficult to study due to a lack of receptor subtype specific agonists and antagonists, however it is possible that multiple supraspinal 5-HT receptor subtypes may be able to modulate the afferent and motor pathways involved in voluntary voiding. For example, it has been shown that 5-HT$_{2C}$ receptor agonists suppress efferent activity in the pelvic nerve and therefore suppress reflex bladder contractions (Steers *et al.*, 1989). Serotonergic activity mediated via 5-HT$_2$ and 5-HT$_3$ receptors also enhances urine storage in the cat by facilitating sphincter reflexes (Danuser *et al.*, 1996). Evidence has also been provided for the existence of an inhibitory 5-HT$_4$ receptor, which suppresses efferent activity to the bladder in the monkey (Waikar *et al.*, 1994), and in human bladder strips 5-HT$_4$ agonists facilitate acetylcholine release (Candura *et al.*, 1996). Recently it was also shown that intracerebroventricular (i.c.v.) administration of 5-HT$_{2A/B/C}$ and 5-HT$_4$ receptor agonists, but not 5-HT$_3$ receptor agonists, decreases bladder capacity and micturition volume, and increases micturition pressure in conscious rats (Ishizuka *et al.*, 2002). The use of receptor agonists in this study demonstrates the presence of these receptors supraspinally, but does not determine if these receptors are actively involved in the physiological control of voiding. Further studies using selective receptor antagonists are required to confirm the possible physiological role for 5-HT$_2$ and 5-HT$_4$ receptors in the control of micturition.

Recently, supraspinal 5-HT_7 receptors have been demonstrated to be involved in the control of micturition (Read *et al.*, 2003). Intracerebroventricular, but not intrathecal, administration of the selective 5-HT_7 receptor antagonists SB-269970 and SB-656104 increase the pressure and volume thresholds (i.e. bladder capacity) in anaesthetized rats. It is intriguing that both 5-HT_7 and 5-HT_{1A} receptor antagonists block the micturition reflex despite differing cellular effector systems, and it suggests a similar overall physiological function in the reflex control of the bladder for both these receptors. It is possible that these two 5-HT receptor systems may interact to obtain optimal control of the micturition reflex, however this remains to be determined.

1. 2 – The serotonergic signalling system.

5-hydroxytryptamine (5-HT; serotonin) produces its effects through a variety of membrane-bound receptors. 5-HT and its receptors are found in both the central and peripheral nervous systems, as well as some non-neuronal tissues in the cardiovascular system, gut and blood and the peripheral. In 1986 the pharmacology of 5-HT was reviewed and the existence of at least three 5-HT receptor families ($5\text{-}HT_{1\text{-}3}$) was proposed (Bradley *et al.*, 1986). In the seventeen years since this classification, improved molecular biological techniques have aided in the discovery of many additional 5-HT receptors. The 5-HT family of receptors is now divided into seven main classes ($5\text{-}HT_{1\text{-}7}$), largely on the basis of their structural and operational characteristics, and comprises of a total of 14 structurally and pharmacologically distinct 5-HT receptor subtypes (Hoyer *et al.*, 1994). Apart from the $5\text{-}HT_3$ receptor subtype, which is a ligand gated ion channel, the 5-HT family are G-protein couple receptors (GPCRs) with seven putative transmembrane spanning domains.

The degree of physical diversity between 5-HT receptors clearly underscores the physiological importance of 5-hydroxytryptamine, however evidence for an even greater degree of operational diversity continues to emerge (see Hoyer *et al.*, 2002). For example, post-translational modifications, such as alternate splicing and RNA editing, increase the number of proteins available, oligomerisation and heteromerisation increase the number of complexes of receptors, and the presence of multiple G-proteins suggest receptor trafficking (see section 1.5.7 for further detail), allowing phenotypic switching and cross talk within and possibly between receptor families. These observations, coupled with an extremely efficient reuptake system and the vast array of 5-HT receptor

subtypes, theoretically provides the 5-HT system with almost limitless signalling capabilities.

The molecular characterisation, localisation, pharmacology and disease relevance for each family of 5-HT receptors is reviewed in Table 1.3. Since this thesis examines micturition, and the 5-HT_1 and 5-HT_7 receptor subtypes are more relevant in this area than the other subtypes, only these two classes will be reviewed in detail here.

1.2.1 – The 5-HT_1 receptor family

The initial characterisation of the 5-HT_1 receptor came from radioligand binding studies which found high affinity binding sites for [^{3}H]-5-HT in rat cortex with low affinity for spiperone (Peroutka *et al.*, 1979). Subsequent studies have identified further heterogeneity within the 5-HT_1 class, and the 5-HT_1 receptor family now includes 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E} and 5-ht_{1F} receptors (see Hoyer *et al.*, 2002). The receptors of the 5-HT_1 family have high amino acid sequence homology and all couple negatively to adenylate cyclase, among other secondary effector systems, via $G_{i/o}$ proteins. The general pharmacological characteristics of the 5-HT_1 receptors were initially reported by Bradley *et al.* (1986) and included effects mimicking 5-CT, blocked/mimicked by methiothepin/methysergide and not blocked by selective antagonists of the 5-HT_2 and 5-HT_3 receptors. However, recently this classification has been revised to take into account the unusual property of the 5-ht_{1E} and 5-ht_{1F} receptors which have a low affinity for 5-CT and methiothepin (see Barnes *et al.*, 1999). The 5-ht_{1E} and 5-ht_{1F} receptors are given a lower case appellation to denote that endogenous receptors with a physiological role have yet to be found (see Hoyer *et al.*, 2002).

Table 1.3

	5-HT$_{1A}$	**5-HT$_{1B}$**	**5-HT$_{1D}$**	**5-HT$_{1E}$**	**5-HT$_{1F}$**	**5-HT$_{5A}$**	**5-HT$_{5B}$**	**5-HT$_{7}$**
Alternative names	—	5-HT$_{1D\beta}$	5-HT$_{1D\alpha}$	5-HT$_{1E\alpha}$	5-HT$_{1F\beta}$	5-HT$_{1\alpha}$	5-HT$_{1D\beta}$	5-HT$_{1\text{-like}}$, 5-HT$_{\gamma}$
Selective agonists	8-OH-DPAT U92016A	CP-94253	L775606	—	LY-334370 LY-344864	—	—	—
Selective antagonists	WAY-100635 Robalzotan	GR-127935 SB-216641 SB-224289 SB 236057	GR-127935 BRL-15572 ketanserin ritanserin	—	—	Methio-thepin	Methio-thepin	SB-258719 SB-266970 SB-656104 clozapine
Radio-ligands	[^{3}H]-WAY-100635 [^{3}H]-8-OH-DPAT	[^{3}H]-sumatriptan [^{3}H]-GR127935	[^{3}H]-sumatriptan [^{3}H]-GR127935	[^{3}H]-5-HT	[^{3}H]-LY334370 [^{125}I]-I-LSD	[^{3}H]-5-CT [^{125}I]-I-LSD	[^{3}H]-5-CT [^{125}I]-I-LSD	[^{3}H]-5-CT [^{3}H]-SB-266970
G-protein coupling	G_i/G_O	G_i/G_O	G_i/G_O	G_i/G_O	G_i/G_O	G_i/G_O	G_i/G_O	G_s
Expression profile	cerebral cortex, hipp, septum, amygdala, raphé, myenteric plexus	striatum, substantia nigra, globus pallidus, hipp, raphé, autonomic terminals	striatum, substantia nigra, globus pallidus, locus coeruleus, hipp, DRN, autonomic and trigeminal terminals	cerebral cortex, caudate, amyg-dala, hipp, hypothal-amus	Hipp, caudate, raphé, cortex, thalamus, spinal cord, uterus	cortex, SCN, raphé, caudate, habenula, hipp, spinal cord,	cortex, olfactory bulb, hipp, cerebellum, habenula	cortex, globus pallidus, amygdala, substantia nigra, raphé, SCN, hipp, vascular smooth muscle

Table 1.3 cont.

	5-HT$_{1A}$	**5-HT$_{1B}$**	**5-HT$_{1D}$**	**5-HT$_{1E}$**	**5-HT$_{1F}$**	**5-HT$_{5A}$**	**5-HT$_{5B}$**	**5-HT$_{7}$**
Physiological function	Somatodendritic autoreceptor in raphé, somatodendritic at cholinergic terminals of myenteric plexus, control of micturition , heart and lungs	Presynaptic autoreceptor and heteroreceptor, contractions of vascular smooth muscle	Somatodendritic autoreceptor in raphé, vasoconstriction in vascular smooth muscle, inhibition in autonomic neurones	Unclear	Trigeminal neuro-inhibition	Unclear	Unclear	Smooth muscle relaxation, circadian phase shifts, control of micturition
Knockout phenotype	Anxious, less reactive, decreased depressive related behaviours, less aggressive	Aggressive, more reactive, abnormal exploratory behaviour, altered sleep patterns, decreased startle response, increased body weight	—	—	—	Abnormal baseline startle, anxiety related behaviour, increased exploratory activity	—	—
Disease relevance	Depression, schizophrenia, cognitive decline, attention deficit hyperactivity disorder, neurodegeneration	Depression, migraine, Parkinson's disease, schizophrenia, cognitive decline	Depression, migraine, schizophrenia, cognitive decline	Unclear	Migraine	Unclear but possibly cognitive decline, schizophrenia, anxiety and depression	Unclear	Anxiety, depression, sleep disorders

	5-HT$_{2A}$	**5-HT$_{2B}$**	**5-HT$_{2C}$**	**5-HT$_4$**	**5-HT$_6$**
Alternative names	D, 5-HT$_2$	5-HT$_{2F}$	5-HT$_{2C}$	—	—
Selective agonists	(partially selective) DOI, DOB	BW-723C86	m-CPP, Ro600175	BIMU8, RS67506, ML-10302, SC-53116	LSD
Selective antagonists	Ketanserin, spiperone, MDL 100,907	SB-204741, SB-200646, SB-206553	SB-200646, SB-206553, SB-242084, SB-243213	GR-113808, SB-204070, RS-100235	Ro046790, Ro630563, SB-271046, SB-357134
Radioligands	[^{3}H]-ketanserin	[^{3}H]-5-HT	[^{3}H]-mesulergine	[^{3}H]-GR-113808 [^{125}I]-SB-207710	[^{3}H]-LSD, [^{125}I]-SB-258585, [^{3}H]-Ro630563
G-protein coupling	G_q/G_{11}	G_q/G_{11}	G_q/G_{11}	G_s	G_s
Expression profile	Cortex, nucleus accumbens, hipp, caudate, GI tract, vascular and bronchial SM	Cerebellum, lateral septum, hypo, amygdala, stomach, uterus	Cortex, choroid plexus, amygdala, substantia nigra, medulla pons, hipp, hypo, habenula, nucleus accumbens	Cortex, striatum, thalamus, hipp, brainstem, substantia nigra, parasympathetic neurones	Cortex, striatum, olfactory tubercule nucleus accumbens, hipp, superior cervical ganglia
Physiological function	Vascular SM constriction, platelet activation	Endothelium dependent vasorelaxation, vascular SM constriction	Possibly invoved in modulation of transferrin production and regulation of CSF volume	SM relaxation, myenteric cholinergic neuroexcitation, increased EEG amplitude	Possible modulation of CNS acetycholine release
Knockout phenotype	—	Embryonic and neonatal death: heart defects	Spontaneous convulsions, weight gain, cognitive impairment	—	—
Disease relevance	Depression, schizophrenia	Anxiety, eating disorders	Anxiety, schizophrenia, depression, sleep disorders	Cognitive decline, schizophrenia, Parkinson's disease	Cognitive decline

Table 1.3 – 5-hydroxytryptamine receptors: The molecular characterisation, localisation, pharmacology and disease relevance for each 5-HT receptor subtype (modified from Roberts *et al.*, 2002). Abbreviations: hipp, hippocampus; hypo, hypothalamus; SM, smooth muscle; GI, gastrointestinal; CSF, cerebrospinal fluid; DRN; dorsal raphé nucleus; SCN, suprachiasmatic nucleus; EEG, electroencephalography.

1.2.1.1 – 5-HT_{1A} receptors

The 5-HT_{1A} binding site was identified in the early 1980's (Pedigo *et al.*, 1981; Middlemiss *et al.*, 1983) and knowledge of the pharmacology and function of the receptor quickly progressed (Gozlan *et al.*, 1983) mainly due to the early identification of the archetypal selective 5-HT_{1A} high efficacy receptor agonist 8-OH-DPAT (8-Hydroxy-2-(di-*n*-propylamino)-tertralin; (Middlemiss *et al.*, 1983), although it is now known to show activity at 5-HT_7 receptors (To *et al.*, 1995; Wood *et al.*, 2000). Due to these early breakthroughs and the discovery that buspirone and related agonists were anxiolytic and antidepressant in the clinic (Traber *et al.*, 1987; Robinson *et al.*, 1990), the 5-HT_{1A} receptor is the best characterised of the 5-HT receptors to date.

1.2.1.1.1 – 5-HT_{1A} receptor structure

The 5-HT_{1A} receptor was the first 5-HT receptor to be successfully cloned. Both the human and rat 5-HT_{1A} receptors were identified by screening a genomic library for homologous sequences to the β_2-adrenoceptor (Kobilka *et al.*, 1987; Fargin *et al.*, 1988; Albert *et al.*, 1990). In fact, the 5-HT_{1A} receptor shows high affinity for certain β-adrenoceptor ligands and experiments on mutated forms of the receptor have shown that a single amino acid residue (Asn 385) in the 7^{th} transmembrane domain confers this affinity (Guan *et al.*, 1992). The rat 5-HT_{1A} receptor has 422 amino acids and shares 89% homology with the human receptor. The gene is intronless with a tertiary structure typical of a seven transmembrane spanning protein with sites for glycosylation and phosphorylation. The human receptor is localised on chromosome 5 (5q11.2-q13) (see Roberts *et al.*, 2002).

1.2.1.1.2 – 5-HT$_{1A}$ receptor distribution

The distribution of the 5-HT$_{1A}$ receptor in the brain has been extensively mapped by receptor autoradiography using a range of ligands such as [^{3}H]5-HT, [^{3}H]8-OH-DPAT, [^{3}H]ipsapirone, [^{125}I]-BH-8-MeO-N-PAT, [^{125}I]p-MPPI and more recently [^{3}H]WAY-100635 (Pazos *et al.*, 1985b; Weissmann-Nanopoulus *et al.*, 1985; Hoyer *et al.*, 1986; Verge *et al.*, 1986; Radja *et al.*, 1991; Khawaja *et al.*, 1995; Kung *et al.*, 1995). [^{3}H]WAY-100635 has also been widely used for the *in vivo* labelling of 5-HT$_{1A}$ receptors in various transgenic knock-out mice (Fabre *et al.*, 2000; Evrard *et al.*, 2002; Lopez-Gimenez *et al.*, 2002). PET studies using [^{11}C]WAY-100635 have also been used to image 5-HT$_{1A}$ receptors in the living human brain (Pike *et al.*, 1995). All these studies have revealed a high density of 5-HT$_{1A}$ receptors in limbic areas, notably the hippocampus, lateral septum, cortical areas (particularly the cingulate and entorhinal cortex), amygdala and the mesencephalic raphé nuclei (both dorsal and median raphé nuclei). In contrast, levels of 5-HT$_{1A}$ binding sites are barely detectable in the basal ganglia and cerebellum. Immunohistochemical studies revealed a similar 5-HT$_{1A}$ receptor protein distribution, but in addition, identified 5-HT$_{1A}$ receptors in the habenula (el Mestikawy *et al.*, 1990). The distribution of mRNA encoding the 5-HT$_{1A}$ receptor is almost identical to that of the radioligand binding sites, particularly in the raphé (Chalmers *et al.*, 1991; Pompeiano *et al.*, 1992). The overall pattern of 5-HT$_{1A}$ receptor distribution is very similar across all species, although the laminar organisation of 5-HT$_{1A}$ receptors in hippocampal and cortical areas in humans differs from that in the rodent (Burnet *et al.*, 1995).

5-HT_{1A} receptors are located both postsynaptic to 5-HT neurones in forebrain areas (i.e. heteroreceptors which act to control the release of another transmitter), and also presynaptically on the 5-HT neurones themselves at the level of the soma (somatodendritic) and dendrites in the mesencephalic and medullary raphé nuclei where they act as autoreceptors. These autoreceptors mediate a local negative feedback mechanism to control the level of raphé neuronal firing (see section 1.3.1.1). The differing synaptic locations of 5-HT_{1A} receptors were confirmed by lesioning studies using the 5-HT neurotoxin 5,7-dihydroxytryptamine, which produced a decrease in raphé 5-HT_{1A} binding sites (Verge *et al.*, 1986) and mRNA (Miquel *et al.*, 1992), thus confirming that in the raphé nuclei 5-HT_{1A} receptors are located on 5-HT containing neurones. In addition 5-HT_{1A} receptors are also found on cholinergic neurones in the septum (Kia *et al.*, 1996a) and pyramidal neurones (probably glutamatergic) in the cortex and hippocampus (Francis *et al.*, 1992). *In situ* hybridisation and immunocytochemical studies have also demonstrated the presence of 5-HT_{1A} receptors in cortical pyramidal neurones, as well as pyramidal and granular neurones of the hippocampus (Pompeiano *et al.*, 1992; Burnet *et al.*, 1995). Ultrastructurally, 5-HT_{1A} receptors have been shown to be located at synaptic membranes as well as extrasynaptically (Kia *et al.*, 1996b).

1.2.1.1.3 – 5-HT_{1A} receptor pharmacology

The pharmacological characteristics of the 5-HT_{1A} receptor clearly distinguish it from other members of the 5-HT_1 receptor family, and indeed other 5-HT receptors (see Barnes *et al.*, 1999). Several agonists show some selectivity for the 5-HT_{1A} receptor, for example 8-OH-DPAT, is the archetypal full agonist in most systems (Middlemiss *et al.*, 1983), although as mentioned previously, it has now been demonstrated to show some

selectivity for 5-HT_7 receptors, and buspirone, gepirone and MDL 72832 are partial 5-HT_{1A} receptor agonists. The synthesis of selective 5-HT_{1A} receptor antagonists has proven difficult, with several ligands including BMY-7378, NAN-190, MDL 73005 and SDZ 216525 originally identified as clear antagonists in various models of 5-HT_{1A} heteroreceptor function (see Fletcher *et al.*, 1993). However, further studies revealed that these compounds are not particularly selective and also demonstrated partial agonist properties at autoreceptors (Hjorth *et al.*, 1990; Sharp *et al.*, 1990; see Hoyer *et al.*, 1993; Schoeffter *et al.*, 1997). Non-selective antagonists such as pindolol, spiperone and propranolol, were the only tools available before the development of the selective 'silent' 5-HT_{1A} receptor antagonists WAY-100635 (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)-cyclohexanecarboxamide trichloride; (Fletcher *et al.*, 1996) and more recently robalzotan (NAD-299; (*R*)-3-*N,N*-Dicyclobutylamino-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide hydrogen (*2R,3R*)-tartrate monohydrate; (Johansson *et al.*, 1997) which act at both types of 5-HT_{1A} receptors (see Figure 1.5). WAY-100635 is the most potent of these two compounds (K_i = 0.24nM and 0.59nM for WAY-100635 and robalzotan respectively; (Johansson *et al.*, 1997), although robalzotan appears to be slightly more selective (Johansson *et al.*, 1997). Table 1.4 summarises the affinities across the 5-HT receptor family, of a number of 5-HT_{1A} and 5-HT receptor ligands that have proved useful as pharmacological tools.

1.2.1.1 4 – Signal transduction in 5-HT_{1A} receptors

5-HT_{1A} receptors are coupled to the G_i family of G-proteins, which include pertussis toxin-sensitive G_{i1}, G_{i2}, G_{i3} and G_o, and pertussis toxin-insensitive G_z proteins (Raymond *et al.*, 1993; Butkerait *et al.*, 1995; Albert *et al.*, 1996; Barr *et al.*, 1997). The pertussis

toxin-sensitive G-proteins act to cause the inhibition of adenylate cyclase, opening of potassium (K^+) channels or the inhibition of calcium (Ca^{2+}) channels (see Figure 1.6). In the hippocampus (i.e. postsynaptic 5-HT_{1A} receptors), the 5-HT_{1A} receptor is coupled to both adenylate cyclase and the opening of K^+ channels (Andrade *et al.*, 1986; De Vivo *et al.*, 1986; Markstein *et al.*, 1986; Clarke *et al.*, 1987; Colino *et al.*, 1987), whereas in the dorsal raphé 5-HT_{1A} somatodendritic autoreceptor activation opens K^+ channels and inhibits neuronal firing (Innis *et al.*, 1987; Blier *et al.*, 1993; Penington *et al.*, 1993). Activation of somatodendritic 5-HT_{1A} autoreceptors on dorsal raphé neurones also directly inhibits voltage-dependent calcium currents (Penington *et al.*, 1990; Chen *et al.*, 1996).

These second messenger and electrophysiological responses will now be reviewed in further detail.

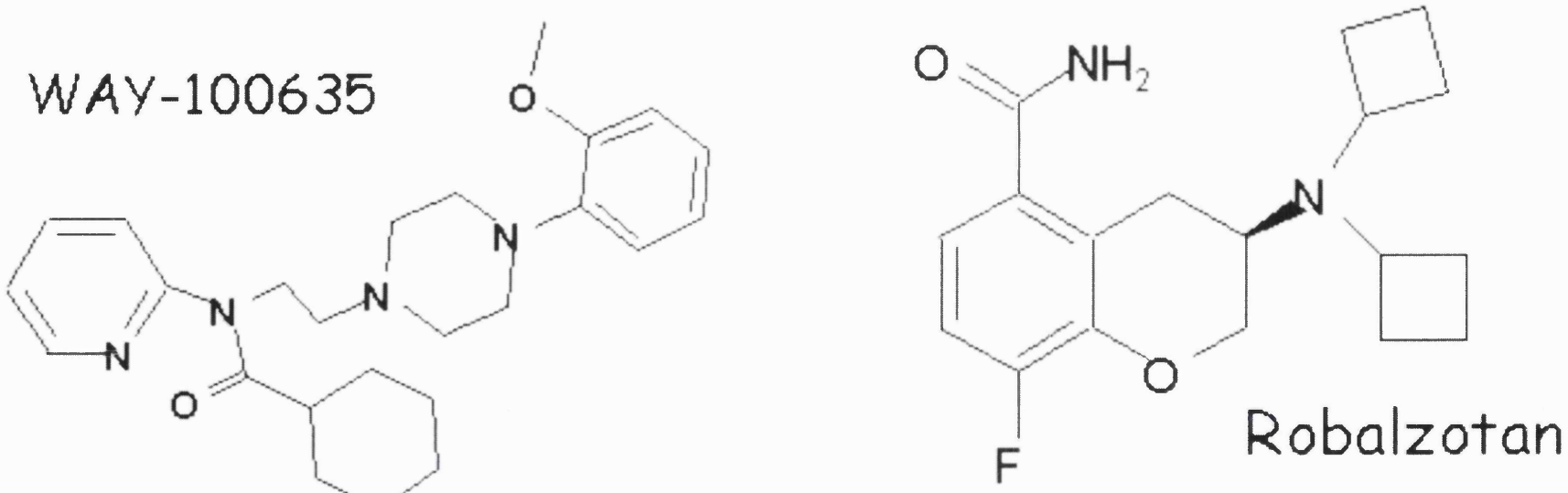


Figure 1.5 – Chemical structures of WAY-100635 and robalzotan. Both ligands are selective 5-HT_{1A} receptor antagonists which act at pre- and postsynaptic receptors, but are from different chemical series.

	5-HT receptor subtype											
	1A	**1B**	**1D**	**1E**	**1F**	**2A**	**2B**	**2C**	**4**	**5A**	**6**	**7**
5-HT	8.4	8.3	8.3	7.9	7.9	6.4	7.8	7.3	6.4	6.7	6.9	8.5
8-OH-DPAT	8.7	6.5	7.4	5.5	5.7	5.0	5.5	4.9	<5.0	5.6	<4.6	6.9
Sumatriptan	6.4	7.7	8.4	5.4	7.6	<5.3	<5.0	<5.0	<5.0	5.3	6.5	5.8
5-CT	9.2	8.9	9.0	4.9	6.1	<5.3	6.4	5.6	5.3	7.7	6.0	9.4
RU-24969	8.1	7.5	7.8	6.6	6.5	5.9	6.9	6.2	5.7	6.0	<5.0	<5.0
LY-334370	7.9	6.9	6.9		8.8	6.8	5.9	5.3				5.8
WAY-100635	9.0	<5.5	6.7	<5.3	<5.3	6.2	7.3	6.2	<4.3	<5.0	<5.1	6.9
SB-224289	<5.5	8.2	6.3	<5.0	<5.0	5.9	<5.5	6.2	5.7	5.3	<5.7	<6.0
SB-236057	<5.0	8.2	6.3	<5.0	<5.0	<5.2	<5.0	<5.3	5.4	<5.2	<5.0	<5.0
SB-216641	6.3	9.0	7.6	5.0	5.9	7.3	5.8	6.8	5.4	<5.0	<5.4	5.7
BRL-15572	7.8	6.4	8.0	5.8	6.0	6.6	7.4	6.2	5.3	5.7	5.9	6.3
GR-127935	7.2	8.7	8.3	5.4	6.4	7.8	6.2	7.0	5.4	5.8	5.7	6.1
SB-272183	8.0	8.1	8.7	5.0	5.1	5.5	6.1	5.7	5.6	5.6	5.7	6.8
SB-266970	<5.0	6.0	5.8	<5.2	<5.5	<5.0	5.0	<5.0	5.9	7.2	5.2	8.9

Table 1.4 – Binding affinities (pK_i) of various 5-HT ligands at human cloned 5-HT receptors. (Taken from Roberts *et al.*, 2001c)

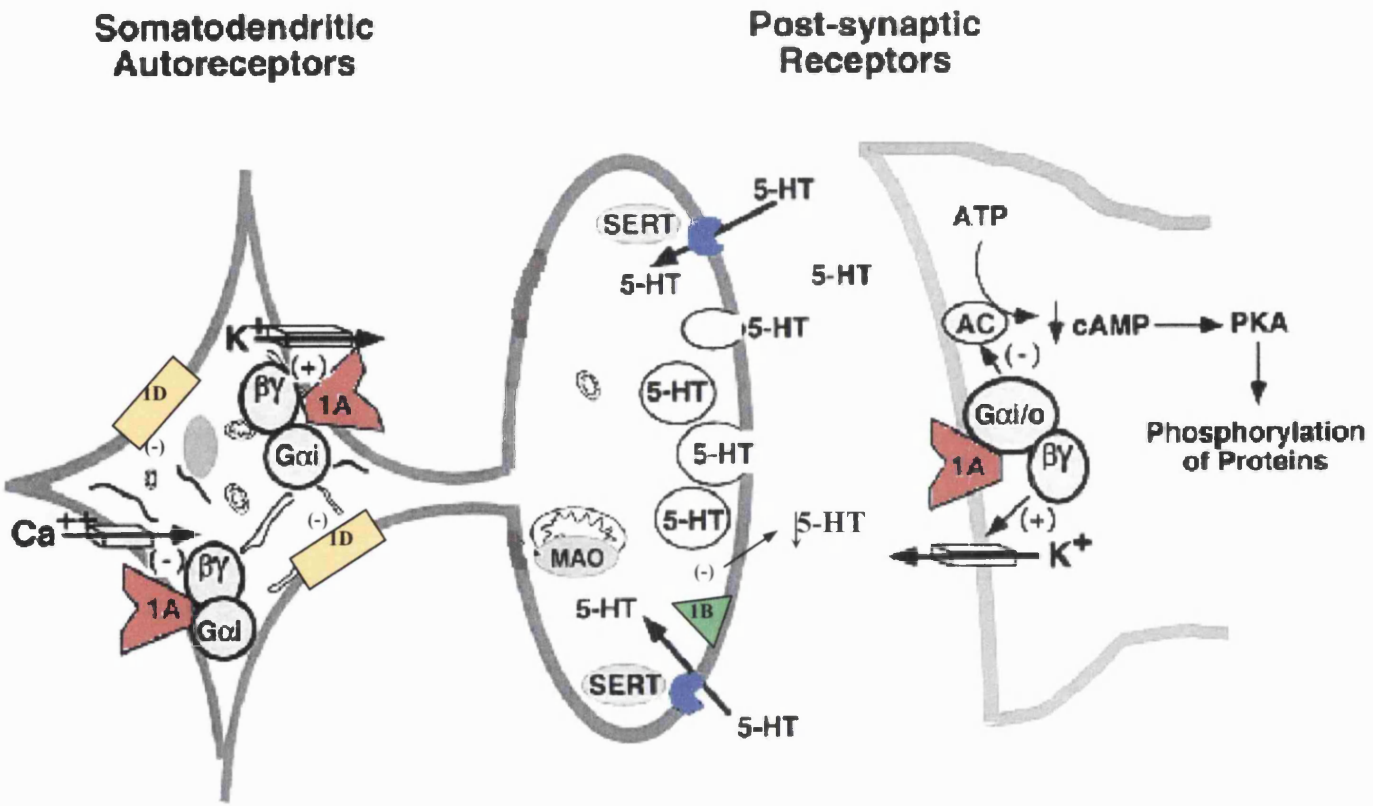


Figure 1.6 – Basic 5-HT_1 receptor control of serotonergic neurotransmission. The 5-HT_{1A} receptor is located on 5-HT-containing cell bodies and dendrites (located in the raphé nuclei) and functions as a somatodendritic autoreceptor. In terminal field areas of serotonergic innervation, the 5-HT_{1A} receptor is also located postsynaptically. 5-HT_{1B} and 5-HT_{1D} autoreceptors, 5-HT_{1B} and 5-HT_{1D} autoreceptors, at terminal and somatodendritic levels respectively, are also involved in regulating the level of 5-HT release (see section 1.3.1. **2** for further detail). (Modified from Hensler, 2003).

1.2.1.1.4.1 – Second messenger responses

The 5-HT_{1A} receptor negatively couples to adenylate cyclase via G proteins (α_i) in both rat and guinea-pig hippocampal tissue and cell lines (pituitary GH4C1 cells, COS-7 cells, HeLa cells) stably expressing the cloned 5-HT_{1A} receptor (see Saudou *et al.*, 1994; see Albert *et al.*, 1996), although the efficiency of coupling can differ markedly depending on whether native or model expression is studied. For example, in hippocampal tissue (under forskolin- or VIP-stimulated conditions) the rank order of potency of many agonists and antagonists correlates with their affinity for the 5-HT_{1A} binding site (De Vivo *et al.*, 1986; Schoeffter *et al.*, 1988), but despite the high density of 5-HT_{1A} receptors in the dorsal raphé, Clarke *et al.* (1996) have suggested that 5-HT_{1A} receptors in this region do not couple to the inhibition of adenylate cyclase. However, this observed lack of effect of 5-HT_{1A} receptor activation on adenylate cyclase activity maybe due to poor coupling of the receptor rather than a complete lack of inhibition of adenylate cyclase by the raphé 5-HT_{1A} receptor, since poor coupling has been demonstrated in wild-type CHO cells expressing 5-HT_{1A} receptors at levels similar to those in the brain (Mendez *et al.*, 1999). There are also reports of positive coupling of the 5-HT_{1A} receptor to adenylate cyclase in hippocampal tissue (Shenker *et al.*, 1983; Markstein *et al.*, 1986; Fayolle *et al.*, 1988), although this response has recently been attributed to 5-HT_7 receptor activation (Thomas *et al.*, 1999). 5-HT_{1A} receptor activation is able to enhance the effects of 5-HT_7 activation, possibly via the action of the $\beta\gamma$ subunits released upon 5-HT_{1A} receptor activation, which also potentiate the G_s stimulation of adenylate cyclase produced by 5-HT_7 receptor agonists (Thomas *et al.*, 1999).

5-HT_{1A} receptors have also been shown to decrease intracellular calcium, activate phospholipase C and increase intracellular calcium in transfected cell lines, although these observations await confirmation in native tissue and are dependent on the type of cells being studied (see Boess *et al.*, 1994; see Albert *et al.*, 1996). The biochemical basis for these diverse effects of the 5-HT_{1A} receptor is thought to reside in both the G protein compliment of the particular cell, and the particular isoforms of the effector enzymes being expressed in that cell (Albert *et al.*, 1996).

The 5-HT_{1A} receptor has also been reported to induce the secretion of the growth factor, protein S-100, from primary astrocyte cultures (Azmitia *et al.*, 1996) and increase markers of growth in neuronal cultures (Riad *et al.*, 1994). These results, and others, suggest that the 5-HT_{1A} receptor may have a neurotrophic role in the developing brain, and even possibly in the adult (see Barnes *et al.*, 1999).

1.2.1.1.4. 2 – Electrophysiological responses

Electrophysiological experiments have demonstrated that 5-HT_{1A} receptor activation causes neuronal hyperpolarisation, an effect which is mediated through G-protein coupled opening of K^+ channels without the involvement of diffusible intracellular messengers such as cAMP (see Aghajanian, 1995). As observed with metabotropic second messengers, there is a degree of plurality in the coupling of 5-HT_{1A} receptors to ion channels with Ca^{2+} channels also being affected by 5-HT_{1A} receptor activation (see Stamford *et al.*, 2000). In the hippocampus and frontal cortex, 5-HT_{1A} receptor agonists (e.g. 8-OH-DPAT) and 5-HT itself inhibit neuronal activity after iontophoretic administration *in vivo* (Sprouse *et al.*, 1988; Ashby *et al.*, 1994), and hyperpolarize

neurones in these regions when bath applied to brain slices (Andrade *et al.*, 1987; Araneda *et al.*, 1991). This inhibitory effect is blocked by WAY-100635 (Corradetti *et al.*, 1996a) and is therefore mediated via 5-HT_{1A} receptors.

Interestingly, when compared to 5-HT and 8-OH-DPAT, a number of high affinity 5-HT_{1A} receptor ligands (including MDL 73005 EF, BMY 7378 and buspirone) have low efficacy in specific forebrain regions (Andrade *et al.*, 1987; Van den Hooff *et al.*, 1991) despite behaving as full agonists in the dorsal raphé nucleus. These data have been explained by electrophysiological (Dong *et al.*, 1997) and neurochemical (Bockaert *et al.*, 1987) evidence that these drugs are partial agonists at 5-HT_{1A} heteroreceptors, and also that 5-HT_{1A} receptor density is significantly lower in the hippocampus and other forebrain regions, than in the raphé nucleus (Meller *et al.*, 1990; Yocca *et al.*, 1992). However, it is also possible that these observations may be due to regional differences in the regulation of 5-HT_{1A} receptors. For example, it has been demonstrated that electrophysiological responses mediated by 5-HT_{1A} receptors in the hippocampus are resistant to desensitization following agonist administration (see Hensler, 2003), even though behavioural and neuroendocrine responses elicited by 5-HT_{1A} heteroreceptor activation are attenuated after 5-HT_{1A} receptor agonist administration (Newman *et al.*, 1992). Thus, this evidence for regional differences in the regulation of 5-HT_{1A} receptor function, combined with the varying pharmacological properties of auto and heteroreceptors and the strong possibility for promiscuous coupling of the 5-HT_{1A} receptor to various G-proteins (see 1.5.7 for further detail), suggests the possible existence of more than one 5-HT_{1A} receptor subtype.

There is much evidence to suggest that stimulation of somatodendritic 5-HT_{1A} autoreceptors in the dorsal raphé nucleus causes neuronal hyperpolarization, and this can be attributed to the opening of a G-protein-coupled K^+ channel on rat raphé neurones (Bayliss *et al.*, 1997; Katayama *et al.*, 1997). The role of these receptors in regulating the firing of raphé neurones is often explained by this effect. Many studies have shown that a wide range of 5-HT_{1A} receptor agonists cause a marked inhibition of 5-HT neuronal firing in the rat dorsal raphé nucleus (Aghajanian, 1972; Haigler *et al.*, 1974; Sprouse *et al.*, 1988) and that this is blocked by selective 5-HT_{1A} receptor antagonists such as WAY-100635 (Craven *et al.*, 1994; Corradetti *et al.*, 1996b) and (S)-UH-301 (Arborelius *et al.*, 1994). Selective 5-HT_{1A} receptor antagonists also reverse the inhibition caused indirectly by 5-HT reuptake inhibitors releasing agents (Gartside *et al.*, 1995; Hajos *et al.*, 1995; Gartside *et al.*, 1997). Reports on the effects of WAY-100635 administered alone on 5-HT neuronal firing are inconsistent, although generally slight increases are detected in anaesthetized rats (Gartside *et al.*, 1995; Wang *et al.*, 1995) and guinea-pigs (Mundey *et al.*, 1996). However, WAY-100635 has a definite stimulatory effect on 5-HT neuronal firing in awake cats (Fornal *et al.*, 1996), suggesting that the 5-HT_{1A} autoreceptor is under physiological tone.

The role of somatodendritic 5-HT_{1A} autoreceptors in limiting the firing of raphé neurones is usually attributed to the opening of G-protein coupled K^+ channels, however, it is possible that inhibition of Ca^{2+} channels may also play a role. Studies with *Xenopus* larvae primary sensory neurones have shown that 5-HT inhibits Ca^{2+} currents by differential inhibition of P/Q-(5-HT_{1A}) and N-(5-HT_{1D}) type Ca^{2+} channels (Sun *et al.*, 1998), with the 5-HT_{1A} effects being voltage-dependent. Interestingly, similar responses have been observed in rat C-like dorsal root ganglion neurones where 5-HT_{1A} receptor

stimulation modulates the depolarisation-mediated influx of Ca^{2+} ions and decreases action potential duration via inhibition of N- and L-type Ca^{2+} channels, which are coupled to two different signalling pathways (Cardenas *et al.*, 1997). Thus, it appears that somatodendritic 5-HT_{1A} autoreceptors in the raphé are coupled to both G-protein coupled K^+ and Ca^{2+} channels.

1.2.1.1. 5 – Neurotransmitter release mediated via 5-HT_{1A} receptors.

5-HT_{1A} receptors have been shown to play a role in the release of 5-HT in the rat forebrain (see Sharp *et al.*, 1990), acetylcholine in the guinea-pig cortex (Bianchi *et al.*, 1990) and hippocampus (Wilkinson *et al.*, 1994), and noradrenaline in a variety of brain areas (see Barnes *et al.*, 1999). However, only the release of 5-HT will be reviewed in detail here.

In accordance with the electrophysiological data, microdialysis studies show that 5-HT_{1A} receptor agonists (administered subcutaneously) cause a decrease in the release of 5-HT in the forebrain of the rat *in vivo* (Sharp *et al.*, 1990; Hjorth *et al.*, 1995) due to the activation of the raphé somatodendritic 5-HT_{1A} autoreceptors (see section 1. 3 for further detail) and these effects are blocked by the selective 5-HT_{1A} receptor antagonist WAY-100635 (0.3 mg kg^{-1} s.c.;(Hjorth *et al.*, 1995). However, selective 5-HT_{1A} receptor antagonists alone do not consistently increase 5-HT release in microdialysis studies under anaesthetized or conscious conditions (Nomikos *et al.*, 1992; Routledge *et al.*, 1993), which contrasts with the electrophysiological data suggesting that the 5-HT_{1A} receptor is under physiological tone (Fornal *et al.*, 1996). The non-selective 5-HT_1 receptor antagonists penbutolol and tertalol have been demonstrated to increase 5-HT release in

the rat (Hjorth *et al.*, 1993; Assie *et al.*, 1996), but additional 5-HT_{1B} receptor blockade is thought to contribute to these effects.

1.2.1.1. 6 – Behavioural and other physiological responses.

The pharmacological involvement of 5-HT_{1A} receptors in a number of physiological and behavioural effects has been established. For example, the administration of 8-OH-DPAT and other 5-HT_{1A} receptor agonists in the rat have been shown to induce a 5-HT behavioural syndrome, characterised by flat body posture, reciprocal forepaw treading and head weaving (Tricklebank *et al.*, 1984; see Green *et al.*, 1985), hyperphagia (Simansky, 1996), hypothermia (Higgins *et al.*, 1988; Hillegaart, 1991), altered sexual behaviour (see Lucki, 1992) and a tail flick response (Millan *et al.*, 1991). In addition 5-HT_{1A} receptor agonists have been implicated in neuroendocrine regulation, with 5-HT_{1A} receptor agonists causing an elevation of plasma adrenocorticotrophic hormone (ACTH) and corticosteriod levels (Gilbert *et al.*, 1988; Gartside *et al.*, 1990) but not prolactin secretion (Jorgensen *et al.*, 2001) as originally thought (Gartside *et al.*, 1990). In man, 5-HT_{1A} receptor agonists have also been shown to increase secretion of growth hormone (Cowen *et al.*, 1990). Although these initial studies with 5-HT_{1A} receptor agonists suggested the pharmacological involvement of 5-HT_{1A} receptors, an actual physiological role for these receptors has been confirmed with more recent studies using the selective 5-HT_{1A} receptor antagonist WAY-100635 which is able to block the 5-HT behavioural syndrome (Fletcher *et al.*, 1996) and elevation of plasma ACTH levels (Critchley *et al.*, 1994) induced by 8-OH-DPAT. However, in some cases controversy exists regarding the involvement of 5-HT_{1A} autoreceptors and/or heteroreceptors. It is generally regarded that the 5-HT behavioural syndrome is mediated via activation of

postsynaptic 5-HT_{1A} heteroreceptors (see Lucki, 1992) as is the tail flick response which is mediated via spinally localized postsynaptic 5-HT_{1A} receptors (Bervoets *et al.*, 1993). Whereas, evidence for a 5-HT_{1A} somatodendritic autoreceptor in the hyperphagia response appears convincing at least at the level of the dorsal raphé, since it has been demonstrated that intra-dorsal raphé injections of 5-HT_{1A} receptor agonists induce this effect (see Simansky, 1996). Conversely, for hypothermia there appears to be some species specific differences with respect to the action of 5-HT_{1A} receptor agonists. In rats, intra-dorsal raphé injection of 5-HT_{1A} receptor agonists evokes hypothermia (Higgins *et al.*, 1988; Hillegaart, 1991), however, inhibition of 5-HT synthesis and 5-HT lesions (induced by 5-7-dihydroxytryptamine) do not prevent hypothermia when the agonists are systemically injected (Bill *et al.*, 1991; O'Connell *et al.*, 1992), thus suggesting that in rats, the response can be mediated via both auto- and heteroreceptors (presumably involving different neural circuits). Whereas, in the mouse, only autoreceptors mediate the hypothermic response since 5-HT lesions abolish the hypothermic response to 5-HT_{1A} receptor agonists (Goodwin *et al.*, 1985; Bill *et al.*, 1991). 5-HT_{1A} receptor agonists have also been shown to induce a strong discriminative stimulus, and both pre- and postsynaptic mechanisms also seem to be able to mediate this response in rats (Schreiber *et al.*, 1993). The elevation of ACTH levels after 5-HT_{1A} receptor agonist administration is still observed in rats with 5-HT lesions, thus suggesting that this response is mediated by postsynaptic 5-HT_{1A} receptors (see Fuller, 1996). The functional responses associated with the activation of 5-HT_{1A} auto- and heteroreceptors are summarised in Table 1.5.

5-HT_{1A} receptors also play a role in modulating anxiety-related behaviours, and there is a large literature of basic and clinical data revealing the anxiolytic and antidepressant

activity of 5-HT_{1A} receptor agonists (see Handley, 1995; see Den Boer *et al.*, 2000). Recent studies using 5-HT_{1A} receptor knock out (KO) mice have supported this role for 5-HT_{1A} receptors, since these KO animals demonstrate increased anxiety in a number of experimental paradigms (Heisler *et al.*, 1998; Parks *et al.*, 1998), for example, decreased exploratory activity and increased fear of aversive environments (open or elevated spaces), and decreased immobility in the forced swim test which is an effect commonly associated with antidepressant-like drug efficacy (Ramboz *et al.*, 1998). The modulation of 5-HT_{1A} receptors in the treatment of affective disorders such as anxiety and depression will be reviewed more thoroughly in 1.2.2.

Level	*Response*	*Mechanism*
Cellular	Adenylate cyclase (-)	Heteroreceptor
Electrophysiological	Hyperpolarisation	Heteroreceptor
Behavioural	5-HT syndrome	Heteroreceptor
	Hypothermia	Auto-/ heteroreceptor
	Hyperphagia	Autoreceptor
	Anxiolysis	Auto-/ heteroreceptor
	Sexual behaviour (+)	Auto-/ heteroreceptor
	Discriminative stimulus	Auto-/ heteroreceptor
Neurochemical	5-HT release (-)	Autoreceptor
	Noradrenaline release	Heteroreceptor
	Acetycholine release	Heteroreceptor
	Glutamate release	?
Neuroendocrine	ACTH (+)	Heteroreceptor
	Prolactin (+)	Heteroreceptor

Table 1.5 – Summary of the functional responses associated with activation of the rat brain 5-HT_{1A} receptor, and the mechanisms by which the response is mediated. (Taken from Barnes *et al.*, 1999).

1.2.1.1. 7 – The therapeutic possibilities of 5-HT$_{1A}$ receptor intereference

Given the variety of physiological and behavioural responses that 5-HT$_{1A}$ receptors are involved with, it is not surprising that modulating 5-HT$_{1A}$ receptor activity provides therapeutic potential in a number of disease areas, such as Alzheimer's disease, anxiety and depression, ischaemia and schizophrenia (see Roberts *et al.*, 2002).

Alzeimer's disease and cognition

5-HT$_{1A}$ receptors modulate the release of non-serotonergic neurotransmitters, and application of 5-HT$_{1A}$ receptor agonists and antagonists to the cortex decreases or increases glutamate release in the striatum respectively (Dijk *et al.*, 1995). It has also been demonstrated that learning impairments induced in rats and primates by cholinergic antagonists or lesions, can be reversed by WAY-100635 (Harder *et al.*, 1996; Carli *et al.*, 1997). This has led to the suggestion that 5-HT$_{1A}$ receptor antagonists may enhance cognition by removal of a 5-HT$_{1A}$ receptor inhibitory input to cortical pyramidal neurones which compensates for the loss of an excitatory cholinergic input (see Barnes *et al.*, 1999), however, recent electrophysiological recordings of cortical neurones in awake rats have failed to confirm this suggestion (Hajos *et al.*, 1998). 5-HT$_{1A}$ receptor agonists also increase acetylcholine release in the cortex (Bianchi *et al.*, 1990) and hippocampus (Izumi *et al.*, 1994), and it has been suggested that 5-HT$_{1A}$ receptor agonists may be beneficial in the treatment of cognitive dysfunction (see Roberts *et al.*, 2002).

Ischaemia

The 5-HT_{1A} receptor agonists BAY x 3702 and 8-OH-DPAT have been demonstrated to be neuroprotective in rat models of cerebral ischaemia and traumatic brain injury (Suchanek *et al.*, 1998) and NMDA-induced excitotoxicity (Oosterink *et al.*, 1998).

Schizophrenia

Recent post-mortem studies in schizophrenic patients have revealed an increase in 5-HT_{1A} heteroreceptor density in the prefrontal cortex (Bantik *et al.*, 2001). These 5-HT_{1A} receptors are located on pyramidal neurones and could possibly reflect an abnormal glutamatergic network. Further evidence that ligands acting at 5-HT_{1A} receptors may be beneficial in the treatment of schizophrenia comes from the observation that 5-HT_{1A} receptor agonists increase dopamine release in the prefrontal cortex which is thought to improve negative symptoms of schizophrenia (Sakaue *et al.*, 2000).

Anxiety and depression

5-HT_{1A} autoreceptors are known to modulate synaptic 5-HT release and neuronal firing in the raphé nuclei (Aghajanian, 1972). 5-HT_{1A} receptor agonists, such as the azapirone compounds (e.g. buspirone, gepirone, ipsapirone, tandospirone), will decrease extracellular 5-HT release and many studies support both an anxiolytic and antidepressant action for these ligands (Traber *et al.*, 1987; Lucki, 1991; Handley, 1995). Buspirone is also used clinically in the treatment of anxiety disorders (Charney *et al.*, 1990). Studies of the mechanisms underlying the anxiolytic properties of 5-HT_{1A} receptor agonists tend to favour an action at autoreceptors for the reasons noted above, although an involvement of heteroreceptors cannot be ruled out (see De Vry, 1995; see Handley, 1995).

5-HT_{1A} receptor agonists have also been demonstrated to increase noradrenaline release in the locus coeruleus, and this effect is attenuated by WAY-100635 (Suzuki *et al.*, 1995; Hajos-Korcsok *et al.*, 1996). The locus coeruleus projects to the medial pontine reticular formation, which is responsible for the control of REM sleep. Therefore, as would be expected, 5-HT_{1A} receptor agonists also modulate sleep patterns with agonists reducing slow wave and REM sleep whilst increasing wakefulness (Monti *et al.*, 2000), and this may contribute to the antidepressant action of 5-HT_{1A} receptor agonists, since it has been shown that there is a link between sleep regulation and depression (see Feige *et al.*, 2002), with sleep deprivation actually constituting an effective, albeit often short-lived, antidepressant treatment in about 70% of patients with major depressive disorder (Berger *et al.*, 1993; Vollmann *et al.*, 1993; Riemann *et al.*, 1999).

5-HT_{1A} receptor agonists and antagonists have also been proven useful in combination therapy with other antidepressant drugs such as selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase inhibitors and certain tricyclic antidepressants. For example in rats, 5-HT_{1A} receptor antagonists (such as WAY-100635) have been shown to potentiate the 5-HT release observed with the aforementioned antidepressant drugs (Hjorth, 1993; Gartside *et al.*, 1995; Artigas *et al.*, 1996; Romero *et al.*, 1996; Sharp *et al.*, 1997a; Sharp *et al.*, 1997b) by preventing the negative feedback mediated by 5-HT_{1A} autoreceptors at the level of the 5-HT containing neurones in the dorsal raphé. In addition it has also been demonstrated that the therapeutic effect of antidepressants in the clinic is improved in the presence of pindolol, a 5-HT_{1A} receptor antagonist with partial agonist activity (Artigas *et al.*, 1996). Pindolol is thought to be unique in its ability as a 5-HT_{1A} partial agonist to improve the therapeutic effects of antidepressants, and it is thought its mechanism of action could be a result of a lack of selectivity (Artigas *et al.*,

2001). Possible explanations for this effect include, pindolol acting as an antagonist at 5-HT_{1A} autoreceptors in the presence of SSRIs (Hjorth, 1996) and/or acting as an antagonist at terminal 5-HT_{1B} autoreceptors (Dawson *et al.*, 2000) or blockade of β-adrenoceptors since its affinity for β-adrenoceptors is ten times higher than for 5-HT_{1A} receptors (Pazos *et al.*, 1985a) and some antidepressants (not SSRIs) downregulate cortical β-adrenoceptors (see Artigas *et al.*, 2001). These studies suggest that combination therapy with antidepressant drugs and 5-HT_{1A} receptor antagonists (or pindolol) may be beneficial in the treatment of depression – i.e. reduce the delay in onset of antidepressant efficacy of SSRIs.

1.2.1. 2 – 5-HT_{1B} and 5-HT_{1D} receptors

The 5-HT_{1B} receptor was originally characterised as a [^{3}H]-5-HT binding site with low affinity for spiperone in rodent brain tissue (Pedigo *et al.*, 1981) and was originally thought to be a rodent species specific receptor. Another binding site for [^{3}H]-5-HT was detected in bovine brain, and was originally classified as a 5-HT_{1D} binding site on the basis of it being pharmacologically distinguishable from the rodent 5-HT_{1B} site (Heuring *et al.*, 1987). However, similarities in transductional features, function and brain distribution led to the opinion that the rodent '5-HT_{1B}' and nonrodent '5-HT_{1D}' receptors were species homologues (Hoyer *et al.*, 1989a), which was demonstrated without doubt when the two receptors were cloned (Hartig *et al.*, 1992). This initial idea became complicated by the discovery of two human receptor related genes, 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$, which demonstrated the pharmacology of the originally described 5-HT_{1D} site, but not the rodent 5-HT_{1B} site (see Hartig *et al.*, 1992). However, it was subsequently shown that the rodent 5-HT_{1B} and human 5-$HT_{1D\beta}$ receptors shown 96% sequence homology with each

other (Jin *et al.*, 1992) and are rodent and non-rodent species homolgues respectively. These findings, together with the discovery of a rat gene homologous to the human 5-$HT_{1D\alpha}$ receptor which encoded a receptor with a 5-HT_{1D} binding site profile (Hamblin *et al.*, 1992), lead to a revision of the 5-HT receptor nomenclature (Hartig *et al.*, 1996). The 5-$HT_{1D\beta}$ receptor is now known as the 5-HT_{1B} receptor, consistent with the fact that it is the human homologue of the originial rodent 5-HT_{1B} receptor, and the 5-$HT_{1D\alpha}$ receptor is now called the 5-HT_{1D} receptor.

1.2.1.2. 1 – 5-HT_{1B} and 5-HT_{1D} receptor distribution

Autoradiographical studies using a variety of radioligands have demonstrated a high density of 5-HT_{1B} receptors in the CNS, concentrated in the rat basal ganglia (particularly the substantia nigra, globus pallidus, ventral pallidum and entopeduncular nucleus) and various other regions such as the subiculum, superior colliculi and cerebellum (Verge *et al.*, 1986; Bruinvels *et al.*, 1993b). There is evidence for both an auto- and heteroreceptor function for 5-HT_{1B} receptors. Some areas with a high density of 5-HT_{1B} receptor binding, such as the anterior raphé nuclei (Verge *et al.*, 1986), also express a high level of 5-HT_{1B} receptor mRNA (Doucet *et al.*, 1995), thus suggesting that these receptors are present on indigenous neurones where they may act as autoreceptors. However, other areas with a high level of 5-HT_{1B} receptor binding (e.g. globus pallidus and ventral pallidum) have little detectable 5-HT_{1B} receptor mRNA, and therefore the 5-HT_{1B} receptors are likely to be present on afferent non-5-HT nerve terminals (see Barnes *et al.*, 1999), having been synthesised and then transported from cell bodies in other regions (see Boschert *et al.*, 1994). Evidence from 5-HT neuronal lesioning experiments (5,7-dihydroxytryptamine induced lesions) using radioligand binding have generated

contradictory results, with some studies demonstrating upregulation and others downregulation of 5-HT_{1B} receptors in the same brain areas (see Roberts *et al.*, 2002). However, *in situ* hybridisation studies have shown a consistent decrease in 5-HT_{1B} receptor mRNA in the dorsal and median raphé nuclei following neuronal lesioning (Doucet *et al.*, 1995), thus confirming at least, the synthesis of 5-HT_{1B} receptors in 5-HT containing neurones in the raphé, which is consistent with an autoreceptor role at the level of the terminal of these neurones (see Stamford *et al.*, 2000). The role of 5-HT_{1B} receptors in the control of 5-HT release in 5-HT containing neurones in the raphé is reviewed in further detail in section 1. 3.

Due to the relatively low abundance of 5-HT_{1D} receptors in the brain, and the lack of a suitable high affinity and selective radioligand, it has been difficult to determine their distribution in the brain. However, it appears that the 5-HT_{1D} receptor distribution is similar to that of 5-HT_{1B} receptors, with 5-HT_{1D} binding sites present in the basal ganglia (particularly the globus pallidus, substantia nigra and caudate putamen) and also the hippocampus and cortex (Bruinvels *et al.*, 1993a; Castro *et al.*, 1997). *In situ* hybridisation studies have detected 5-HT_{1D} receptor mRNA in various brain regions such as the caudate putamen, nucleus accumbens, olfactory cortex, hippocampus, dorsal raphé nucleus and the locus coeruleus (Bach *et al.*, 1993; Bruinvels *et al.*, 1994a; Bruinvels *et al.*, 1994b). The mRNA was in low abundance in all these regions, but interestingly was undetectable in certain regions such as the globus pallidus and substantia nigra, despite 5-HT_{1D} binding sites being present in these areas (see Barnes *et al.*, 1999). Together, these findings are similar to those observed with the 5-HT_{1B} receptor, and suggest that the 5-HT_{1D} receptor may also be predominantly located on axon terminals of both 5-HT and non-5-HT containing neurones.

1.2.1.2. 2 – 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor pharmacology

There are a large number of ligands with high affinity for the 5-HT$_{1B}$ receptor but most are not selective (see Table 1.4) for example RU-24969, 5-CT, sumatriptan and CP-93129 are potent agonists and methiothepin is a potent antagonist (Hoyer *et al.*, 1994). Since the human 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors show 77% homology in their amino acid sequence (see Barnes *et al.*, 1999), it is unsurprising that many of the ligands with high affinity for 5-HT$_{1B}$ receptors (listed above) also have high affinity for the 5-HT$_{1D}$ binding site (e.g. Pauwels *et al.*, 1996). However, ligands were identified which showed selectivity for both 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors versus other 5-HT$_1$ receptors, which has made them useful tools, for example GR-127935 (Skingle *et al.*, 1996) and GR-125743 (Millan *et al.*, 2002) which are both mixed 5-HT$_{1B/1D}$ receptor antagonists. Despite the similarities between 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors, there are nevertheless some detectable pharmacological differences between them which allows for discrimination between the two receptor subtypes. For example, ketanserin and ritanserin show approximately 15 – 30 fold selectivity for 5-HT$_{1D}$ over 5-HT$_{1B}$ receptors (Pauwels *et al.*, 1996) and the antagonist BRL-15572 is reported to have 60-fold selectivity for 5-HT$_{1D}$ over 5-HT$_{1B}$ receptors (Price *et al.*, 1997). Recently, selective and potent 5-HT$_{1B}$ receptor antagonists, such as SB-216641 (Price *et al.*, 1997), SB-224289 (Gaster *et al.*, 1998) and SB-236057 (Roberts *et al.*, 2000) have also been identified.

1.2.1.2. 3 – 5-HT$_{1B/1D}$ receptor second messengers and functional effects

Both rat and human cloned 5-HT$_{1B}$ receptors have been shown to negatively couple to adenylyl cyclase under forskolin-stimulated conditions (Adham *et al.*, 1992; Levy *et al.*, 1992; Weinshank *et al.*, 1992), and this has also been shown in native tissue, in both rat

(Bouhelal *et al.*, 1988) and calf substantia nigra (Schoeffter *et al.*, 1989). Cloned 5-HT_{1D} receptors have also been demonstrated to negatively couple to adenylyl cyclase (Hamblin *et al.*, 1991; Weinshank *et al.*, 1992), although this has not yet been shown in native tissue. In addition, there are reports that 5-HT_{1D} receptor activation will increase cAMP accumulation in CHO cell lines (Watson *et al.*, 1996).

There is now convincing evidence that both 5-HT_{1B} and 5-HT_{1D} receptors are located on 5-HT and non-5-HT neurones, and function as autoreceptors in both the raphé nuclei and 5-HT nerve terminal regions (see Barnes *et al.*, 1999; see Stamford *et al.*, 2000). 5-HT_{1B} receptors have been demonstrated to act as terminal autoreceptors (Schlicker *et al.*, 1997) as well as being localized in cell body regions, such as the raphé (Davidson *et al.*, 1995b), where they act to regulate 5-HT release from serotonergic neurones. 5-HT_{1B} receptor agonists have been reported to decrease while antagonists increase 5-HT release, although responses may be brain region dependent (Roberts *et al.*, 1998). 5-HT_{1D} receptors have also been identified as autoreceptors, possibly somatodendritic (see Stamford *et al.*, 2000), in the dorsal raphé (Starkey *et al.*, 1994; Davidson *et al.*, 1995b; Pineyro *et al.*, 1996) but not the median raphé (Hopwood *et al.*, 2001) or terminal brain regions (Schlicker *et al.*, 1997). The role of 5-HT_{1B} and 5-HT_{1D} receptors in the serotonergic control of the raphé and the regulation of 5-HT release is examined in more detail in section 1. 3.

A mismatch in the distribution of 5-HT_{1B} binding sites and 5-HT_{1B} receptor mRNA has lead to speculation that in some brain areas the 5-HT_{1B} receptor functions as a 5-HT heteroreceptor having been transported to a non-5-HT nerve terminal (Bruinvels *et al.*, 1994a; Bruinvels *et al.*, 1994b). There is now functional evidence to support this

heteroreceptor role, with 5-HT_{1B} receptors thought to modulate ACH release in the rat hippocampus (Cassel *et al.*, 1995) and rat frontal cortex (Consolo *et al.*, 1996), dopamine release in the frontal cortex (Lyer *et al.*, 1996) and glutamate release in the subiculum (Boeijinga *et al.*, 1996) and raphé (Li *et al.*, 1998). There is limited functional evidence for a heteroreceptor role for the 5-HT_{1D} receptor, probably due to the paucity of selective 5-HT_{1D} receptor ligands. There is some evidence supportive of a heteroreceptor role for these receptors (see Barnes *et al.*, 1999), however selective 5-HT_{1D} ligands such as BRL-15572 need to be tested in native tissue models before a heteroreceptor function can be attributed unequivocally to the 5-HT_{1D} receptor.

As a consequence of their autoreceptor activity, 5-HT_{1B} and 5-HT_{1D} receptor antagonists may be antidepressant, and acute administration of the 5-HT_{1B} receptor inverse agonist, SB-236057, has been shown to increase 5-HT release in the dentate gyrus (Roberts *et al.*, 2000) and may act as a fast-acting antidepressant. The lack of selective 5-HT_{1D} receptor ligands has limited investigations into the importance of the 5-HT_{1D} receptor in the antidepressant field. However, it has been demonstrated that to potentiate the effects of SSRIs and maximise the release of 5-HT in terminal brain regions, 5-HT_{1D} as well as 5-HT_{1B} and 5-HT_{1A} receptors must be blocked simultaneously (Roberts *et al.*, 1999), thus indicating that all these receptors are important in the treatment of depression.

Many clinically effective anti-migraine drugs display 5-HT_{1B} as well as 5-HT_{1D} and 5-HT_{1F} receptor agonist activity (see Hamel, 1999). It has been shown that 5-HT_{1B} receptors are present on meningeal blood vessels and 5-HT_{1B} receptor agonists act to constrict these vessels (see Hamel, 1999). During a migraine attack the cranial vessels are dilated (Pascual, 1998), and therefore the constricting action of 5-HT_{1B} receptor

agonists is thought to be important in the efficacy of these antimigraine drugs. However, the selective 5-HT_{1D} receptor agonist PNU-109291 has been shown to play a significant role in the suppression of meningeal neurogenic inflammation and trigeminal nociception in guinea-pig models (Cutrer *et al.*, 1999), suggesting that this action at 5-HT_{1D} receptors may also play a therapeutic role in the treatment of migraine with 5-$HT_{1B/1D}$ receptor agonists.

1.2.1. 3 – 5-HT_7 receptors

The 5-HT_7 receptor is the most recently identified 5-HT receptor, and some functional responses which have been documented over the years, have since been attributed to this receptor. For example, it is now clear that the 5-HT_7 receptor is the orphan receptor originally described as the '5-HT_1-like' receptor mediating relaxation of the guinea-pig ileum and cat saphenous vein (Feniuk *et al.*, 1983; see Hoyer *et al.*, 2002). The 5-HT_7 receptor has been cloned from the rat, mouse, guinea-pig and human cDNA, and is thought to be the mammalian homologue of the 5-HT_{drol} receptor identified in the fruitfly, *Drosophila melanogaster* (Witz *et al.*, 1990). The 5-HT_7 receptor shows high interspecies homology (>90%; To *et al.*, 1995), but shares a low homology with other members of the 5-HT family (<50%).

1.2.1.3. 1 – 5-HT_7 receptor pharmacology

The 5-HT_7 receptor is characterised by high affinity for the prototypical 5-HT_1 agonists 5-CT, 5-MeOT and 8-OH-DPAT (see Table 1.4; To *et al.*, 1995; Wood *et al.*, 2000), and the atypical antipsychotics, such as clozapine (To *et al.*, 1995), however to date, no selective 5-HT_7 receptor agonist has been identified. The high affinity of 5-HT_7 receptors

for the archetypal 5-HT_{1A} receptor agonist, 8-OH-DPAT, means that many 8-OH-DPAT-induced responses originally attributed to 5-HT_{1A} receptors, such as the hypothermic response, may actually be due to 5-HT_7 receptor modulation as well/instead. Recent studies using fast cyclic voltammetry or measuring *in vitro* [^{3}H]5-HT release in rat cortical and dorsal raphé slices have failed to provide any evidence for an autoreceptor role for 5-HT_7 receptors (Roberts *et al.*, 2001a), however evidence from studies measuring electrically induced efflux of [^{3}H]5-HT from rat midbrain slices have suggested that 5-HT_7 receptors may be involved in regulating 5-HT release in the dorsal and median raphé (Harsing *et al.*, 2001). It has also been shown that co-perfusion of WAY-100635 and SB-269970 increases 5-HT efflux to a greater extent than when perfused alone (Roberts *et al.*, 2001b), thus inferring that there may be an interaction between 5-HT_{1A} and 5-HT_7 receptors in the dorsal raphé. The recent identification of the selective 5-HT_7 receptor antagonists SB-269970 (Hagan *et al.*, 2000; Lovell *et al.*, 2000) and SB-656104 (Thomas *et al.*, 2003) may aid in clarifying any confusion over 5-HT_7 and 5-HT_{1A} mediated responses, and these antagonists have recently been used to demonstrate that supraspinal 5-HT_7 receptors are involved in the control of micturition (Read *et al.*, 2003). Therefore it is possible that the facilitation of micturition observed with intracerebroventricular 8-OH-DPAT may partially be due to 5-HT_7 as well as 5-HT_{1A} receptor activation.

1.2.1.3. 2 – 5-HT_7 receptor distribution

The 5-HT_7 receptor exhibits a distinct distribution in the CNS, and autoradiographical studies have identified relatively high 5-HT_7 receptor expression in the thalamus, hypothalamus and hippocampus, with generally lower levels in areas such as the cerebral

cortex, amygdala, substantia nigra, periaqueductal grey and septum (To *et al.*, 1995; Waeber *et al.*, 1995; Gustafson *et al.*, 1996; Stowe *et al.*, 1998). Immunohistochemical studies confirmed these autoradiographic observations and in addition provided evidence for the existence of 5-HT_7 receptors in the dorsal raphé (see Roberts *et al.*, 2001c). *In situ* hybridisation studies have identified 5-HT_7 receptor mRNA in the hippocampus, hypothalamus, thalamus, amygdala, dorsal and median raphé nuclei, cortex and superior colliculus (Shen *et al.*, 1993; To *et al.*, 1995; Gustafson *et al.*, 1996; Heidmann *et al.*, 1998). These results show that in rat and guinea-pig brain, both 5-HT_7 receptor mRNA and 5-HT_7 receptor binding sites display a similar distribution, suggesting that the receptor is expressed close to the site of synthesis, and hence the receptors may act as autoreceptors on 5-HT-containing neurones. However, currently there is contrasting evidence in establishing whether 5-HT_7 receptors have an autoreceptor role. For example, the 5-HT_7 receptor antagonist SB-269970 has no effect on 5-HT release in the dorsal raphé, nor does it attenuate 5-CT or 8-OH-DPAT induced inhibition of 5-HT efflux in this region (Roberts *et al.*, 2001a), thus providing no evidence for an autoreceptor role. However, other similar studies using SB-258719 have suggested that 5-HT_7 receptors, as well as 5-HT_{1A} and 5-$HT_{1B/1D}$ receptors, may also be involved in somatodendritic 5-HT release in the raphé nuclei (Harsing *et al.*, 2001). Additional investigation with selective 5-HT_7 receptor agonists is required to elucidate an autoreceptor role. Interestingly, the cellular localisation of rat hypothalamic 5-HT_7 receptors has been suggested to be postsynaptic with respect to 5-HT-containing neurones, and regulated by altered synaptic levels of endogenous neurotransmitter (Clemett *et al.*, 1999).

1.2.1.3. 3 – 5-HT$_7$ receptor second messengers and functional responses

Both recombinant and native 5-HT$_7$ receptors have been demonstrated to positively modulate cAMP formation by stimulating adenylyl cyclase (Bard *et al.*, 1993; Lovenberg *et al.*, 1993a; Ruat *et al.*, 1993; Shen *et al.*, 1993; Tsou *et al.*, 1994), and it is thought that this occurs through coupling to G_s (Obosi *et al.*, 1997). However, it should be noted that in recombinant systems, artificial expression of the 5-HT$_7$ receptor has shown that the receptor can also activate the G_s insensitive isoforms of adenylyl cyclase, AC1 and AC8, as well as the G_s sensitive isoform AC5 (Baker *et al.*, 1998). 5-HT$_7$ receptors have also been demonstrated to activate the mitogen-activated protein kinase, ERK, in primary neuronal cultures (Errico *et al.*, 2001).

Due to the relatively recent discovery of 5-HT$_7$ receptors, few functional and physiological responses have been unequivocally attributed to these receptors other than in micturition (Read *et al.*, 2003), in the control of cardiovascular reflexes (Kellett *et al.*, 2003; Kellett *et al.*, 2004) and in the control of REM sleep (Thomas *et al.*, 2003). The original physiological role attributed to these receptors was in the regulation of circadian rhythms (Lovenberg *et al.*, 1993b) and it has been demonstrated that 8-OH-DPAT induced phase shifts in behavioural circadian rhythms are actually mediated by the 5-HT$_7$ receptor and not the 5-HT$_{1A}$ receptor as previously thought, since WAY-100635 is unable to block this response, but it is blocked by ritanserin (a 5-HT$_7$ receptor antagonist; Ying *et al.*, 1997; see Barnes *et al.*, 1999). Circadian rhythms are also disrupted during depression and so drugs acting at this receptor are also potential antidepressants. Further, support for a possible role of 5-HT$_7$ receptors in the pathophysiology of affective disorders stems from the observation that atypical antipsychotics and antidepressants,

such as clozapine, have high affinity for the 5-HT_7 receptor (Roth *et al.*, 1994). Furthermore, a down-regulation of 5-HT_7 receptors occurs after chronic SSRI antidepressant treatment (Sleight *et al.*, 1995; Mullins *et al.*, 1999). 5-HT_7 receptors are also thought to be involved in the regulation of 5-CT-induced hypothermia in guinea-pigs as the response was blocked by both SB-269970 and the nonselective 5-HT_7 receptor antagonist, metergoline (Hagan *et al.*, 2000).

All the evidence reviewed above suggests that 5-HT_7 receptors may act as both auto- and heteroreceptors, and that they also show a similar distribution to 5-HT_{1A} receptors. Therefore, it is possible that both 5-HT_{1A} and 5-HT_7 receptors may have similar roles, at least in the control of micturition and on cardiovascular reflexes, despite their opposing actions on adenylyl cyclase. However, precisely how and where these two 5-HT receptor systems interact remains to be determined.

1.2.2 – Adaptive changes to 5-HT neurotransmission

Due to the importance of 5-HT signalling in so many physiological processes, it is perhaps not surprising that there is a large amount of evidence suggesting that both pharmacological and genetic interference with the 5-HT system leads to a variety of adaptive changes in 5-HT neurotransmission. This plasticity in the 5-HT system has been demonstrated in a number of mice with knock out mutants for various 5-HT receptors/transporters (see 1.2.2.2) and also after chronic administration of various 5-HT ligands (see 1.2.2.3) and has important implications on the clinical efficacy of drugs that interfere with central 5-HT transmission (such as antidepressants – see 1.2.2.1).

1.2.2.1 – The mode of action of selective serotonin reuptake inhibitors and adaptive changes in 5-HT receptors.

Selective serotonin (5-HT) reuptake inhibitors (SSRIs) are believed to exert their antidepressant action by enhancing central 5-HT function (see Stahl, 1998). However, it generally takes 2-3 weeks for the clinical efficacy of SSRIs to develop (see Hjorth *et al.*, 1996). This is thought to be due to the initial indirect activation of raphé somatodendritic 5-HT_{1A} autoreceptors by elevated extracellular 5-HT in the raphé cell body areas after reuptake blockade, which results in decreased levels of raphé neuronal firing (see 1. 3), decreased nerve terminal release of the transmitter and hence a lack of clinical efficacy (see Artigas *et al.*, 1996). This hypothesis has been confirmed by a number of studies, which have demonstrated that the onset of clinical efficacy of SSRIs can be enhanced by co-administration with a compound with 5-HT_{1A} receptor antagonist properties such as pindolol (Artigas *et al.*, 1994; Blier *et al.*, 1995; Perez *et al.*, 1997; Tome *et al.*, 1997; see Artigas *et al.*, 2001) and WAY-100635 (Gartside *et al.*, 1995; Hjorth *et al.*, 1997; Invernizzi *et al.*, 1997).

It is now known that adaptive responses occurring secondarily to enhanced terminal 5-HT release, are ultimately responsible for the onset of the antidepressant effects of SSRIs. Such delayed pharmacological actions of SSRIs include desensitization of 5-HT_{1A} (Blier *et al.*, 1990a; Blier *et al.*, 1994; Invernizzi *et al.*, 1994; Le Poul *et al.*, 1995; see Artigas *et al.*, 1996) and 5-HT_{2C} receptors (see Bristow *et al.*, 2000). The desensitization of 5-HT_{1A} receptors reduces the efficacy of the negative feedback control of raphé firing (see 1. 3), thus the 5-HT mediated neurotransmission is increased, and in the continuous presence of an SSRI, enhanced extracellular 5-HT levels are observed in terminal regions (Bel *et al.*, 1993; see Stahl, 1998). However, it must be noted that other studies failed to observe

such effects, despite using large doses of SSRIs (Hjorth *et al.*, 1994; Auerbach *et al.*, 1995; Invernizzi *et al.*, 1995).

1.2.2.2 – Compensatory changes in serotonergic knock out mice

The recent development of knock out (KO) mice lacking various receptors/transporters involved in 5-HT neurotransmission has emphasised the importance of 5-HT signalling in many physiological processes, as many compensatory changes occur to try and maintain 'normal' 5-HT neurotransmission in these KO animals. For example KO mice lacking the 5-HT transporter (5-HTT) show marked alterations in 5-HT_{1A} (Fabre *et al.*, 2000; Li *et al.*, 2000) and 5-HT_{1B} (Fabre *et al.*, 2000) autoreceptor densities with brain region specific decreases (for example in the dorsal raphé nuclei, hypothalamus and amygdala) thought to underlie the desensitization of 5-HT_{1A} receptors in these animals resulting from the lack of 5-HT reuptake in the cell body regions. Similarly, in monoamine oxidase-A (MAO-A) KO mice, the enhancement of extracellular 5-HT levels induces down-regulation of the 5-HTT, and a desensitzation of 5-HT_{1A} autoreceptors which allows the maintenance of tonic activity of 5-HT neurones in the dorsal raphé nucleus (Evrard *et al.*, 2002). The compensatory changes in the levels of 5-HT_1 autoreceptors in these animals confirm the important role that these receptors play in regulating 5-HT neurotransmission. This is also shown in 5-HT_{1A} and 5-HT_{1B} receptor KO mice, where there appears to be crosstalk between these two receptor subtypes, at least in brain regions where they are co-localised to the same neurones, such as the dorsal raphé nuclei (Ase *et al.*, 2002), since it appears that in 5-HT_{1B} receptor KO mice there is a compensatory decrease in the efficiency of G-protein coupling to 5-HT_{1A} receptors as measured by 8-OH-DPAT induced stimulation of guanosine-5′-*O*-(3-[^{35}S]thio)-

triphosphate ([^{35}S]GTPγS) incorporation into these areas (Ase *et al.*, 2002). In 5-HT_{1B} receptor KO mice there is no change in 5-HT_{1A} receptor agonist radioligand binding (i.e. receptor density; Ase *et al.*, 2001; Ase *et al.*, 2002), but the generalised compensatory decrease in the efficiency of G-protein coupling to the 5-HT_{1A} receptor (Ase *et al.*, 2002) could explain the decreased responsiveness to 8-OH-DPAT observed in the hippocampus of 5-HT_{1B} receptor KO mice (Knobelman *et al.*, 2001). Decreases in the density of the 5-HTT were also found in various brain regions of 5-HT_{1B} receptor KO mice (Ase *et al.*, 2001), although increases were observed in the amygdalo-hippocampal nucleus and ventral hippocampus which has been shown to be due to 5-HT hyper-innervation (Ase *et al.*, 2001), and is thought to play a role in the increased aggressiveness of these animals.

In 5-HT_{1A} receptor KO mice, the loss of the 5-HT_{1A} autoreceptor-mediated negative feedback control of raphé neurones leads to a significant enhancement (~90%) of the basal rate of 5-HT neuronal firing (Richer *et al.*, 2002), although this was measured in chloral hydrate anasethetised animals and therefore it is possible that this observation could be complicated by anaesthetic effects. Surprisingly, however, the extracellular levels of 5-HT in projection areas of 5-HT_{1A} receptor KO mice are unaltered as measured by microdialysis (He *et al.*, 2001; Knobelman *et al.*, 2001) and a brain slice superfusion model (Richer *et al.*, 2002). This surprising observation was originally thought to be due to a compensatory increase in the function of the 5-HT_{1B} autoreceptor (see Ramboz *et al.*, 1998; Gross *et al.*, 2000), however more recent studies using quantitative autoradiography (Ase *et al.*, 2001) and 5-HT_{1B} receptor ligand modulation of electrically evoked [^{3}H]5-HT release from pre-loaded brain slices from 5-HT_{1A} -/- mice (Richer *et al.*, 2002), have suggested that there are no compensatory changes in the efficacy of

5-HT_{1B} receptors in 5-HT_{1A} KO mice. This could be regarded as surprising, especially considering the crosstalk observed between 5-HT_{1A} and 5-HT_{1B} receptors in 5-HT_{1B} receptor KO animals, however since extracellualr 5-HT levels remain unaltered in 5-HT_{1A} receptor KO mice, there clearly must be some compensatory mechanism occurring to compensate for the increased levels of 5-HT molecules being released after increased 5-HT neuron firing. One possibility is that the 5-HTT is up-regulated to compensate for increased synaptic 5-HT levels, however this is not supported by various studies which have shown a normal density and distribution (Heisler *et al.*, 1998) and function (Richer *et al.*, 2002) of the 5-HTT in 5-HT_{1A} receptor KO mice. Further studies are required to determine what compensatory mechanisms occur to allow the maintenance of such an apparent homeostasis in the level of 5-HT released in 5-HT_{1A} receptor KO mice.

Interestingly, in 5-HT_{2C} receptor KO mice the 5-HT system does not exhibit any compensatory up- or down-regulation of the 5-HTT or 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_4 and 5-HT_7 receptors (Lopez-Gimenez *et al.*, 2002). When compared with the compensatory changes observed in the 5-HT systems of 5-HT_{1A} and 5-HT_{1B} receptor KO animals, these results show the relative lack of importance of 5-HT_{2C} receptors in the control of 5-HT neurotransmission in comparison with the vital function of 5-HT_1 receptors.

1.2.2.3 – Adaptive changes to 5-HT signalling after pharmacological intervention

Many compensatory changes in 5-HT neurotransmission are found in KO mice, however the presence of these adaptive changes could be explained by developmental alterations

resulting from altered levels of 5-HT during early development, and therefore of little importance in genetically "normal" animals. However, there are also many examples of compensatory changes occurring after chronic pharmacological interference in 'normal' animals, indicating that the 5-HT system is highly plastic and capable of adaptive changes which are not confined to early development. For example, pharmacological investigations have shown that 5-HT_{1A} autoreceptors in adult rats are down-regulated after chronic treatment with MAO-I (Blier *et al.*, 1985) or SSRIs (Le Poul *et al.*, 2000), and in addition a similar but more pronounced down-regulation of these autoreceptors is observed in 5-HTT (Fabre *et al.*, 1998; Fabre *et al.*, 2000) and MAO-A (Evrard *et al.*, 2002) knock out mice. Thus, the down-regulation of 5-HT_{1A} autoreceptors found in the knock out animals probably results from their tonic stimulation by excess extracellular 5-HT, rather than from some developmental adaptation.

Further examples of adaptive changes after chronic pharmacological interference include the brain region specific changes (both up- and down regulation depending on the area) in the density of 5-HT_{1A} receptors and 5-HTT after chronic clozapine administration (Ase *et al.*, 1999), desensitization of 5-HT_{1A} receptors after 14 days administration of the 5-HT_{1A} receptor agonists flesinoxan (Haddjeri *et al.*, 1999) and alnespirone (Casanovas *et al.*, 1999). Desensitization of 5-HT_{1A} receptors is also observed after chronic administration of SSRIs (see 1.2.2.1)

Given the obvious plasticity of the 5-HT system after pharmacological and genetic interference, it is surprising that studies undertaken to assess the effect of prolonged blockade of 5-HT_{1A} receptors on their own, or in combination with SSRI administration, failed to demonstrate any apparent change in somatodendritic 5-HT_{1A} autoreceptor

sensitivity (Hervas *et al.*, 2001; Dawson *et al.*, 2002). A number of clinical trials have been conducted to examine the effect of combined administration of SSRIs with pindolol, however conflicting results have been obtained, with both some open-label and double-blind placebo controlled trials suggesting little acceleration in the onset time of antidepressant activity after co-administration (Blier *et al.*, 1997; Perez *et al.*, 1997; Berman *et al.*, 1999). Various reasons have been purported for these observations including, variations in the pharmacokinetics of the SSRIs used (Plenge *et al.*, 2003), too low a dosage of pindolol (Rabiner *et al.*, 2001) and problems with the complexity of pindolol acting as a partial agonist at 5-HT_{1A} receptors (Artigas *et al.*, 2001). For these reasons, combined with the potentially fatal consequences of a pindolol overdose (see Olver *et al.*, 2000), drug companies are now looking for more selective 5-HT_{1A} receptor antagonists to use in combination with SSRIs for the enhanced treatment of depression. However, to the author's knowledge there has been no publication of a clinically efficacious trial of 5-HT_{1A} receptor antagonist induced augmentation/early onset of antidepressant effects despite approximately 8 years passing since trials involving pindolol were conducted. It is tempting to speculate that in a clinical setting there is a problem with acceleration of antidepressant effects after 5-HT_{1A} receptor blockade, whether this be due to a lack of augmentation or perhaps clinical relapse after removal of the prolonged blockage of 5-HT_{1A} receptors. Indeed, should prolonged blockade of 5-HT_{1A} receptors result in receptor sensitisation, the withdrawal of the antagonist would increase the efficacy of the 5-HT_{1A} mediated negative feedback control of raphé neuron firing and hence reduce the ability of the SSRI to increase extracellular 5-HT, thus increasing the possibility of a clinical relapse. Interestingly, a recent 8-week clinical trial investigating the use of chronic robalzotan (a 5-HT_{1A} receptor antagonist) in the treatment of depression have shown that this compound lacks antidepressant efficacy

(Ybema *et al.*, 2003) the reason for which is currently unexplained, but it is tempting to speculate that the prolonged blockade of 5-HT_{1A} receptors is resulting in receptor sensitization.

1. 3 – Regulation of transmitter release from 5-HT containing neurones

The central 5-HT system has its centre in clusters of 5-HT-containing cell bodies, known as the raphé nuclei, which are located in the brain stem. From the raphé nuclei there is a distribution of both ascending and descending axonal projections. The ascending projections reach a variety of higher centres of supraspinal control such as the cortex, amygdala, striatum, thalamus, hypothalamus, hippocampus and substantia nigra, whereas the descending axons project to the brain stem and the spinal cord. The ascending 5-HT projections originate almost entirely from the median and dorsal raphé nuclei (MRN and DRN respectively), and although most forebrain regions receive projections from both these nuclei, some structures are preferentially innervated by one or the other, for example, the frontal cortex from the DRN and the dentate gyrus from the MRN (Kosofsky *et al.*, 1987). The DRN contains approximately half of all the brain's 5-HT containing neurones, and the activity of DRN and MRN neurones is controlled mainly by 5-HT uptake sites (i.e. the 5-HT transporter; 5-HTT), 5-HT_1 autoreceptors and 5-HT_7 receptors.

5-HT_1 autoreceptors control both the release of 5-HT in the CNS and/or the activity of 5-HT-containing neurones, and both protein and mRNA localisation data have shown these autoreceptors to be widely distributed within the CNS, including the raphé nuclei. These, inhibitory 5-HT autoreceptors fall into two categories: somatodendritic (or cell

body) autoreceptors and terminal autoreceptors. In addition to their location, these autoreceptors also differ in their pharmacology and in the mechanism through which they inhibit 5-HT release. Somatodendritic autoreceptors inhibit 5-HT release mainly through inhibition of neuronal firing, whereas terminal autoreceptors mediate a direct inhibition of release at the terminal. There are currently three known pharmacologically and functionally distinct 5-HT autoreceptors: 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptors, although 5-HT_{1F}, 5-HT_{5A} and 5-HT_7 receptors remain as potential candidates for additional autoreceptors (see Roberts *et al.*, 2001c).

1.3.1 – 5-HT autoreceptors in the raphé nuclei

In the dorsal and median raphé cell bodies, 5-HT autoreceptors control both the rate of neuronal firing and the release of 5-HT in the raphé nuclei. Until recently, it was thought that somatodendritic 5-HT_{1A} autoreceptors controlled serotonergic firing in the DRN and MRN, whereas 5-$HT_{1B/1D}$ receptors exerted local control of 5-HT release at the terminals. However, there is mounting evidence to suggest that this is an oversimplication and potentially misleading, since it is probable that 5-HT_{1D} receptors also act as somatodendritic autoreceptors involved in the local control of 5-HT release, and 5-HT_{1B} receptors may also be involved (see Stamford *et al.*, 2000). This raises the possibility that neuronal firing and 5-HT release are differentially controlled by particular 5-HT_1 receptor subtypes. Furthermore, it is becoming clear that there are differences between the DRN and MRN in autoreceptor control, with 5-HT_{1B} receptors appearing to play a more important role in the MRN compared to the DRN (see later), thus further increasing the complexity of the system (Adell *et al.*, 2001; Hopwood *et al.*, 2001).

1.3.1.1 – Raphé 5-HT neuronal firing

The firing activity of 5-HT-containing neurones in the DRN and MRN is primarily under 5-HT_{1A} receptor control. The first evidence was produced by Sprouse and Aghajanian who showed that intravenous (i.v.) administration of 5-HT_{1A} receptor agonists (e.g. ipsapirone) but not 5-HT_{1B} receptor agonists (e.g. trifluoromethylphenylpiperazine; TFMPP) inhibit DRN neuronal firing in the anaesthetized rat (Sprouse *et al.*, 1987). Consistent with this, Craven *et al.* (1994) reported that the 5-HT_1 receptor agonists 5-HT, 5-CT and 8-OH-DPAT decreased neuronal firing in guinea-pig DRN slices. These effects were blocked by WAY-100635 (a 5-HT_{1A} receptor antagonist) but not by GR-127935 (a 5-$HT_{1B/1D}$ receptor antagonist), thus confirming 5-HT_{1A} receptor involvement (Craven *et al.*, 1994). 5-HT_{1A} receptor agonists also inhibit neuronal firing in the MRN, but there are some reports that suggest that the 5-HT_{1A} effect is larger in the DRN than in the MRN (Sinton *et al.*, 1988; Blier *et al.*, 1990b). *In vitro* studies such as the study by Craven *et al.* (1994), and the observation that ipsapirone decreased cell firing in rat DRN slices (Haj-Dahmane *et al.*, 1991), suggests that much of this 5-HT_{1A} receptor mediated control is exerted locally, however, *in vivo*, the possibility of long feedback loops involving 5-HT_{1A} receptors located on other 5-HT neurones cannot be excluded, since it has been demonstrated that frontal cortex lesions attenuate systemic 8-OH-DPAT-induced inhibition of DRN neuronal firing (Ceci *et al.*, 1994). Furthermore, the effect of systemic 8-OH-DPAT has been shown to involve a polysynaptic loop through the medial prefrontal cortex (Hajos *et al.*, 1999; Celada *et al.*, 2001). A 5-HT_{1A} receptor-mediated feedback loop has also been demonstrated from the central nucleus of the amygdala to the caudal linear raphé nucleus (Bosker *et al.*, 1997).

Circumstantial evidence exists for the involvement of other 5-HT_1 receptors in the control of DRN neuronal firing. For example, an indirect effect of 5-$HT_{1B/1D}$ receptors on neuronal firing has been suggested following the observations that GR-127935 does not affect DRN neuronal firing in anaesthetized guinea-pigs (Sprouse *et al.*, 1997), nor in a superfused slice *in vitro* (Craven *et al.*, 1994), but RU-24969 (a mixed 5-$HT_{1B/1A}$ receptor agonist) and CP-94253 (a 5-HT_{1B} receptor agonist) both increase DRN neuronal firing in wild type, but not in 5-HT_{1B} receptor knockout mice, an effect which was blocked by GR-127935 (Evrard *et al.*, 1999). This agonist-induced increase in neuronal firing can be explained by the 5-HT_{1B} receptor agonist inhibiting 5-HT release, due to the autoreceptor role of 5-HT_{1B} receptors, thus reducing endogenous 5-HT tone and reducing the 5-HT_{1A} receptor-induced decrease in neuronal firing. Further evidence for an indirect effect of 5-$HT_{1B/1D}$ receptors on neuronal firing was demonstrated by the observation that systemic administration of GR-127935 decreased extracellular 5-HT in the frontal cortex of the freely moving guinea-pig (Roberts *et al.*, 1997b; Roberts *et al.*, 1998), despite local administration to the frontal cortex causing an increase in 5-HT levels (Skingle *et al.*, 1996). The systemic effect of GR-127935 can be reversed by WAY-100635 (Roberts *et al.*, 1999), and it was therefore hypothesised that systemic administration of 5-$HT_{1B/1D}$ receptor antagonists shut down neuronal firing by increasing tone at somatodendritic 5-HT_{1A} autoreceptors.

Recent data from Adell *et al.* (2001) reported that systemic administration of the 5-HT_{1B} receptor agonist, CP-94253, increased neuronal firing in the MRN but had no effect in the DRN. This was an indirect effect since perfusion of 5-HT_{1B} receptor agonists into the raphé decreases 5-HT levels (more marked in the MRN; Adell *et al.*, 2001). Thus this data suggests that the control of neuronal firing differs in the two raphé regions, with

5-HT_{1B} autoreceptors perhaps playing a more important role in the MRN rather than the DRN.

1.3.1. 2 – Raphé 5-HT release

In addition to controlling neuronal firing, somatodendritic 5-HT_{1A} receptors are also known to regulate raphé 5-HT release. 5-HT_{1A} receptor agonists have been shown to decrease basal 5-HT release *in vivo* in the DRN (Casanovas *et al.*, 1996; Matos *et al.*, 1996) and the MRN (Casanovas *et al.*, 2000). However, considerable evidence now suggests the involvement of additional 5-HT_1 receptor subtypes in the negative modulation of intra-raphé 5-HT release. Early *in vitro* voltammetry data using rat DRN slices, demonstrated that the 5-$HT_{1B/1D}$ receptor agonist sumatriptan, inhibits electrically evoked 5-HT release (O'Connor *et al.*, 1992). This was later duplicated in the guinea-pig DRN and was also shown to be blocked by the 5-$HT_{1B/1D}$ receptor antagonist GR-127935 (Starkey *et al.*, 1994), thus providing the first evidence for a 5-HT_1 receptor, other than the 5-HT_{1A} receptor, in the control of 5-HT efflux. Davidson and Stamford (1995) subsequently showed that GR-127935 could potentiate the effect of the SSRI, paroxetine, significantly elevating 5-HT efflux. This was attributed to the blockade of the effects of the released 5-HT activating 5-$HT_{1B/1D}$ receptors, which would usually limit further increases in 5-HT efflux (Davidson *et al.*, 1995a). Sumatriptan and GR-127935 have low affinity at 5-HT_{1A} receptors, but show appreciable affinity at both 5-HT_{1B} and 5-HT_{1D} subtypes, thus although this data provides evidence for the role of 5-HT receptors other than 5-HT_{1A}, it does not clarify whether 5-HT_{1B} or 5-HT_{1D} (or both) receptors have a role in the control of DRN 5-HT release.

However, there is *in vivo* and *in vitro* data that strongly indicates 5-HT_{1D} receptors to be involved in the control of DRN 5-HT release. For example, sumatriptan was shown to decrease locally stimulated endogenous 5-HT release in rat DRN *in vitro*, an effect which was blocked by GR-127935, but not by WAY-100635 or isamoltane (a 5-HT_{1B} receptor antagonist; Davidson *et al.*, 1995b). In another study, differential normal pulse voltammetry and nafion-coated electrodes were used to measure extracellular 5-hydroxyindole levels in the DRN of anesthesized rats, and systemic administration of 8-OH-DPAT and TFMPP (0.5 mg kg^{-1}; a non-selective 5-HT_1 receptor agonist) both reduced extracellular 5-hydroxyindole concentrations (Pineyro *et al.*, 1995b). The effect of TFMPP was abolished by pre-treatment with mianserin (an antagonist with high affinity for 5-HT_{1D} receptors) but not by WAY-100635, thus suggesting a role for 5-HT_{1D} receptors in the modulation of 5-HT release at the cell body level of 5-HT neurones. Measurement of tritium release from rat mesencephalic raphé slices pre-incubated in [^{3}H]5-HT showed that sumatriptan (1 nM – 1 μM) decreased the tritium efflux (Pineyro *et al.*, 1995b), and this effect was blocked by GR-127935 and mianserin (both 1 μM) but not by S-UH-301 (a 5-HT_{1A} receptor antagonist). As sumatriptan has limited selectivity for 5-HT_{1D} receptors, it must be ensured that its actions are not mediated via 5-HT_{1B} sites. This was confirmed by Pineyro *et al.* (1995) who showed that sumatriptan could inhibit 5-HT release in midbrain slices of both wild type and 5-HT_{1B} receptor knock-out mice. This effect was blocked by GR-127935 in the DRN but not in terminal areas (Pineyro *et al.*, 1995a). Collectively, this data suggests that, in addition to 5-HT_{1A} receptors, 5-HT_{1D} receptors are autoreceptors in the DRN, and although they do not appear to influence neuronal firing, they are clearly involved in the negative modulation of somatodendritic release of 5-HT in the DRN. However, as mentioned previously, there is evidence that

the 5-HT receptors which modulate 5-HT release differ between the DRN and MRN. This was first suggested by Roberts *et al.* (1998) who demonstrated indirect inhibition of 5-HT release in the frontal cortex by mixed 5-$HT_{1B/1D}$ and non-selective 5-HT_{1B} receptor antagonists. However, the idea is also supported by data which shows that although the effects of the selective 5-HT_{1B} receptor antagonist SB-216641 are clear in the MRN, effects of the 5-HT_{1D} receptor antagonist BRL-15572 are not observed (Hopwood *et al.*, 2001). Therefore, this data suggests that 5-HT release is modulated by 5-HT_{1D} receptors in the DRN, but not in the MRN.

The role of 5-HT_{1B} receptors in the control of 5-HT release in the DRN is an area of much debate. Initial studies using microdialysis in guinea-pigs showed that the selective 5-HT_{1B} receptor antagonists (SB-220272, SB-224289 and SB-216641) do not modify frontocortical release (Roberts *et al.*, 1997a), although the 5-$HT_{1B/1D}$ receptor antagonist GR-127935 caused significant increases in the cortical extracellular level of 5-HT in guinea-pigs as measured by microdialysis (Skingle *et al.*, 1995). This led to the suggestion that DRN 5-HT release was modulated by 5-HT_{1D} but not 5-HT_{1B} receptors. However, by contrast, another microdialysis study in guinea-pig DRN showed that, when applied locally, the 5-$HT_{1B/1D}$ receptor agonist naratriptan decreased 5-HT release (Moret *et al.*, 1997). This was unaffected by WAY-100635 or ketanserin (which shows higher affinity for 5-HT_{1D} than 5-HT_{1B} receptors). Similarly, locally applied methiothepin causes an increase in DRN 5-HT release, whilst neither sumatriptan nor GR-127935 had any effect (Hervas *et al.*, 1998). These data suggest a possible modulation of DRN 5-HT release by 5-HT_{1B} receptors. *In vitro* studies have also been used to try and elucidate the role of 5-HT_{1B} receptors, and have resulted in conflicting results. Initially, Pineyro *et al.* (1995a and 1995b) showed that sumatriptan decreased evoked tritium release from

preloaded rat (Pineyro *et al.*, 1995b) and mouse (Pineyro *et al.*, 1995a) midbrain slices, but they failed to detect an inhibitory response to CP-93129 (a selective 5-HT_{1B} receptor agonist), despite observing inhibition of tritium release with similar concentrations of CP-93129 in hippocampal slices (Pineyro *et al.*, 1995b), and were therefore unable to confirm a role for 5-HT_{1B} receptors in the control of DRN 5-HT release. However, it has since been suggested that this absence of evidence might result from methodological drawbacks (see Stamford *et al.*, 2000) with the Pineyro studies. Davidson and Stamford (1995) have since shown using more anatomically precise and physiologically less extreme conditions, that in the rat DRN 5-HT efflux is decreased by CP-93129 and this effect can be antagonized by the selective 5-HT_{1B} receptor antagonist isomoltane, but not by WAY-100135. Recently it has also been shown that the effect of CP-93129 on DRN 5-HT efflux is blockable by the 5-HT_{1B} receptor antagonist SB-216641, but not by the 5-HT_{1D} receptor antagonist BRL-15572 (see Stamford *et al.*, 2000). Therefore, it appears that 5-HT_{1B} receptors also play an important role in the control of 5-HT release in the DRN, and that much of the controversy surrounding this idea results from the initial lack of ligands with specificity at 5-HT_{1B} versus 5-HT_{1D} receptors, which has made their pharmacological differentiation difficult. The recent and continuing identification of selective 5-HT receptor subtype ligands will aid with the future functional characterisation of the receptors involved in the autoregulation of 5-HT release in the DRN.

In conclusion, it appears that multiple 5-HT_1 subtypes exist in the raphé nuclei, and it is apparent that neuronal firing and 5-HT release might be independently governed by individual subtypes of 5-HT_1 autoreceptors depending on the location of the 5-HT neurone (i.e. MRN or DRN). Neuronal firing appears to be mediated mainly, if not

completely, by 5-HT_{1A} receptors in both the DRN and MRN. Whereas, 5-HT release is influenced by 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptors in the DRN, but only by 5-HT_{1A} and 5-HT_{1B} receptors in the MRN.

1.3. 2 – 5-HT autoreceptors in terminal regions

There is a large amount of evidence indicating that 5-HT_{1B} receptors regulate 5-HT release in areas such as the hippocampus, cortex (Buhlen *et al.*, 1996), inter geniculate nucleus and striatum in various species (Hoyer *et al.*, 1989b; Radja *et al.*, 1991; Schlicker *et al.*, 1997). However, recently several studies have raised the possibility of multiple terminal 5-HT autoreceptors. *In vitro* studies measuring [^{3}H]5-HT release have suggested the presence of both 5-HT_{1B} and 5-HT_{1D} terminal autoreceptors in the rat (Limberger *et al.*, 1991; Davidson *et al.*, 1996) and guinea-pig brain (Wilkinson *et al.*, 1992; Roberts *et al.*, 1996), and studies using SB-216641, a selective 5-HT_{1B} receptor antagonist, and BRL-15572 have demonstrated that both 5-HT_{1B} and 5-HT_{1D} receptors control the release of 5-HT in the inter geniculate nucleus (Davidson *et al.*, 1996). *In vivo* microdialysis data has demonstrated that systemic administration of 5-$HT_{1B/1D}$ receptor antagonists, such as SB-236057, are able to attenuate sumatriptan-induced inhibition of 5-HT release (Roberts *et al.*, 2000). In addition, perfusion of 5-$HT_{1B/1D}$ receptor antagonists directly into the terminal regions produces an increase in extracelluar 5-HT levels (Roberts *et al.*, 1997b). However, further studies using the recently identified selective 5-HT_{1B} and 5-HT_{1D} receptor ligands are required to further characterise 5-HT_1 autoreceptor subtypes in terminal brain regions.

1.3.3 – Location of 5-HT$_1$ autoreceptors in the DRN

All of the above evidence implicates the presence of 5-HT$_{1A}$, 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors in the DRN, however these autoreceptor subtypes are not necessarily located on the same neuronal structures. For example, they could be located on the cell body, on dendrites or on axon collaterals, since it is known that the DRN contains many serotonergic axon terminals due to collateral innervation and intra-raphé connectivity (Chazal *et al.*, 1987). It is important to know the location of each autoreceptor subtype as this has obvious implications on the function of the receptor, although ascribing a particular subcellular location to each receptor subtype is difficult.

5-HT$_{1A}$ autoreceptors are known to be somatodendritic in their location, and there are a large number of studies including immunocytochemical (Sotelo *et al.*, 1990), *in situ* hybridisation (Chalmers *et al.*, 1991), receptor autoradiography (Chalmers *et al.*, 1991) and immunogold electron microscopy (Riad *et al.*, 2000) studies confirming that 5-HT$_{1A}$ receptors are localized to the soma and dendrites of DRN neurones. Studies using 5,7-DHT lesions almost eliminated the *in situ* hybridisation signal (Miquel *et al.*, 1992), indicating that 5-HT$_{1A}$ receptor mRNA was expressed in 5-HT-containing neurones, and hence also implicating a presynaptic location for these receptors. A second population of 5-HT$_{1A}$ receptors are found throughout the dendritic field and modulate release of 5-HT.

The location of 5-HT$_{1B}$ receptors is thought to be restricted to terminal regions and, in particular, to pre-terminal axons since immunocytochemical studies have shown little 5-HT$_{1B}$-like immunoreactivity in the DRN (Sari *et al.*, 1999; Riad *et al.*, 2000). This suggests that 5-HT$_{1B}$ receptors might have a role in regulating the release of transmitters

through 'paracrine' actions of 5-HT. Given that there is much data to suggest a terminal localization for 5-HT_{1B} receptors (Pineyro *et al.*, 1995a; Pineyro *et al.*, 1995b), but not a somatodendritic location, it appears that 5-HT_{1B} receptors are located upon terminals. These might be either afferent terminals from other brainstem 5-HT-containing neuron groups such as the MRN, or axon collaterals of DRN 5-HT neurones (see Figure 1.7). There is also evidence to suggest that there may be presynaptic 5-HT_{1B} receptors located on afferent glutamatergic terminals (Li *et al.*, 1998).

There is little information on the localization of 5-HT_{1D} receptors in the DRN due a lack of selective ligands. However, there is indirect evidence for a somatodendritic location since 5-HT_{1D} receptor agonists reduced the duration of suppression of 5-HT neuron firing (a 5-HT_1 autoreceptor mediated response) induced by the 5-HT-pathway stimulation of the ventromedial tegmentum (Pineyro *et al.*, 1996). Because, both *in vitro* and *in vivo*, 5-HT_{1D} receptor agonists do not have any effect on neuronal firing, but the 5-HT_{1D} receptor is known to be involved in modulating DRN 5-HT release, it has been suggested that the 5-HT_{1D} receptors may only be located on the dendrites, in particular the distal dendrites (see Stamford *et al.*, 2000).

5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptors show similar affinities for their natural ligand, 5-HT, and although there are differences in the coupling of these receptors to second messengers in model systems, it is unclear whether these differences occur to the same extent in native tissues. Therefore, initially there appears little need for multiple 5-HT_1 receptor subtypes in the DRN. However, one explanation could be if the receptors are differentially located, hence enabling independent regulation of 5-HT cell firing and 5-HT release (see Stamford *et al.*, 2000). Figure 1.7 shows the possible location of

5-HT_1 receptors in the DRN assuming that only 5-HT_{1A} receptors control 5-HT neuronal firing, 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptors all modulate 5-HT release and that only 5-HT_{1A} and 5-HT_{1D} receptors are somatodendritic. In this scheme, 5-HT_{1A} receptors are located on the soma and dendrites and it is possible that the 5-HT_{1A} receptors controlling neuronal firing may be preferentially located in the soma and proximal dendrites and centred around the axon hillock, which is an area involved in action potential generation, and known to show 5-HT_{1A} receptor immunoreactivity in brainstem motorneurones (Azmitia *et al.*, 1996). 5-HT_{1A} receptors involved in the control of 5-HT release are distributed in the wider dendritic field. It is possible that 5-HT_{1A} receptors in these two sites might be coupled to different G proteins and second messenger systems, and therefore also possible that drugs may affect the two loci differently. 5-HT_{1D} receptors appear to control 5-HT release but not neuronal firing, and as noted before, the localization of these receptors is thought to be somatodendritic (Pineyro *et al.*, 1996) although a role in controlling 5-HT release would suggest localization primarily on the distal dendrites (see Stamford *et al.*, 2000). 5-HT_{1B} receptors are found on three putative loci: serotonergic afferents and axon collaterals arising from other nuclei of the raphé complex, glutamatergic terminals and pre-termianl portions of axons. The scheme in Figure 1.7 provides theoretical locations for 5-HT_1 receptors in the DRN based on the current available data. However, it must be noted that autoreceptor control in the MRN is different, and is still being investigated.

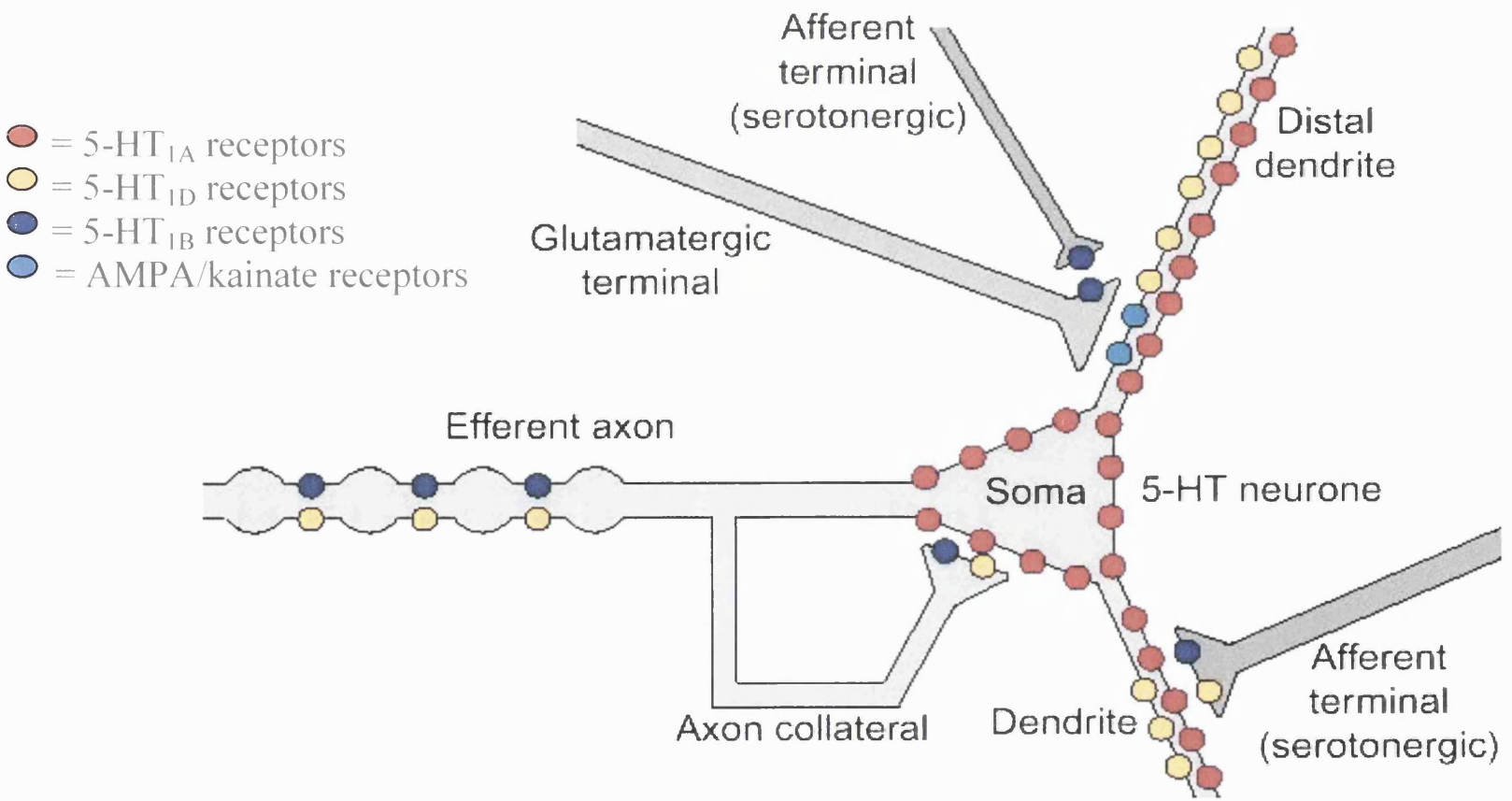


Figure 1.7 – Theoretical location of 5-HT_1 autoreceptors in the DRN. The 5-HT_{1A} receptors in the soma and proximal dendrites comtrol DRN neuronal firing, whilst the second population of 5-HT_{1A} receptors localized throughout the dendritic field modulate release of 5-HT. 5-HT_{1D} receptors are found on the dendrites and pre-terminal axons where they modulate release of 5-HT. 5-HT_{1B} receptors are found at three different loci: serotonergic afferents, glutamatergic terminals and pre-terminal portions of axons. See text for further detail. (Diagram taken from Stamford *et al.*, 2000).

1.4 – G-protein coupled receptor (GPCR) systems and the concept of constitutive activity and inverse agonism.

1.4.1 – G-protein coupled receptors: mechanism of action

G-protein coupled receptors (GPCRs) are the largest family of cell surface receptors involved in signal transduction. These membrane spanning receptors are heptahelical and possess seven transmembrane domains (TM I – TM VII), three extracellular loops and three intracellular loops. The N-terminus is located extracellularly and a ligand binding site is located on the extracelluar loops, whilst the C-terminus is located intracelluarly and the intracellular loops couple to G-proteins which regulate the activity of effector systems (Gilman, 1987; Birnbaumer *et al.*, 1990; Bockaert *et al.*, 1999). These G-proteins are heterotrimeric and consist of α, β and γ subunits, of which the α subunit binds guanine nucleotides (GDP and GTP) when the receptor is activated. The G-protein cycle has six main steps and is described in Figure 1.8. There are many types of Gα protein subunits that can couple with GPCRs (see 1.5.7), and many GPCRs couple to multiple cellular effector systems, for example 5-HT_{2C} receptors (see Berg *et al.*, 2003) therefore allowing GPCRs a wide level of control over intracellular signalling.

1.4.1.1 – Classical receptor occupancy theory and the two-state model of receptor action.

In the early 20th century, the effects of ligands on putative receptor proteins were based on models and equations used to describe mass-action kinetics of molecules and inert surfaces (the law of mass action) i.e. the Langmuir equation (Figure 1.9a), although this equation is primarily useful in finding the affinity of drugs in binding or functional studies, it was soon realised that the action of an agonist cannot be described by an

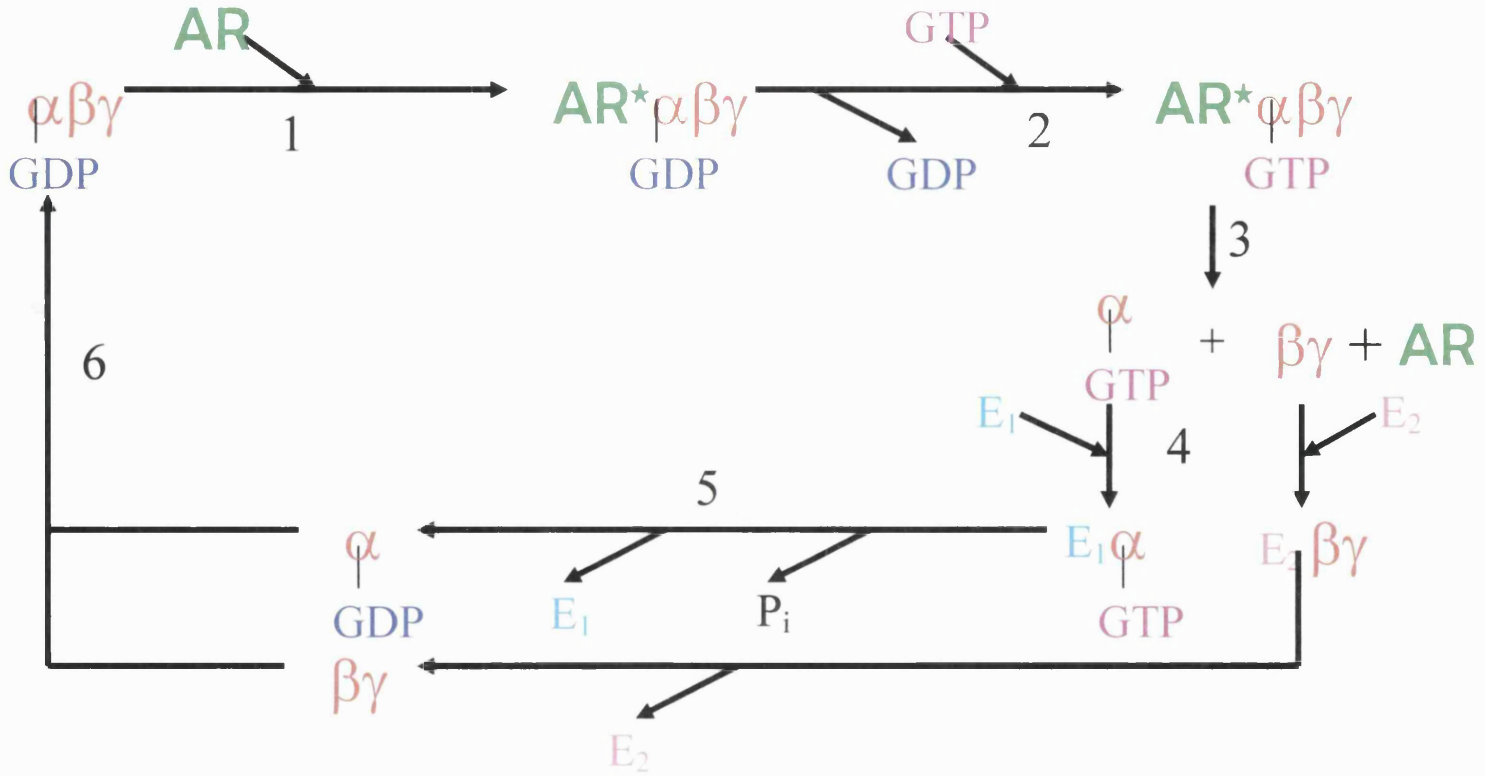


Figure 1.8 – The functional cycle of G-proteins. Step 1 is the association of the resting G-protein heterotrimer αβγ (with GDP bound to α) with the agonist-liganded receptor (AR) – binding of the G-protein to the receptor allows the receptor to take up its active state (R*). Step 2, the α subunit releases GDP and GTP binds in its place. This is the rate-limiting step in the G-protein cycle. Step 3 is the dissociation of AR*G, releasing α-GTP and βγ. Step 4 is the association of both α and Bγ to effectors (E_1, E_2 etc.) which are enzymes or ion channels involved in initiating biochemical cascades corresponding to cellular responses to the agonist. Step 5 is the hydrolysis of GTP bound to α (due to intrinsic GTPase activity of α), consequently the affinity of α for the effector is decreased and the effector (E_1) is released. The mechanism for the release of effector from βγ is unclear. Step 6 is the reassociation of the G-protein trimer, thus allowing the cycle to start again with a newly activated receptor (see Daeffler *et al.*, 2000).

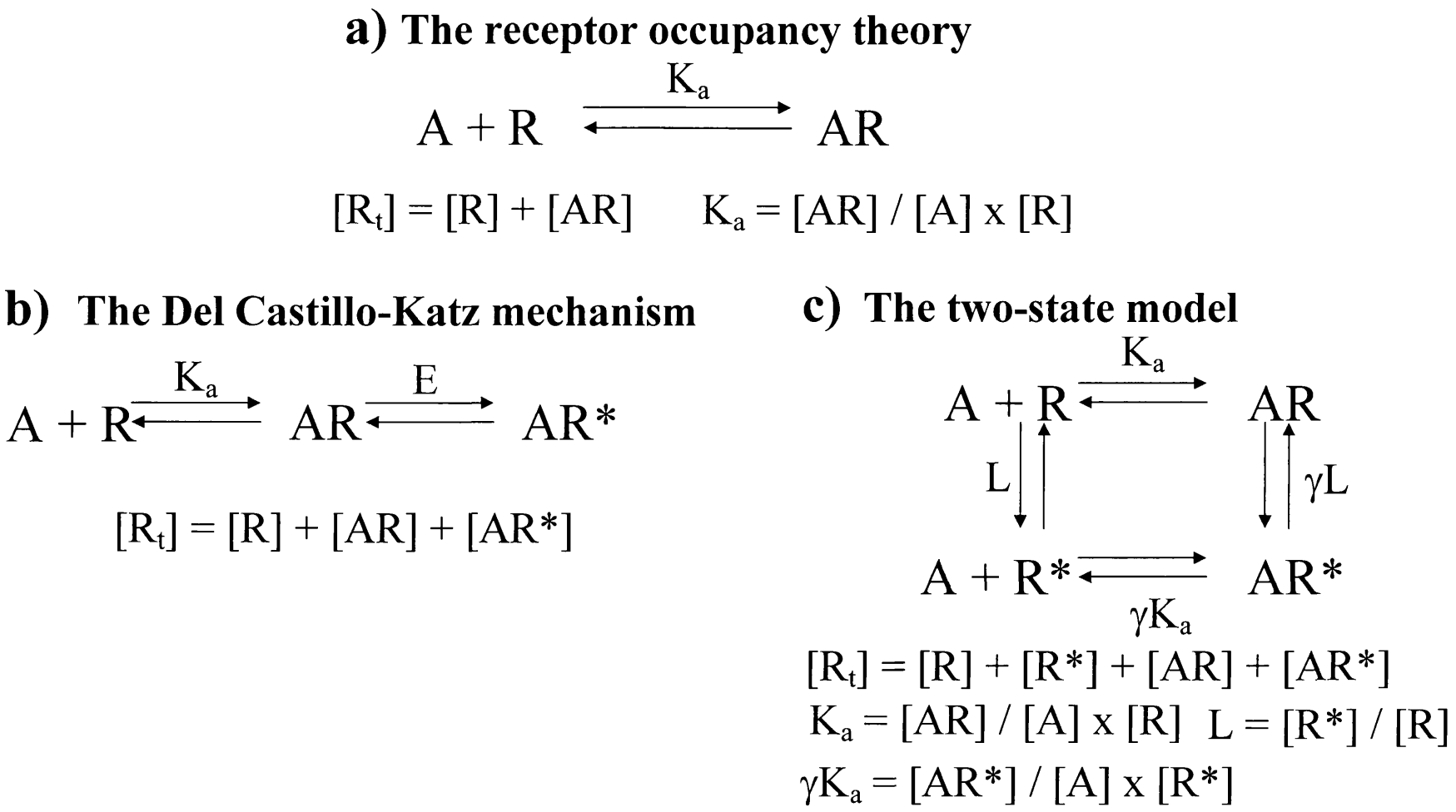


Figure 1.9 – Equations describing ligand (A)- receptor (R) interactions. $[R_t]$ is the total concentration of receptor. a) In the receptor occupancy theory, K_a is the equilibrium association constant. b) In the Del-Castillo-Katz mechanism, K_a is the equilibrium association constant for R-AR and E is the equilibrium constant for the R-R* isomerization of the receptor. c) In the two-state model the active (R*) and the inactive (R) state of the receptor co-exist. K_a is the equilibrium association constant for R-AR and γK_a the equilibrium association constant of R*-AR*, L is the equilibrium allosteric constant of R-R*, and γL the equilibrium allosteric constant of AR-AR*.

affinity constant alone. In the 1950's it became clear that some proteins could undergo conformational changes and that the ligand affinity for the two conformations might vary (Wyman, 1951), and in 1957, following work on the properties of nicotinic receptors to bind choline derivatives, Del Castillo and Katz postulated that receptors exist in a single 'quiescent' state (R) to which agonists bind inducing a conformational change of the receptor to an activated 'functional' state (R*; (Del Castillo *et al.*, 1957). Given this theory, it was believed that in the absence of an agonist, 100% of the receptor population was considered to be in the R conformation, and antagonists worked by interacting with the receptor to prevent agonist binding without inducing any conformational change themselves (i.e. the receptor remained in the R state). However, following the suggestion that co-operative enzymes can exist in inactive and active states in the absence of substrate (Monod *et al.*, 1965), it was then considered that in the absence of an agonist, GPCRs may exist in equilibrium between the R and R* states. This model is the two-state model (see Figure 1.9c) and in the R state, GPCRs are uncoupled from G-proteins whereas in the R* state, GPCRs can couple to, and activate, G-proteins (Leff, 1995). When viewed from the intracellular space, the transmembrane domains of GPCRs are arranged in a clockwise arrangement (Bockaert *et al.*, 1999), and it has been shown that rotation of TM III relative to TM VI constitutes a conformational switch for R to R* isomerization (Bockaert *et al.*, 1999) which is highly conserved among different GPCR families, such as β_2-adrenoceptors (Sheikh *et al.*, 1999). This R to R* isomerization of GPCRs can occur spontaneously, so a GPCR can adopt an active conformation in the absence of an agonist. The presence of this unliganded active state R*, enables a GPCR to show 'constitutive activity'. A ligand (A) may have distinct affinities for the two receptor states – if the ligand has a higher affinity for R* ($\gamma > 1$) then it is an agonist, and

according to the two-state model (Figure 1.9c) shifts the equilibrium towards R* (see Figure 1.10a). The conformational change in GPCRs associated with R to R* isomerization enables GPCRs to promote the dissociation of GDP from G-proteins (Figure 1.8), which is the rate limiting step in the G-protein cycle (Gilman, 1987), therefore agonists increase the basal GDP/GTP exchange rate at G-proteins and increase (or, occasionally decrease) effector system activity (Figure 1.10b). Partial agonists are less efficient than full agonists at stabilizing the R* state and are therefore unable to increase GDP/GTP exchange to the same extent, resulting in lower effector system activity. If a ligand has a higher affinity for the inactive state R ($\gamma < 1$), it shifts the prevailing equilibrium to R and reduces basal GDP/GTP exchange (see Figure 1.10a & b). Ligands of this type are known as inverse agonists since they show the opposite activity to that of agonists and they are also able to block the effects of agonists. Neutral antagonists do not alter the equilibrium between R and R* and do not change basal G-protein activity, however they block both the inhibitory effects of inverse agonists and the stimulatory effects of agonists. In most quiescent systems, equilibrium in the absence of a drug is already shifted toward R, so a drug with $\gamma < 1$ (i.e. an inverse agonist) produces no directly visible effect, but will still block the effects of agonists. This explains why most inverse agonists were originally classified as antagonists. It is highly likely that many drugs, originally identified as antagonists in the literature, may in fact show inverse agonist activity. Clearly, in a constitutively active system where the equilibrium is shifted toward R*, the effects of an inverse agonist will be more easily observed as a reduction of basal receptor activity. Therefore, many constitutively active mutants (CAM) of GPCRs are being developed and expressed in cell lines, which allows further investigation into the 'antagonistic' properties of particular ligands on these GPCRs, for

example β_2-adrenoceptors (Samama *et al.*, 1993), 5-HT_{1A} receptors (Newman-Tancredi *et al.*, 1997a) and histamine H_2 receptors (Smit *et al.*, 1996; see 1.5.1).

1.4.1.2 – Multi-state receptor models of action: ternary complex models

The two-state model as described above has been largely applied to transmitter-gated ion channels whose transduction mechanism (gating) was clearly defined. However, in the case of heptahelical receptors it is incorrect to describe GPCR function simply in terms of the receptor (i.e. two-state theory) – the G-protein is an interactive and essential part of the GPCR system, and the behaviour of the receptor can be modified by the G-protein and vice versa. Therefore, interaction of the receptor and receptor-ligand complex with the G-protein must be taken into account in an accurate model of GPCR systems. This observation led to the establishment of the simple ternary complex model which was similar to the two-state model, but R* was replaced by RG and the two conformational states of the receptor were not clearly distinguished. In order to interpret the molecular properties of constitutively active receptors with high affinity for agonists even in the absence of G-proteins, the ternary complex model was extended to propose the 'allosteric ternary complex model' (Lefkowitz *et al.*, 1993; Samama *et al.*, 1993). In this model (see Figure 1.11a), receptors exist spontaneously in either the R* or R form and ligands can interact with either form. G-proteins interact with the R* state whether or not it is occupied by ligands. The process of receptor activation comprises of at least two distinct steps, conversion of R to R* and binding of R* to G – the presence of the ligand (A) can influence both steps of activation. A more thermodynamically complete version of the system allows the inactive receptor (R) to interact with the G-protein, and the

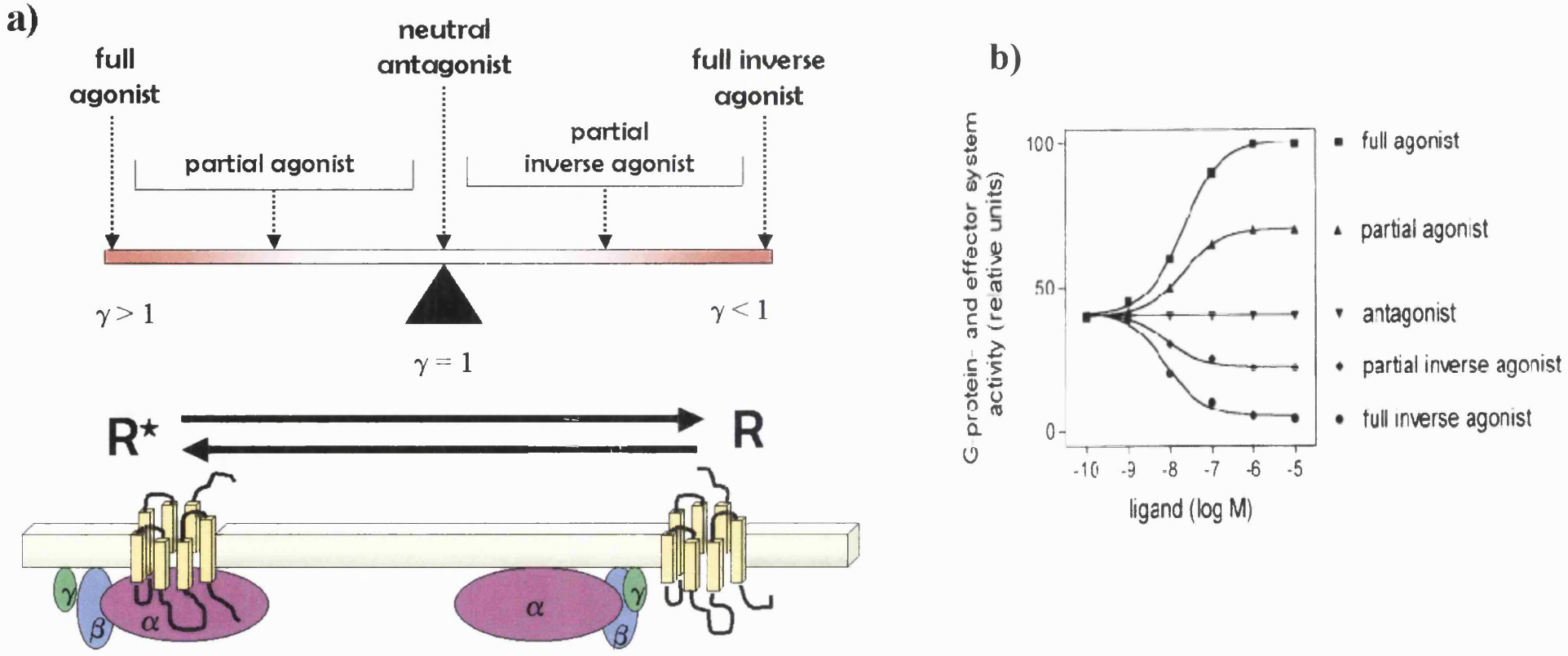


Figure 1.10 – Classification of different receptor ligand types according to the two-state model of GPCR activation. **a)** The two-state model assumes that GPCRs isomerize from an inactive (R) state to an active (R*) state. Different receptor ligands stabilize these different states of the receptor to varying degrees (see text for details). Modified from (Seifert *et al.*, 2002) . **b)** R to R* isomerization in GPCRs occurs, to a different extent, in the absence of an agonist and is referred to as constitutive activity. This figure shows the effects of various ligand types on the basal G-protein and effector activity in a system where the GPCR is linked to effector system activation. (Taken from Seifert *et al.*, 2002).

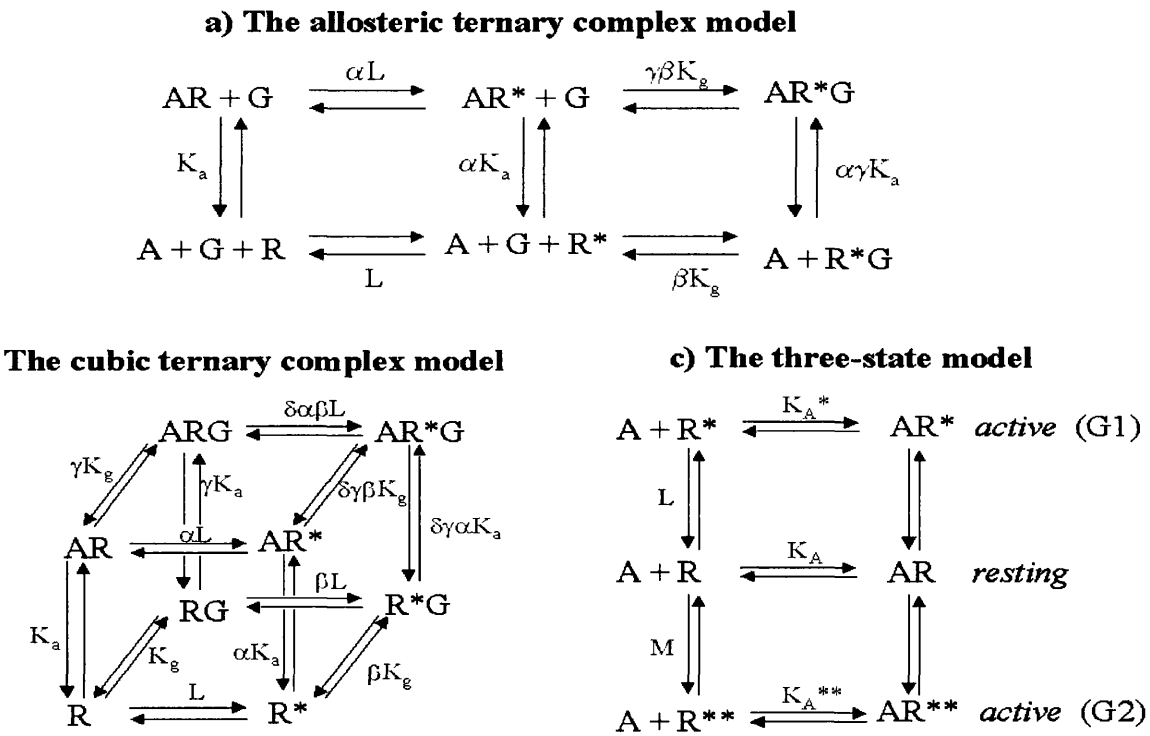


Figure 1.11 - Multi-state models for GPCR systems. a) The allosteric ternary complex model assumes that only the active-state receptor (R*) can interact with the G-protein either spontaneously (in the absence of ligand to form R*G) or through ligand binding (to form AR*G). The significance of the different equilibrium constants and the effects of different ligand types are explained in the text (see Daeffler *et al.*, 2000) b) The cubic ternary complex is very similar to the allosteric ternary complex model except that it allows the inactive state, R, to interact with the G-protein as well (see Kenakin, 2001). c) The three-state model hypothesizes that the receptor exists in three states, an inactive/resting conformation (R), an active conformation , R*, which interacts with G protein G1, and another active conformation, R**, which interacts with G protein G2. In the absence of agonist (A) the distribution of the receptors into the three states is governed by the equilibrium constants, L and M. The activity of an agonist is governed by the equilibrium dissociation constants K_A, K_A* and K_A**, which determine its affinity for R, R* and R** respectively (see Leff *et al.*, 1997). For all three models, the association constants are K_A (ligand to receptor) and βK_g (receptor to protein). L is the allosteric constant and α and γ the modifiers of affinity once the receptor is active or ligand bound respectively.

thermodynamically complete model for GPCR systems is termed the cubic ternary complex model (Weiss *et al.*, 1996) and is shown in Figure 1.11b. The usefulness of such a model is questionable as many of the proposed constants cannot be experimentally determined. The allosteric ternary complex model can be regarded as a subset of the cubic ternary complex model and is adequate for GPCR systems for which the interaction of R (inactive state) with the G-protein is thought to be minimal (i.e. in most GPCR systems).

One further level of complexity has been added to the ternary complex model to allow for the interaction of the same receptor to couple to different G-proteins. Increasing evidence suggests that the same receptor, when coupled to different G-proteins can exhibit different agonist potency orders or affinity/efficacy levels (Milligan, 1993; Kenakin, 1995a); see also 1.5.7), and this may be more likely in systems with high levels of receptor expression, since the primary G proteins may be saturated and overflow to other receptor-G protein couples may ensue. Therefore, in order for receptor promiscuity to result in altered agonist pharmacology it is logically necessary to propose that more than one active state of the receptor exists, and a three-state receptor model has been proposed by Leff *et al.* with the inactive conformation R and two active states, R* and R** interacting with two different types of G-protein (Leff *et al.*, 1997).

1.4.1.2. 1 – Limitations of the ternary complex models

Recently, evidence has suggested that heptahelical receptors can interact with each other to form dimers or oligomers in the cell membrane (Hebert *et al.*, 1998). It is possible that this dimerization/oligomerization may be linked to the activation of the receptors, since

agonists of β_2 adrenoceptors have been shown to shift the equilibrium towards the dimeric form of the receptor, which is though to be the active and high-affinity state of the receptor (Gouldson *et al.*, 1998). Clearly the models proposed above do not take into account any changes in the equilibrium of the oligomeric state of the receptor, which must be considered when interpreting experimental results.

It must also be remembered that other interacting proteins, which may influence ligand induced activation of intracellular effector systems, tightly surround the ligand-receptor-G protein complex. For example, effectors that interact with G proteins such as RGS (regulators of G protein signalling) proteins which combine with α subunits to activate the GTPase activity of G proteins(Berman *et al.*, 1998), or proteins which interact selectively with the receptor itself such as G protein receptor kinases (GRK) and arrestins which are involved in the process of homologous desensitization (Bunemann *et al.*, 1999); see 1.5.3 for further detail). It has also been shown that NHERF (Na^+/H^+ exchanger regulatory factor) interacts with the C terminal tail of β_2 adrenoceptors to transduce the message of agonists (Hall *et al.*, 1998), suggesting that some cellular responses to the stimulation of heptahelical receptors might be G protein independent. Therefore, it must be remembered that the effects of ligands on GPCRs and subsequent intracellular signalling cannot be predicted solely from the ternary complex model.

1.5 – Inverse agonism

1.5.1 – History and mechanisms of inverse agonist action

For almost a quarter of a century, ligands that interacted with GPCRs were classified as either agonists or antagonists. Agonist binding was thought to produce an activated state of the receptor which induced cellular signalling, whilst antagonists had no cellular signalling ability on their own, but did bind to the receptor preventing agonists from binding and activating the receptor. However, in 1989 Costa and Herz demonstrated that δ-opioid receptors could be manipulated into a constitutively or spontaneously active state, which could produce cellular signalling in the absence of agonist binding (Costa *et al.*, 1989). In the same manuscript, evidence was provided that certain 'antagonists', such as ICI 174864, could 'switch-off' or inactivate these spontaneously active receptors, and these compounds were named 'negative antagonists'. The term 'negative antagonist' has now been largely replaced by the term 'inverse agonist'. It was the creation of these genetically engineered constitutively active GPCR systems that was pivotal for the development of the concept of inverse agonism, since in natural systems the amount of spontaneously formed active-state (R*) receptor species is often not sufficient to demonstrate visible constitutive activity. Such manipulations include the construction and expression of constitutively active mutant receptors (CAM), which show higher agonist-independent activity and have been reported for various receptor subtypes, including β_2-adrenoceptors which was the first CAM receptor to be described (Samama *et al.*, 1993). Other manipulations include overexpression of the receptor, since constitutive activity is positively correlated to receptor density and various classes of GPCRs display constitutive activity upon overexpression, such as β_2-adrenoceptors in

Sf9 insect cells (Chidiac *et al.*, 1994). Overexpression of the G protein involved in the receptor signalling may also increase the level of basal activity of the receptors, by favourably altering the receptor-G protein ratio. For example, high levels of $G_{\alpha q}$ cotransfected with various muscarinic receptor subtypes in NIH3T3 cells resulted in increased constitutive activity of the receptors which was reversed completely by muscarinic 'antagonists', indicating that they were behaving as inverse agonists (Burstein *et al.*, 1997). Table 1.6 is a partial list of GPCRs for which evidence has been generated, using the various manipulations noted above, to suggest the existence of spontaneously active states. This table also shows the subsequent classification of various ligands as a result of the emergence of constitutive activity at that receptor.

Using the allosteric ternary complex model of receptor activity described previously (see 1.4.1.2), an inverse agonist can be described as a ligand which displays a higher affinity for uncoupled receptor states, R or R* (inactive/active states), over the coupled receptor state, R*G (active state binding to G protein), resulting in a reduction in the basal regulation of the effector system. There are three mechanisms whereby this could be achieved. The first model states that *an inverse agonist shifts the R:R* equilibrium in favour of R.* Constitutively active mutants of GPCRs have been very useful in the proposition of this model – since the R* state is favoured by these mutants and the inverse agonist binds preferentially to R over R*, it might be assumed that the affinity of the inverse agonist would be reduced by the mutation. Such mutations in α_2- and β_2-adrenoceptors have resulted in an approximate twofold decrease in inverse agonist affinity for these receptors (Samama *et al.*, 1993; Wade *et al.*, 2001). These effects are consistent with model 1 of inverse agonism, with ~50% of the receptor in the R* state

Table 1.6

Ligand and receptor subtype		*Model*	*Inverse agonist*	*Tested for inverse agonism*	*Reference*
Amine					
Acetylcholine	M2	Native receptor	pirenzapine	Antag: atropine and AF-DX 116	(Daeffler *et al.*, 1999)
Adrenoceptor	α_{2A}	CAM	rauwolscine, yohimbine, RX821002, MK912	n.d.	(Wade *et al.*, 2001)
	β_2	in vivo mice	ICI 118-551	Antag: alprenolol	(Bond *et al.*, 1995)
Dopamine	D_{1A}	Native receptor	(+)-butaclamol, flupentixol		(Tiberi *et al.*, 1994)
	D_{1A}	Native receptor	clozapine, fluphenazine	Antag: SCH23390	(Cai *et al.*, 1999)
	D3	Native receptor	haloperidol, radopride, (+)-UH-232, (+)-AJ-76	n.d.	(Malmberg *et al.*, 1998)
Histamine	H1	Native receptor	mepyramine, cetrizine, epinastine, acrivastine	no endogenous agonist	(Bakker *et al.*, 2000)
	H2	Native receptor	cimetidine, ranitidine	Antag: burimamide	(Smit *et al.*, 1996)
	H3	Native receptor	ciproxifan, thioperamide, FUB 465; clobenpropit, iodophenpropit	Antag: proxyfan	(Morisset *et al.*, 2000a) (Wieland *et al.*, 2001) (Rouleau *et al.*, 2002)
Serotonin	$5\text{-}HT_{1A}$	Native receptor	spiperone	Antag: WAY	(Newman-Tancredi *et al.*, 1997a)
	$5\text{-}HT_{2C}$	Native overexpressed	SB 206553, clozapine, mianserin	Antag: chloropromazine, thioridazine, thiothixene, 5-methoxygramine	(Herrick-Davis *et al.*, 2000) (Berg *et al.*, 1999)
	$5\text{-}HT_{4A}$	Native receptor, CAM	GR 125487, SB 207266	n.d.	(Claeysen *et al.*, 2000)

Table 1.6 (*Continued*)

Ligand and receptor		*Model*	*Inverse agonist*	*Tested for inverse agonism*	*Reference*
Peptide					
Angiotensin	AT_1	CAM	Losartan, DUP 753, LF 7-0156. LF 8-0129	n.d.	(Groblewski *et al.*, 1997)
Bradykinin	B2	Native receptor;	NPC17731, HOE140	no endogenous agonist	(Fathy *et al.*, 1999)
Formyl peptide	FPR	Native receptor	cyclosporin H	Antag: BocPLPLP	(Wenzel-Seifert *et al.*, 1998)
CCK-BR		CAM	(R)-L-740,093	Antag: YM022	(Beinborn *et al.*, 1998)
Neurotensin	NTS1	CAM	SR 48692	n.d.	(Barroso *et al.*, 2002)
	NTS2	Native receptor	JMV 457	Antag: neurotensin	(Richard *et al.*, 2001)
Opioid	κ	CAM	naloxone	n.d.	(Li *et al.*, 2001)
	μ	Native receptor	naloxone, β-chlornaltrexamine	Antag: CTAP	(Liu *et al.*, 2001)
	δ	Native receptor	BNTX, NTB, ICI 174864, clocinnamox	Antag: naltrindole	(Neilan *et al.*, 1999)
	δ	Native receptor	ICI 174864	Antag: naloxone	(Szekeres *et al.*, 1997)
	δ_2	in vivo mice	Leu-Enkephalin	Antag: naltriben	(Rady *et al.*, 2001)
Nucleotide-like					
Adenosin	A1	Native overexpressed	8-cyclopentyl-1,3-dipropylxanthine, WRC 0571	Antag: N-0861	(Shyrock *et al.*, 1998)
Cannabis	CB1	Native receptor	SR 114716A	no endogenous agonist	(Bouaboula *et al.*, 1997)
	CB2	Native receptor	SR 144528	Antag: Δ9-THC	(Bouaboula *et al.*, 1999b)

Table 1.6 – Partial list of constitutively active GPCR systems and their inverse agonists (Modified from Bond *et al.*, 2003)

in the CAM. However, it must be remembered that there will only be a large change in the affinity of the inverse agonist if the mutation causes the R* state to be strongly favoured. For many GPCRs the R* state is normally a very unfavourable state, and although these constitutively active mutations do increase the stability of R* the affinity and potency of agonists is correspondingly increased. For some GPCR CAM's, the extent of the increase in the stability of R* is such that the effects on the affinity of inverse agonists are not great, which means this can be an insensitive test for this mechanism of action (Samama *et al.*, 1994). Further evidence for model 1 emerges from studies on the phosphorylation of the β_2-adrenoceptor (Samama *et al.*, 1994). There is a good correlation between the ability of β-adrenoceptor kinase (βARK) to phosphorylate the agonist-occupied β_2-adrenoceptor and the intrinsic activity of the agonist to stimulate adenylyl cyclase (Benovic, 1988), and in the absence of agonist very little phosphorylation of the native receptor was observed. However for the constitutively active mutant of the β_2-adrenoceptor, which might favour the R* conformation, significant βARK-catalyzed phosphorylation was observed in the absence of agonist (i.e. there was constitutive phosphorylation) (Samama *et al.*, 1994). This constitutive phosphorylation was 50% suppressed by the inverse agonist ICI118551 ((±)-1-[2,3-(dihydro-7-methyl-1*H*-indeny-4yl)oxyl]-3-[1-methylethyl)amino]-2-butanol), the affinity of which is also reduced by the constitutively active mutation (Samama *et al.*, 1994). This work was performed in a reconstituted system in the absence of G protein, thus suggesting that the inverse agonist acts by binding to the R state of the receptor in preference to the R* state. Thus evidence in favour of model 1 for the mechanism of inverse agonism exists for some receptors, however, for others, such as α_{1b}-adrenoceptors (Rossier, 1999), constitutively active mutations result in no change of inverse agonist

affinity despite these receptors exhibiting substantial constitutive activity. Thus, suggesting there maybe other mechanisms of inverse agonist action.

Model 2 states that *inverse agonists bind to the uncoupled (R and R*) states of the receptor in preference to the coupled (R*G)* state. One of the earliest descriptions of inverse agonism for GPCRs was for opiate receptors (Costa *et al.*, 1989). By measuring the sensitivity of the binding of inverse agonists to the effects of guanine nucleotides, it was suggested that inverse agonists were switching the receptor between the R*G and R states of the receptor, since agonist affinity was decreased by GTP whereas inverse agonist affinity was increased. It was assumed that GTP was destabilising the R*G state, and that whereas agonists bound more tightly to the R*G state of the receptor, inverse agonists bound more tightly to the R state. Further evidence in favour of this model has been obtained by observing the effects of guanine nucleotides on the binding of antagonists/inverse agonists for 5-HT_{1A} (Sundaram *et al.*, 1993) and 5-HT_{2C} receptors (Westphal *et al.*, 1994), cannabinoid CB_1 receptors (Bouaboula *et al.*, 1997), adenosine A_1 receptors (Freissmuth *et al.*, 1991) and dopamine D2 receptors (De Lean *et al.*, 1982). In the case of 5-HT_{1A} receptors, spiperone has been shown to be an inverse agonist due to its suppression of agonist-independent [^{35}S]GTPγS binding (McLoughlin *et al.*, 2000), and is also sensitive to the effects of guanine nucleotides since spiperone exhibits a higher B_{max} in the presence of GTP (Sundaram *et al.*, 1993). Therefore, it could be suggested that spiperone stabilises the R state of the receptor at the expense of the R*G state (i.e. model 2). However, this observation, and similar observations in other receptor systems where guanine nucleotide sensitivity of inverse agonist binding has been shown, cannot be used definitively to identify the mechanism of inverse agonism, since binding

of the inverse agonist would also exhibit sensitivity to GTP if it were stabilizing R over R* (model 1). Model 1 or 2 could therefore apply and further work is required to differentiate the models. In the case of 5-HT_{1A} receptors, this has been attempted by making mutations of the 5-HT_{1A} receptor which might favour the R* state (Malmberg *et al.*, 2000). However, this work was not useful in elucidating the mechanism of inverse agonism at 5-HT_{1A} receptors since the mutations did not elicit constitutive activity and there were effects on the coupling of the receptor to different G proteins.

Another tool which has been used to probe the different states of the receptor (i.e. R, R* or R*G) and elucidate the mechanism of inverse agonist binding is the evaluation of inverse agonist affinity in radioligand binding competition assays. According to the allosteric ternary complex model if models 1 or 2 hold, the radiolabelled agonist should label R*G with high affinity, a radiolabelled antagonist will label all states with equal affinity, and a radiolabelled inverse agonist will label the uncoupled states (R or R*, depending on the model) with higher affinity than the R*G state (Strange, 2002). Therefore, there may be a difference in inverse agonist affinity when measured in competition assays versus radiolabelled agonists and inverse agonists, although this will only be observed if there is an excess of R over G and substantial precoupling of R and G. This test has been applied to the 5-HT_{1A} receptor, and spiperone shows a clear difference in affinity for the 5-HT_{1A} receptor when assessed in the presence of a radiolabelled agonist compared with a radiolabelled inverse agonist (McLoughlin *et al.*, 2000), which is in agreement with the sensitivity of the binding of this ligand to guanine nucleotides (McLoughlin *et al.*, 2000). However, as noted before with the guanine nucleotide sensitivity binding experiments, a difference in affinity would be observed whether the inverse agonist bound preferentially to R over R*, or R or R* over R*G

(Models 1 and 2), therefore further experiments are still required to distinguish between models 1 and 2, although these studies do indicate if an inverse agonist works via a mechanism that does not redistribute the affinity states (see Model 3 explained below). Methiothepin, like spiperone, has been characterized as a full inverse agonist, however it does not show any difference in affinity when measured versus radiolabelled agonists and inverse agonists (McLoughlin *et al.*, 2000). This indicates that more than one mechanism of inverse agonism can occur at a particular receptor when different compounds are administered, and suggests that some mechanisms of inverse agonist action do not involve a redistribution of the different receptor states (i.e. models 1 and 2), and that another model of action may exist. This has led to the proposition of Model 3 – *the binding of the inverse agonist to the receptor switches the receptor to an inactive conformation that can exist in G protein-coupled and uncoupled forms but is unable to signal.* This concept of switching the receptor to an inactive state is partly a restatement of the cubic ternary complex model (Figure 1.11b), which allows for an inactive receptor conformation that is able to couple to G proteins. Evidence for the idea that receptors can switch to an inactive state has been shown for muscarinic acetylcholine receptors (Vogel *et al.*, 1995) and cannabinoid receptors (Bouaboula *et al.*, 1997), where it has been suggested that the inverse agonist, SR141716A, promotes an 'active negative state' of the receptor that sequesters the Gα protein into an inactive form. Recently, Costa *et al.* have suggested that inverse agonists that work via this mechanism (i.e. by blocking the G protein in an inactive form to prevent signalling) should be distinct from other inverse agonists which work by actively reducing spontaneous association of the receptor with the G protein, and they termed these ligands 'negative antagonists' (Costa *et al.*, 2003).

1.5.2 – Agonists, antagonists and inverse agonists.

In the majority of systems, the R* state of the receptor is energetically highly unfavourable, and therefore the equilibrium between the two states of the receptor favours the inactive (R) state. In these circumstances, the effects of an inverse agonist will appear to be qualitatively similar to those effects observed with a neutral antagonist, since antagonists bind to both states with equal affinity, but will bind to R most strongly in this situation, as will inverse agonists (due to their inherent preference for the R state). This explains why many ligands, historically described as antagonists, may actually act as inverse agonists if tested in a constitutively active system, and it is important to discriminate the 'effects' of antagonists from those of inverse agonists. Despite the vast number of ligands which have been described as 'neutral antagonists', it is quickly becoming clear that such a class of compounds does not really exist, or if so, is extremely limited. The accurate definition of a neutral antagonist is a ligand that has equal affinity for both the active and inactive receptor conformations, and ligands which fit this description are very rare. Instead, it is more accurate to describe antagonists as 'partial inverse agonists' with various degrees of efficacy and variable affinity for the two receptor states. Thus, it is tempting to speculate that there is only one class of ligands after all – i.e. agonists, whether they are full, partial, partial inverse ('antagonists') or full inverse. However, for the sake of clarity and for consistency, the remainder of this thesis will continue to use the term 'antagonist' and 'neutral antagonist' as previously described in the literature.

Agonists preferentially bind to and enrich the numbers of receptors in the R* state, whereas inverse agonists preferentially bind to and enrich the number of receptors in the

R state. Thus, agonists and inverse agonists appear to modulate cellular activity in a reciprocal manner (see Table 1. 7), for example inverse agonists move 'baseline' activity in the opposite direction to an agonist. This reciprocity implies that inverse agonists 'actively' alter signalling on their own, rather than just preventing agonist activity as is true for antagonists. Following the observation that, after chronic administration, only β-adrenoceptor antagonists with inverse agonist activity are beneficial in treating patients with heart failure (Marleau *et al.*, 1989), it has recently been speculated that the reciprocity of agonists and inverse agonists may extend to their ability to induce and regulate intracellular signalling (Bond *et al.*, 2003). Acute administration of agonists increases signalling by receptor activation, and chronic agonist activation produces desensitization and decreases in signalling, whereas inverse agonists acutely decrease signalling and may possibly chronically increase signalling due to an up regulation of receptor number after long-term treatment (see 1.5.3). Since neutral antagonists do not alter the expression of receptors, the long-term effects of an antagonist and an inverse agonist are very different, and could have significant therapeutic implications, thereby reinforcing the importance of discriminating between true antagonists and inverse agonists.

Agonist	*Inverse Agonist*
• Promotes formation of *active* state of receptor (R*)	• Promotes formation of *inactive* sate of receptor (R)
• Increases 'baseline' activity	• Decreases 'baseline' activity
• Promotes phosphorylation by GRK	• Prevents phosphorylation by GRK
• Promotes endocytosis and downregulation of receptor	• Promotes upregulation of cell surface receptors
• Promotes conformational changes (decrease fluorescence emission) (Gether *et al.*, 1995)	• promotes conformational changes (increase fluorescence) (Gether *et al.*, 1995)
• Homologous desensitization	• Homologous sensitization
• Heterologous desensitization	• Heterologous sensitization
• Acutely increase signalling	• Acutely decrease signalling (act as antagonist)
• Chronically decrease signalling	• Chronically increase signalling

Table 1. 7 – The reciprocal relationship between agonists and inverse agonists (modified from Bond *et al.*, 2003)

1.5.3 – Inverse agonism and the regulation of receptor number

Cellular populations of GPCRs are not static and since they are the initial recognition point for the presence, variation and intensity of hormonal and neurotransmitter encoded information, it is not surprising that they are regulated at many levels, including transcriptional, translational, post-translational and degradative stages. One facet of this regulation is the observation of chronic agonist-induced desensitization and downregulation of receptor number. Agonists have the capacity to cause internalisation or sequestration of GPCRs over a relatively short period of exposure, and this can be followed by recycling to the cell surface, or if the stimulus is both prolonged and intense (i.e. a high efficacy agonist), degradation of the GPCR, resulting in an overall reduction in cellular receptor levels. The cellular mechanisms involved in agonist-induced desensitization of heptahelical receptors have been extensively studied and it is mediated by receptor phosphorylation (by G-protein receptor kinases and second-messenger kinases), interaction of phosphorylated receptors with arrestins and receptor uncoupling from G proteins (see Krupnick *et al.*, 1998). Agonist-induced receptor endocytosis also contributes to desensitization by depleting the cell surface of high-affinity receptors (see Bohm *et al.*, 1997). Receptor downregulation is characterized by a decrease in the total number of receptors in a cell, and is possibly caused by enhanced degradation and/or reduced synthesis of the receptors. It is observed after long-term exposure to agonists for hours or days, and is responsible for the subsequent tolerance and tachyphylaxis (Bohm *et al.*, 1997). For most systems, downregulation and desensitization of receptors is associated with ligands that function to increase G protein coupling (i.e. agonists). Thus the receptor conformation that interacts with G proteins (i.e. R*) is often also a substrate for desensitization and downregulation. For example, a study using human embryonic

kidney cells (HEK293) transfected with the β_2-adrenoceptor showed that the same rank order of agonist potency was observed for adenylate cyclase activation, desensitization and receptor internalisation (January *et al.*, 1997), thus suggesting that the conformational requirements of the receptor are similar for all three processes. However, studies on insect Sf9 cells expressing the 5-HT_{2C} receptor have shown that downregulation of receptors is not only caused by agonists, since a range of antagonist and/or inverse agonist ligands produced receptor downregulation (Labrecque *et al.*, 1995). However, there was no strong correlation between inverse efficacy and the degree of effect, suggesting that these two features are not intrinsically linked, although it appears that in the majority of GPCR systems downregulation is indeed associated with ligands with an agonist type action.

Given the reciprocity of agonists and inverse agonists (see Table 1. 7), based on the selective stabilization of inactive and active GPCR conformations, if prolonged exposure to agonist ligands frequently results in GPCR downregulation, it could be speculated that equivalent treatment with inverse agonists would result in their upregulation (Milligan *et al.*, 1997). Indeed, the mechanisms noted above for agonist-induced desensitization and downregulation also occur for spontaneously active receptors in the absence of agonist. Thus, by selectively stabilizing the inactive state of the receptor, inverse agonists could halt the spontaneous receptor degradation, whilst leaving receptor synthesis unaltered, thereby upregulating GPCR number (see Daeffler *et al.*, 2000; see Kenakin, 2001). There is a large body of accumulating evidence for various receptor types, that long-term treatment with inverse agonists is associated with upregulation of the receptor involved, and this increase in receptor density may explain associated drug tolerance and withdrawal effects (see de Ligt *et al.*, 2000). For example, following expression of the

rat histamine H_2 receptor in CHO cells, Smit *et al.*, observed a time- and concentration-dependent upregulation in levels of H_2 receptor after the cells were exposed to ranitidine and cimetidine, although these observations were not replicated by burimamide nor ligands with good selectivity for the histamine H_1 or H_3 receptors (Smit *et al.*, 1996). Both ranitidine and cimetidine function as inverse agonists at H_2 receptors, whereas burimamide is an antagonist (Smit *et al.*, 1996), which proves that inverse agonists are capable of inducing receptor upregulation in this system. Since, burimamide had no effect, this study also shows that the inverse agonist-induced upregulation resulted directly from the nature of the ligand rather than indirectly by blocking a degree of GPCR downregulation that usually occurs due to the presence of circulating endogenous agonist (see Milligan *et al.*, 1997). Similar results have been observed for the wild-type human β_2-adrenoceptor following expression in NG108-15 neuroblastoma X glioma hybrid cells, where exposure to the inverse agonists, betaxolol and sotalol, resulted in almost a doubling in membrane levels of the receptor, in contrast to equivalent treatment with the antagonist alprenolol (MacEwan *et al.*, 1996).

The upregulation elicited by inverse agonists may have therapeutic implications for receptors that display significant levels of constitutive activity such as the H_2 and β_2 receptors, which are both classical targets for antagonist drugs. For the H_2 receptor, the inverse agonists cimetidine, ranitidine and famotidine were used to treat peptic ulcers (Deakin *et al.*, 1992), however, the upregulation these drugs produced was not desirable as it caused clinical tolerance to the drug and withdrawal effects (Smit *et al.*, 1996). In the treatment of heart failure the upregulation of β_1-adrenoceptors resulting from chronic administration of the inverse agonists metoprolol and carvedilol, has proved

advantageous (see Bond *et al.*, 2003), with the patient showing clinical improvement of cardiac function within 2-4 months of continued therapy. This inverse agonist-induced upregulation of β_1-adrenoceptors overcomes the decreased density observed in failing hearts, allowing endogenous agonists an increased agonist response, which improves cardiac muscle contraction and hence cardiac output. Upregulation of opiate receptors has also been reported in rats, with a one week chronic infusion of naloxone (a δ and μ opioid inverse agonist) resulting in an upregulation of the δ, κ and μ receptors in the brain as measured by quantitative autoradiography (Morris *et al.*, 1988). This upregulation of opiate receptors was also accompanied by a sensitization of the animals towards opioid agonists (Morris *et al.*, 1988). Further studies with naloxone have been carried out to try and elucidate the mechanism of opiate tolerance/dependence and withdrawal (Cruz *et al.*, 1996), with its effects assayed *in situ* in both morphine-treated and control guinea-pig ilea. Naloxone counteracted morphine-induced neurodepression, but it also caused an abstinence response, with this withdrawal phenomenon being observed particularly after bolus dosing. It was hypothesised that dependence can be viewed as an overexpression of the R* state of opioid receptors and withdrawal as an abrupt change from R* to R (Cruz *et al.*, 1996).

In addition to changes in the targeted receptor density, long-term treatment with inverse agonists might have effects on other receptors and alter levels of G proteins as well. It has been postulated that if other receptors share the same signalling pathway, or G proteins, as the target receptor then these receptors may also be affected (de Ligt *et al.*, 2000). For example, SR144,528 not only acts as an inverse agonist on the cannabinnoid CB_2 receptor, but also prevents MAPK activation in response to insulin or

lysophophatidic acid by inhibiting G_i activity (Bouaboula *et al.*, 1999a). These observations provide evidence for cross talk between different receptors via the G_i proteins, and a possible explanation is the existence of an inactive CB_2/G_i protein complex, in which the G_i protein is physically trapped and thus unavailable for other receptors (Bouaboula *et al.*, 1999a). Further evidence for cross talk between receptors has been reported by Berg *et al.* (1999), who reported that the prolonged incubation of CHO cells expressing the 5-HT_{2C} receptor at low levels, to SB206553 (an inverse agonist at 5-HT_{2C} receptors) led to an increased responsiveness of the receptor to agonists (homologous sensitization) as would be expected with an upregulation of 5-HT_{2C} receptors. This was measured as an increase in maximal inositol phosphate production induced by the agonist DOI. However, inositol phosphate production was also enhanced by ATP, mediated via endogenous purinergic P_2 receptors (heterologous sensitisation; (Berg *et al.*, 1999), thus suggesting that treatment with an inverse agonist for 5-HT_{2c} receptors may also alter signalling at other receptors.

1.5.4 – Physiological relevance of inverse agonism.

Many descriptions of inverse agonism have relied on effects in recombinant systems in which constitutive activity has been engineered, however, it is important to determine whether inverse agonism actually has any physiological relevance, and if so, any resulting therapeutic potential. Inverse agonism has also been shown to occur in systems with a more direct link to *in vivo* pharmacology and physiology such as examining the effects of inverse agonists in native tissues, cell systems with 'normal' levels of expression and in tissue/organ bath preparations, thus providing evidence for a physiological role.

Various authors have described inverse agonism and constitutive activity on wild type receptors expressed in artificial cell lines but at more or less 'physiological' levels of expression. Examples of some such constitutively active wild type receptors are listed in Table 1.8 (native receptor model), and some are described below. Both rat (Smit *et al.*, 1996) and human (Alewijnse *et al.*, 1998) histamine H_2 receptors have been stably transfected into CHO cells, and have shown pronounced basal activity which was not altered by neutral antagonists, thus indicating that the receptors show inherent constitutive activity rather than endogenous agonist-induced activity. The basal activity was reduced by exposure of the receptors to the inverse agonists cimetidine and ranitidine. Histamine H_3 receptors have also been shown to be constitutively active in native rodent brain, and this constitutive activity can be suppressed by inverse agonists (Morisset *et al.*, 2000b). In this case, inverse agonists enhance histamine release from histamine-containing neurones by acting on H_3 autoreceptors, providing a clear example of the acute effects of inverse agonists in a native system. Newman-Tancredi *et al.*, (1997) studied [^{35}S]-GTPγS binding to 5-HT_{1A} receptors expressed in CHO cells at physiological levels (1.6 pmol mg^{-1}), and identified spiperone as an inverse agonist since it decreased [^{35}S]-GTPγS binding. WAY-100635 showed no effect on basal [^{35}S]-GTPγS binding by itself, but was able to block both 5-CT-induced stimulation and spiperone-induced inhibition of [^{35}S]-GTPγS binding, thus identifying itself as a neutral antagonist (Newman-Tancredi *et al.*, 1997a). Further examples of inverse agonist activity in wild type receptors at 'normal' levels have been found for cannabinoid receptors, both CB_1 (Bouaboula *et al.*, 1997) and CB_2 (Bouaboula *et al.*, 1999b), various dopamine receptors, D_{1A} and D_{1B} (Tiberi *et al.*, 1994) and D_3 (Griffon *et al.*, 1996), the human formyl peptide

(FP) receptor (Wenzel-Seifert *et al.*, 1998) and human calcitonin receptors have been observed to show constitutive activity (Cohen *et al.*, 1997).

Unfortunately, there is little data related to inverse agonism obtained in intact animals (*in vivo*), apart from in transgenic mice with modest β_2-adrenoceptor overexpression (Nagaraja *et al.*, 1999), which is not a particularly useful physiological model due to the complications of compensatory changes associated with receptor overexpression. Despite the paucity of *in vivo* data, other *ex* vivo studies using cell lines endogenously expressing the receptor of interest and tissue preparations, provide receptors at physiological and pathophysiological levels and have yielded evidence that inverse agonism is physiologically relevant. Table 1.8 lists examples and brief descriptions of some of these 'physiological' studies. For example, the α_{2D}-adrenoceptor endogenously expressed in RIN5AH cells revealed inverse agonistic effects of rauwolscine in [^{35}S]-GTPγS binding assays (Tian *et al.*, 1994) and organ bath preparations of rat thoracic aorta detected inverse agonist effects at α_1-adrenoceptors by benoxathian and WB4101 (Noguera *et al.*, 1996).

The existence of naturally occurring or endogenous inverse agonists is another line of evidence which favours the physiological relevance of inverse agonism. Recently, it has been shown that agouti related protein (AgRP) is an endogenous inverse agonist and acts as a control mechanism to fine-tune the control of body weight (see Adan *et al.*, 2003). AgRP binds to brain melanocortin receptors and in addition to antagonizing the effects of

Cell line/tissue	*Receptor*	*Readout*	*Reference*
NG108-15	δ opiod	GTPase	(Costa *et al.*, 1989)
Porcine atrial membranes (enriched)	mACh	[^{35}S]-GTPγS-biding	(Hilf *et al.*, 1992)
Guinea-pig /human cardiac myocytes	β-adrenoceptor	Calcium current (I_{ca})	(Mewes *et al.*, 1993)
Frog /rat cardiac cells	mACh	Calcium current (I_{ca})	(Hanf *et al.*, 1993)
Rat /bovine myometrial membranes	bradykinin BK_2	IP-production	(Leeb-Lundberg *et al.*, 1994)
Turkey erythrocytes	β-adrenoceptor	AC-activity	(Gotze *et al.*, 1994)
Rat RIN5AH	α_{2D}-adrenoceptor	[^{35}S]-GTPγS-biding	(Tian *et al.*, 1994)
Rat cardiomyocytes	mACh (M_2)	cyclic AMP	(Jakubik *et al.*, 1995)
Rat thoracic aorta strips	α_1-adrenoceptor	↑ resting tone (I_{ca})	(Noguera *et al.*, 1996)
Guinea-pig ileum	κ opiod	contraction (twitch and abstinence response)	(Cruz *et al.*, 1996)
NG108-15	δ opiod	[^{35}S]-GTPγS-biding	(Szekeres *et al.*, 1997)
H.E.L. 92.1.17	α_{2A}-adrenoceptor	↑[Ca^{2+}], cyclic AMP	(Jansson *et al.*, 1998)

Table 1.8 – Examples of 'physiological' studies showing inverse agonism and/or constitutive activity (modified from de Ligt *et al.*, 2000).

the endogenous melanocortin receptor agonist, α-melanocyte-stimulating hormone (α-MSH), it also suppresses the constitutive activity of melanocortin M_4 receptors (Nijenhuis *et al.*, 2001), therefore characterising it as an inverse agonist (Haskell-Luevano *et al.*, 2001). To date, the melanocortin system is the only system in which an endogenous ligand with inverse agonist properties (AgRP) has been identified to contribute to the physiological regulation of receptor activity. However, many receptors show constitutive activity and thus more endogenous agonists should exist. Natural ligands known to possess inverse agonist activity exist, and examples of such natural inverse agonists are shown in Table 1. 9. However, the majority of these natural ligands do not originate from the same species as the receptors on which they act, and therefore possible roles as endogenous regulators of receptor activity in the systems they are synthesized in remain to be proven.

1.5.5 – Tonically active systems: a connection with constitutive receptor activity?

In physiology there are many examples of receptor systems tonically regulating effects. This 'tone' has traditionally been attributed to the presence of the endogenous agonist of the receptor system involved that continuously interacts with its receptors. However, with the relatively recent concept of two-(or more) state receptors in equilibrium (i.e. R and R*) and the subsequent possibility of constitutive activity, it has been speculated that constitutive activity of the receptor system maybe a potential mechanism for receptor-mediated tone instead of – or in addition to - the role of endogenous agonists (de Ligt *et al.*, 2000). An example of a tonically active system which is attributed to constitutive activity rather than endogenous agonists, is the cannabinoid receptor system which regulates thermal nociceptive thresholds in mice. SR141716A is an inverse agonist at

CB_1 receptors (MacLennan *et al.*, 1998) and produces hyperalgesia in mice by inhibiting this tone, in contrast to agonists that produce analgesia in this system (Richardson *et al.*, 1997). The same compound increased voltage-dependent Ca^{2+} currents in neurones microinjected with cloned CB_1 receptor RNA, reversing the tonic CB_1 receptor activity (Pan *et al.*, 1998). For an 'antagonist' to elicit such an effect some receptors must be tonically active, and since care was taken to ensure the absence of endogenous agonist in the experimental set-up, the 'tone' could only be attributed to constitutive receptor activity.

1.5.6 – Therapeutic potential of inverse agonists.

In the majority of situations in which antagonists are used clinically, there is no theoretical reason to believe that inverse agonists should have any intrinsic benefit over an antagonist. As a drug, an antagonist is used simply to compete with and hence limit the action, or to regulate the release of an endogenous agonist, and an inverse agonist will produce a similar outcome despite having a different mechanism of action (see 1.5.2). However, it is now clear that the side-effect profile may significantly vary for the two classes of ligand, especially in pathological conditions, and therefore it is imperative in drug design and therapy to clearly identify whether therapeutic agents are antagonists or inverse agonists. In some cases desired effects may be obtained by treatment with an antagonist, whilst in other situations an inverse agonist might be more effective.

Inverse agonist	*Species inverse agonist isolated from*	*Receptor*	*Species where receptor is located*	*Reference*
Retinal	-	Rhodopsin	e.g. bovine	(Han *et al.*, 1998)
AgRP	Mouse	Melanocortin MC_3 and MC_4 receptors	Mouse	(Nijenhuis *et al.*, 2001) (Haskell-Luevano *et al.*, 2001)
IP10	Mouse	ORF74	Herpesvirus 8	(Geras-Raaka *et al.*, 1998)
SDF-1α	Mouse	ORF74	Herpesvirus 8	(Rosenkilde *et al.*, 1999)
vMIP-II	Mouse	ORF74	Herpesvirus 8	(Rosenkilde *et al.*, 1999)
Fractalkine	Human	US28	Human cytomegalovirus	(Casarosa *et al.*, 2001)
Exendin-(9-39)	Lizard	Glucagon-like peptide 1 receptor	Mouse	(Serre *et al.*, 1998)

Table 1. 9 – Natural inverse agonists and their receptors (modified from Adan *et al.*, 2003). Abbreviations: AgRP, agouti related protein; IP10, γ-interferon-inducible protein 10; ORF74, open reading frame 74; SDF-1α, stromal cell-derived factor 1α; vMIP-11, viral macrophage inflammatory protein II; US28, receptor encoded by human cytomegalovirus.

The extent to which inverse agonism could be a therapeutic advantage depends on the role of constitutive GPCR activity in pathology. For example, in certain diseases, such as retinitis pigmentosa or Jansen's metaphyseal chondrodysplasia, somatic receptor mutations lead to constitutively active receptors (see Spiegel, 1996). Inverse agonists may be beneficial in treating these complaints since they would decrease the high basal activity induced by the mutation, while antagonists would have no effect. Inverse agonists may also play an important role in the treatment of autoimmune diseases, as it has been reported that autoantibodies against the second extracellular loop of certain GPCRs are involved in the pathology of human autoimmune diseases (see de Ligt *et al.*, 2000). These autoantibodies are believed to recognise an epitope that is only presented on the active conformation (R*) of the receptor. An inverse agonist would shift the equilibrium towards the inactive conformation (R) of the receptor, covering up the epitope recognized by the antibody, resulting the antibody being unable to activate the receptor. Other potential therapeutic areas where inverse agonists may be clinically useful are cancer (see Kenakin, 2001) and viral infections which can lead to constitutively active GPCR pathology. For example, infection with Kaposi's sarcoma-associated herpes virus leads to expression of a constitutively active chemokine receptor which is linked to cell proliferation (Arvanitakis *et al.*, 1997).

Despite the apparent advantageous clinical use of inverse agonists, potential concerns exist over the use of their sustained treatment. For example, the upregulation of receptor number associated with long-term administration means that upon removal of the drug, careful monitoring would be required to avoid the potential onset of subsequent short-term supersensitivity to endogenous agonists (see Milligan *et al.*, 1995). However, an alternative view is that upregulation of a GPCR by inverse agonist treatment might

actually result in tachyphylaxis due to endogenous agonists subsequently having access to a larger GPCR population, thus stimulating the cellular desensitization machinery more effectively (Milligan *et al.*, 1997).

Thus, the presence of constitutive activity in *in vivo* systems suggests a physiological role for inverse agonists, and whether the full therapeutic potential of inverse agonists is achieved or not, the extra dynamic range that they can provide for regulation of receptor function must be considered.

1.5.7 – 5-HT_{1A} receptors: differential G-protein subunit coupling and inverse agonism

The GDP/GTP exchange step of the G-protein cycle (see Figure 1.8) is of key interest in the study of ligand efficacy and can be investigated by use of the hydrolysis-resistant GTP analogue, guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS). Studies of this kind have yielded extensive information regarding the efficacy of ligands at certain receptors, including 5-HT_{1A} receptors (Stanton *et al.*, 1997; Newman-Tancredi *et al.*, 1998b; Cosi *et al.*, 2001). Different cell lines are known to express differing subpopulations of G-proteins, for example HeLa cells predominantly express $G\alpha_{i3}$ subunits whereas CHO cells express $G\alpha_{i2}$ subunits. Studies using recombinant human 5-HT_{1A} receptors expressed in various cell lines have shown that h5-HT_{1A} receptors couple to G-proteins with the following order of preference: $G\alpha_{i3} > G\alpha_{i2} \geq G\alpha_{i1} \geq G_o > G_Z$ (see Raymond *et al.*, 1999), and it has been shown that differences in coupling to G-protein subtypes may influence ligand profiles at 5-HT_{1A} receptors. For example, WAY-100635 acts as a neutral antagonist in [^{35}S]GTPγS assays in CHO cells (Newman-

Tancredi *et al.*, 1997b), but as an inverse agonist in a study employing HeLa cells (Cosi *et al.*, 2000), thus suggesting that the differing populations of G proteins ($G\alpha_{i2}$ and $G\alpha_{i3}$ respectively) were influencing the observed efficacy of the ligand. Coupling of 5-HT_{1A} receptors to differential G-proteins also occurs physiologically, with rat brain 5-HT_{1A} autoreceptors in the raphé nuclei coupling preferentially to $G\alpha_{i3}$ subunits, whereas postsynaptic 5-HT_{1A} receptors in the hippocampus couple preferentially to G_o (Mannoury-La Cour *et al.*, 2001). It has also been shown that 5-HT_{1A} receptors in the rat dorsal raphé are not coupled to adenylate cyclase inhibition, although there is a strong coupling observed with 5-HT_{1A} heteroreceptors in the hippocampus (Clarke *et al.*, 1996), and despite similar densities in these brain regions, agonist-induced [^{35}S]GTPγS labelling is considerably lower in regions containing autoreceptors than in those containing heteroreceptors (Newman-Tancredi *et al.*, 1999; but see Meller *et al.*, 2000; Hensler *et al.*, 2001). It is possible that these contrasting responses of 5-HT_{1A} auto- and heteroreceptors may be explained by differential coupling to intracellular G-proteins, and since WAY-100635 acts as an inverse agonist at 5-HT_{1A} receptors expressed in HeLa cells (Cosi *et al.*, 2000) which are coupled to $G\alpha_{i3}$ subunits, it is possible it may also act as an inverse agonist at 5-HT_{1A} receptors in the rat dorsal raphé.

Since the above evidence shows that some receptors can differentially couple to G-protein subunits, it has been suggested that ligands may selectively direct receptor signalling towards specific intracellular pathways (Kenakin, 1995b; Clarke, 1998; Kenakin, 2001). In the case of 5-HT_{1A} receptors, there is increasing evidence for agonist-dependent differential coupling, for example S14506 (1-[2-(4-fluorobenzoylamino)ethyl]-4-(7-methoxynaphtyl)piperazine) and clozapine selectively

bind to receptors which are preferentially coupled to a tightly associated G-protein subtype (Newman-Tancredi *et al.*, 2001b) which is in contrast to other agonists such as 8-OH-DPAT and buspirone whose binding affinity is greater than that of S14506 and clozapine after receptor-G-protein dissociation is induced by GTP (Newman-Tancredi *et al.*, 2001a). Further, Gettys *et al.* showed that certain agonists (such as 8-OH-DPAT and 5-HT) more potently activated $G\alpha_{i3}$ than $G\alpha_{i2}$, whereas rauwolscine (a partial $5\text{-}HT_{1A}$ receptor agonist) activated both subunits with similar potency (Gettys *et al.*, 1994). Therefore, it is possible that the ligand directed activation of G-protein subunits may further regulate intracellular signalling to other transduction cascades (Newman-Tancredi, 2003), thus enabling ligands to have varying intracellular effects despite being specific for a particular receptor. Recently a novel approach, involving antibody capture of activated G-proteins (i.e. [^{35}S]GTPγS) and a scintillation proximity assay (De Lapp *et al.*, 1999), has been used to reveal complex interactions in receptor-G-protein coupling at human $5\text{-}HT_{1A}$ ($h5\text{-}HT_{1A}$) receptors (Newman-Tancredi *et al.*, 2002). These studies using $h5\text{-}HT_{1A}$ receptors in CHO cells ($G\alpha_{i3}$ subunits) showed that 5-HT and other high efficacy agonists such as 8-OH-DPAT yielded bell-shaped isotherms for $G\alpha_{i3}$ activation (Newman-Tancredi *et al.*, 2002), thus indicating that the direction of the response to agonists (i.e. stimulation or inhibition) was dependent on the ligand concentration used. This suggests that altering the ligand concentration can induce a conformational change by which $h5\text{-}HT_{1A}$ receptors 'switch' signalling to another G-protein subtype, possibly $G\alpha_{i2}$ (Newman-Tancredi, 2003). This hypothesis is supported by the fact that in classical [^{35}S]GTPγS binding assays which are unable to distinguish G-protein subtypes, 5-HT induced stimulation is biphasic, suggesting the sequential activation of at least two G-protein subtypes (Newman-Tancredi *et al.*, 2002). Interestingly, (-)pindolol which has

previously been described as a partial agonist in classical [^{35}S]GTPγS binding assays (Newman-Tancredi *et al.*, 1998a), displays sigmoidal stimulation isotherms for $G\alpha_{i3}$ activation, suggesting that it is not capable of inducing the conformational switch observed with the higher efficacy agonists, which may explain its partial agonist activity.

The above evidence suggests that h5-HT_{1A} receptors show promiscuous coupling to multiple G-protein subtypes which may enable ligand-directed trafficking of receptor signalling, thus allowing phenotypic switching and crosstalk within and possibly between 5-HT receptor families. This may be the case for many other GPCRs since adenosine A_1 receptors have been shown to activate various G-protein subtypes (Browning *et al.*, 2000). The observation that 5-HT_{1A} receptors appear to show promiscuous coupling to multiple G-protein subtypes, coupled with other mechanisms that increase the complexity of the 5-HT system (e.g. posttranslational modifications, oligomerisation) and the vast array of 5-HT receptor subtypes, suggests that there are almost limitless signalling capabilities for the 5-HT system.

1.6 – Aims of this study

The primary objective for this study was to confirm the role of central 5-HT_{1A} receptors in the control of micturition, and to determine whether 5-HT_{1A} receptor antagonists possess any therapeutic potential in the treatment of overactive bladder.

Therefore, the initial work investigated the role of 5-HT_{1A} receptors in the control of micturition in an anaesthetized rat model using the 5-HT_{1A} receptor antagonist, WAY-100635 , and confirmed that there are two central sites for 5-HT_{1A} receptors in the control of micturition. However, during this initial work an interesting phenomenon was observed after repeated administration of WAY-100635, which suggested that WAY-100635 was less effective at suppressing micturition after repeat administration. Therefore, the next part of the study investigated the effect of acute and chronic administration of WAY-100635 and robalzotan (a structurally distinct 5-HT_{1A} receptor antagonist) on micturition in a conscious rat model. Since, the results from these studies suggested that chronic administration of 5-HT_{1A} receptor antagonists appeared to cause the onset of tolerance, thus preventing these 5-HT_{1A} receptor ligands from exerting any further effect on micturition, additional aims of the study emerged which included further confirmation and investigation into the time of onset of this phenomenon. This was achieved using a radiotelemetry model in conscious freely moving rats, to monitor bladder activity in the absence and presence of chronic i.v. administration of WAY-100635. Finally, a possible explanation for the observation of tolerance after chronic WAY-100635 administration was sought using quantitative autoradiography to provide a measure of the density of 5-HT_{1A} and 5-$HT_{1B/1D}$ receptors and the serotonin transporter (5-HTT) in various brain regions of chronically treated rats.

Chapter 2

Central 5-HT$_{1A}$ receptor sites of action:

Anaesthetized studies

2. 1 – Introduction

The storage and periodic elimination of urine (micturition) is dependent upon the co-ordination of the activities of the bladder and urethra. The excretory function of these structures is regulated by a complex neural control system in the brain and lumbosacral spinal cord. 5-hydroxytryptamine (5-HT), along with various other neurotransmitters, has been implicated in this central neural regulation of the lower urinary tract (see section 1.5.1). Histochemical studies have confirmed that autonomic nuclei in the lumbosacral spinal cord, which are known to contain preganglionic neurones innervating the LUT, include a collection of 5-HT-containing nerves which originate in the raphé nucleus in the caudal brain stem (Dahlstrom *et al.*, 1965). Transneuronal tracing studies with pseudorabies virus have also shown that raphé neurones in the brainstem of the rat are labelled after injection of the virus into either the bladder (Nadelhaft *et al.*, 1992), urethra (Vizzard *et al.*, 1995) or EUS (Marson, 1997) and both electrical and chemical stimulation of these raphé neurones inhibits the micturition reflex in the rat and cat (McMahon *et al.*, 1982; Lumb, 1986a; Morrison *et al.*, 1986; Chen *et al.*, 1993).

5-HT receptors are widely distributed in the CNS including various areas concerned with the control of micturition. For example, 5-HT$_{1A/1B}$, 5-HT$_2$ and 5-HT$_3$ receptors are all present in rat and cat lumbosacral spinal cord (Monroe *et al.*, 1983; Glaum *et al.*, 1988; Murphy *et al.*, 1988; Thor *et al.*, 1993) in addition to 5-HT receptors being present in various brain regions known to be important in the control of micturition, for example, 5-HT$_{1A}$ receptors are found on 5-HT-containing neurones in the raphé nucleus (Aghajanian *et al.*, 1986). Several 5-HT receptor types, including 5-HT$_{1A}$, 5-HT$_2$, 5-HT$_3$,

5-HT_4 and 5-HT_7, have been studied with regard to their role in controlling micturition (see 1.1.5.1), however, due to the early availability of various selective 5-HT_{1A} ligands, the role of 5-HT_{1A} receptors in the control of micturition is slightly better understood than that of other 5-HT receptors. Previous studies in the rat have revealed that activation of spinal and supraspinal 5-HT_{1A} receptors using the 5-HT_{1A}-receptor agonist 8-OH-DPAT facilitates the micturition reflex (Lecci *et al.*, 1992), whereas administration of the 5-HT_{1A} neutral antagonists WAY-100635 and NAD-299 both intravenously (Testa *et al.*, 1999; Conley *et al.*, 2001; Pehrson *et al.*, 2002) and intrathecally (Kakizaki *et al.*, 2001; Pehrson *et al.*, 2002) suppress the micturition reflex. 5-HT_{1A} receptor antagonists that also show partial agonist activity do not inhibit the micturition reflex (Testa *et al.*, 1999).

All the above evidence suggests the possible presence of an inhibitory descending raphé-spinal pathway which is activated by afferent input from the bladder, and inhibits the micturition reflex (Testa *et al.*, 1999). Since inhibitory 5-HT_{1A} receptors play an important role in the control of raphé firing (Aghajanian *et al.*, 1986) it is not surprising that modulating 5-HT_{1A} receptor activity can have significant effects on the micturition reflex. Studies using intrathecal administration of WAY-100635 in the urethane anaesthetized rat have shown that 5-HT_{1A} receptors at the L6/S1 level of the spinal cord have an important physiological role in the tonic control of the descending limb of the micturition reflex pathway (Kakizaki *et al.*, 2001). Further studies have also shown intracerebroventricular administration of 8-OH-DPAT to facilitate the micturition reflex suggesting that supraspinal 5-HT_{1A} receptors are present and a potential modulatory mechanism (Lecci *et al.*, 1992), however an *in vivo* physiological role needs to be confirmed using a 5-HT_{1A} receptor antagonist. Therefore the aim of the present study was to confirm a physiological role for supraspinal 5-HT_{1A} receptors in the control of

micturition by examining the effects of i.c.v. and i.t. administration of WAY-100635 on the micturition reflex in female urethane anaesthetized rats. The effects of WAY-100635 were examined on the bladder and urethral responses evoked by saline infusion into the bladder.

2. 2 - Methods

All experiments were carried out under the Animals (Scientific Procedures) Act, 1986. After completion of experiments animals were killed by an overdose of pentobarbitone sodium (i.v.).

2.2. 1 – General preparation

Experiments were carried out in 23 female anaesthetized spontaneously breathing Sprague Dawley rats (210-285 g). Anaesthesia was induced and maintained during initial surgery with isoflurane in oxygen (4% reduced to 1.5% as necessary). The right jugular vein and left carotid artery were cannulated to permit intravenous (i.v.) injection of drugs and measurement of arterial blood pressure (and heart rate) respectively. The trachea was also cannulated to maintain a patent airway. Isoflurane administration was then stopped and anaesthesia was maintained for the remainder of the experiment with i.v. injections of 1.2 g kg^{-1} urethane (Maggi *et al.*, 1986a; 25% solution dissolved in saline). Supplementary doses of urethane (0.1 g kg^{-1} i.v.) were given where necessary. Depth of anaesthesia was assessed by cardiovascular and respiratory parameters, and by an absence of limb withdrawal in response to paw pinch. Body temperature was monitored using a rectal probe placed under the animal (to prevent interference with the urethral cannula) and maintained between 36 and 38°C using a homeothermic blanket system (Harvard). The animals were infused (0.03 ml min^{-1} i.v.) with a solution comprising 10ml plasma substitute (Gelofusine), 10 ml distilled water, 0.04 g glucose and 0.168 g sodium bicarbonate to maintain blood volume and prevent the development of non-respiratory acidosis. Surgical preparation was followed by a stabilisation period of

1 h, during which and for the remainder of the experiment, inspired air was enriched with oxygen (0.05 – 0.10 l min^{-1}) and blood gases and pH were monitored and maintained between 90 – 130 mmHg P_{O_2}, 35 – 45 mmHg P_{CO_2} and pH 7.3 – 7.4 (using a Ciba Corning pH/blood gas analyser (Model 238)).

2.2. 2 – Measurement of bladder and urethral pressures

The ureters were exposed by retroperitoneal incisions and the proximal ends of each ureter leading from the kidney were cannulated to prevent the bladder filling with urine during experiments. The distal ends of the ureters were tied to ensure no backflow and hence leakage of saline from the bladder. The rats were placed in a stereotaxic frame and the head tilted approximately 10 –15° to allow the animal to lie in the supine position to prevent the weight of the animal affecting the bladder and urethral pressure recordings. The urinary bladder was exposed by a midline abdominal incision and a cut made in the bladder dome. A double lumen cannula with its tip embedded in a Gilson eppendorf pipette tip (1000 µl) was inserted through the cut in the bladder dome and wedged in the bladder neck, permitting separation of the bladder and urethra without surgical interruption (see Figure 2.1). This double lumen cannula was used to measure urethral pressure – the outer cannula (0.86 mm internal and 1.52 mm external diameter) was connected to a syringe pump for the continuous infusion of saline (0.9% wv^{-1}) through the urethra (0.09 ml min^{-1}) which drained freely from the urethral opening. The inner cannula was connected to a pressure transducer (Gould Statham P23Db), such that urethral pressure was measured as the resistance to flow of saline through the urethra. Two cannulae (0.52 mm internal and 1.2 mm external diameter) were also inserted into the bladder, one was connected to a pressure transducer (Gould Statham P23Db) to

record intravesical pressure, the other connected to a syringe pump for the infusion of saline (0.9% wv^{-1}) to evoke the micturition reflex. The rate (0.05 ml min^{-1}) of infusion into the bladder was chosen to stimulate the maximal hourly diuresis rate (see Klevmark, 1974). All three cannulae inserted into the bladder were secured with a suture around the top of the dome of the bladder. This system, based on the method described by Kakizaki *et al.* (1997) is shown in Figure 2.1.

2.2. 3 – Cannulation of the right lateral cerebral ventricle (intracerebroventricular injections)

The rat was placed in a stereotaxic apparatus and a stainless steel guide cannula (22 gauge) was implanted into the right lateral cerebral ventricle according to the following Paxinos and Watson coordinates (Paxinos *et al.*, 1998), 1 mm posterior to bregma, 1.5 mm lateral to bregma and 3 mm ventral to the surface of the skull. Drugs and vehicle solutions (5 µl volume) were administered through an i.c.v. injection cannula (28 gauge) attached via a length of polythene tubing (pre-loaded) to a 10 µl syringe (Hamilton). At the end of the experiment, the cannula position was confirmed by the administration of 3 µl of 2% pontamine sky blue dye.

2.2. 4 – Cannulation of the subarachnoid space (intrathecal injections)

The rat was placed in a stereotaxic apparatus and positioned with a straight, taught spine and head bent forward. The atlanto-occipital membrane was exposed and a small incision made at the midline using the tip of an 18-gauge needle as a cutting edge. A stretched saline filled PE10 catheter was inserted through the incision into the subarachnoid space and advanced caudally until the tip reached the required level of the spinal cord. The length of the catheter required was calculated after measurement against

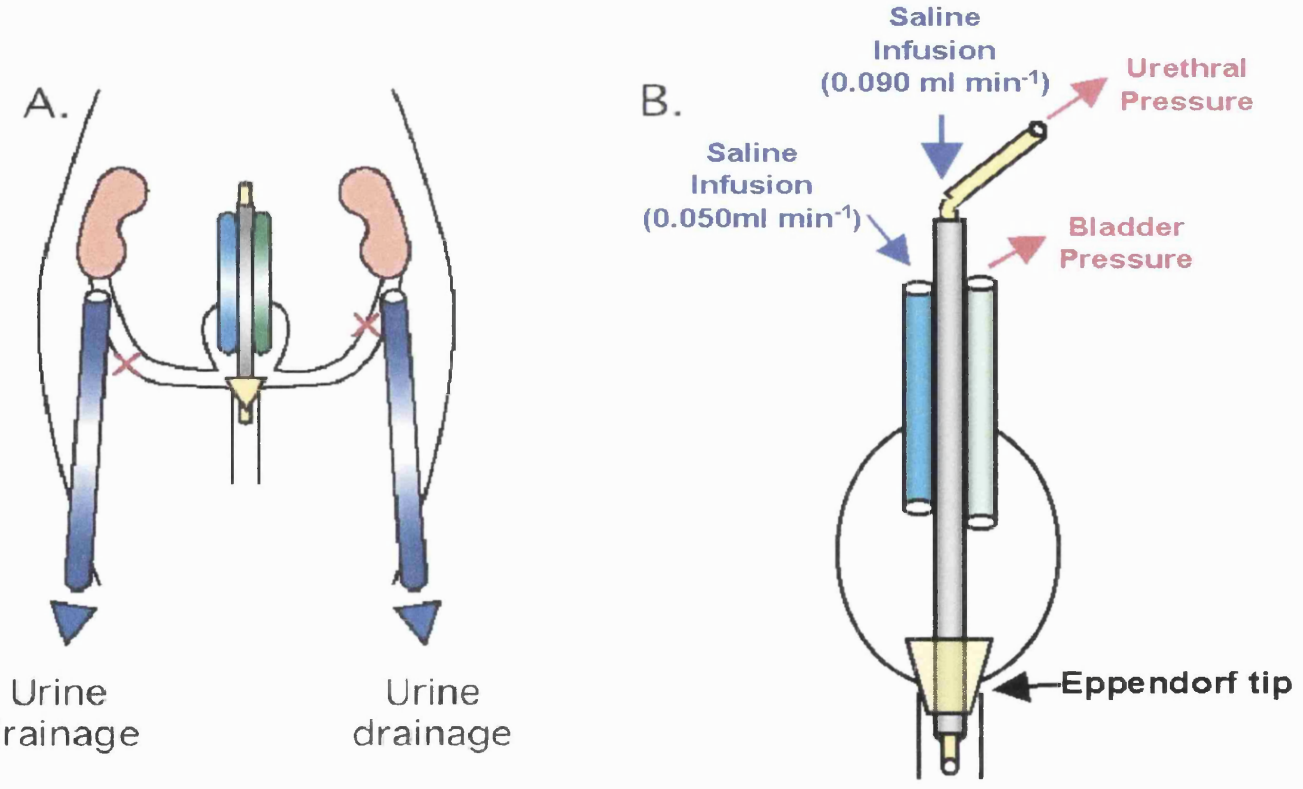


Figure 2.1 - Schematic representation of the experimental methods used. (A) The ureters were tied and cut at the level of the bladder and cannulated at the level of the kidneys. (B) A double lumen cannula was embedded in an eppendorf tip, which was wedged in the bladder neck and allowed simultaneous measurement of bladder and urethral pressures. Two cannulae inserted into the bladder allowed measurement of intravesical bladder pressure and saline infusion to evoke the micturition reflex. (Diagram adapted from Wibberley *et al.*, 2002).

the rat prior to surgery, ~9.5 cm for the L6/S1 region and ~4.5 cm for the T5/T6 region. Compound was administered in a 5 µl volume followed by a flush of saline (volume dependent on length of catheter, ~ 10 µl). At the end of the experiment a laminectomy was performed to verify the position of the catheter tip.

To confirm the direction of flow in the subarachnoid space, end of day experiments were carried out using 5 µl pontamine sky blue dye injected intrathecally at the L6/S1 and T5/T6 levels. The majority of the dye (after local diffusion from the injection site) travelled in a supraspinal direction and the time taken to reach the caudal brain stem from the T5/T6 site was measured and correlated with the onset time observed for suppression of micturition after injection of WAY-100635 at the mid thoracic level.

2.2. 5 – Experimental protocols

Effects of test substances on continuous micturition reflexes.

All animals were left for 1 hr to stabilise after completion of surgery. In this protocol (see Figure 2.2) a 'priming' micturition reflex was evoked by infusion of saline (0.9% w v^{-1}) into the bladder (0.050 ml min^{-1}). After the appearance of three consecutive reflex-evoked bladder contractions of similar amplitude, saline infusion was discontinued. The infused volume of saline was left in the bladder for a 5 min period during which time the reflex was ongoing with isovolumetric bladder contractions. The bladder was then emptied and the cannula used to fill the bladder left open whilst the preparation was left to stabilise for a further 20 min. Another saline infusion in to the bladder was then initiated and stopped once three consecutive bladder contractions of the same amplitude were obtained. These micturition reflexes were allowed to continue

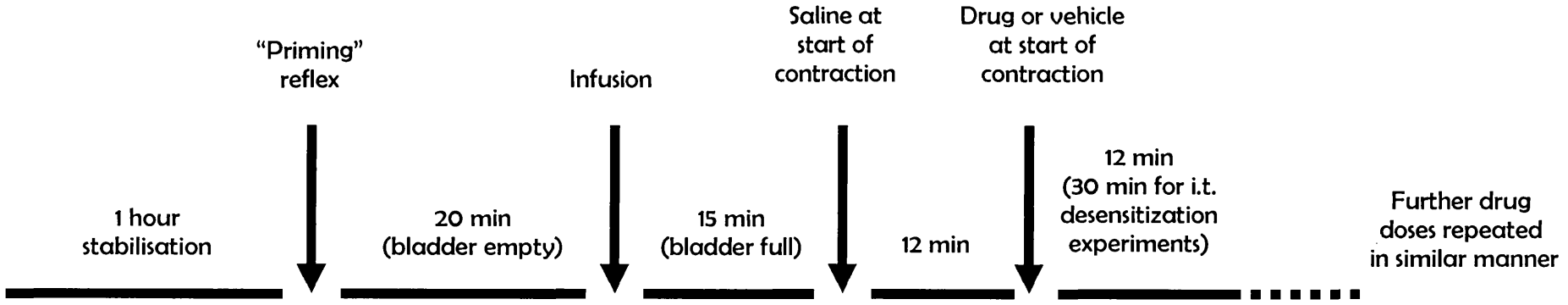


Figure 2.2 - Diagram showing the experimental protocol used

indefinitely and if consistent the test compounds were then administered. After 15 min of stable bladder contractions vehicle was administered at the start of one of the contractions and the contractions observed for the next 12 min (the approximate plasma half-life of WAY-100635 in rat; Pfizer Ltd, personal communication). After this time the test compound was then administered at the start of a contraction and the contractions monitored as before. In some experiments further repeated doses of drug were administered in a similar manner. For i.c.v. administration doses were administered at 12 min intervals, and for i.t. administration there were 30 min intervals.

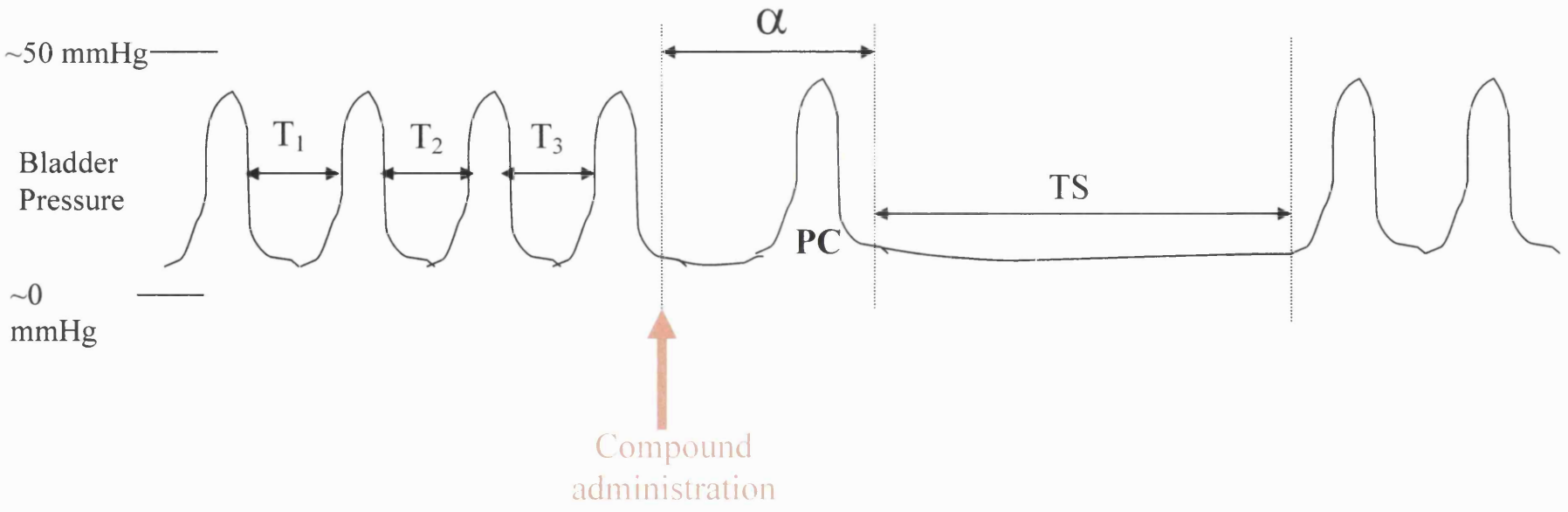
2.2.6 – Data Capture and Analysis

Arterial blood pressure, bladder and urethral pressures were continuously displayed on a chart recorder (Grass instruments) and captured by the Ponemah Physiology Platform version 3.0 (Gould Instrument Systems, Inc.) to allow data to be acquired and analysed off-line using P3 Analysis Modules (Gould Instrument Systems, Inc.). The heart rate (HR) was derived electronically on-line from the blood pressure signal using this software.

Analysis of reflex bladder and urethral responses

Saline infusion into the bladder evoked rhythmic bladder contractions with corresponding urethral relaxations, each of which represents a micturition reflex (Maggi *et al.*, 1986a; see Figure 2.4a). The volume of saline in the bladder remained constant once the experiment had started, so bladder contractions (and associated urethral relaxations) were continuous and all reflexes measured were under isovolumetric conditions. Baseline parameters (bladder pressure, urethral pressure, MAP and HR) were measured, once

values had stabilised between contractions, over 3 x 30 s periods between micturition reflexes at least 4 min after drug administration, and the mean value taken. This time was chosen as it was adjudged that effects evoked on these variables had stabilised. The duration of suppression of the micturition reflex (i.e. time of suppression, TS) was measured as the time taken from the end of the contraction which preceded a longer than average interval before the next contraction (i.e. the preceding contraction, PC) to the start of the next contraction after the PC (see Figure 2.3). If there was no change in the interval between contractions (e.g. after vehicle administration) then the time of suppression was measured as the time taken from the end of the first contraction after compound administration to the start of the following contraction. The onset time (OT) for the compound effect was measured as the time taken from the end of the contraction during which compound was administered, to the end of the PC + the average interval between the 3 contractions immediately prior to compound administration (see Figure 2.3). Since we are unable to determine exactly when the compound takes effect during the time of suppression this is the most accurate method of calculating the onset time for the drug, although obviously it could be slightly longer than the actual onset time. The frequency of bladder contractions (and hence the micturition reflex) was measured as the time taken for the first three reflex bladder contractions after the reflex reappeared, this value was then divided into three and frequency expressed as bladder contractions per min. The mean amplitude (mmHg) and duration (s) of the first three bladder contractions and urethral relaxations after the TS were also measured. High frequency oscillations (HFO's) in urethral pressure occurred with each reflex bladder contraction (see Figure 2.4b), and the mean amplitude (mmHg) and duration (s) of these HFO's were also measured.



$$\text{Where: } OT = \alpha + \frac{(T_1 + T_2 + T_3)}{3}$$

Figure 2.3 – Diagram explaining how the time of suppression of micturition (TS) and onset time (OT) were calculated. See text for further detail. All time measurements were measured in seconds (s). Abbreviations: PC, preceding contraction (i.e. contraction prior to suppression of micturition); $T_{1,2+3}$, interval between contractions; TS, Time of suppression of micturition; OT , onset time for suppression of micturition.

Statistical Analysis

Changes in all of the above variables were measured after drug administration and all values are expressed as mean ± s.e.mean. General drug evoked changes were compared to vehicle/time match control using repeated measures ANOVA to observe an overall drug treatment effect, and this was followed by post-hoc paired Student's t-test to analyse individual dose effects. For all statistical tests, $P < 0.05$ was considered to be statistically significant.

2.2. 7 – Drugs and solutions

Drugs and chemicals were obtained from the following sources: urethane from Sigma Aldrich Chemicals, Poole, Dorset, U.K.; *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)-cyclohexanecarboxamide trichloride (WAY-100635, 1 μg kg^{-1} – 100 μg kg^{-1}) a gift from Wyeth Research U.K., Maidenhead; pentobarbitone sodium from Rhône Mérieux Ltd., Harlow, Essex; isoflurane from Abbott Labs, Queenborough, Kent; gelofusine from Braun Medical Ltd., Aylesbury, Bucks; sodium chloride, glucose, sodium bicarbonate from Merck/BDH, Poole, Dorset; heparin from CP Pharmaceuticals Ltd., Wrexham. WAY-100635 was dissolved in 0.9% wv^{-1} saline and for i.c.v. and i.t. administration the pH was altered to ~6.5 by addition of 0.1M NaOH. All i.c.v. agents were prepared in a 5 μl dose volume and administered over a 20 s period. All i.t. agents were also prepared in a 5 μl dose volume and administered over a 10 s period and followed by ~10 μl flush of saline. All drugs were given as their salts.

2. 3 – Results

2.3. 1 – Saline induced reflex-evoked responses

Infusion of saline into the bladder in 23 female rats caused distension of the bladder, and when the volume threshold was reached evoked the micturition reflex, characterised by the appearance of continuous rhythmic bladder contractions (see Figure 2.4a). In the majority of animals these bladder contractions remained rhythmic throughout the remainder of the experiment with time match controls showing that contractions were still consistently regular after at least 1 hour.

Each rhythmic bladder contraction was accompanied by a fall in urethral pressure that returned to baseline, or in some cases above baseline after each bladder contraction. In 17 out of these 19 animals, high frequency oscillations (HFO's) in urethral pressure occurred just after the start of the bladder contraction (see Figure 2.4b) in the remaining 2 animals HFO's were not observed probably due to variations in the position of the urethral cannula. Each reflex bladder contraction was also accompanied by a slight increase in MAP and HR in some animals (see Figure 2.4a).

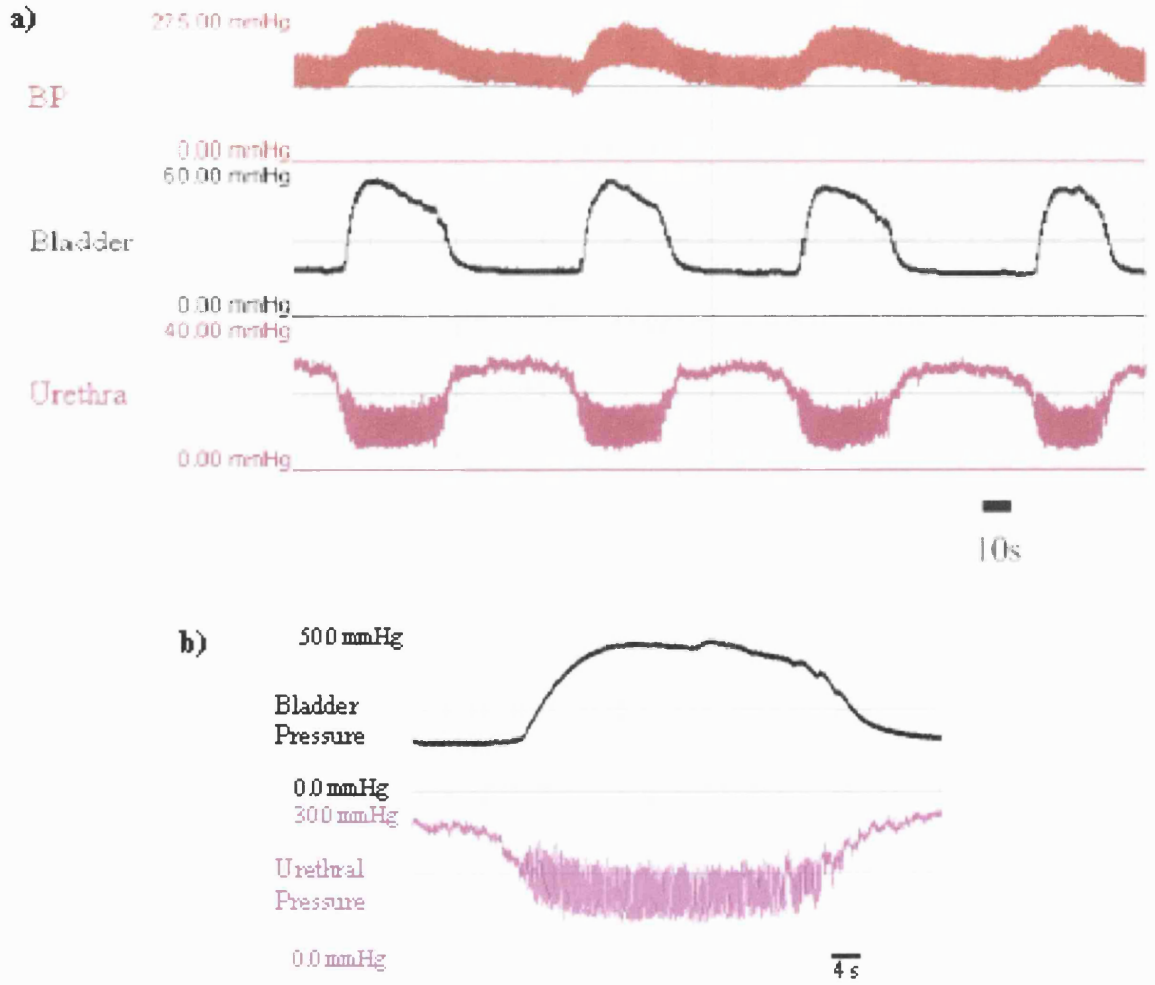


Figure 2.4 - **a)** Typical trace showing distension-induced micturition reflexes characterised by changes in bladder and urethral pressures and MAP. **b)** An individual reflex-evoked bladder contraction showing accompanying urethral relaxation and high frequency oscillations in urethral pressure.

2.3.2 – Effects of i.c.v. administration of WAY-100635 on reflex evoked responses

Administration of the vehicle for WAY-100635 (0.9 % wv^{-1} saline, i.c.v.) had no significant effect on reflex evoked changes in bladder and urethral pressures, MAP or HR (see Table 2.1 and Table 2.2). WAY-100635 i.c.v. (1, 10 & 100 μg kg^{-1}, n = 5) dose dependently and significantly (10 and 100 μg kg^{-1} only) suppressed the micturition reflex for 259 ± 86s, 526 ± 114s and 739 ± 176s respectively, with a rapid mean onset time of 73, 77 and 32 s (see Table 2.2 & Figure 2.5). Figure 2.5 clearly shows that at the end of the period of inhibition of the micturition reflex the rhythmic reflex abruptly reappeared with the bladder contractions having the same amplitude and frequency as in the pre-drug control period. WAY-100635 i.c.v. (1, 10 & 100 μg kg^{-1}, n = 5) had no effect on bladder contraction amplitude or associated urethral variables during micturition (see Table 2.2).

2.3.3 – Effects of i.t. administration of WAY-100635 on reflex evoked responses

I.t. administration of vehicle (0.9 % wv^{-1} saline) had no significant effect on the micturition reflex at either the L6/S1 or the T5/T6 level of the spinal cord (see Table 2.2). I.t. administration of WAY-100635 (10 μg kg^{-1}, n = 5) at the L6/S1 level caused an immediate suppression (onset time = 59 ± 12s) of the micturition reflex for 823 ± 174s (see Table 2.2). To confirm two distinct sites for central 5-HT$_{1A}$ receptors in the control of micturition, WAY-100635 (10 μg kg^{-1}, n = 4) was also injected intrathecally at the mid thoracic T5/T6 level and caused mean suppression of the micturition reflex for 638s but only after an onset time of 375 ± 74s (see Figure 2.6).

Experimental group	*n*	*Bladder Pressure (mmHg)*	*Urethral Pressure (mmHg)*	*MAP (mmHg)*	*Heart Rate (beats min^{-1})*
Vehicle (saline) i.c.v.	5	16.3 ± 1.4	15.4 ± 2.6	152.4 ± 3.9	233 ± 5
WAY-100635 1μg kg^{-1} i.c.v.	5	15.8 ± 1.6	13.5 ± 1.8	152.8 ± 3.5	232 ± 5
WAY-100635 10 μg kg^{-1} i.c.v.	5	15.8 ± 1.9	13.3 ± 1.8	157.4 ± 3.9	230 ± 5
WAY-100635 100 μg kg^{-1} i.c.v.	5	15.9 ± 2.0	13.1 ± 2.0	153.1 ± 4.6	227 ± 4
Vehicle (saline) i.t. L6/S1 level	5	17.6 ± 1.3	15.6 ± 0.5	134.5 ± 3.3	256 ± 9
WAY-100635 10 μg kg^{-1} i.t. L6/S1	5	17.6 ± 1.6	14.7 ± 0.5	137.4 ± 3.5	267 ± 11
Vehicle (saline) i.t. T5/T6 level	4	13.9 ± 1.1	13.9 ± 0.4	134.0 ± 2.5	218 ± 2
WAY-100635 10 μg kg^{-1} i.t. T5/T6	4	13.7 ± 0.8	14.1 ± 0.7	141.0 ± 4.4	221 ± 5
Time match control (i.c.v.) 10 min	4	11.6 ± 5.2	14.2 ± 0.7	163.8 ± 1.0	217 ± 4
Time match control (i.c.v.) 60 min	4	16.3 ± 9.3	13.3 ± 1.2	163.2 ± 2.1	221 ± 4

Table 2.1 - Baseline values of urethral and bladder pressures, mean arterial blood pressure (MAP) and heart rate (HR) for all experimental groups in urethane-anaesthetized female rats. All baselines are a mean measure of baseline values over 3 x 30s periods in between micturition reflexes at least 4 min after compound administration.

Experimental group	*n*	*Suppression (s)*	*Onset time (s)*	*Freq (cont min^{-1})*	*Bladder contractions*		*Urethral relaxations*		*HFO's*	
					Amp (mmHg)	*Dur (s)*	*Amp (mmHg)*	*Dur (s)*	*Amp (mmHg)*	*Dur (s)*
Vehicle (saline) i.c.v.	5	84 ± 42	34 ± 5	0.9 ± 0.1	29.5 ± 1.2	36 ± 3	12.3 ± 0.5	37 ± 2	10.9 ± 0.7	23 ± 2
WAY 1μg kg^{-1} i.c.v.	5	259 ± 86	73 ± 36	0.8 ± 0.1	29.6 ± 1.1	40 ± 3	12.3 ± 0.7	42 ± 2 *	10.7 ± 0.9	26 ± 3
WAY 10 μg kg^{-1} i.c.v.	5	526 ± 114 *	77 ± 10 *	1.0 ± 0.2	30.1 ± 1.5	37 ± 3	12.3 ± 0.8	38 ± 3	10.9 ± 1.0	23 ± 3
WAY 100 μg kg^{-1} i.c.v	5	739 ± 176 *	32 ± 10	1.1 ± 0.2	29.8 ± 1.5	39 ± 3	12.9 ± 1.2	37 ± 3	11.9 ± 1.5	24 ± 4
Vehicle (saline) i.t. L6/S1 level	5	125 ± 58	41 ± 16	0.7 ± 0.1	36.3 ± 1.9	53 ± 4	13.2 ± 0.6	55 ± 3	10.2 ± 0.6	30 ± 3
WAY 10 μg kg^{-1} i.t. L6/S1	5	823 ± 174 *	59 ± 12	0.6 ± 0.1	35.4 ± 1.6	57 ± 3	11.9 ± 0.4	55 ± 5	9.3 ± 0.7	29 ± 3
Vehicle (saline) i.t. T5/T6 level	4	23 ± 8	36 ± 9	1.0 ± 0.2	39.7 ± 1.8	47 ± 4	13.0 ± 0.5	51 ± 5	11.7 ± 0.6	32 ± 4
WAY 10 μg kg^{-1} i.t. T5/T6	4	638 ± 180 *	375 ± 74 *	0.9 ± 0.2	36.1 ± 1.2	52 ± 7	11.4 ± 0.7	46 ± 8	10.5 ± 0.4	24 ± 4
Time match control (i.c.v.) 60 min	4	-	-	0.9 ± 0.1	29.4 ± 1.9	33 ± 2	12.9 ± 1.2	33 ± 2	12.8 ± 1.2	21 ± 1

Table 2.2 - Effects of i.c.v. administration of WAY-100635 (1,10 & 100 μg kg^{-1}) and i.t. (L6/S1 & T5/T6) administration of WAY-100635 (10 μg kg^{-1}) on various micturition reflex parameters. * $P < 0.05$ when compared to vehicle (saline) control using paired Student's *t*-test. Abbreviations: Freq, frequency; cont, contractions; Amp, amplitude; Dur, duration; HFO's, high frequency oscillations.

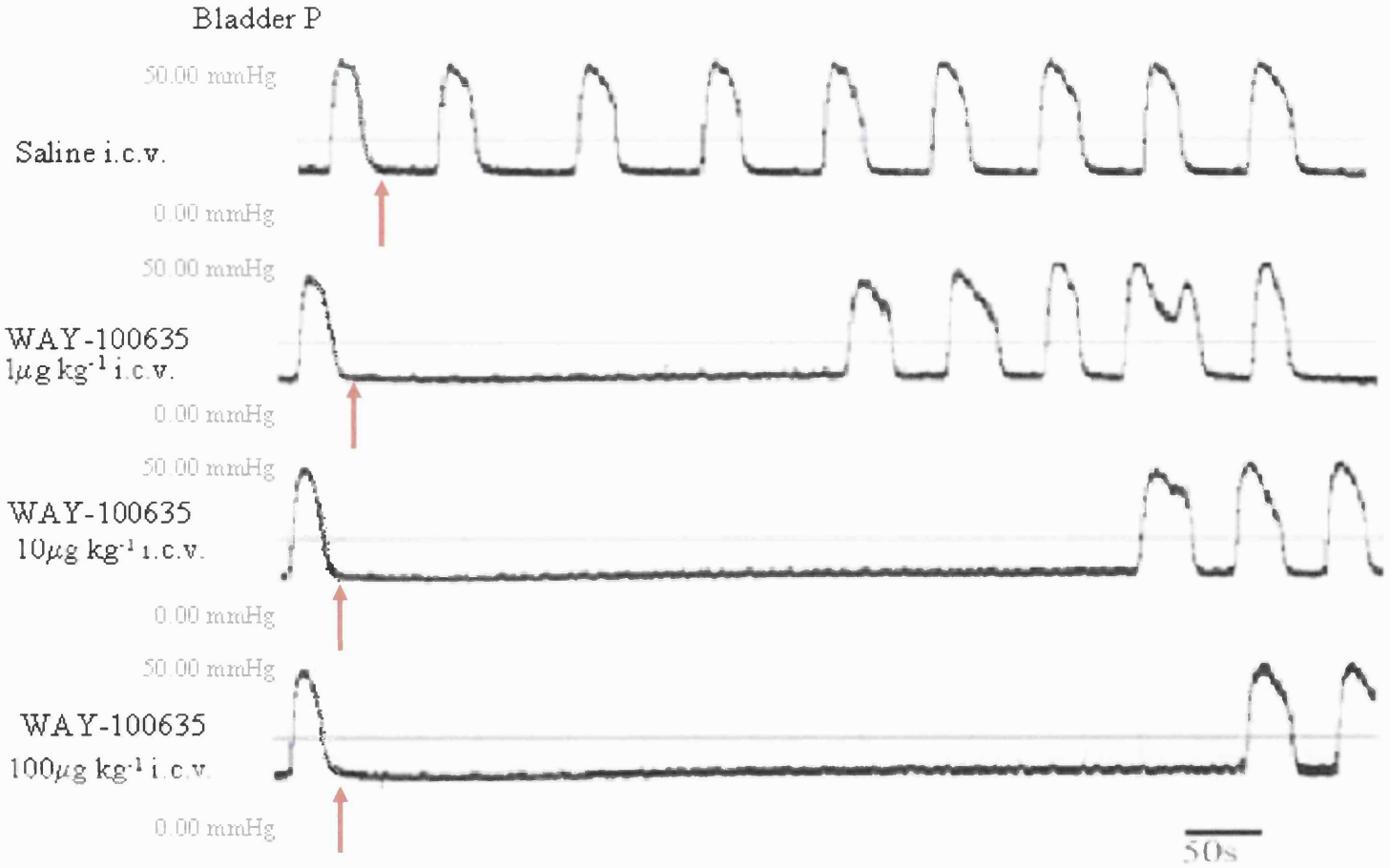


Figure 2.5 - Trace showing WAY-100635 (1, 10 & 100 μg kg^{-1}, i.c.v.,) induced suppression of reflex-evoked bladder contractions in the urethane anaesthetized female rat. The urethral relaxations which accompany the bladder contractions during the micturition reflex were also inhibited but for the sake of clarity this is not shown in this trace. The red arrows mark the point at which compound was administered (i.e. at the end of the preceding contraction (PC) see 2.2.6).

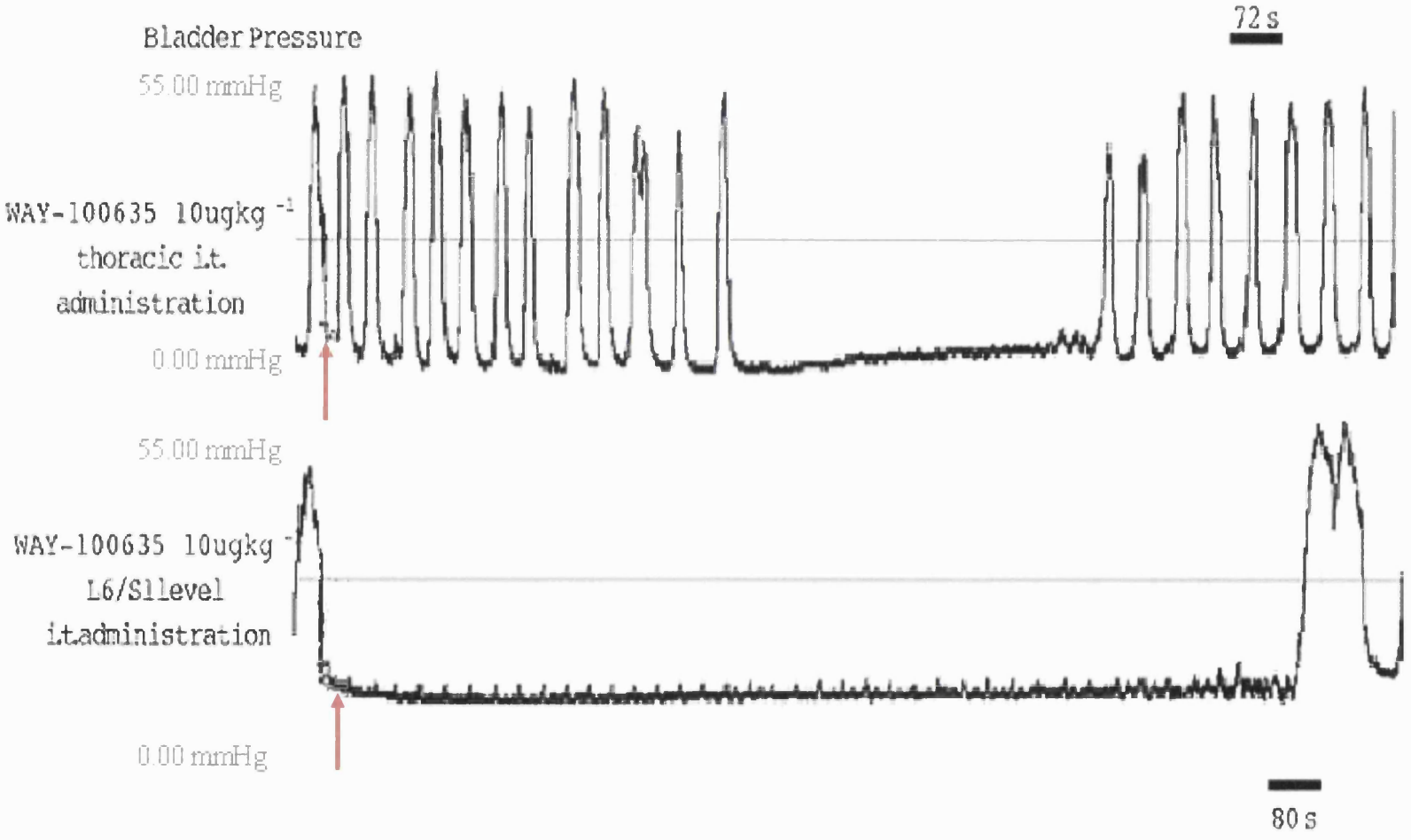


Figure 2.6 - Trace showing the effect of thoracic (T5/T6, n = 4) and lumbosacral (L6/S1, n = 4) i.t. administration of WAY-100635 on bladder contractions in female urethane anaesthetized rats. The red arrows mark the point at which compound was administered (i.e. at the end of the preceding contraction (PC) see 2.2.6).

2.3.4 – Effect of multiple dosing of WAY-100635 after i.c.v. and i.t. administration

A second dose of i.c.v. WAY-100635 (10 μg kg^{-1}, n = 5) 12 min after the initial dose suppressed micturition for a significantly shorter period of time than the initial dose. The second dose was only 39% as effective as the initial dose at suppressing the micturition reflex with the initial dose suppressing micturition for an average of 567 ± 117 s as opposed to 234 ± 82 s with the second dose (see Figure 2.7). The same phenomenon was also observed after intrathecal administration at the L6/S1 level with the second dose (administered 30 min after the initial dose) only 26% as effective as the first dose – the first dose elicited suppression of micturition for 823 ± 174 s as opposed to 217 ± 134 s with the second dose (see Figure 2.7). Vehicle and time match controls for both routes of administration were carried out by administering saline at 12 or 30 min intervals for a time period of at least 1 h, and as regular micturition reflexes were obtained throughout this time (see Table 2.2), these controls confirmed that the above phenomenon is specific to repeated administration of WAY-100635 rather than time–induced. The repeated administration of WAY-100635 was also carried out at a longer time interval (60 min) between doses and the above phenomenon was still observed (data not shown, n = 2). To try and further characterise this phenomenon and establish whether the 5-HT_{1A} receptors were still functional after the initial dose of WAY-100635, 8-OH-DPAT (100 μg kg^{-1}, i.c.v., n = 2) was administered after an initial WAY-100635 dose and the typical facilitation of micturition by 5-HT_{1A} receptor agonists was still observed (data not shown).

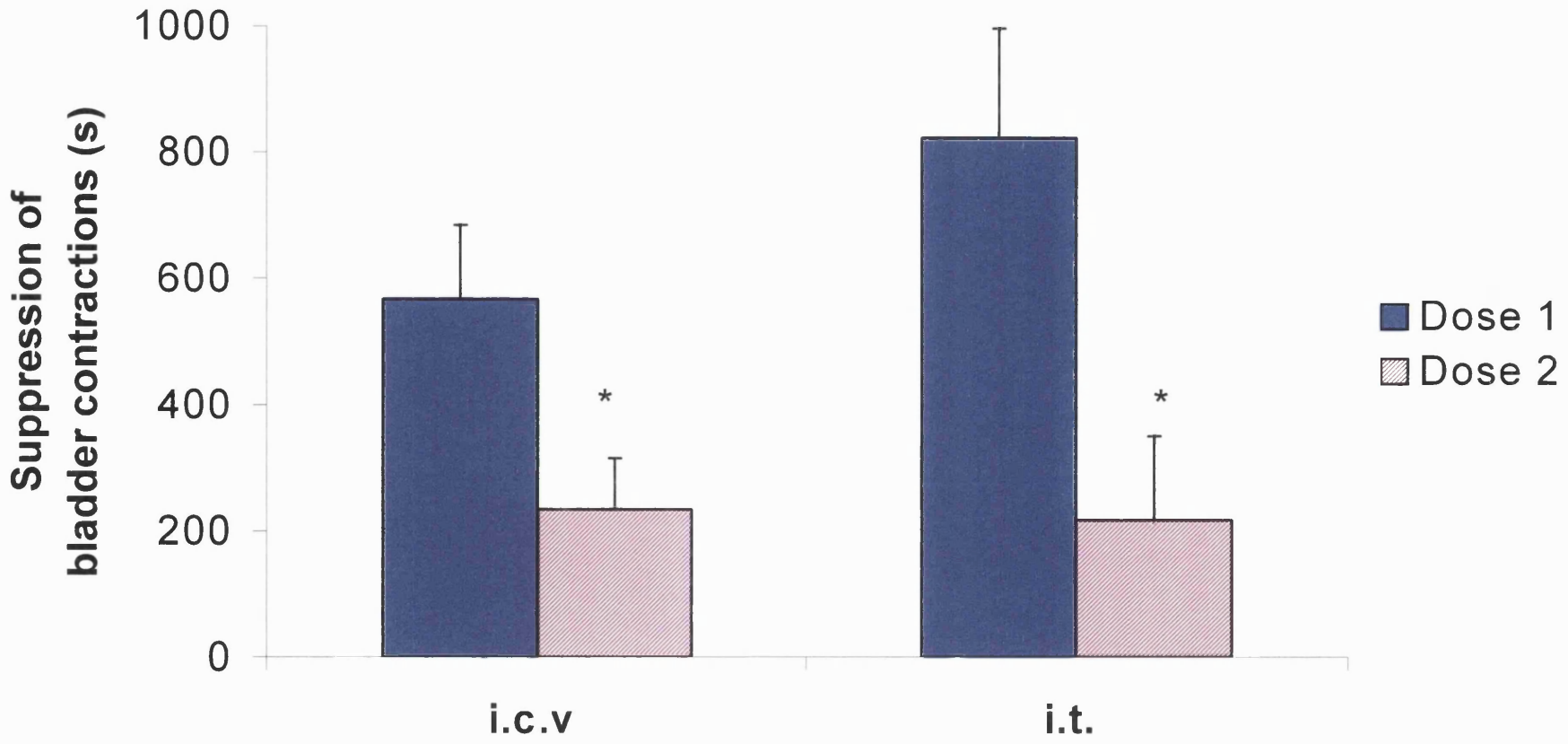


Figure 2.7 - Graph showing that a second dose of WAY-100635 (10 μg kg^{-1}) only suppresses micturition for 39 % (i.c.v., n = 5) and 26 % (i.t at the L6/S1 level., n = 5) of the time of suppression (TS) observed with the initial dose. * $P < 0.05$ when compared to the TS for initial dose using paired Students *t*-test. All values mean ± s.e.mean.

2.4 – Discussion

The results from this study have shown that WAY-100635 i.c.v. (1, 10 & 100 μg kg^{-1}, n = 5) is able to dose dependently and significantly suppress the micturition reflex with a rapid mean onset time (see Figure 2.5) in urethane anaesthetized female rats. Intrathecal administration of WAY-100635 (10 μg kg^{-1}) also caused an immediate suppression of the micturition reflex but for a longer period of time than that observed after i.c.v. administration (see Figure 2.6). Interestingly, mid-thoracic i.t. administration (T5/T6 level) of WAY-100635 (10 μg kg^{-1}) had a longer onset time for the suppression of micturition than that observed after i.t. administration at the L6/S1 level, thus confirming that there are two distinct sites for 5-HT$_{1A}$ receptors involved in the control of micturition (see 2.4.1). Data from the present study also suggests that WAY-100635 may be less effective at suppressing micturition after repeated administration, since a second dose of WAY-100635, after both i.c.v. and i.t. administration, was only 39% or 26% respectively, as effective as the initial dose at suppressing micturition (see Figure 2.7).

2.4.1 – Sites of action for central 5-HT$_{1A}$ receptors involved in micturition

The present study investigated the role and sites of action of central 5-HT$_{1A}$ receptors involved in the control of micturition in the female urethane anaesthetized rat. Micturition in the rat is meditated by a spinobulbospinal reflex, which consists of an ascending afferent limb passing from the lumbosacral spinal cord to the PAG which acts as a co-ordinating centre for afferent information arriving from the LUT and activates the PMC in the rostral pons. The descending efferent limb passes from the PMC back down to lumbosacral parasympathetic pre-ganglionic neurones which send axons in the pelvic

nerve to convey efferent excitatory outflow to the bladder (de Groat *et al.*, 1993). Since 5-HT$_{1A}$ receptors have been identified at the lumbosacral level (Thor *et al.*, 1993) and are also present on medullary raphé neurones in the brainstem (Barnes *et al.*, 1999), it is possible that WAY-100635 could exert its effects at multiple levels of the neuraxis.

Until the very recent publication of a study investigating the effect of intracerebroventricular administration of WAY-100635 in the anaesthetized female rat (Yoshiyama *et al.*, 2003), an actual physiological role for supraspinal 5-HT$_{1A}$ receptors in the control of micturition had yet to be demonstrated. However, the results of this study confirm that there are two distinct sites for 5-HT$_{1A}$ receptors actively involved in the control of voiding, with both supraspinal and lumbosacral spinal cord 5-HT$_{1A}$ receptors playing a physiological role. Intracerebroventricular administration of WAY-100635, a selective silent 5-HT$_{1A}$ receptor antagonist, suppresses micturition in an isovolumetric anaesthetized model for a period of time which is dose dependent (see Figure 2.5). This is consistent with previous reports of a depressant effect of intravenous and intrathecal administration of WAY-100635 on bladder activity (Testa *et al.*, 1999; Conley *et al.*, 2001; Kakizaki *et al.*, 2001) and reports that intracerebroventricular administration of the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, facilitates micturition by activating supraspinal 5-HT$_{1A}$ receptors (Lecci *et al.*, 1992). Results from this present study showing that intrathecal (L6/S1 level) administration of WAY-100635 suppresses micturition, also confirmed that 5-HT$_{1A}$ receptors at the L6/S1 level of the spinal cord have an excitatory role in the control of micturition, in agreement with previous studies (Lecci *et al.*, 1992; Kakizaki *et al.*, 2001).

The present study investigated the role of 5-HT$_{1A}$ receptors using WAY-100635 which has been reported in the literature as being a highly selective and potent silent 5-HT$_{1A}$ receptor antagonist (Craven *et al.*, 1994; Forster *et al.*, 1995; Corradetti *et al.*, 1996; Fletcher *et al.*, 1996; Fornal *et al.*, 1996). Central 5-HT$_{1A}$ receptors exist as two functionally distinct populations. 5-HT$_{1A}$ autoreceptors are located on the dendrites and cell bodies of 5-HT-containing neurones and mediate serotonergic negative-feedback inhibition in these neurones, whereas 5-HT$_{1A}$ heteroreceptors are located on other non 5-HT-containing neurones. The difference between these two populations of 5-HT$_{1A}$ receptors in terms of their receptor reserve and receptor-effector coupling has lead to some conflicting pharmacological results with certain 5-HT$_{1A}$ receptor compounds which have opposing actions at the two types of 5-HT$_{1A}$ receptor. The 'silent' antagonistic properties of WAY-100635 distinguish it from other compounds, such as NAN-190, which were initially described as selective 5-HT$_{1A}$ receptor antagonists but have since been shown to have partial agonist activity at somatodendritic 5-HT$_{1A}$ autoreceptors (Hjorth *et al.*, 1990). Thus, the present data is unable distinguish which type of 5-HT$_{1A}$ receptor (i.e. auto- or heteroreceptor) is involved in the control of micturition.

The present data shows that i.c.v. administration of WAY-100635 potently inhibits the frequency of isovolumetric reflex bladder contractions in anaesthetized rats. The rapid saline induced distension of the urinary bladder caused a series of rhythmic reflex bladder contractions which have been observed in previous studies (Guarneri *et al.*, 1993; Testa *et al.*, 1999; Kakizaki *et al.*, 2001; Yoshiyama *et al.*, 2003). It has been suggested that the frequency of these contractions is associated with the sensory afferent limb of the micturition reflex pathway in the spinal cord and the correct functioning of the PMC, whereas the amplitude is a property of the descending efferent arm of the reflex (Maggi

et al., 1984; Maggi *et al.*, 1986b). I.c.v. administration of WAY-100635 in this current study caused an immediate inhibition of reflex bladder contractions followed by a recovery of the contractions exhibiting the same amplitude and frequency as the pre-drug level, in agreement with similar studies using i.v. and i.t. administration of WAY-100635 (Testa *et al.*, 1999; Kakizaki *et al.*, 2001). This suggests that i.c.v. WAY-100635 influences the timing of the micturition reflex by suppressing the afferent limb of the reflex pathway but has no effect on the descending efferent limb. These results are in contrast to those of Yoshiyama *et al.*, (2003) who found that i.c.v. administration of a high dose of WAY-100635 (100μg) caused a similar inhibition of reflex contractions but upon recovery of the contractions there was a marked reduction in their amplitude and frequency. This behaviour is shared by several antimuscarinic drugs (Guarneri *et al.*, 1993) but has never before been observed with 5-HT$_{1A}$ receptor antagonists and is therefore somewhat unexpected. Since a dose of 100μg per animal is approximately four times higher (dependant on rat weight) than the 100μg kg^{-1} dose used in this study, it is possible that the dose used by Yoshiyama *et al.* is not selective for 5-HT$_{1A}$ receptors and maybe acting on α_1–adrenoceptors which are the next receptor family, after 5-HT$_{1A}$ receptors, that WAY-100635 has affinity for (K_i (mM) of 0.33 and 18.8 for 5-HT$_{1A}$ receptors and α_{1A}-adrenoceptors respectively; Testa *et al.*, 1999). α_1–adrenoceptors are involved in the control of bladder and urethral activity (Yoshiyama *et al.*, 2000; Conley *et al.*, 2001) and α_{1A}-adrenoceptors have been shown to facilitate modulation of the efferent limb of the micturition reflex with the α_{1A}-adrenoceptor antagonist RS-100329 significantly reducing bladder contraction amplitude (Yoshiyama *et al.*, 2001), although this was not observed by Conley *et al.* (2001). Therefore lack of selectivity with action at

α_{1A}-adrenoceptors may explain the observation of a reduction in amplitude of contractions after a high dose of WAY-100635 (100μg) by Yoshiyama *et al.* (2003).

Further evidence for the presence of two distinct sites of action for central 5-HT$_{1A}$ receptors involved in the control of micturition was provided by the fast onset times and effects of intrathecal administration in the current study. I.c.v. administration of WAY-100635 (1, 10 and 100μg kg^{-1}) into the right lateral ventricle caused an immediate suppression of reflex bladder contractions. Since the effects of the drug had such a rapid time of onset it is very unlikely that the drug was acting directly on spinal 5-HT$_{1A}$ receptors, however this was confirmed by the intrathecal administration of WAY-100635 (10μg kg^{-1}) at various levels of the spinal cord. I.t. administration at the L6/S1 level caused an immediate suppression of reflex bladder contractions due to the presence of 5-HT$_{1A}$ receptors at this level of the spinal cord. However, mid-thoracic administration at the T5/T6 level had a mean onset time of 375s. The direction of flow of cerebrospinal fluid in the subarachnoid space is towards the brain, so it can be reasoned that the onset time for the effects of WAY-100635 after mid thoracic administration is the time taken for the compound to reach supraspinal 5-HT$_{1A}$ receptors. Post-mortem examination of dye distribution after mid thoracic administration helped confirm this supposition. The observation that immediate suppression of reflex bladder contractions occurs after both i.c.v. and i.t. (L6/S1) administration but not mid-thoracic administration (T5/T6) confirms that there are two distinct central sites for 5-HT$_{1A}$ receptors involved in the control of micturition. The eventual suppression of reflex bladder contractions 375 s after mid-thoracic administration is in contrast to observations by Kakizaki *et al.* (2001) who surprisingly found that intrathecal administration of WAY-100635 (30 μg) at the

thoracic or cervical levels had no effect on rhythmic bladder contractions. This may simply be due to not waiting a sufficient period of time to observe the onset of suppression of reflex bladder contractions, since the delay in onset could be unexpected considering the immediate suppression observed at the L6/S1 level.

It is interesting to note that the length of time of suppression of the micturition reflex is significantly longer after intrathecal (L6/S1) administration of WAY-100635 than after i.c.v. administration, suggesting that the 5-HT$_{1A}$ receptors at the different central sites may act via two distinct mechanisms. Although i.c.v. administration delivers WAY-100635 directly into the right lateral ventricle of the brain, it does not necessarily mean that the effects of the drug are restricted only to a direct effect at supraspinal sites – it is possible to speculate that WAY-100635 could indirectly change transmission at synapses in the spinal cord by altering activity in bulbospinal pathways due to blockade of somatodendritic 5-HT$_{1A}$ autoreceptors in raphé neurones (see discussion below). This hypothesis is supported by studies showing that intracerebroventricular pre-treatment of 5,7-dihydroxytryptamine, which destroys 5-HT-containing neurones, blocks the effects of i.c.v. but not i.t. 8-OH-DPAT (Lecci *et al.*, 1992), which also suggests that the effects of intrathecal 8-OH-DPAT are mediated by actions on 5-HT$_{1A}$ heteroreceptors on spinal neurones which are functionally independent from the supraspinal 5-HT input. However, it must be noted that i.c.v. 5,7-dihydroxytryptamine does not completely deplete spinal 5-HT, mainly because 5-HT neurones which are present in the autonomic areas of the spinal cord (Newton *et al.*, 1986) are likely to be spared by the i.c.v. injection of the neurotoxin, and this could explain why the effects of i.t. 8-OH-DPAT are not blocked after this pre-treatment (Lecci *et al.*, 1992). However, the possibility of 5-HT$_{1A}$ heteroreceptors on spinal neurones which are functionally independent from the

supraspinal 5-HT input is still conceivable since it has been shown that the excitatory response to intrathecal 8-OH-DPAT is still observed in chronic spinal rats (Lecci *et al.*, 1992). Further investigation in to the effects of antagonising spinal 5-HT$_{1A}$ receptors has been undertaken using electrophysiological techniques (Kakizaki *et al.*, 2001) which revealed that intrathecal administration of WAY-100635 appears to suppress the descending pathway from the brainstem to the spinal cord, since WAY-100635 (30 & 100μg i.t.) dose dependently reduced the amplitude of bladder contractions evoked by electrical stimulation of the PMC (Kakizaki *et al.*, 2001). However, WAY-100635 (30 & 100μg i.t.) was unable to alter the amplitude or latency of pelvic nerve-evoked potentials in the periaqueductal gray (Kakizaki *et al.*, 2001), thus suggesting that WAY-100635 does not alter the ascending afferent pathway from the bladder to the PAG/PMC. Therefore, it is possible that in the present study, the action of intrathecal WAY-100635 on spinal postsynaptic 5-HT$_{1A}$ receptors suppresses micturition for longer periods of time than i.c.v. administration due to a direct inhibition of the efferent drive to the bladder, whereas the effects on micturition via supraspinal 5-HT$_{1A}$ receptors after i.c.v. administration are indirect and could be shortened due to increased competition with endogenous 5-HT in the dorsal raphé (see discussion below). It would be interesting to examine the effect of intrathecal administration of NAN-190 which has antagonistic properties at 5-HT$_{1A}$ heteroreceptors but is a partial agonist at autoreceptors (Hjorth *et al.*, 1990). However, it must also be remembered that the blood flow is slow at the spinal level and the longer period of suppression of micturition after intrathecal administration may be due to slower metabolism of WAY-100635 after administration via this route.

Several studies provide evidence supporting the idea that WAY-100635 acts on supraspinal 5-HT_{1A} receptors to unmask an inhibitory pathway that is tonically suppressed by supraspinal serotonergic mechanisms. 5-HT_{1A} receptors are universally present in the rat brain (Barnes *et al.*, 1999), however several 5-HT-containing neuronal clusters located in the brainstem raphé nuclei have been identified which contain abundant somatodendritic 5-HT_{1A} autoreceptors (Barnes *et al.*, 1999), and it has been shown that 5-HT within the dorsal horn of the spinal cord arises primarily from these 5-HT neurones in the raphé nuclei (Bowker *et al.*, 1981). Electrical stimulation of the brainstem nucleus raphé magnus inhibits bladder contractions in rats and cats (McMahon *et al.*, 1982; Sugaya *et al.*, 1998) and also inhibits the firing of spinal dorsal horn neurones which are activated by afferents in the pelvic nerve (Lumb, 1986b). This, along with the observation that raphé neurones are activated by bladder distension (Lumb, 1986b; Oh *et al.*, 1986), indicates that raphé neurones might be involved in a spinobulbospinal negative-feedback circuit in which ascending afferent input from the bladder triggers a reflex loop resulting in the activation of spinal-spinal inhibitory projections back to the sacral spinal cord which suppress micturition. Some of these projections are 5-HT-containing, and their activity is controlled by abundant somatodendritic 5-HT_{1A} autoreceptors which mediate a local negative-feedback mechanism (Aghajanian *et al.*, 1986). After stimulation, these 5-HT_{1A} autoreceptors inhibit the firing of spinal neurones (Sprouse *et al.*, 1987) thus decreasing 5-HT turnover in the spinal cord (Gobert *et al.*, 1995). In contrast, it has been shown that antagonism by WAY-100635 increases the firing rate of raphé, and hence activity of spinal cord 5-HT-containing neurones both *in vitro* (Corradetti *et al.*, 1996) and *in vivo* (Fornal *et al.*, 1996; Mundey *et al.*, 1996), presumably by blocking the feedback action of endogenous 5-HT at 5-HT_{1A} autoreceptors. Therefore, it is possible that the inhibitory action of

WAY-100635 on the micturition reflex after i.c.v. administration could be due to blockade of 5-HT_{1A} autoreceptors in the raphé, which increases the firing rate of raphé neurones, resulting in increased levels of spinal 5-HT and thus inhibiting spinal processing of afferent inputs from the bladder. Citalopram (a selective serotonin reuptake inhibitor; SSRI) potentiates the inhibitory effect of neutral 5-HT_{1A} receptor antagonists on the micturition reflex (Leonardi *et al.*, 2001) helping to confirm the above hypothesis by indicating that 5-HT release is involved in the mechanism of action of these antagonists. Assuming the above mechanism of action for WAY-100635 to be correct, then it is also possible that in the present study, after administration of WAY-100635, the ever increasing synaptic concentrations of 5-HT (due to the negation of the usual negative-feedback control mediated by 5-HT_{1A} autoreceptors) eventually leads to competition between endogenous 5-HT and WAY-100635 for the 5-HT_{1A} receptors sites, hence the return of the regulation of raphé firing and the sudden resumption of reflex bladder contractions. This may help explain the observation that the time of suppression of micturition is longer after i.t. administration (L6/S1) than i.c.v. administration of WAY-100635 (10 $\mu g\ kg^{-1}$; 823 and 526s respectively). Since WAY-100635 at the spinal level directly inhibits parasympathetic preganglionic neurones innervating the bladder, there is unlikely to be competition with increasing levels of 5-HT and therefore reflex bladder contractions only reappear after WAY-100635 has been metabolised. This is feasible since the half-life of WAY-100635 in rats is very short (~ 12-15 min; Pfizer Ltd., personal communication). Despite this evidence suggesting an inhibitory raphé-spinal pathway is involved in the control of micturition, the present data cannot rule out possible interactions with other supraspinal structures such as the locus coeruleus (Barnes *et al.*, 1999).

2.4.2 – Species variation in the role of 5-HT$_{1A}$ receptors in the control of micturition?

Recently, it has been suggested that 5-HT$_{1A}$ receptor agonists have opposite effects in rats and cats (see de Groat, 2002; Thor *et al.*, 2002). In rats, it has been demonstrated that intravenous (i.v.), i.c.v. and i.t. administration of the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, facilitates micturition and causes a decrease in bladder capacity and a reciprocal increase in voiding frequency (Lecci *et al.*, 1992), and data from the present study has confirmed that blockade of 5-HT$_{1A}$ receptors by WAY-100635 suppresses micturition in rats. However, systemic administration of 8-OH-DPAT (0.1-1 mg kg^{-1}) has been shown to cause an increase in bladder capacity and a decrease in voiding frequency in cats in which the bladder was irritated by intravesical infusion of acetic acid (Thor *et al.*, 2002). This observation has led to the suggestion that "the effects of 5-HT$_{1A}$ receptor activation in rats and cats show opposite effects" (Thor *et al.*, 2002) and hence the idea of species variation in the response of the LUT to 5-HT$_{1A}$ receptor activation. However, it must be remembered that 8-OH-DPAT only has a significant inhibitory effect in cats under the conditions of intravesical acetic acid infusion, and not saline infusion (Thor *et al.*, 2002). The afferent information arising from an irritated bladder is increased, and results in altered control of micturition under these conditions, therefore the data obtained from studies conducted under irritated or non-irritated conditions are not directly comparable. Assuming 8-OH-DPAT acts at the level of 5-HT$_{1A}$ autoreceptors in the raphé nuclei, it would be expected that administration of this 5-HT$_{1A}$ receptor agonist would have a facilitatory effect on micturition, as observed in rats, since activation of the autoreceptors would reduce raphé neuronal firing, and concomitantly reduce 5-HT levels in projection areas resulting in a removal of the inhibitory serotonergic control. The observations by Thor *et al* (2002) are also surprising since it

has been shown in cats, that increasing raphé neuronal firing by chemical (Chen *et al.*, 1993) or electrical stimulation (McMahon *et al.*, 1982) has an inhibitory effect on bladder function, and therefore a similar response would be expected after increasing raphé neuronal firing by removal of the negative feedback control by 5-HT$_{1A}$ autoreceptor activation.

Apart from the contradictory observations by Thor *et al* (2002) explained above, the majority of evidence indicates that activation of the central serotonergic system by endogenous 5-HT in projection areas, can suppress voiding in both rats and cats, by enhancing efferent control to the urethral outlet and inhibiting the parasympathetic excitatory input to the urinary bladder (see de Groat, 2002).

2.4.3 – 'Tachyphylaxis' after repeated administration of WAY-100635

In the current study, repeated administration (two doses) of i.c.v. WAY-100635 appeared to cause the second dose to be significantly less effective than the initial dose at suppressing micturition. This 'tachyphylaxis' phenomenon was not restricted to i.c.v. administration as it was also observed after L6/S1 intrathecal administration (see Figure 2.7) and after i.v. administration (Pfizer Ltd., personal communication). These results are surprising given that WAY-100635 is considered to be a neutral or silent antagonist, and this sort of 'desensitization' effect is often associated with agonist-induced down regulation of receptor number. The observation of this phenomenon also directly contradicts results published in the literature. Kakizaki *et al.* (2001) found that reproducible inhibition of rhythmic bladder contractions could be evoked after multiple injections (3-4 times, 30 min intervals) of i.v. WAY-100635 at higher doses (0.3 and 3

mg kg^{-1}) than those used in the current study. Yoshiyama *et al.* (2003) also investigated the possibility of a tachyphylactic effect with repeated administration of WAY-100635 using various dosing regimens (WAY-100635 administered at 60 min intervals), but did not observe any significant difference in the time of inhibition of bladder contractions after multiple administration. The reason for these conflicting results is unknown, but it is noteworthy to mention that in the current study the phenomenon was statistically significant and highly reproducible, and although there was only a 12 min interval between i.c.v. doses, the phenomenon was still observed after intrathecal administration at 30 min intervals, and could even be observed after a 60 min interval (data not shown). It is also interesting that although not significant, Yoshiyama *et al.* (2003) did observe a considerable decrease in the time of inhibition of isovolumetric bladder activity with a second dose of i.c.v. WAY-100635 (for example a second dose of 10 μg WAY-100635 had a time of suppression of 9.5 min as opposed to 15.6 min with the initial dose), thus possibly suggesting the presence of a similar phenomenon. The mechanism for this phenomenon is unknown, but interestingly studies with administration of 8-OH-DPAT after WAY-100635 have shown that 5-HT_{1A} receptors are still functional after blockade with WAY-100635 (see section 2.3.4) and therefore the 'desensitization' effect cannot be attributed to receptor internalisation after the initial dose of WAY-100635. There are range of possible explanations for the presence of this phenomenon, including an exhaustion in of the releasable stores of 5-HT, a conformational change in the receptors, increased metabolic degradation of the drug or an increased homeostatic response which nullifies the drug's effect. However, the apparent speed of the onset of the phenomenon is surprising and further studies are required to further characterise and confirm its presence (see section 2.4.5).

2.4.4 – The 'eppendorf tip double lumen cannula' isovolumetric method in rat: a good model for micturition?

The model of micturition used in the present study allows simultaneous measurement of bladder and urethral pressures, and is characterised by a series of rhythmic isovolumetric reflex contractions which the bladder, as a closed system, undergoes. Although the insertion of the eppendorf tip is a potentially irritating experimental procedure, it is less invasive than the use of ligatures around the bladder neck to functionally separate bladder and urethral pressures as used by previous investigators (Bennett *et al.*, 1995), and the advantage of allowing simultaneous measurement of bladder and urethral pressures, thus enabling investigation of the complex interactions between these functional components, outweighs the above limitation. The use of this model allowed us to observe that upon the return of the micturition reflex after suppression by WAY-100635 there was no direct effect of WAY-100635 on the urethral relaxations (see Table 2.2), but obviously urethral activity stopped whilst micturition was suppressed. Despite the above advantage of this model, it is inevitable that it is not particularly physiological since the bladder never empties and the resulting rhythmic reflex bladder contractions occur much more frequently than in an anaesthetized cystometry model. It is therefore probable that there is a higher than normal afferent drive from the bladder, and it could be argued that this may allow WAY-100635 to have a greater suppressant effect than in a more physiological model. However, other studies have shown WAY-100635 to significantly increase the bladder capacity in an anaesthetized rat cystometry model (Testa *et al.*, 1999) and acute i.v. administration of WAY-100635 in conscious rats has been shown to significantly decrease the frequency of voiding over a 3 h period (see Chapter 3), therefore confirming that the suppressant effect observed with WAY-100635 in the present study is not unique to this model. Since the aim of the present study was to

investigate the sites of action for 5-HT$_{1A}$ receptors, the use of this model also had the advantage of allowing a more accurate estimate for the onset time of suppression as the interval between micturition reflexes was greatly reduced from that observed during cystometry.

Another interesting observation with this model is that the length of time of suppression after i.c.v. WAY-100635 administration appears to be much shorter (10 μg kg^{-1} ; 8.7 min) than that observed (10 μg ;15.6min) by Yoshiyama *et al.* (2003) who ligated the urethral outlet and did not measure urethral pressure. This observation could be due to differences in levels of anaesthesia, or due to the increased dose used by Yoshiyama *et al.* (approximately 4 times higher, dependent on rat weight, than the dose used in the present study). However, it is possible that the constant flow of fluid through the urethra (to enable measurement of urethral pressure) may initiate an urethrovesical reflex, which although suppressed initially by WAY-100635, causes an increased afferent drive which eventually shortens the suppression by WAY-100635. The presence of a urethrovesical reflex was first suggested by Barrington (1931) who showed that in cats the flow of urine through the urethra can actually elicit a detrusor contraction, and it has been intimated that it maybe involved in maintaining the detrusor contraction during voiding and is required for efficient bladder emptying (Barrington, 1931). The presence of a urethrovesical reflex has since been confirmed in a number of species, including cats (Garry, 1959), sheep (Robain *et al.*, 2001), rats (Jung *et al.*, 1999) and even recently in humans (Shafik *et al.*, 2003). It has been shown that the main component of a detrusor response to flow along the urethra is a spinobulbospinal reflex involving the brainstem with its afferent limb in the pudendal nerve and efferent in the pelvic nerve (Barrington,

1941). Perhaps, therefore, it is not surprising that the increased afferent drive resulting from this reflex shortens the time of suppression by i.c.v. WAY-100635. Interestingly, the flow of saline through the urethra does not appear to shorten the time of suppression by WAY-100635 after intrathecal administration, when compared to a similar study by Kakizaki *et al.* (2001) who ligated the urethral outlet. This maybe due to the proposed different mechanism of action of spinal 5-HT_{1A} receptors, by which blockade with WAY-100635 directly inhibits parasympathetic preganglionic neurones innervating the bladder. Therefore the increased afferent drive resulting from the urethrovesical reflex is nullified due to the inhibition of efferent drive to the bladder.

2.4.5 – Concluding Remarks

The present study has confirmed that there are two central sites for 5-HT_{1A} receptors involved in the control of micturition. 5-HT_{1A} receptors at both supraspinal and sacral spinal sites have a physiological role in modulating micturition with blockade of these receptors causing suppression of the reflex. The ability of 5-HT_{1A} antagonists to successfully suppress micturition raises the possibility that these drugs may have a therapeutic application for the treatment of overactive bladder. However, the presence of a possible 'tachyphylaxis' phenomenon after repeated administration of WAY-100635 in the current study, raises the possibility that chronic administration of these compounds may not be clinically efficacious. Further studies are therefore required to confirm the existence of this phenomenon, investigate whether it is attributable solely to WAY-100635 or 5-HT_{1A} receptor antagonists in general (i.e. a peculiarity of the system) and to elucidate the mechanism for this phenomenon. These studies form the basis of the remainder of this thesis and are examined in chapters 4, 5 and 6.

Chapter 3

Acute administration of 5-HT$_{1A}$ receptor ligands: Conscious studies

3.1 - Introduction

The inhibitory effects of 5-HT$_{1A}$ receptor antagonists on micturition in anaesthetized rats have been described previously in chapter 2 and also in the literature (Testa *et al.*, 1999; Conley *et al.*, 2001; Kakizaki *et al.*, 2001; Pehrson *et al.*, 2002; Yoshiyama *et al.*, 2003). The effects of i.v. WAY-100635 (Testa *et al.*, 1999; Pehrson *et al.*, 2002) and robalzotan (Pehrson *et al.*, 2002) on cystometric variables have also been examined in conscious animals, and it has been suggested that both these ligands are efficacious in anaesthetized animals at doses which have no effect in conscious rats (Pehrson *et al.*, 2002). The anaesthetized studies were conducted under urethane anaesthesia, which may interact with glutamate receptors (Yoshiyama *et al.*, 1994) known to be involved in the control of micturition, and therefore urethane may have contributed to the increased sensitivity in anaesthetized studies. Therefore, one of the aims of the present study was to further investigate, and confirm, the effects of WAY-100635 and robalzotan on micturition in conscious animals, and to determine efficacious dose levels of these ligands in conscious rats, for use in subsequent chronic dosing studies (see Chapter 4). This study also investigates whether acute i.v. administration of WAY-100635 has similar effects on micturition in both male and female conscious rats.

A further complication with these studies, which to the author's knowledge has yet to be addressed in the literature, is the unusually short half-lives of these 5-HT$_{1A}$ receptor ligands. WAY-100635 and robalzotan have half-lives in rats of ~ 12 to 15 min (Pfizer Ltd., 2000; personal communication), which complicates the investigation of the effects of these ligands on micturition variables in conscious animals, since acquisition of data at

accurate dose levels for a period longer than ~15 min requires continuous infusion of the compound to achieve steady state free plasma concentrations. Therefore, the present study uses a novel model that allows the monitoring of conscious micturition variables, under more physiological conditions than cystometry, whilst simultaneously enabling continuous i.v. infusion of the test compound.

3.2 - Methods

All experiments were carried out under the Animals (Scientific Procedures) Act, 1986. After completion of all studies, animals were killed by an approved schedule 1 method.

3.2.1 – Measuring conscious micturition variables during continuous i.v. infusion of test compound.

The pharmacokinetics of the compounds needing to be tested meant that the conscious micturition variables had to be monitored during continuous i.v. infusion of the compound. This was achieved by tethering the rats, via an exteriorised jugular vein catheter, to an infusion pump system whilst housing the animals in metabolic cages for three hours to allow monitoring of the urine output (see Figure 3.1).

3.2.1.1– Jugular vein catheter implantation

Jugular vein catheters were implanted and exteriorised in the dorsal neck region to allow continuous i.v. infusion during study periods. All surgery was performed under aseptic conditions, using sterile instruments and with body temperature maintained by a standard homeothermic control unit and blanket. Briefly, 12 female (250 – 350g) and 6 male (350 – 450 g) Sprague Dawley rats were initially anaesthetized using 3–5% isoflurane in oxygen in an induction chamber. Carprofen analgesia (5 mg kg^{-1}) was administered subcutaneously and ocular lubrication was applied (Lacri-Lube®, Allergan Inc, USA). After shaving and disinfecting (using Pevidine surgical scrub and antiseptic solution) the right ventral region of the neck, the animal was transferred to theatre and anaesthesia maintained using 2% isoflurane in oxygen. Depth of anaesthesia was assessed by

respiratory parameters, and by an absence of limb withdrawal in response to paw pinch. The right external jugular vein was exposed by a 1cm incision and a 3 French silicone catheter (UNO Roestvastall BV, Netherlands; modified with a 4 French retention bead (UNO) 3 cm from the implanted tip and a silicone ball with a Dacron cuff 3.5 cm from the exteriorised tip, Pfizer U.K.) was inserted into the vessel and tied in place. A subcutaneous tunnel was blunt dissected from the ventral neck region to the dorsal neck where the catheter was exteriorised, and the incisions closed. The rat was allowed to return to consciousness in a heated recovery box. Carprofen analgesia (a nonsteroidal anti-inflammatory drug; 5 mg kg^{-1}) was administered once daily for three days post-surgery. Post-surgery animals were housed singly and in a reverse light/dark cycle (12h:12h) so animals were always studied whilst at their most active. The jugular vein catheters were flushed at least once a week with sterile saline and heparinized glucose to maintain the patency and lifetime of the catheter.

3.2.1.2– Tethering the rats

The animals were tethered via their jugular vein catheters to an infusion pump using a system devised by Instech laboratories that allows free movement of the rat with simultaneous continuous i.v. infusion of compound. Briefly, polyethylene tubing (Portex) was connected to the jugular vein catheter and held in place by the tethering jacket. The tubing passed up through a spring, which was connected to a swivel (Instech laboratories) and allowed free movement of the rat. The swivel was clamped in place above the cage and the tubing passed from the swivel to an infusion pump (see Figure 3.1). As far as possible aseptic techniques were used to minimise the risk of infection associated with infusing into the jugular vein.

3.2.1.3 – Variables measured using the metabolic cage set-up

Urine voided by the animal whilst in the metabolic cage (for three hours) was funnelled into a collecting pot, which was connected to a force displacement transducer (FT03 D, Grass Instruments; see Figure 3.1). This allowed measurement (by weight) of the volume of urine voided (to an accuracy of 0.1 ml) and this information was continuously captured every 2 seconds by the Ponemah Physiology Platform version 3.0 (Gould Instrument Systems, Inc.), thus allowing additional identification of the frequency of voiding and average volume per void. The collected data was condensed into a volume voided every minute of the study period using a specially designed macro in Microsoft Excel, and a void was defined by an increase of ≥ 0.2 ml in the total volume voided which lasted for at least 2 min. The average volume per void was calculated by dividing the total volume voided by the number of voids over that period. Since normal rats (i.e. a non-pathological model for overactive bladder) only void approximately once an hour, the rate of voiding was artificially increased in all rats by enhancing diuresis using furosemide (2 mg kg^{-1}) and a saline load (20 ml kg^{-1}) administered i.p. in a single injection just prior to observation in the metabolic cage. This widened the window of the rate of voiding, thus enabling any changes between vehicle and compound dosed animals to be more easily detected. Whilst housed in the metabolic cages the rats had free access to drinking water.

3.2.1.4 – Infusion protocols

Following in house studies at Pfizer Ltd. investigating the pharmacodynamics and metabolism of both WAY-100635 and robalzotan, the infusion protocols shown in Table 3.1a and Table 3.1b were formulated to enable accurate free plasma concentrations (nM)

to be quickly reached and maintained over the infusion period. The loading dose was administered i.v. in a volume of 1 ml kg^{-1}, and then the maintenance infusion was continuously administered i.v. through the jugular vein catheter at a rate of 0.0335 ml min^{-1}. For the vehicle control animals a loading dose of vehicle was still administered i.v. prior to a maintenance infusion of vehicle.

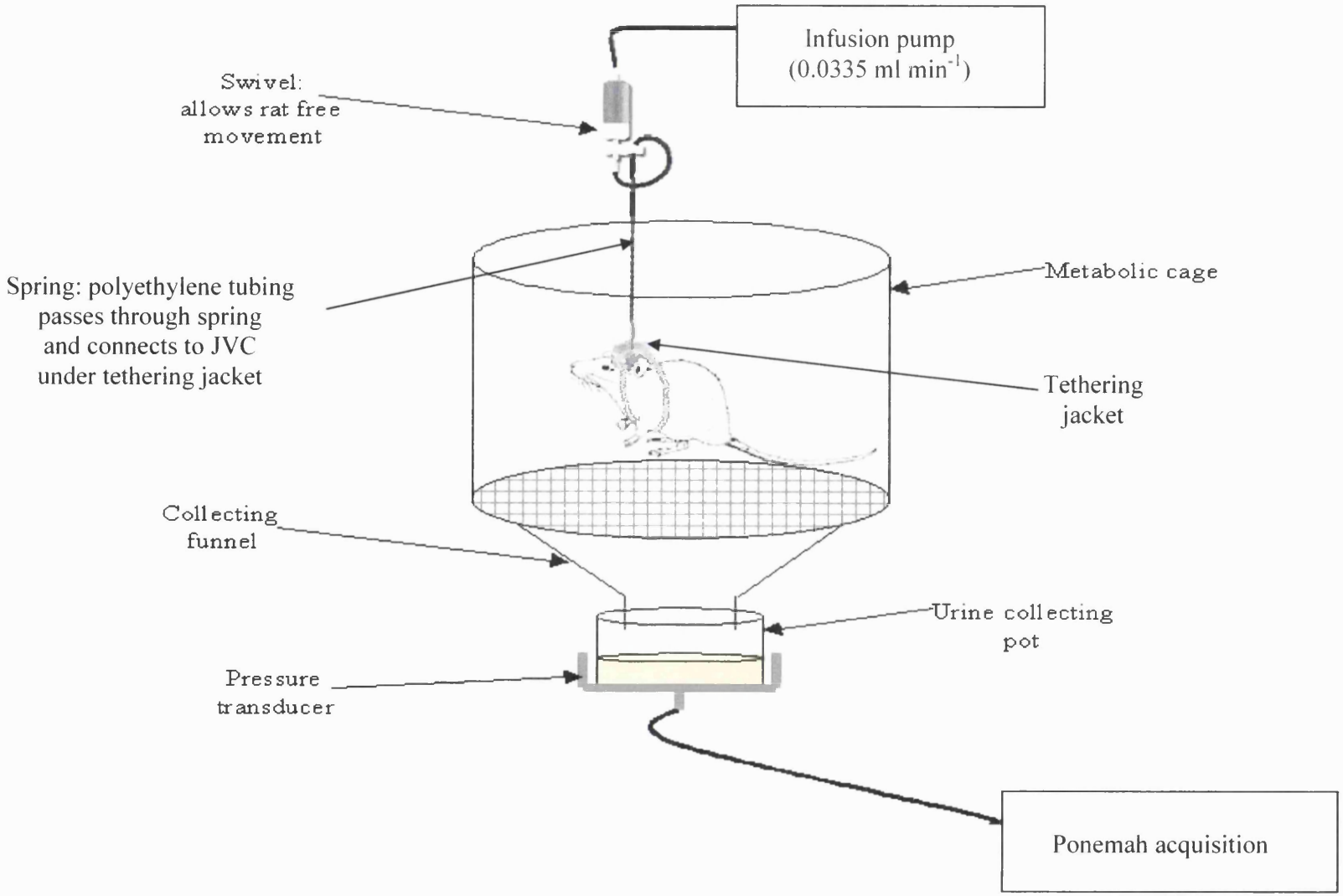


Figure 3.1 – A diagram to show the basic method used during these studies to enable measurement of conscious micturition variables during continuous i.v. infusion of test compound. See text for more detail.

a) Infusion protocol for WAY-100635

Target Free Plasma Concentration (nM)	*Loading Dose ($\mu g\ kg^{-1}$)*	*Maintenance Infusion ($\mu g\ kg^{-1}min^{-1}$)*
0.01	0.21	0.003
0.1	2.1	0.03
1	21	0.33
10	210	3.3

b) Infusion protocol for robalzotan

Target Free Plasma Concentration (nM)	*Loading Dose ($\mu g\ kg^{-1}$)*	*Maintenance Infusion ($\mu g\ kg^{-1}min^{-1}$)*
0.25	0.63	0.02
2.5	6.3	0.2
25	63	2.0
250	630	20.0

Table 3.1 - Infusion protocols for **a)** WAY-100635 and **b)** robalzotan. Formulated from in-house data at Pfizer Ltd.

The infusion was maintained for three hours whilst the urine output of the animals was monitored using the metabolic cage set-up. After the infusion had been switched off the jugular vein catheter was flushed with saline and heparinized glucose to help maintain the patency of the jugular vein catheter and the rat returned to its home cage. Rats were selected at random from the group which had jugular vein catheter surgery to study the effects of differing concentrations of the test compounds (in dose order) but were allowed at least a 7-day wash-out period between studies. On test days both vehicle and compound were tested to minimise any risk of introducing day-to-day variability.

3.2.2 – Statistical analysis

Changes in all of the above variables, i.e. number of voids, total volume voided (ml) and average volume per void (ml) were measured over the three hour compound infusion time and all values are expressed as mean ± s.e.mean. Individual drug dose evoked changes were compared to vehicle/time match control using a two-sided unpaired Student's *t*-test and adjusting for unequal variances if necessary. For all statistical tests, $P < 0.05$ was considered to be statistically significant.

3.2.3 – Drugs and solutions

Drugs and chemicals were obtained from the following sources: Isoflurane (ISOCARE™) from Animalcare Ltd., U.K.; Carprofen (Rimadyl™ Large Animal Solution) from Pfizer Ltd., Sandwich, U.K.; Furosemide from Sigma Aldrich Chemicals, Poole, Dorset, U.K.; *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)-cyclohexanecarboxamide trichloride (WAY-100635, 0.1 – 10 nM) and (*R*)-3-*N,N*-Dicyclobutylamino-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide hydrogen

(*2R,3R*)-tartrate monohydrate (robalzotan; NAD-299, 2.5 nM) were gifts from Pfizer Ltd., Sandwich, U.K.). Furosemide was dissolved in ~ 5% PEG and 95% saline and warmed. WAY-100635 was dissolved in 0.9% wv^{-1} saline and robalzotan in acidified saline (~ pH 5.0).

3.3 - Results

3.3.1 – Effect of WAY-100635 on conscious micturition variables in female rats

Administration of a 3 h i.v. infusion of the vehicle for WAY-100635 (0.9 % wv^{-1} saline) to female rats (n = 11) undergoing furosemide and saline load induced diuresis caused a mean number of voids (over 3 h) of 6.9 ± 0.8, with a mean total volume voided of 7.7± 0.5 ml and an average volume per void of 1.22 ± 0.12 ml (see Table 3.2). A 3 h infusion of 0.1 nM WAY-100635 (i.v.; n = 8) was ineffective at altering any of the micturition parameters measured (see Table 3.2 and Figure 3.2) however a similar infusion of 1 nM WAY-100635 (n = 7) caused a significant ($p < 0.05$) reduction in the number of voids over 3 h (5.0 ± 1.1 voids) and a significant increase in the average volume per void to 1.83 ± 0.25 ml, with the total volume voided over the 3 h period remaining unaltered (see Table 3.2 and Figure 3.2) when compared to vehicle control.

A similar study carried out in house at Pfizer Ltd. (Hall, 2002; personal communication) also shows a 3 h infusion of 10 nM WAY-100635 (n = 8) to cause a significant reduction in the number of voids over a 3 h period (5.4 ± 1.1 to 2.3 ± 0.6 voids) and a significant increase in the average volume per void (1.10 ± 0.17 to 1.30 ± 0.13 ml) with no significant change in the total volume voided (see Table 3.2 and Figure 3.3) when compared to vehicle control. It must be noted that due to this study being conducted in a different batch of animals, different baseline values were obtained for the various micturition parameters measured and therefore these results with a 10 nM infusion of WAY-100635 cannot be directly compared with the results obtained for infusions of 0.1 and 1 nM WAY-100635 detailed above. Accordingly, these results are graphically represented on separate graphs (see Figure 3.2 and Figure 3.3).

3.3.2 – Effect of robalzotan on conscious micturition variables in female rats

A 3 h infusion of 2.5 nM robalzotan (i.v.; n = 4) caused a significant ($p < 0.01$) reduction in the number of voids over 3 h (4.5 ± 0.3 voids) when compared to the number of voids over a 3 h infusion of vehicle (acidified saline ~ pH 5.0) in control animals (n = 3; 8.7 ± 0.6 voids; see Table 3.2. However, robalzotan administration caused no significant changes to the average volume per void (2.09 ± 0.52 ml compared with 1.25 ± 0.09 ml) nor the total volume voided (9.0 ± 1.7 ml compared with 10.6 ± 0.6 ml) when compared to the vehicle control (see Table 3.2 and Figure 3.4).

	Number of voids over 3 h	*Total volume voided (ml)*	*Average volume per void (ml)*
Vehicle (saline; n = 11)	6.9 ± 0.8	7.7 ± 0.5	1.22 ± 0.12
0.1 nM WAY-100635 (n = 8)	6.0 ± 0.6	7.9 ± 0.5	1.35 ± 0.14
1 nM WAY-100635 (n = 7)	5.0 ± 1.1 *	8.1 ± 1.0	1.83 ± 0.25 *
Vehicle (acidified saline; n = 3)	8.7 ± 0.6	10.6 ± 0.6	1.25 ± 0.09
2.5 nM robalzotan (n = 4)	4.5 ± 0.3 **	9.0 ± 1.7	2.09 ± 0.52
Vehicle (saline; n = 8)	5.4 ± 1.1	5.2 ± 0.8	1.10 ± 0.17
10 nM WAY-100635 (n = 8)	2.3 ± 0.6 *	3.1 ± 1.1	1.30 ± 0.13 *

Table 3.2 – Effects of vehicle, WAY-100635 and robalzotan on conscious micturition measurements in female rats after a 3 h i.v. infusion. NB – results obtained for 10 nM WAY-100635 were conducted in house at Pfizer Ltd. (Hall, 2002; personal communication) using a different batch of animals. All measurements are mean ± s.e.mean. * = $P < 0.05$; ** = $p < 0.01$ compared to vehicle control by unpaired Student's *t*-test.

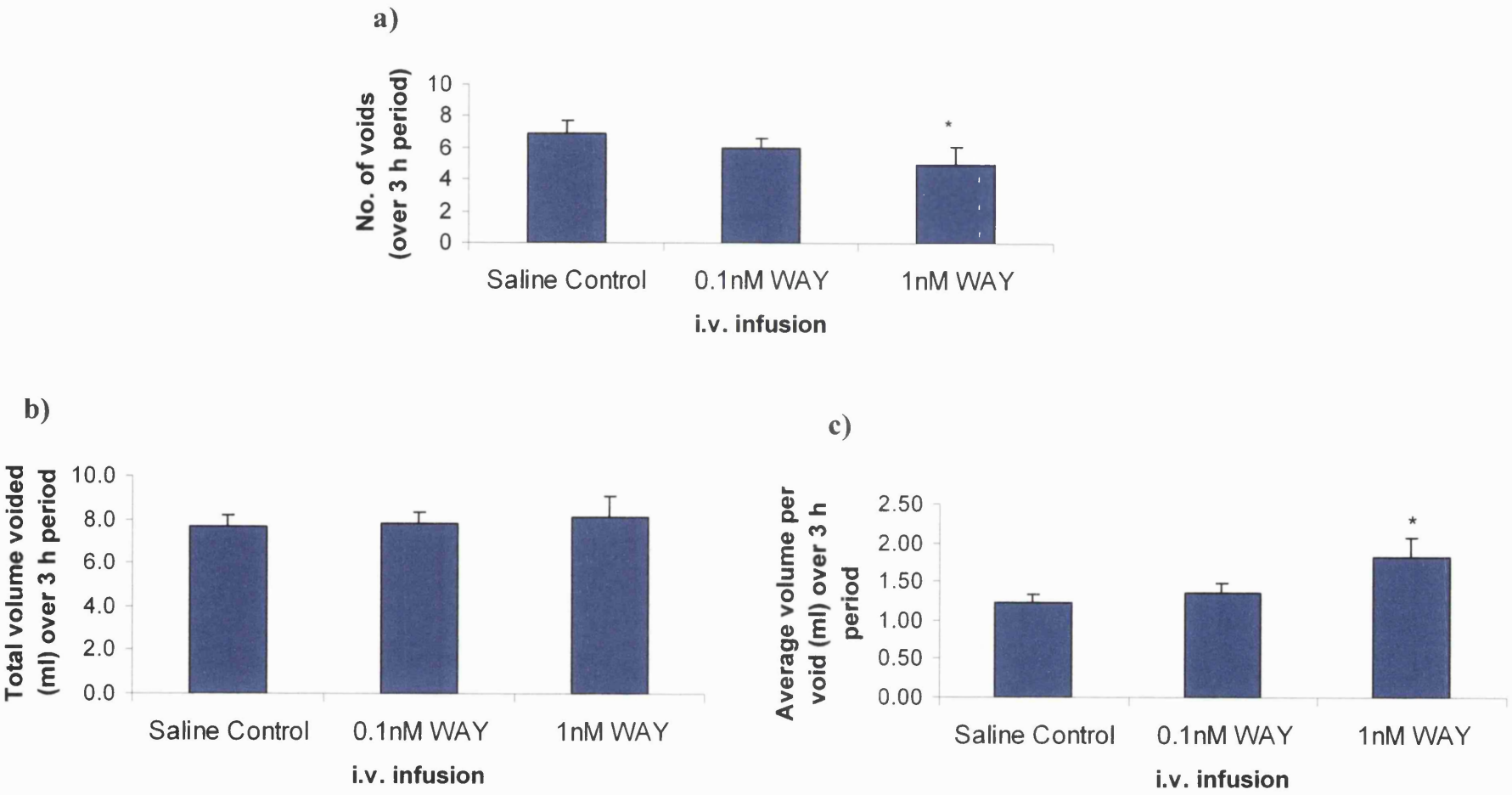


Figure 3.2 – Graphical representation of the effects of vehicle (saline; n = 11), 0.1 nM (n = 8) and 1 nM WAY-100635 (n = 7) on **a)** the number of voids over a 3 h period, **b)** the total volume voided (ml) over 3 h and **c)** the average volume per void (ml) in conscious female rats. Abbreviations: WAY, WAY-100635. * $P < 0.05$ compared to vehicle control by unpaired Student's *t*-test.

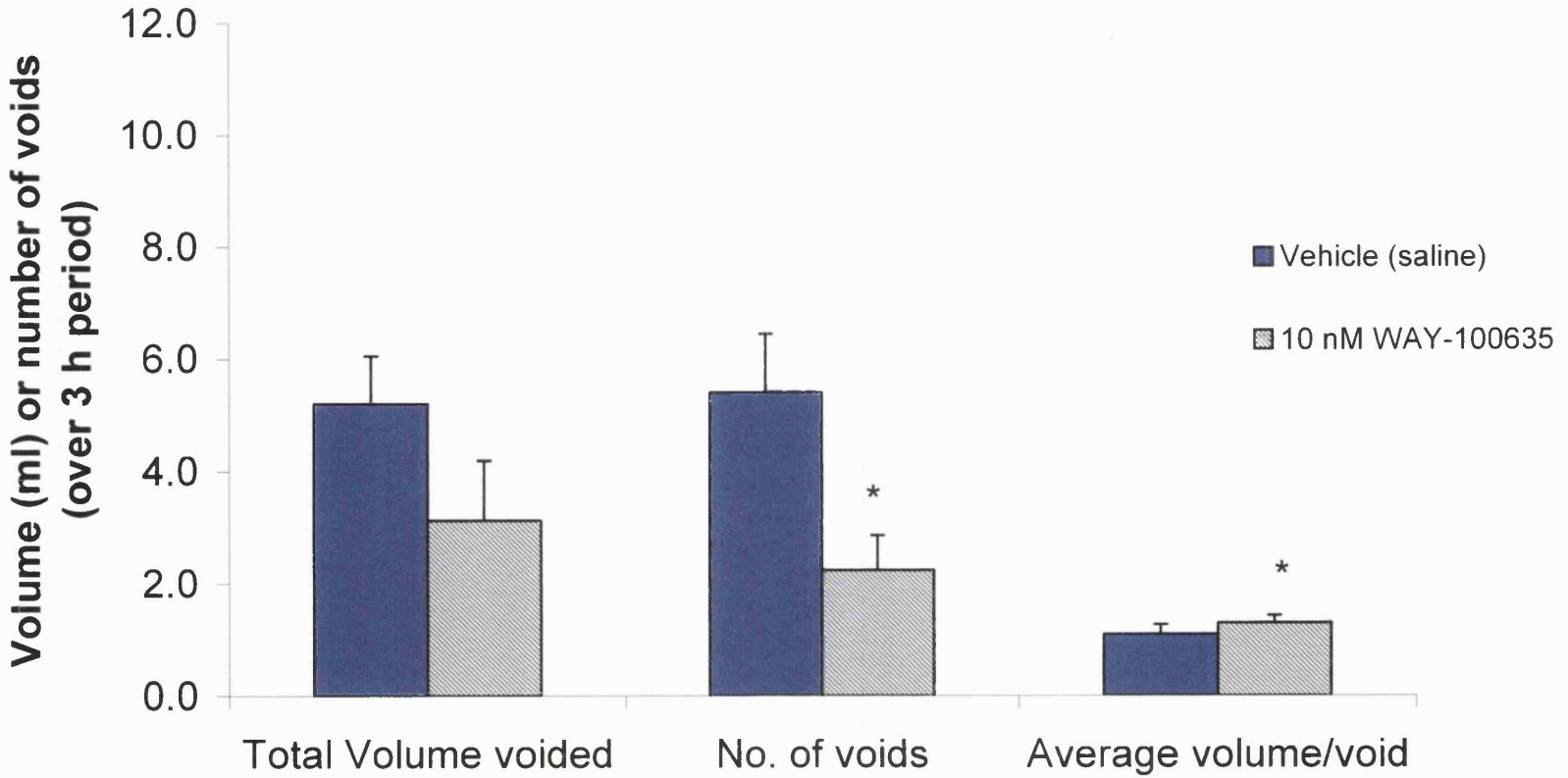


Figure 3.3 – Graphical representation of the effects of vehicle (saline; n = 8) and 10 nM WAY-100635 (n = 8) on various conscious micturition variables in female rats over a 3 h period. It must be noted that these results were obtained in house at Pfizer Ltd. (Hall, 2002; personal communication) using different animals to those used to obtain the results shown in Figure 3.2. * $P < 0.05$ compared to vehicle control by unpaired Student's *t*-test.

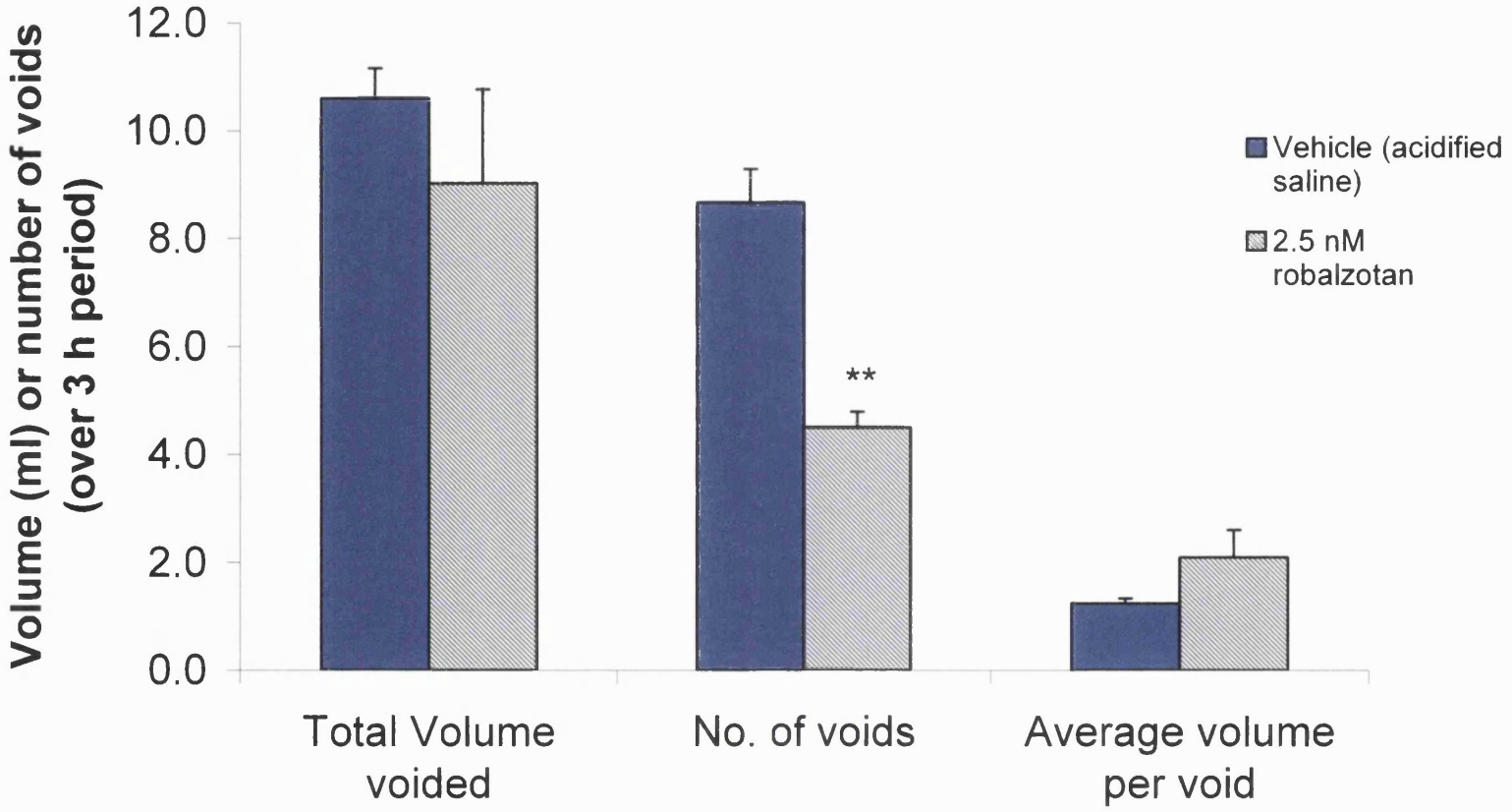


Figure 3.4 - Graphical representation of the effects of vehicle (acidified saline; n = 3) and 2.5 nM robalzotan (n = 4) on various conscious micturition variables in female rats over a 3 h period. ** $P < 0.01$ compared to vehicle control by unpaired Student's *t*-test.

3.3.3 – Effect of WAY-100635 on conscious micturition variables in male rats

Administration of a 3 h i.v. infusion of 0.9 % wv^{-1} saline (vehicle for WAY-100635) to male rats (n = 7) undergoing furosemide and saline load induced diuresis caused a mean number of voids (over 3 h) of 7.7 ± 0.4, with a mean total volume voided of 5.5 ± 0.4 ml and an average volume per void of 0.70 ± 0.08 ml (see Table 3.3). A 3 h infusion of 0.1 nM WAY-100635 (i.v.; n = 8) was ineffective at altering any of the micturition parameters measured however similar infusions of 1 nM (n = 7) and 10 nM WAY-100635 (n = 11) caused significant ($p < 0.05$) dose-dependent reductions in the number of voids over 3 h (6.0 ± 0.6 and 5.3 ± 0.9 voids respectively; see Table 3.3 and Figure 3.5) when compared to vehicle control. After both 1 and 10 nM WAY-100635, there was no significant change in the total volume voided over the 3 h period, however there was an unusual decrease (not significant) observed with 10 nM WAY-100635 which may explain why there was no significant increase in the average volume per void with 10 nM WAY-100635 infusion (0.91 ± 0.13 ml) despite a significant increase being observed in the average volume per void after a 1 nM WAY-100635 infusion (1.05 ± 0.1 ml; see Table 3.3 and Figure 3.5).

	Number of voids over 3 h	*Total volume voided (ml)*	*Average volume per void (ml)*
Vehicle (saline; n = 7)	7.7 ± 0.4	5.5 ± 0.4	0.70 ± 0.08
0.1 nM WAY-100635 (n = 7)	8.0 ± 1.1	6.9 ± 0.8	0.89 ± 0.08
1 nM WAY-100635 (n = 7)	6.0 ± 0.6 *	6.1 ± 0.6	1.05 ± 0.10 *
10 nM WAY-100635 (n = 11)	5.3 ± 0.9 *	4.3 ± 0.7	0.91 ± 0.13

Table 3.3 – Effects of vehicle (saline) and WAY-100635 (0.1, 1 and 10 nM) on conscious micturition measurements in male rats after a 3 h i.v. infusion. All measurements are mean ± s.e.mean. * $P < 0.05$ compared to vehicle control by unpaired Student's *t*-test.

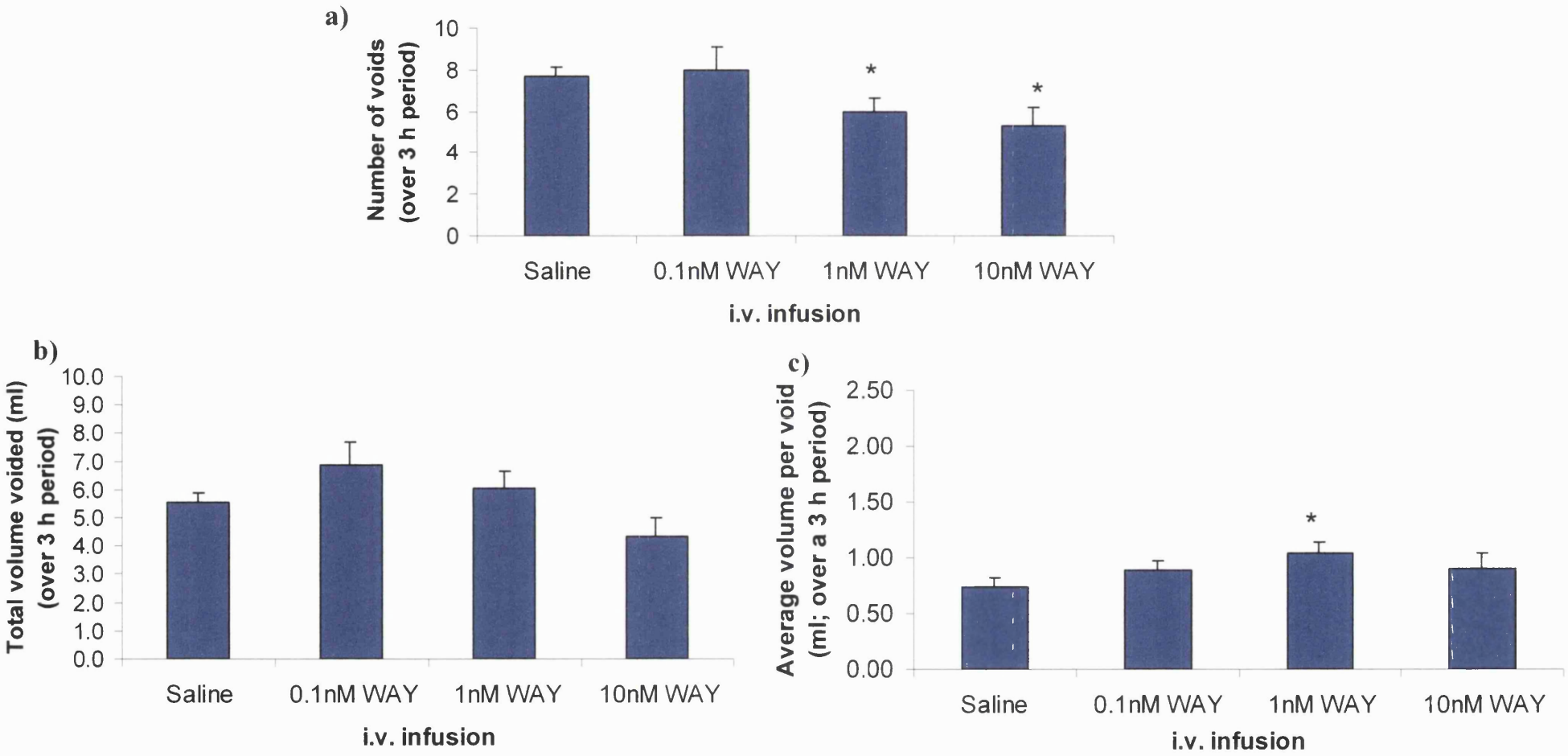


Figure 3.5 - Graphical representation of the effects of vehicle (saline; n = 7), and 0.1 (n = 7), 1 (n = 7) & 10 nMWAY-100635 (n = 11) on **a)** the number of voids over a 3 h period, **b)** the total volume voided (ml) over 3 h and **c)** the average volume per void (ml) in male rats. Abbreviations: WAY, WAY-100635. * $P < 0.05$ compared to vehicle control by unpaired Student's *t*-test.

3.3.4 – Hourly analysis of the number of voids over the three hour infusion period in conscious rats after WAY-100635 (male and female rats) and robalzotan infusions (female rats only).

Further detailed breakdown, into individual h, of the 3 h infusion data noted previously reveals that a 2.5 nM robalzotan infusion causes a significant ($p < 0.05$) reduction, when compared to vehicle control, in the number of voids during the 1st h of infusion (1.5 ± 0.7 voids compared with 4.0 ± 0.0 voids) as expected. However, during the 2nd and 3rd h of the infusion there is no significant difference between the number of voids in vehicle treated and robalzotan treated animals (see Table 3.4 and Figure 3.6). A similar phenomenon was observed after 10 nM infusion of WAY-100635 in both female and male rats, with a significant decrease in the number of voids observed in the 1st h of WAY-100635 infusion (0.9 ± 0.3 compared with 2.5 ± 0.6 voids and 3.4 ± 0.6 compared with 5.4 ± 0.7 voids respectively for female and male rats), but no significant difference in the number of voids between vehicle and compound treated animals during the 2nd and 3rd h of the infusion (see Table 3.4 and Figure 3.7).

		Number of voids over 1st hour	*Number of voids over 2nd hour*	*Number of voids over 3rd hour*
Female	Vehicle (saline; n = 8)	2.5 ± 0.6	1.1 ± 0.3	1.8 ± 0.4
	10 nM WAY-100635 (n = 8)	0.9 ± 0.3 *	0.4 ± 0.3	1.0 ± 0.3
Male	Vehicle (saline; n = 8)	5.4 ± 0.7	2.1 ± 0.7	2.0 ± 0.7
	10 nM WAY-100635 (n = 11)	3.4 ± 0.6 *	1.1 ± 0.3	0.8 ± 0.3
Female	Vehicle (acidified saline; n = 3)	4.0 ± 0.0	2.0 ± 0.3	2.0 ± 0.7
	2.5 nM robalzotan (n = 4)	1.5 ± 0.7 *	1.3 ± 0.5	1.8 ± 0.5

Table 3.4 – A more detailed hourly breakdown of the effect of 3 h infusions of 2.5 nM robalzotan (female rats only) and 10 nM WAY-100635 (female and male rats) on the number of voids during the infusion. All measurements are mean ± s.e.mean; * $P < 0.05$ compared to vehicle control for that hour by unpaired Student's *t*-test.

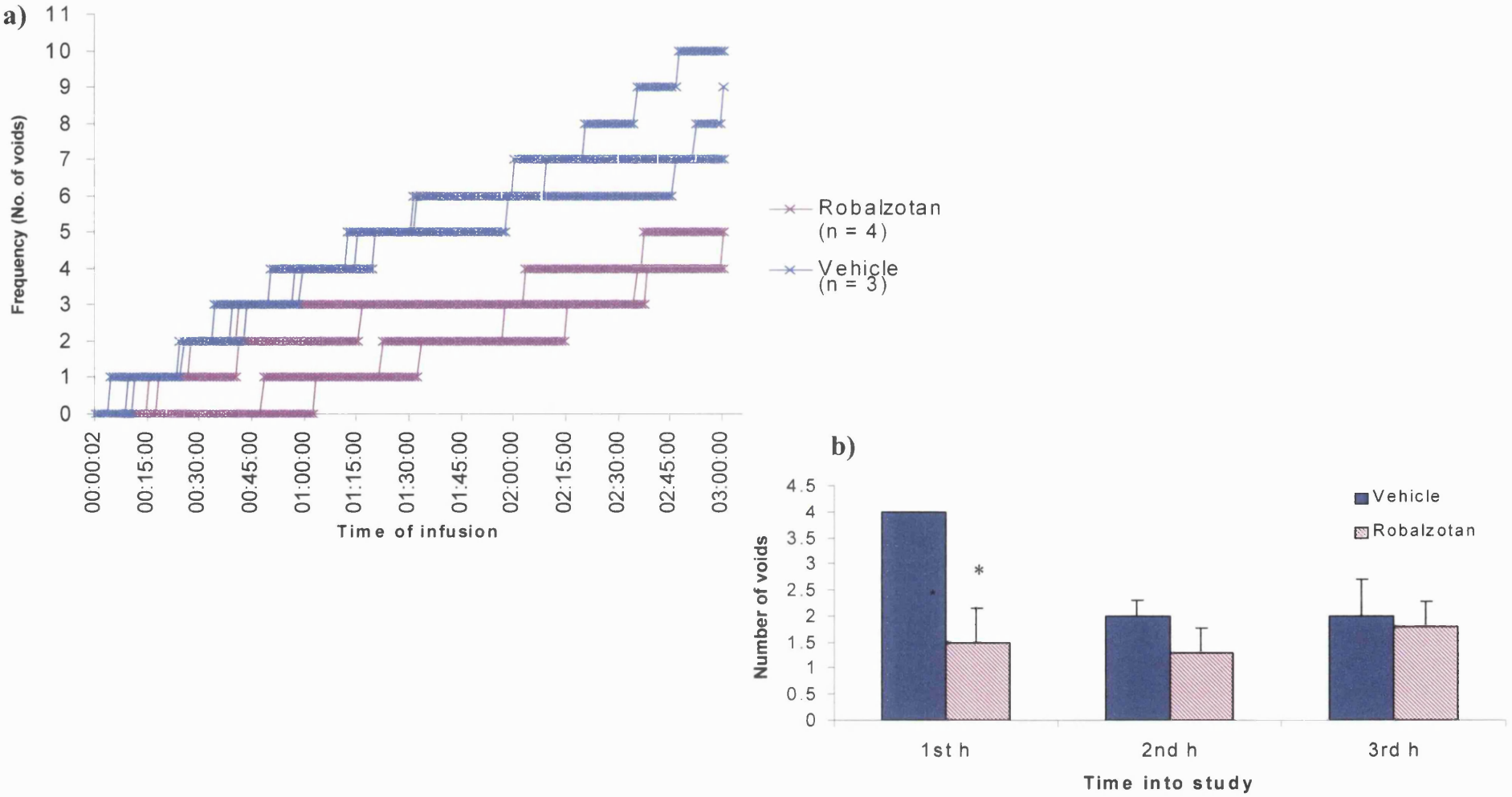


Figure 3.6 – The effect of a 3 h infusion of robalzotan (2.5 nM) on rate of voiding in female rats. **a)** Traces of the voiding pattern of individual rats during a 3 h infusion of vehicle (acidified saline, n = 3) or robalzotan (n = 4). **b)** Graphical representation of the number of voids over individual hours during the 3 h infusion. * $P < 0.05$ compared to vehicle control during that hour by unpaired Student's *t*-test.

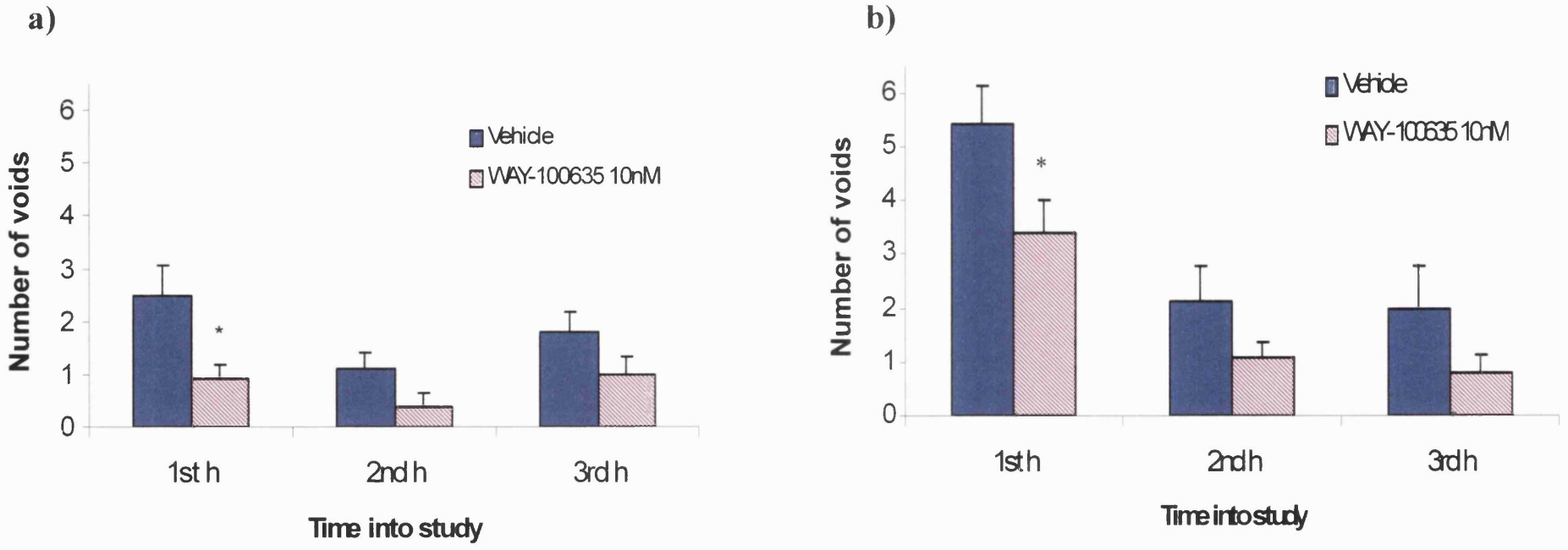


Figure 3.7 - The effect of a 3 h infusion of WAY-100635 (10 nM) on rate of voiding in female rats. Graphical representation of the number of voids over individual hours during the 3 h infusion in **a)** female and **b)** male rats. * $P < 0.05$ compared to vehicle control during that hour by unpaired Student's *t*-test.

3.4 – Discussion

The results from the present study have shown that in both female (see Table 3.2) and male (see Table 3.3) conscious rats, acute 3 h i.v. infusions of WAY-100635 (1 and 10 nM free plasma concentration) cause a dose dependent reduction in the number of voids over the infusion period, although a 0.1 nM dose of WAY-100635 is ineffective at altering any of the micturition variables. A similar effect was observed after a 3 h i.v. infusion of robalzotan (2.5 nM free plasma concentration) in female rats, with administration of this compound significantly reducing the number of voids over the 3 h infusion period when compared to the vehicle control (see Table 3.2). Interestingly, further hourly analysis of the data obtained over the 3 h infusion period revealed that the reduction in voiding caused by administration of the 5-HT$_{1A}$ receptor antagonists is only observed in the first hour of the infusion, thus suggesting that these ligands become less effective at suppressing micturition as the infusion progresses.

3.4.1 – Effect of WAY-100635 and robalzotan on conscious micturition variables in male and female rats.

The present study investigated the effects of a continuous acute i.v. infusion of the 5-HT$_{1A}$ receptor ligands WAY-100635 and robalzotan on conscious micturition parameters in both female and male (WAY-100635 only) rats. The results show that WAY-100635 infusions (1 and 10 nM free plasma concentration) cause a dose dependent reduction in the number of voids over the infusion period, although a 0.1 nM dose of WAY-100635 is ineffective at altering any of the micturition variables. These results are in agreement with previous observations in anaesthetized animals, which showed

WAY-100635 (1, 10 and 100 μg kg^{-1}) to significantly suppress micturition both i.c.v. (see Chapter 2) and i.t. (Kakizaki *et al.*, 2001). Comparison of the effective dose levels between anaesthetized and conscious animals indicates that in anaesthetized rats, WAY-100635 inhibits micturition at a dose (1 μg kg^{-1}) which has no effect in conscious rats (0.1 nM) – these doses are roughly comparable since the loading dose for a 0.1 nM free plasma concentration is 2.1 μg kg^{-1} (see Table 3.1) which is only twice the 1 μg kg^{-1} dose administered to the anaesthetized animals. Similar observations have been made by Pehrson *et al.* (2002), however, it must be noted that in the anaesthetized studies in chapter 2, WAY-100635 was administered i.c.v. as opposed to the i.v. infusion of WAY-100635 in the current conscious studies – this may account, in part, for the higher efficacy observed with low doses of WAY-100635 in anaesthetized animals since the rate of metabolism is slower after i.c.v. administration.

The present conscious studies used a metabolic cage set-up to monitor urine output, which has the advantage over regular conscious cystometric investigations (e.g. Testa *et al.*, 1999; Leonardi *et al.*, 2001; Testa *et al.*, 2001) of enabling investigation of the effect of test compounds on kidney function. Indeed these studies revealed that neither WAY-100635 nor robalzotan significantly alter the total volume voided over the infusion period which suggests that these ligands do not have any effect on the rate of diuresis, and confirms that the reduction in the rate of voiding observed with these compounds is not as a result of a decrease in urine production. Since the total volume voided does not alter, but the number of voids decreases, it is not surprising that the average volume per void increases after WAY-100635 (1 and 10 nM) administration (see Table 3.2). However, unexpectedly this increase was not significant after robalzotan administration,

probably due to a smaller sample size. Similar increases in the average volume per void/bladder capacity have been observed after i.v. administration of WAY-100635 (Testa *et al.*, 1999; Pehrson *et al.*, 2002) and robalzotan (Pehrson *et al.*, 2002) in conscious rats.

Another advantage of this tethering/metabolic cage model is the fact that micturition variables can be monitored during continuous i.v. infusion of test compounds. This is of particular importance for ligands such as WAY-100635 and robalzotan which have been shown to have very short half lives, ~12 min to 15 min (Pfizer Ltd., personal communication), and therefore require continuous infusion to maintain compound levels at the dose required whilst natural voiding is monitored. A further advantage of this model is the observation of micturition variables under more physiological conditions than those obtained with conscious cystometry. Conscious cystometric investigations require invasive surgery of the bladder and continuous saline infusion into the bladder, neither of which are required with the current model. However, a drawback with this model is a reduction in the number of variables that can be measured when compared to cystometry models which are able to measure pressure variables within the bladder. One further drawback is that since this model measures natural voiding (i.e. not voiding induced by saline infusion into the bladder) the inter-animal variation appears to be greater, which results in a slightly increased sample number being required to achieve statistical significance.

3.4.2 – Similarities in the effects of 5-HT$_{1A}$ receptor ligands on micturition in male and female rats

Interestingly, these results have shown that WAY-100635 has exactly the same effects on micturition in male rats as observed in female rats, with WAY-100635 in male rats causing a dose dependent decrease in the number of voids over a 3 h infusion period, and an increase in average volume per void (see Table 3.3). Although female rats are historically used for studies on micturition due to their shorter urethra and less complicated pattern of oscillatory voiding (see Streng *et al.*, 2002), to the author's knowledge there is no evidence of gender differences in the spinal and supraspinal control of micturition and therefore, it is not surprising that WAY-100635 has similar effects in both male and female rats. Similar effects with 5-HT$_{1A}$ receptor antagonists in conscious male rats have been observed (Testa *et al.*, 1999; Conley *et al.*, 2001; Leonardi *et al.*, 2001; Testa *et al.*, 2001).

3.4.3 – Possible 'tachyphylaxis' after three hour i.v. infusion of 5-HT$_{1A}$ receptor antagonists?

Further detailed hourly analysis of the variables measured over the three hour infusion period indicate that the inhibitory effect of WAY-100635 (10 nM) and robalzotan (2.5 nM) on the rate of voiding is only observed during the first hour of compound infusion, with there being no significant change in the variables over the second and third hours when compared to time matched vehicle controls (see Table 3.4). There are a couple of possible explanations for this observation. Firstly, since these 5-HT$_{1A}$ receptor ligands are only effective during the first hour of infusion, it suggests the presence of a 'tachyphylaxis' phenomenon. The possibility of a 'tachyphylaxis' phenomenon after

repeated administration of WAY-100635 was also previously indicated in the studies in anaesthetized rats, where it was shown that successive doses of WAY-100635 are less than half as effective as the initial dose at suppressing micturition (see 2.4.3). Therefore, these results in conscious animals provide further corroboration for the concept that 5-HT$_{1A}$ receptor antagonists lose efficacy after repeated administration. However, it must also be noted that in these conscious studies the furosemide/saline load induced increase in the rate of voiding was most pronounced during the first hour after administration, and started to wear off over the final two hours of the infusion. Therefore, it is possible that the smaller window in the rate of voiding was an additional factor in the lack of any significant effect by WAY-100635 or robalzotan in the final two hours of the infusion. However, the fact that in female rats with WAY-100635 or robalzotan on board, the number of voids in the third hour was greater (although not significantly) than in the first hour (despite the facilitatory influence of the furosemide/saline load in the first hour; see Table 3.4), suggests that a 'tachyphylactic' effect of the test compound is also involved.

3.4.4 – Concluding remarks

The present study in conscious animals, confirmed the observations previously suggested from anaesthetized rats, that acute dosing of 5-HT$_{1A}$ receptor antagonists are effective at inhibiting micturition, without altering the levels of urine production. Interestingly, the present study also suggests that the 'tachyphylaxis' phenomenon (defined as a lack of effect of the compound on suppression of micturition) observed with repeated dosing of WAY-100635 in anaesthetized animals, is also observed in conscious animals after the first hour of a three hour i.v. infusion of either WAY-100635 or robalzotan.

Results from this study have also provided details on effective doses of WAY-100635 and robalzotan in conscious animals, and this information was used to determine the dose levels used in subsequent chronic dosing studies (see Chapter 4).

Chapter 4

Chronic administration of 5-HT_{1A} receptor ligands: Conscious and anaesthetized studies.

4.1 - Introduction

Results from the previous two chapters and the literature have shown that the 5-HT_{1A} receptor antagonists WAY-100635 (Testa *et al.*, 1999; Conley *et al.*, 2001; Kakizaki *et al.*, 2001; Pehrson *et al.*, 2002; Yoshiyama *et al.*, 2003) and robalzotan (Pehrson *et al.*, 2002) are effective after acute administration at suppressing micturition in both anaesthetized and conscious rats. However, the observations that in anaesthetized animals after repeated administration, WAY-100635 is less than half as effective at suppressing micturition (see chapter 2), and in conscious animals both WAY-100635 and robalzotan only appear to decrease the rate of voiding during the first hour of a three hour infusion (See chapter 3), suggest there may be a fast onset tachyphylaxis/tolerance phenomenon developing after repeated administration. Therefore, the aim of the present studies was to investigate the effect of chronic administration of WAY-100635 on the conscious micturition variables measured in the previous chapter, and also to confirm the presence/absence of this phenomenon in these rats using an anaesthetized cystometry model. A similar study was also carried out to investigate the effect of chronic administration of robalzotan. Since WAY-100635 and robalzotan are structurally distinct 5-HT_{1A} receptor antagonists, any similarities in the phenomenon observed after administration of each compound would indicate that the phenomenon is related to its ability to alter 5-HT_{1A} receptor signalling, rather than a distinct, unusual pharmacological property of these compounds. These present studies also investigated the effect of chronic administration of WAY-100635 on spontaneously hypertensive rats (SHR), which have been shown to be a pathological model for overactive bladder (Persson *et al.*, 1998).

The doses of WAY-100635 (10 nM free plasma concentration) and robalzotan (2.5 nM free plasma concentration) used for chronic administration were known from previous acute administration to be effective at suppressing micturition in conscious rats (see Chapter 3). However, the present study also investigated the effect of chronic administration of a non-effective dose of WAY-100635 to examine whether this dose is still capable of causing the tolerance/desensitization phenomenon despite being non-effective at suppressing micturition, since it is possible that the serotonergic signalling system is sensitive to low doses of 5-HT_{1A} receptor antagonists considering that similar doses of 5-HT_{1A} receptor agonists are known to cause desensitization (Seth *et al.*, 1997; Casanovas *et al.*, 1999).

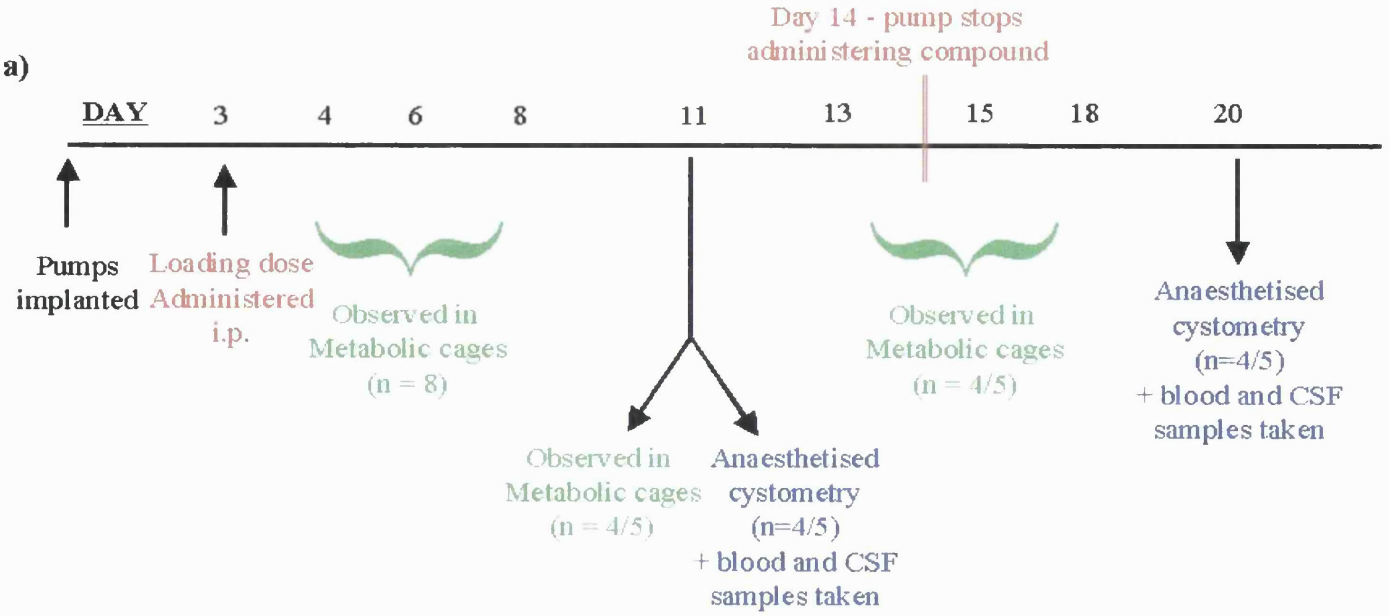
Due to the unusually short half-lives of these 5-HT_{1A} receptor antagonists, once or twice daily dosing with the compound would, in the author's view, not constitute 'true' chronic administration since the compound would only be active in the system for approximately 30 min per day. Therefore, to overcome this problem, the present studies used subcutaneously implanted osmotic pumps fitted with a catheter into the jugular vein to constantly administer compound intravenously at a fixed rate per hour. This is despite a number of studies in the literature using once (Cao *et al.*, 1998) or twice (Dawson *et al.*, 2002) daily dosing to examine the effect of 'chronic' WAY-100635 administration in the field of depression.

4.2 - Methods

All experiments were carried out under the Animals (Scientific Procedures) Act, 1986. After completion of anaesthetized experiments, animals were killed by an overdose of pentobarbitone sodium (i.v.).

4.2.1 – Study overview

A general overview and time line of the study is shown in Figure 4.1a. 64 normal rats and spontaneously hypertensive rats (SHR) were allocated as shown in Figure 4.1b and implanted subcutaneously with osmotic pumps (ALZET; Model 1002, 14 day administration, 0.25µl hr^{-1}) fitted with a catheter placed in the jugular vein for constant i.v. administration of WAY-100635 (10 or 0.01 nM), robalzotan (2.5nM, free plasma concentrations) or their vehicles (saline and acidified saline respectively). Rats were placed in metabolic cages on days 4, 6, 8, 11, 13, 15 & 18 to measure conscious micturition variables as previously described in chapter 3. On days 11 (D11; i.e. during WAY-100635/robalzotan administration) and 20 (D20; i.e. 14 days WAY-100635/robalzotan administration plus a sufficiently long (6 day) wash-out period) half the animals were observed in an anaesthetized cystometry model in which the usual cystometry profile was monitored, followed by the effects of an acute 30 min i.v. infusion of WAY-100635 (10 nM free plasma concentration) or robalzotan (2.5 nM free plasma concentration). During anaesthetized cystometry blood samples were taken prior to the additional WAY-100635/robalzotan infusion and the levels of WAY-100635/robalzotan were measured using HPLC analysis and mass spectrometry to ensure that the osmotic



b)

10 x	normal rats with vehicle (saline) osmotic pump
10 x	normal rats with WAY-100635 (10 nM) osmotic pump
8 x	normal rats with vehicle (acidified saline) osmotic pump
8 x	normal rats with robalzotan (2.5 nM) osmotic pump
10 x	SHR with vehicle (saline) osmotic pump
10 x	SHR with WAY-100635 (10 nM) osmotic pump
8 x	SHR with WAY-100635 (0.01 nM) osmotic pump

Figure 4.1 – **a)** Overview and timeline of the study. See text for further detail. **b)** Rat allocation within the study. Abbreviations; SHR, spontaneously hypertensive rats; CSF, cerebrospinal fluid

pump had been functioning correctly, and that the free plasma concentration was approximately (± 2nM) at the target concentration.

4.2.1.1 – Osmotic pump implantation

Osmotic pump preparation

20 of the normal rats were implanted with model 2ML2 osmotic pumps (ALZET, Durect corporation, CA, USA) which have a pump reservoir capacity of 2 ml and administer compound at a rate of 5 μl hr^{-1} for 14 days, the remaining animals (including the SHR which have a smaller body weight) were implanted with model 1002 osmotic pumps (ALZET) which have a pump reservoir capacity of 100 μl and administer compound at a rate of 0.25 μl hr^{-1} for 14 days.

Prior to implantation the osmotic pumps were filled in a sterile environment, according to the manufacturers instructions, with a concentration of compound which would achieve the target free plasma concentration when continuously administered at the pumping rate of the osmotic pump. This concentration of compound required in the pump reservoir was calculated using the following formula:

$$K_o = Q \times C_d$$

where K_o = mass delivery rate (μg hr^{-1})
C_d = [drug] required (μg μl^{-1})
Q = pumping rate of the pump (μl hr^{-1})

Using the infusion protocols for WAY-100635 and robalzotan detailed in Table 3.1a and 3.1b respectively, K_o was calculated individually for each rat depending on its weight. If necessary, the pH of the compound solution being used to fill the pumps was adjusted to a pH greater than 1.8 using NaOH.

Once filled, the osmotic pump was attached to a stretch of sterile polyethylene tubing (ALZET; PE-60, internal diameter = 0.76 mm) approximately 9 cm in length, which was pre-loaded with the compound solution. Osmotic pumps were filled and then primed overnight, prior to surgery, by placing in sterile saline at 37°C to ensure immediate pumping upon implantation into the rat.

Osmotic pump implantation surgery

All surgery was performed under aseptic conditions, using sterile instruments and with body temperature maintained by a standard homeothermic control unit and blanket. Briefly, 36 female (250 – 350g) normal Sprague Dawley rats and 28 female (185 – 250 g) spontaneously hypertensive rats (SHR; Harlan, genetic background WKY) were initially anaesthetized using 3 –5% isoflurane in oxygen in an induction chamber. Carprofen analgesia (5 mg kg^{-1}) was administered subcutaneously and ocular lubrication was applied (Lacri-Lube®, Allergan Inc, USA). After shaving and disinfecting the right ventral region of the neck using dilute povidone iodine (Pevidine®, C-Vet Veterinary products, Leyland, UK), the animal was transferred to theatre and anaesthesia maintained using 2% isoflurane in oxygen. Depth of anaesthesia was assessed by respiratory variables, and by an absence of limb withdrawal in response to paw pinch. The right external jugular vein was exposed by a 1cm incision and the sterile polyethylene catheter connected to the osmotic pump was inserted into the vessel and tied in place. A subcutaneous tunnel was blunt dissected from the ventral neck region to the dorsal neck over the right shoulder where the osmotic pump was placed in a subcutaneous pocket, and all the incisions closed. The rat was allowed to return to consciousness in a heated recovery box. Carprofen analgesia (5 mg kg^{-1}) was administered once daily for three

days post-surgery. Post-surgery animals were housed singly and in a reverse light/dark cycle (12h:12h) so animals were always studied whilst at their most active.

4.2.1.2 – Measurement of conscious micturition variables.

Animals were allowed at least a 48 h recovery period from surgery before any procedures were conducted, and therefore the loading dose was administered i.p on study day 3 to bring the free plasma concentration of compound up to the target level. On days 4, 6, 8, 11, 13, 15 and 18 of the study the animals were monitored in metabolic cages to observe any changes in their micturition variables. The methods used, and variables measured, were exactly as noted in section 3.2.1.3 of chapter 3. However, since SHR void more frequently than normal rats, these rats did not require any pharmacological intervention via furosemide/saline load administration, to increase the rate of voiding over the 3 h observation periods.

4.2.1.3 – Measurement of anaesthetized cystometry variables

On day 11 (D11) of each study, half the animals implanted with osmotic pumps were advanced to anaesthetized cystometry to monitor their regular cystometric profile and the effect of an additional 30 min i.v. infusion of WAY-100635/robalzotan. The remaining animals were monitored under anaesthetized cystometry on D20 of the study.

Anaesthesia was induced and maintained via i.p. administration of urethane (1.2 g kg^{-1}) (25% solution dissolved in saline), however during initial surgery supplementary administration of isoflurane in oxygen (~1.5%) was given where necessary. The right jugular vein and left carotid artery were cannulated to permit intravenous (i.v.) injection

of drugs and measurement of arterial blood pressure respectively. The trachea was also cannulated to maintain a patent airway. Once the jugular vein had been cannulated, supplementary doses of urethane (0.1 g kg^{-1} i.v.) were given where necessary. Depth of anaesthesia was assessed by cardiovascular and respiratory parameters, and by an absence of limb withdrawal in response to paw pinch. Body temperature was monitored using a rectal probe placed under the animal (to prevent interference with the urogenital system) and maintained between 36 and 38°C using a homeothermic blanket system (Harvard). The urinary bladder was exposed by a midline abdominal incision and a cut made in the bladder dome. A cuffed cannula (0.86 mm internal and 1.52 mm external diameter) was inserted into the bladder, secured with a suture around the top of the dome of the bladder and then pulled back up to the suture so the cannula was positioned as close to the edge of the bladder as possible. Saline infusion (at a rate of 0.05 ml min^{-1}) through the cannula was started and once it was checked that there was no leakage from the bladder, the abdominal incision was closed. The animal remained in dorsal recumbency for the remainder of the experiment. Surgical preparation was followed by a stabilisation period of 1 h.

Saline infusion into the bladder induced a rhythmic voiding pattern, with each void characterised by a typical rat cystometric profile (see Streng *et al.*, 2002) consisting of a sudden rise in bladder pressure (the opening pressure) once the threshold volume is reached. This is followed by a second phase where the bladder pressure starts to decline but high-frequency oscillations appear (the emptying phase) and then the void is completed when urinary flow stops due to another sudden rise in bladder pressure (the closing pressure) which is followed by a drop in the bladder pressure to the low level observed before the micturition contraction. Bladder pressure was measured by

connection of the bladder cannula to a pressure transducer (via a three-way tap), thus allowing simultaneous measurement of bladder pressure and saline infusion into the bladder through the same cannula. Arterial blood pressure and bladder pressures were continuously displayed on a chart recorder (Grass instruments) and captured by the Ponemah Physiology Platform version 3.0 (Gould Instrument Systems, Inc.) to allow data to be acquired and analysed off-line using P3 Analysis Modules (Gould Instrument Systems, Inc.).

The protocols used for each study during the anaesthetized cystometry experiments are shown in Figure 4.2. In these experiments, the inter-void interval was measured as the time (s) from the bladder pressure falling back down to baseline level after the previous void to the point at which the bladder pressure increased sharply at the start of the next void. After the initiation of a 30min i.v. infusion of WAY-100635/robalzotan the time to first void (s) was measured as the time from the start of the infusion (which was always initiated right at the end of a void) to the point at which the bladder pressure increased sharply at the start of the next void.

4.2.2 – Statistical Analysis

4.2.2.1 – Conscious variables

Changes in all of the conscious variables, i.e. number of voids, total volume voided (ml) and average volume per void (ml) were measured over a three hour period on each study day and all values are expressed as mean ± s.e.mean. Chronic drug evoked changes were compared to vehicle/time match control using one-way ANOVA followed by unpaired Student's *t*-test and $P < 0.05$ was considered to be statistically significant.

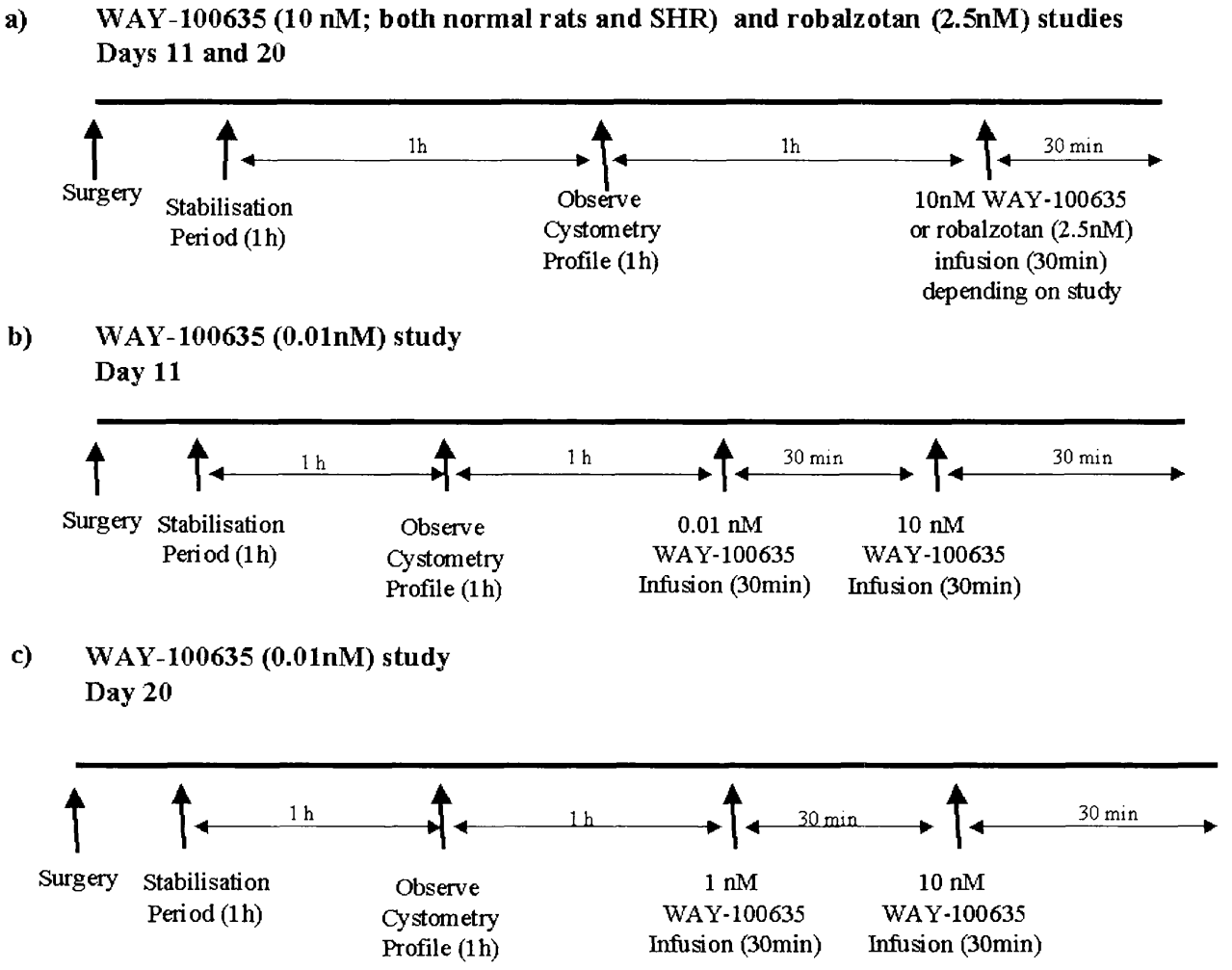


Figure 4.2 – Protocols used during the anaesthetized cystometry in each study **a)** the studies observing the effect of chronic administration of WAY-100635 (10nM) and robalzotan (2.5nM). **b** and **c)** the study observing the effect of chronic administration of WAY-100635 (0.01nM) – NB this study also took a slightly different timeline to all the other studies in that conscious variables were only monitored on days 4,6 & 8.

4.2.2.2 – Anaesthetized cystometry variables

The intervoid interval during the regular cystometric profile (prior to any subsequent infusion) of chronically compound treated animals was compared to vehicle/time match control using a two-sided unpaired Student's *t*-test and adjusting for unequal variances if necessary. The time to first void after the administration of a subsequent i.v. infusion of test compound was compared to the average intervoid interval of three voids prior to the infusion in that particular animal using a paired Student's *t*-test ($p < 0.05$ considered significant).

4.2.3 – Drugs and solutions

Drugs and chemicals were obtained from the following sources: Isoflurane (ISOCARE™) from Animalcare Ltd., U.K.; Carprofen (Rimadyl™ Large Animal Solution) from Pfizer Ltd., Sandwich, U.K.; urethane from Sigma Aldrich Chemicals, Poole, Dorset, U.K.; pentobarbitone sodium from Rhône Mérieux Ltd., Harlow, Essex; Furosemide from Sigma Aldrich Chemicals, Poole, Dorset, U.K.; *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)-cyclohexanecarboxamide trichloride (WAY-100635, 0.1 – 10 nM) and (*R*)-3-*N,N*-Dicyclobutylamino-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide hydrogen (*2R,3R*)-tartrate monohydrate (robalzotan; NAD-299, 2.5 nM) were chemically synthesized by Pfizer Ltd., Sandwich, U.K.). Furosemide was dissolved in ~ 5% PEG and 95% saline and warmed. WAY-100635 was dissolved in 0.9% wv^{-1} saline and robalzotan in acidified saline (~ pH 5.0).

4.3 - Results

4.3.1– Effect of chronic i.v. administration of WAY-100635 (10 nM) on normal rats

Chronic i.v. administration of WAY-100635 (10 nM free plasma concentration; see Table 4.1) had no significant effect on any of the conscious micturition variables measured on any of the study days, when compared to vehicle/time match control animals (see Table 4.2 and Figure 4.3; one exception was on day 18 when an increase in average volume per void was observed), despite this dose being effective at reducing the rate of voiding and increasing the average volume per void after acute administration in conscious rats (see Figure 3.3).

This lack of effect by chronic administration of WAY-100635 (10nM) in conscious animals was confirmed when the animals were advanced to anaesthetized cystometry - on both days 11 and 20 there was no significant difference in the inter-void interval in chronic WAY-100635 pre-treated animals (17.6 ± 24.2% & –33.4 ± 7.9% respectively) when compared to vehicle control (see Table 4.4 and Figure 4.4a & b). In addition, a subsequent i.v. infusion of WAY-100635 (10 nM) caused a significant increase in the time to first void in vehicle pre-treated animals on both day 11 (308.3 ± 49s to 564.8 ± 83s) and day 20 (535.6 ± 104s to 935.9 ± 188s), however, it had no effect in WAY-100635 pre-treated animals on either day (362.7 ± 75s to 502.4 ± 164s and 343.5 ± 40s to 450.0 ± 123.6 s respectively; see Figure 4.4a & b).

Rat Type	*Study Day*	*Target WAY-100635 free plasma concentration (nM)*	*Actual WAY-100635 free plasma concentration (nM)*
Normal	11 (n = 5)	10	9.36 ± 0.82
	20 (n = 5)	0	0.84 ± 0.22
SHR	11 (n = 4)	10	10.48 ± 2.19
	20 (n = 4)	0	6.51 ± 1.48

Table 4.1 – Actual free plasma concentrations of WAY-100635 (nM) as measured by HPLC analysis and mass spectrometry at various time points (study days) throughout the studies in both normal and spontaneously hypertensive rats. All values are mean ± s.e.mean.

Rat type	*Pre-treatment*	*Day*	*Number of voids over 3 h*	*Total volume voided (ml)*	*Average volume per void (ml)*
Normal	Vehicle (saline) days 4,6 & 8; n = 8 days 11,13,15 & 18; n = 5	4	7.0 ± 1.4	4.7 ± 0.7	0.945 ± 0.28
		6	5.0 ± 1.2	2.4 ± 0.5	0.676 ± 0.204
		8	6.0 ± 0.9	4.3 ± 0.6	0.832 ± 0.129
		11	6.0 ± 1.1	3.1 ± 0.6	0.705 ± 0.292
		13	10.0 ± 1.8	4.6 ± 1.0	0.455 ± 0.051
		15	6.2 ± 0.9	3.7 ± 0.7	0.657 ± 0.157
		18	9.4 ± 1.8	3.5 ± 0.5	0.401 ± 0.044
	WAY-100635 (10 nM) Days 4,6 & 8; n = 8 Days 11,13,15 & 18; n = 5	4	7.1 ± 0.8	6.4 ± 1.2	0.946 ± 0.162
		6	2.1 ± 0.3	3.0 ± 0.6	0.573 ± 0.165
		8	4.9 ± 0.7	3.8 ± 0.2	0.939 ± 0.184
		11	4.4 ± 0.9	2.6 ± 0.8	0.577 ± 0.055
		13	6.8 ± 1.2	4.2 ± 0.7	0.642 ± 0.096
		15	5.4 ± 0.4	3.4 ± 0.4	0.644 ± 0.104
		18	5.2 ± 0.2	3.4 ± 0.4	0.661 ± 0.088 *

Table 4.2 – Effect of WAY-100635 (10 nM) and vehicle (saline) on the conscious micturition variables measured at various time points throughout the study (days) in normal rats. None of the variables in WAY-100635 pre-treated rats were significantly different to the vehicle control values when compared by unpaired Student's *t*-test.

Rat type	*Pre-treatment*	*Day*	*Number of voids over 3 h*	*Total volume voided (ml)*	*Average volume per void (ml)*
SHR	Vehicle (saline) days 4,6 & 8; n = 8 days 11,13,15 & 18; n = 5	4	6.8 ± 1.1	2.1 ± 0.5	0.291 ± 0.02
		6	8.6 ± 1.1	2.3 ± 0.4	0.265 ± 0.013
		8	6.9 ± 1.4	2.0 ± 0.6	0.228 ± 0.019
		11	9.2 ± 2.7	3.1 ± 1.2	0.316 ± 0.033
		13	8.0 ± 2.7	2.4 ± 0.7	0.315 ± 0.052
		15	9.6 ± 2.2	3.0 ± 0.8	0.315 ± 0.015
		18	11.6 ± 2.2	4.8 ± 1.4	0.388 ± 0.043
	WAY-100635 (10 nM) Days 4,6 & 8; n = 8 Days 11,13,15 & 18; n = 4	4	10.0 ± 1.7	2.8 ± 0.4	0.288 ± 0.007
		6	5.9 ± 1.4	2.0 ± 0.4	0.338 ± 0.032
		8	5.5 ± 1.1	2.1 ± 0.4	0.404 ± 0.034
		11	8.3 ± 1.1	2.2 ± 0.3	0.284 ± 0.057
		13	9.5 ± 0.7	3.0 ± 0.2	0.312 ± 0.014
		15	7.3 ± 2.0	2.3 ± 0.6	0.318 ± 0.051
		18	13.3 ± 0.9	4.5 ± 0.3	0.254 ± 0.088

Table 4.3 - Effect of WAY-100635 (10 nM) and vehicle (saline) on the conscious micturition variables measured at various time points throughout the study (days) in spontaneously hypertensive rats (SHR)

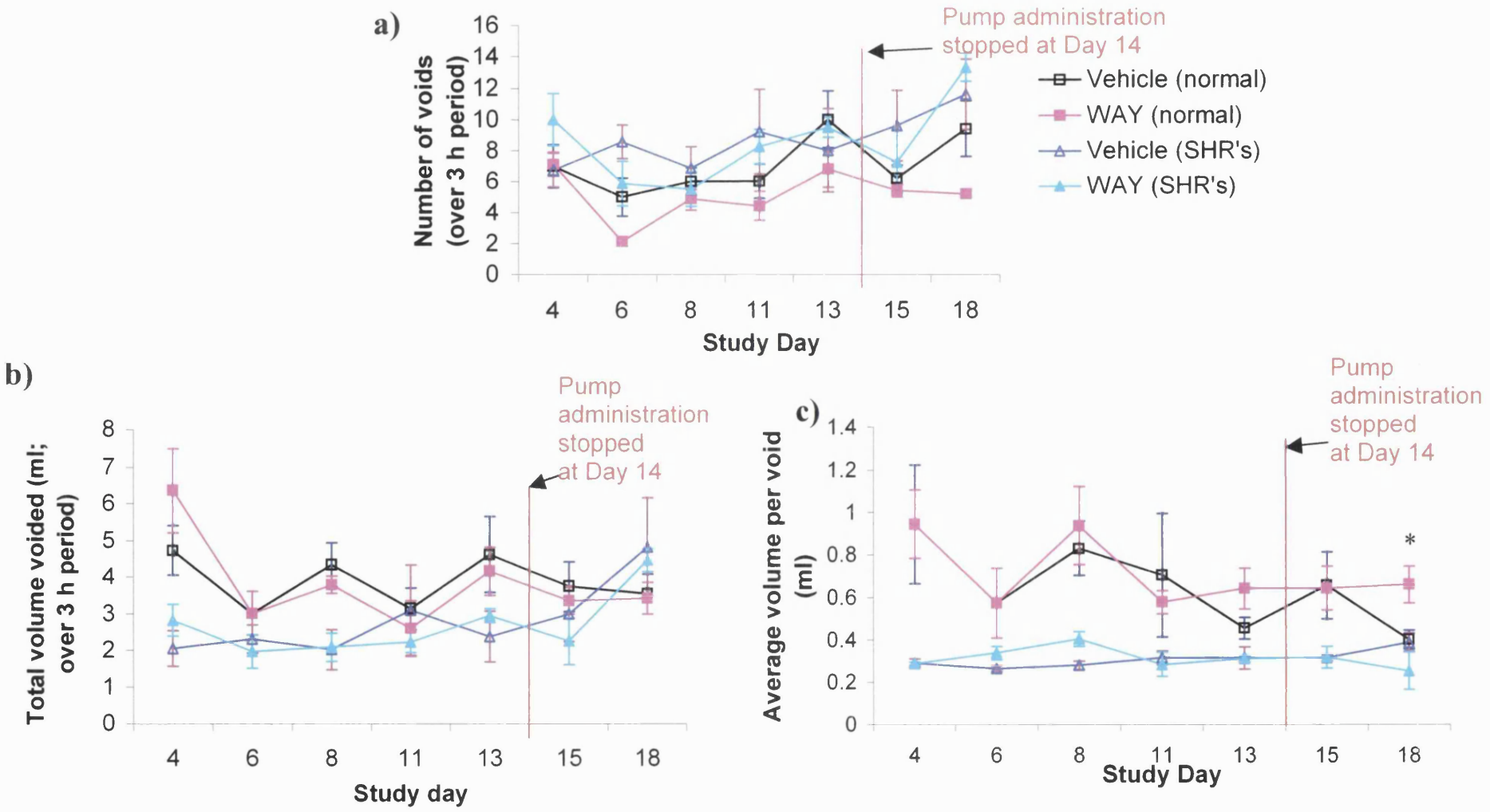


Figure 4.3 – Effect of WAY-100635 (10 nM) and vehicle on conscious micturition variables in both normal and SHR at various time points throughout the study. **a)** Number of voids over 3 h period; **b)** Total volume voided (ml) over 3 h period; **c)** Average volume per void (ml) over 3 h period. It is important to note that the osmotic pumps stopped delivering compound at day 14. n = 8 at days 4, 6 & 8; n = 4/5 at days 11, 13 ,15 & 18. *P < 0.05 for WAY-100635 when compared to vehicle control (normal rat) using unpaired Student's *t*-test.

Rat type	*Pre-treatment*	*Day*	*Intervoid interval prior to WAY (10 nM) infusion (s)*	*Time to first void after WAY (10 nM) Infusion (s)*
Normal	Vehicle (saline; n = 5)	11	308.3 ± 49.2	564.8 ± 83.0 *
	WAY-100635 (n = 4)	11	362.2 ± 74.5	502.4 ± 164.1
	Vehicle (saline; n = 4)	20	535.6 ± 103.6	935.9 ± 188.0 *
	WAY-100635 (n = 4)	20	343.5 ± 40.0	450.0 ± 123.6
SHR	Vehicle (saline; n = 5)	11	104.1 ± 26.0	272.0 ± 22.2 **
	WAY-100635 (n =4)	11	167.7 ± 32.0	185.0 ± 18.4
	Vehicle (saline; n = 4)	20	224.3 ± 77.0	437.0 ± 88.7 *
	WAY-100635 (n = 4)	20	192.4 ± 13.0	186.0 ± 30.3

Table 4.4 – Anaesthetized micturition variables measured before and after an additional WAY-100635 (10nM) i.v. infusion in normal rats and spontaneously hypertensive rats (SHR) that have been previously chronically administered i.v. with WAY-100635 (10nM) or vehicle via an osmotic pump for 11 days (Day 11) or 14 days (Day 20). * $P < 0.05$ and ** $p < 0.01$ when compared to intervoid interval prior to WAY-100635 infusion by paired student's *t*-test.

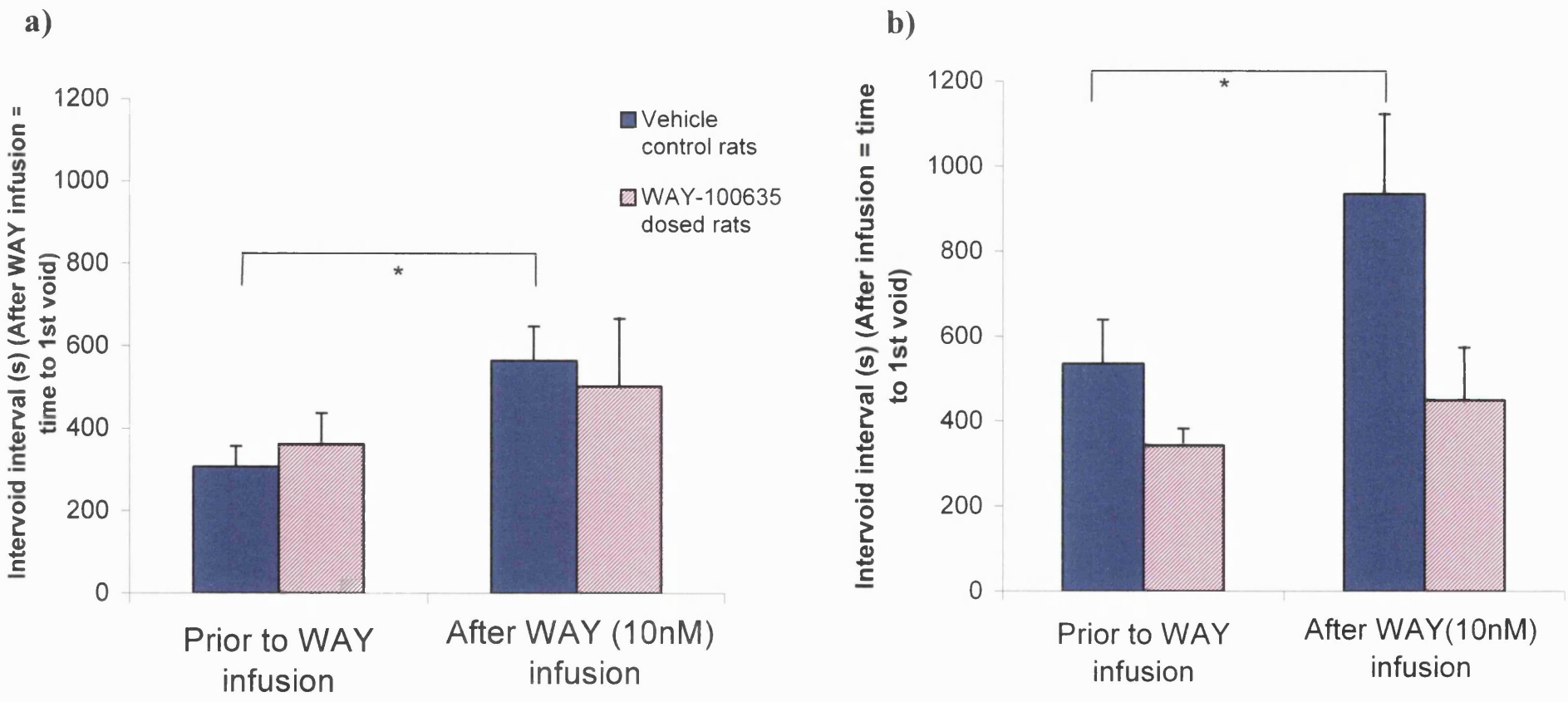


Figure 4.4 – Effect of previous **a)** 11 day and **b)** 14 day (with 6 day washout period) chronic i.v. administration of WAY-100635 (10nM) or vehicle on an additional 30 min i.v. infusion of WAY-100635 on the intervoid interval/ time to first void in anaesthetized normal rats. * $P < 0.05$ when compared to intervoid interval prior to WAY-100635 infusion by paired student's *t*-test.

4.3.2 - Effect of chronic i.v. administration of WAY-100635 (10 nM) on spontaneously hypertensive rats (SHR)

Chronic i.v. administration of WAY-100635 (10 nM free plasma concentration; see Table 4.1) had no significant effect on any of the conscious micturition variables measured on any of the study days, when compared to vehicle/time match control animals (see Table 4.3 and Figure 4.3). It is interesting to note the lower average volume per void observed with SHR when compared to normal conscious rats (see Figure 4.3c), thus confirming the presence of bladder hyperactivity.

When these pre-treated animals were monitored using anaesthetized cystometry very similar results were obtained to those observed with the normal rats. On both days 11 and 20 there was no significant difference in the inter-void interval in chronic WAY-100635 pre-treated animals (61.1 ± 30.7% & −14.2 ± 5.8% respectively) when compared to vehicle control (see Table 4.4 and Figure 4.5a & b). In addition, a subsequent i.v. infusion of WAY-100635 (10 nM) caused a significant increase in the time to first void in vehicle pre-treated animals on both day 11 (104.1 ± 26s to 272.0 ± 22s) and day 20 (224.3 ± 77s to 437.0 ± 89s), however, it had no effect in WAY-100635 pre-treated animals on either day (167.7 ± 32s to 185.0 ± 18s and 192.4 ± 13s to 186.0 ± 30s respectively; see Figure 4.5a & b). Unsurprisingly, the anaesthetized inter-void interval in the SHR was also lower than that observed in normal rats (see Table 4.4).

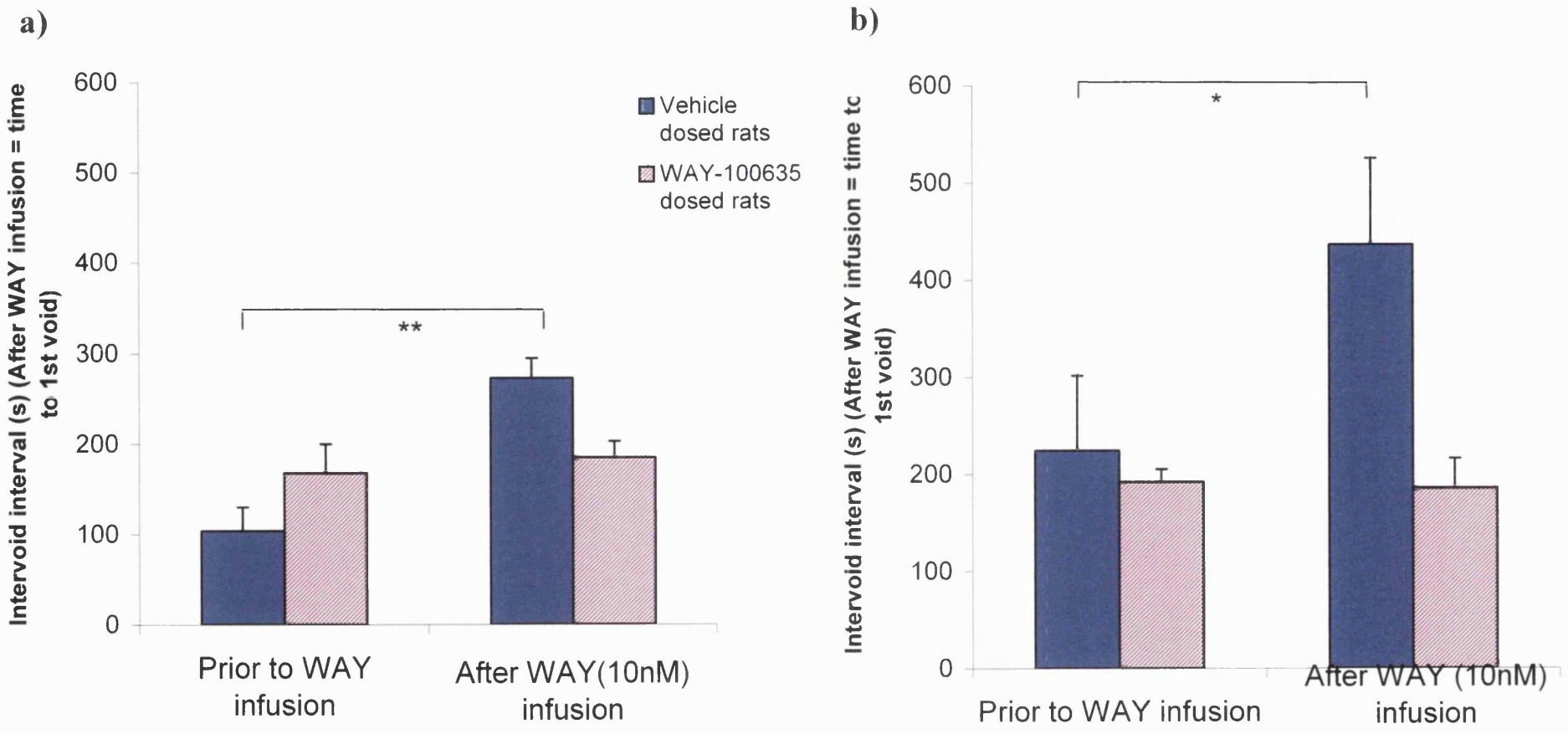


Figure 4.5 - Effect of previous **a)** 11 day and **b)** 14 day (with 6 day washout period) chronic i.v. administration of WAY-100635 (10nM) or vehicle on an additional 30 min i.v. infusion of WAY-100635 on the intervoid interval/ time to first void in anaesthetized female spontaneously hypertensive rats (SHR). * $P < 0.05$ and ** $p < 0.01$ when compared to intervoid interval prior to WAY-100635 infusion by paired student's *t*-test.

4.3.3 - Effect of chronic i.v. administration of a non-effective dose of WAY-100635 (0.01 nM) on spontaneously hypertensive rats (SHR)

As previously shown in chapter 3, 0.1nM WAY-100635 administered acutely is not effective at altering any of the conscious micturition variables measured in this model. Therefore, it is not surprising that chronic i.v. administration of 0.01 nM WAY-100635 in SHR has no effect on any of the conscious micturition variables when compared with vehicle control on days 4, 6 and 8 of the study (see Table 4.5 and Figure 4.6).

However, surprisingly it appears that chronic i.v. administration of a non-effective dose (0.01nM) of WAY-100635, prevents effective doses of WAY-100635 (e.g. 10 nM) from having any effect in anaesthetized rats. In SHR which had been previously administered i.v. with WAY-100635 (0.01nM) or vehicle (saline) and then progressed to anaesthetized cystometry, a subsequent i.v. infusion of WAY-100635 (10 nM) caused a significant increase in the time to first void in vehicle pre-treated animals on both day 11 (104.1 ± 26s to 272.0 ± 22s) and day 20 (224.3 ± 77s to 437.0 ± 89s; se2 Table 4.6), however, it had no significant effect in WAY-100635 (0.01nM) pre-treated animals on either day (272.0 ± 50s to 352.0 ± 44s and 262.0 ± 31s to 302.0 ± 50s respectively; see Table 4.6 and Figure 4.7).

Rat type	*Pre-treatment*	*Day*	*Number of voids over 3 h*	*Total volume voided (ml)*	*Average volume per void (ml)*
SHR	Vehicle (saline) (n = 8)	4	6.8 ± 1.1	2.1 ± 0.5	0.291 ± 0.02
		6	8.6 ± 1.1	2.3 ± 0.4	0.265 ± 0.013
		8	6.9 ± 1.4	2.0 ± 0.6	0.228 ± 0.019
	WAY-100635 (0.01nM) (n = 7/8)	4	9.2 ± 2.7	3.1 ± 1.2	0.316 ± 0.033
		6	8.0 ± 2.7	2.4 ± 0.7	0.315 ± 0.052
		8	9.6 ± 2.2	3.0 ± 0.8	0.315 ± 0.015

Table 4.5 - Effect of WAY-100635 (0.01nM) and vehicle (saline) on the conscious micturition variables in female spontaneously hypertensive rats (SHR) measured on days 4, 6 & 8 of the study.

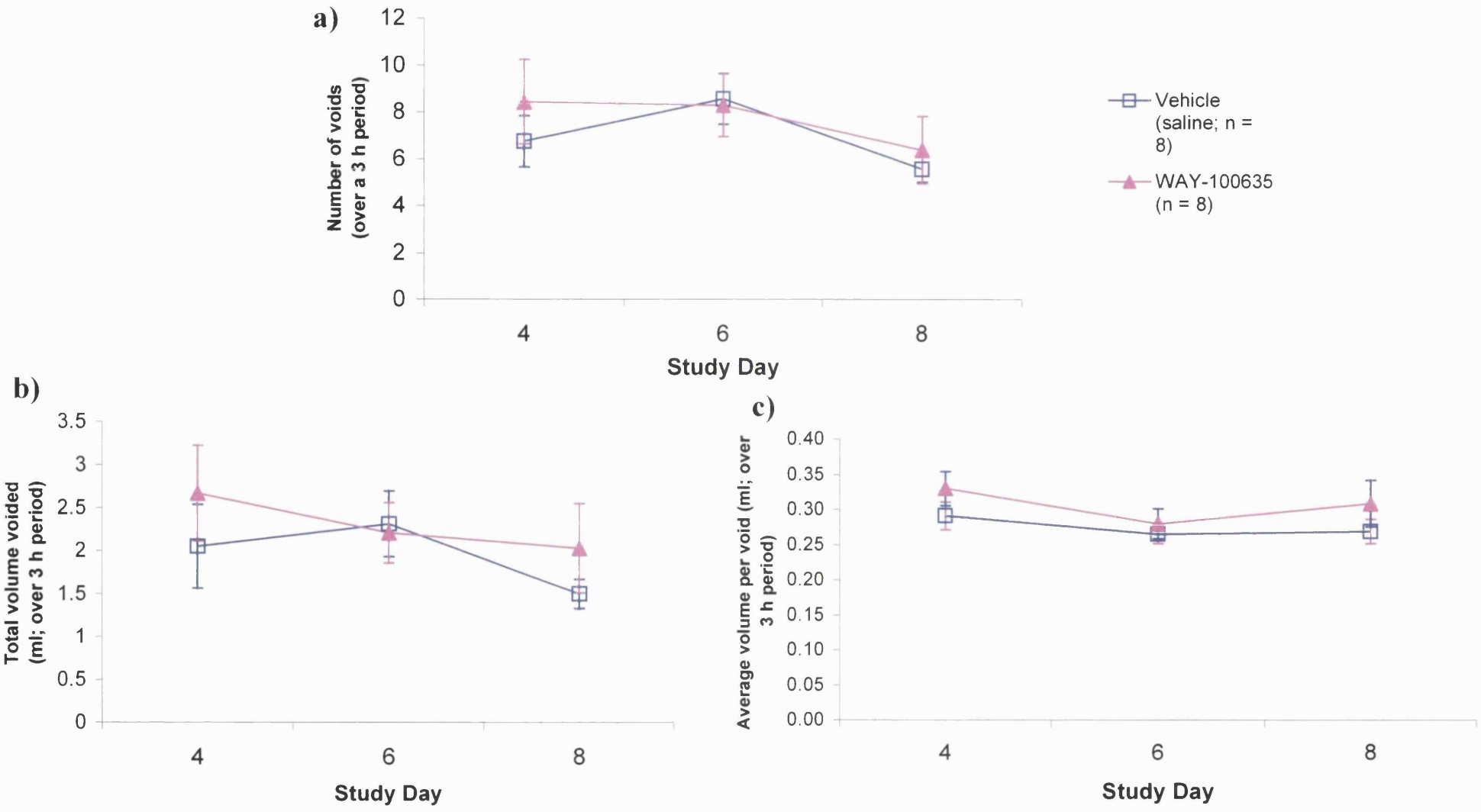


Figure 4.6 - Effect of WAY-100635 (0.01nM) and vehicle on conscious micturition variables in SHR at days 4,6 & 8 of the study. **a)** Number of voids over 3 h period; **b)** Total volume voided (ml) over 3 h period; **c)** Average volume per void (ml) over 3 h period.

Rat type	*Pre-treatment*	*Day*	*Intervoid interval prior to WAY infusion (s)*	*Time to first void after WAY infusion (s)*
SHR	WAY-100635 (0.01nM)	11 (n = 3)	272.0 ± 50.3	352.0 ± 44.3
		20 (n = 4)	262.0 ± 30.8	302.0 ± 50.3
	Vehicle (saline)	11 (n = 5)	104.1 ± 26.0	272.0 ± 22.2 **
		20 (n = 4)	224.3 ± 77.0	437.0 ± 88.7 *

Table 4.6 - Anaesthetized inter-void interval/time to first void measured prior to and after an additional WAY-100635 (10nM) i.v. infusion in spontaneously hypertensive rats (SHR) that have been previously chronically administered i.v. with WAY-100635 (0.01nM) via an osmotic pump for 11 days (Day 11) or 14 days with a 6 day washout period (Day 20). *P < 0.05 and **P < 0.01 when compared to intervoid interval prior to WAY-100635 infusion by paired Student's *t*-test.

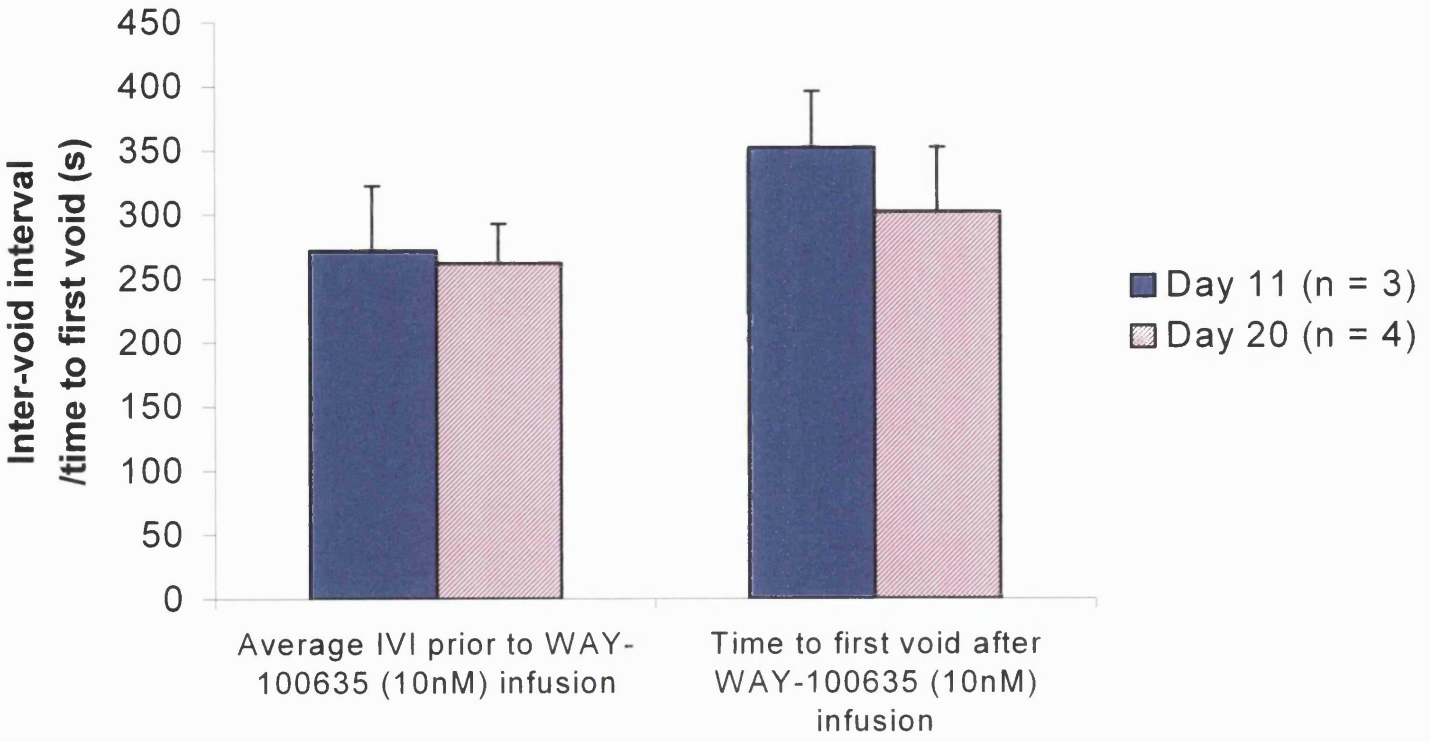


Figure 4.7 - Effect of previous 11 day and 14 day (with 6 day washout period) chronic i.v. administration of WAY-100635 (0.01nM) on an additional 30 min i.v. infusion of WAY-100635 (10nM) on the intervoid interval/ time to first void in anaesthetized spontaneously hypertensive rats. The effect of a similar 30 min i.v. infusion of WAY-100635 (10nM) on SHR with vehicle pre-treatment is shown in Figure 4.5. Abbreviations:, IVI, intervoid interval.

4.3.4 - Effect of chronic i.v. administration of robalzotan (2.5 nM) on normal rats

The target free plasma concentration of robalzotan after chronic dosing using the osmotic pump was 2.5 nM. On study day 11 the actual free plasma concentration of robalzotan, as measured by HPLC analysis and mass spectrometry, was 2.8 ± 0.6 nM (n = 3) and by study day 20 (i.e. after a 6 day wash-out period) the actual free plasma concentration of robalzotan was 0.7 ± 0.4 nM (n = 4).

Chronic i.v. administration of robalzotan (2.5 nM free plasma concentration) had no significant effect on any of the conscious micturition variables measured on any of the study days, when compared to vehicle/time match control animals (see Table 4.7 and Figure 4.8), despite this dose being effective at reducing the rate of voiding after acute administration in conscious rats (see Figure 3.4).

This lack of effect by chronic administration of robalzotan (2.5 nM) in conscious animals was confirmed when the animals were advanced to anaesthetized cystometry - on both days 11 and 20 there was no significant difference in the inter-void interval in chronic robalzotan pre-treated animals (23.9 ± 21.2% & 105.3 ± 52.1% respectively) when compared to vehicle control (see Table 4.8 and Figure 4.9). However, in the anaesthetized cystometry model, a robalzotan infusion has a different profile to that of a WAY-100635 infusion, in that it generally causes a complete suppression of micturition with the bladder pressure artificially raised during the infusion period, which causes continuous dribbling rather than distinct voiding (see Figure 4.10). This is in comparison to a 30 min i.v. infusion of WAY-100635, which initially causes an increase in the time to first void after the start of the infusion, but efficient emptying is still maintained and a

regular cystometric profile gradually returns throughout the rest of the infusion period (see Figure 4.11). Due to the lack of regular voiding observed during an infusion of robalzotan , the 'robalzotan' effect cannot be measured from the time to first void, and instead is expressed as the change in the number of complete/regular voids over the 30 min infusion period. As Figure 4.10 shows, in rats pre-treated with i.v. robalzotan (2.5nM), a subsequent 30 min i.v. infusion of robalzotan (2.5nM) in the anaesthetized cystometry model, does not prevent micturition in the same manner observed in vehicle pre-treated animals. In vehicle pre-treated rats on Day 20, there were 0.5 ± 0.50 voids during a subsequent 30 min i.v. infusion of robalzotan (2.5 nM), whilst in robalzotan pre-treated animals there was a significantly increased number of voids (2.3 ± 0.48; see Table 4.8), thus indicating that robalzotan (2.5 nM) is unable to produce its usual suppression of micturition in rats pre-treated with chronic i.v. administration of robalzotan (2.5nM). However, it was observed that in these robalzotan pre-treated rats a subsequent infusion of 250 nM robalzotan was able to suppress micturition, although not to the same extent or with the same profile as observed after a 2.5 nM infusion of robalzotan in vehicle pre-treated rats (see Figure 4.12).

Rat type	*Pre-treatment*	*Day*	*Number of voids over 3 h*	*Total volume voided (ml)*	*Average volume per void (ml)*
Normal	Vehicle (saline) days 4,6 & 8; n = 8 days 11,13,15 & 18; n = 4	4	3.6 ± 1.1	3.2 ± 0.4	1.19 ± 0.26
		6	3.9 ± 0.6	2.7 ± 0.4	0.80 ± 0.14
		8	3.7 ± 0.6	1.9 ± 0.2	0.58 ± 0.12
		11	7.5 ± 1.5	4.1 ± 1.1	0.55 ± 0.12
		13	3.8 ± 0.3	2.2 ± 0.5	0.57 ± 0.12
		15	6.3 ± 1.0	4.0 ± 1.4	0.62 ± 0.14
		18	4.0 ± 0.9	2.0 ± 0.5	0.57 ± 0.22
	Robalzotan (2.5 nM) Days 4,6 & 8; n = 8 Days 11,13,15 & 18; n = 4	4	3.4 ± 0.5	2.3 ± 0.3	0.79 ± 0.14
		6	6.1 ± 1.1	2.9 ± 0.4	0.52 ± 0.08
		8	3.3 ± 0.3	1.6 ± 0.2	0.55 ± 0.11
		11	5.7 ± 1.2	3.3 ± 1.3	0.58 ± 0.12
		13	4.0 ± 0.4	2.3 ± 0.2	0.61 ± 0.13
		15	4.3 ± 0.8	1.7 ± 0.2	0.43 ± 0.08
		18	4.8 ± 0.9	2.5 ± 0.6	0.56 ± 0.11

Table 4.7 - Effect of robalzotan (2.5 nM) and vehicle (acidified saline) on the conscious micturition variables in normal rats measured at various time points throughout the study (days).

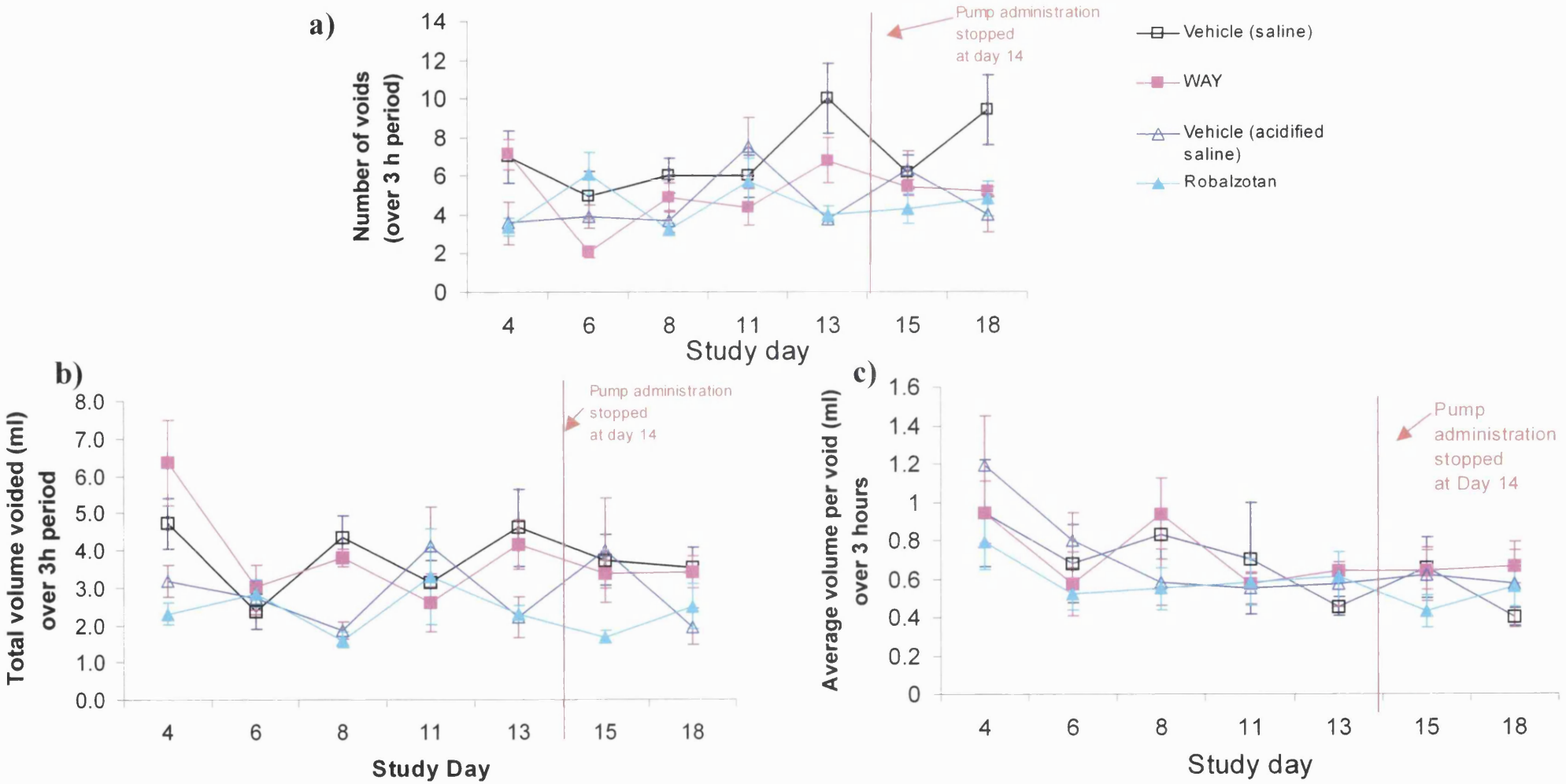


Figure 4.8 - Effect of robalzotan (2.5 nM), WAY-100635 (10 nM) and vehicle (acidified saline/ saline) on conscious micturition variables in normal female rats at various time points throughout the study. **a)** Number of voids over 3 h period; **b)** Total volume voided (ml) over 3 h period; **c)** Average volume per void (ml) over 3 h period. It is important to note that the osmotic pumps stopped delivering compound at day 14. n = 8 at days 4, 6 & 8; n = 4/5 at days 11, 13 ,15 & 18.

Rat type	*Pre-treatment*	*Day*	*Intervoid interval prior to robalzotan infusion (s)*	*Number of voids during 30 min robalzotan infusion (s)*
Normal	Vehicle (n = 3)	11	402.0 ± 56.4	-
	Robalzotan (n = 4)	11	498.0 ± 85.2	-
	Vehicle (n = 4)	20	261.4 ± 80.2	0.5 ± 0.50
	Robalzotan (n = 4)	20	536.6 ± 136.3	2.3 ± 0.48 *

Table 4.8 – Effect of chronic i.v. robalzotan (2.5nM) and vehicle (acidified saline) pre-treatment on the cystometric profile of normal anaesthetized female rats, and on the number of voids observed during a 30 min i.v. infusion of robalzotan (2.5 nM). * $P < 0.05$ when compared to vehicle control by unpaired Student's *t*-test.

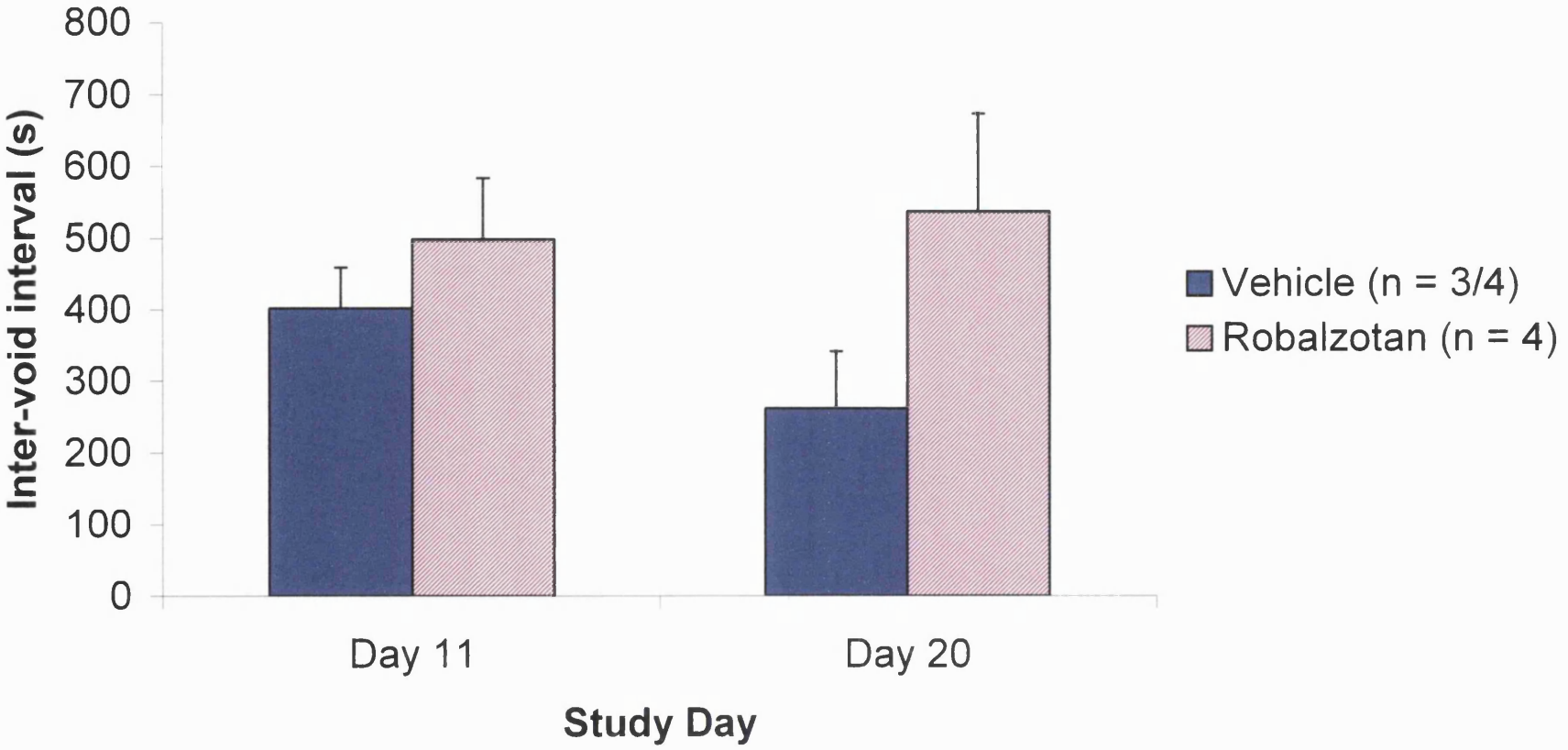


Figure 4.9 - Effect of chronic i.v. robalzotan (2.5nM) and vehicle (acidified saline) pre-treatment on the regular cystometric profile of anaesthetized normal female rats on Day 11 and Day 20 of the study.

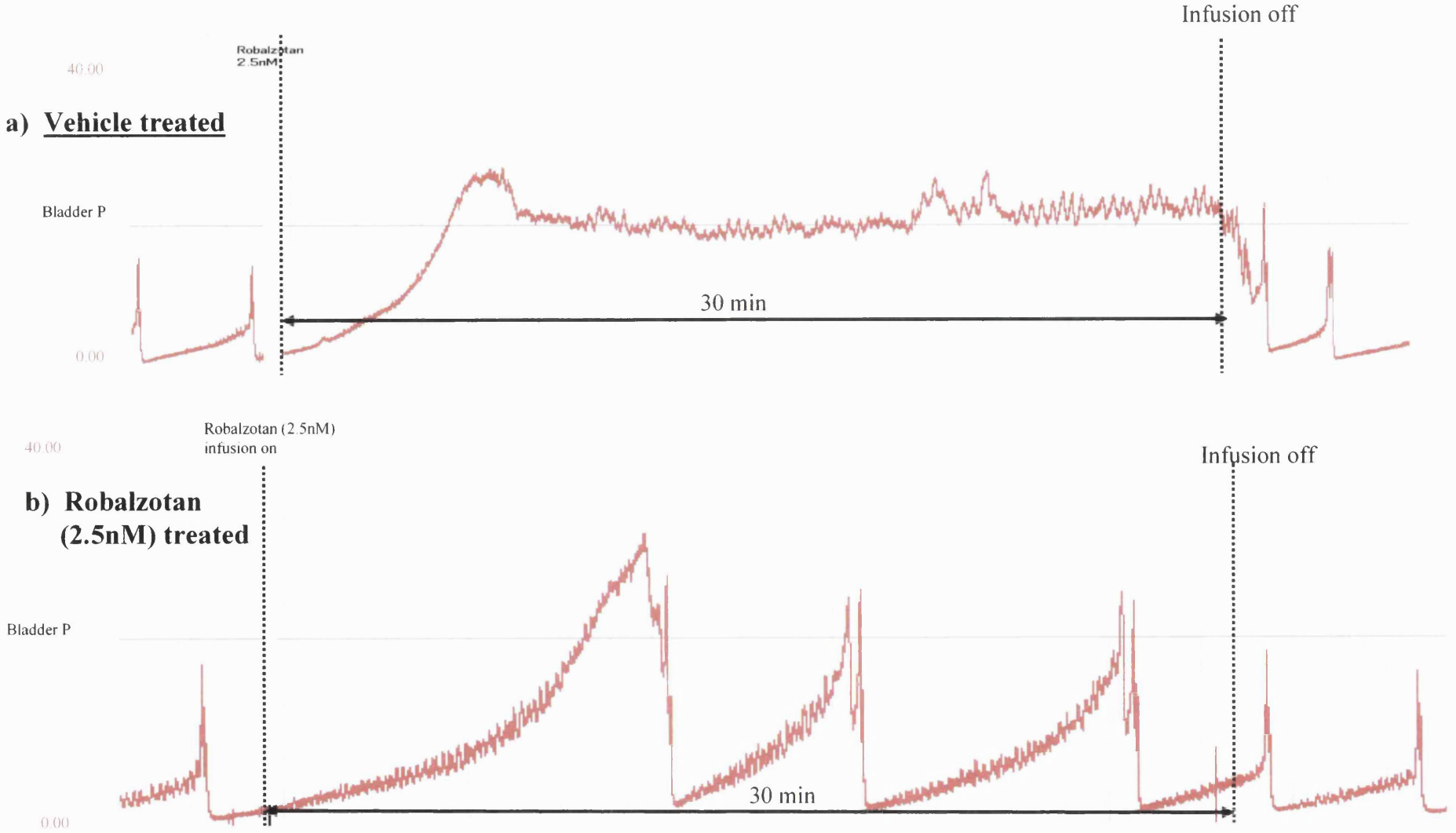


Figure 4.10 – Typical trace of the effect of a 30 min i.v. infusion of robalzotan (2.5 nM free plasma concentration) in **a)** vehicle (acidified saline) pre-treated and **b)** robalzotan (2.5 nM; 14 days with 6 day washout period) pre-treated anaesthetized normal female rats.

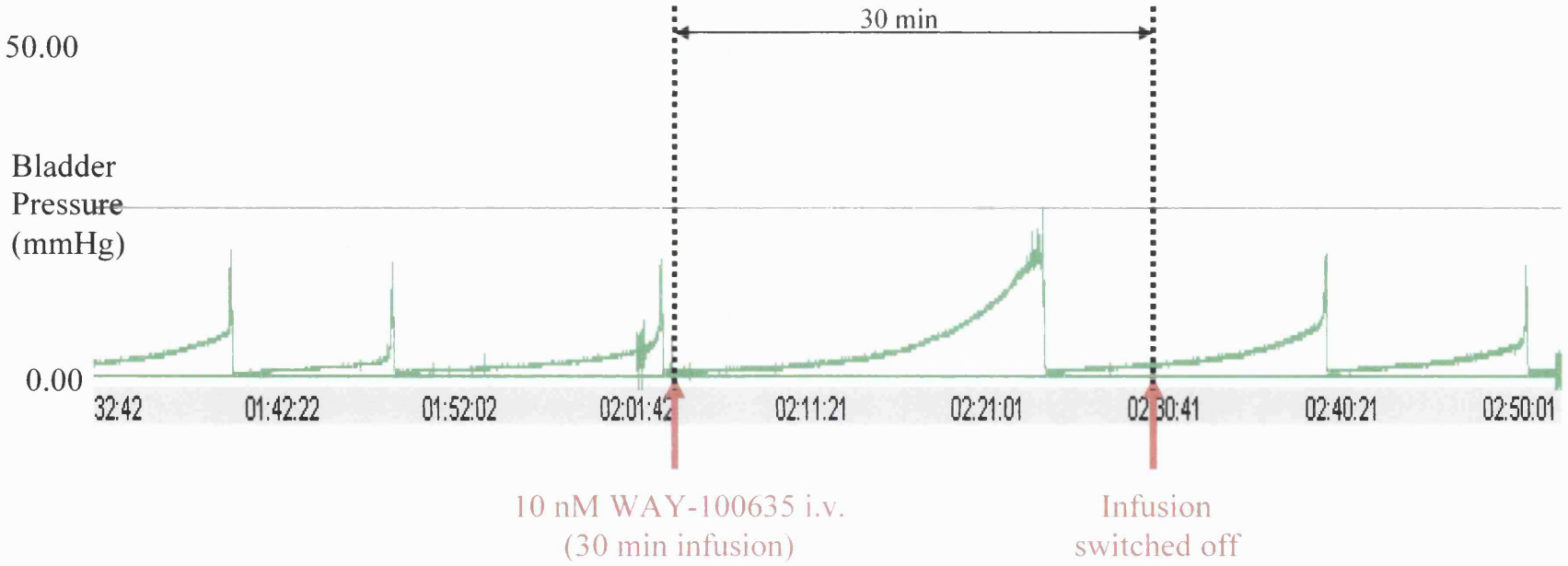


Figure 4.11 - Typical cystometry trace of the effect of a 30 min i.v. infusion of WAY-100635 (10 nM free plasma concentration) in vehicle (saline) pre-treated anaesthetized normal female rats.

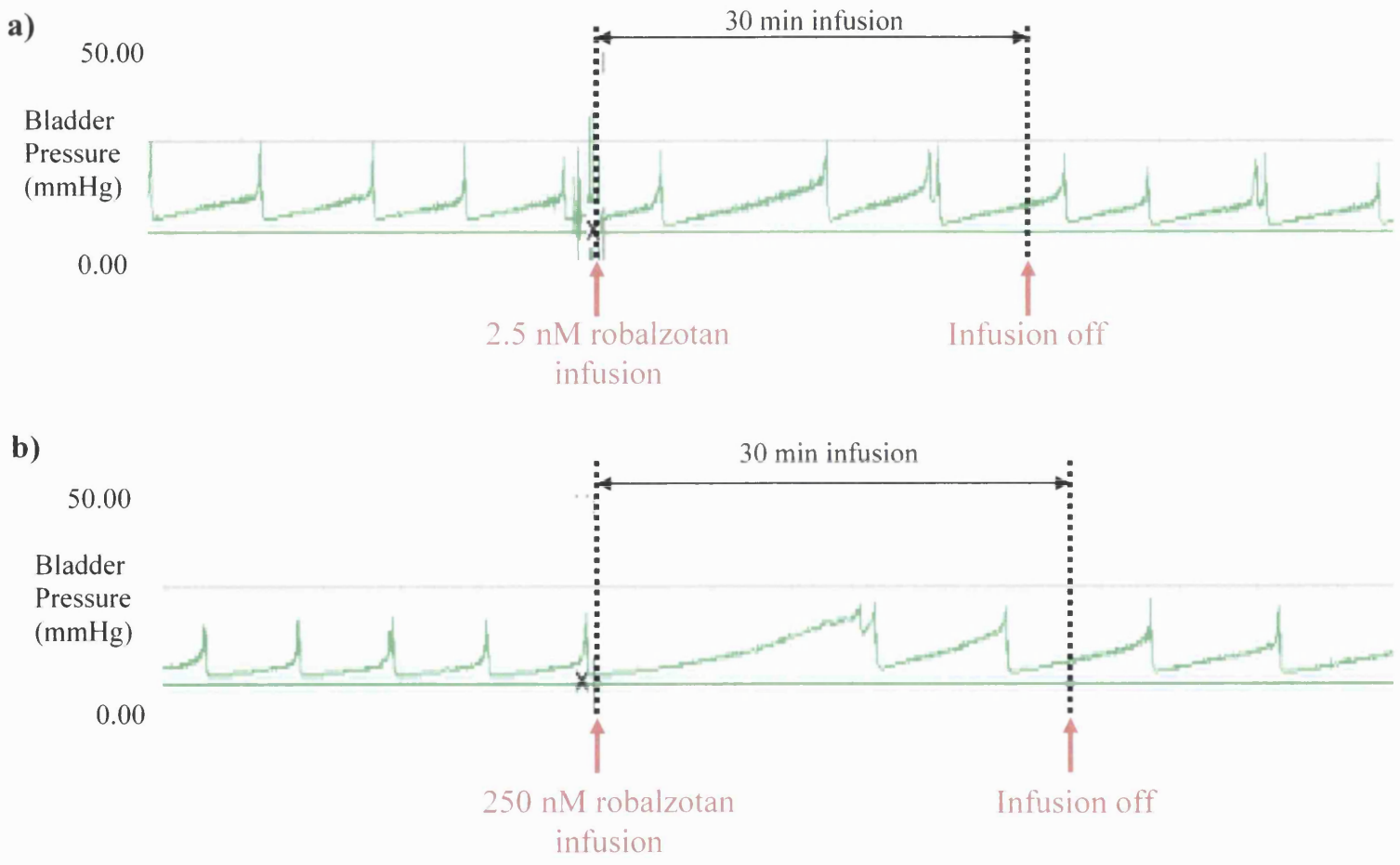


Figure 4.12 – Typical anaesthetized cystometry trace to show the effect on the bladder presure of a) 2.5 nM robalzotan (free plasma concentration) and b) 250 nM robalzotan (free plasma concentration) 30 min i.v. infusions in the same rat which has been pre-treated with chronic i.v. robalzotan (11 days; 2.5 nM free plasma concentration). Note that the tolerance to robalzotan observed after the 2.5 nM infusion is overcome when the concentration of robalzotan is increased to 250 nM.

4.4 – Discussion

The results from the present study have shown that in both normal and spontaneously hypertensive rats, WAY-100635 at a constant free plasma level of 10 nM has no effect on conscious micturition variables after chronic i.v. administration (see Figure 4.3), and this lack of effect on micturition was confirmed with anaesthetized cystometry studies which showed that an infusion of WAY-100635 (10 nM free plasma concentration) has no effect on micturition in rats chronically pre-treated with WAY-100635 (see Figures 4.4 and 4.5). Data from this study have also shown a similar phenomenon with the structurally distinct 5-HT_{1A} receptor antagonist, robalzotan, with chronic i.v. administration at a free plasma concentration of 2.5 nM having no effect on conscious micturition variables (see Figure 4.8) or anaesthetized cystometric variables (see Figure 4.9) despite this dose having been previously shown to be effective at suppressing micturition after acute administration (see chapter 3, section 3.3.2). Interestingly, anaesthetized cystometry studies showed that the 'tachyphylaxis' phenomenon induced by chronic robalzotan pre-treatment can be overcome by increasing the challenge dose of robalzotan to 250 nM (see Figure 4.12).

4.4.1 – Effect of chronic i.v. administration of WAY-100635 (10 nM)

The results from this study have shown that WAY-100635 at a constant free plasma level of 10 nM has no effect on conscious micturition variables after chronic i.v. administration (see Figure 4.3), despite this dose having been shown to decrease the rate of voiding and increase the average volume per void after acute administration in both male and female conscious rats. This suggests the presence of a tolerance/desensitization of 5-HT_{1A}

receptors to WAY-100635, which was confirmed with the anaesthetized cystometry studies which showed that a subsequent i.v. infusion of WAY-100635 (10 nM) is unable to significantly alter the time to first void in animals pre-treated with chronic WAY-100635, although a significant increase is observed in rats pre-treated with vehicle control (see Figure 4.4). This was observed on both D11 (i.e. during chronic WAY-100635 administration) and surprisingly also on D20 of the study (i.e. 14 day administration plus a six day wash out period), thus indicating that this phenomenon is long-lasting and still present at least 6 days after chronic WAY-100635 administration is stopped. It also appears that this phenomenon has a fast onset time since WAY-100635 administration had no effect on conscious variables by day 4 of the study (the first day rats could be monitored after osmotic pump implantation surgery). However, from this model we are unable to identify anymore accurately the onset time of this phenomenon. Further studies are required to examine the onset time of the phenomenon and are addressed in Chapter 5.

This 'tachyphylaxis' phenomenon was also observed in conscious and anaesthetized spontaneously hypertensive rats (SHR) chronically administered i.v. with WAY-100635 (10 nM). This provides further confirmation for the presence of this phenomenon as a 'real' effect, since it has been observed in two strains of rat, one of which (SHR) is used as model for overactive bladder (Persson *et al.*, 1998; Cefalu *et al.*, 2002); Pfizer Ltd. 2002, personal communication). SHR exhibit increased voiding frequency, decreased void volume and bladder instability (in the form of non-voiding contractions) when compared to their normotensive control strain (Wistar-Kyoto; Cefalu *et al.*, 2002) and these characteristics are thought to be associated, in part, to changes in the noradrenergic control of the micturition reflex as well as increased smooth muscle and decreased

neuronal responsiveness to noradrenaline (Persson *et al.*, 1998) and elevated nerve growth factor (NGF) levels. The characteristic urodynamics of SHR were also observed in the present studies, with a significant decrease in the average volume per void observed in conscious animals (see Figure 4.3c) and a decrease in the average inter-void interval (i.e. an increased voiding frequency) in anaesthetized SHR when compared to normal Sprague Dawley rats (see Figure 4.5). The observation that a 30 min i.v. infusion of WAY-100635 (10 nM) in anaesthetized SHR pre-treated with vehicle in the osmotic pump (i.e. a control SHR) was able to increase the time to first void and hence the bladder capacity (see Figure 4.5), and decrease the number of non-voiding contractions, indicates that 5-HT_{1A} receptor antagonists also exhibit their effects on micturition in a rat model of overactive bladder. The fact that 5-HT_{1A} receptor modulation influences SHR bladder hyperactivity, which is mediated by changes in noradrenergic control (Persson *et al.*, 1998), once again confirms the high level of interplay between the noradrenergic and 5-HT systems in the control of micturition.

The initial studies in this present chapter examined the effect of chronic administration of WAY-100635 (10 nM), which had previously been shown to be an effective dose at suppressing micturition in conscious rats. However, interestingly a further study using a dose of WAY-100635 (0.01 nM), which is non-effective at suppressing micturition, was still able to prevent an additional i.v infusion of WAY-100635 (10 nM) from having any suppressive effect on micturition in anaesthetized rats (see Figure 4.7). This was observed on both D11 and D20 of the study, indicating once again that the 'tachyphylaxis' phenomenon is long lasting, however, it must be noted that the protocol for anaesthetized infusions on D20 resulted in the 10 nM infusion of WAY-100635 being administered after a 1 nM infusion (see Figure 4.2), which may have already desensitized

the system to WAY-100635. It is surprising that such a low dose of WAY-100635 is capable of causing this phenomenon, however, this highlights the sensitivity of the 5-HT signalling system to pharmacological intervention, and the intrinsic activity, and hence importance, of 5-HT_{1A} receptors in the control of a number of physiological processes which have been previously discussed in chapter 1. Therefore, it is possible that after chronic 5-HT_{1A} receptor blockade (even with low doses of antagonist), which disrupts 'normal' 5-HT_{1A} receptor control, there may be compensatory changes in other 5-HT_1 receptors to allow the maintenance of correct physiological functioning (see 1.2.2 for examples). If this is the case, then these results emphasize the complexities involved in using the 5-HT signalling system as a target in drug development.

4.4.2 – Effect of chronic i.v. administration of the structurally diverse 5-HT_{1A} receptor ligand, robalzotan.

Robalzotan is a 5-HT_{1A} receptor antagonist from a different chemical series to WAY-100635 (Johansson *et al.*, 1997; see Figure 1.5). However, as observed with WAY-100635, data from the present study has shown that chronic i.v. administration of robalzotan (2.5 nM) also has no significant effect on any of the conscious micturition variables monitored (see Figure 4.8), despite this dose being able to significantly reduce the rate of voiding after acute (3 h) administration in conscious rats. Similarly, a subsequent 30 min i.v. infusion of robalzotan (2.5 nM) in anaesthetized rats chronically pre-treated with robalzotan was unable to significantly prevent voiding over the infusion period, as observed in vehicle pre-treated animals (see Figure 4.10). Thus confirming the presence of the 'tachyphylaxis' phenomenon in rats chronically treated with robalzotan as well as WAY-100635. Since these two 5-HT_{1A} receptor antagonists are structurally

different, yet cause a similar effect, it suggests that this phenomenon results from modulation of 5-HT_{1A} receptors, rather than being drug specific.

Interestingly, WAY-100635 and robalzotan appear to have very different profiles in cystometrograms of normal rats after 30 min i.v. infusions. Administration of WAY-100635 (10 nM) initially lengthens the time to first void after the start of the infusion, however efficient voiding still occurs and the rate of voiding gradually returns to pre-infusion values over the remainder of the infusion period (see Figure 4.11a). However, the effect of a 30 min infusion of robalzotan (2.5 nM) is much more disruptive to the regular voiding pattern, with robalzotan significantly reducing (or in a number of cases completely preventing) any functional voids throughout the duration of the infusion (see Figure 4.10) – the bladder pressure initially increases as it would for a 'normal' void and then remains artificially high which prevents efficient emptying of the bladder and results in 'dribbling' throughout the infusion period. As soon as the robalzotan infusion is switched off the regular voiding pattern returns almost immediately and efficient voiding is maintained, therefore confirming the short half-life of robalzotan. The reasons for the differences in the cystometric profile between the two ligands are unknown, however, they could be related to the differing chemical structure of the two compounds (Johansson *et al.*, 1997) which results in each compound having varying effects on other receptors, some of which could modulate the cystometric profile.

4.4.3 – Possible explanation for the 'tachyphylaxis' phenomenon

Interestingly it was noted during anaesthetized cystometry studies, that the 'tachyphylaxis' phenomenon observed towards further robalzotan challenges (2.5 nM

free plasma concentration) after chronic robalzotan dosing could be overcome if the (2.5 nM free plasma concentration) challenge dose was increased to 100 times that of the original challenge dose (i.e. to 250 nM; see Figure 4.12). This observation indicates that 5-HT_{1A} receptors are still capable of being blocked after chronic 5-HT_{1A} receptor antagonist dosing, and thus suppressing micturition, but require an increased dose of antagonist for this to occur. Therefore this suggests that the 5-HT_{1A} receptors are actually showing tolerance to the antagonist rather than desensitization/tachyphylaxis, since desensitized receptors would not respond to an increased antagonist challenge dose. However, it must be noted that this high dose of robalzotan is at the limits of the selectivity of this ligand for 5-HT_{1A} receptors, since the K_i of robalzotan for α_1-adrenoceptors is 260 ± 40 nM (Johansson *et al.*, 1997), therefore it is possible the suppression of micturition observed after 250 nM challenge of robalzotan may have been due to blockade of α_1-adrenoceptors, although this would be surprising since suppression of micturition has not been observed after administration of α_1-adrenoceptor antagonists in other studies (Conley *et al.*, 2001). Therefore, this data suggests that the phenomenon previously referred to in this thesis as a 'tachyphylaxis' phenomenon may actually be due to the 5-HT_{1A} receptors showing tolerance to further antagonist challenges, thus for the remainder of this thesis this phenomenon will be referred to as 'tolerance' rather than 'tachyphylaxis'. As mentioned previously (see 2.4.3), it is surprising for an antagonist to elicit this kind of phenomenon and further investigation is required to try and more accurately clarify the speed of the onset and a possible mechanism for this tolerance. Both these issues are addressed in chapters 5 and 6 respectively.

4.4.4 – Concluding Remarks.

The present study has confirmed that the chronic i.v. administration of the 5-HT_{1A} receptor antagonists, WAY-100635 and robalzotan, are unable to suppress micturition in conscious normal and spontaneously hypertensive rats, despite these ligands significantly reducing the rate of micturition after acute administration. It appears that this lack of effect after chronic administration may be due to 5-HT_{1A} receptors showing tolerance to further compound administration, with this tolerance phenomenon developing quickly (within at least 4 days of the start of chronic administration) and being long-lasting (for at least 6 days after the end of chronic administration). These results suggest that the chronic administration of 5-HT_{1A} receptor antagonists will not be clinically efficacious in the treatment of overactive bladder.

Chapter 5

Investigation into the effects of chronic i.v. administration of WAY-100635 in conscious rats: A radiotelemetry study.

5.1 – Introduction

The data from chapters 3 and 4 have suggested that chronic administration of the 5-HT_{1A} receptor antagonist WAY-100635 causes a tolerance phenomenon, resulting in further WAY-100635 administration having no effect on the rate of micturition. Studies in the previous chapter indicated that this tolerance phenomenon has an onset time of less than 4 days (see section 4.3.1), although restrictions due to the Animals (Scientific Procedures) Act, 1986, prevented a more accurate onset time from being determined. Observations during a study investigating the effect of a 3 h acute infusion of WAY-100635 (see section 3.3.4) as well as measurements made after repeated administration of WAY-100635 in anaesthetized rats (see section 2.3.4) also suggested the possibility of tolerance occurring over this short time period. Therefore, it is likely that the onset time of the tolerance phenomenon after chronic administration of WAY-100635 is much faster than 4 days. The aim of the present study was to further investigate the onset time for the tolerance phenomenon associated with chronic WAY-100635 administration. This was achieved through the use of VIP refillable pumps, instead of osmotic pumps, for the chronic administration of WAY-100635. Since these pumps are injectable once implanted, the animal can recover from implantation surgery, and then have the pump filled with the test compound, enabling the immediate observation of compound evoked changes in micturition. This study employed a radiotelemetry system to measure bladder activity, core body temperature and rat activity thus allowing measurements to be made in conscious, freely moving rats in their home cages.

The use of the radiotelemetry system enabled changes in the physiological parameters noted above to be measured quickly and simply and therefore a further aim of the present study was to investigate whether animals exhibiting the tolerance phenomenon after chronic WAY-100635 administration would show a differing response to the hypothermia and increased locomotor activity associated with activation of 5-HT_{1A} receptors by 8-OH-DPAT administration. This study also provided some validation into the use of radiotelemetry as a tool for measuring bladder activity and hence as a novel model for use in investigating the effects of compounds on the micturition system in freely moving conscious rats.

5. 2 – Methods

All experiments were carried out under the Animals (Scientific Procedures) Act, 1986. Due to the long lifetime of the telemetry probes and VIP injectable pumps, the animals used in the following experiments were not culled at the end of the study, instead they were used for further urological investigations.

5.2.1 – Study overview

A general overview and time line of the study is shown in Figure 5.1. 16 female normal Sprague Dawley rats (300 - 400g) were implanted subcutaneously with injectable refillable constant flow pumps (Model 69-0106, nominal flow rate 0.170 ml 24 h^{-1}; Veterinary implantable products, VIP, Texas, U.S.A) fitted with a silicone catheter placed in the jugular vein for constant i.v. administration of WAY-100635 (10nM free plasma concentration) or vehicle (saline). These rats were also implanted intraperitoneally with a radiotelemetry transmitter probe to measure bladder pressure and rat activity (n = 7; Model TA11PA-C40, Data Sciences International, MN, U.S.A.) or to measure bladder pressure, rat activity, core body temperature and heart rate from 2 lead ECG (n = 9; Model TA11-C50-PXT, Data Sciences International). The combined use of refillable injectable pumps with the measurement of particular physiological variables by radiotelemetry allowed a more accurate measurement of the onset time for the tolerance phenomenon observed after chronic WAY-100635 administration. This was achieved by monitoring the effect of chronic WAY-100635 (10 nM free plasma concentration) i.v. administration on the frequency of voiding on 4 consecutive days after the start of WAY-100635 administration (see section 5.2.1.2 for further detail).

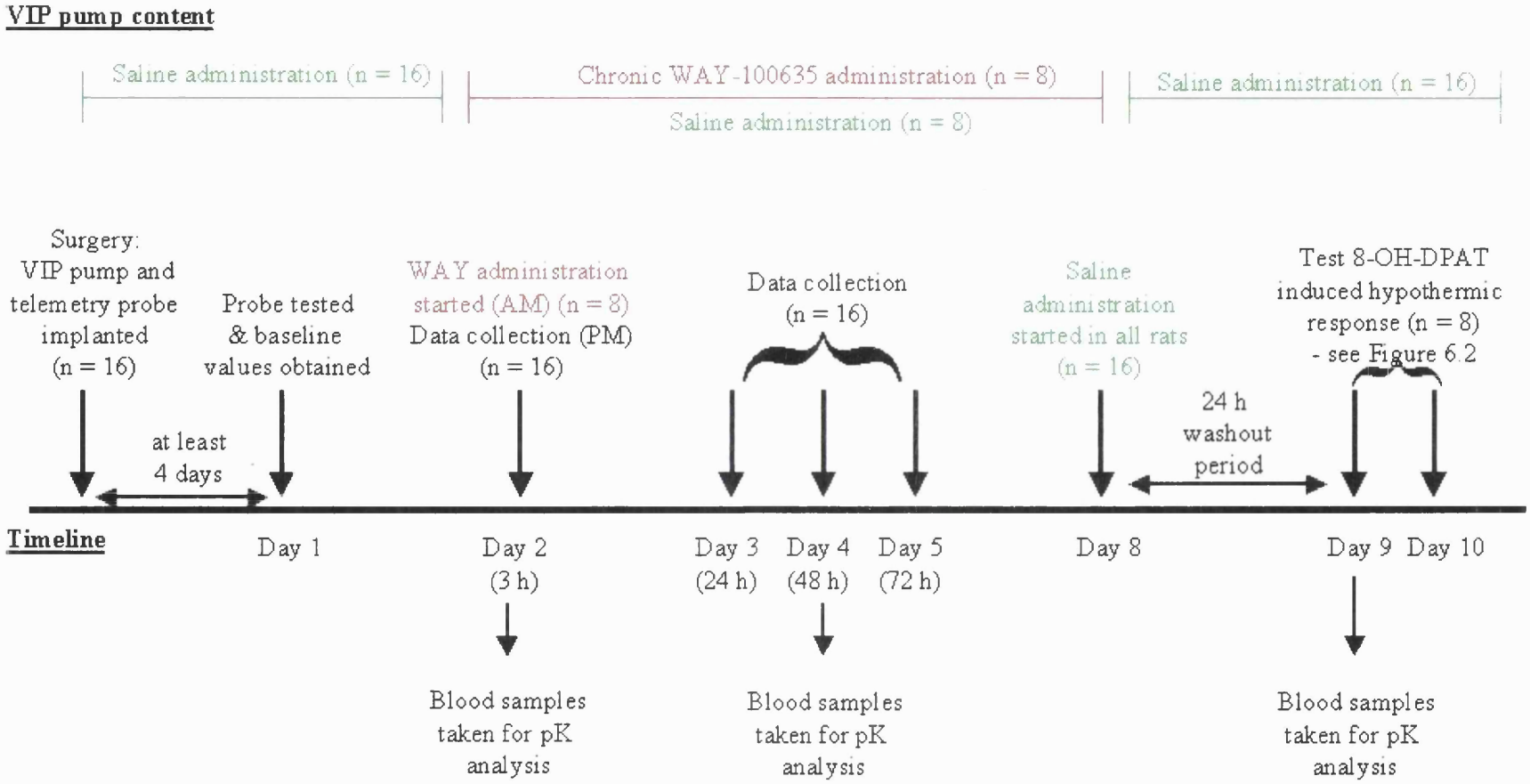


Figure 5.1 – General overview and timeline of radiotelemetry study. See text for further detail.

After a further 2 days of chronic WAY-100635 administration (6 days in total) it was stopped and followed by a 24 h wash-out period. After this wash-out period, the effects of s.c. 8-OH-DPAT on hypothermia and locomotor activity were investigated (see section 5.2.1.3 for further detail).

5.2.1.1 – Surgery: VIP implantable pump and telemetry probe implantation

All surgery was performed under aseptic conditions, using sterile instruments and with body temperature maintained by a standard homeothermic control unit and blanket. Briefly, 16 female normal Sprague Dawley rats (300 – 400g) were initially anaesthetized using 3 –5% isoflurane in oxygen in an induction chamber. Carprofen analgesia (5 mg kg^{-1}) was administered subcutaneously and ocular lubrication was applied (Lacri-Lube®, Allergan Inc, USA). After shaving and disinfecting the right ventral region of the neck and the top half of the back using dilute povidone iodine (Pevidine®, C-Vet Veterinary products, Leyland, UK), the animal was transferred to theatre and anaesthesia maintained using 2% isoflurane in oxygen. Depth of anaesthesia was assessed by respiratory parameters and by an absence of limb withdrawal in response to paw pinch. A subcutaneous pocket for the pump to be anchored in was made mid-way on the back of the animal by making an incision traverse to the backbone and posterior to the intended pump pocket location followed by blunt dissection to create the pocket. The pump was anchored in the pocket through the use of 3 non-absorbable sutures sewing the suture band of the pump to the tissue over the backbone. The right external jugular vein was then exposed by a 1cm incision and a subcutaneous tunnel was blunt dissected from the subcutaneous pump pocket to the ventral neck region where the sterile silicone catheter

connected to the VIP implantable pump was inserted into the jugular vein and tied in place. All incisions were then closed.

Immediately after VIP pump implantation, the telemetry probe (models as noted previously in section 5.2.1) was implanted through a 2-inch incision into the peritoneal cavity and sutured to the abdominal wall. The 3mm modified pressure tip connected to the probe was implanted in the bladder and secured with a purse string suture. Model TA11-C50-PXT telemetry probes (n = 9) also contained 2 ECG sensor leads, which were tunnelled from the abdominal cavity and placed in a lead II conformation across the chest just under the skin and sutured in place to the underlying muscles. All incisions were then closed and sprayed with pevidine. The rat was allowed to return to consciousness in a heated recovery box. Carprofen analgesia (5 mg kg^{-1}) was administered once daily for three days post-surgery. Post-surgery animals were housed singly and in a reverse light/dark cycle (12h:12h) so animals were always studied whilst at their most active. No sham operations were undertaken as this form of validation has been examined previously (Sgoifo *et al.*, 1996).

5.2.1.2 – Experiment to investigate the onset time for the tolerance phenomenon associated with chronic WAY-100635 administration.

After at least a 4-day recovery period from surgery, baseline values for all the variables measured by the telemetry probe were obtained (i.e. Day 1 of the study). This was achieved by placing the home cage of the rat on a receiver pad (model RPC-1, Data Sciences International, MN, U.S.A.), after the transmitter inside the animal had been switched on (by use of a magnet). The receiver pad, via two receiving antennae, detected

changes in the required variables which were converted to digital pulses and forwarded to the Dataquest ®- A.R.T. system for recording. Variables were monitored over a 3 h time period.

The following day (Day 2), chronic WAY-100635 administration (10 nM free plasma concentration) was started in 8 rats by refilling the VIP implantable pump (according to the manufacturers instructions) with the correct concentration of WAY-100635 to maintain a free plasma concentration of 10 nM with a pump infusion rate of 0.17ml 24 h^{-1} (see equation noted in section 4.2.1.1). Following a 3 h period, which was calculated to be sufficient for WAY-100635 levels to have reached the desired free plasma concentrations (confirmed using by taking blood samples and checking WAY-100635 levels) given the dead space of the catheter from the infusion pump, animals were injected intraperitoneally with furosemide (2 mg kg^{-1}) and a saline load (20 ml kg^{-1}) in a single injection to artificially increase the rate of voiding, and then monitored for 3 h by placing on the radiotelemetry receiver pad. Animals were monitored in a similar fashion (i.e. under furosemide/saline load induced diuresis) on days 3, 4 and 5, ensuring that each rats was always monitored at the same time each day. Chronic WAY-100635 i.v. administration was continued throughout this time (and up until day 8) via the VIP implantable pump, which was refilled when required via a subcutaneous injection. WAY-100635 free plasma levels were checked by measuring the levels of WAY-100635 in tail vein blood samples taken on Day 4 (see Figure 5.1).

5.2.1.3 - Experiment to investigate the effect of chronic pre-treatment with WAY-100635 on the effect of 8-OH-DPAT induced hypothermia and increases in activity.

On Day 8 of the study, chronic WAY-100635 administration was stopped by refilling the implantable pumps with saline. After a 24 h wash-out period (i.e. on Day 9 of the study), the rats implanted with model TA11-C50-PXT telemetry probes (n = 9) were used in an experiment to investigate the effect of 8-OH-DPAT (0.5 mg kg^{-1}, s.c. in the right flank) on the various variables measured by the telemetry probes. A number of different protocols were used for specific groups of rats and these are shown in Figure 5.2 a, b & c.

Tail vein blood samples were also taken on Day 9 to check for possible presence of WAY-100635 from the previous chronic administration, however given the short half-life of WAY-100635 it was expected that all the WAY-100635 would have been metabolised after a 24 h wash-out period.

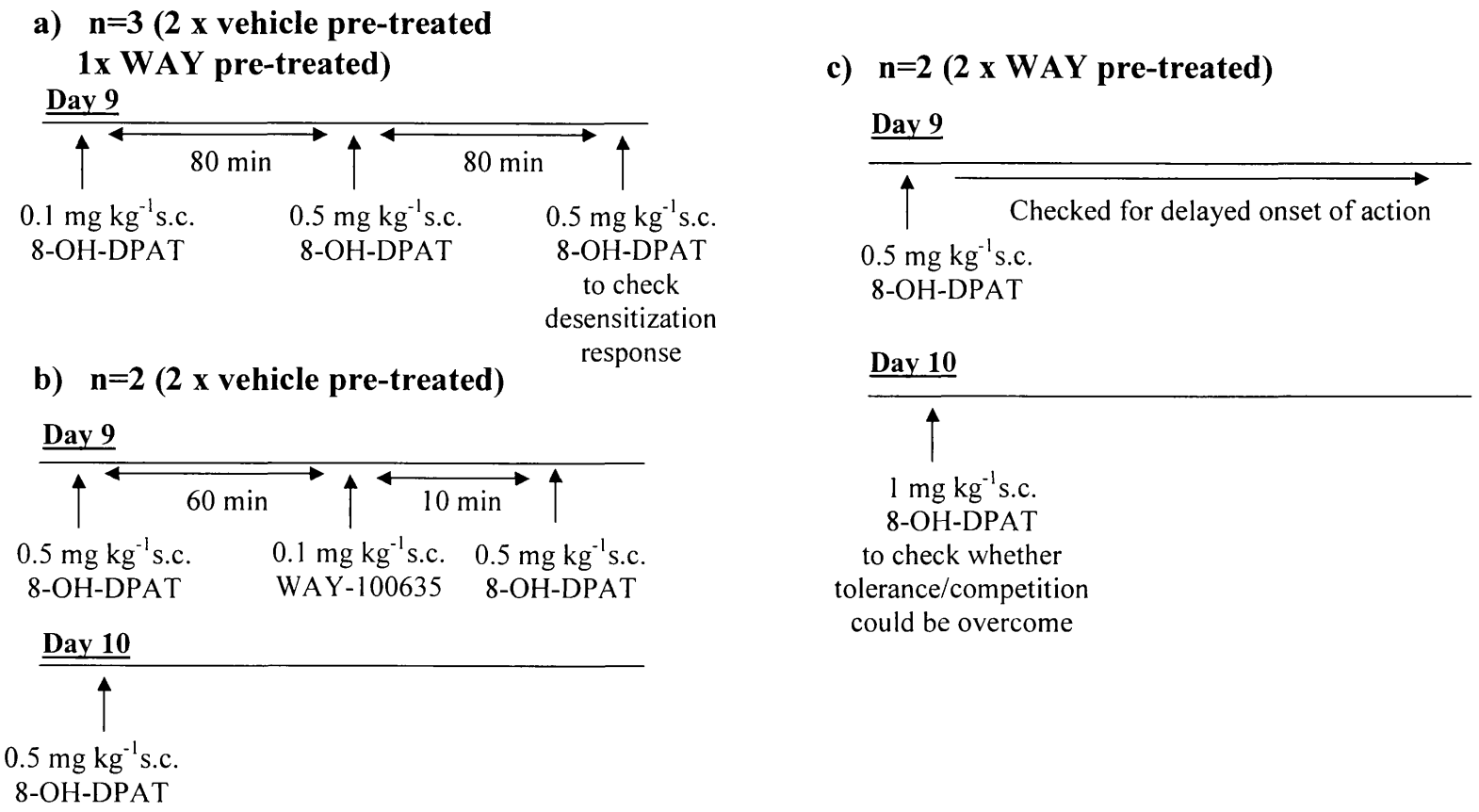


Figure 5.2 – Protocols used for different groups of rats to determine the effect of chronic WAY-100635 administration on 8-OH-DPAT induced hypothermia (n = 8). All protocols conducted on days 9 and 10 of study after previous chronic WAY-100635 administration (see Figure 5.1). **a)** Initial protocol used to determine dose of 8-OH-DPAT to be used in subsequent protocols. **b)** Protocol used to confirm whether the hypothermia observed after 8-OH-DPAT administration is mediated via 5-HT_{1A} receptor modulation (day 9) and whether hypothermic effect returns 24 h after acute WAY-100635 administration (day 10). **c)** Protocol used to confirm effect of various doses of 8-OH-DPAT (0.5 and 1 mg kg^{-1}) administration in rats after chronic pre-treatment with WAY-100635.Abbreviation: WAY, WAY-100635.

5.2.2 – Data Analysis

5.2.2.1 – Bladder activity

The number of micturition events occurring over 1 h and over 3 h from the time of furosemide/saline load administration was counted from the bladder pressure trace. Most voids were easy to identify for two reasons, firstly they often shared a common profile (similar to that observed in anaesthetized cystometry; see Figure 5.3a) consisting of a large rise in pressure due to the opening pressure, followed by a period of high frequency oscillation in bladder pressure and finally a high closing pressure (more exaggerated than that observed with anaesthetized cystometry). Secondly, most voids also shared a common pattern of activity, with the rat active immediately prior to and after the void, but still during the micturition event itself (see Figure 5.3a). However, it was observed that some increases in bladder pressure were due to increases in abdominal pressure associated with particular activities of the rat, especially when the rat reached up on its hind legs (see Figure 5.3 b), rather than changes in bladder activity. Therefore, to remove the possibility of erroneously labelling a void as an increase in bladder pressure due to activity of the rat or vice versa, the rat activity trace was superimposed on the bladder activity trace and increases in bladder pressure clearly associated with activity of the rat were not included as micturition events (see Figure 5.3b for an example).

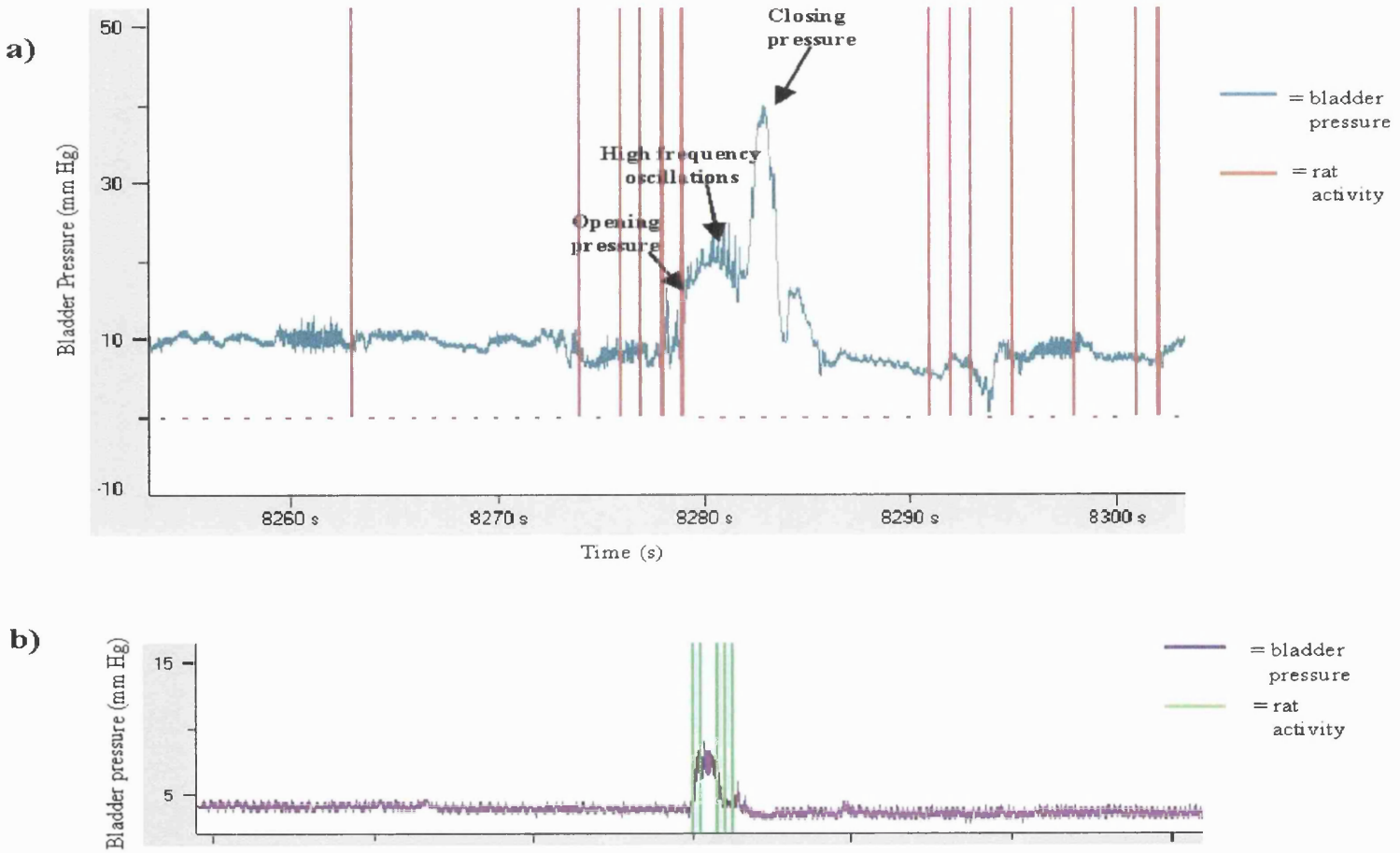


Figure 5.3 – Typical traces obtained by radiotelemetry in a conscious female rat. **a)** Bladder pressure trace obtained by radiotelemetry showing a typical micturition event – see text for more details. Also superimposed on the trace is the activity of the rat (represented as red bars) – note that the rat is completely still during the micturition event. **b)** Bladder pressure trace with activity superimposed (green bars) to show an increase in bladder pressure due to the increase in abdominal pressure associated with the activity of the rat.

5.2.2.2 – Core body temperature

The core body temperature data was only analysed in the experiments investigating the effect of 8-OH-DPAT administration s.c. in rats chronically pre-treated with WAY-100635 or vehicle. The core body temperature trace was replayed for each rat and the temperature measured at one-minute intervals for 45 min from the time of 8-OH-DPAT administration.

5.2.2.3 – Rat activity

The rat activity was only quantitatively measured in the experiments investigating the effect of 8-OH-DPAT administration in rats chronically pre-treated with WAY-100635 and vehicle. The activity counts of each rat were derived from changes in the signal strength between the transmitter and receiver as the animal moved about the cage and the changes were due to either locomotor movement or changes in orientation of the rat. The activity counts were scaled to counts per minute and the sum of the activity counts was calculated over a 30 min period from the time of 8-OH-DPAT administration.

5.2.3 – Statistical analysis

Drug evoked changes in all of the variables measured were compared to vehicle/time match controls by unpaired Student's *t*-test adjusting for unequal variances if necessary. When evaluating the effect of 8-OH-DPAT on rat activity, the 8-OH-DPAT induced changes were compared with the level of activity over 30 min prior to 8-OH-DPAT administration using paired Student's *t*-test. All values are mean ± s.e.mean and $P<0.05$ is considered significant.

5.2.4 – Drugs and solutions

Drugs and chemicals were obtained from the following sources: Isoflurane (ISOCARE™) from Animalcare Ltd., U.K.; Carprofen (Rimadyl™ Large Animal Solution) from Pfizer Ltd., Sandwich, U.K.; Furosemide from Sigma Aldrich Chemicals, Poole, Dorset, U.K.; *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)-cyclohexanecarboxamide trichloride (WAY-100635) was a gift from Pfizer Ltd., Sandwich, U.K. (±) 8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT) from Tocris Cookson, Ltd., Bristol, U.K. Furosemide was dissolved in ~ 5% PEG and 95% saline and warmed. WAY-100635 was dissolved in 0.9% wv^{-1} saline. 8-OH-DPAT was dissolved in 0.9% wv^{-1} saline and gently warmed.

5.3 – Results

5.3.1 – Effect of chronic WAY-100635 administration on bladder activity as measured by radiotelemetry.

An approximate 3 h i.v. infusion of WAY-100635 (10 nM free plasma concentration) on study day 2 (i.e. 3 h after the start of chronic WAY-100635 administration) caused a significant reduction in the number of voids over both 1 h (4.6 ± 0.8 compared to 11.0 ± 1.7) and 3 h (9.4 ± 1.1 compared to 22.3 ± 4.8) time periods after furosemide/saline load administration, when compared to the vehicle/time match control animals (n = 8; see Table 5.1). The frequency of voiding in these WAY-100635 treated animals was also reduced when compared to the baseline values obtained the previous day, although this reduction was not significantly different (see Table 5.1). However, 24 h later (on study day 3) there was no significant difference in the number of voids over a 1 h or 3 h period between the animals chronically administered with WAY-100635 and the vehicle control animals (see Table 5.1 and Figure 5.4), and when compared with the previous day (i.e. study day 2 after 3 h of WAY-100635 administration) the animals chronically administered with WAY-100635 showed a significant increase in the frequency of voiding (see Figure 5.4). The frequency of voiding in rats chronically administered with WAY-100635 continued at a similar level for the remainder of the time points measured (i.e. on study days 4 and 5; see Table 5.1).

	Vehicle pre-treated rats		*WAY-100635 pre-treated rats*	
Study Day & length of WAY-100635 administration (h)	***Number of voids over 1 h***	***Number of voids over 3 h***	***Number of voids over 1 h***	***Number of voids over 3 h***
1 (Baseline; n = 4)	6.5 ± 2.4	13.8 ± 3.8	8.8 ± 2.3	16.3 ± 3.6
2 (3 h; n = 8)	11.0 ± 1.7	22.3 ± 4.8	4.6 ± 0.8 **	9.4 ± 1.1 *
3 (24 h; n = 8)	9.6 ± 1.9	20.6 ± 4.0	8.1 ± 0.7 †	14.8 ± 1.0 ††
4 (48 h; n = 8)	6.4 ± 1.1	12.5 ± 2.1	6.0 ± 0.6 †	12.6 ± 1.7
5 (72 h; n = 8/7)	5.8 ± 0.8	13.0 ± 1.9	6.1 ± 1.5	12.3 ± 2.4

Table 5.1 – Effect of chronic i.v. WAY-100635 (10 n M free plasma concentration) and vehicle administration on the frequency of voiding in conscious rats as measured by radiotelemetry at various time points throughout administration. On Day 1 baseline values were obtained prior to WAY-100635 administration and on days 2, 3, 4 and 5 the duration of WAY-100635 administration was approximately 3, 24, 48 and 72 h respectively. All values are mean ± s.e.mean. * $P<0.05$ and ** $P<0.01$ when compared to vehicle control at that time point by an unpaired Student's *t*-test. † $P< 0.05$ and †† $P< 0.01$ when compared to values in WAY-100635 pre-treated animals 24 h previous (i.e. on the previous day) by paired Student's *t*-test.

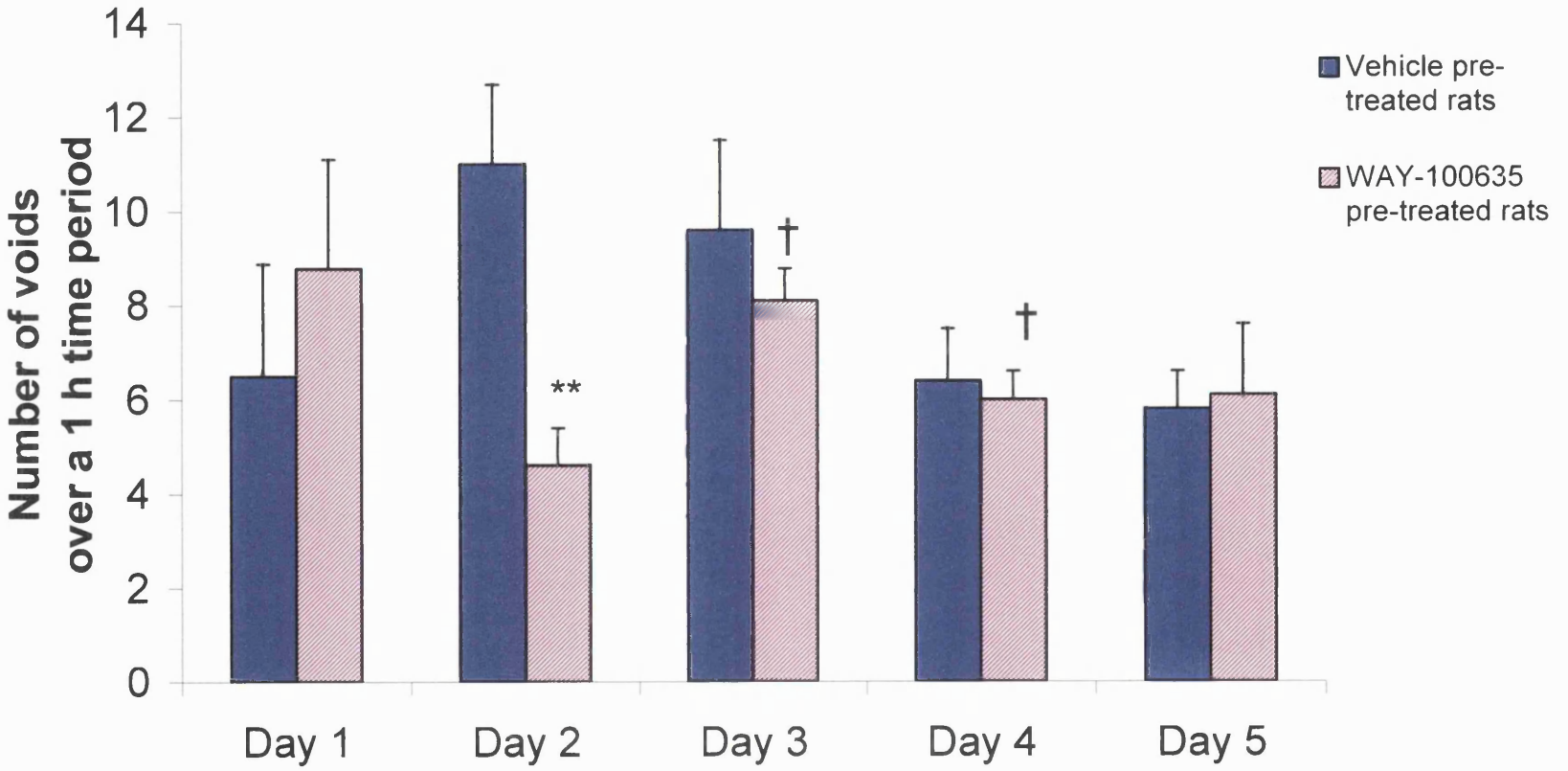


Figure 5.4 – The effect of varying durations of chronic i.v. WAY-100635 administration on the frequency of furosemide/saline load induced voiding in conscious female rats as measured by radiotelemetry over a 1 h time period after diuretic administration. On Day 1 baseline values were obtained prior to WAY-100635 administration and on days 2, 3, 4 and 5 the duration of WAY-100635 administration was approximately 3, 24, 48 and 72 h respectively. **P<0.01 when compared to vehicle control at that time point by an unpaired Student's *t*-test. † P< 0.05 when compared to values in WAY-100635 pre-treated animals 24 h previous (i.e. on the previous day) by paired Student's *t*-test.

5.3.2 – Effect of chronic WAY-100635 administration on 8-OH-DPAT-induced hypothermia in female rats as measured by radiotelemetry.

Chronic WAY-100635 administration appeared to completely block 8-OH-DPAT (0.5 mg kg^{-1}) induced hypothermia (see Figure 5.5 and Figure 5.6) with a mean core body temperature of 37.7 ± 0.2 °C in WAY-100635 pre-treated rats (n = 4) which was significantly different ($P<0.001$; unpaired Student's *t*-test) compared with 34.6 ± 0.3 °C in vehicle control animals (n = 4) 30 min after 8-OH-DPAT administration (see Table 5.2). Further experiments showed that the hypothermic effect induced by 8-OH-DPAT (0.5 mg kg^{-1}; s.c.) was reproducible after a second dose of 8-OH-DPAT (0.5 mg kg^{-1}) with a core body temperature of 34.1 ± 0.2 °C reached 30 min after the first dose and 34.3 ± 0.3 °C reached after the second dose (see Figure 5.7). In vehicle pre-treated animals (n = 2) the 8-OH-DPAT (0.5 mg kg^{-1}) induced hypothermia was blocked by WAY-100635 (0.1 mg kg^{-1}; s.c.) administration 10 min prior to the 8-OH-DPAT challenge (see Figure 5.8). Interestingly, it also appeared that even increasing the dose of the 8-OH-DPAT challenge to 1 mg kg^{-1} s.c. in WAY-100635 pre-treated animals did not result in a drop in temperature of even 50% of that observed after a 0.5 mg kg^{-1} 8-OH-DPAT in vehicle pre-treated animals (see Table 5.3 and Figure 5.9), although it must be noted that the core body temperature following the 1 mg kg^{-1} 8-OH-DPAT challenge in WAY-100635 pre-treated animals was significantly lower (at time points 35, 40 and 45 min after 8-OH-DPAT administration) than that observed after a 0.5 mg kg^{-1} 8-OH-DPAT challenge in the same animals (see Table 5.3). The onset time for this 8-OH-DPAT induced hypothermic effect in WAY-100635 pre-treated animals was longer than that observed with vehicle pre-treated animals, with the drop in core

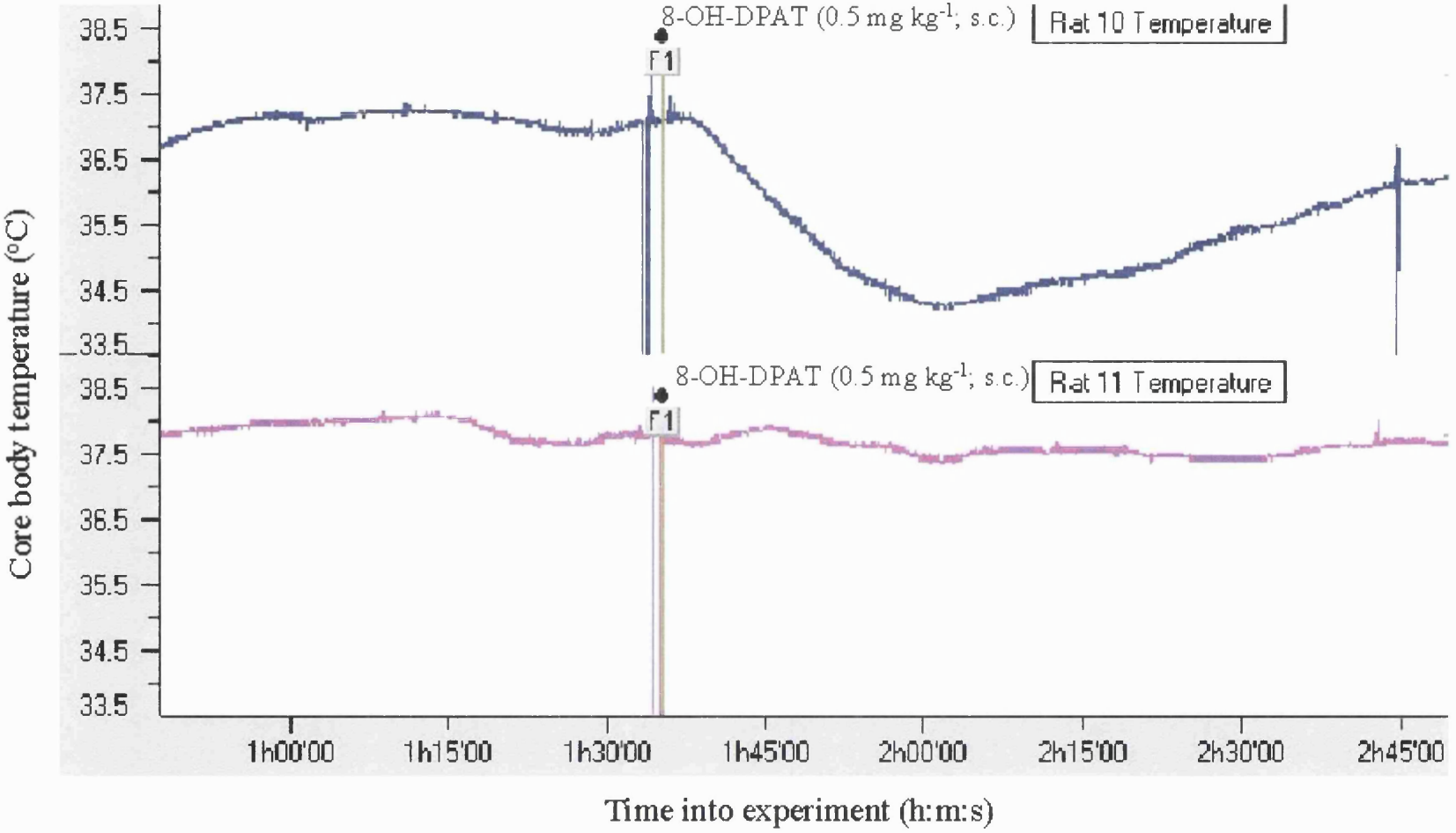


Figure 5.5 – A typical radiotelemetry trace showing the effect of 7 day chronic WAY-100635 (10 nM free plasma concentration; Rat 11 - bottom trace) and vehicle (Rat 10 - top trace) administration on 8-OH-DPAT (0.5 mg kg^{-1}) induced hypothermia as determined by radiotelemetric measurement of the core body temperature in conscious freely moving female rats.

Time after 8-OH-DPAT administration (min)	*Core body temperature (^{0}C) in vehicle pre-treated rats (n = 4)*	*Core body temperature (^{0}C) in WAY-100635 pre-treated rats (n = 4)*
0	37.2 ± 0.1	38.1 ± 0.1 **
5	36.8 ± 0.2	38.1 ± 0.2 **
10	36.0 ± 0.2	38.2 ± 0.2 ***
15	35.2 ± 0.3	38.2 ± 0.2 ***
20	34.8 ± 0.3	38.0 ± 0.2 ***
25	34.6 ± 0.4	37.8 ± 0.2 ***
30	34.6 ± 0.3	37.7 ± 0.2 ***
35	34.7 ± 0.3	37.7 ± 0.2 ***
40	34.9 ± 0.3	37.7 ± 0.2 ***
45	35.1 ± 0.4	37.7 ± 0.2 ***

Table 5.2 – The effect of 7-day chronic WAY-100635 (10 nM free plasma concentration) treatment on 8-OH-DPAT (0.5 mg kg^{-1}) induced hypothermia as determined by radiotelemetric measurement of the core body temperature in conscious freely moving female rats. All values are mean ± s.e.mean. **P<0.01 and *** P<0.001 when compared to vehicle control at those time points by an unpaired Student's *t*-test.

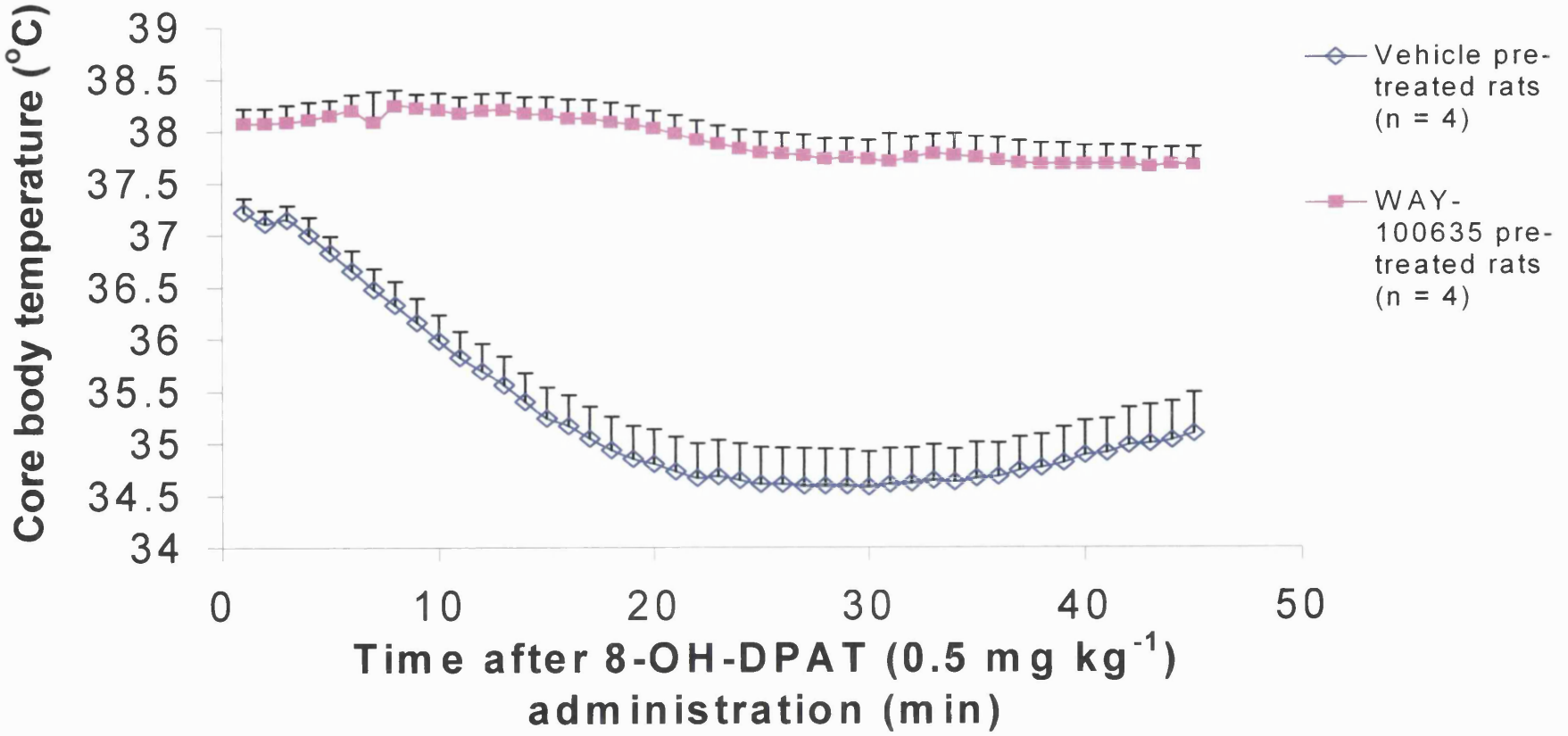


Figure 5.6 – Graphical representation of the effect of 7 day chronic WAY-100635 (10 nM free plasma concentration; n = 4) and vehicle (n = 4) administration on 8-OH-DPAT (0.5 mg kg^{-1}) induced hypothermia as determined by radiotelemetric measurement of the core body temperature in conscious freely moving female rats. At all time points measured the core body temperature in rats pre-treated with WAY-100635 was significantly higher than the vehicle control (using unpaired Student's *t*-test) – not shown on graph.

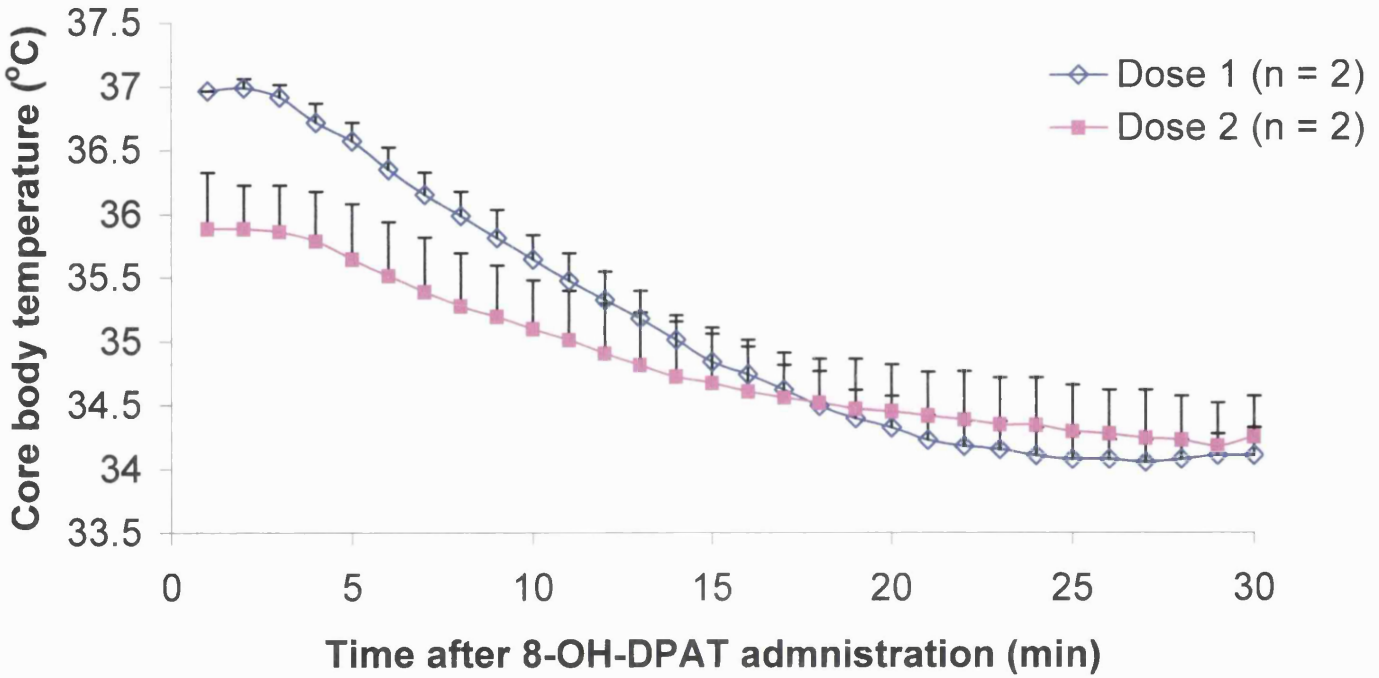


Figure 5.7 – The effect of repeat administration of 8-OH-DPAT (0.5 mg kg^{-1}; s.c.) on core body temperature in conscious freely moving female rats (n = 2; vehicle pre-treated) as measured by radiotelemetry.

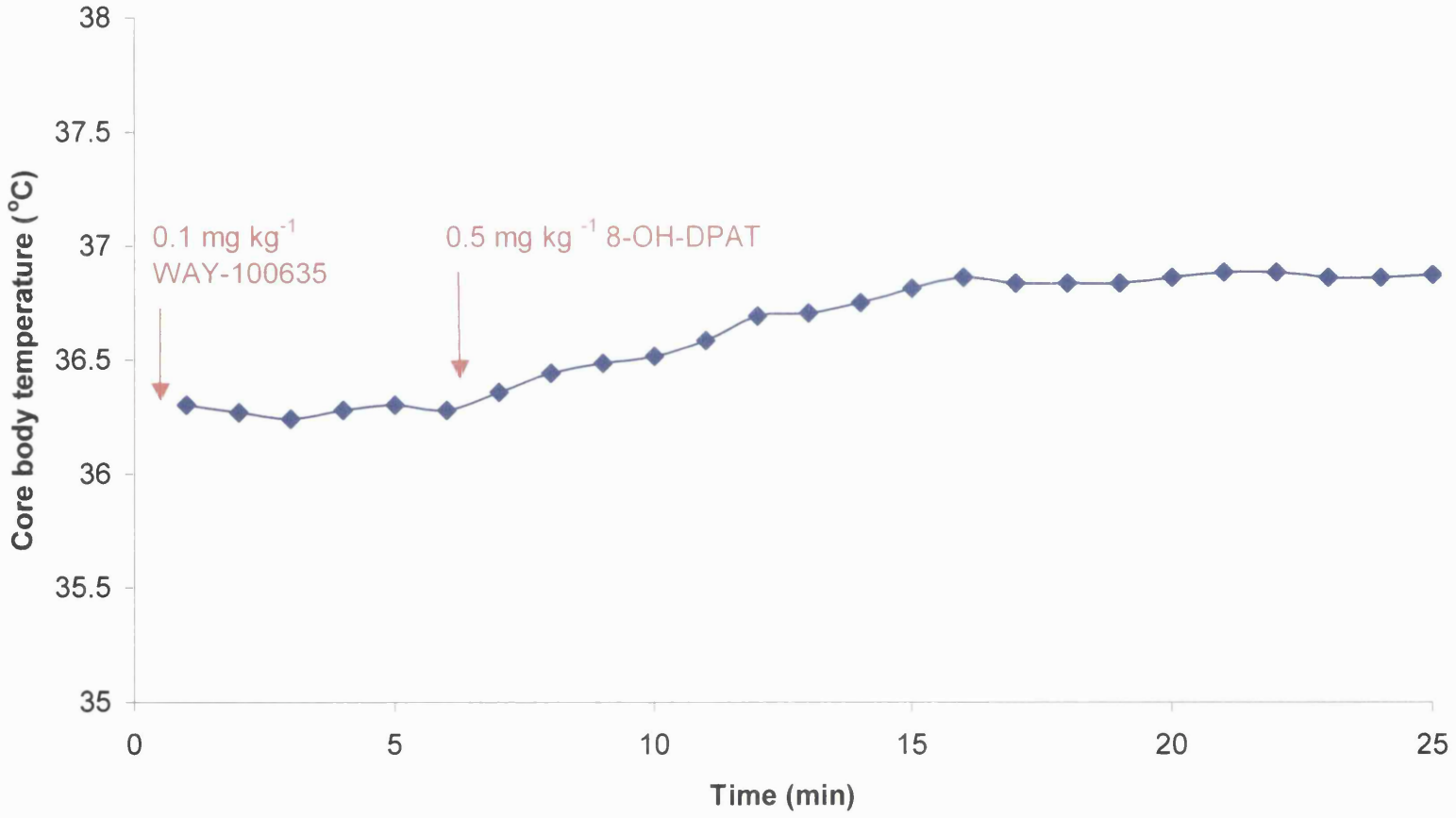


Figure 5.8 – 8-OH-DPAT (0.5 mg kg^{-1}) induced hypothermia was blocked by administration of WAY-100635 (0.1 mg kg^{-1}) 10 min prior to the 8-OH-DPAT challenge in vehicle pre-treated conscious freely moving female rats (n = 2), as determined by radiotelemetric measurement of the core body temperature (°C).

	WAY-100635 pre-treated rats (n = 3)		*Vehicle pre-treated rats (n = 4)*
Time after 8-OH-DPAT administration (min)	***Core body temperature (°C) after 0.5 mg kg^{-1} 8-OH-DPAT challenge***	***Core body temperature (°C) after 1 mg kg^{-1} 8-OH-DPAT challenge***	***Core body temperature (°C) after 0.5 mg kg^{-1} 8-OH-DPAT challenge***
0	38.2 ± 0.0	37.8 ± 0.1 †	37.2 ± 0.1
5	38.3 ± 0.0	37.9 ± 0..1 ††	36.8 ± 0.2
10	38.3 ± 0.2	38.0 ± 0.1 ††	36.0 ± 0.2
15	38.3 ± 0.1	37.8 ± 0.2 ††	35.2 ± 0.3
20	38.2 ± 0.1	37.4 ± 0.3 ††	34.8 ± 0.3
25	38.0 ± 0.2	37.1 ± 0.3 ††	34.6 ± 0.4
30	37.8 ± 0.2	36.9 ± 0.3 ††	34.6 ± 0.3
35	37.8 ± 0.3	36.8 ± 0.3 * ††	34.6 ± 0.3
40	37.7 ± 0.2	36.6 ± 0.3 ** †	34.9 ± 0.3
45	37.7 ± 0.2	36.7 ± 0.3 ** †	35.1 ± 0.4

Table 5.3 – The effect of 8-OH-DPAT (0.5 and 1 mg kg^{-1} s.c.) on core body temperature of conscious freely moving female rats pre-treated with 7-day chronic i.v. administration of WAY-100635 (10 nM free plasma concentration; n = 3) or vehicle (saline; n = 4). All values are mean ± s.e.mean. *P < 0.05 and **P < 0.01 when compared by a paired Student's *t*-test to the core body temperature at the same time point after 0.5 mg kg^{-1} 8-OH-DPAT in WAY-100635 pre-treated animals. †P < 0.05 and †† P < 0.01 when compared by unpaired Student's *t*- test to the core body temperature at the same time point after 0.5 mg kg^{-1} 8-OH-DPAT in vehicle pre-treated animals.

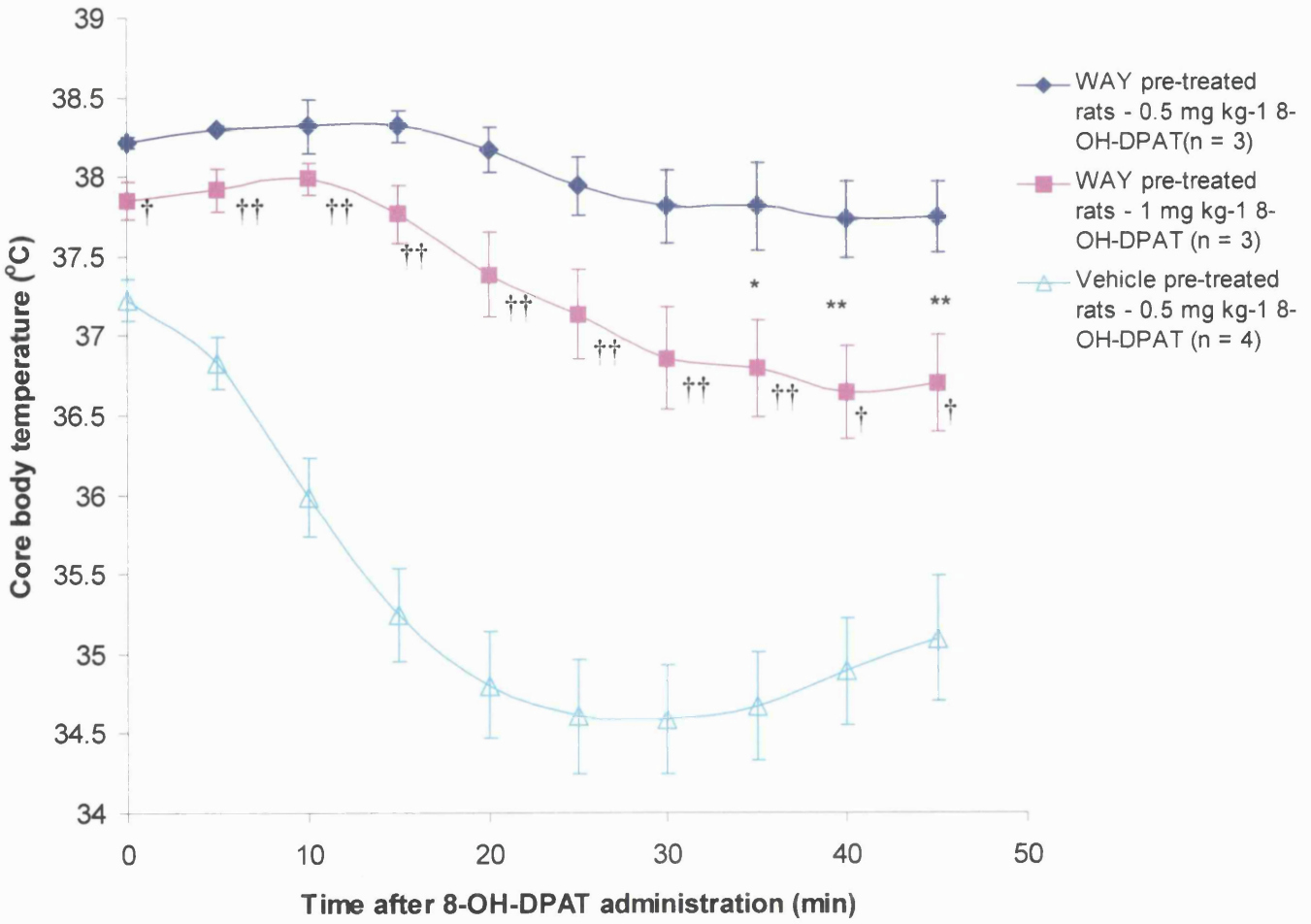


Figure 5.9 – Graphical representation of the effect of 8-OH-DPAT (0.5 and 1 mg kg^{-1} s.c.) on core body temperature of conscious freely moving female rats pre-treated with 7-day chronic i.v. administration of WAY-100635 (10 nM free plasma concentration; n = 3) or vehicle (saline; n = 4). *P < 0.05 and **P < 0.01 when compared by a paired Student's *t*-test to the core body temperature at the same time point after 0.5 mg kg^{-1} 8-OH-DPAT in WAY-100635 pre-treated animals. †P < 0.05 and †† P < 0.01 when compared by unpaired Student's *t*- test to the core body temperature at the same time point after 0.5 mg kg^{-1} 8-OH-DPAT in vehicle pre-treated animals.

body temperature only becoming apparent at least 10 min after 8-OH-DPAT administration, as opposed to an immediate drop in temperature in vehicle pre-treated animals (see Figure 5.9).

8-OH-DPAT (0.5 mg kg^{-1}, s.c.) also caused a significant increase in the level of activity of the rat in both vehicle (n = 4) and WAY-100635 (n = 4) pre-treated animals when compared to activity levels prior to 8-OH-DPAT administration in each group of rats (5855 ± 2538 to 40388 ± 2412 activity counts and 4264 ± 1219 to 22446 ± 2132 activity counts respectively; see Figure 5.10). However, it must be noted that in WAY-100635 pre-treated rats the increase in activity associated with 8-OH-DPAT administration was significantly lower than that observed in vehicle pre-treated rats ($P < 0.01$; unpaired Student's *t*-test).

5.3.3 – Plasma levels of WAY-100635 at various time points throughout the study

The average free plasma concentration for WAY-100635 during the period of chronic administration was 11.00 ± 1.84 nM assuming 80% plasma protein binding (n = 8; calculated from blood samples taken on Day 2 and Day 4 of the chronic administration period, see Figure 5.1), thus confirming that the VIP refillable pumps were functioning correctly. However, on study day 9 (i.e. 24 h after WAY-100635 administration had been stopped and replaced with saline administration) the free plasma concentration of WAY-100635 was 9.45 ± 1.43 nM (n = 6) thus suggesting that WAY-100635 levels still remained at the level observed during chronic administration despite the 24 h wash-out period.

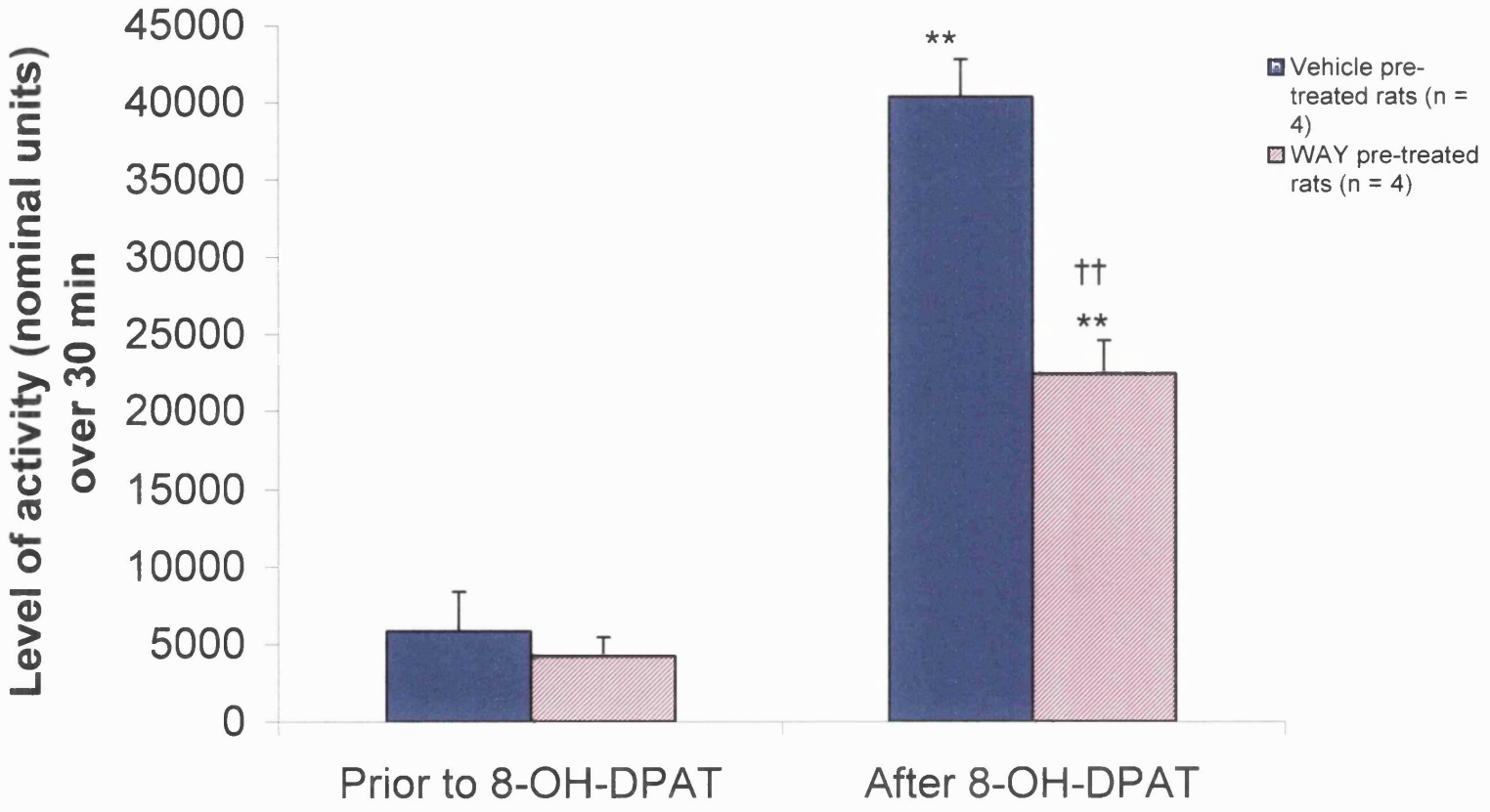


Figure 5.10 – Effect of 8-OH-DPAT (0.5 mg kg^{-1}) on the activity level of conscious freely moving female rats pre-treated with either vehicle (saline; n = 4) or WAY-100635 (10 nM free plasma concentration; n = 4). **P < 0.01 when compared to activity level prior to 8-OH-DPAT administration (paired Student's *t*-test). †† P < 0.01 when compared to activity level after 8-OH-DPAT administration in vehicle pre-treated rats (unpaired Student's *t*-test).

5.4 - Discussion

The results from this study have shown that chronic WAY-100635 administration (10 nM free plasma concentration) can initially (i.e. after 3 h of administration) suppress micturition and reduce the frequency of voiding (see Figure 5.4). However, within 24 h of the start of chronic WAY-100635 administration, this suppression of micturition is no longer observed (see Figure 5.4), thus suggesting the development of tolerance to further WAY-100635 administration, with an onset time for this phenomenon of less than 24 h. Further experiments also showed that chronic pre-treatment with WAY-100635 appears to block 8-OH-DPAT (0.5 mg kg^{-1}) induced hypothermia and attenuate 8-OH-DPAT induced increases in locomotor activity. This blockade of 8-OH-DPAT induced hypothermia in WAY-100635 pre-treated animals could not be overcome by increasing the dose of 8-OH-DPAT (1 mg kg^{-1}).

5.4.1 – Onset time of tolerance during chronic WAY-100635 administration.

In the present study, the observation that tolerance develops within 24 h of the start of chronic WAY-100635 administration provides further evidence, in a more physiological model, that long-term treatment with WAY-100635 is unable to suppress micturition and therefore suggests that blocking 5-HT_{1A} receptors is an unsuitable target to treat OAB. Unfortunately due to a restriction in the number of telemetry receiver pads, each rat could only be tested once per day at a 24 h interval (each rat was tested at the same time each day to reduce any circadian rhythm effects), thus the onset time for the tolerance phenomenon could not be assessed more accurately than within 24 h of the start of

administration. However, this data confirms that the onset of the tolerance phenomenon is fast and corroborates the lack of effect of chronic WAY-100635 administration observed on the micturition variables of conscious rats in the osmotic pump and metabole study in chapter 4. The speed of onset for this tolerance phenomenon is perhaps surprising given that the clinical tolerance observed with long-term administration of many drugs, such as ranitidine and famotidine (H_2 receptor antagonists/inverse agonists), metoprolol and carvedilol (β_2-adrenoceptor antagonists/inverse agonists) and naloxone (a δ and μ opioid inverse agonist) have been shown to develop within 14 days (Komazawa *et al.*, 2003), 7 days (Morris *et al.*, 1988) and 2-4 months (see Bond *et al.*, 2003) respectively. However, it is possible that the actual time of onset may be faster than the times chosen for observation in these studies, and further investigation measuring the development of tolerance immediately after compound administration is required to obtain more accurate estimations of the time of onset for tolerance. Interestingly studies investigating the onset of desensitization due to receptor downregulation have shown that 24 h exposure of human lung mast cells to the β_2-adrenoceptor agonists salbutamol and terbutaline is a sufficient length of exposure to result in desensitization due to a decrease in the β_2-adrenoceptor density in the cell membranes (Chong *et al.*, 2003), therefore suggesting the a similar time of onset for the tolerance phenomenon in the present study may be feasible. It must also be remembered that tolerance to repeated administration of WAY-100635 in anaesthetized rats also appears to have a particularly fast onset time, with attenuation of the suppression of micturition observed after 12 min (i.c.v. administration) or 30 min (i.t. administration; see Chapter 2), thus providing further evidence for a fast onset for the tolerance phenomenon (see Chapter 7 for further discussion).

5.4.2 – Effect of chronic WAY-100635 pre-treatment on 8-OH-DPAT induced hypothermia and increases in locomotor activity.

8-OH-DPAT (0.5 mg kg^{-1}) administration in vehicle pre-treated animals resulted in significant drop in core body temperature which peaked at approximately 30 min postinjection as noted previously in the literature (Hjorth, 1985; Bill *et al.*, 1991; Deveney *et al.*, 1998; Li *et al.*, 1999). Since 8-OH-DPAT is also known to show affinity for 5-HT_7 receptors (Hoyer *et al.*, 1994; To *et al.*, 1995; Wood *et al.*, 2000) as well as 5-HT_{1A} receptors (Middlemiss *et al.*, 1983) it is possible that the observed hypothermic effect could be mediated via both receptor subtypes. However, in the present study it was shown that the hypothermia was blocked by administration of WAY-100635 (0.1 mg kg^{-1}; s.c.) prior to 8-OH-DPAT administration indicating that the effect was mediated solely via activation of 5-HT_{1A} receptors since WAY-100635 has little affinity for the 5-HT_7 receptor (over 74-fold selectivity over 5-HT_{1A} receptors (Forster *et al.*, 1995)). This is in agreement with the literature (Zuideveld *et al.*, 2002). The protocol from the present study led to the effect of WAY-100635 administration prior to the 8-OH-DPAT challenge being tested in animals which had already received one 8-OH-DPAT dose (0.5 mg kg^{-1}), therefore it was possible that the lack of effect after WAY-100635 administration was due to a desensitization of the 5-HT_{1A} receptors to 8-OH-DPAT rather than blockade by WAY-100635. However, this possibility can be excluded since it was shown that the hypothermic effect in response to a repeated dose of 8-OH-DPAT (0.5 mg kg^{-1}) was not attenuated when compared to the initial dose (see Figure 5.7).

In rats pre-treated with WAY-100635, the hypothermia induced by 8-OH-DPAT was completely blocked and there was no drop in core body temperature in these animals (see Figure 5.6). This observation was unexpected, especially since it was possible that if the animals had developed tolerance to WAY-100635 due to an increase in the level of 5-HT_{1A} receptors (see Chapter 6), there would be an increased sensitivity to 8-OH-DPAT. However, despite a 24 h wash-out period, the average plasma concentration of WAY-100635 in the WAY-100635 pre-treated animals was 9.5 nM. Therefore the blockade of 8-OH-DPAT induced hypothermia in WAY-100635 pre-treated animals can be explained by simple competition for the 5-HT_{1A} receptors between residual WAY-100635 and the 8-OH-DPAT challenge. This hypothesis is further supported by the observation that a higher dose of 8-OH-DPAT (1 mg kg^{-1}) was able to lower the core body temperature further than a 0.5 mg kg^{-1} dose (see Figure 5.9), although still not to the same extent as observed with the lower dose of 8-OH-DPAT in vehicle pre-treated animals. The fact that circulating levels of WAY-100635 were still present, and at such high concentrations, despite a 24 h wash-out period was surprising since the half life of WAY-100635 was thought to be very short (approximately 12-15 min; Pfizer Ltd., personal communication). The reason for the apparent lack of metabolism of WAY-100635 cannot be explained, but suggests that the pharmacodynamics of the metabolism of WAY-100635 may alter after chronic administration. The length of time taken to calculate the plasma levels of WAY-100635 resulted in the data only being obtained after the completion of the radiotelemetry study and therefore the author was unaware that WAY-100635 was still present in the system when the investigation into the effects of 8-OH-DPAT was being conducted. Unfortunately, the author was unable to repeat the 8-OH-DPAT experiments in the

WAY-100635 pre-treated animals once WAY-100635 had been completely metabolised, and it therefore still remains to investigate whether the tolerance caused by pre-treatment with WAY-100635 will alter the response to 8-OH-DPAT.

The data from the present study clearly shows that 8-OH-DPAT (0.5 mg kg^{-1}) causes an increase in the activity of the rat in vehicle pre-treated animals (see Figure 5.10). In rats pre-treated with WAY-100635 the activity level after 8-OH-DPAT administration was significantly lower than that observed in vehicle control animals. Given the now realised presence of competing WAY-100635 in these animals this observation is to be expected, however it is surprising that the activity level was still significantly higher than prior to the 8-OH-DPAT challenge. This may suggest that the 8-OH-DPAT induced increase in activity is not solely mediated via $5\text{-}HT_{1A}$ receptors, since the presence of WAY-100635 (in the WAY-100635 pre-treated animals) appears to only block 47% of the increase in activity, indicating that the remaining increase may be due to activation of another receptor type. Several microdialysis studies have shown that 8-OH-DPAT increases brain extracellular levels of noradrenaline in the conscious rat in a dose dependent manner (Done *et al.*, 1994; Chen *et al.*, 1995; Suzuki *et al.*, 1995; Hajos-Korcsok *et al.*, 1996) and it has also been shown that WAY-100635 is unable to block the increase in noradrenaline induced by higher doses of 8-OH-DPAT (1 mg kg^{-1}; s.c.) although WAY-100635 did abolish the response to a lower dose of 8-OH-DPAT (0.1 mg kg^{-1}; (Hajos-Korcsok *et al.*, 1996). Increases in extracellular noradrenaline levels, for example by repeated administration of reboxetine (a NA reuptake inhibitor), have been shown to increase exploratory behaviour and potentiate 8-OH-DPAT induced locomotor activity (Rogoz *et al.*, 2002) and stimulation of α_1 and α_2-adrenergic heteroreceptors has also

been shown to amplify dopamine mediated locomotor activity (Villegier *et al.*, 2003). Since 8-OH-DPAT has also been shown to increase dopamine neuronal-firing and stimulate the release of dopamine in the rat cortex (Arborelius *et al.*, 1993) it is possible that the increase in locomotor activity observed after 8-OH-DPAT administration in WAY-100635 pre-treated rats could be mediated via increases in noradrenaline or dopamine. However, it must be noted Hajós-Korcsok *et al.* (1996) did not observe any increase in locomotor activity after 8-OH-DPAT administration in rats acutely pre-treated with WAY-100635. Reasons for these apparently contradicting results could be due to technical differences in recording the activity levels since Hajós-Korcsok *et al.* (1996) used an activity box which would not include rearing of the animals in the activity level, whereas the present study used a telemetric receiver which determines 'activity' as a change in the vectoral orientation of the probe inside the animal with regard to the receiver, therefore including rearing in the activity count of the animal may account for the increase in activity observed in the present study. A further reason for the contradiction with Hajós-Korcsok *et al.* (1996) maybe due to the chronic nature of WAY-100635 pre-treatment in the present study, which may have resulted in the development of a long term adaptation of the noradrenergic system, such as sensitization of α-adrenoceptors, which could account for the increase in activity after 8-OH-DPAT administration being observed in the present study but not by Hajós-Korcsok *et al.* after acute WAY-100635 administration.

5.4.3 – Radiotelemetry – a novel model for urological investigation?

To the author's knowledge the present study is the first example of radiotelemetered monitoring of bladder pressure in small laboratory animals, although similar systems

have been used to measure bladder pressure/activity in pigs (Mills *et al.*, 2000), rhesus monkeys (Ghoniem *et al.*, 1996; Ghoniem *et al.*, 1997) and humans (e.g.Yeung, 1998). Despite the present study failing to provide a complete and accurate validation for this telemetry model by directly comparing results with those obtained from other more conventional rat urodynamic studies, it has proven that observation of bladder activity in conscious freely moving rats can be reliably achieved using a radiotelemetry system. Micturition events were usually easily identifiable, especially due to the common pattern of activity of the rat prior to, and after the void (see Figure 5.3), and in animals showing slight bladder hyperactivity superimposing the activity trace on the bladder trace aided in identification of "true" micturition events. Mills *et al.* (2000) provided a thorough validation for the use of radiotelemetry to measure cystometric bladder pressure in conscious freely moving pigs, thus providing further evidence to support the use of this model in urodynamic investigations. The use of radiotelemetry to measure blood pressure is not novel and a number of studies have confirmed that the radiotelemetric system is capable of providing reliable, accurate long-term (days to months depending on the battery life of the implanted transmitter probe) measurement in rats, which can be validated when compared to the more conventional methods of measuring blood pressure in the rat such as indwelling catheters and tail cuff (Brockway *et al.*, 1991; Guiol *et al.*, 1992; Brooks *et al.*, 1996; Hess *et al.*, 1996; Deveney *et al.*, 1998; Van Vliet *et al.*, 2000). Further validation of the radiotelemetry system includes pharmacological validation of the measurement of core body temperature by comparing 8-OH-DPAT induced hypothermia in telemetry rats with rectal temperatures obtained from normal rats (Deveney *et al.*, 1998). Deveney *et al.* also showed a good correlation between the activity counts from an automatic activity box and those from a telemetric probe.

A principal advantage of the radiotelemetered pressure monitoring system is its ability to obtain continuous, high-fidelity recordings in conscious, unrestrained animals in their home cage. This reduces the level of stress experienced by the animal resulting in the collection of meaningful data under more physiological conditions. An example of this can be shown by comparing the body temperature in normal saline treated control rats in a study by Hjorth (1985) who measured the rectal temperature with the temperatures measured via telemetry by Deveney *et al.* (1998). In the study of Hjorth the body temperature of the saline treated control rats stayed at 38.8°C throughout the study, whereas in the Deveney study the temperature was initially raised to the same level but fell throughout a 1 h period towards the normal temperature of the animals at rest. This probably reflects the restraint necessary in the Hjorth study to take the rectal temperature, whereas in the telemetry study only one handling was required to administer the injection (which accounts for the initial rise in temperature) but for the remainder of the experiment the rat was not disturbed and its temperature fell to rest levels, thus highlighting the effect of stress induced by handling on the body temperature of rats. Although potential stress was minimized in this study, it is unlikely that the rats were completely unstressed since it is possible that lower urinary tract function might have been slightly affected by the perivesical dissection, vesical and peritoneal catheters, and the sutures in the wall of the bladder.

A clear advantage of the telemetry system for the measurement of bladder pressure in conscious rats over the more conventional cystometry studies is the ability to obtain pressure readings without infusing saline into the bladder, hence enabling measurement of natural filling of the bladder. This is much more physiological since it has been shown

that the more rapid stretching of the detrusor smooth muscle during artificial bladder filling can negatively affect its contractility and can activate a reflex sympathetic inhibition which decreases parasympathetic ganglionic transmission (see Mills *et al.*, 2000). It is also possible that detrusor contractility may be promoted by sensory information arising from the ureter or uretovesical junction, which is absent during the artificial filling required for cystometrical recording. One disadvantage of the continuous ambulatory telemetry model, compared to usual conscious cystometry studies, is that urine collection and measurement of flow rate is impossible due to the freely moving nature of the animals in their home cage.

A further advantage of the radiotelemetry model is that it permits prolonged monitoring of experimental rats thus allowing the assessment of the long-term effects of drugs on the urological system for longer than conventional methods. Furthermore, use of the implantable radiotelemetry device could minimize the number of animals required, since it can extend the period over which an individual animal can be studied, and the long-term patency of the catheter is improved thus reducing the morbidity associated with conventional urodynamic line maintenance. Associated with this, is the much reduced day-to-day maintenance, of telemetered animals compared with conventional cystometry animals.

5.4.4 – Concluding remarks

The data from the present study has confirmed that chronic i.v. administration of WAY-100635 is ineffective at suppressing micturition owing to tolerance to the compound quickly developing within 24 h from the onset of chronic administration.

Observations from this study have also suggested that the metabolism of WAY-100635 after chronic administration may differ from that after acute administration. Finally, this study has provided support for the use of radiotelemetry as a novel model to measure urodynamic variables in rats.

Chapter 6

Quantitative autoradiographical analysis of supraspinal 5-HT receptor and 5-HT transporter sites after chronic 5-HT_{1A} receptor antagonist administration.

6.1 - Introduction

The data described in the previous two chapters has indicated that 5-HT_{1A} receptor antagonists are unable to suppress micturition after chronic administration. This phenomenon appears to have a fast onset time in addition to being very persistent, given that it is still present at least 6 days after chronic administration has stopped. There are a number of possible explanations for this surprising phenomenon, one of which is the possibility that chronic administration of 5-HT_{1A} receptor antagonists may alter the number of functional 5-HT_{1A} receptors in the brain. The aim of the present study is to use quantitative autoradiography to measure the binding of [^{3}H]WAY-100635 to 5-HT_{1A} receptors in the brains of rats which have been chronically treated with WAY-100635 (10nM) or vehicle (saline).

Recent studies with knock out mice lacking particular 5-HT receptors/transporters involved in 5-HT signalling, have shown significant compensatory changes in various other elements involved in the 5-HT signalling system (Fabre *et al.*, 2000; Li *et al.*, 2000; Ase *et al.*, 2001; Ase *et al.*, 2002; Evrard *et al.*, 2002). Therefore, it may be possible that chronic pharmacological modification of 5-HT_{1A} receptors may also lead to compensatory changes in other receptors/transporters involved in the 5-HT signalling system. The present study also aims to further investigate this possibility by measuring any changes in 5-$HT_{1B/1D}$ receptor binding of [^{3}H]GR-125743 and 5-HTT binding of [^{3}H]citalopram in brains from rats chronically pre-treated with WAY-100635 (10 nM) or vehicle (saline).

Previous experiments have also suggested that the 'tolerance' phenomenon associated with chronic administration of 5-HT_{1A} receptors persists even after administration has stopped. Therefore, this present study will also investigate whether changes in the level of radioligand binding remain after chronic administration has stopped, by comparing levels from during and after chronic WAY-100635 administration.

6.2 – Methods

All experiments were carried out under the Animals (Scientific Procedures) Act, 1986. Immediately prior to brain removal, animals were killed by an overdose of pentobarbitone sodium (i.v.).

6.2.1 – General preparation

6.2.1.1 – Animal dosing

The rat brains used in this autoradiographic analysis were removed from the normal Sprague Dawley rats previously used in the i.v. osmotic pump study investigating the effect of chronic WAY-100635 (10 nM) on conscious and anaesthetized micturition variables (as previously described in chapter 4 - see section 4.2.1). The 10 rats underwent constant chronic i.v. administration of WAY-100635 (as previously described – see section 4.2.1.1) and 5 were killed on study day 11 (i.e. after 11 days administration of WAY-100635) and the remaining 5 were killed on study day 20 (i.e. after 14 days administration plus a six day wash-out period; see Figure 6.1). The same timeline was used for the 10 rats that had been chronically administered with vehicle (saline). Unfortunately due to problems during the freezing/sectioning of the brains, a few brains were unable to be used for autoradiographical analysis. Therefore, the actual number of brains used for each part of the study is as follows: Day 11, WAY-100635 (n = 3) and vehicle (n = 4) and for Day 20, WAY-100635 (n = 3) and vehicle (n = 4).

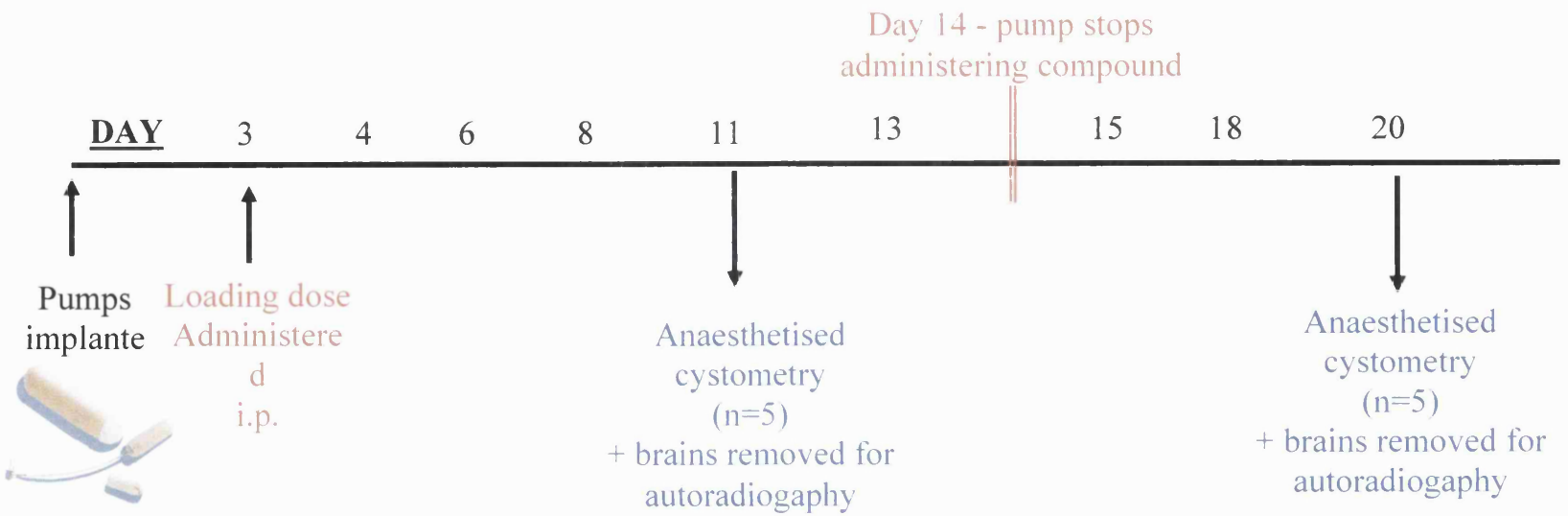


Figure 6.1 – Timeline used for the removal of brains for autoradiographical analysis from rats pre-treated with WAY-100635 (10 nM) or vehicle (saline).

6.2.1.2 – Organ removal

Animals were killed at the end of the anaesthetized cystometry experiments by an overdose of pentobarbitone sodium (i.v.). The brain, kidneys, bladder and spinal cord were rapidly dissected, frozen in plastic bags in N-methylbutaneisopentane cooled on dry ice and stored at –80°C until further use.

6.2.1.3 – Section Preparation

Brain sections (16 μm thick) were cut on a microtome-cryostat (Leica CM1900), thaw mounted onto poly-lysine coated slides (BDH), and kept at –80°C until used. Sections were cut at three different levels of the brain to allow observation of radioligand binding to particular brain regions known/thought to be involved in the control of micturition. These levels were at Bregma –7.60mm to enable observation of binding to the raphé nuclei (dorsal and median), Bregma –5.60mm for the hippocampus and Bregma –3.60mm for the hypothalamus and amygdala. Multiple slides (at least 22) were required for each brain since all incubations were carried out in duplicate to enable determination of total binding (TB) and non-specific binding (NSB), and multiple incubations were conducted with each radioligand type to eliminate the possibility of introducing an unsubstantiated error from just one incubation. Extra slides were also prepared and stained with haematoxylin and eosin to aid in anatomical localization. Adjacent sections were cut and placed on consecutive slides with the slides prepared for each incubation run as shown in Figure 6.2.

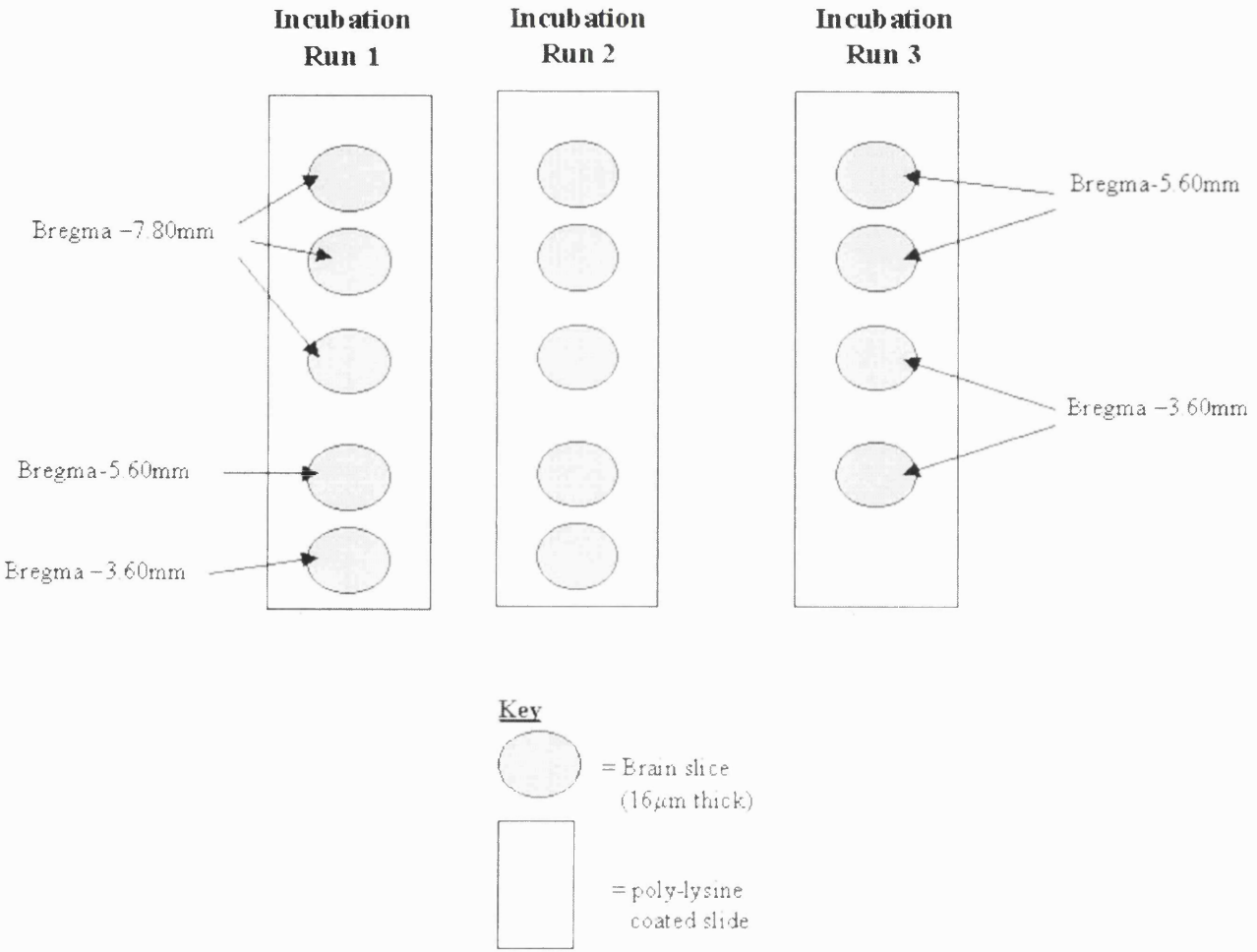


Figure 6.2 – Diagram showing the brain levels of slices on each slide used in each incubation run (for all three radioligands).

6.2.2 – Pilot Studies

To identify the most effective concentration of radioligand to use in the incubations, the literature was consulted and concentrations of 2 nM and 5 nM were decided upon for [^{3}H]GR-125743 (Domenech *et al.*, 1997; Lopez-Gimenez *et al.*, 2002; Millan *et al.*, 2002) and [^{3}H]citalopram (Ase *et al.*, 2001) respectively, after testing to ensure that sufficient levels of specific radioligand binding were obtained (data not shown). However, in the case of [^{3}H]WAY-100635 various different concentrations have been reported to be used in the literature to label 5-HT$_{1A}$ receptors (Fabre *et al.*, 2000; Evrard *et al.*, 2002; Lopez-Gimenez *et al.*, 2002), therefore a pilot study was carried out to identify the most effective concentration of [^{3}H]WAY-100635 to use in the present study. This was achieved by varying the concentration of radioligand and conducting a full incubation protocol (see section 6.2.3) and choosing the concentration which emitted sufficient levels of radioactivity to allow good visualisation of the binding profile whilst keeping non-specific binding to a minimum (see section 6.3.1) for chosen concentrations).

Pilot experiments were also conducted to determine the optimal preincubation times, to ensure that residual drug from the chronic pre-treatment would not interfere with the binding of the radioligands to the 5-HT$_{1A}$ receptors. In brief, sections were preincubated for different times (30, 60, 90, 120 and 180 min) and processed as usual as described in section 6.2.3. A preincubation time of 120 min was finally chosen (see section 6.3.1). Pilot studies with pre-incubations carried out at +4°C and room temperature (RT) were also conducted to assess the most favourable temperature conditions for residual drug dissociation, thus allowing improved radioligand binding during the incubation period.

Initially, the binding of [^{3}H]WAY-100635 and [^{3}H]GR-125743 to bladder, kidney and spinal cord (L6/S1) sections was also investigated, however, due to a lack of sufficient radioligand binding, any chronic WAY-100635 induced changes would have been undetectable and therefore this line of examination was not continued throughout the rest of the study.

6.2.3 – Incubation protocol

An overview of the incubation protocol is shown in Figure 6.3. Sections were preincubated for 120 min at room temperature in buffer A for [^{3}H]WAY-100635 and [^{3}H]GR-125743 or buffer B for [^{3}H]citalopram, where buffers A and B consist of:

Buffer A	Buffer B
170mM Tris-HCl (pH 7.6)	50mM Tris-HCl (pH 7.6)
4mM $CaCl_2$	120mM NaCl
0.01% ascorbic acid	5mM KCl
0.5% bovine serum albumin (BSA)	0.01% ascorbic acid
	0.5% BSA

Sections were then incubated for a further 120 min at +4°C in the same buffers supplemented with either 10μM pargyline and 10nM [^{3}H]WAY-100635 to label 5-HT_{1A} receptors, 10μM pargyline and 2nM [^{3}H]GR-125743 to label 5-$HT_{1B/1D}$ receptors or 5nM [^{3}H]citalopram to label 5-HTT sites. Non-specific binding was defined in the presence of 10μM 5-HT for [^{3}H]WAY-100635 and [^{3}H]GR-125743 binding, and 10μM fluoxetine for [^{3}H]citalopram binding. Sections were washed twice (5 min each) in buffer C

([^{3}H]WAY-100635 and [^{3}H]GR-125743) or buffer D ([^{3}H]citalopram) at +4°C, where buffers C and D consist of:

Buffer C	Buffer D
170mM Tris-HCl (pH7.6)	50mM Tris-HCl
4mM $CaCl_2$	120mM NaCl
0.01% ascorbic acid	5mM KCl
10μM pargyline	0.01% ascorbic acid

and then sections were washed twice (5 min each) in distilled H_2O at +4°C. Finally sections were dried in a stream of cold air.

6.2.4 – Imaging and analysis

The dried, labelled slides were imaged using a Biospace 2000 Beta imager (Biospace, Paris, France) over a 12 h period. To obtain a higher resolution image (for localisation purposes) individual sections were imaged using a Biospace micro-imager for a 72 h period, which produces images of a resolution of approximately 100μm. Unfortunately, due to problems with the supply of tritiated hyperfilm, confirmation of the Biospace beta-imager analyses was unable to be obtained through exposure of the sections to film.

Quantitative analyses of the autoradiographic images produced by the beta-imager were performed using a computerized image analysis system associated with the imager (β-vision, Biospace). Radioactive counts per mm^2 were obtained for each brain region showing radioligand binding on the 'total binding' slides, with the radioactive counts per

mm^2/brain region also calculated using the same sized area limits on the duplicate 'non-specific binding' slide. Specific binding was calculated by subtracting the non-specific binding values from the total binding. Data from this radioligand autoradiography was converted into fmol mg^{-1} brain tissue using formulae obtained from the brain paste standard studies detailed in the next section.

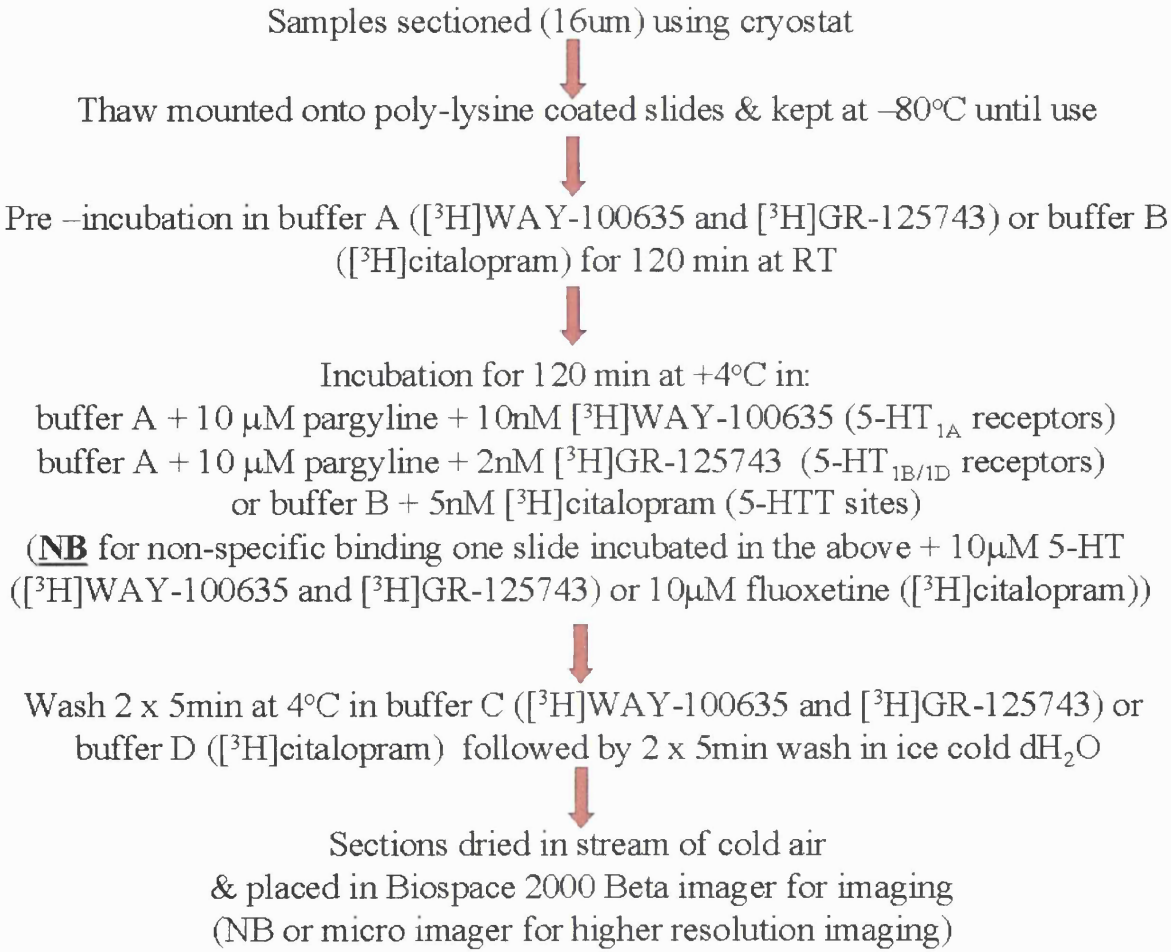


Figure 6.3 – Overview of protocol for labelling of sections with radioligands. See text for details of buffers A,B,C and D. Abbreviations: RT, room temperature.

6.2.5 – Brain paste standards

To enable conversion of the level of radioligand binding from counts per mm^2 (obtained from the β-vision software package) to fmol mg^{-1} brain tissue, various brain paste standards which contained known levels of radioactivity per mg of tissue homogenate (0.1, 1, 10 and 100 nM) were constructed for each radioligand. The homogenate was made by homogenising a brain without the cerebellum (to try and more closely mirror the ratio of white: grey matter found in the sections studied and hence reduce the possibility of variation from tritium quenching (see Geary *et al.*, 1985)) from a control animal, and suspending the homogenate in either buffer A or buffer B (depending on the radioligand to be tested) at a concentration of 100mg ml^{-1}. These brain paste standards were frozen 1 cm diameter holes in blocks of frozen Tissue Tek ™ OCT embedding medium (R. A. Lamb, London, U.K.), sectioned using the cryostat at the same thickness as the previous sections (16μm) and imaged using the Biospace 2000 Beta imager. These values (cp mm^2) obtained from the β-vision software combined with those obtained from the measurement (disintegrations per minute) of the level of radioactivity in various concentrations (0.1, 1, 10 and 100 nM) of radioligand dissolved in brain tissue homogenate (100 mg ml^{-1}) enabled the construction of standard curves for each radioligand, from which the counts per mm^2 for each brain area could be converted into fmol mg^{-1} brain tissue.

6.2.6 – Statistical analysis

Multiple sections (≥4) for each incubation run, with 3 incubation runs in total, were used to obtain levels of radioligand binding in the various regions for each animal. For areas that are found in both the left and right hemispheres of the brain, such as the

hippocampus, values were obtained from both hemispheres and the mean used as the value for that section. Data were analysed by two-way ANOVA with the treatment (vehicle or WAY-100635) and brain structure as factors, followed by a two-tailed unpaired Student's *t*-test (using an average value for replicates from the same animal) to compare the treatment effect in each individual brain area. The above analysis was carried out individually for data from day 11 and day 20. All values are given as mean ± s.e.mean. $P < 0.05$ considered significant.

6.2.7 – Drugs and solutions

Drugs and chemicals were obtained from the following sources: 2-methylbutane (isopentane), Tris-HCl (1M stock, pH 7.60), $CaCl_2$ (1M stock solution), ascorbic acid, bovine serum albumin (BSA), KCl, NaCl, pargyline, 5-HT creatinine sulphate, fluoxetine HCl all from Sigma Aldrich Chemicals, Poole, Dorset, U.K.; [^{3}H]WAY-100635 (81.0 Ci/mmol), [^{3}H]GR-125743 (N-[4-Methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl)benzamide); 79.0 Ci/mmol) and [^{3}H]citalopram (81.0 Ci/mmol) from Amersham Biosciences, Bucks, U.K.

6. 3 - Results

6.3.1 – Pilot studies

6.3.1.1 – Radioligand concentrations and levels of non-specific binding

To identify the most effective concentration of [^{3}H]WAY-100635 to use in the incubations, pilot studies were undertaken using varying concentrations of [^{3}H]WAY-100635. As Figure 6.4 shows, 10 nM [^{3}H]WAY-100635 showed the highest level of binding in the hippocampus (1163 fmol mg^{-1} brain tissue) whilst still remaining specific for 5-HT$_{1A}$ receptors since only areas known to contain 5-HT$_{1A}$ receptors showed [^{3}H]WAY-100635 binding, and levels of non-specific binding in the presence of 10μM 5-HT remained negligible (see Figure 6.5). Therefore, 10 nM [^{3}H]WAY-100635 was chosen as the incubation concentration to label 5-HT$_{1A}$ receptors in the remainder of the study.

As Figure 6.6 shows 2 nM [^{3}H]GR-125743 labels 5-HT$_{1B/1D}$ receptors in the brain (detailed further in section 6.3.3) but not the kidney, bladder or spinal cord (L_6/S_1 level). There were negligible levels of non-specific binding in the brain with 2 nM [^{3}H] GR-125743 in the presence of 5-HT, however there appears to be non-specific binding to receptors, other than 5-HT receptors, in the kidney particularly the cortex (see Figure 6.6) which accounts for the apparent binding of 2nM [^{3}H]GR-125743 to the kidney.

5 nM [^{3}H]citalopram was used to label 5-HTT and showed 5-HTT expression in the brain (detailed further in section 6.3.4), low levels of expression in the kidney (see Figure 6.7)

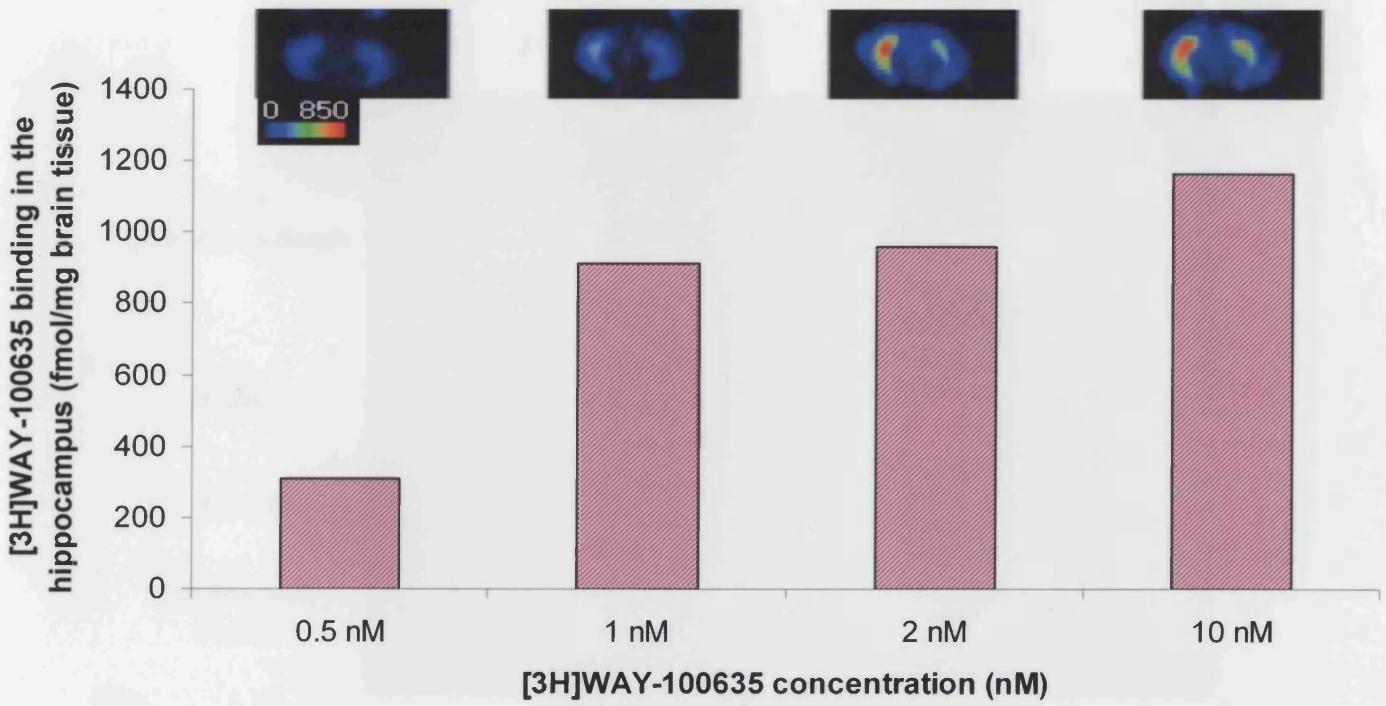


Figure 6.4 – Graph to show the level of [^{3}H]WAY-100635 binding in the hippocampus (fmol mg^{-1} brain tissue) after incubations with varying concentrations of [^{3}H]WAY-100635 (n = 1). Superimposed on the graph are the visual radiographical images of the level of radioactivity emitted in coronal sections of the brain at Bregma –5.60mm for each concentration of [^{3}H]WAY-100635 tested.

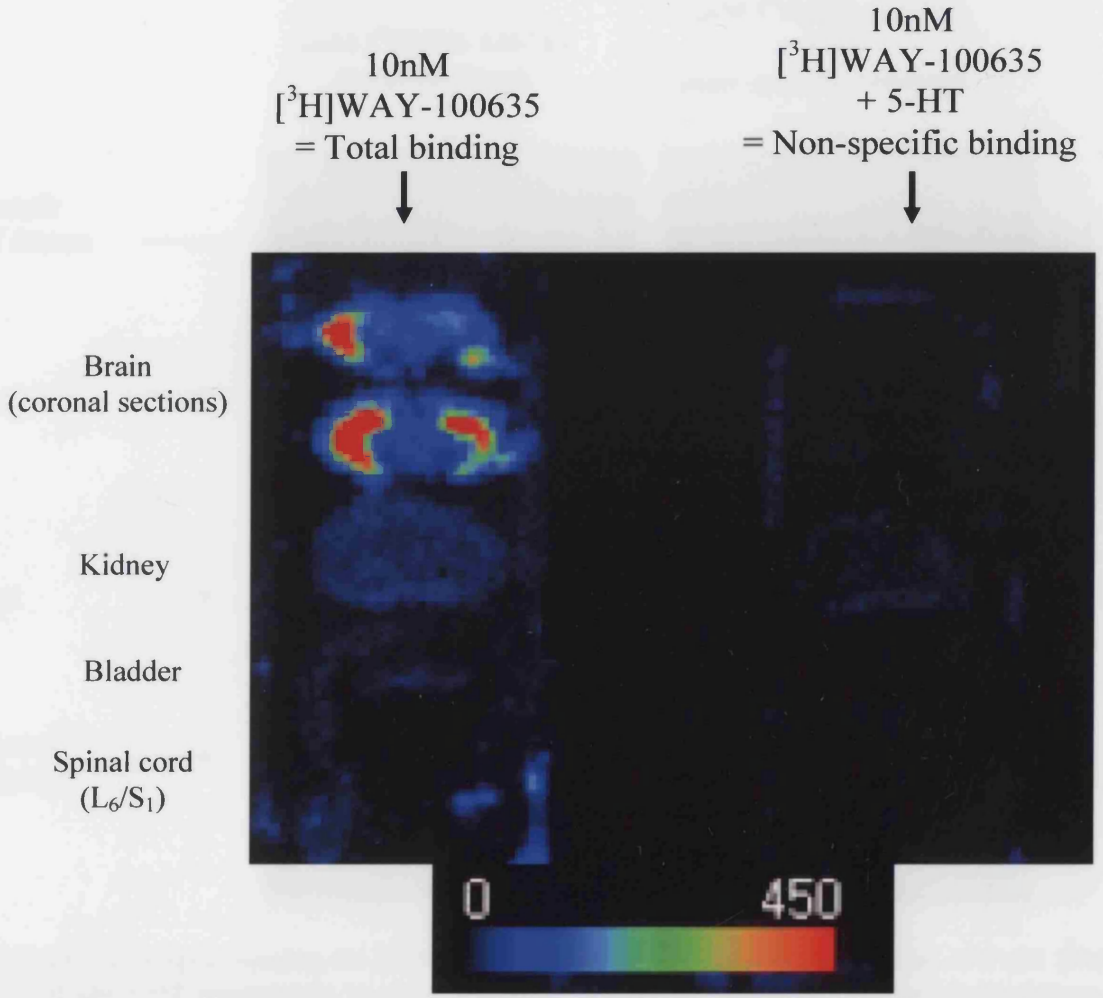


Figure 6.5 – Autoradiographical images showing the levels of total binding and non-specific binding (negligible) of 10 nM [^{3}H]WAY-100635 in the absence and presence of 10 μM 5-HT respectively, in coronal sections of the brain (Bregma – 7.80 and –5.60mm), the kidney, bladder and spinal cord (L_6/S_1 level) from a single rat. The scale is a nominal measure of the radioactivity emitted and is for visualisation purposes only.

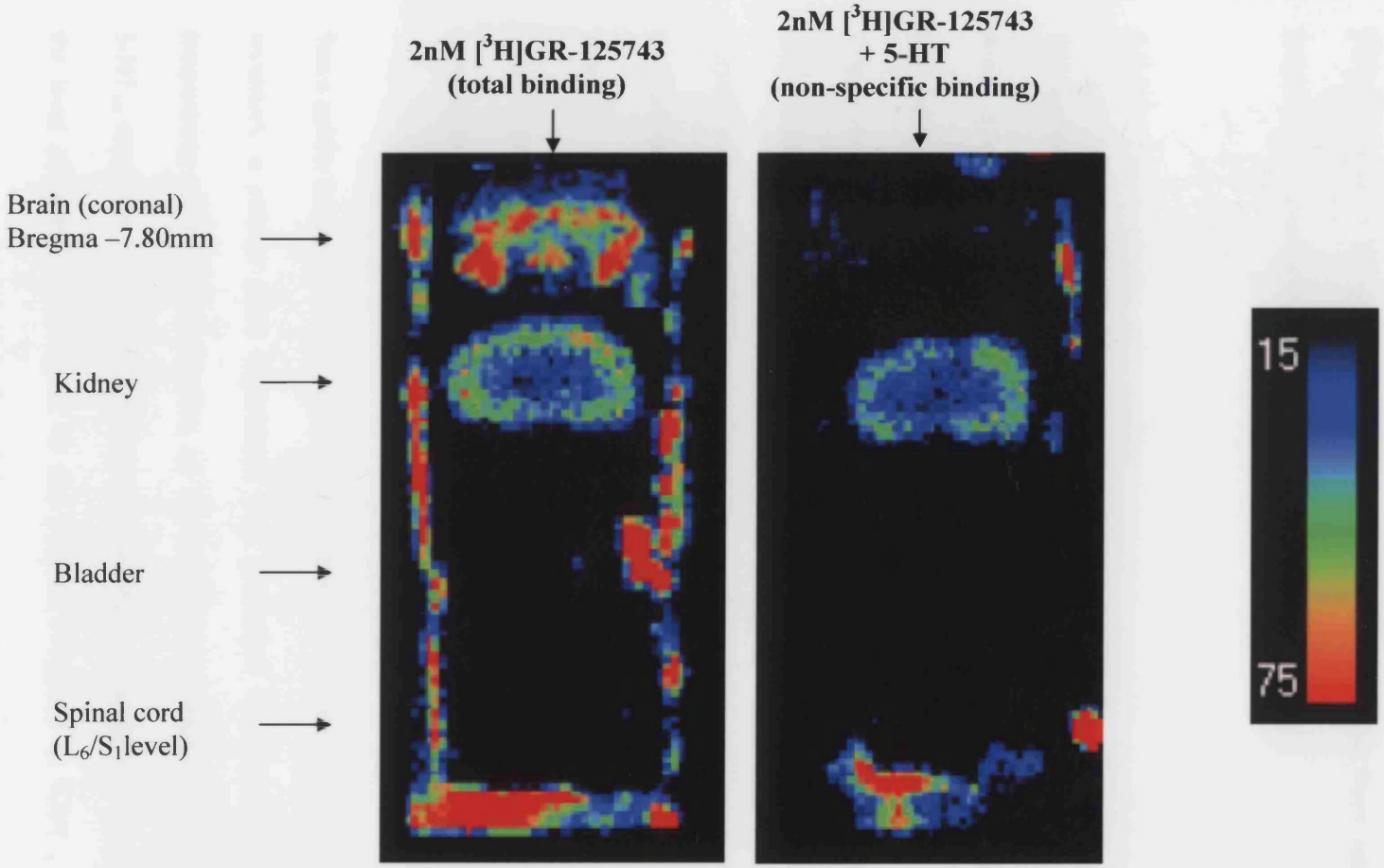


Figure 6.6 – Autoradiographical images showing the levels of total binding and non-specific binding of 2 nM [^{3}H]GR-125743 in the absence and presence of 10 μM 5-HT respectively, in coronal sections of the brain (Bregma – 7.80 mm), the kidney, bladder and spinal cord (L_6/S_1 level) from a single rat. The scale is a nominal measure of the radioactivity emitted and is for visualisation purposes only. The apparent high levels of radioactivity around the edge of the slide is due to sparking from the edge of the slide which causes artificially high readings from the β-imager.

and possibly the spinal cord (L_6/S_1 level), but no expression in the bladder. There were negligible levels of non-specific binding in the brain, bladder or spinal cord with 5 nM [^{3}H]citalopram in the presence of 10 μM fluoxetine, although there appears to be slight levels of non-specific binding in the kidney (see Figure 6.7).

6.3.1.2 – Pre-incubation pilot studies

Tissues were pre-incubated to wash out any residual drug from the chronic challenge dose, or endogenous 5-HT, which could potentially interfere with the binding of the radioligands to 5-HT_{1A} receptors. Optimal pre-incubation times (at +4°C) were determined from preliminary experiments using sections of brain containing the hippocampus from two animals, one which had been pre-dosed with vehicle in the osmotic pump and the other with WAY-100635 (10 nM), differing the length of pre-incubation time and then labelling 5-HT_{1A} receptors using 10 nM [^{3}H]WAY-100635 . As shown in Figure 6.8, specific binding increased in the hippocampus with a pre-incubation time of approximately 120 min, showing a plateau at 90-120 min. Therefore, a pre-incubation time of 120 min was chosen for all remaining experiments.

Since colder temperatures are known to slow the rate of dissociation of ligands from their receptors, a similar pilot experiment was conducted to determine the effect of the temperature of the pre-incubation on the subsequent binding of [^{3}H]WAY-100635 to 5-HT_{1A} receptors. As Figure 6.9 shows, when the pre-incubation was carried out at RT, the level of specific binding of 10 nM [^{3}H]WAY-100635 to 5-HT_{1A} receptors in the hippocampus was higher in both vehicle (n = 1) and WAY-100635 (n = 1) pre-dosed rats. Therefore the pre-incubation for all remaining experiments was carried out at RT.

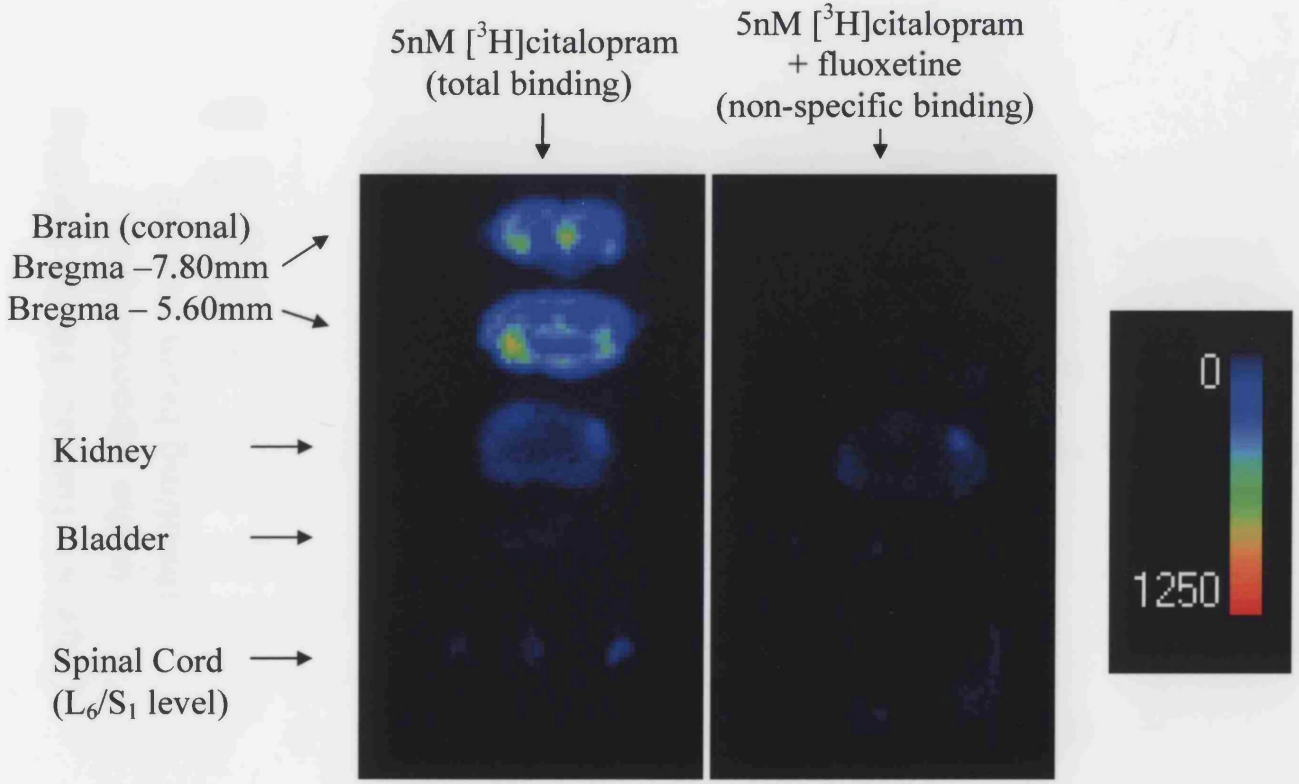


Figure 6.7 - Autoradiographical images showing the levels of total binding and non-specific binding of 5 nM [^{3}H]citalopram in the absence and presence of 10 μM fluoxetine respectively, in coronal sections of the brain (Bregma – 7.80 and –5.60mm), the kidney, bladder and spinal cord (L_6/S_1 level) from a single rat. The scale is a nominal measure of the radioactivity emitted and is for visualisation purposes only.

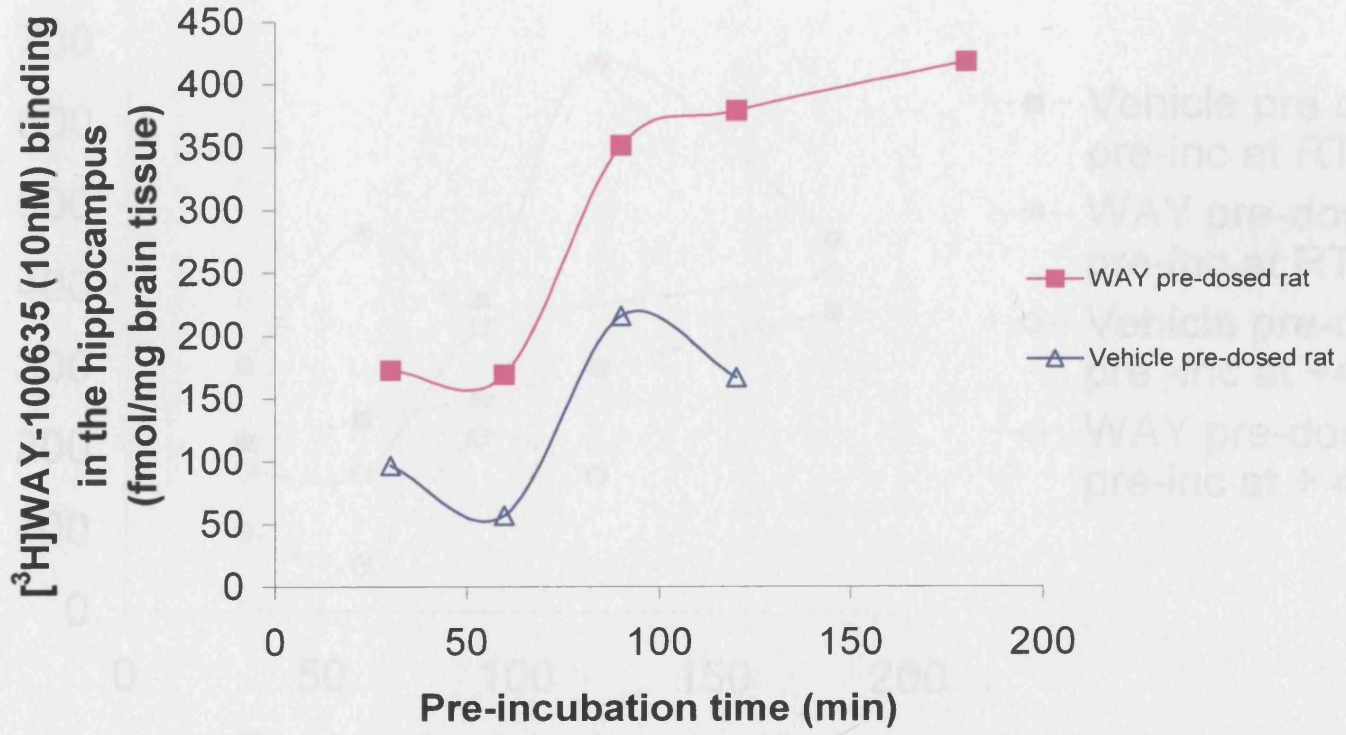


Figure 6.8 – Effect of pre-incubation (at +4°C) time on binding of [^{3}H]WAY-100635 to rat hippocampus in rats chronically pre-dosed with either vehicle (n = 1) or WAY-100635 (n = 1).

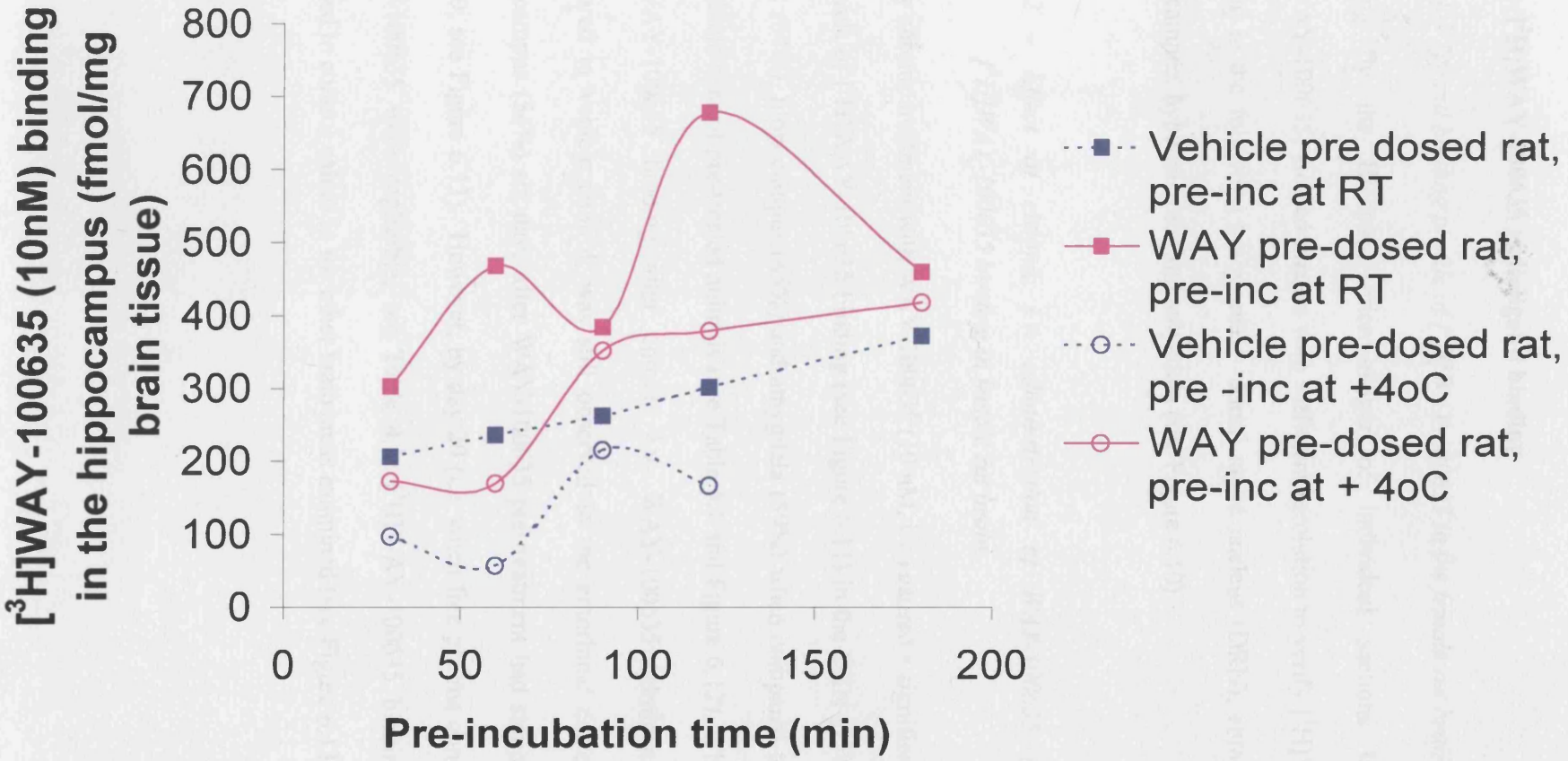


Figure 6.9 – Effect of temperature of the pre-incubation on the binding of [^{3}H]WAY-100635 to rat hippocampus in rats chronically pre-dosed with either vehicle (n = 1) or WAY-100635 (n = 1). Abbreviations: RT, room temperature; pre-inc, pre-incubation.

6.3.2 – [^{3}H]WAY-100635 radioligand binding

6.3.2.1 – Typical binding profile of [^{3}H]WAY-100635 in the female rat brain

Imaging by the Biospace micro-imager of individual sections labelled with [^{3}H]WAY-100635, yielded images with sufficient resolution to verify [^{3}H]WAY-100635 binding in the following brain areas: dorsal raphé nucleus (DRN), entorhinal cortex, hippocampus, hypothalamus and amygdala (see Figure 6.10).

6.3.2.2 – Effect of chronic i.v. administration of WAY-100635 (10 nM) on [^{3}H]WAY-100635 binding in female rat brain.

11 day chronic treatment with WAY-100635 (10 nM, i.v.) caused a significant increase in the levels of [^{3}H]WAY-100635 binding (see Figure 6.11) in the DRN (73%), entorhinal cortex (64%), hippocampus (45%) and amygdala (59%) when compared to brains from the vehicle control pre-treated animals (see Table 6.1 and Figure 6.12). This increase in [^{3}H]WAY-100635 binding after chronic i.v. WAY-100635 administration, when compared to vehicle control, was still observed in the entorhinal cortex (46%) and hippocampus (54%) six days after WAY-100635 pre-treatment had stopped (i.e. study day 20; see Figure 6.13). However, by day 20 (i.e. when free plasma concentrations of WAY-100635 were negligible; see Table 4.1) [^{3}H]WAY-100635 binding levels had returned to control values in the other brain areas examined (see Figure 6.13).

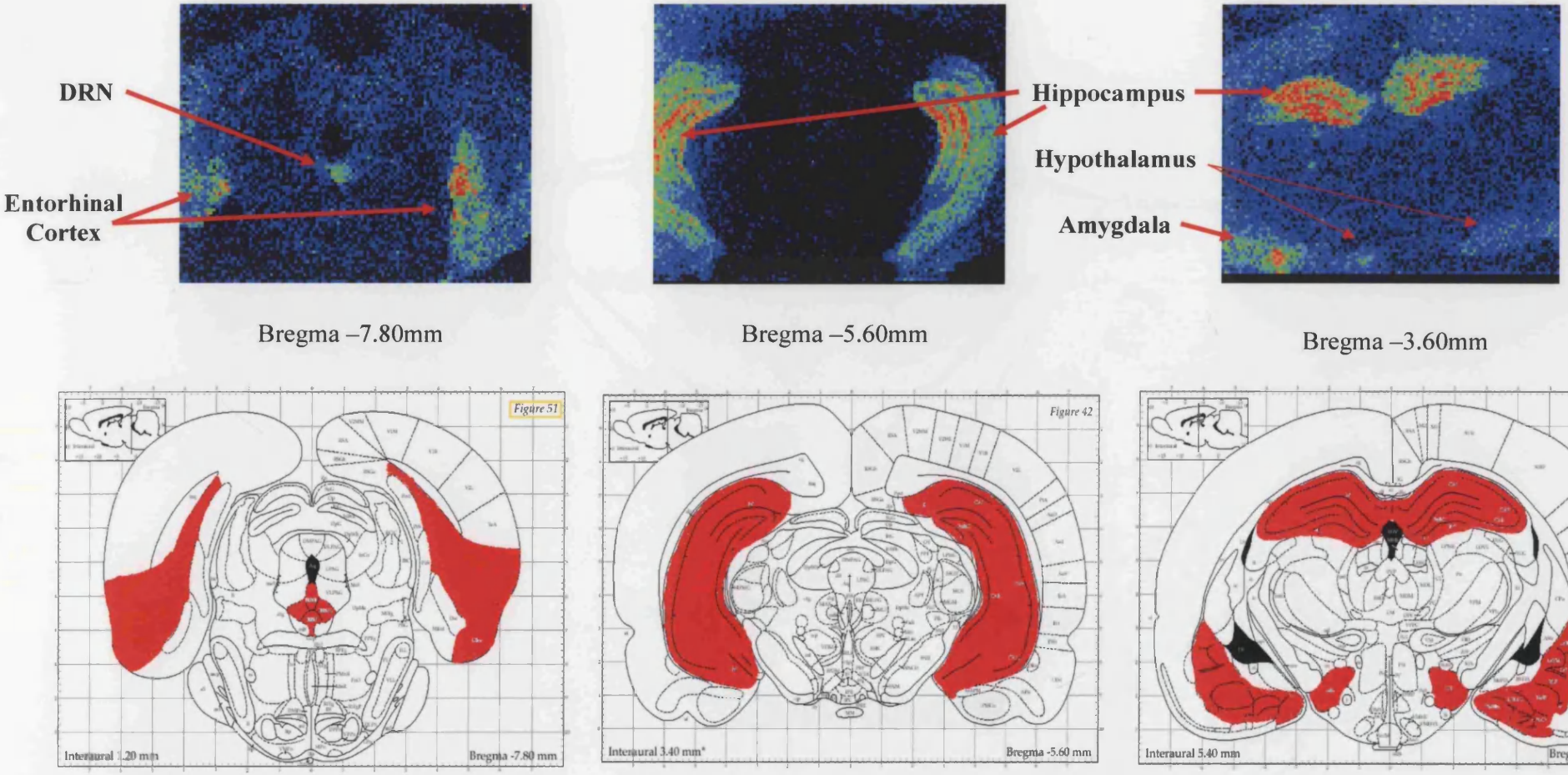


Figure 6.10 – Binding of [^{3}H]WAY-100635 in different brain regions. (Brain maps taken from Paxinos *et al.*, 1998)

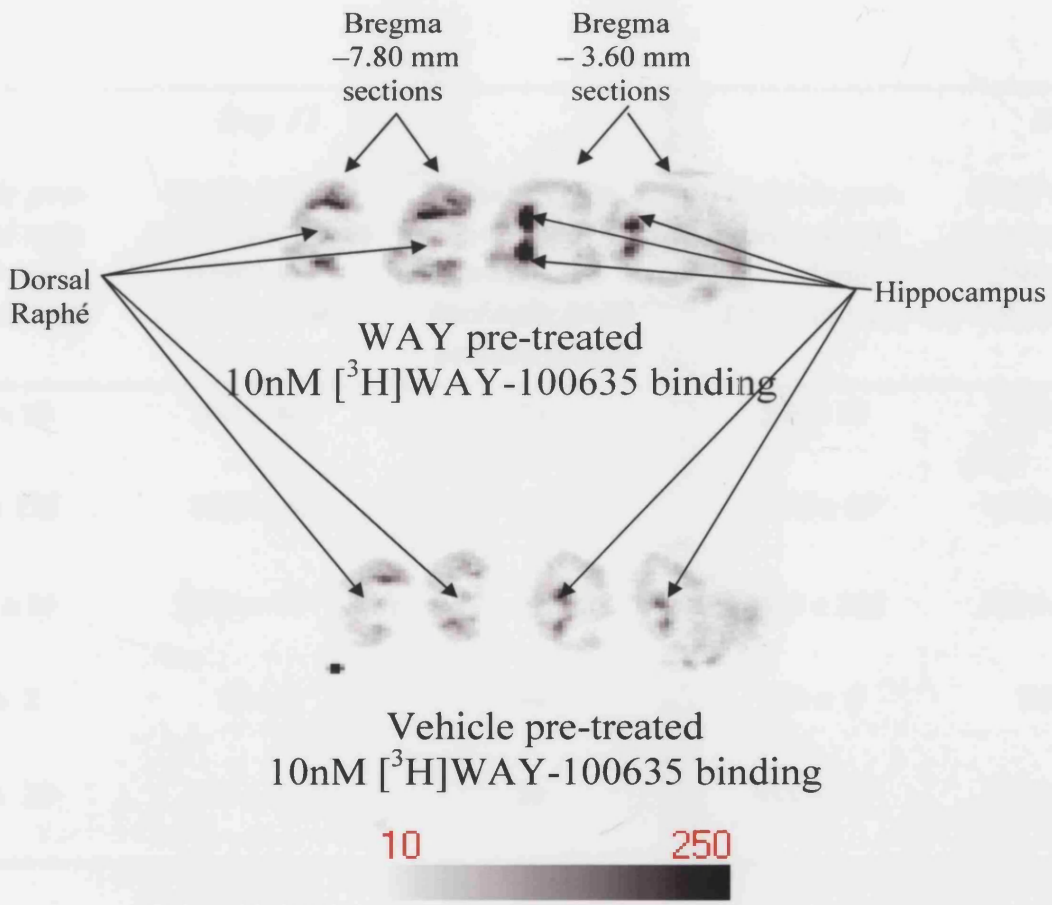


Figure 6.11 – Autoradiographical image of the binding of [^{3}H]WAY-100635 in the DRN and hippocampus after 11-day infusion (i.v.) of WAY-100635 (10 nM; top) or vehicle (bottom). The scale is a nominal measure of the radioactivity emitted and is for visualisation purposes only. Note the darker level of radioactive staining in the brain sections from rats pre-treated with WAY-100635 when compared to vehicle control.

	Day 11			*Day 20*		
	Vehicle pre-treated rats (fmol mg^{-1} brain tissue)	*WAY-100635 pre-treated rats (fmol mg^{-1} brain tissue)*	*Binding in WAY-brains expressed as % increase from vehicle levels*	*Vehicle pre-treated rats (fmol mg^{-1} brain tissue)*	*WAY-100635 pre-treated rats (fmol mg^{-1} brain tissue)*	*Binding in WAY-brains expressed as % increase from vehicle levels*
Dorsal raphé nucleus	198 ± 19	342 ± 40 *	73	358 ± 18	419 ± 47	17
Entorhinal cortex	787 ± 135	1287 ± 67*	64	1155 ± 89	1686 ± 203 *	46
Hippocampus	1045 ± 85	1514 ± 99 **	45	1455 ± 200	2236 ± 233 *	54
Hypothalamus	51 ± 2	84 ± 3	64	104 ± 6	130 ± 7	25
Amygdala	254 ± 20	404 ± 41 *	59	488 ± 54	651 ± 102	33

Table 6.1 - Binding of [^{3}H]WAY-100635 (fmol mg^{-1} brain tissue)in animals chronically pre-treated with vehicle or WAY-100635 (n = 2/3, multiple replicates). Values are mean ± s.e.mean. * $P < 0.05$; ** $P < 0.01$ when compared to vehicle/time match control by unpaired Student's *t*-test.

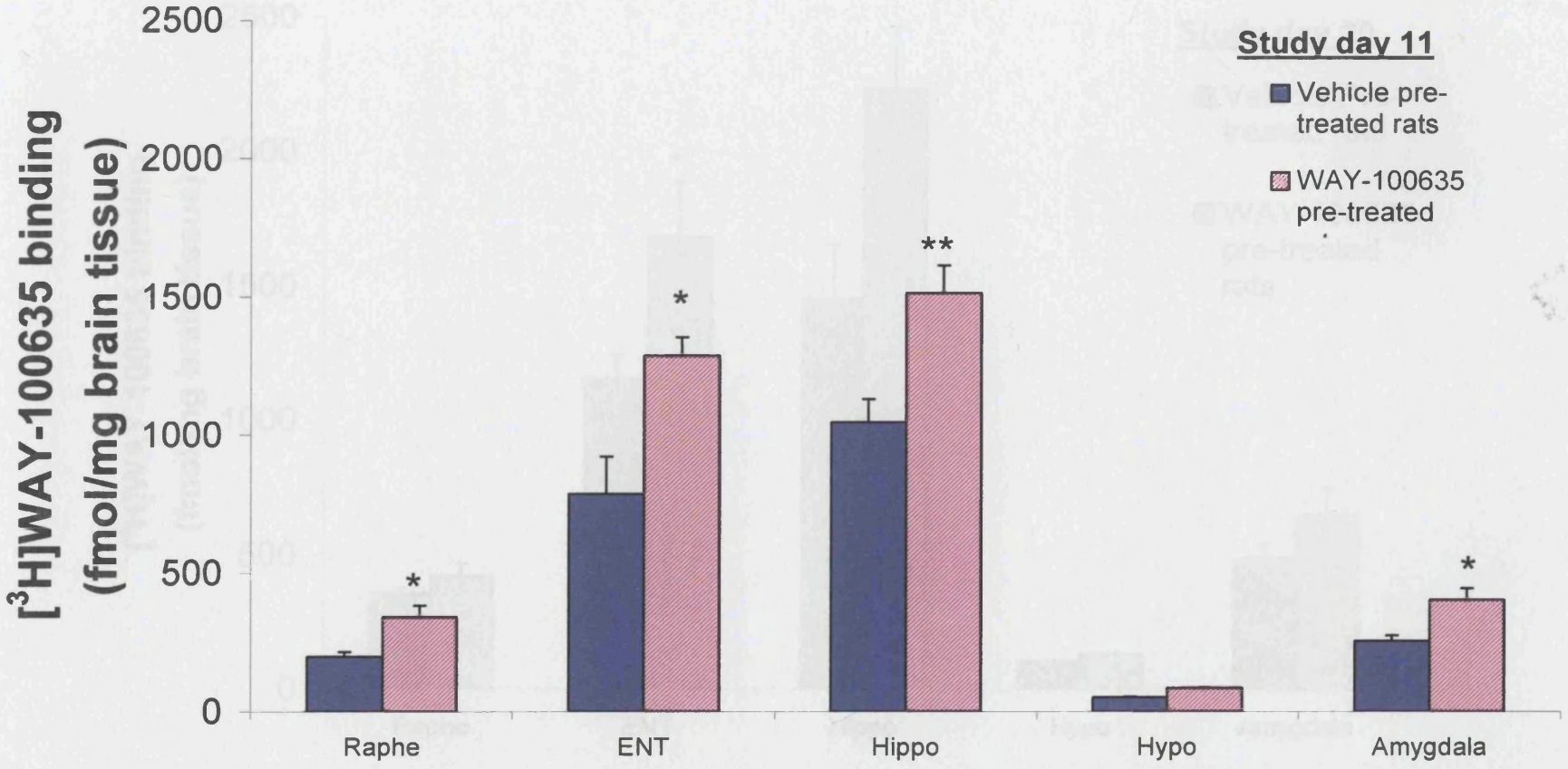


Figure 6.12 – [3H]WAY-100635 binding (fmol mg-1 brain tissue) in rat brains after 11-day chronic i.v. administration of WAY-100635 (10 nM) or vehicle control. * $P < 0.05$; ** $P < 0.01$ when compared to vehicle/time match control by unpaired Student's *t*-test; n = 2/3, multiple replicates. Abbreviations: ENT, entorhinal cortex; hippo, hippocampus; hypo, hypothalamus.

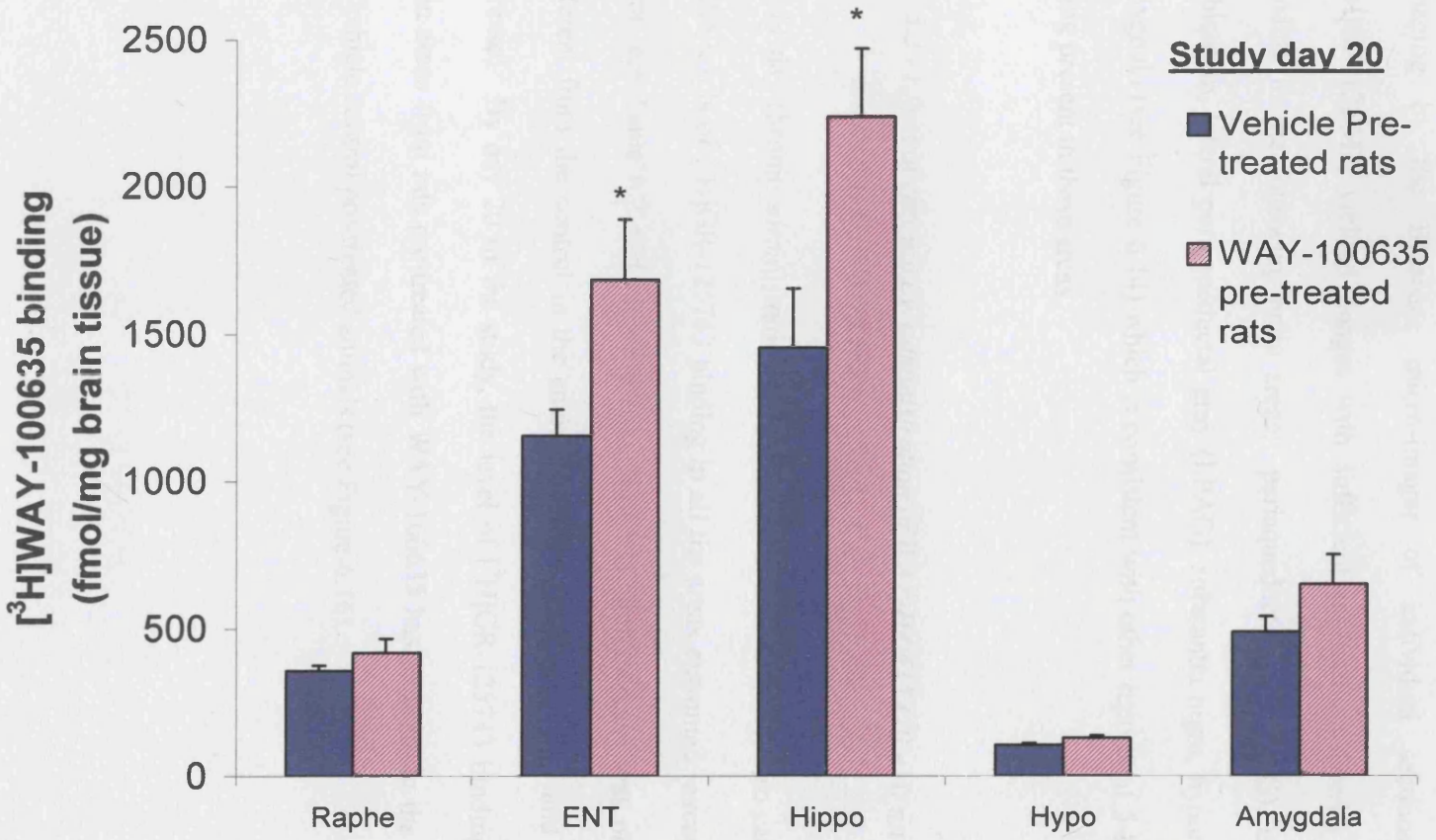


Figure 6.13 – [^{3}H]WAY-100635 binding (fmol mg^{-1} brain tissue) in rat brains 6 days after cessation of 14 days chronic administration of WAY-100635 or vehicle control (i.e. on study day 20). * $P < 0.05$ when compared to vehicle/time match control by unpaired Student's *t*-test; n = 2/3, multiple replicates. Abbreviations: ENT, entorhinal cortex; hippo, hippocampus; hypo, hypothalamus.

6.3.3 - [^{3}H]GR-125743 radioligand binding

6.3.3.1 - Typical binding profile of [^{3}H]GR-125743 in the female rat brain

Imaging by the Biospace micro-imager of individual sections labelled with [^{3}H]GR-125743, yielded images with sufficient resolution to verify [^{3}H]GR-125743 binding in the following brain areas: periaqueductal gray (PAG), entorhinal cortex, subiculum, lateral periaqueductal gray (LPAG), substantia nigra, hypothalamus and the amygdala (see Figure 6.14) which is consistent with other reports of 5-$HT_{1B/1D}$ receptors being present in these areas.

6.3.3.2 - Effect of chronic i.v. administration of WAY-100635 (10 nM) on [^{3}H]GR-125743 binding in female rat brain.

An 11-day chronic administration of WAY-100635 (10 nM, i.v.) also caused an increase in the levels of [^{3}H]GR-125743 binding in all the areas examined (except the substantia nigra; see Table 6.2 and Figure 6.15). However, this increase was only significantly different from the control in the entorhinal cortex (50% increase) and the PAG (74% increase). By day 20 of the study, the level of [^{3}H]GR-125743 binding in the various brain areas from rats pre-treated with WAY-100635 has returned to the levels found in the vehicle control pre-treated animals (see Figure 6.16).

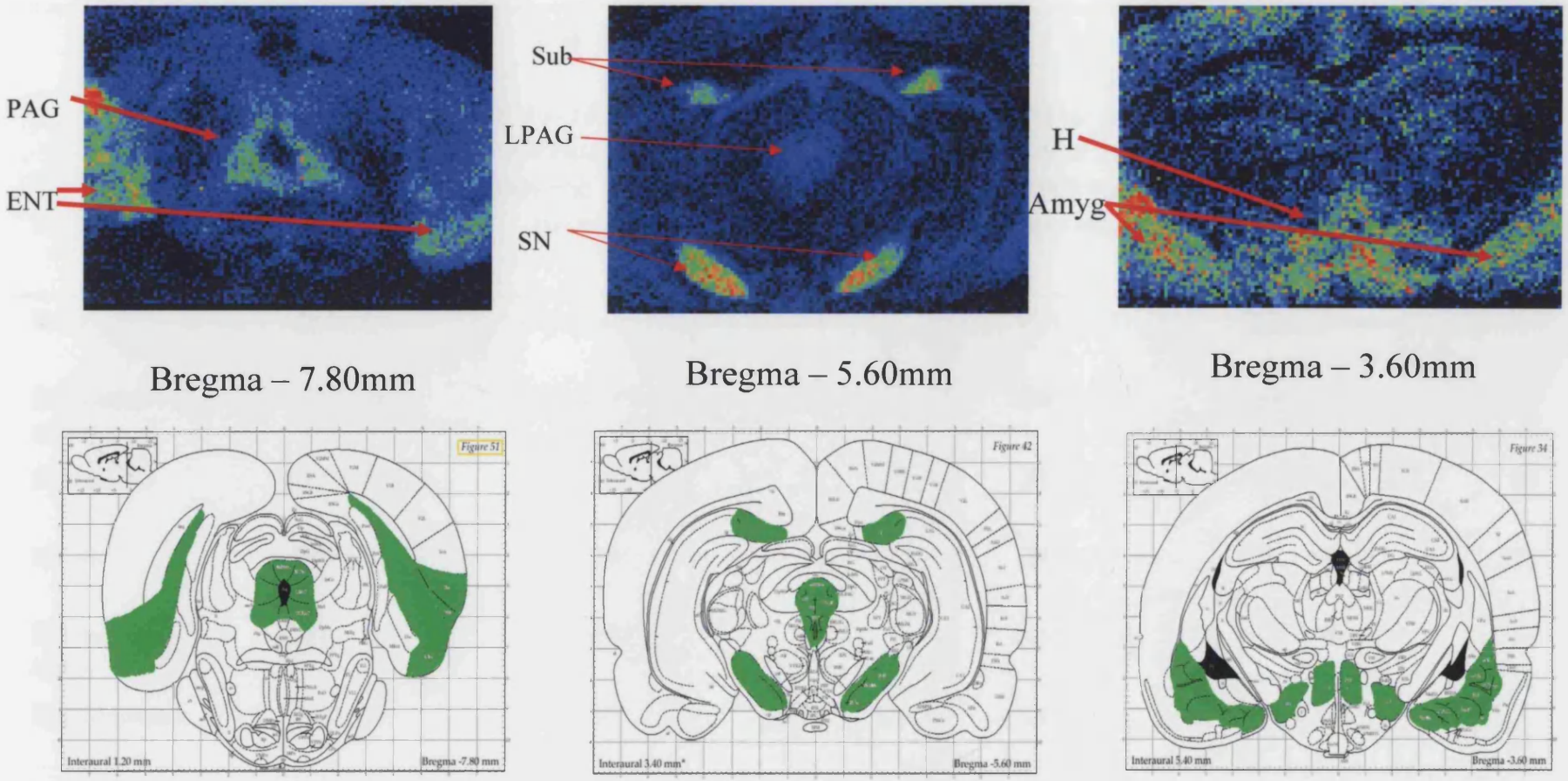


Figure 6.14 - Binding of [^{3}H]GR-125743 in different brain regions. (Brain maps taken from Paxinos *et al.*, 1998). Abbreviations: PAG, periaqueductal gray; ENT, entorhinal cortex; Sub, subiculum; LPAG, lateral periaqueductal gray; SN, substantia nigra; H, hypothalamus; Amyg, amygdala.

	Day 11			*Day 20*		
	Vehicle pre-treated rats (fmol mg^{-1}brain tissue)	*WAY-100635 pre-treated rats (fmol mg^{-1}brain tissue)*	*Binding in WAY-brains expressed as % increase from vehicle levels*	*Vehicle pre-treated rats (fmol mg^{-1}brain tissue)*	*WAY-100635 pre-treated rats (fmol mg^{-1}brain tissue)*	*Binding in WAY-brains expressed as % increase from vehicle levels*
PAG	267 ± 0	466 ± 44 *	74	293 ± 14	302 ± 31	3
Entorhinal cortex	552 ± 39	825 ± 42 **	50	580 ± 34	548 ± 83	-6
Subiculum	915 ± 59	1162 ± 167	27	842 ± 64	1013 ± 93	20
LPAG	299 ± 6	382 ± 28	28	292 ± 11	352 ± 28	20
Substantia nigra	1544 ± 138	1385 ± 152	-10	1438 ±103	1609 ± 89	12
Hypothalamus	269 ± 16	338 ± 32	26	287 ± 23	291 ± 9	1
Amygdala	480 ± 26	678 ± 83	41	550 ± 33	545 ± 28	-1

Table 6.2 - Binding of [^{3}H]GR-125743 (fmol mg^{-1} brain tissue)in animals chronically pre-treated with vehicle or WAY-100635 (n = 2/3, multiple replicates). Values are mean ± s.e.mean. * $P < 0.05$; ** $P < 0.01$ when compared to vehicle/time match control by unpaired Student's *t*-test.

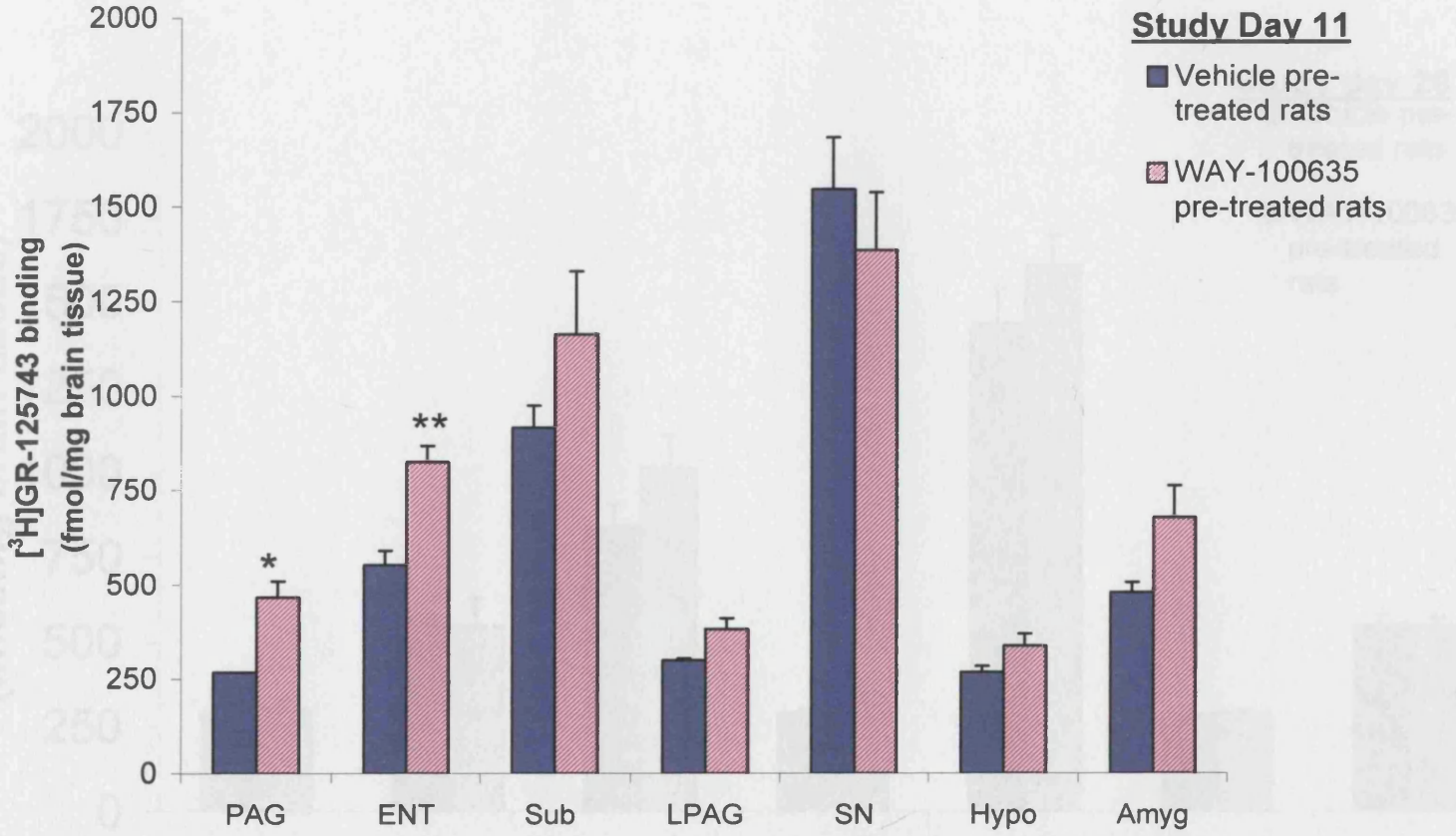


Figure 6.15 - [^{3}H]GR-125743 binding (fmol mg^{-1} brain tissue) in rat brains after 11-day chronic i.v. administration of WAY-100635 (10 nM) or vehicle control. . * $P < 0.05$; ** $P < 0.01$ when compared to vehicle/time match control by unpaired Student's *t*-test; n = 2/3, multiple replicates. Abbreviations: PAG, periaqueductal gray; ENT, entorhinal cortex; Sub, subiculum; LPAG, lateral periaqueductal gray; SN, substantia nigra; Hypo, hypothalamus; Amyg, amygdala.

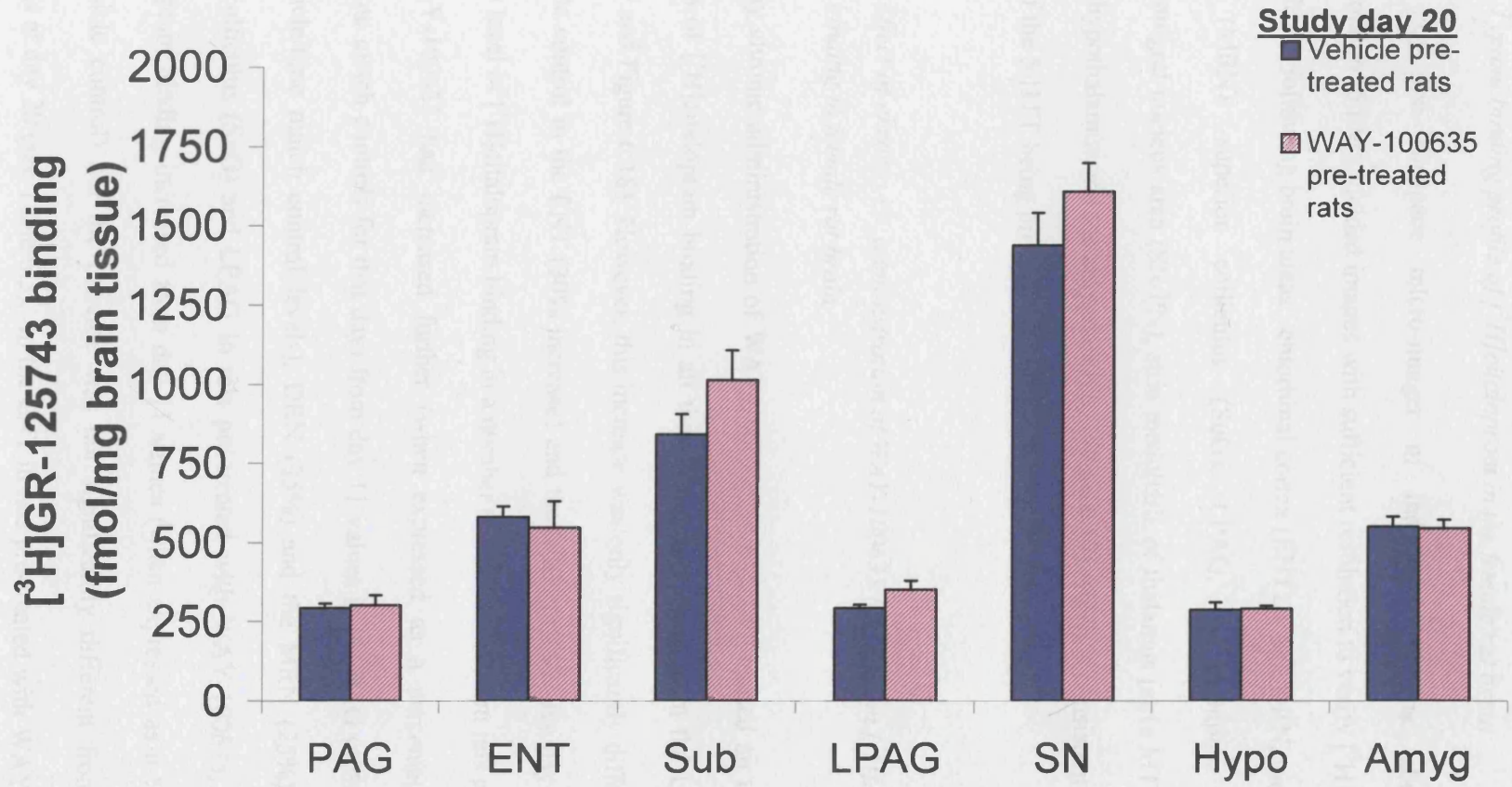


Figure 6.16 - [^{3}H]GR-125743 binding (fmol mg^{-1} brain tissue) in rat brains 6 days after cessation of 14 days chronic administration of WAY-100635 or vehicle control (i.e. on Study day 20). n = 2/3, multiple replicates. Abbreviations: PAG, periaqueductal gray; ENT, entorhinal cortex; Sub, subiculum; LPAG, lateral periaqueductal gray; SN, substantia nigra; Hypo, hypothalamus; Amyg, amygdala.

6.3.4 – [^{3}H]citalopram radioligand binding

6.3.4.1 - Typical binding profile of [^{3}H]citalopram in the female rat brain

Imaging by the Biospace micro-imager of individual sections labelled with [^{3}H]citalopram (5 nM), yielded images with sufficient resolution to verify [^{3}H]citalopram binding in the following brain areas: entorhinal cortex (ENT), PAG, DRN, median raphé nucleus (MRN), superior colliculus (SuG), LPAG, hippocampus, substantia nigra/paranigral nucleus area (SN/PN), stria medullaris of thalamus (stria MT), thalamic nucleus, hypothalamus and the amygdala (see Figure 6.17) which is consistent with other reports of the 5-HTT being present in these areas.

6.3.4.2 - Effect of chronic i.v. administration of WAY-100635 (10 nM) on [^{3}H]citalopram binding in female rat brain.

An 11 day chronic administration of WAY-100635 (10 nM, i.v.) caused an increase in the levels of [^{3}H]citalopram binding in all the areas examined (except the LPAG; see Table 6.3 and Figure 6.18) However, this increase was only significantly different from the vehicle control in the ENT (30% increase) and the MRN (19%). By day 20 of the study, the level of [^{3}H]citalopram binding in a number of brain areas from rats pre-treated with WAY-100635 had increased further (when expressed as a percentage of the vehicle/time match controls for that day) from day 11 values in the PAG (42% increase from vehicle/time match control levels), DRN (25%) and the MRN (25%). In the superior colliculus (SuG) and LPAG in rats pre-treated with WAY-100635, levels of [^{3}H]citalopram binding increased from day 11 values (when expressed as a % of time match/vehicle control) but the increase was not significantly different from vehicle control rats at day 20 (see Table 6.3). In the ENT in rats pre-treated with WAY-100635,

the level of [^{3}H]citalopram binding had dropped from day 11 values back to time match control values by day 20. In all other areas examined at day 20 the levels of [^{3}H]citalopram binding decreased from day 11 values (see Figure 6.19).

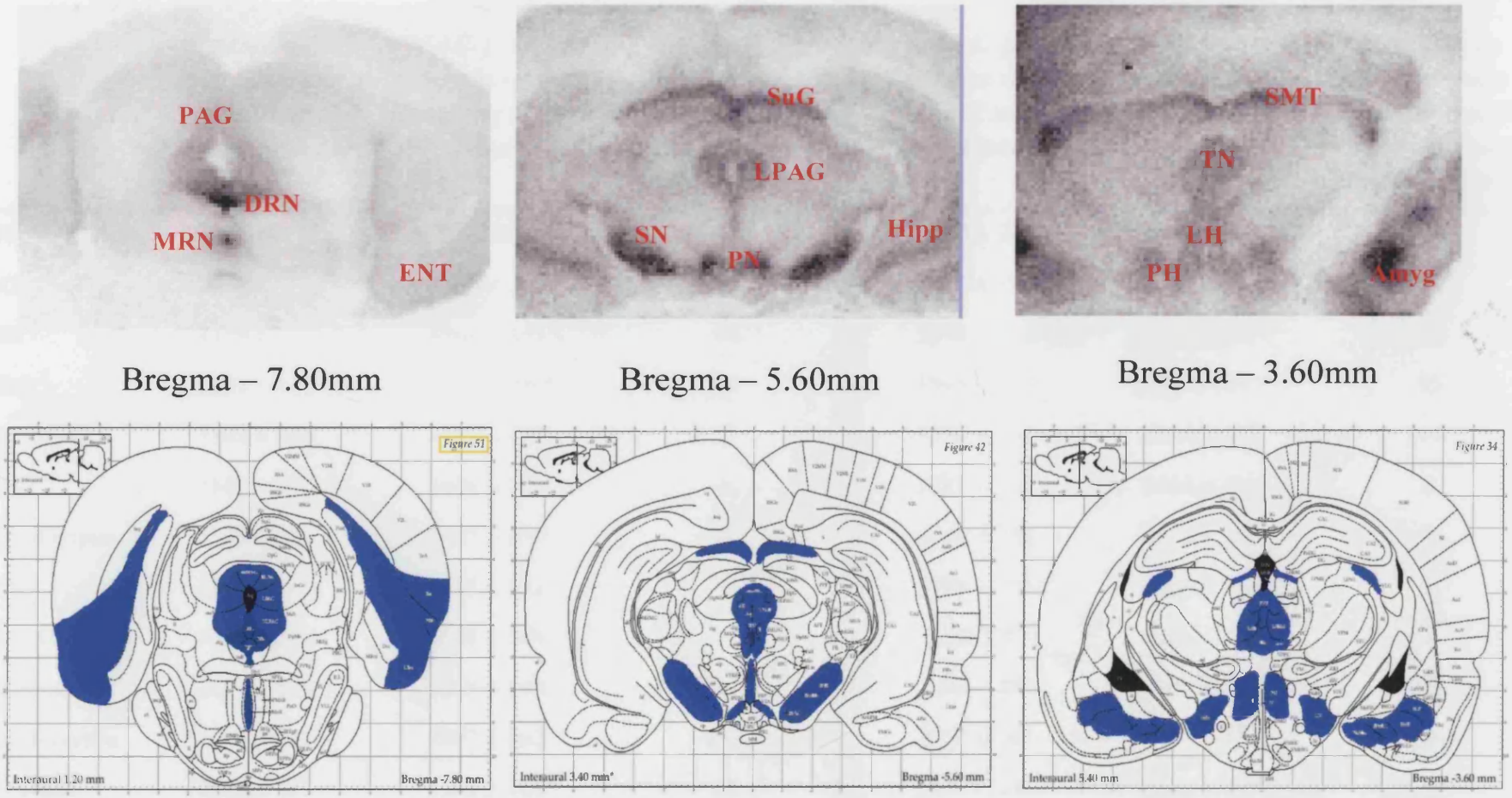


Figure 6.17 - Binding of [^{3}H]citalopram in different brain regions. (Brain maps taken from Paxinos *et al.*, 1998). Radiographical images are in greyscale to aid in visualisation. Abbreviations: ENT, entorhinal cortex; PAG, periaqueductal gray; DRN, dorsal raphé nucleus; MRN, median raphé nucleus; SuG, superior colliculus; LPAG, lateral periaqueductal gray; SN/PN, substantia nigra/paranigral nucleus area; Hipp, hippocampus; SMT, stria medullaris of thalamus; TN, thalamic nucleus; LH, lateral hypothalamus; PH, posterior hypothalamus; Amyg, amygdala.

	Day 11			*Day 20*		
	Vehicle pre-treated rats (fmol mg^{-1}brain tissue)	*WAY-100635 pre-treated rats (fmol mg^{-1}brain tissue)*	*Binding in WAY-brains expressed as % increase from vehicle levels*	*Vehicle pre-treated rats (fmol mg^{-1}brain tissue)*	*WAY-100635 pre-treated rats (fmol mg^{-1}brain tissue)*	*Binding in WAY-brains expressed as % increase from vehicle levels*
ENT	3923 ± 197	5083 ±175 ***	30	3188 ± 282	3633 ± 293	14
PAG	2718 ± 68	2898 ± 283	7	1849 ± 195	2633 ± 250 *	42
DRN	4339 ± 295	4966 ± 451	14	3078 ± 218	3859 ± 288 *	25
MRN	2633 ± 129	3125 ± 149 *	19	1948 ± 147	2442 ± 181 *	25
Sup Collic.	3342 ± 274	3409 ± 168	2	2230 ± 161	2545 ± 177	14
LPAG	3143 ± 250	2949 ± 218	-6	2281 ± 165	2464 ± 184	8
Hippocampus	5149 ± 246	5447 ± 199	6	4163 ± 258	4418 ± 286	6
SN/PN	3114 ± 110	3472 ± 122	12	2578 ± 175	2374 ± 188	-8
Stria MT	1860 ± 115	2068 ± 109	11	1598 ± 81	1630 ± 90	2
Thalamic N	2741 ± 198	2849 ± 149	4	2313 ± 190	2185 ± 142	-6
Hypothalamus	2611 ± 220	2897 ± 162	11	2377 ± 162	2517 ± 112	6
Amygdala	4872 ± 411	5701 ± 344	17	4810 ± 302	4923 ± 408	2

Table 6.3 - Binding of [^{3}H]citalopram (fmol mg^{-1} brain tissue) in animals chronically pre-treated with vehicle or WAY-100635 (n = 2/3, multiple replicates). Values are mean ± s.e.mean. * $P < 0.05$; *** $P < 0.001$ when compared to vehicle/time match control by unpaired Student's *t*-test. Abbreviations: ENT, entorhinal cortex; PAG, periaqueductal gray; DRN, dorsal raphé nucleus; MRN, median raphé nucleus; SuG, superior colliculus; LPAG, lateral periaqueductal gray; SN/PN, substantia nigra/paranigral nucleus area; Stria MT, stria medullaris of thalamus; Thalamic N, thalamic nucleus.

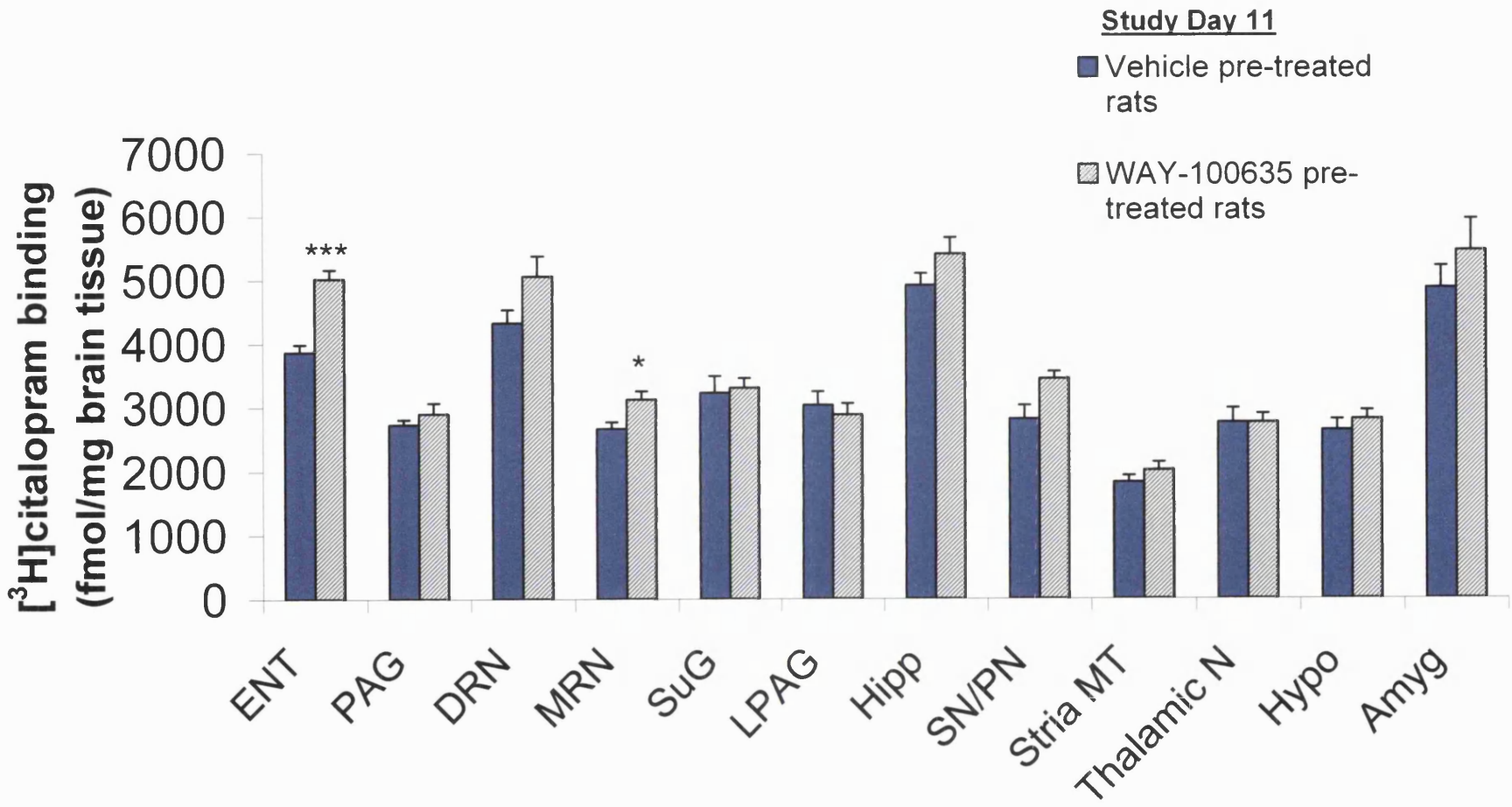


Figure 6.18 - [^{3}H]citalopram binding (fmol mg^{-1} brain tissue) in rat brains after 11-day chronic i.v. administration of WAY-100635 (10 nM) or vehicle control. * $P < 0.05$; *** $P < 0.001$ when compared to vehicle/time match control by unpaired Student's *t*-test; n = 2/3, multiple replicates. Abbreviations: ENT, entorhinal cortex; PAG, periaqueductal gray; DRN, dorsal raphé nucleus; MRN, median raphé nucleus; SuG, superior colliculus; LPAG, lateral periaqueductal gray; SN/PN, substantia nigra/paranigral nucleus area; Stria MT, stria medullaris of thalamus; Thalamic N, thalamic nucleus; Hipp, hippocampus; Hypo, hypothalamus; Amyg, amygdala.

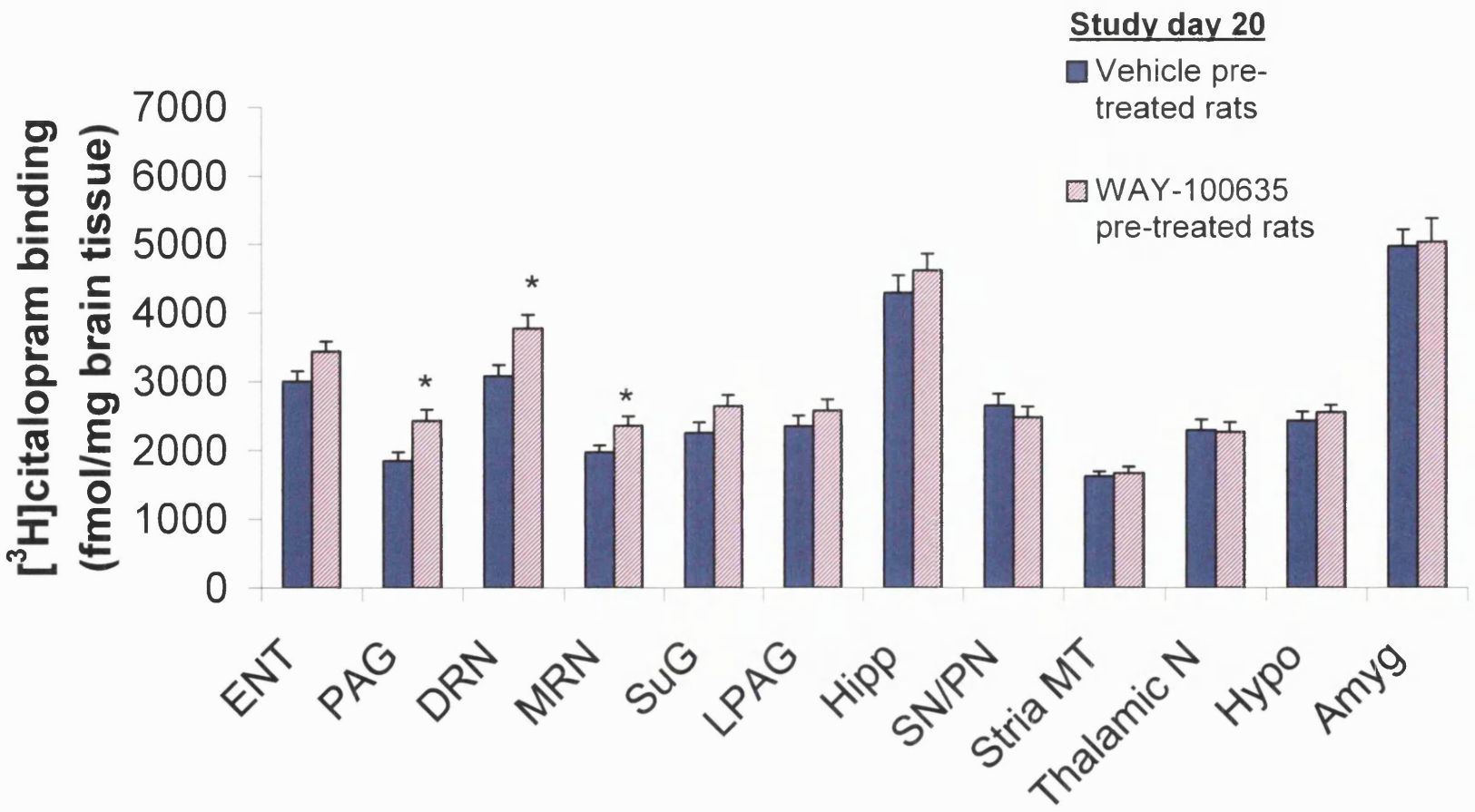


Figure 6.19 - [^{3}H]citalopram binding (fmol mg^{-1} brain tissue) in rat brains 6 days after cessation of 14 days chronic administration of WAY-100635 or vehicle control (i.e. on study day 20). * $P < 0.05$ when compared to vehicle/time match control by unpaired Student's *t*-test; n = 2/3, multiple replicates. Abbreviations: ENT, entorhinal cortex; PAG, periaqueductal gray; DRN, dorsal raphé nucleus; MRN, median raphé nucleus; SuG, superior colliculus; LPAG, lateral periaqueductal gray; SN/PN, substantia nigra/paranigral nucleus area; Stria MT, stria medullaris of thalamus; Thalamic N, thalamic nucleus; Hipp, hippocampus; Hypo, hypothalamus; Amyg, amygdala.

6.4 – Discussion

The present experiments demonstrate that chronic i.v. administration of the 5-HT_{1A} receptor antagonist WAY-100635 (10 nM) causes an approximately 50% increase in the number of [^{3}H]WAY-100635 binding sites in all the brain areas examined (DRN, ENT, hippocampus, hypothalamus and amygdala, see Table 6.1), thus suggesting an increase in the density of 5-HT_{1A} receptors. This increase in 5-HT_{1A} receptor binding sites is observed after 11 days chronic WAY-100635 (10 nM) administration, and still observed in brain areas containing 5-HT_{1A} heteroreceptors even after chronic administration of WAY-100635 has ceased (6 days previous, see Figure 6.13). Interestingly, it appears that chronic administration of a 5-HT_{1A} receptor antagonist may also affect other receptors/transporters involved in 5-HT signalling, since significant changes were also observed in the number of [^{3}H]GR-125743 binding sites (probably 5-$HT_{1B/1D}$ receptors) and [^{3}H]citalopram binding sites (the 5-HTT). It appears that there is a transitory increase in 5-$HT_{1B/1D}$ receptor levels in the PAG and ENT during chronic 5-HT_{1A} receptor antagonist administration, with levels having returned to control values by day 20, i.e. 6 days after chronic administration is suspended (see Table 6.2). Conversely, 5-HTT levels appear to increase further in animals even after ceasing WAY-100635 treatment (i.e. by study day 20), particularly in brain areas known to be involved in micturition (DRN,MRN and PAG, see Table 6.3). Figure 6.20 summarises the changes in 5-HT_{1A} receptor, 5-$HT_{1B/1D}$ receptor and 5-HTT levels in the main brain areas associated with micturition after chronic WAY-100635 (10 nM) administration (on both days 11 and 20 of the study).

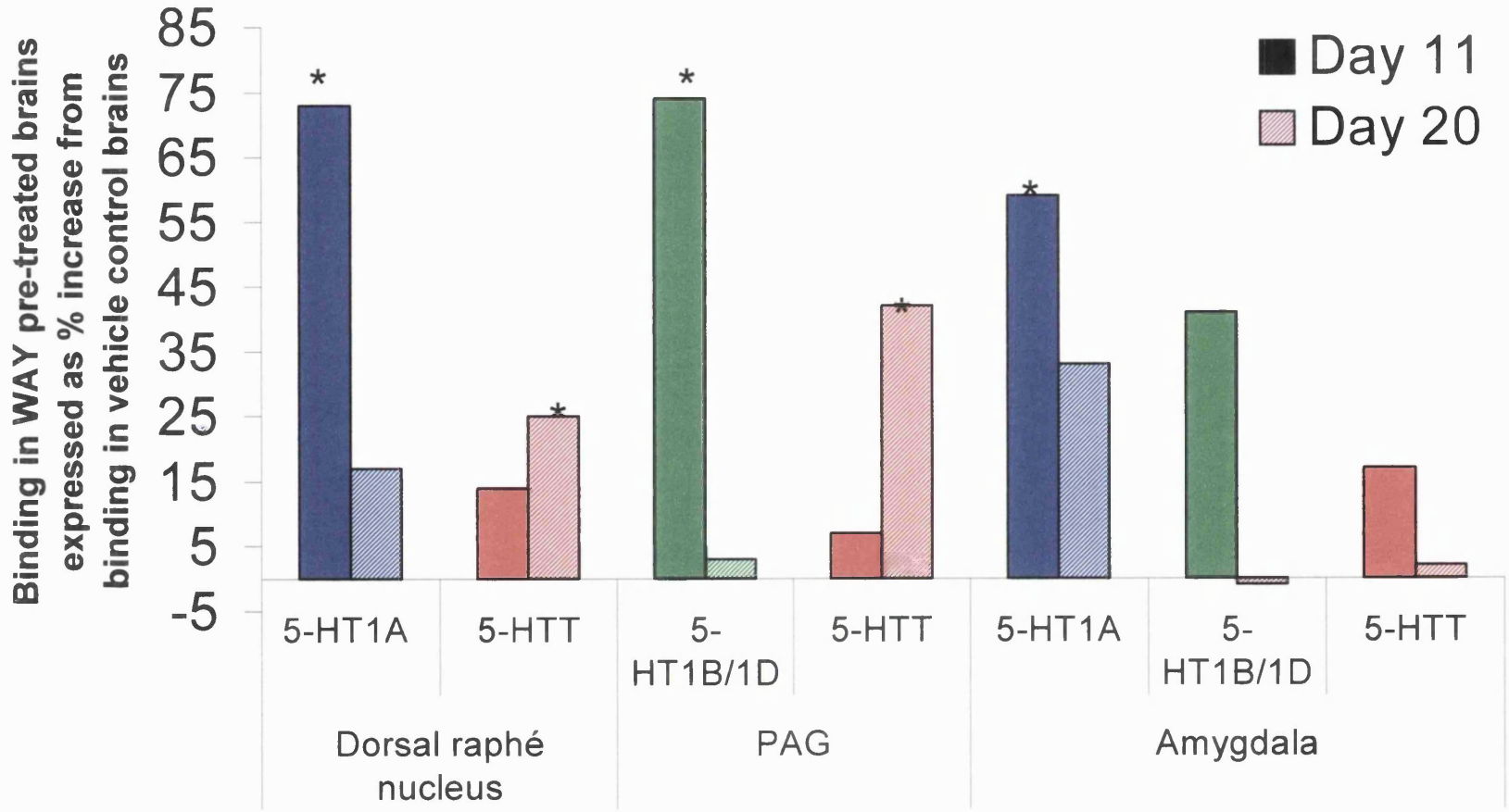


Figure 6.20 – Summary diagram to indicate the changes in [^{3}H]WAY-100635 (for 5-HT$_{1A}$ receptors; blue), [^{3}H]GR-125743 (for 5-HT$_{1B/1D}$ receptors; green) and [^{3}H]citalopram (for 5-HTT sites; red) radioligand binding levels in brain areas associated with micturition in rats after 11 day (i.e. study day 11) and 14 days + 6day wash-out period (i.e. study day 20) chronic administration with WAY-100635 (10 nM free plasma concentration). All values are expressed as the percentage increase from binding in vehicle/time match control animals. *P < 0.05 when compared to vehicle/time match control animals by unpaired Student's *t*-test.

6.4.1 – Tolerance associated with chronic 5-HT_{1A} receptor antagonist administration may be due to an upregulation of 5-HT_{1A} receptors.

The observation that 11 days of chronic administration of WAY-100635 induces an increase in the number of [^{3}H]WAY-100635 binding sites, suggests that the number of supraspinal 5-HT_{1A} receptors is upregulated. This is a highly surprising observation since WAY-100635 is thought to be a neutral antagonist, and would therefore be expected to have no effect on the number of 5-HT_{1A} receptors. However, the upregulation of 5-HT_{1A} receptors would explain the unexpected attenuation of the suppression of micturition observed after chronic 5-HT_{1A} receptor antagonist administration (see chapters 2, 3 and 4). Given that chronic WAY-100635 administration appears to upregulate 5-HT_{1A} receptors, it is possible that this phenomenon is actually due to tolerance of 5-HT_{1A} receptors to WAY-100635, since an increase in receptor number would result in a similar dose of WAY-100635 being less efficacious. This hypothesis is given further support from the observation that the tolerance to robalzotan (2.5 nM) observed after chronic robalzotan (2.5 nM) administration could be overcome by increasing the robalzotan concentration to 250 nM (see 4.3.4), although it is possible that the robalzotan effect at this concentration was not specific to 5-HT_{1A} receptors. Further evidence for the association between 5-HT_{1A} receptor upregulation and the tolerance phenomenon is the observation that both the tolerance remains present, and 5-HT_{1A} receptor levels remain elevated, 6 days after chronic WAY-100635 administration has stopped. In the present study it is clear that this upegulation in 5-HT_{1A} receptor number has occurred after 11 days of chronic WAY-100635 administration, however, it is highly likely that the onset time for this observation is faster than 11 days, since it has been shown previously that tolerance to chronic WAY-100635 administration develops

quickly – within 24 h in conscious rats (see Chapter 5). Therefore, further investigation into the speed of receptor upregulation is required, and would aid confirmation into whether the tolerance observed after chronic WAY-100635 administration is due to an increase in 5-HT_{1A} receptor number.

To the author's knowledge there is only one other report on the effect of chronic WAY-100635 administration on the density of brain 5-HT_{1A} receptors (Hervas *et al.*, 2001a), which found that chronic treatment with WAY-100635 did not modify [^{3}H]8-OH-DPAT labelling in the DRN, dentate gyrus or the hippocampus. This contradicts the results found in the present study, however it must be noted that Hervas *et al* used [^{3}H]8-OH-DPAT (0.5 nM) to label the 5-HT_{1A} receptors instead of ^{3}H]WAY-100635. It is generally thought that radiolabelled antagonists give better estimations of the receptor abundance (De Lean *et al.*, 1980) since they recognize every available receptor with the same affinity, independently of their coupling to G-proteins, whereas agonists have a higher affinity for the receptor population that is coupled to G-proteins and are therefore more likely to have their labelling influenced by the G-protein abundance and the extent of the G-protein coupling to receptors. It must also be noted that [^{3}H]8-OH-DPAT may also label 5-HT_7 receptors (Bonaventure *et al.*, 2002) and since these receptor levels may show a compensatory changes after chronic blockade of 5-HT_{1A} receptors it is possible that these changes may have masked 5-HT_{1A} receptor level increases. However, it must be noted that Hervas *et al* also measured 5-HT_{1A} receptor mRNA levels and these remained unaltered by chronic WAY-100635 administration (Hervas *et al.*, 2001b), but this does not necessarily convincingly indicate that 5-HT_{1A} receptor levels remained unaltered since it has been shown that downregulation of DRN 5-HT_{1A} receptors by chronic alnespirone treatment is not

paralleled by a decrease in 5-HT_{1A} receptor mRNA in the DRN (Casanovas *et al.*, 1999b).

The present study only used one concentration of [^{3}H]WAY-100635 to label 5-HT_{1A} receptors, and therefore to ensure that the level of [^{3}H]WAY-100635 binding in the current study does represent the accurate number of 5-HT_{1A} receptors present, a saturation analysis is required to determine the B_{max} of [^{3}H]WAY-100635 for 5-HT_{1A} receptors. Unfortunately due to a lack of time this has been unable to be conducted, however the present study still provides substantial evidence to suggest that chronic WAY-100635 administration induces upregulation in brain 5-HT_{1A} receptors.

A further consideration is whether an approximately 50 % increase in 5-HT_{1A} receptor levels would be sufficient to result in the observed tolerance phenomenon. Studies using CHO cells stably transfected with rat H_2 receptors to investigate the upregulation of H_2 receptors by cimetidine, showed a 180% upregulation when compared to control cells (Alewijnse *et al.*, 1998), which is substantially higher increase than the current study, although Alewijnse *et al* used a less physiological model. However, even with H_2 receptors, there is currently a paucity of data correlating the level of upregulation with clinical effects of tolerance, therefore it is difficult to predict whether the levels of upregulation observed in the present study would be sufficient to cause tolerance. However, it is interesting to note that autoradiographical studies investigating the association of receptor downregulation with desensitization have found that desensitization of 5-HT_{1A} receptors due to chronic alnespirone administration is associated with only an approximate 25-30% reduction in [^{3}H]8-OH-DPAT labelling in the DRN (Casanovas *et al.*, 1999a), and the compensatory desensitization of 5-HT_{1A}

receptors in 5-HTT knock out mice is attributable to an approximate 50% reduction (female rats; 30% in male rats) in the density of 5-HT_{1A} receptors (Li *et al.*, 2000).

6.4.2 – WAY-100635 and robalzotan – possible inverse agonists at 5-HT_{1A} receptors?

Since WAY-100635 and robalzotan are both though to be neutral 5-HT_{1A} receptor antagonists, the observation that chronic administration of WAY-100635 appears to cause upregulation of brain 5-HT_{1A} receptors is highly surprising. Receptor upregulation has been associated with the chronic administration of inverse agonists (Smit *et al.*, 1996; Milligan *et al.*, 1997; see Daeffler *et al.*, 2000) since inverse agonists selectively stabilize the inactive state of the receptor, thereby halting spontaneous receptor degradation, whilst leaving receptor synthesis unaltered, resulting in upregulating GPCR number. Therefore it is tempting to speculate that WAY-100635 may cause upregulation of 5-HT_{1A} receptors, and hence tolerance, by acting as an inverse agonist in this system. Recent cell based assay evidence to support this hypothesis, has shown both WAY-100635 and robalzotan to act as inverse agonists in a constitutively active system (Pfizer Ltd., 2003; personal communication).

If WAY-100635 and robalzotan are to act as inverse agonists in this system, a further consideration to be made is that the 5-HT_{1A} receptors acted upon by these 'antagonists' must show spontaneous constitutive activity. Given the important role of 5-HT_{1A} autoreceptors in consistently regulating raphé neuronal firing, perhaps it would not be surprising if these receptors showed constitutive activity in addition to, or instead of, endogenous agonist induced tonic activity. To confirm this, studies are required to show

that 5-HT_{1A} autoreceptors from the DRN show basal activity that is not altered by a neutral antagonist, however to the author's knowledge a true neutral antagonist at 5-HT_{1A} receptors has yet to be discovered. The presence of constitutively active 5-HT_{1A} heteroreceptors maybe less likely due to the role these receptors play in regulating more specific physiological processes – i.e. if these receptors were constitutively active there would be less neurotransmitter control over the response. However, it is interesting to note that 6 days after chronic administration of WAY-100635 had stopped, 5-HT_{1A} autoreceptor levels had returned to baseline values, whereas 5-HT_{1A} heteroreceptor levels in the hippocampus and entorhinal cortex remained elevated, perhaps suggesting that these receptors also play an important role in the observed tolerance to WAY-100635 in suppressing micturition.

6.4.3 – Compensatory changes to 5-HT signalling after chronic 5-HT_{1A} receptor antagonist administration.

Due to the importance of 5-HT signalling in so many physiological processes, it is perhaps not surprising that there is a large amount of evidence suggesting that both pharmacological and genetic interference with the 5-HT system can lead to a variety of compensatory adaptations to a number of receptors/transporters involved in 5-HT neurotransmission (see section 1.2.2). Such compensatory mechanisms can include changes in the density of receptors/transporters (for example Li *et al.*, 2000; Ase *et al.*, 2001) and altered G-protein coupling for receptors (Ase *et al.*, 2002). The data from the present study has suggested that chronic treatment with a 5-HT_{1A} receptor antagonist is able to induce changes in the density of 5-$HT_{1B/1D}$ receptors and the 5-HTT, in addition to 5-HT_{1A} receptors themselves. During chronic WAY-100635 administration, there is an

increase in the level of [^{3}H]GR-125743 labelling in the PAG (44%) and the ENT (30%). Given the fact that both 5-HT_{1A} and 5-$HT_{1B/1D}$ receptors may have similar autoreceptor roles (see Stamford *et al.*, 2000), and all these subtypes have been shown to negatively couple to adenylyl cyclase via $G_{(i)}$-type G-proteins, it is perhaps not surprising that these receptors may also be affected by long-term treatment with a ligand which only targets 5-HT_{1A} receptors. Indeed, it has been previously suggested that long-term treatment with inverse agonists for a particular receptor might have effects on other receptors which share the same G-protein or the same signalling pathway (see de Ligt *et al.*, 2000). Recently it has been shown that 5-HT_{1A} receptors couple to both isoforms $G_{i\alpha1}$ and $G_{i\alpha2}$, whereas 5-HT_{1B} and 5-HT_{1D} receptors couple primarily through isoform $G_{i\alpha2}$ (Lin *et al.*, 2002), therefore there is a crossover between the isoform of G-protein used by these 5-HT_1 receptor subtypes and it is possible that the increase in 5-HT_{1A} receptors observed after chronic WAY-100635 administration might result in a depletion of $G_{i\alpha2}$ levels and hence 5-$HT_{1B/1D}$ receptor levels increase in response to increased competition for $G_{i\alpha2}$. However, since the increase in 5-$HT_{1B/1D}$ receptor levels is transitory and not observed after chronic WAY-100635 administration is stopped (i.e. on day 20 of the study), despite 5-HT_{1A} receptor levels remaining elevated, it suggests that the compensatory changes observed in 5-$HT_{1B/1D}$ receptor levels maybe more likely due to their role as terminal heteroreceptors in maintaining functional 5-HT neurotransmission, especially since the increases were brain region specific and found in the PAG and ENT where 5-HT_{1B} receptors are probably located on non-5-HT containing neurones. Interestingly, there is conflicting evidence as to the changes observed with 5-HT_{1B} receptors in 5-HT_{1A} KO mice. Ramboz *et al* (1998) found an increase in the density of 5-HT_{1B} receptors in 5-HT_{1A} receptor KO mice which was associated with an increased sensitivity to the

5-HT_{1B} receptor agonist, CP-93129 (Ramboz *et al.*, 1998), whereas more recent studies using autoradiography (Ase *et al.*, 2001) and the effect of 5-HT_{1B} receptor agonists on the electrically evoked release of [^{3}H]5-HT from pre-loaded brain slices (Richer *et al.*, 2002) have suggested a lack of any compensatory changes in 5-HT_{1B} receptor density in 5-HT_{1A} receptor KO mice.

The present data also showed significant brain region specific increases in the density of [^{3}H]citalopram binding in which could be indicative of an increase in 5-HTT number. The observation of compensatory changes in the level of 5-HTT was not surprising, given that alterations in 5-HTT density have been observed in both 5-HT_{1A} and 5-HT_{1B} KO mice (Ase *et al.*, 2001). However, these studies showed a decrease in 5-HTT density in 5-HT_{1A} receptor KO mice as opposed to the increase observed in the present study after chronic WAY-100635 administration. This apparent contradiction can be explained because the decrease in 5-HTT density was found in the limbic areas of 5-HT innervation where the reduction might decrease 5-HT clearance thus contributing to enhanced 5-HT neurotransmission in the absence of 5-HT_{1A} receptors. However in the present study the increases in 5-HTT density were mainly found in areas where 5-HT_{1A} autoreceptors mediate negative feedback control (i.e. the DRN and MRN), and hence a possible explanation is that the blockade of 5-HT_{1A} receptors by chronic WAY-100635 administration lead to an increase in synaptic levels of 5-HT which were removed by increased levels of the 5-HTT, thus maintaining normal 5-HT neuronal firing. The further increases in 5-HTT density after chronic WAY-100635 administration was stopped (i.e. on day 20 of the study) cannot be explained but it is interesting to note that as 5-$HT_{1B/1D}$ receptor levels in the PAG and ENT returned to baseline values by day 20, 5-HTT levels increased in both these areas.

Given the compensatory changes in 5-$HT_{1B/1D}$ receptor and 5-HTT levels that have been observed in the present study, it is possible that there maybe alterations in the levels of other receptors, transporters and intracellular effectors (e.g. enzymes) which have not been measured in the present study. Of particular interest would be possible compensatory changes in 5-HT_7 receptor levels, since these receptors appear to have a brain expression profile similar to 5-HT_{1A} receptors (Gustafson *et al.*, 1996; see Vanhoenacker *et al.*, 2000), and seem to have similar roles in the control of physiological processes, at least in micturition (Read *et al.*, 2003). Therefore it is highly likely that these receptors would also show compensatory changes after chronic administration of a 5-HT_{1A} receptor antagonist, but due to the lack of a commercially available radioligand selective for 5-HT_7 receptors this was unable to be examined in the present study.

6.4.4 – Concluding remarks

The data from the present study has shown that chronic administration of the 5-HT_{1A} receptor antagonist, WAY-100635, causes an upregulation of [^{3}H]WAY-100635 binding in a number of different brain regions, thus suggesting an increase in the density of 5-HT_{1A} receptors in these areas. This upregulation suggests that WAY-100635 may be acting as an inverse agonist in this system, and the increase in 5-HT_{1A} receptor density may explain the tolerance to WAY-100635 observed as a lack of effect of WAY-100635 on suppressing micturition after chronic administration (see chapter 4). [^{3}H] WAY-100635 binding remained elevated even 6 days after chronic WAY-100635 administration was stopped, indicating the upregulation to be persistent as observed with the tolerance phenomenon. Interestingly, chronic administration with a 5-HT_{1A} receptor antagonist caused compensatory alterations in the level of 5-$HT_{1B/1D}$ receptors and the

5-HTT, thus indicating that the regulation of the 5-HT signalling system is highly complex. This complexity coupled with the evidence that chronic administration of a 5-HT_{1A} receptor 'antagonist' will be ineffective at suppressing micturition due to tolerance, suggest that targeting central 5-HT signalling to treat OAB may be highly problematic.

Chapter 7

General Discussion

7.1 – General aims of the present study

The primary objective for the present study was to confirm the role of 5-HT_{1A} receptors in the control of micturition, and to determine whether 5-HT_{1A} receptor antagonists possess any therapeutic potential in the treatment of overactive bladder. Since chronic administration of 5-HT_{1A} receptor antagonists appeared to cause the onset of tolerance, thus preventing these 5-HT_{1A} receptor ligands from exerting any further effect on micturition, additional aims of the study emerged which included further investigation into the time of onset of this phenomenon and to provide a possible mechanistic explanation.

7.2 – Role of 5-HT_{1A} receptors in the control of micturition

The present study has confirmed that there are two central sites for 5-HT_{1A} receptors involved in the control of micturition in rats. 5-HT_{1A} receptors at both supraspinal and sacral (L6/S1) spinal sites have a physiological role in modulating micturition (see Chapter 2) and blockade of these receptors by the 5-HT_{1A} receptor antagonists, WAY-100635 and robalzotan, causes suppression of the reflex. This is consistent with previous reports of a depressant effect of intravenous and intrathecal administration of WAY-100635 on bladder activity (Testa *et al.*, 1999; Conley *et al.*, 2001; Kakizaki *et al.*, 2001), however, until the very recent publication of a study investigating the effect of intracerebroventricular administration of WAY-100635 in the anaesthetized female rat (Yoshiyama *et al.*, 2003), an actual physiological role for supraspinal 5-HT_{1A} receptors in the control of micturition had yet to be demonstrated. The present study has also shown that, when taking into account the short half-lives of WAY-100635 and robalzotan, these compounds can also suppress micturition during an acute i.v. infusion in conscious rats

(see Chapter 3). Since both WAY-100635 and robalzotan are regarded as 'silent' 5-HT_{1A} receptor antagonists, results from the present anaesthetized study are not able to directly determine whether somatodendritic 5-HT_{1A} autoreceptors or 5-HT_{1A} heteroreceptors are involved in the control of micturition. However, it has been speculated that 5-HT_{1A} receptor antagonists may exert their suppressive effect by acting on raphé 5-HT_{1A} autoreceptors, which acts to unmask the usually tonically suppressed descending raphé-spinal inhibitory pathway to the bladder, hence suppressing micturition (McMahon *et al.*, 1982; Lumb, 1986; Kakizaki *et al.*, 2001; Yoshiyama *et al.*, 2003). The observation in the present study that the length of time of suppression of the micturition reflex is significantly longer after i.t administration of WAY-100635 when compared with i.c.v. administration, possibly suggests that both 5-HT_{1A} autoreceptors and heteroreceptors are involved in the control of micturition, with spinal 5-HT_{1A} heteroreceptors involved in the direct control of the efferent drive to the bladder, whilst supraspinal 5-HT_{1A} autoreceptors also pay a role in modulating the afferent information arising from the bladder (see 2.4.1 for further discussion). Despite the present study clearly showing that 5-HT_{1A} receptors play an excitatory role in the control of micturition in rats, it must be remembered that species variation may exist, with 5-HT_{1A} receptors appearing to have an inhibitory role in micturition in cats (see de Groat, 2002; Thor *et al.*, 2002; see 2.4.2 for further discussion). However, the control of other parasympathetic outflows in this species indicates that these receptors are excitatory (Ramage, 2001).

7.3 – The development of tolerance after chronic and repeated acute administration of 5-HT_{1A} receptor antagonists.

A variety of different models and techniques have been used in the present study to confirm that the chronic administration, and repeat acute administration, of 5-HT_{1A} receptor antagonists results in the development of tolerance to further antagonist administration and hence a lack of effect at suppressing micturition. This phenomenon was first observed in anaesthetized rats after repeat acute administration of WAY-100635 at both the i.c.v. and i.t. levels (see Chapter 2), and also after acute administration (3 h infusion) of both WAY-100635 and robalzotan in conscious rats (see Chapter 3). Studies investigating the effect of chronic WAY-100635 and robalzotan administration in conscious rats also showed that these 5-HT_{1A} receptor antagonists are unable to suppress micturition after chronic administration (see Chapter 4), and further confirmation for the presence of this phenomenon was obtained using radiotelemetric measurements of bladder activity in the presence and absence of chronic WAY-100635 administration (see Chapter 5). The fact that this phenomenon was observed in both anaesthetized and conscious rats, using a variety of models, and with two structurally distinct 5-HT_{1A} receptor antagonists indicates that this is a real effect associated with the chronic blockade/modulation of 5-HT_{1A} receptors, rather than a peculiarity related to a particular compound or model/method of administration. The observation that the 5-HT_{1A} receptor density appears to increase as tolerance to 5-HT_{1A} receptor antagonists develops (see Chapter 6) also provides a possible mechanistic explanation for the observed phenomenon.

Despite the large amount of evidence from the present study to indicate that chronic, and repeated acute, administration of 5-HT_{1A} receptor antagonists results in the development

of tolerance and hence has no suppressive effect on micturition, this observation contradicts the literature. For example Kakizaki *et al.* (2001) found that reproducible inhibition of rhythmic bladder contractions could be evoked after multiple injections (3-4 times, 30 min intervals) of i.v. WAY-100635 at higher doses than those used in the current study, and Yoshiyama *et al.* (2003) also investigated the possibility of a tachyphylactic effect with repeated administration of WAY-100635 using various dosing regimens, but did not observe any significant difference in the time of inhibition of bladder contractions after multiple administration. The reason for this contradiction between the literature and the present study cannot be explained, however it is interesting to note that, although not significant, Yoshiyama *et al.* (2003) did observe a considerable decrease in the time of inhibition of isovolumetric bladder activity with a second dose of i.c.v. WAY-100635 (for example a second dose of 10 μg WAY-100635 had a time of suppression of 9.5 min as opposed to 15.6 min with the initial dose), thus possibly suggesting the presence of a similar phenomenon. Despite the lack of corroboration from the literature, the author believes that the large amount of evidence from the present study is sufficient to determine that the development of tolerance after chronic 5-HT_{1A} receptor antagonist administration is a real effect, at least in rats.

A further point to note regarding the development of the tolerance phenomenon is the speed of onset upon chronic 5-HT_{1A} receptor antagonist administration, and the persistence of the phenomenon after chronic administration had stopped. The studies in conscious rats (see Chapters 4 & 5) showed that the speed of onset of tolerance, as measured by the effect on the rate of micturition, is very fast with tolerance developing within 24 h of the start of chronic administration, and within 12-30 min in anaesthetized

rats (see Chapter 2). It also appears that the tolerance is long-lasting and is present at least 6 days after the cessation of chronic 5-HT_{1A} receptor antagonist administration – further studies are required to determine a more accurate length of time for the persistence of this phenomenon.

The presence of this tolerance phenomenon was surprising given that WAY-100635 and robalzotan are considered to be 'neutral' antagonists, and this sort of 'attenuation' effect is usually associated with agonist administration. However, there are a number of possible explanations for the presence of this phenomenon after chronic/repeated 5-HT_{1A} receptor antagonist administration including, an exhaustion in the releasable stores of 5-HT due to the incapacitation of the 5-HT_{1A} autoreceptor mediated negative feedback control of raphé neuronal firing, which would result in the levels of raphé neuronal firing returning to pre-compound administration levels and hence a 'normal' rate of micturition, although this is unlikely given the persistence of the phenomenon, and the fact that regulation of the serotonergic system is highly controlled and compensatory changes would be likely to replenish releasable stores of 5-HT relatively quickly. Other possible explanations are an increase in the speed of metabolism of the compound after chronic administration, which would result in lower/non-existent free circulating levels of the test compound, or metabolism after chronic administration resulting in a metabolite which is ineffective at blocking 5-HT_{1A} receptors, however both these possibilities can be ruled out by the observation that circulating free plasma levels after 11 days of chronic administration of WAY-100635 and robalzotan were at the target levels (see Chapter 4), thus indicating that chronic administration had not caused the immediate metabolism of these compounds. Since tolerance to compounds in a clinical setting is often due to an upregulation of receptor number (see Milligan *et al.*, 1997), it is possible that the

phenomenon observed in the present study could be due to an upregulation in 5-HT_{1A} receptor density after chronic 5-HT_{1A} receptor antagonist administration. This was investigated in the present study using quantitative autoradiography, which showed an increase in the levels of [^{3}H]WAY-100635 binding in the brains of rats showing tolerance to 5-HT_{1A} receptor antagonists after chronic administration (see Chapter 6), thus suggesting that an increase in the number of 5-HT_{1A} receptors may explain the observed tolerance phenomenon. These changes in the binding of [^{3}H]WAY-100635 were also observed in the hippocampus and entorhinal cortex in rats 6 days after the cessation of chronic WAY-100635 administration indicating that changes in the number of 5-HT_{1A} receptors mirror the persistence of the tolerance phenomenon, thus providing further evidence that this phenomenon could be due to an increase in 5-HT_{1A} receptor density.

However, despite this quantitative autoradiography study suggesting that 5-HT_{1A} receptor number may have increased after chronic WAY-100635 administration, this present study does not provide a completely accurate measure of the density of 5-HT_{1A} receptors since only one concentration of [^{3}H]WAY-100635 was used, and therefore the B_{max} of [^{3}H]WAY-100635 for 5-HT_{1A} receptors in these pre-treated rat brains is unknown. It would be interesting to carry out *in situ* hybridisation or competitive RT-PCR studies to confirm whether the increase in receptor number is mirrored by an increase in 5-HT_{1A} receptor mRNA. It must also be remembered that although there is an increase in [^{3}H]WAY-100635 binding in brains from rats pre-treated with chronic WAY-100635 administration, the increased number of receptors binding the radioligand may not actually be functional, for example changes in the G-protein coupling to the receptor or further downstream changes to the effector systems involved may render the 'newly expressed' receptor useless. The present study did attempt to examine the functional

status of the 5-HT_{1A} receptors in rats chronically pre-treated with WAY-100635 by examining the hypothermic effect of 8-OH-DPAT, since it would be expected that if the density of 5-HT_{1A} receptors had increased, and these receptors were functional, then there would be an increased sensitivity to 8-OH-DPAT, however due to the problems discussed in section 5.4.2, the functional status of these receptors was unable to be assessed. It would be interesting, to further examine the functional status of these 5-HT_{1A} receptors *in vivo*, for example by investigating the capacity of 5-HT_{1A} autoreceptor stimulation in chronically pre-treated rats to trigger inhibition of 5-HT synthesis (5-HT_{1A} receptor agonists should exert a negative effect on brain 5-HT synthesis because of their inhibitory action on 5-HT neuron firing; Hamon *et al.*, 1988). Given the evidence for changes in G-protein coupling of 5-HT_{1A} receptors in serotonergic knock out mice (Fabre *et al.*, 2000; Ase *et al.*, 2002), it would also be very interesting to investigate whether chronic WAY-100635 administration induces changes in the G-protein coupling of 5-HT_{1A} receptors, for example by measuring the incorporation of [^{35}S]GTPγS induced by agonist stimulation (e.g. Ase *et al.*, 2002). Given the recent speculation that 5-HT_{1A} receptors may show promiscuous coupling to multiple G-protein subtypes, hence enabling their ligand profile to be altered and perhaps explaining the contrasting responses of 5-HT_{1A} auto- and heteroreceptors (see section 1.5.7), it is also possible that chronic WAY-100635 administration may induce switching in the G-protein coupling to 5-HT_{1A} receptors, thus preventing further blockade of the 5-HT_{1A} receptor from having any effect. It would be very interesting to examine this possibility further, for example by measuring the levels of $G\alpha_{i3}$, $G\alpha_{i2}$, $G\alpha_{i1}$ and G_o proteins (using immunoblots as described by Li *et al.*, 2000) in various brain regions in control and chronic WAY-100635 treated animals and comparing the expression profile and levels of each of

these G-proteins. Given the very fast onset time of the tolerance phenomenon (< 24 h after chronic administration and < 12 min after repeated administration in anaesthetized rats), it is highly possible that there may be a number of explanations for this phenomenon, since it is unlikely that an increase in the density of 5-HT_{1A} receptors can explain the tolerance observed after 12 min as it is doubtful that the number of 5-HT_{1A} receptors could increase to a sufficient level to cause tolerance after this short period of time. Therefore, it is possible that changes in G-protein coupling may underlie the initial tolerance and are followed by an increase in the number of 5-HT_{1A} receptors, which accounts for the observed persistence of the tolerance phenomenon.

7.4 – WAY-100635 and robalzotan – possible inverse agonists?

Since WAY-100635 and robalzotan are both thought to be neutral 5-HT_{1A} receptor antagonists, the observation that chronic administration of WAY-100635 appears to cause upregulation of brain 5-HT_{1A} receptors is highly surprising since neutral antagonists do not usually alter the expression of receptors (as opposed to agonists which are known to induce receptor downregulation after chronic administration). Thus, suggesting that these 5-HT_{1A} receptor ligands may not be acting as 5-HT_{1A} receptor antagonists in this system.

Agonists are known to preferentially bind to and enrich the numbers of receptors in the R* state (active), whereas inverse agonists preferentially bind to and enrich the number of receptors in the R state (inactive) and antagonists bind to both states with equal affinity (see 1.5 for further explanation). In a constitutively active system where the receptor is able to produce cellular signalling in the absence of agonist binding (i.e. the R* state is

favoured) an inverse agonist will shift the equilibrium in favour of the R state, resulting in a decrease in the constitutive signalling and hence moving the 'baseline' activity in the opposite direction to an agonist. This implies that inverse agonists 'actively' alter signalling on their own, rather than just preventing agonist activity as is true for antagonists. Receptor upregulation has been associated with the chronic administration of inverse agonists (Smit *et al.*, 1996; Milligan *et al.*, 1997; see Daeffler *et al.*, 2000) since inverse agonists selectively stabilize the inactive state of the receptor, thereby halting spontaneous receptor degradation, whilst leaving receptor synthesis unaltered, resulting in upregulating GPCR number. Therefore it is tempting to speculate that WAY-100635 may cause upregulation of 5-HT_{1A} receptors, and hence tolerance, by acting as an inverse agonist in this system. This is possible since recent cell based assays, have shown both WAY-100635 and robalzotan to act as inverse agonists in a constitutively active system (Pfizer Ltd., 2003; personal communication) and WAY-100635 has been shown to act as an inverse agonist in a study employing HeLa cells (Cosi *et al.*, 2000).

The observation, after chronic administration, that WAY-100635 and robalzotan may actually be acting as inverse agonists rather than true antagonists is perhaps not surprising when considering the fact that in the majority of systems, the R* state of the receptor is energetically highly unfavourable, and therefore the equilibrium between the two states of the receptor favours the inactive (R) state. In these circumstances, the effects of an inverse agonist will appear to be qualitatively similar to those effects observed with a neutral antagonist, since antagonists bind to both states with equal affinity, but will bind to R most strongly in this situation, as will inverse agonists (due to their inherent preference for the R state). Therefore, it is possible that many ligands originally thought

to be acting as 'antagonists' may actually be inverse agonists. This does not alter the effect these compounds will have in the short term, but it may have therapeutic implications, since long-term administration of inverse agonists will lead to an increase in receptor number, and hence possibly tolerance, whereas a true 'antagonist' would have no effect on levels of receptor expression. The accurate definition of a neutral antagonist is a ligand that has equal affinity for both the active and inactive receptor conformations, and ligands which fit this description are very rare. Instead, it is more accurate to describe antagonists as 'partial inverse agonists' with various degrees of efficacy and variable affinity for the two receptor states. Thus, it is tempting to speculate that there is only one class of ligands after all – i.e. agonists, whether they are full, partial, partial inverse ('antagonists') or full inverse. It will be interesting to observe whether, in time, more 'antagonists' will actually be shown to be acting as inverse agonists.

Assuming the above hypothesis explains the observation of tolerance after chronic WAY-100635 administration, it would suggest that a true 'neutral antagonist' would not show tolerance after chronic administration and may therefore be of use in the treatment of overactive bladder. Therefore, it would be very interesting to test the effect of a neutral 5-HT_{1A} receptor antagonist on the micturition reflex, and to also observe the effects after chronic administration. However to the author's knowledge, a true neutral 5-HT_{1A} receptor antagonist which does not show any inverse agonist activity in a constitutively active system, has yet to be discovered.

7.5 – Therapeutic implications and concluding remarks

The results arising from this present study have significant therapeutic implications, since they indicate that the long-term administration of 5-HT_{1A} receptor 'antagonists' are unable to suppress micturition and alter the rate of voiding due to the development of tolerance. Therefore, this class of compound is unlikely to be clinically efficacious in the treatment of overactive bladder and has little therapeutic potential. The data from the present study has also emphasized the importance, particularly in a drug development setting, of clearly distinguishing between antagonists and inverse agonists, since this will be a determining factor in the potential long-term efficacy of a drug.

Chapter 8

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Appendix: Publications arising from this thesis

GLEW, A.G. (NEE SECKER), CLARKE, N., THURLOW, R.J., WESTBROOK, S.L. & RAMAGE, A.G. (2003). Evidence that chronic administration of the 5-HT_{1A} receptor antagonist, WAY-100635, upregulates 5-HT_{1A} receptors in the brain of female rats: a quantitative autoradiography study. *Br. J. Pharmacol. (pA2 online)*, **1,** 109P (at www.pa2online.org/Vol1Issue4abst109P.html).

GLEW, A.G. (NEE SECKER), HALL, D., WESTBROOK, S.L. & RAMAGE, A.G. (2003). Evidence that the selective 5-HT_{1A} receptor antagonists WAY-100635 and robalzotan (NAD-299) do not suppress micturition during or after chronic administration in female rats. *Br. J. Pharmacol. (pA_2 online)*, **1,** 108P (at www.pa2online.org/Vol1Issue4abst108P.html).

SECKER, A.G., WESTBROOK, S.L. & RAMAGE, A.G. (2003). Evidence that central 5-HT_{1A} receptors control micturition at both a supraspinal and sacral spinal sites in female urethane anaesthetised rats. *Br. J. Pharmacol.*, **140,** 20P.

SECKER, A.G., NAYLOR, A.M. & RAMAGE, A.G. (2002). A role for supraspinal 5-HT_{1A} receptors in the control of micturition in female urethane anaesthetized rats. *Pharmacologist*, **44,** A186.