Serotonin & Ejaculation:

a psychopharmacological and neuroanatomical approach

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Serotonin & Ejaculation:
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Serotonine & Ejaculatie:
een psychofarmacologische en neuroanatomische benadering

(met een samenvatting in het Nederlands)

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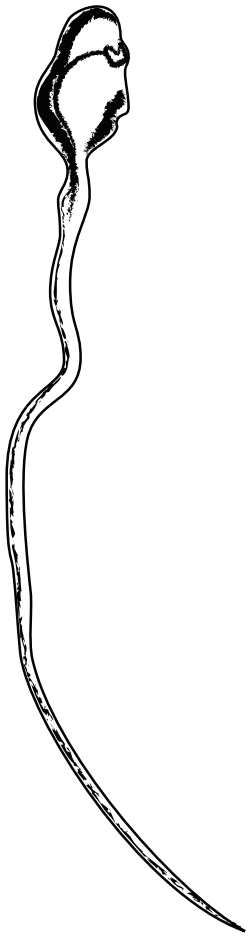
You can't hurry love
No, you'll just have to wait
Just trust in a good time
No matter how long it takes

Phil Collins, SkyRadio, 2002-2005

Contents

Chapter 1



General Introduction

TR de Jong

Aim of the thesis

Ejaculatory dysfunctions, such as lifelong premature or retarded ejaculation, are fairly common human disorders (Waldinger, 2005; Waldinger et al., 2005b). Although it was long thought that ejaculatory disorders are based on psychological problems (Masters and Johnson, 1970), the neurobiological origin became evident when antidepressant drugs that alter serotonergic neurotransmission appeared to relieve premature ejaculation (Waldinger, 2005).

The large impact that ejaculatory disorders can have on the quality of life of both patients and their partners (Hartmann et al., 2005; Waldinger and Schweitzer, 2005) encourages the search for new drugs that specifically raise or lower the ejaculatory threshold. In order to find such drugs, the neuroanatomical, physiological and pharmacological aspects of ejaculation need to be determined.

The present thesis focuses on the psychopharmacology and neuroanatomy of the serotonergic control over ejaculation. The practical and ethical limitations to conduct experiments in this line of research in humans necessitate the use of animal models. Male Wistar rats are commonly used in (sexual) behavioural studies, and were therefore used in all experiments in this thesis. The psychopharmacological experiments were performed using various drugs that alter serotonergic neurotransmission, in particular selective serotonin reuptake inhibitors (SSRIs), and immunohistochemical staining of the immediate early gene c-fos was used for neuroanatomical investigations.

Male rat sexual behaviour

The sexual behaviour of male Wistar rats can be quantified easily and reliably in laboratory settings. For a standard test in our lab, male rats are allowed to copulate freely with a receptive female for 30 min in a red-lighted room.
Rats with normal sexual behaviour always display the same components of copulation. During the first few seconds the male shows appetitive behaviour, such as sniffing the female anogenital region to obtain pheromonal cues of sexual receptivity, and pursuing the female that typically hops and darts away from the male. Following a short chase, the female rat halts and assumes the lordosis position, consisting of an arched back and deflection of the tail to one side allowing the male access to the vagina. The male responds by mounting the female, which is scored by the observer as a 'mount'. If the male rat has a penile erection while mounting, vaginal penetration might be achieved. This is behaviourally distinguished from a mount by an active, jumping dismount and is scored by the observer as an 'intromission'. The latency times from the introduction of the female to the first mount or intromission, which are respectively called the mount latency and intromission latency, can be used as a measurement of motivation (Agmo, 1997; 1999; Bitran and Hull, 1987; Meisel and Sachs, 1994; Pfaus, 1996).
During the next few minutes a pattern occurs of mounts and intromissions, separated by bouts of resting, autogrooming and anogenital investigation and pursuit of the female. This consummatory behaviour usually culminates in an ejaculation, which is distinguishable from mounts and intromissions by the length of the pelvic thrust and the typical lifting of the forepaws directly after ejaculating. The ejaculation latency is measured as the time between the first mount, with or without intromission, and the ejaculation. An ejaculation is always followed by a post ejaculatory interval: a period of rest that ends at the moment of the next mount with or without intromission (Agmo, 1997; Bitran and Hull, 1987; Meisel and Sachs, 1994; Pfaus, 1996).
Male Wistar rats usually display a learning curve over the first sexual behaviour tests (Pfaus *et al.*, 2001), with increasing ejaculation frequencies and decreasing ejaculation latencies (unpublished observations). Therefore, each rat needs to be trained in order to display stable ejaculatory behaviour prior to the start of an experiment. Experienced untreated male Wistar rats show a fairly stereotyped pattern of copulation and ejaculation as illustrated in figure 1. This figure depicts the sexual behaviour of male rats that have gained sexual experience in three similar tests in the preceding weeks. The males display approximately ten mounts and ten intromissions prior to the first ejacu-

lation, which usually takes place within eight minutes, and subsequently rest for five minutes before starting a new ejaculatory cycle.
Interestingly, there are large individual differences in ejaculation latency and mount frequency compared to the highly stereotyped intromission frequency and post ejaculatory interval in this population of rats. Male Wistar rats are apparently able to display delayed or rapid ejaculation under natural circumstances, equivalent to humans (Waldinger *et al.*, 2005a). In the present thesis, rats displaying stable, normal ejaculation frequencies and ejaculation latencies were selected for the psychopharmacological and neuroanatomical experiments, whereas rats displaying persistent retarded or rapid ejaculation were analyzed as a possible animal model for ejaculatory disorders.

Serotonin and sexual behaviour
The neurotransmitter serotonin (5-hydroxytryptamine or 5-HT) is synthesized in neurons in the brainstem, predominantly located in the midline raphe nuclei. Serotonergic fibres are widely distributed throughout the central nervous system and spinal cord (Steinbusch, 1981). When serotonin is released it can bind to at least 14 different serotonergic receptor subtypes located on pre- or postsynaptic soma, dendrites or axon terminals (Barnes and Sharp, 1999). In turn, serotonergic receptors modulate neurotransmission in postsynaptic cells (Raymond *et al.*, 2001), or autoregulate serotonergic neurotransmission via negative feedback loops (Pineyro and Blier, 1999).
Serotonin has been implicated in many behavioural and physiological processes, such as blood pressure, body fluid homeostasis, locomotion, food intake, nociception, cognition, arousal, stress responses and mood. In addition, serotonin is thought to play a significant role in sexual behaviour. Elevated serotonin levels are generally found to inhibit ejaculation, and 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2C} receptors appear to exert control over ejaculation (Giuliano and Clement, 2005). A detailed overview of the existing literature investigating the role of serotonin in the ejaculatory threshold is given in chapter 2.

Staining of Fos

Immediate early genes are expressed rapidly upon cell stimulation, and predominantly encode transcription factors that modify the expression of target genes. Subsequently, target gene expression can alter the phenotype of the cell by shifting the affinity or number of specific receptors, or regulating the synthesis of certain enzymes

and neurotransmitters (Curran and Morgan, 1995; Herrera and Robertson, 1996; Pfaus and Heeb, 1997).

The immediate early gene *c-fos* is widely used as a functional anatomical mapping tool for various reasons: its transcription levels are low under basal conditions, transcription can be induced upon a wide range of stimuli, and the protein Fos that is expressed by the *c-fos* gene is nuclear and can be easily detected and quantified using immunohistochemistry. In addition, the expression of the Fos protein reaches its maximum between one and three hours following a stimulus, which is a reasonable time frame to combine with behavioural studies. Taken together, *c-fos* can be used to identify cells and extended circuitries that become activated in response to various stimuli (Hoffman and Lyo, 2002; Kovacs, 1998).

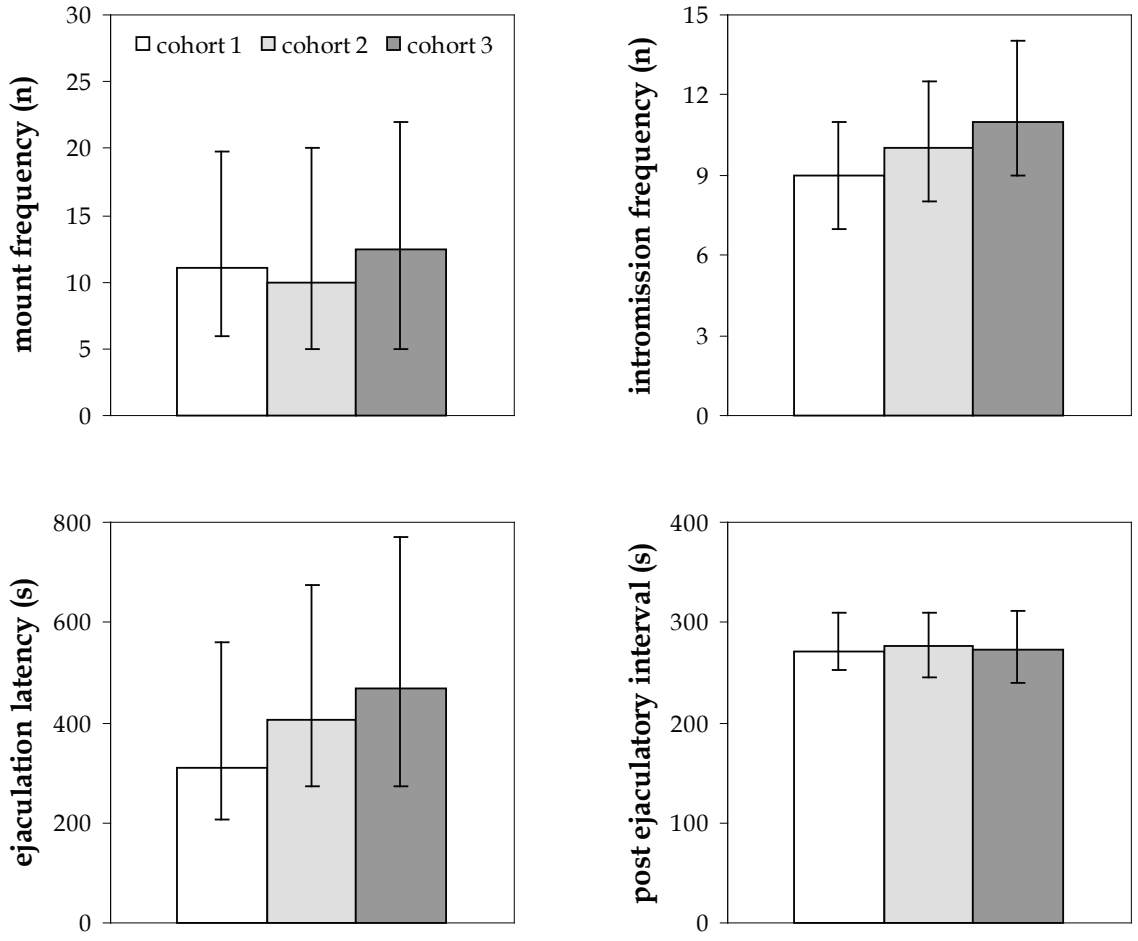


Figure 1. The sexual behaviour of three cohorts of untreated, experienced male Wistar rats (white bars: n=99; light grey bars: n=100; dark grey bars: n=99) in their fourth weekly 30 min training session with a receptive female rat. The bars represent medians; the error bars represent the first and third quartile ranges.

The most important final common pathways that induce *c-fos* expression are increases of intracellular calcium^{2+} or cyclic adenosine monophosphate (cAMP) levels, leading to the phosphorylation of cAMP response-element binding protein (Herrera and Robertson, 1996; Hoffman and Lyo, 2002). Since these pathways are predominantly engaged by stimuli that evoke increased firing of the neuron, Fos is often profiled as a marker for cell excitation. However, depolarization without *c-fos* expression can occur, for instance because a specific temporal pattern of action potentials is required or the appropriate second messenger systems are not implicated. Reversely, depolarization is not always needed, since some cells express *c-fos* permanently and others express *c-fos* in response to hyperpolarization (Fields *et al.*, 1997; Hoffman and Lyo, 2002; Kovacs, 1998; Pfaus and Heeb, 1997). Taken together, the Fos expression in response to sexual behaviour or serotonergic drugs, as investigated in this thesis, should be interpreted with care.

Fos and sexual behaviour

Many parameters of sexual behaviour strongly induce the expression of *c-fos* in the male rat brain (Pfaus and Heeb, 1997). Fos-immunoreactivity increases consistently in response to pheromones of a receptive female in the medial preoptic area (MPOA), the posterodorsal part of the medial amygdala (MeApd) and the posterior part of the medial bed nucleus of the stria terminalis (BNSTpm) of male rats, and this expression is further enhanced in response to mounts and intromissions (Baum and Everitt, 1992; Bressler and Baum, 1996; Coolen *et al.*, 1996). In addition, *c-fos* is strongly expressed following the occurrence of an ejaculation in a specific subset of neurons in the MeApd and BNSTpm, as well as an area in the central tegmental field: the medial parvocellular subparafascicular thalamic nucleus or mSPFp (Baum and Everitt, 1992; Coolen *et al.*, 1997a; Coolen *et al.*, 1996; Greco *et al.*, 1996). The pattern of Fos-immunoreactive cells in response to ejaculation is shown in figure 2 (unpublished photographs from our lab).

The combination of data obtained using *c-fos* expression, lesions (Claro *et al.*, 1995; Hansen *et al.*, 1982; Heeb and Yahr, 2000; Kondo and Yamanouchi, 1995; Liu *et al.*, 1997b; Meisel and Sachs, 1994; Paredes *et al.*, 1993) and tract-tracing studies (Coolen *et al.*, 1998; Coolen *et al.*, 2003a; Heeb and Yahr, 2001) in male rats and gerbils has lead to the theory that the MPOA integrates sensory information induced by pheromones, reaching the MPOA via the vomeronasal organ, medial amygdala and bed nucleus of the stria terminalis, and induced by genital stimulation, reaching the MPOA via the lumbosacral spinal cord and the mSPFp. The MPOA subsequently promotes copu-

latory behaviour and ejaculation. Once a rat has ejaculated, the specific clusters of Fos-positive neurons in the MeApd, BNSTpm and mSPFp might play a role in the inhibition of sexual behaviour during the post ejaculatory interval (Coolen *et al.*, 2004b; Coolen *et al.*, 1997a).

Copulation, oestrous female odours or exposure to sex-related environmental cues also causes an increase in Fos-immunoreactivity in the ventral tegmental area (VTA) and the core and shell of the nucleus accumbens (AcbSh and AcbC). Since the VTA and Acb are associated with reinforcement and reward, this increased Fos expression might reflect the motivational and rewarding aspects of sexual behaviour (Balfour *et al.*, 2004; Kippin *et al.*, 2003; Robertson *et al.*, 1991).

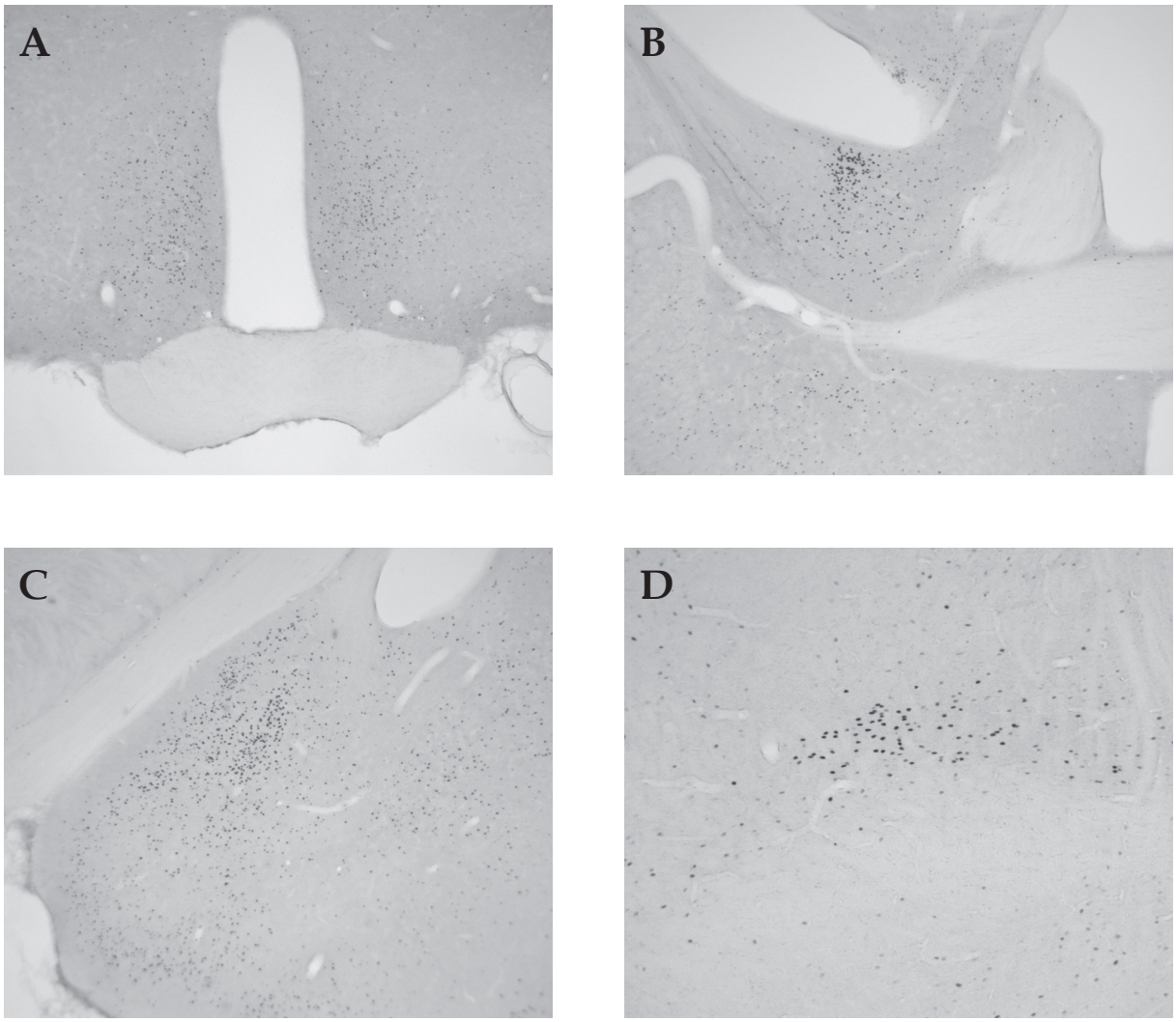


Figure 2. Fos-immunoreactivity in the medial preoptic area (A; bregma –0.12), the rostral posteromedial bed nucleus of the stria terminalis (B; bregma –0.60), the posterodorsal medial amygdala (C; bregma –3.14) and the medial parvocellular subparafascicular thalamic nucleus (D; bregma –4.44) of male Wistar rats perfused 1h after a 30-min sexual behaviour test including ejaculations with a receptive female.

Fos and serotonin

In addition to behavioural studies, the expression of *c-fos* can also be studied in response to psychopharmacological drugs in order to locate brain areas affected by these drugs. The effect of increased 5-HT neurotransmission on the expression of *c-fos* has been investigated using the serotonin precursor 5-hydroxytryptophan (5-HTP), the serotonin releaser (dex)fenfluramine, and various SSRIs. The effects of selective 5-HT_{1A} and 5-$HT_{2A/2C}$ receptor agonists on the expression of *c-fos* have been studied as well. The results of these investigations are summarized in table 1.

Although alterations in serotonin levels cause strong increases in *c-fos* expression, the underlying neurobiological substrates are not yet understood. Some effects might be attributed to direct activation of local serotonin receptors that cause an increase of intracellular calcium^{2+} or cAMP levels (Raymond *et al.*, 2001), whereas other effects are possibly indirect effects of the stress or nausea induced by the drugs (Kovacs, 1998; Veening *et al.*, 1998).

The sensitivity of *c-fos* expression to both sexual behaviour and serotonergic drugs is used in this thesis to find brain and spinal cord areas in which alterations in serotonergic neurotransmission induced by psychopharmacological drugs might have caused changes in sexual behaviour of the male Wistar rat.

Outline of the thesis

The present thesis focuses on the psychopharmacology and neuroanatomy of the serotonergic control over ejaculation.

In **chapter 2**, the existing literature regarding the influence of serotonin on the ejaculatory threshold is reviewed. The emphasis lies on the current knowledge about the effects of psychopharmacological drugs that alter serotonergic neurotransmission on the ejaculatory threshold, and the underlying neuroanatomical substrate.

In **chapter 3**, it is investigated whether 5-HT_{1A} receptor activation can compensate for the effects of SSRI-induced increased serotonin levels on ejaculation. Therefore, sexual behaviour is studied in rats treated with the SSRI citalopram, the selective 5-HT_{1A} receptor antagonist WAY-100635 or both. Possible brain and spinal cord areas involved in the effects of citalopram and/or WAY-100635 on ejaculation are determined using Fos-immunoreactivity.

In **chapter 4** it is investigated whether desensitization of 5-HT_{1A} receptors plays a role in SSRI-induced delayed ejaculation. Therefore, the degree to which the 5-HT_{1A} recep-

Table 1. Overview of the literature reporting an increased *c-fos* expression in response to various serotonergic drugs in the cortex (Cx), prefrontal cortex (PFC), nucleus accumbens core and shell (AcbC and AcbSh), caudate putamen (CPu), ventral lateral septum (LSV), (dorsolateral) bed nucleus of the stria terminalis (BNST(dl)), medial preoptic nucleus (MPN), medial parvocellular and posterior magnocellular paraventricular hypothalamic nucleus (PVNmp and PVNpm), supraoptic nucleus (SON), ventromedial and dorsomedial hypothalamic nucleus (VMH/DMH), central amygdala (CeA), paraventricular thalamic nucleus (PV), locus coeruleus (LC), external lateral parabrachial nucleus (LPBE) and (medial) nucleus of the solitary tract (NST(m)).

Brain area	(D)Fen-fluramine	5-HT precursor	Citalo-pram	Fluoxetine	Fluvox-amine	5-HT$_{1A}$ agonists	5-HT$_{2A/2C}$ agonists
Cx	7,8,10,17	15				21	
PFC		9				3,4	
AcbC		15					
AcbSh				14			
CPu	7,8,10,17	15		22			
LSV				19,22			
BNST(dl)	6,7,8,10		16,20	19,20,22	24	1,2	
MPN		15		22			
PVNmp	6,7,8	15		11,22		1,2	12,23
PVNpm	6,7,13,18			11,22			23
SON	6,8		9	22			23
VMH/DMH				22			23
CeA	6,7,8,10,18		9,16,20	14,19,20,22	24	1,2	
PV	8	15	9				
LC						4,5	
LPBE	7,8,10,17			19	24		
NST(m)	7,8,10,17				24		

1 (Compaan *et al.*, 1996); 2 (Compaan *et al.*, 1997); 3 (Hajos *et al.*, 1999); 4 (Hajos-Korcsok and Sharp, 1999); 5 (Hamamura *et al.*, 1997); 6 (Javed *et al.*, 1999); 7 (Javed *et al.*, 1998); 8 (Javed *et al.*, 1997); 9 (Jongsma *et al.*, 2002); 10 (Li and Rowland, 1996); 11 (Lino-de-Oliveira *et al.*, 2001); 12 (Mikkelsen *et al.*, 2004); 13 (Mikkelsen *et al.*, 1999); 14 (Miyata *et al.*, 2005); 15 (Moorman *et al.*, 1995); 16 (Morelli and Pinna, 1999); 17 (Rouillard *et al.*, 1996); 18 (Rowland *et al.*, 2001); 19 (Salchner and Singewald, 2002); 20 (Thomsen and Helboe, 2003); 21 (Tilakaratne and Friedman, 1996); 22 (Torres *et al.*, 1998); 23 (Van de Kar *et al.*, 2001); 24 (Veening *et al.*, 1998)

tor agonist 8-OH-DPAT is able to facilitate ejaculation is assessed in rats chronically pre-treated with vehicle, paroxetine or fluvoxamine, two SSRIs that show marked differences in their effects on ejaculation latency.

In **chapter 5** the pattern of *c-fos* expression in the brain induced by sexual behaviour under the influence of 8-OH-DPAT or saline is described. This pattern is compared between rats chronically pretreated with paroxetine or vehicle, in order to correlate the different effects of the drug treatments on neuronal activity with their effects on ejaculation.

Activation of 5-HT_{1A} receptors causes the release of oxytocin in the blood plasma, and this is inhibited by chronic paroxetine treatment. Since oxytocin is known to lower the ejaculatory threshold, increased oxytocin release induced by 5-HT_{1A} receptor activation might prevent delayed ejaculation following acute treatment with paroxetine. Therefore, the effects of paroxetine co-administrated with the selective peripheral oxytocin receptor antagonist L-368899 on ejaculation were compared with the effects of paroxetine co-administered with WAY-100635, as desribed in **chapter 6**.

Although experiments with psychopharmacological drugs are able to provide useful knowledge to implement in the treatment of human ejaculatory dysfunctions, an animal model of naturally occurring delayed or premature ejaculation would greatly add to the understanding of these disorders. Such an animal model is presented and analyzed in **chapter 7**.

In **chapter 8** all preceding chapters are summarized, integrated and discussed.

Chapter 2

Serotonin and the neurobiology of the ejaculatory threshold

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Submitted to Neuroscience and Biobehavioral Reviews

Abstract

Disorders of the ejaculatory threshold, for example lifelong premature ejaculation, are fairly common in humans and can have a great impact on the quality of life.

Research in humans and rats have indicated that increased serotonin levels in the central nervous system elevate the ejaculatory threshold, probably via 5-HT_{1B} and 5-HT_{2C} receptors, whereas depletion of serotonin decreases the ejaculatory threshold. 5-HT_{1A} receptor activation strongly lowers the ejaculatory threshold, probably mediated by both the reduction of serotonin levels via presynaptic 5-HT_{1A} receptors and yet unknown effects of postsynaptic 5-HT_{1A} receptors.

The present review attempts to integrate psychopharmacological data on serotonergic control over ejaculation with the knowledge of the neuroanatomical substrate of ejaculation, indicating the importance of the lumbosacral spinal cord, the nucleus paragigantocellularis, the lateral hypothalamic area and several other supraspinal areas. In addition, the gaps in our understanding of the role of serotonin in the ejaculatory threshold are discussed. Filling in those gaps might help to design specific drugs that alter the ejaculatory threshold, thereby alleviating ejaculatory disorders.

Introduction

Male sexual behaviour differs widely between mammalian species, but it has two common factors: the stimulation of the genitals by insertion of the penis into the vagina (intromission) and the expulsion of semen in the female genital tract (ejaculation) (Meisel and Sachs, 1994). The ejaculatory threshold, which can be defined as the number of intromissions preceding ejaculation (intromission frequency) and/or the latency time from the start of copulation to ejaculation (ejaculation latency), can therefore be determined for all male mammals including rats and humans (Bitran and Hull, 1987; Waldinger, 2003).

Investigating the ejaculatory threshold is of great importance, since disorders of this threshold such as lifelong premature and retarded ejaculation are fairly common in human males and can have a great impact on the quality of life (Hartmann *et al.*, 2005; Waldinger, 2005; Waldinger and Schweitzer, 2005; Waldinger *et al.*, 2005b). Premature ejaculation is now often successfully treated with antidepressant drugs that alter serotonergic neurotransmission (Waldinger, 2005), however, drugs designed specifically to treat ejaculatory disorders are called for. The neural substrate of the ejaculatory threshold, including all neuroanatomical, physiological and pharmacological aspects, needs to be determined in order to find such drugs.

Although some research on ejaculation has been performed in men (Waldinger *et al.*, 1998b; Waldinger *et al.*, 2001), the practical and ethical limitations to conduct neuroanatomical and psychopharmacological experiments in humans require the use of animal models. The vast majority of sexual behaviour research has been performed in rats. Therefore, all experiments discussed in this review were conducted in rats unless stated otherwise. The reasons to use rats in sexual behaviour research are various (Pfaus, 1996), and include the fact that their intromissions and ejaculations are clearly discernable (Bitran and Hull, 1987). In addition, rats have an average ejaculation latency of about five minutes, just as humans, although large individual differences exist within both human and rat populations (Olivier *et al.*, 2005; Pattij *et al.*, 2005; Waldinger *et al.*, 2005a). Other aspects of sexual behaviour obviously differ between male humans and rats: rats are much more influenced by olfactory cues than humans, male rats have little physical contact with the female between each mount and intromission, and rats have multiple ejaculations during copulation. A schematic overview of male rat sexual behaviour is given in figure 1, which is an adaptation of the figure in the review of Larsson and Ahlenius (Larsson and Ahlenius, 1999).

The neurotransmitter serotonin (5-HT) has been implicated in the central regulation

of blood pressure, body fluid homeostasis, locomotion, food intake, nociception, cognition, arousal, stress responses, mood and many other autonomic and behavioural functions. The ubiquitous presence of 5-HT fibres throughout the central nervous system (Steinbusch, 1981), the many different 5-HT receptor subtypes (Barnes and Sharp, 1999), the variety of signal transduction mechanisms activated by each 5-HT receptor subtype (Raymond *et al.*, 2001) and the diversity in autoregulatory mechanisms in the 5-HT system (Pineyro and Blier, 1999) make it highly complicated to unravel the precise role of serotonin in behaviour. The specific role of 5-HT in the ejaculatory threshold has been investigated since the early 1970s, and it was soon established that 5-HT, in contrast to dopamine, inhibits ejaculation. Since then, increasingly sophisticated neuroanatomical and psychopharmacological tools have revealed more specific roles of 5-HT and its receptor subtypes in the ejaculatory threshold.

Neuroanatomical Substrate

The autonomic and somatic motor neurons that execute the different phases of ejaculation are located in the thoracolumbar and lumbosacral spinal cord. These motor neurons are activated in a coordinated manner when sufficient sensory input to reach the ejaculatory threshold has entered the central nervous system. Interneurons in the lumbar spinal cord as well as neurons originating from various supraspinal areas are thought to modulate the ejaculatory threshold, possibly using serotonin as neurotransmitter.

Spinal Cord

Ejaculation occurs in two stages, referred to as emission and expulsion (ejection), which are executed via noradrenergic sympathetic and cholinergic parasympathetic and somatic motor neurons originating in the spinal cord. Emission of spermatozoa from the testes and seminal fluids from the seminal vesicles and prostate is induced by sympathetic motor neurons in the thoracolumbar intermediolateral cell column (IML) and parasympathetic motor neurons in the sacral parasympathetic nucleus (SPN). Somatic motor neurons in the dorsolateral and dorsomedial ventral horn of the lumbosacral spinal cord cause rhythmic contractions of the striated bulbospongiosus and ischiocavernosus muscles in the pelvic floor, which leads to the forceful expulsion of semen from the urethra (Coolen *et al.*, 2004b; Marson and McKenna, 1996; McKenna, 2000; Steers, 2000; Waldinger *et al.*, 1998a). A schematic overview of the relevant spinal

cord areas is given in figure 2, which is an adaptation from the figures in the atlas of Paxinos and Watson (Paxinos and Watson, 2005).

The motor neurons involved in ejaculation are triggered, amongst others, by sensory input from the genitals. This genitosensory input is predominantly generated by intromissions and reaches the dorsal horns and dorsal grey commissure of the lumbosacral spinal cord via the dorsal penile nerve, a branch of the pudendal nerve (McKenna and Nadelhaft, 1986; Ueyama *et al.*, 1987). In anesthetized rats with a transection of the spinal cord at the T6 level, urethral distension, which stimulates the dorsal penile nerve, elicits an 'urethrogenital reflex'. This reflex includes rhythmic contractions of the striated muscles and expulsion of the urethral contents, and is therefore used as a model for ejaculation (Carro-Juarez and Rodriguez-Manzo, 2000; Chung *et al.*, 1988; Duran *et al.*, 1997; McKenna *et al.*, 1991).

Apparently, the relay of genitosensory input to ejaculatory motor output takes place at the level of the spinal cord in the form of a reflex arc. A group of galaninergic interneurons in the border area of laminae 7 and 10 at the lumbar 3 and 4 levels of the spinal cord, called the lumbar spinothalamic cells (LSt cells), is the most likely candidate for such a relay centre (Truitt and Coolen, 2002). Galaninergic fibres originating from the LSt cells project to all areas in the spinal cord that contain motor neurons involved in ejaculation. Selective lesion of the LSt cells eliminates ejaculation without affecting other parameters of sexual behaviour (Truitt and Coolen, 2002; Xu *et al.*, 2005). Therefore, the activation of LSt cells probably plays an important role in the ejaculatory threshold.

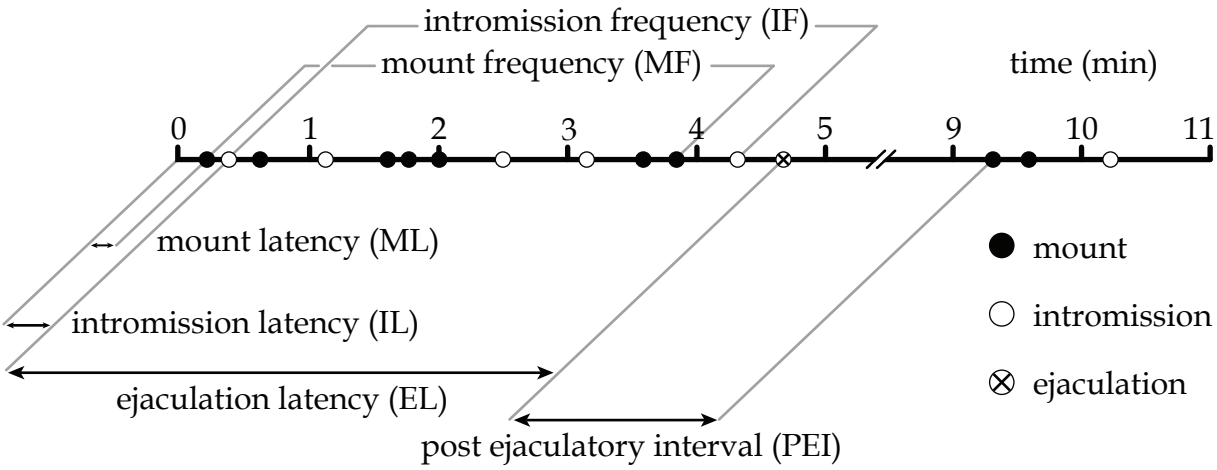


Figure 1. The temporal pattern of male rat sexual behaviour, adapted from Larsson and Ahlenius, 1999.

Serotonergic fibres have been found in all spinal cord areas containing sensory axons and motor neurons involved in ejaculation. Serotonergic fibres are present in the dorsal and ventral horns, dorsal commissural grey and IML and SPN of the lumbosacral spinal cord (Maxwell *et al.*, 1996; Ranson *et al.*, 2003; Tang *et al.*, 1998), they were found in close association with cell bodies in the IML showing Fos-expression in response to the urethrogenital reflex (Marson and Gravitt, 2004), and they make synaptic contact with neurons in the SPN labelled by retrograde tracers injected in the pelvic nerve as well as lumbosacral somatic motor neurons labelled by retrograde tracers injected in the bulbospongiosus and ischiocavernosus muscles (Tang *et al.*, 1998). In addition, serotonergic fibres have been found in close apposition to the LSt cells (Coolen *et al.*, 2004a). Serotonin might affect ejaculation via any of these possible connections.

Supraspinal Areas

A modulating role for supraspinal areas in the ejaculatory threshold was indicated by the finding that the urethrogenital reflex cannot be elicited in intact rats, but requires either thoracal spinal transection or lesion of the nucleus paragigantocellularis (nPGi), an area in the ventrolateral medulla in the brainstem (Marson and McKenna, 1990). Lesioning of the nPGi also facilitates ejaculation in copulating rats (Yells *et al.*, 1992). Neurons in the nPGi are consistently labelled when retrograde transneuronal tracers are injected into the penis, bulbospongiosus muscle, epididymis or prostate (Gerendai *et al.*, 2001; Marson *et al.*, 1993; Orr and Marson, 1998; Tang *et al.*, 1999), and the nPGi is thought to exert a tonic inhibition over ejaculation via these connections, that are relayed in the spinal cord. Serotonin probably mediates this inhibition, since a large portion of neurons in the nPGi that project to the motor neurons innervating the bulbospongiosus muscles are serotonergic (Marson and McKenna, 1992). The medial preoptic area (MPOA), a brain area in the hypothalamus that integrates the sensory information induced by female pheromones and genital stimulation (Bressler and Baum, 1996; Coolen *et al.*, 1998; Coolen *et al.*, 2003b) and is a crucial structure for the performance of sexual behaviour (Hansen *et al.*, 1982; Liu *et al.*, 1997b; Meisel and Sachs, 1994; Paredes *et al.*, 1993), projects heavily to the nPGi via relays in the periaqueductal grey. The MPOA might lower the ejaculatory threshold by removing the tonic serotonergic inhibition exerted by the nPGi (Marson, 2004; Marson and McKenna, 1994a; Murphy and Hoffman, 2001; Murphy *et al.*, 1999).

Besides the MPOA-PAG-nPGi-spinal cord pathway, there is at least one other serotonergic pathway that influences sexual behaviour. Serotonin release in the anterior lateral hypothalamic area, most likely from axons originating from the median and

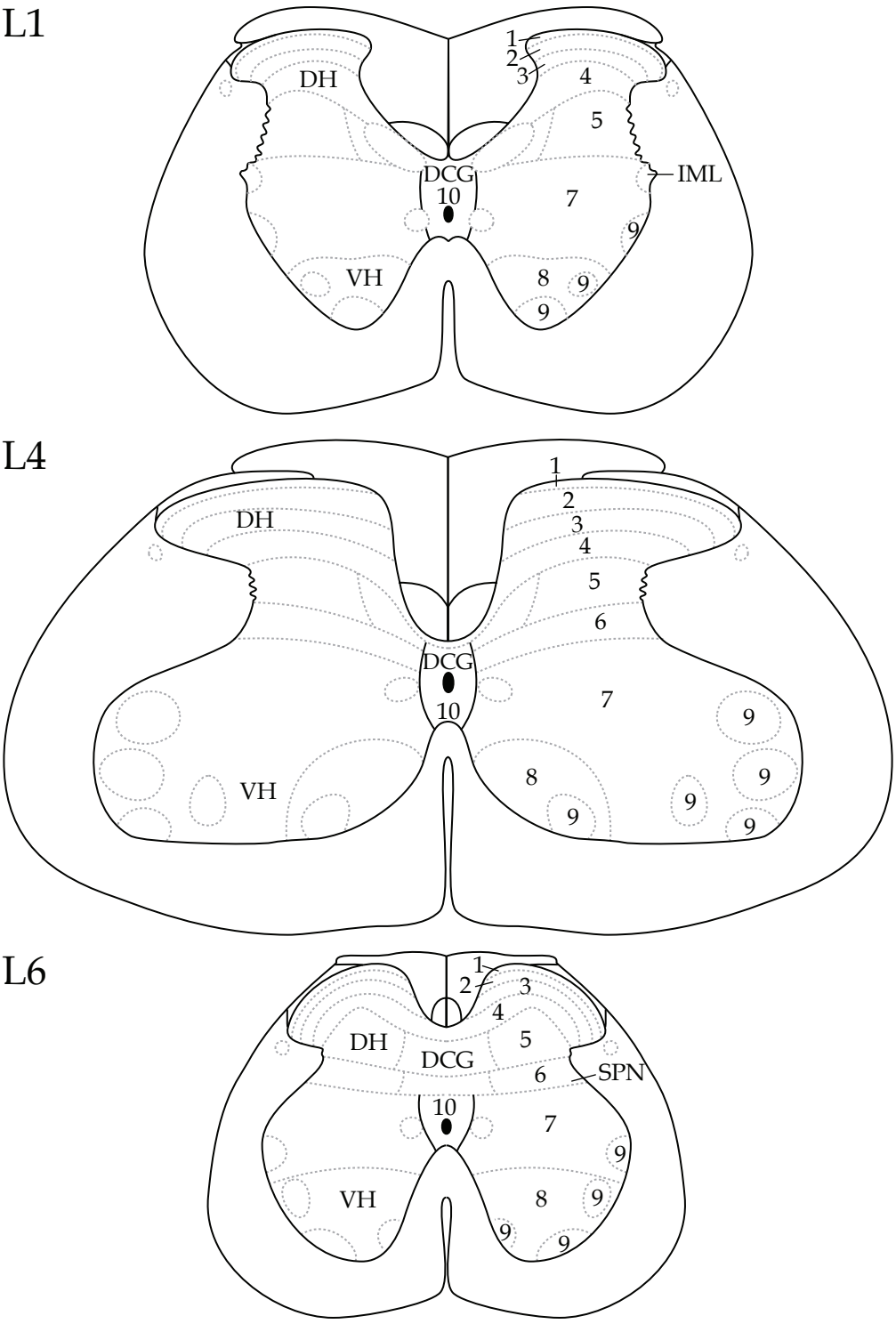


Figure 2. Schematic representation of coronal spinal cord sections at the lumbar 1, 4 and 6 levels. Numbers represent the laminae; DH: dorsal horn; VH: ventral horn; DCG: dorsal commissural grey; IML: intermediate lateral cell column; SPN: sacral parasympathetic nucleus. Adapted from Paxinos and Watson, 2005.

dorsal raphe nuclei and travelling through the medial forebrain bundle (van de Kar and Lorens, 1979), increases sharply in response to an ejaculation (Lorrain *et al.*, 1997). This is thought to induce the suppression of copulation during the post-ejaculatory interval, and could be partly mediated by an inhibition of dopaminergic neurotransmission in the nucleus accumbens (Lorrain *et al.*, 1999). Indeed, electrolytic lesions of the median raphe nucleus and, less consistently, the dorsal raphe nucleus lowered the ejaculatory threshold by reducing intromission frequency and ejaculation latency (Albinsson *et al.*, 1996; McIntosh and Barfield, 1984). Selective degeneration of serotonergic fibres in the medial forebrain bundle by local injection of the serotonergic toxin 5,7-dihydroxytryptamine (5,7-DHT) lead to an increased percentage of rats ejaculating and a decreased intromission frequency compared to sham-lesioned rats (Rodriguez *et al.*, 1984).

In these two pathways, the link between serotonergic neurotransmission and ejaculation is evident. In many other brain areas that are known to influence ejaculation, a mediating role of serotonin is possible but not yet demonstrated. Experiments using lesions (Kondo and Yamanouchi, 1995; Liu *et al.*, 1997b) or the staining of Fos (Baum and Everitt, 1992; Coolen *et al.*, 1997a; Coolen *et al.*, 1996; Greco *et al.*, 1996) in rats, gerbils and hamsters have implicated the medial amygdala, the posterior medial bed nucleus of the stria terminalis and the medial parvocellular subparafascicular thalamic nucleus in ejaculation and the post-ejaculatory interval, possibly via their reciprocal connections with the MPOA (Coolen *et al.*, 1998; Coolen *et al.*, 2003a; Heeb and Yahr, 2001; Parfitt and Newman, 1998). These areas contain some serotonergic fibres (Steinbusch, 1981) that might play a role in the effects on ejaculation.

Furthermore, the nucleus accumbens, paraventricular hypothalamic nucleus and arcuate hypothalamic nucleus receive serotonergic input (Casu *et al.*, 2004; Larsen *et al.*, 1996; Steinbusch, 1981). The nucleus accumbens is thought to play a role in sexual motivation and reward (Balfour *et al.*, 2004) and its lesion disrupts ejaculation (Kippin *et al.*, 2004). The paraventricular hypothalamic nucleus contains oxytocin that lowers the ejaculatory threshold (Stoneham *et al.*, 1985) and its lesion increases the ejaculatory threshold (Liu *et al.*, 1997a). The arcuate hypothalamic nucleus is connected with the medial preoptic area, the medial amygdala, the bed nucleus of the stria terminalis and the paraventricular nucleus, and is thought to integrate information about metabolism with reproductive activity (Gottsch *et al.*, 2004; Magoul *et al.*, 1994).

In conclusion, the understanding of neuroanatomical associations between serotonin and the ejaculatory threshold is far from complete and needs to be studied further.

Serotonin Levels

In the last four decades, many researchers have demonstrated that pharmacological manipulation of serotonergic neurotransmission markedly changed parameters of sexual behaviour, in particular the intromission frequency and ejaculation latency.

Increased 5-HT levels

Since 5-HT does not cross the blood brain barrier, the net effects of serotonin on the ejaculatory threshold have been investigated using systemic injection of the 5-HT precursor 5-hydroxytryptophan (5-HTP) that does cross the blood brain barrier. 5-HTP has been found to increase 5-HT release from serotonergic neurons in the lateral hypothalamic area and the lumbar spinal cord for as long as three hours (Gartside *et al.*, 1992; Kimura *et al.*, 1983; Samathanam *et al.*, 1989; Shimizu *et al.*, 1992). Systemic injection of 5-HTP increased the intromission frequency and ejaculation latency in rats (Ahlenius and Larsson, 1984; 1985; 1991b; 1998; Ahlenius *et al.*, 1980; Fernandez-Guasti and Rodriguez-Manzo, 1992), and mongrel dogs treated with 5-HTP failed to ejaculate upon genital stimulation (Kimura *et al.*, 1977). In addition, serotonin levels can be elevated throughout the central nervous system by acute systemic administration of the 5-HT releasers para-chloroamphetamine (p-CA) or fenfluramine (Gardier *et al.*, 1994; Schwartz *et al.*, 1989; Series *et al.*, 1994). This lead to an increased ejaculation latency as well (Foreman *et al.*, 1992).

5-HT can be injected directly in the brain or the cerebrospinal fluid to avoid the blood brain barrier. Local injection of 5-HT into the nucleus accumbens, medial preoptic area or amygdala, as well as intracerebroventricular or intrathecal injections, increased the intromission frequency and ejaculation latency (Drago *et al.*, 1999; Fernandez-Guasti *et al.*, 1992; Hillegaart *et al.*, 1991) or decreased the percentage of rats that reached an ejaculation (Svensson and Hansen, 1984; Verma *et al.*, 1989). Thus, the elevation of serotonin levels in many brain areas and the spinal cord increases the ejaculatory threshold.

Conversely, local injection of low doses of 5-HT into the median or dorsal raphe nuclei lowered the ejaculatory threshold (Hillegaart *et al.*, 1989), interpreted by assuming that feedback systems, which inhibit cell firing and decrease 5-HT levels in projection areas (Pineyro and Blier, 1999), were activated. However, injection of higher doses of 5-HT into the dorsal and median raphe nuclei had no effect on ejaculation (Fernandez-Guasti *et al.*, 1992; Hillegaart *et al.*, 1989).

SSRIs

The Selective Serotonin Reuptake Inhibitors (SSRIs) fluoxetine, paroxetine, citalopram, fluvoxamine and sertraline are widely used and effective antidepressants. They all act similarly by blocking 5-HT transporters, thereby preventing the reuptake of 5-HT from the synaptic cleft into the presynaptic serotonergic neuron. This leads to elevated extracellular 5-HT levels, as shown by microdialysis studies following acute systemic administration of fluoxetine (Bymaster *et al.*, 2002; Felton *et al.*, 2003; Hervas and Artigas, 1998; Malagie *et al.*, 1995), paroxetine (Hajos-Korcsok *et al.*, 2000; Malagie *et al.*, 2000; Nakayama, 2002), fluvoxamine (Ago *et al.*, 2005; Bosker *et al.*, 1995a; Denys *et al.*, 2004), citalopram (Invernizzi *et al.*, 1995; Moret and Briley, 1996; Wegener *et al.*, 2003; Yoshitake *et al.*, 2003) and sertraline (Sprouse *et al.*, 1996; Zhang *et al.*, 2000).

Besides an increased activation of postsynaptic 5-HT receptors, elevated 5-HT levels also turn on negative feedback systems via serotonin autoreceptors, leading to a reduced release of serotonin from nerve terminals. This probably attenuates acute effects of SSRI treatment on mood disorders. During chronic SSRI-treatment (3 to 4 weeks) autoreceptors, in particular 5-HT_{1A} receptors, become desensitized and this has been proposed to enable the antidepressant effects to occur (Blier *et al.*, 1998; Elena Castro *et al.*, 2003; Hensler, 2003; Invernizzi *et al.*, 1996; Le Poul *et al.*, 2000; Newman *et al.*, 2004; Pineyro and Blier, 1999).

Treatment with SSRIs often causes sexual problems, of which delayed ejaculation and the inability to ejaculate are the most commonly reported (Gregorian *et al.*, 2002; Montgomery *et al.*, 2002; Rosen *et al.*, 1999). These side effects are generally perceived as negative, but SSRI-induced delayed ejaculation has turned out very useful in the treatment of lifelong premature ejaculation (Chia, 2002; Kara *et al.*, 1996; Kim and Seo, 1998; McMahon *et al.*, 2004; Moreland and Makela, 2005; Waldinger *et al.*, 2004a).

The use of the so-called Intravaginal Ejaculation Latency Time (IELT) as measured with a stopwatch (Waldinger, 2003) has greatly increased the amount of objective data on the effects of SSRIs on the ejaculatory threshold. Interestingly, this method revealed marked differences between SSRIs in their ability to delay ejaculation in patients suffering from premature ejaculation: paroxetine delayed ejaculation more strongly than the other SSRIs, whereas citalopram and fluvoxamine affect ejaculation much less (Waldinger *et al.*, 1998b; Waldinger *et al.*, 2001; Waldinger *et al.*, 2004a). In addition, the effects of paroxetine and fluoxetine became clinically relevant after a few weeks of chronic treatment and increased over time (Waldinger *et al.*, 1998b; Waldinger *et al.*, 2001). There have been some studies dealing with the effects of SSRIs on sexual behaviour in rats. These studies often failed to find a significant effect of acute systemic

injection of several SSRIs on the ejaculatory threshold (Ahlenius *et al.*, 1979; Ahlenius and Larsson, 1999; Cantor *et al.*, 1999; de Jong *et al.*, 2005a; de Jong *et al.*, 2005c; Mos *et al.*, 1999), although acutely administered fluoxetine or paroxetine sometimes delayed ejaculation (Waldinger *et al.*, 2002; Yells *et al.*, 1994), and acute local injection of the SSRI alaproclate into the lateral hypothalamic area increased both local serotonin levels and ejaculation latency (Lorrain *et al.*, 1997). Apparently, the ejaculatory threshold is somewhat less sensitive to acute systemic injection of an SSRI compared to acute systemic injection of 5-HTP, despite their shared ability to elevate serotonin levels. This might be explained by the difference in pharmacology between the two drugs: 5-HTP increases 5-HT release, whereas SSRIs prevent 5-HT reuptake. However, a direct comparison between the two compounds on 5-HT levels in brain areas relevant for ejaculation has not yet been performed.

Delayed ejaculation reliably occurs in rats in response to chronic treatment with the SSRIs paroxetine or fluoxetine (de Jong *et al.*, 2005c; Vega *et al.*, 1998; Waldinger *et al.*, 2002), but less or not in response to citalopram or fluvoxamine (de Jong *et al.*, 2005a; de Jong *et al.*, 2005c; Waldinger *et al.*, 2002), which resembles the situation in humans (Waldinger *et al.*, 1998b; Waldinger *et al.*, 2001). The difference between acute and chronic treatment suggests that desensitization mechanisms play a role in the effects of paroxetine and fluoxetine on ejaculation. In addition, the lack of effects of citalopram and fluvoxamine indicate that these desensitization mechanisms vary from one SSRI to the other. More evidence in that direction is discussed in the paragraph about 5-HT_{1A} receptors

5-HT depletion

Multiple systemic injections of para-chlorophenylalanine (p-CPA), which strongly depletes 5-HT in the central nervous system (Kimura *et al.*, 1977; Qureshi *et al.*, 1989) cause a decrease in ejaculation latency (Ahlenius *et al.*, 1971; Dahlof and Larsson, 1979; Gessa and Tagliamonte, 1974; Qureshi *et al.*, 1989; Salis and Dewsbury, 1971; Yamanouchi and Kakeyama, 1992) and intromission frequency (Fernandez-Guasti and Escalante, 1991), and an increase in ejaculation frequency (Tsutsui *et al.*, 1994; Yamanouchi and Kakeyama, 1992). Intracerebroventricular injection of 5,7-DHT, which decreased serotonin levels in the hypothalamus, brainstem and spinal cord, enabled the urethrogenital reflex to occur upon urethral distension in intact rats. A similar result was found when 5,7-DHT was injected intrathecally, which decreased serotonin levels only in the spinal cord (Marson and McKenna, 1994b). Apparently, a decrease in serotonin levels in the spinal cord and supraspinal areas lowers the ejaculatory threshold.

Serotonin Receptors

The abundant evidence that serotonin is involved in the mechanisms mediating the ejaculatory threshold encouraged researchers to determine which serotonin receptors contribute to this process. The increasing availability of selective serotonin receptor agonists and antagonist greatly advanced the knowledge about the roles of specific receptor subtypes. So far, 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2C} receptors are the only serotonin receptor subtypes that have been shown to affect the ejaculatory threshold. All three receptor subtypes are located in the thoracolumbar intermediolateral cell column, the lumbar dorsal commissural grey and laminae 7 and 10, the lumbosacral dorsal and ventral horns and the sacral parasympathetic nucleus (Bancila *et al.*, 1999; Fonseca *et al.*, 2001; Thor *et al.*, 1993), indicating that serotonin may modulate the ejaculatory threshold directly via these receptors in the spinal cord. In addition, the presence of these 5-HT receptor subtypes in supraspinal areas involved in ejaculation may play a role as well.

5-HT_{1A} receptors

5-HT_{1A} receptors are positioned presynaptically on the soma and dendrites of serotonergic neurons as well as postsynaptically on neurons containing a wide variety of neurotransmitters (Barnes and Sharp, 1999). Activation of somatodendritic 5-HT_{1A} autoreceptors leads to a potent inhibition of the firing frequency of serotonergic neurons (Hajos *et al.*, 1999), constituting a negative feedback system through inhibition of 5-HT release in projection areas (Hjorth and Sharp, 1991; Pineyro and Blier, 1999). Activation of postsynaptic 5-HT_{1A} heteroreceptors can lead to a various actions, depending on the electrophysiological properties, projection areas and neurotransmitters used by the postsynaptic neuron.

The staining of 5-HT_{1A} receptor proteins or mRNA has shown that these receptors are located in the raphe nuclei (Kia *et al.*, 1996; Li *et al.*, 1997a; Pompeiano *et al.*, 1992; Wright *et al.*, 1995) and the nucleus paragigantocellularis (Helke *et al.*, 1997; Kia *et al.*, 1996; Pompeiano *et al.*, 1992), where they probably act as autoreceptors on serotonergic neurons. Postsynaptic 5-HT_{1A} receptors are distributed throughout the brain, amongst others in nuclei implicated in ejaculation such as the medial preoptic area (Aznar *et al.*, 2003; Pompeiano *et al.*, 1992), lateral hypothalamic area (Collin *et al.*, 2002; Kia *et al.*, 1996; Li *et al.*, 1997a), medial amygdala (Aznar *et al.*, 2003; Li *et al.*, 1997a; Pompeiano *et al.*, 1992), bed nucleus of the stria terminalis (Kia *et al.*, 1996; Pompeiano *et al.*, 1992), nucleus accumbens (Aznar *et al.*, 2003; Wright *et al.*, 1995), paraventricular hypotha-

lamic nucleus (Collin *et al.*, 2002; Li *et al.*, 1997a; Zhang *et al.*, 2004) and arcuate hypothalamic nucleus (Aznar *et al.*, 2003; Collin *et al.*, 2002).

Selective activation of 5-HT_{1A} receptors has a remarkably strong effect on the ejaculatory threshold. The first report that systemic administration of the 5-HT_{1A} receptor agonist 8-OH-DPAT reduced the intromission frequency and ejaculation latency was published in 1981 (Ahlenius *et al.*, 1981), and this finding has been reproduced many times (Ahlenius and Larsson, 1984; Coolen *et al.*, 1997b; Fernandez-Guasti and Rodriguez-Manzo, 1997; Mendelson and Gorzalka, 1986; Morali and Larsson, 1984; Rehman *et al.*, 1999; Schnur *et al.*, 1989; Sura *et al.*, 2001). Although 8-OH-DPAT has some affinity for 5-HT_7 receptors (Bard *et al.*, 1993; Neumaier *et al.*, 2001), the findings that systemic injection of other 5-HT_{1A} receptor agonists had similar effects on ejaculation (Ahlenius and Larsson, 1991b; Andersson and Larsson, 1994; Haensel and Slob, 1997; Mathes *et al.*, 1990) and that these effects could be reversed completely by systemic injection of selective 5-HT_{1A} receptor antagonists (Ahlenius and Larsson, 1998; Hillegaart and Ahlenius, 1998) indicate that 5-HT_{1A} receptor activation is responsible for the lowering of the ejaculatory threshold.

The somewhat puzzling opposite effects of 5-HT, which is the natural ligand of 5-HT_{1A} receptors, versus 5-HT_{1A} receptor agonists on the ejaculatory threshold might be explained by their activation of somatodendritic 5-HT_{1A} autoreceptors, which causes a reduction of 5-HT levels in projection areas and thus mimics the effects of serotonin depletors like p-CPA (Hjorth and Sharp, 1991; Hughes *et al.*, 2005; Invernizzi *et al.*, 1995; 1996). Indeed, micro-injection of 8-OH-DPAT into the median raphe nucleus reduced the ejaculation latency and intromission frequency (Hillegaart *et al.*, 1991), although a similar injection into the dorsal raphe nucleus failed to affect sexual behaviour (Fernandez-Guasti *et al.*, 1992; Hillegaart *et al.*, 1991).

On the other hand, the reduction of intromission frequency and ejaculation latency following depletion of 5-HT levels by systemic injection of p-CPA or intracerebroventricular injection of 5,7 DHT was further decreased by systemic administration of 8-OH-DPAT (Fernandez-Guasti and Escalante, 1991), suggesting that both pre- and postsynaptic 5-HT_{1A} receptors are involved in the lowering of the ejaculatory threshold. In addition, intrathecal injection of 5-HT_{1A} receptor agonists strongly reduced the intromission frequency and ejaculation latency (Lee *et al.*, 1990; Mathes *et al.*, 1990; Svensson and Hansen, 1984). Since there are no serotonergic cell bodies and no 5-HT_{1A} autoreceptors in the spinal cord, spinal postsynaptic 5-HT_{1A} receptors probably mediated these effects. Moreover, micro-injection of 5-HT_{1A} receptor agonists in the nucleus accumbens and medial preoptic area lowered the ejaculatory threshold as well

(Fernandez-Guasti *et al.*, 1992; Hillegaart *et al.*, 1991; Matuszewich *et al.*, 1999), suggesting an additional role for supraspinal 5-HT_{1A} receptors. However, the low affinity of 8-OH-DPAT for dopamine D_2 receptors might be responsible for some of the effects in these nuclei (Matuszewich *et al.*, 1999).

The effects of 5-HT_{1A} receptor agonists on the ejaculatory threshold are not universal. Systemic injection of 8-OH-DPAT inhibits ejaculation in mice (Rodriguez-Manzo *et al.*, 2002b), rabbits (Paredes *et al.*, 2000), dogs (Yonezawa *et al.*, 2004) and ferrets (Paredes *et al.*, 1994), and either lowers or elevates the ejaculatory threshold in rhesus monkeys, depending on dose (Pomerantz *et al.*, 1993b). Since 5-HT_{1A} autoreceptors probably have the same location and function in different mammalian species (Price *et al.*, 1996), a difference in distribution of postsynaptic 5-HT_{1A} receptors in brain and spinal cord areas might explain this 8-OH-DPAT induced elevation of the ejaculatory threshold.

Interestingly, systemic injection of the selective 5-HT_{1A} receptor antagonist WAY-100635 does not increase the ejaculatory threshold by itself, indicating that activation of 5-HT_{1A} receptors is not necessary to reach ejaculation during normal copulation (Ahlenius and Larsson, 1998; de Jong *et al.*, 2005a). However, systemic injection with WAY-100635 strongly enhances the increased ejaculatory threshold induced by 5-HTP (Ahlenius and Larsson, 1998) or SSRIs (Ahlenius and Larsson, 1999; de Jong *et al.*, 2005a). This could be mediated by the blockade of 5-HT_{1A} autoreceptors that normally limit the increase in 5-HT levels induced by 5-HTP or SSRIs, or blockade of postsynaptic 5-HT_{1A} receptors that lower the ejaculatory threshold via the activation or perhaps inhibition of neurons in brain and spinal cord areas involved in ejaculation.

These findings imply that 5-HT_{1A} receptor activation becomes increasingly important to reach the ejaculatory threshold when serotonin levels are elevated, and that a combination of elevated serotonin levels and impaired 5-HT_{1A} receptor functioning strongly inhibits ejaculation. This might underlie SSRI-induced delayed ejaculation, since chronic treatment with the SSRI paroxetine, which delays ejaculation, reduced the facilitation of ejaculation induced by 8-OH-DPAT in rats (de Jong *et al.*, 2005c), probably through 5-HT_{1A} receptor desensitization (Le Poul *et al.*, 1995; Li *et al.*, 1997b). Chronic treatment with fluvoxamine, an SSRI that has no sexual side effects in humans, failed to delay ejaculation and to reduce the effects of 8-OH-DPAT on ejaculation in rats (de Jong *et al.*, 2005c). Further research might elucidate whether desensitization of pre- or postsynaptic 5-HT_{1A} receptors plays a role in SSRI-induced delayed ejaculation.

5-HT$_{1B}$ receptors

5-HT$_{1B}$ receptors are located on pre- and postsynaptic axon terminals, where they act as autoreceptors and inhibit serotonin release (Barnes and Sharp, 1999; Raymond *et al.*, 2001; Sari, 2004), or as heteroreceptors by inhibiting the release of various neurotransmitters (Clark and Neumaier, 2001; Sari, 2004). In addition to the spinal cord, 5-HT$_{1B}$ receptors are found in the raphe nuclei, lateral hypothalamic area, bed nucleus of the stria terminalis, nucleus accumbens, paraventricular hypothalamic nucleus and arcuate hypothalamic area (Makarenko *et al.*, 2002; Neumaier *et al.*, 1996).

Systemic injection of the selective 5-HT$_{1B}$ receptor agonist anpirtoline elevated the ejaculatory threshold by increasing the ejaculation latency and intromission frequency, and this could be reversed by several 5-HT$_{1B}$ receptor antagonists (Hillegaart and Ahlenius, 1998). In addition, systemic injection of the mixed 5-HT$_{1B/2C}$ receptor agonist N-[3-(trifluoromethyl)phenyl] piperazine (TFMPP) strongly reduced the percentage of rats ejaculating (Fernandez-Guasti *et al.*, 1989), and local injection of TFMPP in the nucleus accumbens or medial preoptic area increased the ejaculation latency (Fernandez-Guasti *et al.*, 1992). However, since 5-HT$_{1B}$ receptors have not been found in the medial preoptic area, these effects might have been mediated partly by 5-HT$_{2C}$ receptors.

The elevation of the ejaculatory threshold by systemic injection of 5-HTP could be prevented by the 5-HT$_{1B}$ receptor antagonist isamoltane (Ahlenius and Larsson, 1998), indicating that 5-HT$_{1B}$ receptors are responsible for the inhibition of ejaculation induced by serotonin. However, systemic injection of 5-HT$_{1B}$ receptor antagonists did not or very weakly facilitate ejaculation (Ahlenius and Larsson, 1998; Hillegaart and Ahlenius, 1998), indicating that during normal copulation the ejaculatory threshold is not maintained solely by 5-HT$_{1B}$ receptor activation. In addition, the ejaculatory threshold is increased in 5-HT$_{1B}$ knockout mice compared to wild type mice, indicating that the absence of 5-HT$_{1B}$ receptors does not facilitate ejaculation in mice.

Possible mechanisms by which 5-HT$_{1B}$ receptors inhibit ejaculation have not yet been demonstrated. A role of 5-HT$_{1B}$ autoreceptors seems unlikely, since these receptors cause a reduction of serotonin release that would be expected to lower the ejaculatory threshold. 5-HT$_{1B}$ heteroreceptor activation possibly inhibits the release of neurotransmitters that facilitate ejaculation, such as acetylcholine (Duran *et al.*, 2000; Sarhan and Fillion, 1999), glutamate (Chambille and Rampin, 2002; Powell *et al.*, 2003; Sari, 2004) or perhaps galanin (Coolen *et al.*, 2004a), in brain and spinal cord areas involved in the ejaculatory threshold.

5-HT$_{2C}$ receptors

5-HT$_{2C}$ receptors are found on postsynaptic dendrites where they generally cause cell excitation. They have not been implicated in autoregulatory feedback mechanisms (Barnes and Sharp, 1999). 5-HT$_{2C}$ receptors are widely distributed in the central nervous system, including the raphe nuclei, medial preoptic area, medial amygdala, bed nucleus of the stria terminalis, nucleus accumbens and arcuate hypothalamic nucleus, in addition to the spinal cord (Abramowski *et al.*, 1995; Clemett *et al.*, 2000).

Systemic injection of the non-selective 5-HT$_2$ receptor agonist [+/-]-2,5-dimethoxy-4-iodoamphetamine (DOI) strongly decreased the percentage of rats ejaculating and increased the ejaculation latency, which could be reversed by several 5-HT$_2$ receptor antagonists (Foreman *et al.*, 1989; Klint *et al.*, 1992; Klint and Larsson, 1995; Watson and Gorzalka, 1991). Systemic administration of the 5-HT$_{2C}$ agonist m-CPP produced a dose-dependent decline in the percent of rats and male rhesus monkeys achieving ejaculation (Mendelson and Gorzalka, 1990; Pomerantz *et al.*, 1993a).

The elevation of the ejaculatory threshold induced by systemic injection of the serotonin-releasers p-CA and fenfluramine could be prevented by pretreatment with the 5-HT$_2$ receptor antagonist LY53857 (Foreman *et al.*, 1992). Conversely, the increased ejaculation latency induced by 5-HTP could not be reversed by the 5-HT$_2$ receptor antagonist ritanserin (Ahlenius and Larsson, 1998). Systemic injection of the 5-HT$_2$ receptor antagonist LY53857 reduced the ejaculation latency (Foreman *et al.*, 1989), whereas ritanserin had no such effect (Watson and Gorzalka, 1991). Possibly, yet unknown differences in the neuropharmacological properties of these antagonists could explain the differences in their effect on the ejaculatory threshold. So far, there are no reports on the possible mechanisms by which 5-HT$_{2C}$ receptor activation elevates the ejaculatory threshold. The presence of 5-HT$_{2C}$ receptors in many (supra-)spinal areas involved in ejaculation indicates a great amount of options that should be investigated.

Ex copula ejaculation

Some studies on the effects of 5-HT on the ejaculatory threshold used the occurrence of spontaneous, *ex copula* ejaculations as a model. These seminal emissions are not dependent on genital stimulation, and can be evoked by the injection of the 5-HT releaser p-CA (Humphries *et al.*, 1981; Renyi, 1985; Yonezawa *et al.*, 2000) or the non-selective 5-HT receptor agonist Me-ODMT (Mas *et al.*, 1985), which respectively increase (Foreman *et al.*, 1992) or decrease (Ahlenius and Larsson, 1991b; Fernandez-Guasti *et al.*,

1986) the ejaculatory threshold during copulation. Depletion of serotonin induced by p-CPA, which by itself did not affect spontaneous ejaculation (Humphries *et al.*, 1981), prevented the *ex copula* ejaculations caused by p-CA (Renyi, 1985; Yonezawa *et al.*, 2000). The effects of p-CA and 5-MeODMT on spontaneous ejaculation could not be reversed by thoracic spinal transection (Mas *et al.*, 1985; Yonezawa *et al.*, 2000). These results suggest that activation of spinal serotonin receptors can directly activate the motor neurons involved in seminal emission. 5-HT_{1A} receptors seem likely candidates, but both systemic and intrathecal injection of 8-OH-DPAT (Lee *et al.*, 1990; Rehman *et al.*, 1999; Schnur *et al.*, 1989) and buspirone (Mathes *et al.*, 1990; Rehman *et al.*, 1999), which strongly decrease the ejaculatory threshold *in copula*, inhibited spontaneous *ex copula* ejaculations. This indicates that the occurrence of spontaneous *ex copula* ejaculations is not representative of the situation during copulation, and should not be used as a model for the ejaculatory threshold.

Summary

Neuroanatomical studies have shown that there are at least two serotonergic pathways involved in the ejaculatory threshold. The tonic release of serotonin in the lumbosacral spinal cord originating from neurons in the nPGi inhibits ejaculation until sensory input overrules this tonic inhibition. Serotonin release in response to ejaculation in the anterior lateral hypothalamic area, and perhaps the medial amygdala and medial bed nucleus of the stria terminalis, mediates the inhibition of copulation during the post-ejaculatory interval.

Psychopharmacological experiments revealed that injection of drugs increasing 5-HT levels in the central nervous system, including the lumbosacral spinal cord, lateral hypothalamic area, medial preoptic area, amygdala and nucleus accumbens, elevates the ejaculatory threshold. 5-HT_{1B} and 5-HT_{2C} receptors possibly mediate this, because activation of these receptors inhibits ejaculation whereas their blockade prevents the inhibition of ejaculation by elevated serotonin levels.

Depletion of 5-HT in the central nervous system decreases the ejaculatory threshold. This might occur naturally during copulation, when pheromonal and genital stimulation are thought to trigger the medial preoptic area to inhibit the serotonergic cell firing in the nPGi, which reduces serotonin release in the lumbosacral spinal cord. Reduced activation of inhibitory postsynaptic 5-HT_{1B} and/or 5-HT_{2C} receptors in the lumbosacral spinal cord might be responsible for the facilitation of ejaculation following 5-HT

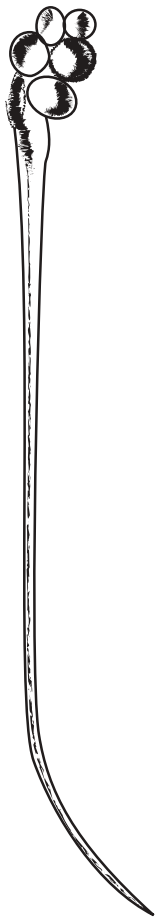
depletion, although systemic injection of 5-HT_{1B} or 5-HT_{2C} receptor antagonists alone do not consistently lower the ejaculatory threshold.
The role of 5-HT_{1A} receptors in the ejaculatory threshold is somewhat more complicated. Activation of 5-HT_{1A} receptors strongly lowers the ejaculatory threshold, and this is thought to be mediated by both reduction of 5-HT levels via 5-HT_{1A} autoreceptors and yet unknown effects of spinal or supraspinal postsynaptic 5-HT_{1A} receptors. Although 5-HT_{1A} receptors are not necessary for ejaculation during normal copulation, their activation becomes crucial for ejaculation when serotonin levels are elevated. Since serotonin levels can fluctuate under many circumstances, it is possible that this mechanism developed to favour successful copulation when serotonin levels are increased.

Further Research

Although much information has been gathered by either neuroanatomical studies or psychopharmacological experiments, the constructive combination of both fields of science is needed to improve the knowledge of the effects of serotonin on the ejaculatory threshold.
For example, the neuropharmacology of serotonergic neurons in the nPGi involved in the ejaculatory threshold is barely known. It is unclear what triggers the firing of these neurons and what their electrophysiological properties are, and whether they contain 5-$HT_{1A/1B}$ autoreceptors on their soma or axon terminals. Moreover, it is unknown whether the serotonergic neurons in the nPGi make direct or indirect functional contact with the LSt cells. Interestingly, serotonergic neurons and fibres in the nPGi and lumbosacral spinal cord co-express substance P (Hokfelt *et al.*, 2000; Maxwell *et al.*, 1996), a neurotransmitter that binds to neurokinin-1 receptors, which are abundantly present on the LSt cells (Truitt and Coolen, 2002). Substance P has been demonstrated to facilitate ejaculation when micro-injected in the medial preoptic/anterior hypothalamic area (Dornan and Malsbury, 1989). Further research to investigate the functional consequences of this co-expression is required.
It is not yet understood how serotonin influences the ejaculatory threshold in supraspinal areas. Inhibition of dopaminergic neurotransmission might play a role, since dopamine release in the medial preoptic area mediated by the medial amygdala (Dominguez *et al.*, 2001), and in the nucleus accumbens mediated by the lateral hypothalamic area (Lorrain *et al.*, 1999), strongly facilitate ejaculation (Hull *et al.*, 2004). Experiments

investigating how serotonin decreases dopamine release in these areas are required.
There are surprisingly few data on the exact role of 5-HT_{1B} and 5-HT_{2C} receptors in the ejaculatory threshold. The existence of increasingly selective receptor agonists and antagonists and the improved techniques to make local injections enable innovative experiments on this subject. In addition, the use of knockout mice or creation of knockout rats that lack the 5-HT_{1A}, 5-HT_{1B} or 5-HT_{2C} receptor could further elucidate the role of serotonin on the ejaculatory threshold.
Ultimately, new findings in all these directions might help to design drugs that elevate the ejaculatory threshold in men suffering from lifelong premature ejaculation, or perhaps relieve other ejaculatory disorders.

CHAPTER 3



Citalopram combined with WAY-100635 inhibits ejaculation and ejaculation-related Fos immunoreactivity

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Abstract

The role of 5-HT (5-hydroxytryptamine, 5-HT)$_{1A}$ receptor activation in the sexual side effects, in particular delayed ejaculation, of selective serotonin reuptake inhibitors (SSRIs) was studied. Male Wistar rats were treated for 15 days with vehicle, the SSRI citalopram (10 mg/kg/day p.o.), the 5-HT$_{1A}$ receptor antagonist N-[2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-N-(2-pyridinyl) cyclohexane carboxamide 3HCL (WAY-100635, 0.1 mg/kg/ day s.c.), or both drugs combined. Sexual behaviour was assessed weekly. One h after the last sexual behaviour test, rat brains were processed for Fos-immunohistochemistry. Acute and chronic citalopram mildly inhibited ejaculation, which was strongly augmented by co-administration of WAY-100635. WAY-100635 alone did not alter sexual behaviour. Brain sites associated with ejaculation showed reduced Fos-immunoreactivity in rats treated with both citalopram and WAY-100635. Citalopram reduced Fos-immunoreactivity in the arcuate hypothalamic nucleus, an area that might link serotonergic neurotransmission to ejaculation.

Introduction

The Selective Serotonin Reuptake Inhibitors (SSRIs) citalopram, fluoxetine, paroxetine, fluvoxamine and sertraline are useful therapeutic agents in the treatment of depression, but are also associated with a high incidence of sexual dysfunction including delayed ejaculation (Rosen *et al.*, 1999).

The antidepressant effect of all SSRIs occurs through blockade of the serotonin (5-hydroxytryptamine, 5-HT) transporter, which results in elevated extracellular serotonin levels (Hiemke and Hartter, 2000). Since serotonin inhibits sexual behaviour (Ahlenius and Larsson, 1998; Lorrain *et al.*, 1997; Marson and McKenna, 1992), elevated serotonin levels are thought to cause the SSRI-induced delayed ejaculation.

However, although all SSRIs inhibit serotonin reuptake and increase serotonin levels in a similar manner (Nutt *et al.*, 1999), they vary in their degree of delaying ejaculation: chronic treatment with paroxetine or fluoxetine strongly delays ejaculation in humans (Waldinger *et al.*, 1998a; Waldinger and Olivier, 1998) and rats (Cantor *et al.*, 1999; Frank *et al.*, 2000; Vega *et al.*, 1998; Waldinger *et al.*, 2002), whereas citalopram and fluvoxamine appear to affect sexual function to a lesser extent in humans (Waldinger and Olivier, 1998; Waldinger *et al.*, 2001) and rats (Ahlenius and Larsson, 1999). These findings led to the theory that fluoxetine and paroxetine affect the neurobiological substrate involved in ejaculation somehow different from citalopram and fluvoxamine.

The 5-HT_{1A} receptor might be involved in this difference, since 5-HT_{1A} receptor agonists such as 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and flesinoxan strongly facilitate ejaculation in rats (Ahlenius *et al.*, 1981; Coolen *et al.*, 1997b; Haensel and Slob, 1997). Possibly, the degree of 5-HT_{1A} receptor activation during chronic SSRI-treatment determines the severity of potential sexual side effects.

To test the hypothesis that activation of 5-HT_{1A} receptors prevents inhibition of ejaculation during acute and chronic treatment with citalopram, we co-administered citalopram with the silent and selective 5-HT_{1A} receptor antagonist WAY-100635 (Fletcher *et al.*, 1996) and studied the effects on sexual behaviour.

The brain areas where serotonin and 5-HT_{1A} receptors influence ejaculation are not yet known. The staining of Fos, the protein product of the immediate-early gene *c-fos,* has been used to investigate neural activation following copulation and ejaculation (Coolen *et al.*, 1996; Greco *et al.*, 1996; Greco *et al.*, 1998; Pfaus and Heeb, 1997; Veening and Coolen, 1998) and acute and chronic administration of SSRIs (Jongsma *et al.*, 2002; Lino-de-Oliveira *et al.*, 2001; Veening *et al.*, 1998). To investigate how the various drug-treatments altered the activation pattern in the CNS following sexual behaviour,

the pattern and number of Fos immunoreactive cell nuclei throughout the brain and spinal cord were studied.

Materials and methods

Animals

Adult male (n=60, 250-300 g and 3 months of age at the start of the experiment) and female (n=100) Wistar rats (Harlan, Zeist, the Netherlands) were used. The animals arrived at the laboratory at least 14 days prior to the start of the experiments, in order to adapt to the laboratory environmental condition and a reversed light/dark cycle (12:12h, lights off at 6.30 am). Food and tap water were available *ad libitum*. Males were housed individually and the females two per cage. Females were sterilized by ligation of the oviducts and served as stimulus animals. Sexual receptivity was reliably induced by subcutaneous injection of 50 µg estradiol benzoate dissolved in 0.1 ml arachidis oil, 36 h prior to testing. Following the Dutch law on the Protection of Animals, the Animal Ethical Committee of the University of Nijmegen approved of the studies.

Drugs

N-[2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide 3HCL (WAY-100635, Wyeth-Ayerst, Princeton, NJ) was dissolved in 0.9% NaCl and injected subcutaneously in a dose of 0.1 mg/kg and a volume of 1 ml/kg.
Citalopram hydrobromide (kindly provided by Lundbeck, Copenhagen, Denmark) was dissolved in 1% methylcellulose and administered orally in a dose of 10 mg/kg and a volume of 5 ml/kg.

Behavioural observations

All sessions were performed between 10.00h and 15.30h, in a red-lighted room. In all sessions the same procedure was used: male rats were placed in a rectangular mating arena (40x50x65 cm) with wood shavings on the floor and a Perspex front. After ten min of habituation a receptive female entered the arena and free contact was allowed for thirty min. The first two weekly sessions were used as training and no observer was present. In the next two weekly sessions the number of ejaculations was counted. From the animals that had reached 2 or 3 ejaculations in 30 min, 32 rats were selected for the experiment and randomly assigned to the four experimental groups (n=8 per group). Every day between 16.00h and 18.00h, each rat received two injections within

Table 1. The effects of vehicle, WAY-100635 (0.1 mg/kg/day s.c.), citalopram (10/mg/kg/day p.o.) or co-administration of WAY-100635 and citalopram after 1-day, 8-day and 15-day treatment on the ejaculation frequency (EF), ejaculation latency (EL), post-ejaculatory interval (PEI), mount frequency (MF), intromission frequency (IF) and intromission latency (IL) of sexually experienced male rats in a 30-min sexual behaviour test with a receptive female.

Day:	Parameter:	Vehicle (n=8)	WAY 100635 (n=8)	Citalopram (n=8)	WAY + citalopram (n=7)
1	EF (n)	3.00 ± 0.26	2.50 ± 0.33	2.00 ± 0.00 **a**	0.00 ± 0.14 **abc**
	EL (s)	340.16 ± 69.06	444.00 ± 101.91	454.90 ± 54.40	1800.00 ± 38.78 **abc**
	PEI (s)	282.45 ± 10.48	320.71 ± 20.20	364.98 ± 8.73 **a**	-
	MF (n)	13.00 ± 2.11	12.00 ± 4.23	12.00 ± 0.85	-
	IF (n)	11.50 ± 1.85	10.00 ± 0.71	7.00 ± 0.99	-
	ML (s)	26.80 ± 12.85	8.02 ± 0.33 **a**	9.53 ± 2.92	17.63 ± 25.52 **abc**
	IL (s)	7.50 ± 3.67	11.90 ± 1.33	18.13 ± 12.69	231.78 ± 283.18 **abc**
8	EF (n)	3.00 ± 0.33	2.50 ± 0.26	2.00 ± 0.13	0.00 ± 0.14 **abc**
	EL (s)	288.69 ± 61.54	319.06 ± 57.73	486.45 ± 48.27	1800.00 ± 14.75 **abc**
	PEI (s)	269.85 ± 24.20	280.95 ± 30.16	355.56 ± 25.02	-
	MF (n)	9.50 ± 3.23	8.50 ± 1.39	19.50 ± 3.96	-
	IF (n)	7.00 ± 1.06	8.50 ± 0.59	6.50 ± 1.39	-
	ML (s)	6.37 ± 5.39	5.80 ± 1.47	10.36 ± 4.45	28.67 ± 7.96
	IL (s)	8.24 ± 1.16	9.92 ± 2.52	6.73 ± 4.82	37.68 ± 52.88 **abc**
15	EF (n)	3.00 ± 0.26	3.00 ± 0.26	2.00 ± 0.59	0.00 ± 0.00 **abc**
	EL (s)	371.41 ± 47.22	382.53 ± 49.00	624.20 ± 374.16	1800.00 ± 0.00 **abc**
	PEI (s)	276.99 ± 10.31	331.70 ± 19.28	322.03 ± 16.00	-
	MF (n)	12.50 ± 2.64	16.00 ± 2.37	21.00 ± 6.01	-
	IF (n)	11.00 ± 12.25	8.00 ± 0.86	8.00 ± 0.67	-
	ML (s)	5.11 ± 0.88	5.91 ± 1.29	10.36 ± 2.89 **a**	12.63 ± 32.20 **ab**
	IL (s)	8.38 ± 1.03	29.20 ± 11.68	10.60 ± 2.33	42.18 ± 292.82 **ac**

Data are medians ± standard error of the median. a=different from the vehicle-treated group, b=different from the WAY-treated group, c=different from the citalopram-treated group; $P<0.05$.

one min in one of four combinations: saline (s.c.) + methylcellulose (p.o.), WAY-100635 (s.c.) + methylcellulose (p.o.), saline (s.c.) + citalopram (p.o.) or WAY-100635 (s.c.) + citalopram (p.o.). On testing days, both injections were given 55-60 min prior to the behavioural test. One rat was discarded from the co-administration group because of a failed injection.
In total three behavioural tests of 30 min were run one h after the 1st, the 8th and the 15th injection (days 1, 8 and 15). The total number of mounts, intromissions and ejaculations was counted using event recording software of The Observer (Noldus, the Netherlands). The mount latency (time from the start of the test to the first mount), intromission latency (time from the start of the test to the first intromission), mount frequency (number of mounts prior to ejaculation), intromission frequency (number of intromissions prior to ejaculation), ejaculation latency (time from first mount or intromission to ejaculation) and post-ejaculatory interval (time from ejaculation to next mount or intromission) were calculated. All parameters were analyzed for the first and second ejaculatory cycle.

Immunohistochemistry
One h after the end of the behavioural test on day 15, males were anesthetized using an overdose of sodium pentobarbital (60 mg/ml, 0.2 ml/kg, i.p.) and perfused transcardially with 0.1 M phosphate buffered saline (PBS, pH 7.3) followed by fixative (4% paraformaldehyde in PBS, pH 7.2). Brains were removed and post fixed for 24h at 4°C before the paraformaldehyde was replaced by 30% sucrose in PB.
Coronal sections (40 µm) were cut using a freezing microtome and collected in PBS-containing tubes. All steps of the immunohistochemistry described below were performed at room temperature.
First, sections were rinsed in PBS, soaked in 30% H_2O_2 for 30 min and rinsed 3 x 20 min in PBS. After 30 min of preincubation with PBS containing 0.1% bovine serum albumin and 0.5% Triton-X-100, sections were incubated overnight in the same medium with an *c-fos* antiserum raised in rabbit (Santa Cruz, USA, dilution 1:20000). The next day, the sections were rinsed 3 x 20 min in PBS and incubated for ninety min in donkey anti-rabbit antibody (Biotin SP conjugated, Jackson Immuno Research, USA, diluted 1:400). Sections were rinsed 3 x 20 min and incubated for ninety min in ABC-elite (Vector elite 1:800 in PBS, prepared 60 min in advance, Brunschwig Chemie, the Netherlands). Again, the sections were rinsed 3 x 20 min in PBS. Then, sections were stained using a chromogen solution consisting of 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.03% Nickel-Ammonium in 0.05M Tris-buffer (pH 7.6): exactly 10 min of

incubation without, and 10 min with 30% H_2O_2. This resulted in a blue-black coloured staining. All sections were rinsed for 3 x 20 min in PBS and mounted on gelatin chrome alum-coated glass slides, dried overnight, cleared in xylene, embedded with Entellan (Merck, Germany) and coverslipped.

Immunoreactive cell nuclei were quantified using the software program Neurolucida (Brightfield, USA). Numbers of Fos-immunoreactive nuclei were counted in homologous square fields (using a grid size of 100x100 µm) that displayed a representative density of stained cells. Some series of brain slices did not yield representative staining and were removed from quantification.

Fos expression was quantified in the following brain areas known to be involved in sexual behaviour: the medial preoptic nucleus, rostral and caudal posteror medial bed nucleus of the stria terminalis, dorsal parvocellular paraventricular hypothalamic nucleus, posterodorsal medial amygdala, medial parvocellular subparafascicular thalamic nucleus and sacral parasympathetic nucleus at the L6-S1 level of the spinal cord. Since these areas are activated in relation with ejaculation, the citalopram-treated rats that did not ejaculate in the last sexual behaviour test were excluded from the comparison.

Fos expression was further quantified in all areas showing substantial fos-immunoreactivity in most of the rats in at least one experimental group: the prelimbic cor-

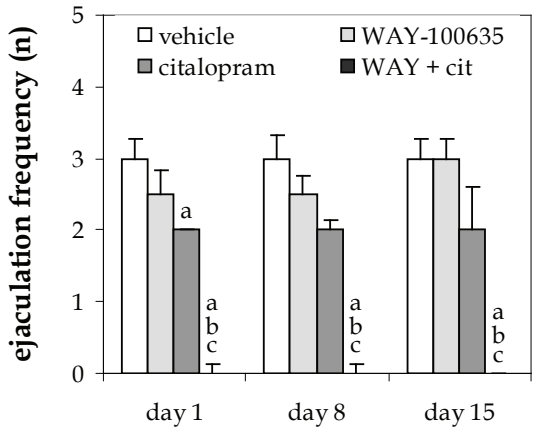

Figure 1. The effects of vehicle (white bars), WAY-100635 (0.1 mg/kg/day s.c., light grey bars), citalopram (10/mg/kg/day p.o., grey bars) or co-administration of WAY-100635 and citalopram (dark grey bars) after 1-day, 8-day and 15-day treatment on the ejaculation frequency of rats in a 30-min sexual behaviour test with a receptive female. Data are medians ± standard error of the median; a=different from the vehicle-treated group, b=different from the WAY-treated group, c=different from the citalopram-treated group; $P<0.05$.

tex, ventral lateral septum, dorsal lateral bed nucleus of the stria terminalis, medial parvocellular paraventricular hypothalamic nucleus, arcuate hypothalamic nucleus, lateral central amygdala, dorsomedial ventromedial hypothalamic nucleus, ventral premammillary nucleus, apical interpeduncular nucleus, ventrolateral periaqueductal grey, compact part of the nucleus incertus, locus coeruleus and medial nucleus of the solitary tract.

Statistical Analysis

All behavioural data were analyzed using the Kruskall Wallis test and, in case of overall significant differences, with the Mann Whitney test. The immunohistochemical data were analyzed using Univariate Analysis of Variance (ANOVA), further post-hoc comparisons were made using the Student-Neuman-Keuls test.

In order to find common changes in activated areas of individual animals, a correlation analysis was performed on the numbers of Fos-immunoreactive cell nuclei using Pearson's correlation coefficient. The level of significance in all tests was $P<0.05$.

Results

Sexual behaviour

An overview of the sexual behaviour of the treatment groups is shown in table 1.

Analysis with the Kruskall Wallis test showed that there were group differences on the first treatment day in ejaculation frequency ($\chi^2=15.857$; $P=0.001$), ejaculation latency ($\chi^2=13.225$; $P=0.004$), post ejaculatory interval ($\chi^2=9.534$; $P=0.023$), mount latency ($\chi^2=9.188$; $P=0.027$) and intromission latency ($\chi^2=15.366l$; $P=0.002$). Further analysis with the Mann-Whitney test revealed that the group treated with both WAY-100635 and citalopram had a reduced ejaculation frequency ($P<0.01$, figure 1) and an increased ejaculation latency and intromission latency ($P<0.01$) than all the other groups, and a higher mount latency than the group treated with WAY-100635 only ($P=0.015$). The WAY-100635-treated group had a lower mount latency compared to the vehicle-treated group ($P=0.021$). Citalopram alone reduced the ejaculation frequency ($P=0.036$) and increased the post ejaculatory interval compared to vehicle on day 1 ($P=0.004$).

After one week of drug-treatment, the Kruskall Wallis test showed group differences in ejaculation frequency ($\chi^2=18.521$; $P=0.000$), EL ($\chi^2=16.873$; $P=0.001$) and intromission latency ($\chi^2=8.621$; $P=0.035$). Further analysis with the Mann Whitney test showed that the co-administration group had a reduced ejaculation frequency ($P<0.005$) and an

increased ejaculation latency (P<0.005) and intromission latency (P<0.05) compared to all other groups. There were no differences between the other groups.
Two weeks of treatment resulted in differences in ejaculation frequency (χ^2=17.318; P=0.001), ejaculation latency (χ^2=15.102; P=0.002), mount latency (χ^2=10.245; P=0.017) and intromission latency (χ^2=9.337; 0.025). According to the Mann Whitney test, the co-administration group had a reduced ejaculation frequency (P<0.05) and an increased ejaculation latency (P<0.05) compared to all other groups. The co-administration group differed in mount latency from the vehicle- and WAY-100635-treated groups (P<0.05) and in intromission latency from the vehicle- and citalopram treated groups (P<0.05). Citalopram increased mount latency compared to vehicle-treatment (P=0.036).
No significant differences on any day existed between the experimental groups in the second ejaculatory cycle (table 2).

Table 2. The effects of vehicle, WAY-100635 (0.1 mg/kg/day s.c.) or citalopram (10/mg/kg/day p.o.) after 1-day, 8-day and 15-day treatment on the second ejaculation latency (EL), post-ejaculatory interval (PEI), mount frequency (MF) and intromission frequency (IF) of sexually experienced male rats in a 30-min sexual behaviour test with a receptive female.

Day:	Parameter:	Vehicle (n=8)	WAY 100635 (n=8)	Citalopram (n=8)
1	EL (s)	190.65 ± 25.92	193.67 ± 32.29	378.87 ± 63.27
	PEI (s)	377.40 ± 4.37	429.82 ± 13.46	429.94 ± 25.87
	MF (n)	7.50 ± 2.31	4.50 ± 2.74	11.00 ± 1.97
	IF (n)	4.50 ± 0.59	3.00 ± 0.00	4.00 ± 0.56
8	EL (s)	150.60 ± 30.55	183.65 ± 16.97	297.45 ± 50.18
	PEI (s)	339.49 ± 25.07	430.32 ± 15.77	389.67 ± 6.13
	MF (n)	6.00 ± 1.27	7.00 ± 1.45	11.50 ± 1.22
	IF (n)	4.00 ± 0.71	3.00 ± 0.13	4.00 ± 0.23
15	EL (s)	201.91 ± 37.52	213.94 ± 10.56	546.95 ± 130.74
	PEI (s)	394.42 ± 20.43	437.81 ± 12.60	-
	MF (n)	10.50 ± 4.09	9.00 ± 2.12	29.00 ± 7.34
	IF (n)	4.50 ± 0.59	4.00 ± 0.56	4.00 ± 0.33

Data are medians ± standard error of the median.

Immunohistochemistry

The combination of drug administration and the concomitant performance of sexual behaviour preceding perfusion led to substantial *c-fos* expression. Figure 2, 3 and 4, showing respectively the paraventricular hypothalamic nucleus, the posterodorsal medial amygdala and the apical interpeduncular nucleus, are illustrations of this expression.

The Univariate ANOVA revealed significant differences between the experimental groups in the number of Fos-positive in several areas (figure 5): the rostral ($F=8.052$, $P=0.001$) and caudal ($F=9.462$, $P=0.000$) posterior medial bed nucleus of the stria terminalis, the dorsal parvocellular paraventricular hypothalamic nucleus ($F=5.519$, $P=0.005$), the posterodorsal medial amygdala ($F=6.263$, $P=0.003$), the medial parvocellular subparafascicular thalamic nucleus ($F=6.663$, $P=0.003$), the sacral parasympathetic nucleus at the L6/S1 level ($F=9.784$, $P=0.000$), the medial parvocellular paraventricular hypothalamic nucleus ($F=3.637$, $P=0.034$), the arcuate hypothalamic nucleus ($F=4.965$, $P=0.011$) and the apical interpeduncular nucleus ($F=13,226$, $P=0.000$).

Further analysis with the post-hoc Student-Neuman-Keuls test showed that the number of Fos-immunoreactive nuclei was strongly reduced in the co-administration group compared to all other experimental groups in the rostral and caudal posterior medial bed nucleus of the stria terminalis, the posterodorsal medial amygdala, the medial parvocellular subparafascicular thalamic nucleus and the sacral parasympathetic nucleus at the L6/S1 level ($P<0.05$). Furthermore, the co-administration group showed less *c-fos* expression in the dorsal parvocellular paraventricular hypothalamic nucleus compared to animals treated with either vehicle or WAY-100635 ($P<0.05$), but not to animals treated with citalopram.

Treatment with citalopram alone or combined with WAY-100635 reduced Fos-immunoreactivity in the arcuate hypothalamic nucleus compared to treatment with vehicle alone ($P<0.05$). Animals that had received injections with WAY-100635 alone showed more Fos-immunoreactivity than vehicle-treated rats in the medial parvocellular paraventricular hypothalamic nucleus ($P<0.05$).

WAY-100635 increased the number of Fos-positive neurons in the apical interpeduncular nucleus compared to all other groups, whereas citalopram decreased Fos-immunoreactivity In all other areas quantified, no significant differences existed between the experimental groups (Table 3).

A correlation-analysis (Table 4) applied on the number of Fos-immunoreactivity neurons in the quantified brain areas of individual animals, in order to find co-varying changes, resulted in strong and significant correlations between the medial preoptic

nucleus, the rostral and caudal posterior medial bed nucleus of the stria terminalis, the dorsal parvocellular paraventricular hypothalamic nucleus, the posterodorsal medial amygdala and the medial parvocellular subparafascicular thalamic nucleus ($0.418<R^{2<}0.852$, $P<0.05$). Fos-immunoreactivity in the sacral parasympathetic nucleus at the L6/S1 level was strongly correlated with the dorsal parvocellular paraventricular hypothalamic nucleus ($R^{2=}0.549$, $P<0.01$), less strongly with the other ejaculation-related areas ($0.401<R^{2<}0.466$, $P<0.05$) and not significantly with the caudal posterior medial bed nucleus of the stria terminalis ($R^{2=}0.371$, $P=0.81$).

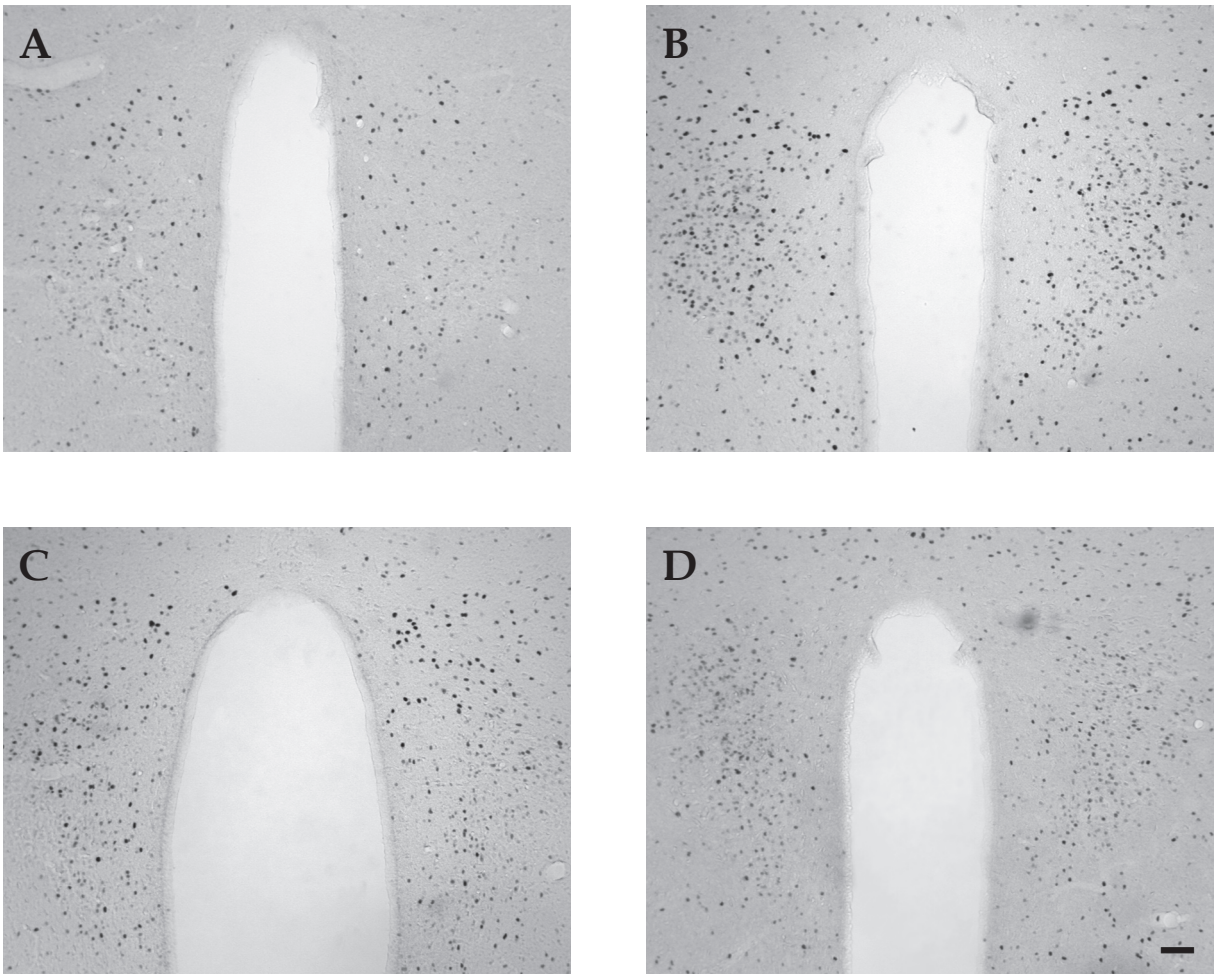

Figure 2. Fos-positive nuclei in medial parvocellular as well as the dorsal parvocellular paraventricular hypothalamic nucleus (Bregma –1.80) of rats that were treated for 15 days with vehicle (A), WAY-100635 (B), citalopram (C) or co-administration of WAY-100635 and citalopram (D) and perfused 1h after a 30-min sexual behaviour test. Scale bar=100µm.

Discussion

Sexual behaviour

Acute and chronic administration of the 5-HT_{1A} receptor antagonist WAY-100635 alone had no relevant effect on sexual behaviour, which is consistent with previous findings using WAY-100635 in doses up to 0.6 mg/kg s.c. (Ahlenius and Larsson, 1999). These results show that under basal conditions 5-HT_{1A} receptors do not play a crucial role in

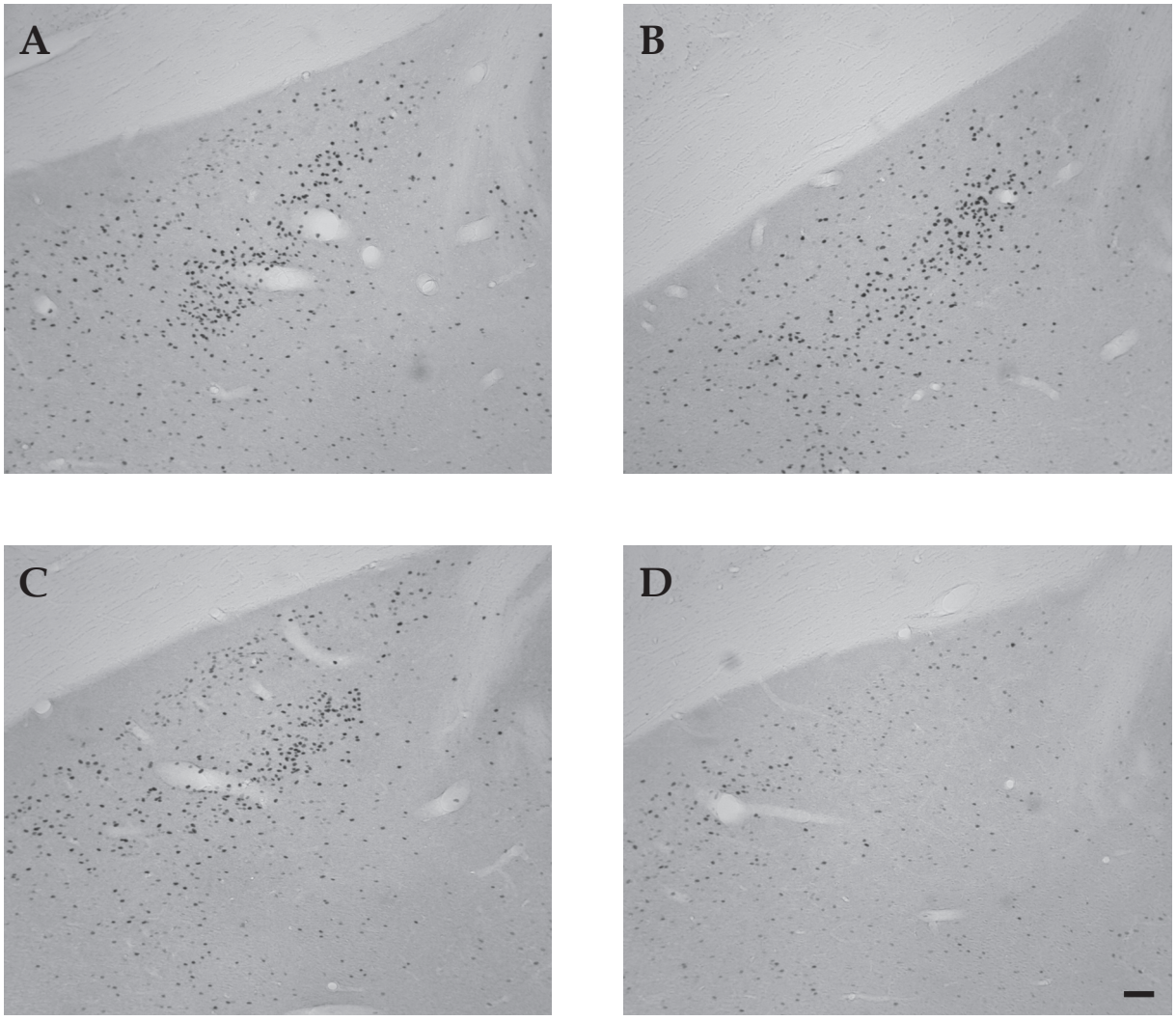

Figure 3. Fos-positive nuclei in the posterodorsal medial amygdala (Bregma -3.14 mm) of rats that were treated for 15 days with vehicle (A), WAY-100635 (B), citalopram (C) or co-administration of WAY-100635 and citalopram (D) and perfused 1h after a 30-min sexual behaviour test. Scale bar=100µm.

sexual behaviour, either because ejaculation can be achieved via other pathways, or because pre- and postsynaptic 5-HT_{1A} receptors are not significantly activated during copulation.

Acute injection with the SSRI citalopram (10 mg/kg p.o.) reduced the ejaculation frequency and increased the post ejaculatory interval in sexually experienced male Wistar rats, but this effect disappeared after 8 and 15 days of treatment. On day 15, citalopram slightly increased the mount latency. These mild sexual side effects might have been

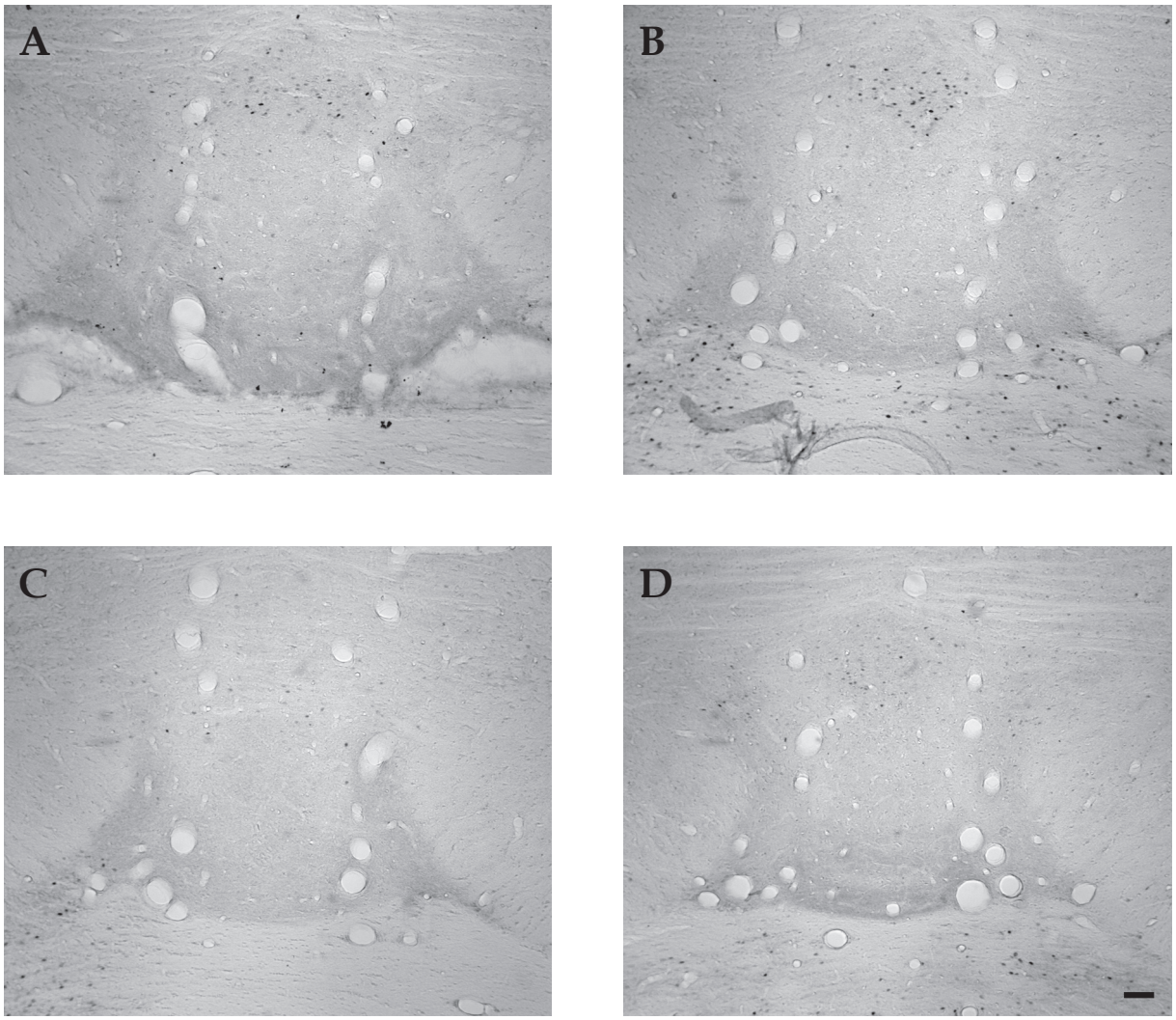

Figure 4. Fos-positive nuclei in the apical interpeduncular nucleus (Bregma –6.72 mm) of rats that were treated for 15 days with vehicle (A), WAY-100635 (B), citalopram (C) or co-administration of WAY-100635 and citalopram (D) and perfused 1 h after a 30-min sexual behaviour test. Scale bar=100µm.

stronger if a higher dose of citalopram was used. However, other groups found that equivalent doses of citalopram at least doubled extracellular 5-HT levels in the ventral hippocampus (Cremers *et al.*, 2000a; Hjorth *et al.*, 1997), indicating a relevant effect of the SSRI on serotonergic neurotransmission. Moreover, Ahlenius *et al.* showed that acute injection with citalopram in doses up to 40 mg/kg s.c. did not change sexual behaviour (Ahlenius and Larsson, 1999).

When citalopram and the 5-HT_{1A} receptor antagonist WAY-100635 (0.1 mg/kg s.c.) were co-administered, 75% of the animals failed to ejaculate within 30 min on day 1 and 8, and 100% failed to ejaculate on day 15. In addition, the intromission latency was strongly increased on all test days and the mount latency was increased on day 15. These results are consistent with, although more pronounced than, the findings of Ahlenius *et al.*, who demonstrated an inhibition of sexual behaviour in male Wistar rats after acute co-administration of WAY-100635 (0.04 and 0.08 mg/kg, s.c.) and citalopram (10 mg/kg, s.c.) (Ahlenius and Larsson, 1999). The present study shows that the effect of treatment with WAY-100635 and/or citalopram on sexual behaviour does not change after chronic treatment, indicating that these drugs fail to induce long-term alterations of the neurobiological substrate underlying sexual behaviour.

Several explanations are possible for the synergistic effects of WAY-100635 and citalopram on sexual behaviour. In vivo microdialysis studies have shown that WAY-100635 significantly facilitates the elevation of serotonin levels by acute citalopram treatment through blockade of the negative feedback signal of the somatodendritic 5-HT_{1A} autoreceptor (Cremers *et al.*, 2000a; Hjorth *et al.*, 1997). It is possible that in order to inhibit ejaculation, serotonin levels need to exceed a certain threshold that is not reached by either citalopram or WAY-100635 alone. However, 5-HT_{1B} receptor antagonists augment the citalopram-induced elevation of serotonin levels even more (Cremers *et al.*, 2000a), while decreasing the inhibition of ejaculation induced by co-administration of WAY-100635 with citalopram (Ahlenius and Larsson, 1999). This suggests that elevation of serotonin levels by blockade of presynaptic receptors is not sufficient to affect ejaculation, and that postsynaptic receptors at least play a role.

By elevating serotonin levels throughout the central nervous system, SSRIs increase the activation of different subtypes of postsynaptic 5-HT receptors. This might result in conflicting effects on sexual behaviour, since ejaculation is facilitated by postsynaptic 5-HT_{1A} receptors (Ahlenius *et al.*, 1981; Coolen *et al.*, 1997b; Fernandez-Guasti and Escalante, 1991; Haensel and Slob, 1997) and inhibited by postsynaptic 5-HT_{1B} and 5-HT_{2C} receptors (Ahlenius and Larsson, 1998; Fernandez-Guasti *et al.*, 1992; Fernandez-Guasti and Rodriguez-Manzo, 1992; Foreman *et al.*, 1989; Hillegaart and Ahlenius,

Table 3. The number of Fos-immunoreactive nuclei in the prelimbic area (PrL); ventral lateral septum (LSV); dorsal lateral bed nucleus of the stria terminalis (BNSTdl); medial preoptic nucleus (MPN); lateral central amygdala (CeAl); dorsomedial ventromedial hypothalamic nucleus (VMHdm); ventral premammillary nucleus (PMV); ventrolateral periaqueductal grey (PAGvl); compact part of the nucleus incertus (NIc); locus coeruleus (LC) and medial nucleus of the solitary tract (NTSm) of sexually experienced rats treated for 15 days with vehicle, WAY-100635 (0.1 mg/kg/day s.c.), citalopram (10/mg/kg/day p.o.) or co-administration of WAY-100635 and citalopram, 1 h after a 30-min sexual behaviour test with a receptive female.

Brain Area:	Vehicle (n=5)	WAY 100635 (n=6)	Citalopram (n=5)	WAY + Citalopram (n=6)
PrL	18.40 ± 2.14	21.17 ± 1.88	22.40 ± 2.39	22.50 ± 1.41
LSV	13.60 ± 2.40	21.20 ± 1.37	16.60 ± 2.33	18.67 ± 2.26
BNSTdl	19.20 ± 2.18	26.33 ± 4.20	23.80 ± 2.51	20.00 ± 1.91
MPN	39.25 ± 4.39	37.25 ± 3.82	37.20 ± 8.01	23.50 ± 2.63
CeAl	24.00 ± 1.76	26.33 ± 2.25	22.00 ± 2.94	22.20 ± 1.76
VMHdm	30.00 ± 4.00	33.17 ± 2.13	29.00 ± 2.78	31.00 ± 2.91
PMV	28.20 ± 2.17	20.80 ± 2.12	26.50 ± 4.84	28.40 ± 1.44
PAGvl	23.00 ± 1.00	23.67 ± 2.86	25.60 ± 3.67	20.33 ± 2.74
NIc	17.00 ± 2.50	22.50 ± 3.96	23.00 ± 2.75	15.80 ± 0.76
LC	20.40 ± 2.82	19.00 ± 2.93	19.60 ± 1.75	16.40 ± 1.50
NTSm	14.80 ± 3.44	17.17 ± 1.04	17.60 ± 2.14	19.00 ± 1.89

Data are means ± standard error of the mean.

Table 4. In brain areas activated by sexual behaviour and ejaculation, the medial preoptic nucleus (MPN); rostral and caudal posterior medial bed nucleus of the stria terminalis (r/cBNSTpm); dorsal parvocellular paraventricular hypothalamic nucleus (PVHdp); posterodorsal medial amygdala (MeApd), medial parvocellular subparafascicular thalamic nucleus (mSPFp) and the sacral parasympathetic nucleus at the L6-S1 level of the spinal cord (SPN), the numbers of Fos-positive cells are correlated.

Correlation	MPN	rBNSTpm	cBNSTpm	PVHdp	MeApd	mSPFp
rBNSTpm	.829 **b**					
cBNSTpm	.591 **b**	.615 **b**				
PVHdp	.796 **b**	.604 **b**	.659 **b**			
MeApd	.715 **b**	.647 **b**	.710 **b**	.852 **b**		
mSPFp	.418 **a**	.525 **a**	.534 **a**	.543 **b**	.565 **b**	
SPN (L6-S1)	.446 **a**	.401 **a**	.371	.549 **b**	.413 **a**	.466 **a**

a=P<0.05, b=P<0.01

1998; Waldinger *et al.*, 1998a). When the facilitating 5-HT_{1A} receptors are blocked, for example by WAY-100635, the netto effect of SSRI-induced elevation of 5-HT levels might be inhibition of ejaculation via 5-$HT_{1B/2C}$ receptors. This could also explain the differences in sexual side-effects between paroxetine and fluoxetine on one hand and citalopram on the other hand: chronic administration of paroxetine and fluoxetine cause desensitization of postsynaptic 5-HT_{1A} receptors (D'Souza *et al.*, 2002; Hensler, 2003; Kantor *et al.*, 2001; Li *et al.*, 1997b), whereas chronic citalopram treatment has less pronounced effects on 5-HT_{1A} receptors (Arborelius *et al.*, 1996; Auerbach and Hjorth, 1995; Chaput *et al.*, 1986; Cremers *et al.*, 2000b; Gundlah *et al.*, 1997; Hjorth and Auerbach, 1999; Invernizzi *et al.*, 1995; Moret and Briley, 1996). Although it is unknown whether SSRI-induced desensitization also affects 5-HT_{1A} receptors involved in ejaculation, it provides an interesting approach for future research.

Immunohistochemistry

Animals treated with both WAY-100635 and citalopram showed significantly less Fos-immunoreactivity compared to the other experimental groups in the rostral and caudal posterior medial bed nucleus of the stria terminalis, the posterodorsal medial amygdala, the medial parvocellular subparafascicular thalamic nucleus and the sacral parasympathetic nucleus at the L6-S1 level of the spinal cord. These areas are known to express *c-fos* when an ejaculation has occurred (Coolen *et al.*, 1996; Coolen *et al.*, 1997b; Coolen *et al.*, 2003b; Greco *et al.*, 1996; Greco *et al.*, 1998), which is consistent with the behavioural results. The dorsal parvocellular paraventricular hypothalamic nucleus showed a similar activation pattern. Moreover, the number of Fos-immunoreactive cell nuclei in the dorsal parvocellular paraventricular hypothalamic nucleus was strongly correlated with all known ejaculation-related areas suggesting that this area participates in the neural circuitry that is activated by ejaculation (Coolen *et al.*, 2003b).

There is evidence to assume that the ejaculation-related areas are activated by the genitosensory signal that an ejaculation has occurred, rather than to cause ejaculation (Coolen *et al.*, 1996; Coolen *et al.*, 1997b; Coolen *et al.*, 2003b; Parfitt and Newman, 1998). In that light, it was not surprising that the amount of Fos-immunoreactivity appeared to be related to the ejaculation frequency. Therefore, this group of brain areas provided no further clues about the location where drugs interact with the neural network to inhibit ejaculation.

Chronic citalopram treatment as well as co-administration reduced Fos-immunoreactivity in the arcuate hypothalamic nucleus, which coincided with increased mount

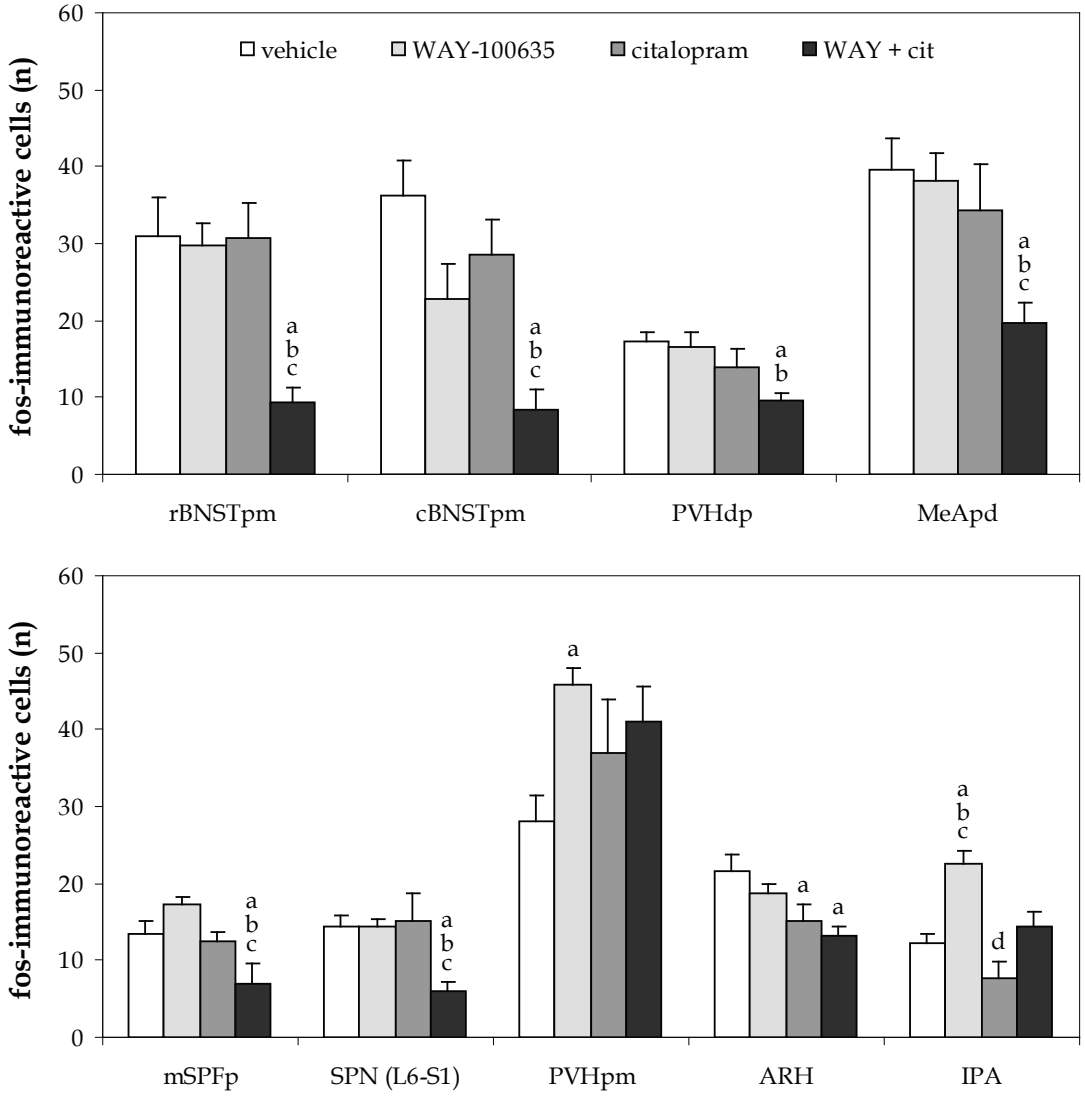

Figure 5. The number of fos-immunoreactive nuclei in the rostral and caudal posterior medial bed nucleus of the stria terminalis (r/cBNSTpm); the dorsal parvocellular paraventricular hypothalamic nucleus (PVHdp); the posterodorsal medial amygdala (MeApd), the medial parvocellular subparafascicular thalamic nucleus (mSPFp), the sacral parasympathetic nucleus (SPN) at the L6-S1 level of the spinal cord, the medial parvocellular paraventricular hypothalamic nucleus (PVHmp), the arcuate hypothalamic nucleus (ARH) and the apical interpeduncular nucleus (IPA) of rats treated for 15 days with vehicle (white bars), WAY-100635 (0.1 mg/kg/day s.c., light grey bars), citalopram (10/mg/kg/day p.o., grey bars) or co-administration of WAY-100635 and citalopram (dark grey bars) and perfused 1 h after a sexual behaviour test. Data are means ± standard error of the mean; a=different from the vehicle-treated group, b=different from the WAY-treated group, c=different from the citalopram-treated group, d=different from the co-administration group; $P<0.05$.

latencies in these experimental groups. The arcuate nucleus is innervated by serotonergic fibres (Paxinos, 1995) and expresses postsynaptic 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2C} receptors (Aznar *et al.*, 2003; Clemett *et al.*, 2000; Collin *et al.*, 2002; Makarenko *et al.*, 2002). It is connected with the medial preoptic nucleus, the medial amygdala, the bed nucleus of the stria terminalis and the paraventricular hypothalamic nucleus, and is thought to integrate information about metabolism with reproductive activity, using galanin-like peptide, neuropeptide Y and gonadotropin-releasing hormone as messengers (Gottsch *et al.*, 2004; Magoul *et al.*, 1994). Taken together, the arcuate hypothalamic nucleus is in an excellent location to connect the serotonergic system with the reproductive system.

The apical interpeduncular nucleus, a brain area that sends projections to the hippocampus, septum and raphe nuclei (Montone *et al.*, 1988) and is dense in 5-HT_{1A} receptor labelling (Kia *et al.*, 1996), was strongly activated by chronic treatment with WAY-100635. Co-administration of citalopram reversed this effect (Fig 4, Table 3). The medial parvocellular paraventricular hypothalamic nucleus showed a similar activation following chronic WAY-100635 treatment, but (co-)administration of citalopram did not alter this activity. Apparently, blocking the 5-HT_{1A} receptor changes activity in these areas, but since WAY-100635 alone did not affect any parameter of sexual behaviour it is unlikely that these areas are involved in male copulation.

Substantial Fos-immunoreactivity was visible in the prelimbic area, the ventral lateral septum, the dorsal lateral bed nucleus of the stria terminalis, the lateral central amygdala, the dorsomedial ventromedial hypothalamic nucleus, the ventral premammillary nucleus, the ventrolateral periaqueductal grey, the compact part of the nucleus incertus, the locus coeruleus and the medial nucleus of the solitary tract. However, the different drug treatments had no effect on the number of immunoreactive nuclei in these brain areas, indicating that they are activated as a result of a common experience such as general activity, sexual arousal or receiving injections.

The distribution of Fos-immunoreactivity and treatment-induced differences in Fos-immunoreactivity do not correspond with previous findings (Jongsma *et al.*, 2002). In that study the effects of acute administration of WAY-100635 and/or citalopram on *c-fos* expression were analyzed. Drug-dependent differences were found in the prefrontal cortex, the central amygdala, the ventromedial hypothalamic nucleus, the dorsal raphe nucleus and other areas. A synergistic effect of WAY-100635 and citalopram was found in the paraventricular hypothalamic nucleus. The differences with the present study can be attributed to the addition of sexual behaviour. Furthermore, (sub)chronic treatment with serotonergic agents has repeatedly been found to attenuate or change

Fos-immunoreactivity compared to acute treatment (Li and Rowland, 1996; Lino-de-Oliveira *et al.*, 2001; Veening *et al.*, 1998), probably because the brain habituates to the treatment, and/or the Fos-inducing mechanisms become desensitized.

In summary, our results show that the weak inhibition of ejaculation by citalopram is strongly augmented by the silent and selective 5-HT_{1A} receptor antagonist WAY-100635. 5-HT_{1A} receptors seem to play a minor role in sexual behaviour when serotonin turnover is normal, but prove to be crucial for ejaculation when serotonin levels are elevated. A combination of elevated serotonin levels and reduced 5-HT_{1A} receptor activation, for example through desensitization of these receptors, could therefore be responsible for the sexual side-effects reported by men treated with SSRIs.

In general, Fos-immunoreactivity reflected the occurrence of an ejaculation rather than revealing putative locations where serotonergic neurotransmission and ejaculatory behaviour interact with each other. However, the arcuate hypothalamic nucleus showed altered Fos-immunoreactivity after chronic citalopram treatment with or without WAY-100635, and needs to be investigated as a possible location for the interaction of serotonergic drugs with male copulatory behaviour.

Acknowledgements

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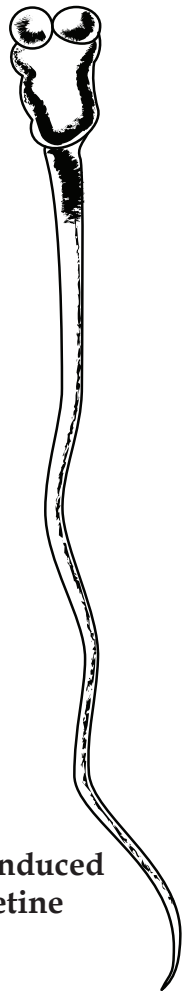
CHAPTER 4



Effects of chronic selective serotonin reuptake inhibitors on 8-OH-DPAT-induced facilitation of ejaculation in rats: comparison of fluvoxamine and paroxetine

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Abstract

Chronic treatment with selective serotonin reuptake inhibitors (SSRIs) can delay ejaculation in humans, but the extent of this effect differs between SSRIs. Involvement of 5-HT_{1A} receptors is likely, since 5-HT_{1A} receptor agonists accelerate ejaculation and chronic SSRI treatment is thought to desensitize 5-HT_{1A} receptors. The objective was to study the effects of chronic pre-treatment with the SSRIs fluvoxamine and paroxetine on the facilitation of ejaculation induced by the 5-HT_{1A} receptor agonist 8-OH-DPAT. Sexually experienced Wistar rats with normal ejaculatory behaviour were treated for 22 days with vehicle, fluvoxamine (30 mg/kg/day p.o.) or paroxetine (10 or 20 mg/kg/day p.o.). On day 22, rats received a challenge with saline or 8-OH-DPAT (0.4 mg/kg s.c.). Sexual behaviour was tested on day 1, 8, 15 and 22 of the SSRI-treatment. Treatment with both doses of paroxetine, but not fluvoxamine, delayed ejaculation. 8-OH-DPAT strongly accelerated ejaculation under vehicle conditions. Pretreatment with paroxetine reduced the effects of 8-OH-DPAT on ejaculation in a dose dependent manner and more strongly than fluvoxamine. We conclude that SSRIs affect 5-HT_{1A} receptors involved in ejaculation. The degree to which this occurs, with paroxetine exerting a stronger effect than fluvoxamine, might determine the extent of SSRI-induced delayed ejaculation.

Introduction

The selective serotonin reuptake inhibitors (SSRIs) paroxetine, fluoxetine, citalopram, fluvoxamine and sertraline are useful therapeutic agents for the treatment of depression, but are also associated with a high incidence of sexual dysfunction (Rosen *et al.*, 1999; Waldinger and Olivier, 1998). In humans suffering from premature ejaculation it has been demonstrated that, of all SSRIs, paroxetine delays ejaculation the most whereas fluvoxamine affects ejaculation the least (Waldinger *et al.*, 1998a; Waldinger *et al.*, 2004a). Comparable results were found in rats (Waldinger *et al.*, 2002).
Fluvoxamine and paroxetine, like all SSRIs, acutely inhibit the reuptake of serotonin in the presynaptic neuron through blockade of the 5-HT transporter. Treatment with fluvoxamine and paroxetine results in elevated extra-cellular serotonin levels (Bosker *et al.*, 1995a; Malagie *et al.*, 2000), and it is thought that the accompanying increase of serotonergic neurotransmission accounts for the antidepressant effects of SSRIs (Nutt *et al.*, 1999).
Serotonergic neurotransmission involves at least fourteen pharmacologically and structurally distinct 5-HT receptor subtypes (Barnes and Sharp, 1999). Among them, postsynaptic 5-HT_{1B} and 5-HT_{2C} receptors have been shown to inhibit ejaculation upon activation (Ahlenius and Larsson, 1998; Fernandez-Guasti *et al.*, 1992; Fernandez-Guasti and Rodriguez-Manzo, 1992; Foreman *et al.*, 1989; Hillegaart and Ahlenius, 1998; Klint *et al.*, 1992; Watson and Gorzalka, 1991) while activation of 5-HT_{1A} receptors has been shown to accelerate ejaculation (Ahlenius *et al.*, 1981; Coolen *et al.*, 1997a; Fernandez-Guasti and Escalante, 1991; Haensel and Slob, 1997; Rehman *et al.*, 1999).
It is not yet known why changes in serotonergic neurotransmission during chronic treatment with paroxetine, but not with fluvoxamine, result in delayed ejaculation. One option is that paroxetine activates inhibiting 5-$HT_{1B/2C}$ receptors more strongly than fluvoxamine, at least in brain areas that mediate sexual behaviour. Another possibility is that chronic paroxetine impairs 5-HT_{1A} receptors involved in ejaculation, while inhibitory 5-$HT_{1B/2C}$ receptors remain functional. Interestingly, while many findings are consistent with the notion that chronic treatment with the SSRIs paroxetine and fluoxetine cause desensitization of pre- and postsynaptic 5-HT_{1A} receptors ((Davidson and Stamford, 1998; D'Souza *et al.*, 2002; Hensler, 2003; Kantor *et al.*, 2001; Le Poul *et al.*, 2000; Le Poul *et al.*, 1995; Li *et al.*, 1997b; Raap *et al.*, 1999), fluvoxamine apparently does not (Bosker *et al.*, 1995b).
If paroxetine-induced desensitization includes 5-HT_{1A} receptors involved in ejaculation, while fluvoxamine leaves these receptors intact, this could contribute to the dif-

ference in sexual side effects between the two SSRIs. To test this hypothesis, we investigated the effects of the potent 5-HT_{1A} receptor agonist 8-OH-DPAT, in a selected dose (0.4 mg/kg s.c.) that has been shown to facilitate ejaculation (Coolen *et al.*, 1997a), on sexual behaviour of male Wistar rats pretreated for 22 days with vehicle, fluvoxamine (30 mg/kg/day) or paroxetine (10 or 20 mg/kg/day).

Materials and methods

Animals

Adult male (250-300 grams and 3 months of age at the start of the experiment) and female Wistar rats (Harlan, Zeist, the Netherlands) were used. The animals arrived at the laboratory at least 14 days prior to the start of the experiments in order to adapt to the laboratory environmental condition and a reversed light/dark cycle (12:12h, lights off at 6.30h). Food and tap water were available *ad libitum*. Males were individually housed and daily handled during the experiment. Females, which served as stimulus animals, were sterilized and housed in couples. Sexual receptivity of females was induced by subcutaneous administration of 50 µg estradiol benzoate dissolved in 0.1 ml arachidis oil 36 h prior to testing. All experiments were approved by the Animal Ethical Committee of the Radboud University Nijmegen and conform national and international laws of animal care.

Behavioural Observations

All behavioural tests were performed between 10.30h and 15.30h, in a red-lighted room. The following procedure was used for all sessions: a male rat was placed in a rectangular mating arena (40x50x65 cm) with a Perspex front and wood shavings on the floor. After 15 min of habituation, a receptive female entered the arena and free contact was allowed for thirty min. The ejaculation frequency (EF) was counted, and the ejaculation latency (EL), mount frequency (MF) and intromission frequency (IF) per ejaculatory cycle were calculated using event recording software The Observer (Noldus Information Technology, Wageningen, the Netherlands). If a rat did not ejaculate in 30 minutes, EL was set at 1800 seconds and MF and IF were not included in the results.

The naïve rats were trained, as described above, during six successive weekly sexual behaviour tests. The results of the last training session before the drug treatment were used as baseline values. Individuals that showed consistent, normal ejaculation fre-

quencies (average ejaculation frequency in the last three training sessions was between 1.33 and 4.33) were selected for the experiment. Their sexual behaviour was observed on days 1, 8, 15 and 22 of the drug treatments.

Drug Treatments

Paroxetine HCl hemihydrate (GlaxoSmithKline, Uxbridge, UK) and fluvoxamine maleate (Solvay Pharmaceuticals, Hannover, Germany) were used. An additional batch of paroxetine-tablets (20 mg paroxetine/tablet, GlaxoSmithKline, UK) was obtained from a local pharmacy; this was used for the high dose of paroxetine (20 mg/kg/day). The rats received oral injections (5 ml/kg) with vehicle (methylcellulose), fluvoxamine (30 mg/kg/day) or paroxetine (10 or 20 mg/kg/day) for 22 consecutive days, either between 15.30 and 17.00h on non-testing days or 1 h prior to the sexual behaviour test. On day 22, an additional subcutaneous injection (1 ml/kg) with saline or (±)-8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT, 0.4 mg/kg, Sigma-Aldrich Chemie, Germany) was given 30 min prior to the sexual behaviour test. This resulted in 8 experimental groups: pretreatment with vehicle and a challenge with saline (n=9) or

Table 1. The mount frequency (MF) and intromission frequency (IF) in the first ejaculatory cycle of sexually experienced male Wistar rats in a 30-min sexual behaviour test with a receptive female before drug treatment (day -6) and on day 1, 8, 15 and 22 of chronic treatment with vehicle, fluvoxamine (30 mg/kg/day p.o.) and paroxetine (10 and 20 mg/kg/day p.o.).

Day:	Parameter:	Vehicle (n = 9)	Fluvoxamine (30) (n = 6)	paroxetine (10) (n = 10)	paroxetine (20) (n = 9)
-6	MF (n)	3.00 ± 0.50	4.00 ± 1.07	4.00 ± 0.94	4.00 ± 0.50
	IF (n)	7.00± 0.25	7.00 ± 0.46	7.50 ± 1.00	7.00 ± 0.25
1	MF (n)	8.00 ± 2.74	10.00 ± 1.98	15.50 ± 5.33	10.50 ± 1.78
	IF (n)	9.00 ± 1.00	6.00 ± 0.69	9.00 ± 1.37	9.50 ± 1.98
8	MF (n)	13.00 ± 1.74	13.00 ± 2.28	21.00 ± 4.98	15.50 ± 1.45
	IF (n)	8.00 ± 1.74	6.50 ± 0.99	10.00 ± 1.24	10.00 ± 1.65
15	MF (n)	14.00 ± 3.48	14.00 ± 1.52	15.50 ± 6.40	16.00 ± 3.23
	IF (n)	11.00 ± 2.74	5.50 ± 1.22	12.50 ± 1.78	10.00 ± 0.99
22	MF (n)	6.00 ± 2.24	14.00 ± 4.42	13.50 ± 4.22	12.00 ± 2.24
	IF (n)	9.00 ± 0.50	6.00 ± 0.91	12.00 ± 1.65	8.00 ± 0.25

Data are medians ± standard error of the median.

8-OH-DPAT (n=10); pretreatment with fluvoxamine and a challenge with saline (n=6) or 8-OH-DPAT (n=6); pretreatment with a low (10 mg/kg) dose of paroxetine and a challenge with saline (n=10) or 8-OH-DPAT (n=8); pretreatment with a high (20 mg/kg) dose of paroxetine and a challenge with saline (n=9) or 8-OH-DPAT (n=9).

Statistical Analyses:
All behavioural data were analyzed using the non-parametric Kruskall Wallis test. In case of significant differences, data were further analyzed with the Mann Whitney test. All statistical analyses were performed using the Statistical Package for the Social Sciences version 12.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when $P<0.05$.

Results

Effects of SSRI treatment on sexual behaviour
Sexual behaviour was analyzed on day -6 (baseline), 1, 8, 15 and 22 of SSRI-treatment. Analysis with the Kruskall Wallis test revealed group differences in ejaculation frequency ($\chi^2=10.191$; $P=0.017$) and latency to the first ejaculation ($\chi^2=9.065$; $P=0.028$) after 8 days of treatment. These differences were present after 22 days of treatment (ejaculation frequency: $\chi^2=9.943$; $P=0.019$ and first ejaculation latency: $\chi^2=9.932$; $P=0.019$). No differences in ejaculation frequency or first ejaculation latency were found between the experimental groups after acute treatment (day 1) and 15 days of treatment (figure 1, data are expressed as medians).
Further post hoc analyses showed that on day 8, treatment with the low and high dose of paroxetine reduced the ejaculation frequency compared to treatment with vehicle and fluvoxamine. Furthermore, in animals pretreated with the low dose of paroxetine, ejaculation latencies were increased compared to vehicle- and fluvoxamine-treated animals, whereas in animals pretreated with the high dose of paroxetine ejaculation latencies were only increased compared to vehicle-treated animals.
On day 22, post hoc analyses revealed that in animals pretreated with fluvoxamine and the low dose of paroxetine the ejaculation frequencies were reduced compared to vehicle-treated animals. Ejaculation latencies were increased in animals pretreated with a low dose of paroxetine compared to vehicle-treated animals, whereas animals pretreated with the high dose of paroxetine had increased ejaculation latencies compared to both vehicle- and fluvoxamine-treated animals. No differences between the

experimental groups were found in first mount and intromission frequency (table 1) or any other parameter in the second ejaculatory cycle (table 2).

Effects of 8-OH-DPAT on sexual behaviour in SSRI-pretreated animals

A challenge with a selected dose of 8-OH-DPAT (0.4 mg/kg) on day 22 affected sexual behaviour in the first and second ejaculatory cycle (figure 2, data are expressed as

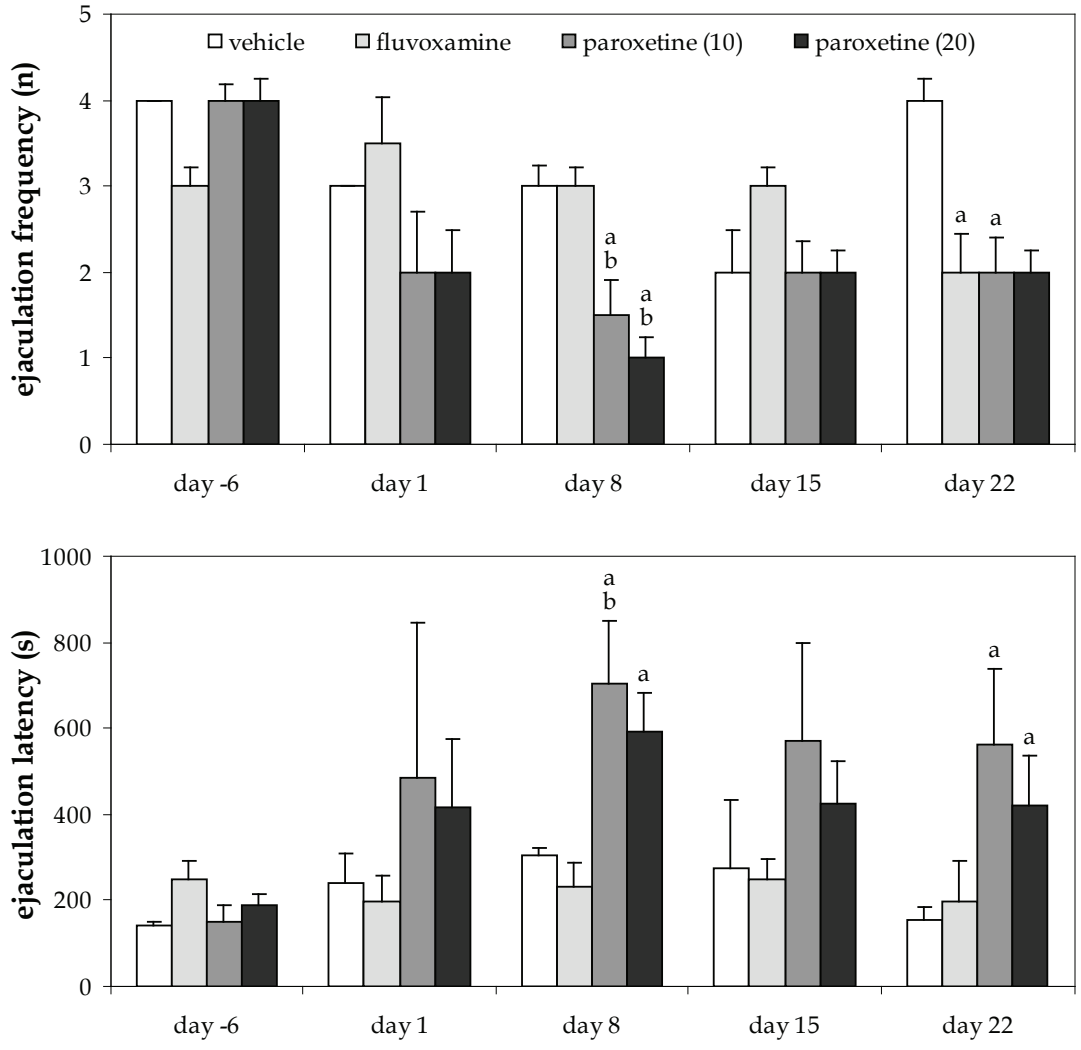

Figure 1. The ejaculation frequency and ejaculation latency of sexually experienced male Wistar rats in a 30-min sexual behaviour test with a receptive female before drug treatment (day -6) and on day 1, 8, 15 and 22 of chronic treatment with vehicle (white bars), fluvoxamine (30 mg/kg/day p.o.; light grey bars) and paroxetine (10 mg/kg/day p.o; grey bars and 20 mg/kg/day p.o.; dark grey bars). Data are medians ± standard error of the median; a=different from the vehicle-pretreated group, b=different from the fluvoxamine-pretreated group; $P<0.05$.

medians). Group differences were found in the latencies until first (χ^2=10.690; P=0.014) and second (χ^2=10.019; P=0.018) ejaculation; the number of mounts needed to achieve the first (χ^2=8.482; P=0.037) and second (χ^2=11.152; P=0.011) ejaculation and the number of intromissions needed to reach the first (χ^2=20.055; P=0.000) and second (χ^2=11.387; P=0.010) ejaculation.

Post hoc analyses showed that in vehicle-pretreated animals, 8-OH-DPAT increased the ejaculation frequency and reduced the first and second ejaculation latencies, the first and second mount frequencies and the first and second intromission frequencies compared to saline. Similarly, in fluvoxamine-pretreated animals, 8-OH-DPAT increased the ejaculation frequency and reduced the first and second mount and intromission frequencies. In animals pretreated with the low dose of paroxetine, 8-OH-DPAT increased the ejaculation frequency and reduced the first ejaculation latency and the first mount frequency. In animals pretreated with the high dose of paroxetine, 8-OH-DPAT reduced the first mount frequency.

Pretreatment with an SSRI altered the effects of 8-OH-DPAT on the first and second ejaculatory cycle compared to pretreatment with vehicle (Fig 2, data are expressed as medians). In animals pretreated with a high dose of paroxetine and challenged with 8-OH-DPAT, the latencies until the first and the second ejaculation and the number of mounts and intromissions needed to reach the first and second ejaculation were increased compared to vehicle-pretreated animals. The lower dose of paroxetine had similar effects, but failed to change the number of mounts to reach the first and second ejaculation compared to vehicle while challenged with 8-OH-DPAT.

Compared to fluvoxamine-pretreated animals, animals that were pretreated with a high dose of paroxetine and challenged with 8-OH-DPAT showed increased latencies until the first ejaculation and an increased number of mounts needed to reach the first ejaculation. In addition, animals pretreated with either the high or the low dose of paroxetine and challenged with 8-OH-DPAT showed an increased intromission frequency needed to reach the first and second ejaculation compared to fluvoxamine-pretreated animals.

Discussion

The present results demonstrate that a 22-day oral treatment with paroxetine, either 10 or 20 mg/kg/day, affects sexual behaviour in male Wistar rats. There were no acute effects of paroxetine, but (sub-)chronic treatment of 8 and 22 days significantly inhibit-

ed and delayed ejaculation compared to vehicle treatment. These effects were present after 15 days of paroxetine-treatment as well, but they were not statistically significant, which was probably due to an unexplained large variability in the sexual behaviour of vehicle-treated rats. Fluvoxamine did not cause delayed ejaculation at any time-point; however, fluvoxamine did reduce the ejaculation frequency after 22 days of treatment. These results are consistent with previous findings indicating that paroxetine delays ejaculation more strongly compared to fluvoxamine in rats (Waldinger *et al.*, 2002) as well as humans (Waldinger and Olivier, 1998; Waldinger *et al.*, 2004a).
Consistent with previous findings, a challenge with a selected dose of the 5-HT_{1A} receptor agonist 8-OH-DPAT strongly facilitated ejaculation in vehicle-pretreated rats by increasing ejaculation frequencies and decreasing ejaculation latencies and the number of mounts and intromissions prior to ejaculation (Ahlenius *et al.*, 1981; Coolen *et*

Table 2. The ejaculation latency (EL), mount frequency (MF) and intromission frequency (IF) in the second ejaculatory cycle of sexually experienced male Wistar rats in a 30-min sexual behaviour test with a receptive female before drug treatment (day -6) and on day 1, 8, 15 and 22 of chronic treatment with vehicle, fluvoxamine (30 mg/kg/day p.o.) and paroxetine (10 and 20 mg/kg/day p.o.).

Day:	Parameter:	vehicle (n = 9)	fluvoxamine (30) (n = 6)	paroxetine (10) (n = 10)	paroxetine (20) (n = 9)
-6	EL (sec)	110.29 ± 5.83	146.82 ± 23.47	143.27 ± 26.43	130.01 ± 14.69
	MF (n)	3.00 ± 0.25	4.50 ± 1.75	5.00 ± 0.77	4.00 ± 0.50
	IF (n)	3.00 ± 0.25	3.50 ± 0.30	3.00 ± 0.24	4.00 ± 0.50
1	EL (sec)	187.68 ± 39.65	124.74 ± 34.59	210.01 ± 23.89	153.25 ± 73.23
	MF (n)	14.00 ± 4.02	4.00 ± 2.67	9.00 ± 3.28	2.50 ± 1.21
	IF (n)	4.50 ± 1.12	3.00 ± 0.33	3.50 ± 0.30	4.00 ± 0.28
8	EL (sec)	194.71 ± 35.83	261.39 ± 38.43	197.95 ± 37.49	228.88 ± 38.44
	MF (n)	10.00 ± 1.99	13.00 ± 1.98	11.00 ± 4.00	10.00 ± 1.96
	IF (n)	4.00 ± 0.25	4.00 ± 0.23	4.00 ± 0.00	4.50 ± 0.65
15	EL (sec)	170.05 ± 57.89	144.07 ± 3.55	356.80 ± 97.76	315.28 ± 67.02
	MF (n)	7.00 ± 1.85	6.00 ± 1.33	10.00 ± 5.22	10.00 ± 3.38
	IF (n)	3.50 ± 0.53	4.00 ± 0.00	5.00 ± 0.71	4.00 ± 0.56
22	EL (sec)	161.75 ± 13.43	277.90 ± 59.43	253.29 ± 62.30	175.59 ± 39.26
	MF (n)	8.00 ± 1.24	12.50 ± 5.22	4.50 ± 1.68	6.00 ± 2.26
	IF (n)	4.00 ± 0.50	4.00 ± 0.09	4.00 ± 0.23	4.00 ± 0.28

Data are medians ± standard error of the median.

al., 1997a; Rehman *et al.*, 1999). 8-OH-DPAT activates somatodendritic 5-HT_{1A} autoreceptors as well as the widely distributed postsynaptic 5-HT_{1A} receptors. Both pre- and postsynaptic 5-HT_{1A} receptors are thought to contribute to the acceleration of ejaculation (Ahlenius and Larsson, 1997; Fernandez-Guasti and Escalante, 1991; Waldinger, 2004). However, 8-OH-DPAT has some affinity for 5-HT_7 (Bard *et al.*, 1993; Neumaier *et al.*, 2001) and dopamine D_2 (Rinken *et al.*, 1999) receptors. The role of 5-HT_7 receptors in sexual behaviour is unclear, but the dopamine D_2 receptor antagonist raclopride has been shown to attenuate the facilitation of ejaculation by 8-OH-DPAT when both drugs were microinjected in the medial preoptic area (Matuszewich *et al.*, 1999). Therefore, dopamine D_2 receptors could be responsible for some of the effects of 8-OH-DPAT on sexual behaviour in the present study.

Pretreatment with fluvoxamine for 22 days slightly attenuated the effects of 8-OH-DPAT on ejaculation latency. Pretreatment with the low dose of paroxetine attenuated the effects of 8-OH-DPAT on the first intromission frequency and on all parameters of the second ejaculatory cycle. The higher dose of paroxetine prevented all effects of 8-OH-DPAT on the first and second ejaculatory cycle, except the reduction in the first mount frequency. Taken together, the present results suggest that chronic SSRI-treatment impairs the functioning of 5-HT_{1A} receptors involved in ejaculation, and the degree of impairment appears to depend on the dose (high dose of paroxetine > low dose of paroxetine) and type (paroxetine > fluvoxamine) of SSRI.

5-HT_{1A} receptor activation is not necessary for ejaculation under basal conditions, since the selective 5-HT_{1A} receptor antagonist WAY-100635 failed to affect any parameter of sexual behaviour (Ahlenius and Larsson, 1998; 1999). Nonetheless, co-administration of WAY-100635 with the SSRI citalopram strongly inhibited ejaculation (Ahlenius and Larsson, 1999). These results indicate that 5-HT_{1A} receptors may play an important role in the regulation of ejaculation when serotonin levels are elevated. If, therefore, chronic treatment with an SSRI leads to a combination of elevated extra-cellular serotonin levels and impairment of relevant 5-HT_{1A} receptors then delayed ejaculation may be expected.

Individual SSRIs tend to be used in experiments as representatives of a homogenous family, rather than being considered as independent drugs with unique properties. Although some differences between SSRIs have been reported in behavioural studies with rodents (Maurel *et al.*, 1999; Sanchez and Meier, 1997; Waldinger *et al.*, 1998a), the pharmacological mechanism underlying these differences including the present data remains speculative. Fluvoxamine and paroxetine are chemically and pharmacologically different, despite their shared mechanism to block the 5-HT transporter

and thereby elevating serotonin levels, (Hiemke and Hartter, 2000). It is possible that their unique physiochemical properties cause a difference in ability to reach or bind to 5-HT transporters in specific brain areas involved in ejaculation. Another possibility is that 5-HT transporters are a heterogeneous group, and paroxetine and fluvoxamine differ in their affinity for specific 5-HT transporters located on distinct serotonergic neurons projecting to brain areas involved in ejaculation. Both options would lead to SSRI-dependent variations in degree of 5-HT_{1A} receptor over-stimulation and subsequent desensitization in relevant brain areas. Fluvoxamine might still desensitize a

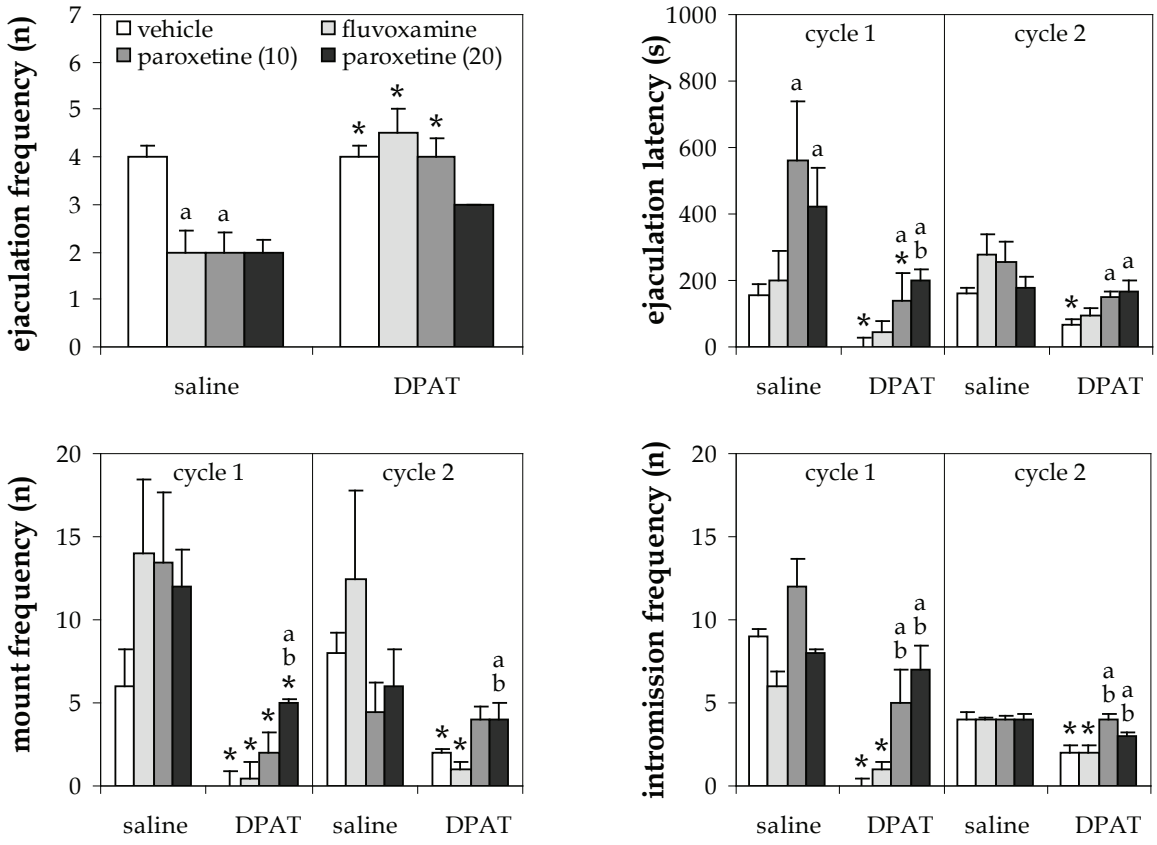

Figure 2. The effect of a challenge with 8-OH-DPAT (0.4 mg/kg s.c.) compared to saline on the ejaculation frequency and first and second ejaculation latency, mount frequency and intromission frequency of male Wistar rats pretreated for 22 days with vehicle (white bars), fluvoxamine (30 mg/kg/day p.o.; light grey bars) and paroxetine (10 mg/kg/day p.o; grey bars and 20 mg/kg/day p.o.; dark grey bars) in a 30-min sexual behaviour test with a receptive female. Data are medians ± standard error of the median; a=different from the vehicle-pretreated group, b=different from the fluvoxamine-pretreated group, *=different from the corresponding group challenged with saline; P<0.05.

subpopulation of 5-HT_{1A} receptors involved in its antidepressant actions, or perhaps exert its antidepressant effect through a sustained increase in 5-HT_{1A} receptor-mediated release of oxytocin (Uvnas-Moberg *et al.*, 1999). Paroxetine, on the other hand, could affect ejaculation via a dose-dependent decrease in 5-HT_{1A} receptor-mediated release of oxytocin (Li *et al.*, 1997b; Raap *et al.*, 1999), a neuropeptide that facilitates sexual behaviour (Argiolas and Melis, 2004) and reverses the inhibition of ejaculation by the SSRI fluoxetine (Cantor *et al.*, 1999). However, future research is required to explore these possibilities.

In conclusion, the present data demonstrate that chronic pretreatment with SSRIs attenuates 8-OH-DPAT-induced acceleration of ejaculation, putatively caused by an SSRI-induced desensitization of pre- and/or postsynaptic 5-HT_{1A} receptors. The degree of this putative 5-HT_{1A} receptor desensitization, depending on the dose and type of SSRI, might determine the occurrence and severity of delayed ejaculation.

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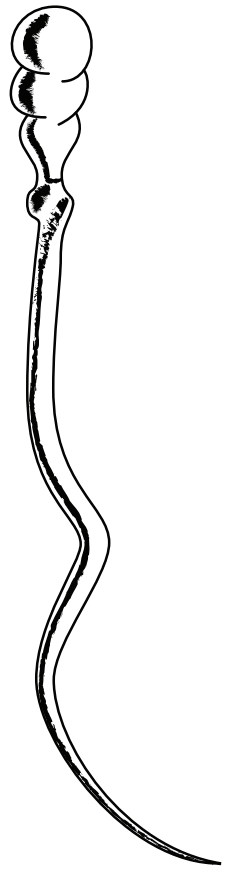
CHAPTER 5



Effects of chronic paroxetine pretreatment on 8-OH-DPAT induced *c-fos* expression following sexual behaviour

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Abstract

Chronic treatment with the selective serotonin reuptake inhibitor (SSRI) paroxetine impairs the functioning of 5-HT_{1A} receptors involved in ejaculation. This could underlie the development of delayed ejaculation often reported by men treated with paroxetine. The neurobiological substrate linking the effects of SSRI-treatment and 5-HT_{1A} receptor activation with ejaculation was investigated. Male Wistar rats that were pretreated with paroxetine (20 mg/kg/day p.o.) or vehicle for 22 days and had received an additional injection with the 5-HT_{1A} receptor agonist 8-OH-DPAT (0.4 mg/kg s.c.) or saline on day 22, 30 min prior to a sexual behaviour test, were perfused 1 hr after the sexual behaviour test. Brains were processed for Fos-, and oxytocin immunohistochemistry. The drug treatments markedly changed both sexual behaviour and the pattern and number of Fos-immunoreactive cells in the brain.

Chronic pretreatment with paroxetine caused delayed ejaculation. Acute injection with 8-OH-DPAT facilitated ejaculation in vehicle-pretreated rats, notably evident in a strongly reduced intromission frequency, whereas 8-OH-DPAT had no effects in paroxetine-pretreated rats. Chronic treatment with paroxetine reduced Fos-immunoreactivity in the locus coeruleus, and prevented the increase in Fos-immunoreactive neurons induced by 8-OH-DPAT in the oxytocinergic magnocellular part of the paraventricular nucleus as well as in the locus coeruleus. Since oxytocin and noradrenalin facilitate ejaculation, the alterations in Fos-IR in these areas could connect SSRI treatment and 5-HT_{1A} receptor activation to ejaculation. Chronic treatment with paroxetine and a challenge with 8-OH-DPAT changed c-fos expression in a number of other brain areas, indicating that Fos-immunohistochemistry is a useful tool to find locations where SSRIs and 8-OH-DPAT exert their effects.

Introduction

Chronic treatment with the selective serotonin reuptake inhibitor (SSRI) paroxetine causes desensitization of pre- and postsynaptic 5-HT_{1A} receptors (Davidson and Stamford, 1998; Le Poul *et al.*, 1995; Li *et al.*, 1997b), which has been implicated in the antidepressant effect of SSRIs (Artigas *et al.*, 1996; Blier *et al.*, 1998).
Since 5-HT_{1A} receptor activation is known to accelerate ejaculation in rats (Ahlenius *et al.*, 1981; Coolen *et al.*, 1997b; Fernandez-Guasti and Escalante, 1991; Haensel and Slob, 1997; Rehman *et al.*, 1999), and treatment with a SSRI combined with a 5-HT_{1A} receptor antagonist strongly inhibits ejaculation (Ahlenius and Larsson, 1999; de Jong *et al.*, 2005a), desensitization of 5-HT_{1A} receptors may also play a role in the delayed ejaculation induced by chronic paroxetine treatment (Rosen *et al.*, 1999; Waldinger *et al.*, 2002; Waldinger *et al.*, 2001; Waldinger *et al.*, 2004b). This theory was supported by the recent findings that chronic pretreatment with paroxetine attenuated the effects of 8-OH-DPAT on rat sexual behaviour (de Jong *et al.*, 2005c).
It is not yet known in which brain areas SSRIs and 5-HT_{1A} receptors exert their effect on depression and/or ejaculation. One approach to tackle this problem is immunohistochemical staining of Fos, the protein product of the immediate-early gene *c-fos*, which is expressed in neurons in response to intracellular signalling cascades and used as a marker of neural activation (Hoffman and Lyo, 2002; Kovacs, 1998; Sng *et al.*, 2004).
Fos-immunoreactivity changes in response to acute and chronic SSRI-treatment (Jongsma *et al.*, 2002; Lino-de-Oliveira *et al.*, 2001; Thomsen and Helboe, 2003; Veening *et al.*, 1998), 5-HT_{1A} receptor agonists (Compaan *et al.*, 1996; Hajos *et al.*, 1999; Hajos-Korcsok and Sharp, 1999; Mikkelsen *et al.*, 2004; Tilakaratne and Friedman, 1996) and copulation and/or ejaculation (Coolen *et al.*, 1996; Greco *et al.*, 1996; Greco *et al.*, 1998; Pfaus and Heeb, 1997; Veening and Coolen, 1998). Taken together, Fos-immunoreactivity can be considered as a useful tool to locate specific brain areas that might link serotonergic neurotransmission to sexual behaviour.
In order to trace these brain areas, 24 rats (n=6 per experimental group) were selected from a previously reported experiment (de Jong *et al.*, 2005c). The selected rats had been pretreated for 22 days with vehicle or paroxetine (20 mg/kg/day p.o.) and on day 22 challenged with saline or 8-OH-DPAT (0.4 mg/kg s.c.), 30 min prior to a sexual behaviour test.
1h after the test the rats were perfused and the pattern and number of Fos-positive cells were quantified throughout the rat brain.

Experimental Procedures

Animals

Adult male (250-300 grams and 3 months of age at the start of the experiment) and female Wistar rats (Harlan, Zeist, the Netherlands) were used. The animals arrived at the laboratory at least 14 days prior to the start of the experiments in order to adapt to the laboratory environmental condition and to a reversed light/dark cycle (12:12h, lights off at 6.30am). Food and tap water were available *ad libitum*. Males were group-housed and 3 weeks before the start of the drug treatment individually housed. They were handled daily from the start of the experiment. The females, which served as stimulus animals, were sterilized by ligation of the oviducts and housed in couples. Their sexual receptivity was fully and reliably induced by subcutaneous injection of oestradiol benzoate (50 µg) dissolved in 0.1 ml arachidis oil, 36 h prior to testing. All experiments were approved by the Animal Ethical Committee of the University of Nijmegen and conform the national and international guidelines on the ethical use of animals. All measures were taken in order to minimize the number of rats used in this study and their suffering.

Drugs

Paroxetine tablets (20 mg paroxetine/tablet; Genthon BV Nijmegen, the Netherlands) were obtained from the local pharmacy and were freshly crushed and dissolved in 1% methylcellulose on every day of the drug treatment. The rats received daily oral injections (5 ml/kg) with vehicle (methylcellulose) or paroxetine (20 mg/kg/day) for 21 consecutive days between 3:30 and 5:00 pm. On the 22nd day, all rats received their paroxetine or vehicle injection one h prior to the sexual behaviour test. In addition, all rats received a subcutaneous injection (1 ml/kg) with saline or 0.4 mg/kg (±)-8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT, Sigma-Aldrich Chemie, Steinheim, Germany) dissolved in saline, 30 min prior to the sexual behaviour test.
This resulted in four experimental groups (n=6 per group): methylcellulose + saline, methylcellulose + 8-OH-DPAT, paroxetine + saline and paroxetine + 8-OH-DPAT.

Behavioural Observations

All rats were trained in at least six sexual behaviour tests prior to the experiment, in order to establish consistent ejaculation frequencies. Individuals showing normal ejaculatory behaviour (an average ejaculation frequency of 1.33≤4.33 in the last three training sessions) were selected for the experiment.

All training and experimental sessions were conducted using the same paradigm, always between 10:30 am and 3:30 pm, in a red-lighted room. The animals were placed in a mating arena (rectangular, 40x50x65 cm) with wood shavings on the floor and a Perspex front. The rats were allowed to habituate to the arena for 15 min. Then, a receptive female was placed in the arena and free contact was allowed for 30 min. In this time frame, the ejaculation frequency (total number of ejaculations), ejaculation latency (time from first mount or intromission to first ejaculation), mount frequency

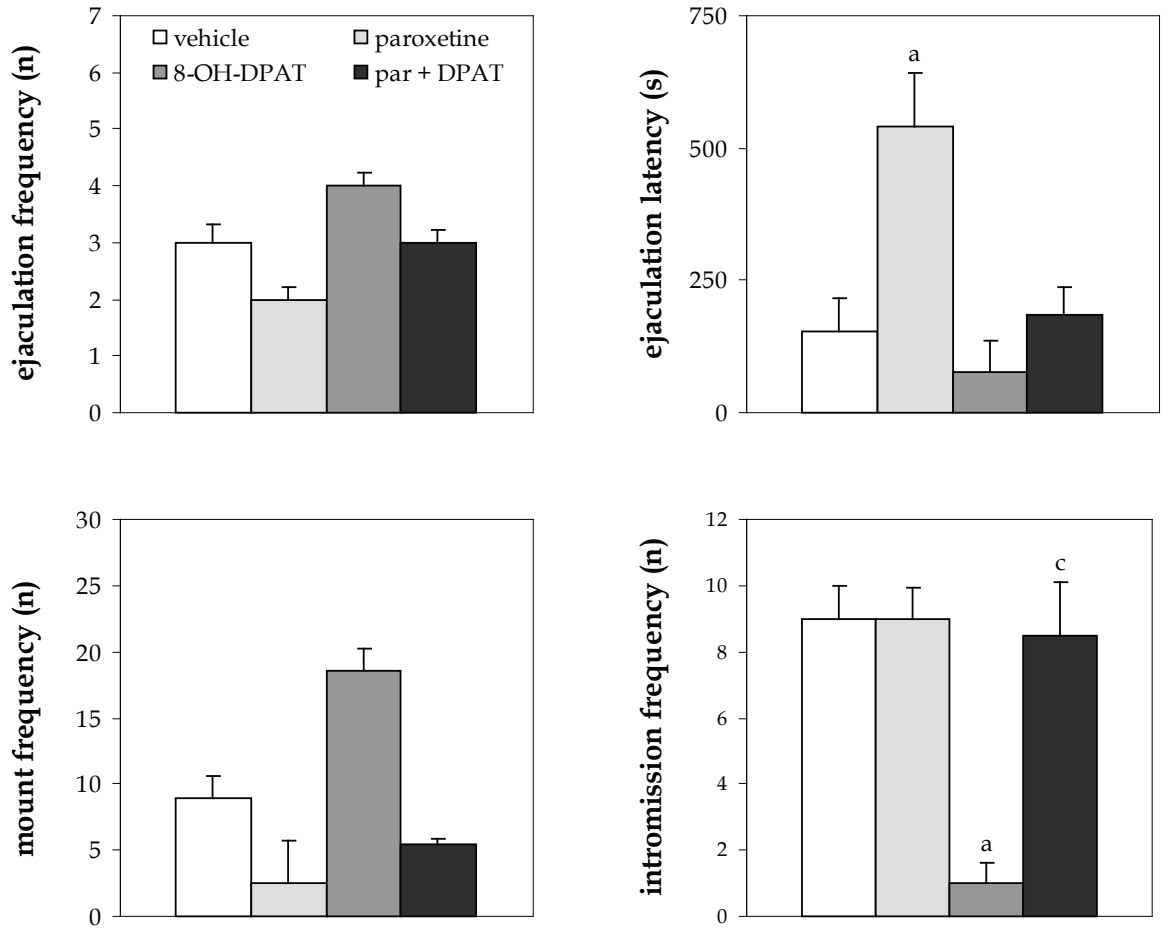

Figure 1. The effect of a challenge with saline following 22 days of pretreatment with vehicle (white bars) and paroxetine (20 mg/kg p.o., light grey bars), or a challenge with 8-OH-DPAT (0.4 mg/kg s.c.) following pretreatment with vehicle (grey bars) or paroxetine (20 mg/kg p.o., dark grey bars) on the ejaculation frequency, mount frequency, intromission frequency and ejaculation latency of male Wistar rats in a 30-min sexual behaviour test with a receptive female. Data are medians ± standard error of the median; a =different from the vehicle + saline group, c=different from the vehicle + 8-OH-DPAT group; $P<0.05$.

(number of mounts prior to the first ejaculation) and intromission frequency (number of intromissions prior to the first ejaculation) were counted using event recording software The Observer (Noldus Information Technology, Wageningen, the Netherlands).

Immunohistochemistry

One h after the end of the sexual behaviour test, the rats were anesthetized using an overdose of sodium pentobarbital and perfused transcardially with 0.1 M phosphate buffered saline (PBS, pH 7.3) followed by fixative (4% paraformaldehyde in PBS, pH 7.2). Brains were removed and postfixed for 1h at 4°C before the paraformaldehyde was replaced by 30% sucrose in phosphate buffer. Each brain was cut in coronal sections (40 µm) with a freezing microtome and collected in a series of six PBS-containing tubes per brain. One tube per brain was immunohistochemically stained. The sections were rinsed in PBS, soaked in 30% H_2O_2 for 30 min and rinsed 3 x 20 min in PBS. After 30 min of preincubation with PBS containing 0.1% bovine serum albumin and 0.5% Triton-X-100 (PBS-BT), sections were incubated overnight in the same medium with *c-fos* antiserum raised in rabbit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, dilution 1:20,000). The next day, the sections were rinsed 3 x 20 min in PBS and incubated for 90 min in donkey anti-rabbit antibody (Biotin SP conjugated, Jackson Immuno Research, West Grove, PA, USA, dilution 1:400) in PBS-BT. Sections were rinsed 3 x 20 min and incubated for 90 min in ABC-elite (Brunschwig Chemie, Amsterdam, the Netherlands, dilution 1:800, prepared 60 min in advance) in PBS-BT. The sections were rinsed 3 x 20 min in PBS and stained with a chromogen solution consisting of 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.03% Nickel-Ammonium in 0.05M Tris-buffer (pH 7.6): exactly 10 min of incubation without, and 10 min with 30% H_2O_2. This resulted in a blue-black staining of Fos-immunoreactive cell nuclei. All sections were rinsed 3 x 20 min in PBS, and the complete process was repeated starting from the pre-incubation with PBS-BT. This time, the overnight incubation was in an oxytocin GP antiserum (Peninsula Laboratories Inc., San Carlos, CA, USA, dilution 1:100,000) raised in rabbit. The staining was established using DAB in 0.05M Tris-buffer (pH 7.6), which resulted in a brown-colored staining of oxytocinergic neurons. All steps of the immunohistochemistry described above were performed at room temperature. The sections were mounted on gelatin chrome alum-coated glass slides, dried overnight, cleared in xylene, embedded with Entellan (Merck & Co., Darmstadt, Germany) and coverslipped.

Immunoreactive cells were quantified using the software program Neurolucida (MicroBrightField, Williston, VT, USA). Numbers of Fos-immunoreactive nuclei were

Table 1. The number of Fos-immunoreactive cells in the prelimbic area (PrL); core and shell of the nucleus accumbens (AcbC and AcbSh); dorsal lateral and posterior medial bed nucleus of the stria terminalis (BNSTdl and BNSTpm); ventral lateral septum (LSV); medial preoptic nucleus (MPN); medial parvocellular and oxytocinergic posterior magnocellular paraventricular hypothalamic nucleus (PVHmp and PVHpm); arcuate hypothalamic nucleus (ARH); central amygdala (CeA); anterodorsal and posterodorsal medial amygdala (MeAad and MeApd); ventral premammillary nucleus (PMV); dorsomedial ventromedial hypothalamic nucleus (VMHdm); lateral hypothalamic area (LHA); medial parvocellular subparafascicular thalamic nucleus (mSPFp); ventrolateral periaqueductal grey (PAGvl); central lateral parabrachial nucleus (PBlc); compact part of the nucleus incertus (NIc); locus coeruleus (LC) and medial nucleus of the solitary tract (NSTm) of rats pretreated for 22 days with vehicle or paroxetine (20 mg/kg/day p.o.) and challenged on day 22 with saline or 8-OH-DPAT (0.4 mg/kg s.c.).

Brain Area	Vehicle + saline	Paroxetine + saline	Vehicle + 8-OH-DPAT	Paroxetine + 8-OH-DPAT
PrL	38.50 ± 7.74	37.00 ± 5.78	39.50 ± 4.94	32.50 ± 6.53
AcbC	35.00 ± 2.67	36.50 ± 1.22	29.00 ± 2.44 **a**	41.50 ± 2.82 **c**
AcbSh	29.00 ± 0.67	33.00 ± 3.12	39.50 ± 5.41	26.50 ± 5.48
BNSTdl	33.00 ± 3.34	45.00 ± 5.41	36.50 ± 1.83	38.00 ± 6.34
BNSTpm	30.50 ± 3.26	27.00 ± 1.00	35.00 ± 2.28	32.00 ± 2.52
LSV	47.00 ± 0.33	55.00 ± 7.84	14.50 ± 2.82 **a**	26.00 ± 2.34 **bc**
MPN	37.00 ± 1.67	31.00 ± 3.05	25.50 ± 1.22	30.00 ± 2.34
PVHpm/OT	2.50 ± 0.47	3.50 ± 0.99	15.00 ± 1.98 **a**	6.00 ± 0.33 **c**
PVHmp	34.00 ± 3.82	38.00 ± 4.49	51.50 ± 4.65 **a**	36.00 ± 5.01 **c**
CeA	40.00 ± 2.67	46.50 ± 2.67	60.50 ± 3.88 **a**	74.50 ± 1.37 **bc**
ARH	37.00 ± 0.67	34.00 ± 1.90	50.00 ± 2.44 **a**	40.5 ± 1.45 **c**
MeAad	39.00 ± 3.00	37.50 ± 2.97	35.50 ± 3.50	35.00 ± 3.43
MeApd	97.00 ± 2.67	96.00 ± 5.01	105.00 ± 6.02	101.00 ± 4.27
VMHdm	78.00 ± 1.33	56.00 ± 3.96	60.00 ± 5.41	46.00 ± 5.64
LHA	36.00 ± 2.67	33.50 ± 4.49	36.50 ± 1.83	36.00 ± 3.58
PMV	74.00 ± 2.34	51.50 ± 5.94 **a**	61.00 ± 5.26 **a**	41.50 ± 2.44 **c**
mSPFp	30.00 ± 1.12	32.00 ± 1.90	29.50 ± 2.36	31.50 ± 3.28
PAGvl	58.00 ± 8.34	57.50 ± 5.56	36.50 ± 3.66	42.00 ± 3.28
PBlc	68.00 ± 10.01	56.00 ± 7.68	51.50 ± 5.56	53.00 ± 2.59
NIc	30.00 ± 0.67	23.50 ± 7.74	68.00 ± 2.52 **a**	39.00 ± 7.01 **c**
LC	12.00 ± 1.67	0.50 ± 0.30 **a**	24.00 ± 1.33 **a**	8.50 ± 0.76 **bc**
NSTm	20.00 ± 2.24	36.50 ± 2.74	59.00 ± 10.35 **a**	44.00 ± 2.00 **bc**

Data are medians ± standard error of the median. a=Different from the vehicle + saline group, b=different form the paroxetine + saline group, c=different from the vehicle + 8-OH-DPAT group; $P<0.05$.

counted in homologous square fields using a grid size of 200x200 µm (except for the compact part of the nucleus incertus: grid size 100x200 µm) displaying a representative density of stained cells. Fos-immunoreactivity was quantified in areas selected for their involvement in sexual behaviour or serotonergic neurotransmission, as well as in areas showing substantial Fos-immunoreactivity in at least one of the experimental groups: the prelimbic area, nucleus accumbens core and shell, dorsal lateral and posterior medial bed nucleus of the stria terminalis, ventral lateral septum, medial preoptic nucleus, medial parvocellular paraventricular hypothalamic nucleus, anterodorsal and posterodorsal medial amygdala, central amygdala, arcuate hypothalamic nucleus, dorsomedial and ventromedial hypothalamic nucleus, ventral premammillary nucleus, lateral hypothalamic area, medial parvocellular subparafascicular thalamic nucleus, ventral lateral periaqueductal grey, central lateral parabrachial nucleus, compact part of the nucleus incertus, locus coeruleus and the medial nucleus of the solitary tract.
In addition, Fos-positive nuclei in oxytocinergic cell bodies were counted in the posterior magnocellular part of the hypothalamic paraventricular nucleus.

Statistics
All data were analyzed using the non-parametric Kruskall Wallis test and, in case of overall significant differences, with the Mann Whitney test.
All statistical analyses were performed with the Statistical Package for the Social Sciences version 12.0 (SPSS Inc., Chicago IL, USA). The level of significance in all tests was set at $P<0.05$.

Results

Behaviour
The effects of chronic pretreatment with vehicle or paroxetine and a challenge with 8-OH-DPAT or saline on the sexual behaviour of male Wistar rats were tested. Analysis with the Kruskall Wallis test revealed significant differences ($P<0.05$) between the experimental groups in ejaculation frequency ($\chi^2=9.389$), mount frequency ($\chi^2=9.123$), intromission frequency ($\chi^2=12.990$) and ejaculation latency ($\chi^2=9.240$) (figure 1). Subsequent analysis with the Mann Whitney test showed that treatment with paroxetine prolonged the ejaculation latency compared to vehicle. In addition, 8-OH-DPAT in combination with vehicle-pretreatment reduced the intromission frequency compared to saline in combination with vehicle-pretreatment as well as 8-OH-DPAT in combina-

tion with paroxetine-pretreatment. Although paroxetine showed a strong tendency to reduce the ejaculation frequency and increase mount frequency, and 8-OH-DPAT showed a weaker tendency to do the opposite, these differences were not statistically significant.

Immunohistochemistry

The combination of drug treatment and sexual behaviour elicited substantial Fos-immunoreactivity in a number of brain regions (table 1 and figure 2). Analysis with the Kruskall Wallis test revealed overall significant differences in the nucleus accumbens core (χ^2=9.263P), ventral lateral septum (χ^2=16.408), oxytocinergic magnocellular paraventricular hypothalamic nucleus (χ^2=14.934, figure 3), medial parvocellular paraventricular hypothalamic nucleus (χ^2=10.261), central amygdala (χ^2=14.808, figure 4), arcuate hypothalamic nucleus (χ^2=12.099, figure 5), ventral premammillary

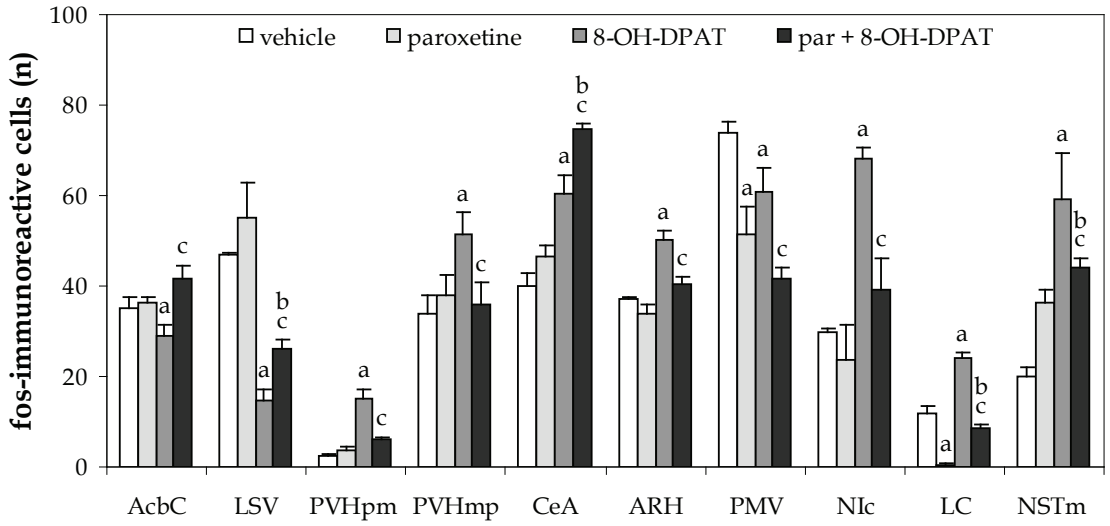

Figure 2. Number of Fos-immunoreactive nuclei induced by a challenge with saline following 22 days of pretreatment with vehicle (white bars) and paroxetine (20 mg/kg p.o., light grey bars), or a challenge with 8-OH-DPAT (0.4 mg/kg s.c.) following pretreatment with vehicle (grey bars) or paroxetine (20 mg/kg p.o., dark grey bars) in the core of the nucleus accumbens (AcbC); ventral lateral septum (LSV); medial parvocellular and oxytocinergic posterior magnocellular paraventricular hypothalamic nucleus (PVHmp and PVHpm/OT); central amygdala (CeA); arcuate hypothalamic nucleus (ARH); ventral premammillary nucleus (PMV); compact part of the nucleus incertus (NIc); locus coeruleus (LC) and medial nucleus of the solitary tract (NSTm) of male Wistar rats perfused 1 h after a 30-min sexual behaviour test with a receptive female. Data are medians ± standard error of the median; a=different from the vehicle + saline group, b=different form the paroxetine + saline group, c=different from the vehicle + 8-OH-DPAT group; P<0.05.

nucleus (χ^2=12.123), compact part of the nucleus incertus (χ^2=10.193), locus coeruleus (χ^2=16.635, figure 6) and medial nucleus of the solitary tract (χ^2=15.357).
Further post-hoc analyses showed that rats pretreated with paroxetine and challenged with saline, Fos-immunoreactivity was reduced in the locus coeruleus compared to rats pretreated with vehicle. In rats pretreated with vehicle, a challenge with 8-OH-DPAT increased Fos-immunoreactivity in the posterior oxytocinergic magnocellular and medial parvocellular paraventricular hypothalamic nucleus, central amygdala, arcuate hypothalamic nucleus, compact part of the nucleus incertus, locus coeruleus and medial nucleus of the solitary tract and decreased Fos-immunoreactivity in the core of the nucleus accumbens, ventral lateral septum and ventral premammillary nucleus compared to a challenge with saline.
In rats pretreated with paroxetine and challenged with 8-OH-DPAT, Fos-immunoreactivity was increased in the central amygdala, locus coeruleus and medial nucleus of the solitary tract and decreased in the ventral lateral septum compared to a challenge with saline. In the same group, but compared to rats pretreated with vehicle and challenged with 8-OH-DPAT, Fos-immunoreactivity was increased in the nucleus accumbens core, ventral lateral septum and central amygdala, and decreased in the oxytocinergic magnocellular and medial parvocellular paraventricular hypothalamic nucleus, arcuate hypothalamic nucleus, ventral premammillary nucleus, compact part of the nucleus incertus and medial nucleus of the solitary tract.

Discussion

Sexual Behaviour

For the present study we used a subset of rats from a previously reported experiment (de Jong *et al.*, 2005c). In that study, the effects of chronic treatment with fluvoxamine and two doses of paroxetine on 8-OH-DPAT-induced facilitation of ejaculation were investigated. Since the higher dose of paroxetine (20 mg/kg p.o.) was the most effective, six rats from that experimental group plus six rats from each appropriate control group were randomly selected for the present Fos-study. The reduction of group size (original group size was n=9/10) attenuated the behavioural differences between the groups compared to the original experiment. In agreement with previous studies in rats (Waldinger *et al.*, 2002) and humans (Waldinger and Olivier, 1998; Waldinger *et al.*, 2004b), chronic treatment with paroxetine caused delayed ejaculation in male Wistar rats. Furthermore, a challenge with 8-OH-DPAT strongly reduced the number

of intromissions required to reach the ejaculatory threshold, which has been demonstrated extensively (Ahlenius *et al.*, 1981; Coolen *et al.*, 1997b; Rehman *et al.*, 1999). 8-OH-DPAT showed a strong trend to increase ejaculation frequency and to decrease ejaculation latency. Chronic pretreatment with paroxetine significantly attenuated the 8-OH-DPAT-induced reduction of intromission frequency, which is thought to reflect desensitization of pre- and/or post-synaptic 5-HT_{1A} receptors caused by chronic paroxetine-treatment (Davidson and Stamford, 1998; Le Poul *et al.*, 1995; Li *et al.*, 1997b).

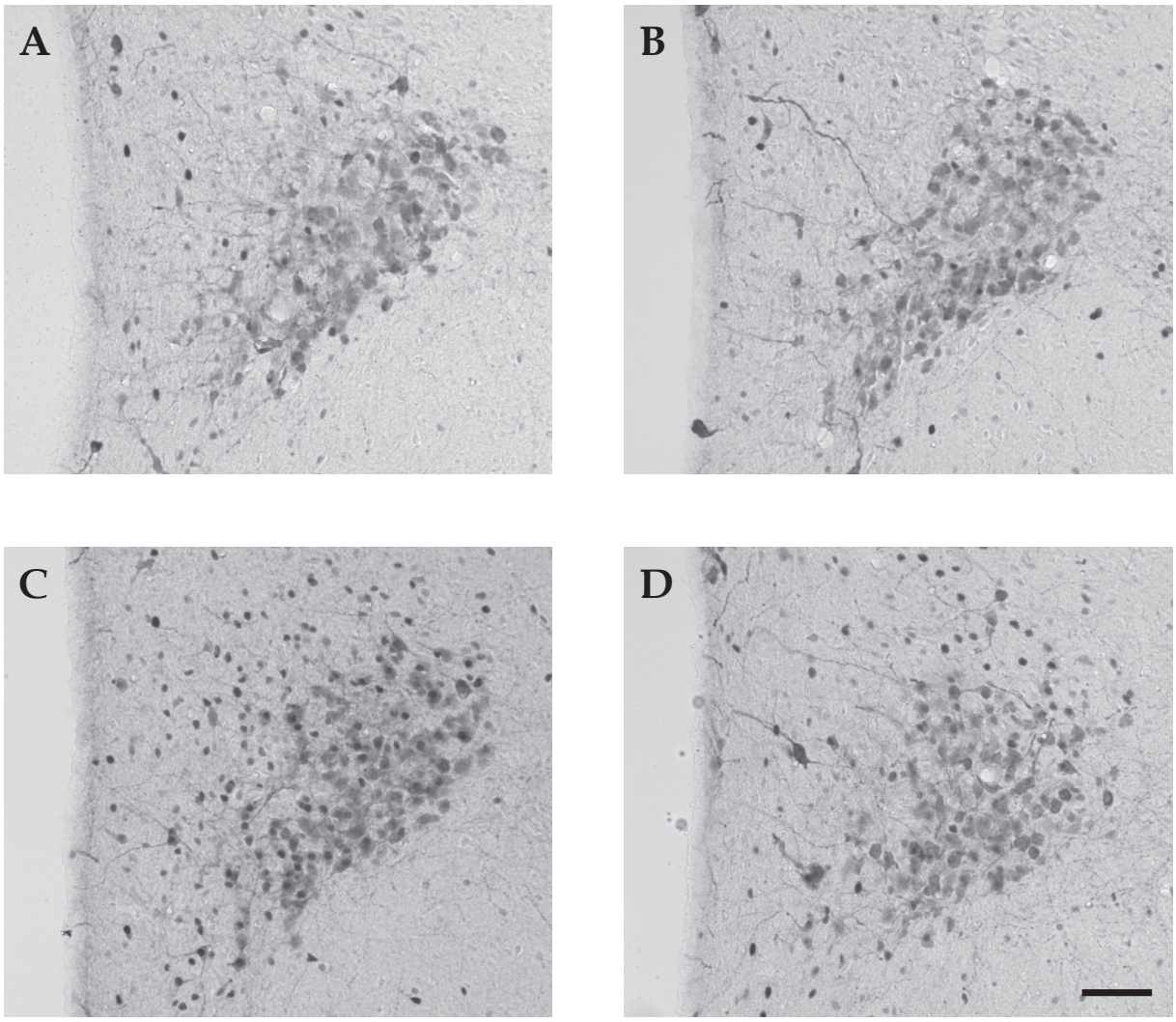

Figure 3. Representative photographs of the paraventricular hypothalamic nucleus (Bregma –1.80), double-stained for Fos-protein (black dots) and oxytocin (grey neuron-shaped staining) of rats challenged with saline after 22 days of pretreatment with vehicle (A) or paroxetine (20 mg/kg p.o., (B)) and rats challenged with 8-OH-DPAT (0.4 mg/kg s.c.) after 22 days of pretreatment with vehicle (C) or paroxetine (D). Scale bar=100µm.

Immunohistochemistry: sexual behaviour

Sexual behaviour and ejaculation caused extensive Fos-immunoreactivity in the posteromedial bed nucleus of the stria terminalis, posterodorsal medial amygdala and medial parvocellular subparafascicular thalamic nucleus (table 1), consistent with previous findings (Baum and Everitt, 1992; Coolen *et al.*, 1997b; Greco *et al.*, 1996). The substantial Fos-immunoreactivity in the prelimbic area, nucleus accumbens shell, anterior dorsal medial amygdala, dorsomedial ventromedial hypothalamic nucleus, lateral hypothalamic area, ventrolateral periaqueductal grey and central lateral parabrachial nucleus was probably caused by the physical exercise (Iwamoto *et al.*, 1996), stress (Kovacs, 1998), social interaction (Salchner *et al.*, 2004) or reward (Balfour *et al.*, 2004) experienced by all rats during the sexual behaviour test. In all these areas, Fos-immunoreactivity was not altered by any drug treatment, indicating that they do not play a crucial role in the integration of serotonergic neurotransmission and sexual behaviour.

Immunohistochemistry: paroxetine

In animals that were chronically treated with paroxetine, the number of Fos-positive cells was decreased in the locus coeruleus (fig. 2), which is consistent with the finding that chronic, but not short-term treatment with paroxetine reduces the spontaneous firing activity in the locus coeruleus (Szabo *et al.*, 1999). In addition, chronic treatment with paroxetine increased Fos-IR in the dorsal lateral bed nucleus of the stria terminalis, central amygdala and medial nucleus of the solitary tract, similar to other studies with SSRIs (Thomsen and Helboe, 2003; Veening *et al.*, 1998), but the increase was not statistically significant. This concurs with reports that (sub-)chronic treatment with serotonergic agents, including SSRIs, induce attenuated or changed Fos-IR compared to acute treatment (Li and Rowland, 1996; Lino-de-Oliveira *et al.*, 2001; Veening *et al.*, 1998), indicating that the rats habituate to possible stressful side-effects of the drugs, and/or the Fos-inducing mechanisms are desensitized by repeated activation.

Immunohistochemistry: 8-OH-DPAT

A challenge with 8-OH-DPAT significantly changed the number of Fos-IR neurons in a number of areas (fig. 2). The increase in Fos-positive oxytocinergic magnocellular neurons in the posterior paraventricular hypothalamic nucleus probably reflects the enhanced release of oxytocin in the blood caused by activation of postsynaptic 5-HT_{1A} receptors on these neurons (Vicentic *et al.*, 1998; Zhang *et al.*, 2004). Oxytocinergic cells in the paraventricular hypothalamic nucleus are activated, as measured with Fos-im-

munoreactivity or electrophysiology, in response to odours of estrous females, copulatory activity and stimulation of the glans penis or dorsal penile nerve in rats (Hillegaart *et al.*, 1998; Nishitani *et al.*, 2004; Yanagimoto *et al.*, 1996). In humans, plasma oxytocin levels are elevated at the time of ejaculation (Carmichael *et al.*, 1987; Murphy *et al.*, 1987). In addition, infusion of oxytocin in the bloodstream reduced the number of intromissions needed for ejaculation in rats (Stoneham *et al.*, 1985). Taken together, the release of oxytocin in the bloodstream might lower the ejaculation threshold, possibly via oxytocin receptors on the smooth muscle cells in the testis, epididymis and vas

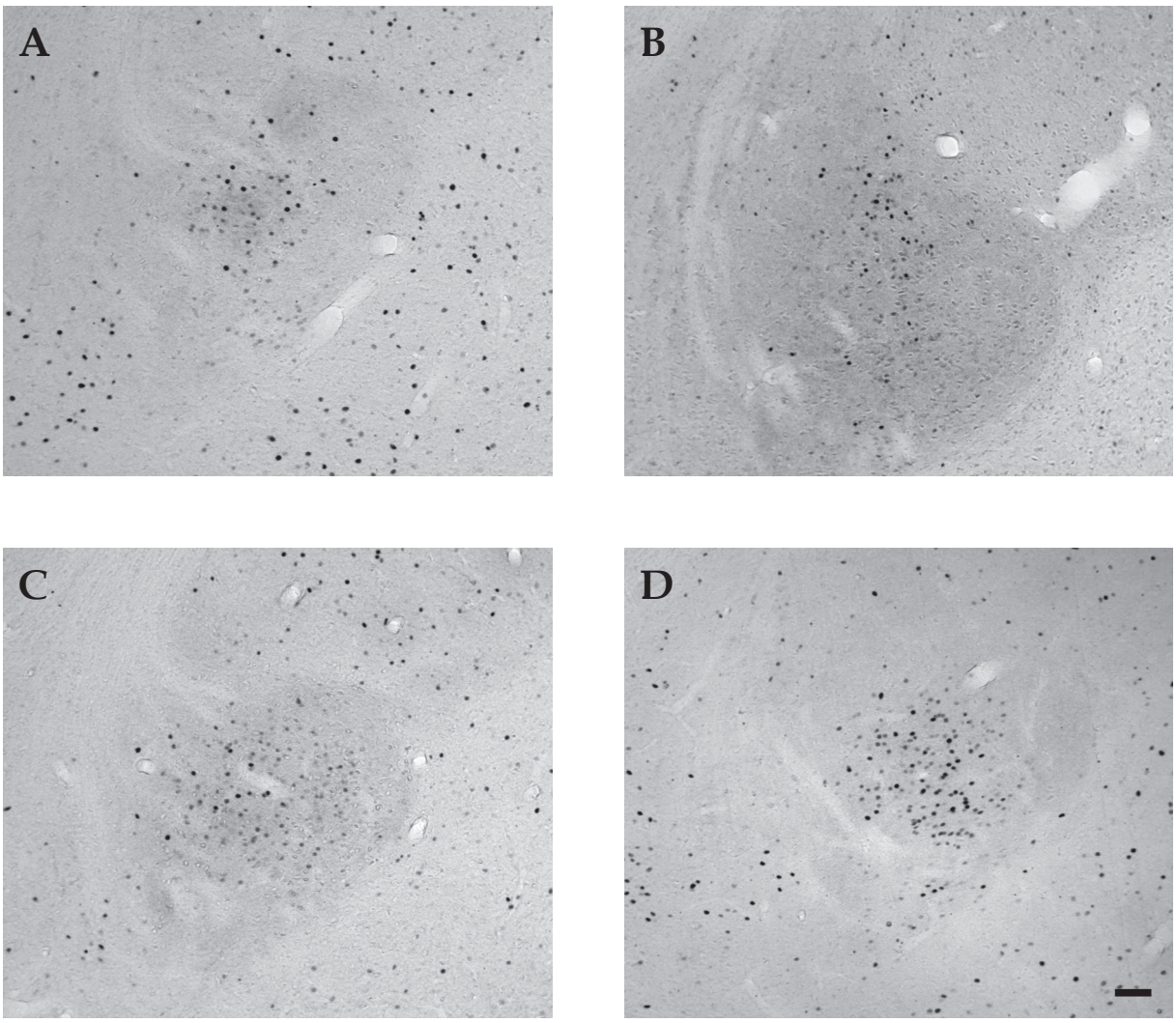

Figure 4. Representative photographs of Fos-immunoreactive cell nuclei in the central amygdala (Bregma –2.80) of rats challenged with saline after 22 days of pretreatment with vehicle (A) or paroxetine (20 mg/kg p.o., (B)) and rats challenged with 8-OH-DPAT (0.4 mg/kg s.c.) after 22 days of pretreatment with vehicle (C) or paroxetine (D). Scale bar=100µm.

deferens (Filippi *et al.*, 2002; Nicholson *et al.*, 1999; Whittington *et al.*, 2001). This might cause the facilitation of ejaculation induced by 5-HT_{1A} receptor agonists.
In the locus coeruleus, a similar increase of Fos-IR in response to 5-HT_{1A} receptor agonists has been reported previously (Hamamura *et al.*, 1997) and is thought to reflect increased noradrenergic transmission at postsynaptic sites (Hajos-Korcsok and Sharp, 1999). This effect has been attributed to presynaptic 5-HT_{1A} autoreceptors (Haddjeri *et al.*, 1997), which is supported by findings that the locus coeruleus receives a dense innervation from the dorsal raphe nucleus (Kaehler *et al.*, 1999), but does not express postsynaptic 5-HT_{1A} receptors (Pompeiano *et al.*, 1992; Wright *et al.*, 1995). Stimulation of noradrenergic neurotransmission has been shown to facilitate copulation and ejaculation (Bitran and Hull, 1987), and to reverse sexual exhaustion (Carro-Juareza and Rodriguez-Manzo, 2003), while lesion of the noradrenergic system antagonizes the facilitation of ejaculation by the non-selective 5-HT_{1A} receptor agonist 5-Methoxy-N,N-dimethyltryptamine (Fernandez-Guasti *et al.*, 1986). Therefore, the noradrenergic system might play a considerable role in the effects of 8-OH-DPAT on sexual behaviour and ejaculation, possibly via connections to neurons in the spinal cord that innervate the penis (Yaici *et al.*, 2002a; Yaici *et al.*, 2002b).
The 8-OH-DPAT-induced increase of Fos-IR neurons in the arcuate hypothalamic nucleus might be mediated by postsynaptic 5-HT_{1A} receptors on these neurons (Collin *et al.*, 2002; Kang *et al.*, 2004). The arcuate nucleus is connected with the medial preoptic area, medial amygdala, bed nucleus of the stria terminalis and the paraventricular nucleus, and is thought to integrate information about metabolism with reproductive activity, using galanin-like peptide, neuropeptide Y and gonadotropin-releasing hormone as messengers (Gottsch *et al.*, 2004; Magoul *et al.*, 1994). The arcuate hypothalamic nucleus is therefore in a suitable location to connect 5-HT_{1A} receptor activation with the reproductive system, although no direct effect of manipulations in this area on sexual behaviour have been reported so far in the male rat.
8-OH-DPAT induced a significant decrease of Fos-positive neurons in the core of the nucleus accumbens. Systemic injection of the 5-HT_{1A} receptor antagonist WAY-100635 has been shown to result in an increase of Fos-immunoreactivity in this area (Jongsma *et al.*, 2002), indicating that 5-HT_{1A} receptors actively inhibit *c-fos* expression in the core of the nucleus accumbens. The nucleus accumbens is involved in sexual behaviour, since an increase in Fos-immunoreactivity has been observed in the core of the nucleus accumbens in response to sex-related odors (Kippin *et al.*, 2003) and copulation (Balfour *et al.*, 2004), and lesion of the core of the nucleus accumbens inhibits non-contact erections, copulation and ejaculation (Kippin *et al.*, 2004). Further investigation is needed

to find out if and how the 8-OH-DPAT-induced decrease in Fos-immunoreactivity in the core of the nucleus accumbens plays a role in the facilitation of ejaculation.
The medial parvocellular paraventricular hypothalamic nucleus, the compact part of the nucleus incertus and the medial nucleus of the solitary tract showed an increase, whereas the ventral lateral septum showed a decrease in Fos-immunoreactivity in response to an 8-OH-DPAT injection. These areas are innervated by serotonergic fibres and often express 5-HT_{1A} receptors (Feldman *et al.*, 2000; Goto *et al.*, 2001; Kia *et al.*, 1996; Pompeiano *et al.*, 1992) and/or are known to express *c-fos* in response to 5-HT_{1A}

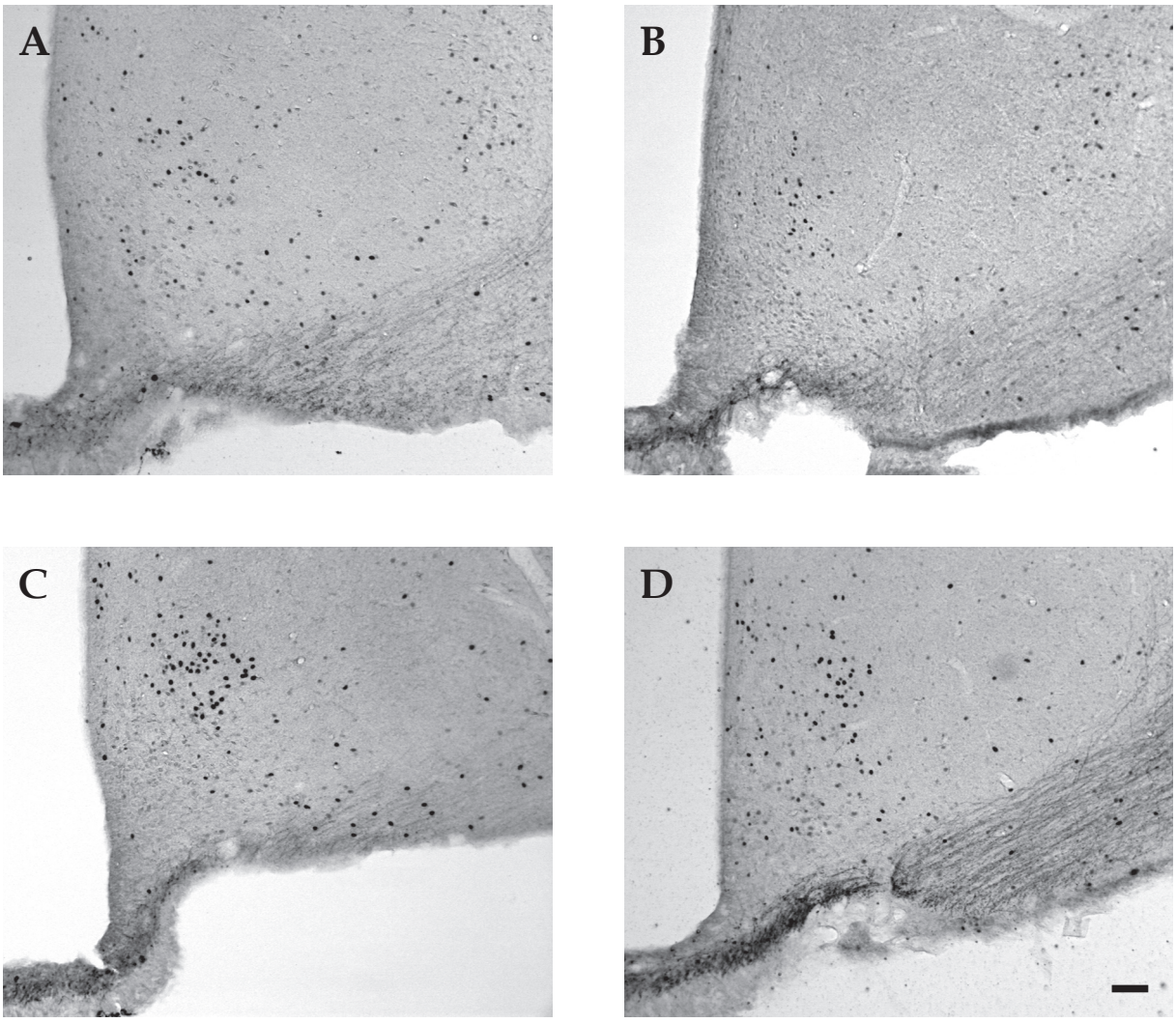

Figure 5. Representative photographs of Fos-immunoreactive cell nuclei in the arcuate hypothalamic nucleus (Bregma –2.80) of rats challenged with saline after 22 days of pretreatment with vehicle (A) or paroxetine (20 mg/kg p.o., (B)) and rats challenged with 8-OH-DPAT (0.4 mg/kg s.c.) after 22 days of pretreatment with vehicle (C) or paroxetine (D). Scale bar=100µm.

agonists and other serotonergic agents (Compaan *et al.*, 1997; Jensen *et al.*, 2001; Jongsma *et al.*, 2002; Mikkelsen *et al.*, 2004; Veening *et al.*, 1998), but are thus far not directly implicated in sexual behaviour and are not likely to play a role in 8-OH-DPAT induced facilitation of ejaculation..

Immunohistochemistry: paroxetine and 8-OH-DPAT

In the oxytocinergic paraventricular hypothalamic nucleus and the locus coeruleus, the 8-OH-DPAT-induced increase in Fos-immunoreactivity was strongly inhibited by chronic pretreatment with paroxetine. Since Fos-immunoreactivity in these areas is thought to represent release of respectively oxytocin and noradrenalin, as discussed above, the results suggest that chronic paroxetine-treatment prevents oxytocin and noradrenalin to be released upon 5-HT_{1A} receptor activation. Although oxytocin and noradrenalin do facilitate ejaculation, it is not known whether inhibition of the release of oxytocin or noradrenalin impairs ejaculation. Oxytocin does reverse the delayed ejaculation caused by chronic treatment with the SSRI fluoxetine (Cantor *et al.*, 1999), indicating that inhibition of oxytocin-release plays a role in the sexual side-effects of SSRIs.

Paroxetine pretreatment also prevented the 8-OH-DPAT-induced alterations in *c-fos* expression in the core of the nucleus accumbens and in the arcuate hypothalamic nucleus, indicating that these areas, which are involved in sexual behaviour, are affected by 5-HT_{1A} receptor desensitization. Whether or not this plays a role in SSRI-induced delayed ejaculation remains to be investigated.

Furthermore, chronic pretreatment with paroxetine attenuated the effects of a challenge with 8-OH-DPAT on the number of Fos-immunoreactive neurons in the medial parvocellular paraventricular hypothalamic nucleus, the compact part of the nucleus incertus and, though somewhat weaker, in the ventral lateral septum and the medial nucleus of the solitary tract. This has been demonstrated before in the paraventricular hypothalamic nucleus (Compaan *et al.*, 1997; Jensen *et al.*, 2001) but not yet in the other areas, and it suggests that these areas are affected by 5-HT_{1A} receptor desensitization.

Conversely, chronic paroxetine-pretreatment augmented the 8-OH-DPAT-induced increase of Fos-immunoreactivity in the central amygdala. In a previous study, repeated injections with the 5-HT_{1A} receptor agonist flesinoxan did not attenuate Fos-immunohistochemistry in the central amygdala compared to a single injection with flesinoxan, in contrast to the paraventricular hypothalamic nucleus, indicating that the central amygdala is not affected by 5-HT_{1A} receptor desensitization (Compaan *et al.*, 1997). Interestingly, systemic injection of the 5-HT_{1A} receptor antagonist WAY-100635

augmented the increase of Fos-immunoreactivity in the central amygdala induced by acute injection of the SSRI citalopram (Jongsma *et al.*, 2002), indicating that activation of 5-HT_{1A} receptors has the same effects as blocking these receptors in the central amygdala. Further research is needed to clarify these seemingly contradicting results. However, since the central amygdala has thus far not been implicated in sexual behaviour, this area is unlikely to underlie SSRI-induced delayed ejaculation.

In the ventral premammillary nucleus, 8-OH-DPAT and paroxetine caused a decrease in Fos-immunoreactivity, and co-administration of both drugs augmented this reduc-

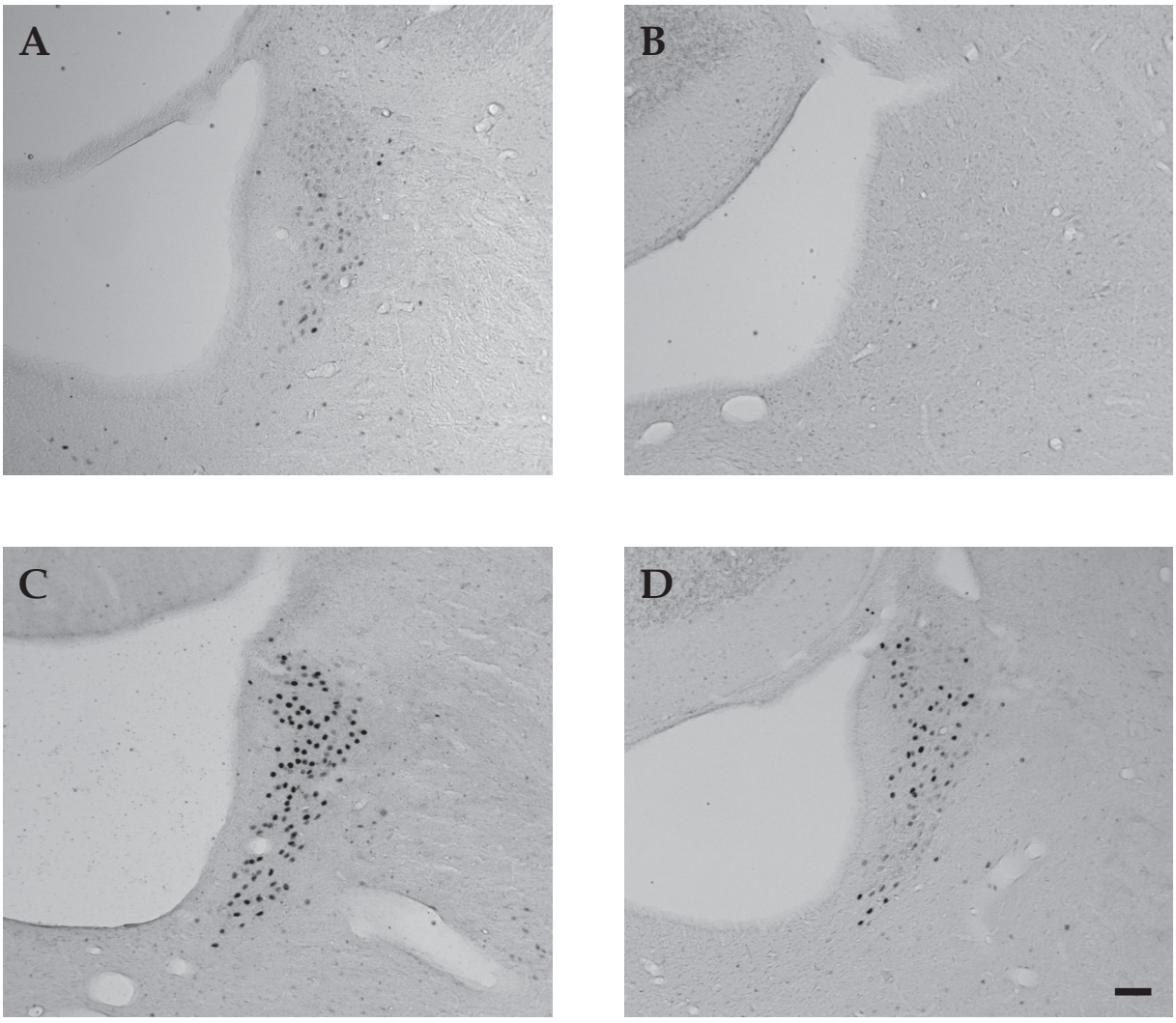

Figure 6. Representative photographs of Fos-immunoreactive cell nuclei in the locus coeruleus (Bregma –10.04) of rats challenged with saline after 22 days of pretreatment with vehicle (A) or paroxetine (20 mg/kg p.o., (B)) and rats challenged with 8-OH-DPAT (0.4 mg/kg s.c.) after 22 days of pretreatment with vehicle (C) or paroxetine (D). Scale bar=100µm.

tion. The ventral premammillary nucleus receives dense serotonergic innervation (Paxinos, 2004), but the role of serotonin in this area is barely investigated. Since the ventral premammillary nucleus is strongly connected with the medial preoptic area (Kocsis *et al.*, 2003), which is crucial for reproductive behaviour, and shows expression of *c-fos* in response to social interaction (Salchner *et al.*, 2004) and female pheromones (Yokosuka *et al.*, 1999), it is possible that serotonin exerts its effects on sexual behaviour partly via this brain area. However, since the drug-induced Fos-immunoreactivity did not concur with the drug-induced alterations in sexual behaviour, it is unlikely that the ventral premammillary nucleus is involved in the effects of SSRIs or 8-OH-DPAT on ejaculation.

Although the main effects of 5-HT neurotransmission on ejaculation are thought to take place in the central nervous system, some influence of the peripheral nervous system cannot be excluded. Noradrenalin-mediated contractions of the vas deferens and seminal vesicles play a crucial role during ejaculation. Paroxetine, fluoxetine and sertraline have been found to inhibit the contractions of rat and human vas deferens induced by noradrenalin or nerve stimulation *in vitro* (Kalyoncu *et al.*, 1999; Medina *et al.*, 2000; Seo *et al.*, 2001; Yaris *et al.*, 2003) and the elevation of intraluminal pressure of the rat vas deferens and seminal vesicles induced by stimulation of the hypogastric or lesser splanchnic nerves *in vivo* (Hsieh *et al.*, 1998; Kim *et al.*, 2000; Kim and Paick, 2004). This inhibition was attributed to the blockade of calcium channels by SSRIs (Kalyoncu *et al.*, 1999; Yaris *et al.*, 2003) or the direct activation of 5-$HT_{1A/1B/2C}$ receptors in the vas deferens and seminal vesicles (Kim and Paick, 2004). In contrast, an equivalent dose of fluoxetine as well as chronic treatment with sertraline and fluoxetine have been found to amplify rather than inhibit noradrenalin-induced contractions of rat vas deferens *in vitro* (Busch *et al.*, 1999; Busch *et al.*, 2000; Ozyavuz *et al.*, 2004), indicating that the role of peripheral effects in SSRI-induced delayed ejaculation are somewhat ambiguous. Moreover, the *in vivo* effects of SSRIs on intraluminal pressure of seminal vesicles and vas deferens seem to originate in the central nervous system (Kim *et al.*, 2000; Seo *et al.*, 2001).

In conclusion, selective activation of 5-HT_{1A} receptors by 8-OH-DPAT strongly changed the number of Fos-positive cells in several brain areas, which was attenuated when rats were pretreated with paroxetine for 22 days. In some brain areas, Fos-immunoreactivity correlated with the effects of paroxetine and 8-OH-DPAT on sexual behaviour and ejaculation. In particular, the oxytocinergic magnocellular paraventricular hypothalamic nucleus and the noradrenergic locus coeruleus are putative candidate areas to link 5-HT_{1A} receptor activation with facilitation of ejaculation, and 5-HT_{1A} receptor

desensitization with SSRI-induced delayed ejaculation. In addition, the results clearly demonstrate that Fos-immunohistochemistry can be used to pinpoint brain areas that are affected by SSRI-induced changes, in order to investigate the central mechanism of these antidepressants.

Acknowledgements

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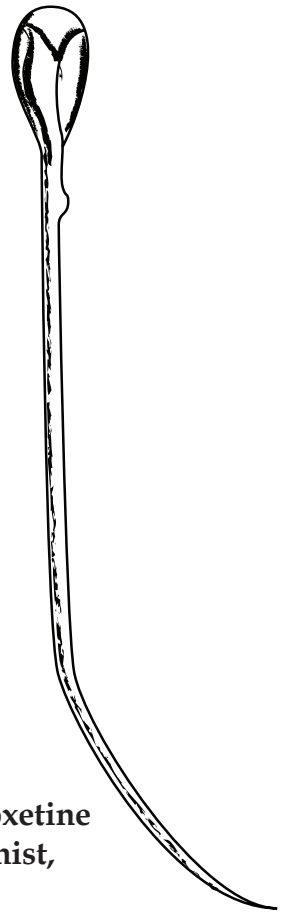
Chapter 6



Acute co-administration of the selective serotonin reuptake inhibitor paroxetine with a 5-HT_{1A} receptor antagonist, but not a peripheral oxytocin antagonist, inhibits ejaculation

TR de Jong, JG Veening, MD Waldinger, N Robinson, AR Cools, B Olivier

Abstract

Chronic treatment with the Selective Serotonin Reuptake Inhibitor (SSRI) paroxetine relieves depression by elevating 5-HT levels in the CNS, but also delays ejaculation as a side effect. In contrast, acute administration of paroxetine does not delay ejaculation. Possibly, activation of pre- and/or postsynaptic 5-HT_{1A} receptors prevents delayed ejaculation under these circumstances. Increased release of oxytocin, a neuropeptide that facilitates ejaculation, induced by postsynaptic 5-HT_{1A} receptor activation might play a role.

Male Wistar rats were injected with several doses of the selective peripheral oxytocin receptor antagonist L-368899 (ranging from 10^{-4} to 10 mg/kg s.c.) or saline and studied in a 30 min sexual behaviour test with a receptive female. A second group of male Wistar rats was injected with paroxetine (10 mg/kg p.o.) or vehicle combined with the selective 5-HT_{1A} receptor antagonist WAY-100635 (0.1 mg/kg s.c.), L-368899 (3 mg/kg s.c.) or saline and studied in a 30 min sexual behaviour test with a receptive female.

Acute systemic injection of L-368899 did not affect any parameter of sexual behaviour in any dose. In addition, acute systemic injection of paroxetine or WAY-100635 did not change sexual behaviour. Co-administration of paroxetine and WAY-100635 strongly inhibited ejaculation. Co-administration of paroxetine and L-368899 did not inhibit ejaculation, although a trend to delay the second and third ejaculation was visible.

Activation of 5-HT_{1A} receptors does prevent delayed ejaculation following acute paroxetine treatment; however, this is apparently not or barely mediated by the release of oxytocin in the bloodstream. Instead, activation of somatodendritic 5-HT_{1A} autoreceptors or postsynaptic 5-HT_{1A} receptors controlling the release or binding of other neurotransmitters might be involved.

Introduction

The Selective Serotonin Reuptake Inhibitors (SSRIs) fluoxetine, citalopram, paroxetine, fluvoxamine, and sertraline are widely used antidepressants that act by blocking 5-HT transporters in the central nervous system, thereby elevating extracellular serotonin levels (Nutt *et al.*, 1999; Vaswani *et al.*, 2003). Although chronic SSRI-treatment induces relatively few side-effects, sexual dysfunctions such as delayed ejaculation or anorgasmia are reported regularly (Gregorian *et al.*, 2002; Rosen *et al.*, 1999).

The neurobiological background of SSRI-induced delayed ejaculation is not yet unravelled. Increased 5-HT neurotransmission probably plays a role, since elevated serotonin levels in brain and spinal cord inhibit ejaculation (Ahlenius and Larsson, 1998; Foreman *et al.*, 1992; Hillegaart *et al.*, 1991; Marson and McKenna, 1992; Verma *et al.*, 1989), most likely via activation of 5-HT_{1B} and/or 5-HT_{2C} receptors (Fernandez-Guasti *et al.*, 1989; Foreman *et al.*, 1989; Hillegaart and Ahlenius, 1998; Klint *et al.*, 1992; Pomerantz *et al.*, 1993a; Watson and Gorzalka, 1991).

Although SSRIs acutely elevate 5-HT levels (Bymaster *et al.*, 2002), the inhibition of ejaculation seems to require chronic SSRI-treatment (Cantor *et al.*, 1999; Mos *et al.*, 1999; Taylor *et al.*, 1996; Vega *et al.*, 1998). Moreover, the degree of delayed ejaculation differs markedly between paroxetine and fluoxetine, which cause a severe delay, and citalopram and fluvoxamine, which hardly impair ejaculation in humans and rats (Waldinger *et al.*, 1998b; Waldinger *et al.*, 2002; Waldinger *et al.*, 2001; Waldinger *et al.*, 2004a), despite their comparable effect on 5-HT levels (Bymaster *et al.*, 2002).

Growing evidence suggests that 5-HT_{1A} receptor functioning plays a crucial role in the occurrence and degree of SSRI-induced delayed ejaculation. It is well known that 5-HT_{1A} receptor activation strongly facilitates ejaculation (Ahlenius *et al.*, 1981; Coolen *et al.*, 1997a; Haensel and Slob, 1997), and it is possible that after acute injection with an SSRI the inhibiting effects of elevated 5-HT levels on ejaculation are compensated by 5-HT_{1A} receptor activation. Consistently, acute treatment with the SSRI citalopram strongly inhibits ejaculation when co-administered with the selective 5-HT_{1A} receptor antagonist WAY-100635 (Ahlenius and Larsson, 1999; de Jong *et al.*, 2005a). Chronic treatment with paroxetine impairs those 5-HT_{1A} receptors that facilitate ejaculation (de Jong *et al.*, 2005c), probably by desensitization (Le Poul *et al.*, 1995; Li *et al.*, 1997b), and together with the elevated serotonin levels this might underlie delayed ejaculation.

It is not yet known whether and how 5-HT_{1A} receptor activation can prevent the inhibition of ejaculation following acute SSRI treatment. The neuropeptide oxytocin might play a role, since both 5-HT_{1A} receptor activation and acute treatment with the SSRI

citalopram induce the release of oxytocin in the blood (Uvnas-Moberg *et al.*, 1999; Vicentic *et al.*, 1998), and intravenous administration of oxytocin facilitates ejaculation in a similar fashion as 5-HT_{1A} receptor agonists (Ahlenius *et al.*, 1981; Stoneham *et al.*, 1985). In addition, the ability of the 5-HT_{1A} receptor agonist 8-OH-DPAT to induce the release of oxytocin in the blood is inhibited in rats treated chronically with the SSRI paroxetine, as measured by oxytocin levels in the blood plasma (Li *et al.*, 1997b) or *c-fos* expression in the magnocellular oxytocin-releasing cells in the paraventricular hypothalamic nucleus (de Jong *et al.*, 2005b). Moreover, systemic administration of oxytocin reverses the inhibition of ejaculation induced by chronic fluoxetine treatment (Cantor *et al.*, 1999). These results suggest that 5-HT_{1A} receptor desensitization induced by chronic SSRI-treatment might mediate delayed ejaculation through inhibition of oxytocin release in the blood.

Since oxytocin is a peptide and cannot easily penetrate the blood-brain barrier (Gimpl and Fahrenholz, 2001), the experiments described above indicate a role for peripheral oxytocin receptors in ejaculation, as opposed to central oxytocin receptors. Indeed, peripheral oxytocin receptors have been located on smooth muscle cells in the epididymis, testis and vas deferens and are known to play a role in the contractions that initiate ejaculation (Filippi *et al.*, 2002; Nicholson *et al.*, 1999; Whittington *et al.*, 2001).

To study the role of oxytocin in normal sexual behaviour and in the effects of acute injection with paroxetine on sexual behaviour, the peripheral acting oxytocin receptor antagonist L-368899 was used. First, a dose response curve of the effects of L-368899 on sexual behaviour was conducted. Second, the effects of acute administration of a selected dose of paroxetine that is known to elevate 5-HT levels (10 mg/kg p.o.) co-administered with either a selected, effective dose of the 5-HT_{1A} receptor antagonist WAY-100635 (0.1 mg/kg s.c.) or a selected dose of L-368899 that fully occupies oxytocin receptors (3 mg/kg s.c.; personal communication with Pfizer, Sandwich, United Kingdom) on sexual behaviour were studied.

Methods

Animals

Adult male (250-300 grams and 3 months of age at the start of the experiment) and female Wistar rats (Harlan, Zeist, the Netherlands) were used. The animals arrived at the laboratory at least 14 days prior to the start of the experiments in order to adapt to the laboratory environmental condition and to a reversed light/dark cycle (12:12h, lights

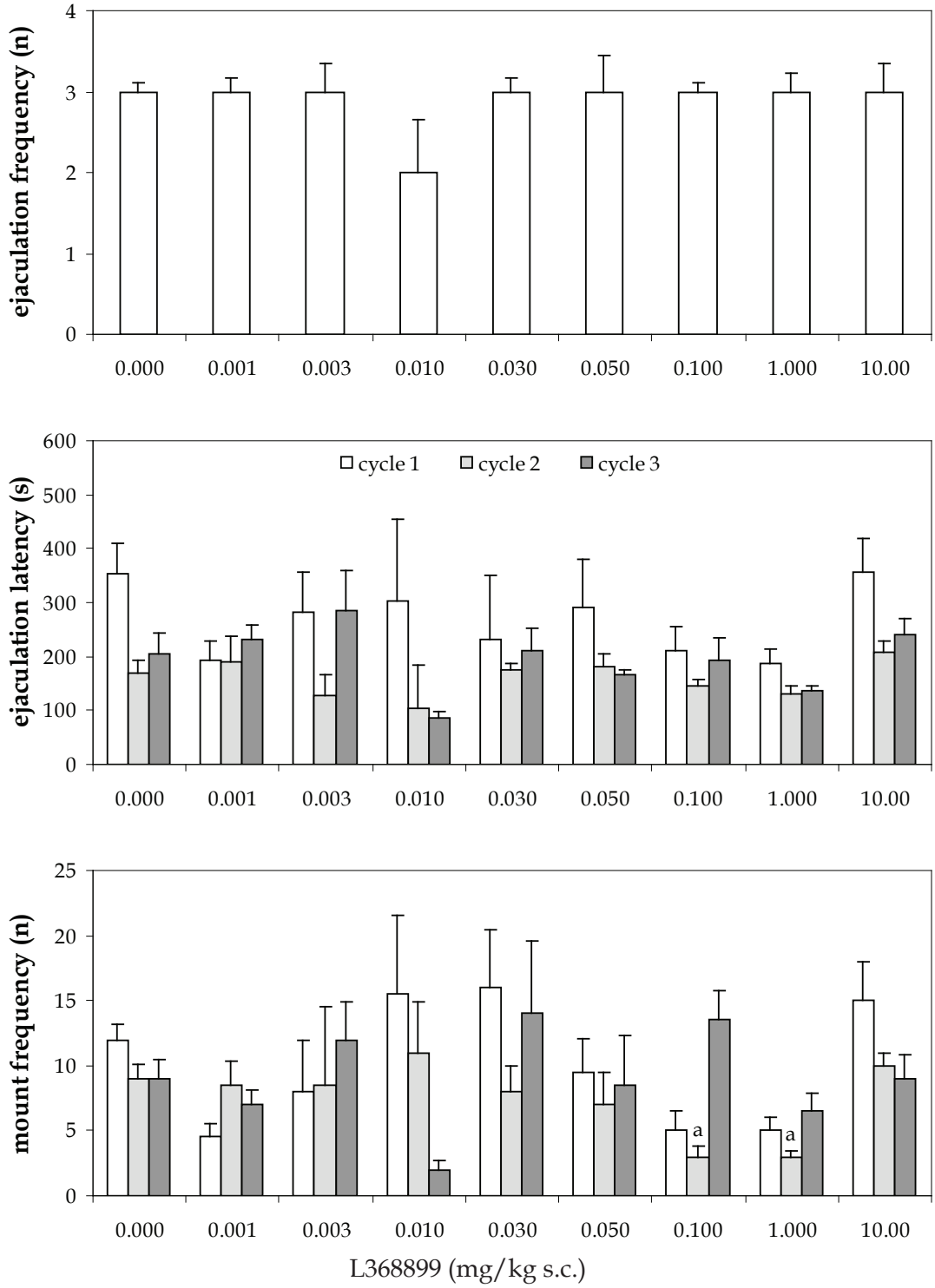


Figure 1. The effects of different doses of the peripheral oxytocin receptor antagonist L-368899 on the ejaculation frequency and the first (white bars), second (light grey bars) and third (dark grey bars) ejaculation latency and mount frequency of male Wistar rats in a 30 min sexual behaviour test with a receptive female. Data are medians ± standard error of the median; a=different from saline; $P<0.05$.

off at 6.30am). Food and tap water were available *ad libitum*. Males were individually housed and handled daily from the start of the experiment. The females, which served as stimulus animals, were sterilized by ligation of the oviducts and housed in couples. Their sexual receptivity was fully and reliably induced by subcutaneous administration of 50 µg estradiol benzoate dissolved in 0.1 ml arachidis oil, 36 h prior to testing. All experiments were approved by the Animal Ethical Committee of the University of Nijmegen and conform the national and internal laws of animal care and welfare.

Behavioural Observations

All rats were trained in four sexual behaviour tests before the start of the experiment, in order to establish consistent ejaculation frequencies. 24 individuals with normal ejaculatory behaviour (an average ejaculation frequency of 2.5 ≤ 3.5 in the last three training sessions) were selected for the experiment.

All training sessions and experimental sessions were conducted using the same paradigm and always between 10.30h and 15.30h, in a red-lighted room. The rats were placed in a rectangular mating arena (40x50x65 cm) with wood shavings on the floor and a Perspex front. The rats were allowed to habituate to the arena for 15 min. Then, a receptive female was placed in the arena and free contact was allowed for 30 min.

In this time frame, the ejaculation frequency (the total number of ejaculations) was counted and the ejaculation latency (time from first mount or intromission to ejaculation), mount frequency (number of mounts prior to ejaculation) and intromission frequency (number of intromissions prior to ejaculation) of three consecutive ejaculations, as well as the post-ejaculatory interval (first mount or intromission following ejaculation) of the first two ejaculations were determined using event recording software The Observer (Noldus Information Technology, Wageningen, the Netherlands).

Drugs

L-368899 (Merck & Co., Inc., Whitehouse Station, USA, kindly provided by Pfizer, Sandwich, United Kingdom) was dissolved in saline. Rats received a subcutaneous injection (1 ml/kg) with saline or L-368899 in doses of 10^{-4}, $3x10^{-4}$, 10^{-3}, $3x10^{-3}$, $5x10^{-3}$, 10^{-2}, 10^{-1} and 10 mg/kg, 10-15 minutes prior to the sexual behaviour test.

The 5-HT_{1A} receptor antagonist *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide 3HCL (WAY-100635, Wyeth-Ayerst, Princeton, NJ) was dissolved in saline. Rats received a subcutaneous injection (1 ml/kg) with saline or WAY-100635 in a dose of 0.1 mg/kg, 20-15 minutes prior to sexual behaviour.

Paroxetine tablets (20 mg paroxetine/tablet; Genthon BV Nijmegen, the Netherlands)

were obtained from the local pharmacy and were crushed and dissolved in 1% methylcellulose. The rats received an oral injection (5 ml/kg) with vehicle (methylcellulose) or paroxetine (10 mg/kg), 55-60 min prior to the sexual behaviour test.

Experimental Design

12 rats were treated weekly with a dose of L-368899 or saline in a within-subject design, so that in nine weeks each individual had received each dose once in random order. The remaining 12 rats were treated weekly with a combination of vehicle or paroxetine with saline, WAY-100635 or L-368899 in a within-subject design, so that in 6 weeks each individual had received each combination once in random order.

Statistical Analyses

All statistical analyses were performed with the Statistical Package for the Social Sciences version 12.0 (SPSS Inc., Chicago IL, USA). Statistical outliers were excluded from testing. The data were not normally distributed; therefore overall significant differences were detected with the non-parametric Kruskall Wallis test and further analyzed with the Mann-Whitney test.

Table 1: The effects of different doses of the peripheral oxytocin receptor antagonist L-368899 on the first, second and third intromission frequency (IF) and the first and second post ejaculatory interval (PEI) of male Wistar rats in a 30 min sexual behaviour test with a receptive female.

L-368899 (mg/kg s.c.)	IF1	IF 2	IF 3	PEI 1	PEI 2
0.000	11.50 ± 1.24	4.00 ± 0.34	4.00 ± 0.38	260.09 ± 8.97	360.25 ± 6.11
0.001	8.50 ± 0.59	4.00 ± 0.35	5.00 ± 0.42	273.53 ± 13.52	380.39 ± 12.79
0.003	12.00 ± 1.18	4.50 ± 0.40	3.00 ± 0.85	253.92 ± 14.46	342.90 ± 9.84
0.010	9.00 ± 2.12	4.00 ± 0.85	3.00 ± 0.09	268.92 ± 15.33	337.07 ± 11.90
0.030	11.00 ± 1.65	5.00 ± 0.25	6.00 ± 1.27	246.51 ± 22.57	351.11 ± 9.25
0.050	9.00 ± 0.83	4.50 ± 0.65	4.50 ± 0.30	266.20 ± 13.00	375.09 ± 14.65
0.100	11.00 ± 1.69	4.00 ± 0.23	4.50 ± 0.65	290.61 ± 14.29	366.08 ± 19.44
1.000	8.00 ± 1.49	4.00 ± 0.53	3.50 ± 0.59	279.46 ± 16.45	341.70 ± 15.88
10.000	11.00 ± 0.75	4.00 ± 0.33	4.50 ± 0.30	308.08 ± 22.43	383.66 ± 24.64

Data are medians ± standard error of the median.

Results

Dose response curve

Analysis with the Kruskall Wallis test revealed that the different doses of L-368899 caused overall significant differences in the mount frequency prior to the second ejaculation, but not in any other parameter of sexual behaviour (figure 1 and table 1). The Mann Whitney Test further showed that, compared to saline, L-368899 reduced the second mount frequency (χ^2 =17.403; P=0.026) when administered in the doses of 10^{-2} mg/kg (P=0.005) and 10^{-1} mg/kg (P=0.006).

Co-administration

In the experiment with co-administration of paroxetine, L-368899 and WAY-100635, significant overall differences were found in ejaculation frequency (χ^2=27.829; P=0.000), latency time to the first ejaculation (χ^2 =28.405; P=0.000) and mount frequency prior to the third ejaculation (χ^2 =10.187; P=0.037) using the Kruskall Wallis test (figure 2 and table 2).

When the effects of co-administration of paroxetine with WAY-100635 were compared with the effects of paroxetine or WAY-100635 administered separately, using the Mann Whitney test, it was revealed that co-administration of paroxetine and WAY-100635 significantly reduced the ejaculation frequency and increased the ejaculation latency compared to treatment with paroxetine (P<0.001) or WAY-100635 (P<0.001) alone. Either paroxetine or WAY-100635 alone did not alter these parameters compared to co-administration of vehicle and saline (P>0.178). No significant differences between the treatments were found in effects on the third mount frequency.

No significant differences were found between the effects of co-administration of paroxetine with L-368899 and the administration of paroxetine or L-368899 separately on any parameter of sexual behaviour.

Discussion

Dose response curve

Administration of the peripheral oxytocin receptor antagonist L-368899 in a wide array of doses barely affected sexual behaviour, although a minor reduction of mount frequency prior to the second ejaculation was found in some doses. This finding was probably an artefact, since the mount frequencies prior to the first and third ejacula-

tion were not affected, and higher and lower doses of L-368899 showed a trend to increase mount frequencies.

L-368899 was administered systemically in the present experiment. The peptidergic antagonist does not readily penetrate the blood brain barrier and probably blocked only peripheral oxytocin receptors, indicating that peripheral oxytocin is not of crucial importance to sexual behaviour and ejaculation. In contrast, oxytocin receptor antago-

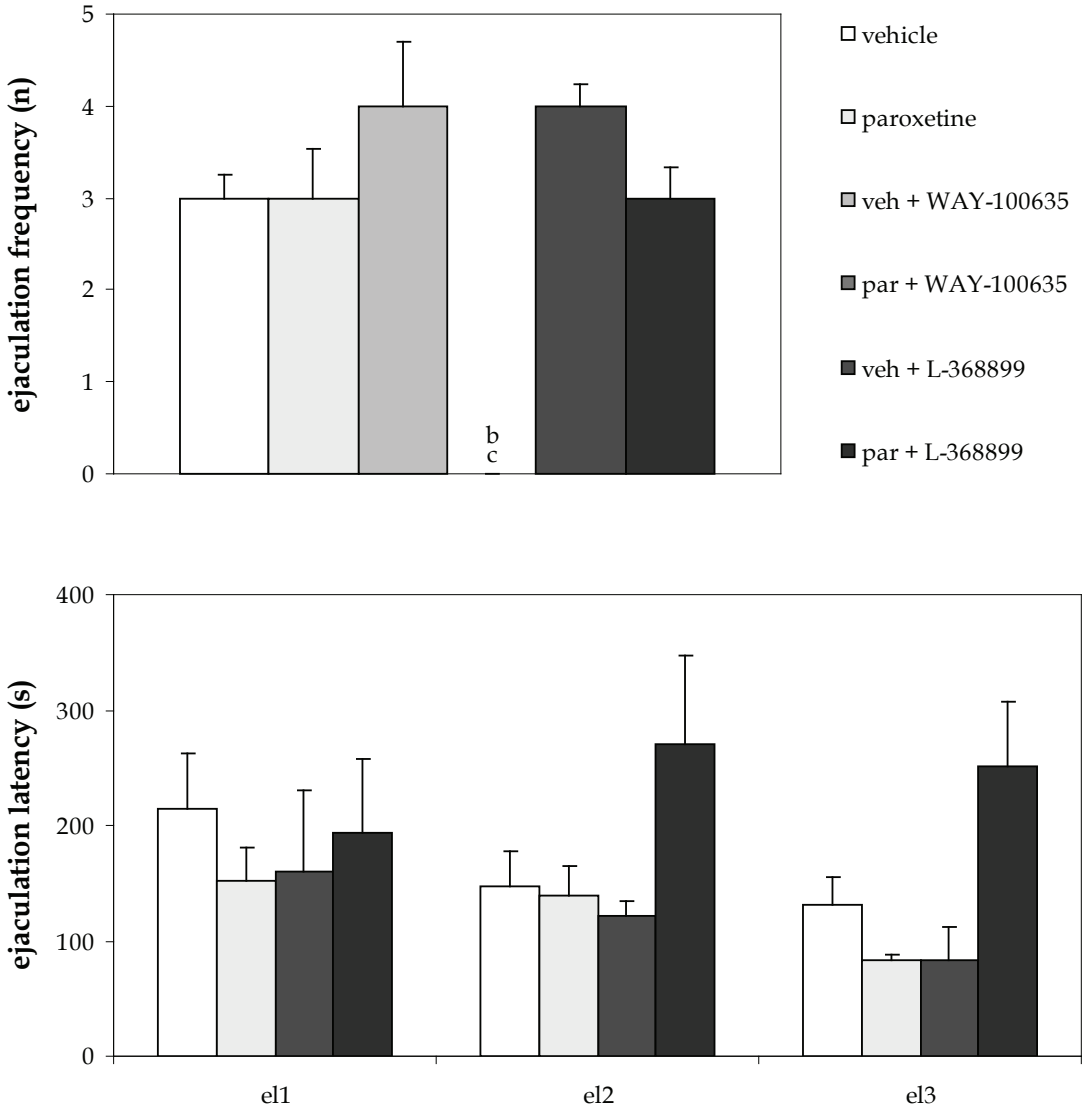


Figure 2. The effects of vehicle or paroxetine (10 mg/kg p.o.), co-administered with WAY-100635 (0.1 mg/kg sc) or L-368899 (3 mg/kg s.c.), on the ejaculation frequency and the first, second and third ejaculation latency of male Wistar rats in a 30 min sexual behaviour test with a receptive female. Data are medians ± standard error of the median; b=different from paroxetine, c=different from WAY-100635; $P<0.05$.

nists injected into the ventricles of male rats have been reported to impair sexual behaviour (Argiolas *et al.*, 1988). Oxytocinergic neurons in the parvocellular paraventricular hypothalamic nucleus might play a role, since these cells release oxytocin within the central nervous system and project to the motor nuclei in the spinal cord involved in sexual behaviour (Tang *et al.*, 1998; Veronneau-Longueville *et al.*, 1999). However, these oxytocinergic neurons are thus far only implicated in the facilitation of erection rather than ejaculation (Argiolas, 1992; Giuliano *et al.*, 2001; Melis *et al.*, 1986). In addition, lesions of the parvocellular paraventricular hypothalamic nucleus failed to prolong the ejaculation latency (Ackerman *et al.*, 1997; Hughes *et al.*, 1987), whereas lesion of both the parvocellular and magnocellular paraventricular hypothalamic nucleus caused delayed ejaculation (Liu *et al.*, 1997a). One alternative that needs to be further investigated is whether and how release of oxytocin from magnocellular paraventricular hypothalamic neurons into the third ventricle (Hughes *et al.*, 1987; Xiao *et al.*, 2005) can affect ejaculation.

Co-administration

Acute treatment with paroxetine (10 mg/kg p.o.) failed to affect any parameter of sexual behaviour. Previous experiments showed that acute treatment with the same dose of paroxetine either slightly delayed ejaculation (Waldinger *et al.*, 2002) or had no effect on the ejaculation latency (de Jong *et al.*, 2005c; Mos *et al.*, 1999).

Acute administration of WAY-100635 (0.1 mg/kg s.c.) did not alter any parameter of sexual behaviour, consistent with previous experiments (Ahlenius and Larsson, 1998; 1999; de Jong *et al.*, 2005a). Co-administration of WAY-100635 and paroxetine strongly inhibited ejaculation: only 2 rats reached one ejaculation. This is consistent with the effects of co-administration of the SSRI citalopram with WAY-100635 (Ahlenius and Larsson, 1999; de Jong *et al.*, 2005a), and confirms the theory that 5-HT_{1A} receptor activation is crucial for ejaculation when serotonin levels are elevated by an SSRI.

The selected dose of the oxytocin receptor antagonist L-368899 (3.0 mg/kg s.c.) did not alter any parameter of sexual behaviour, consistent with the dose-response curve performed in this study. In contrast, intracerebroventricular injections of the oxytocin receptor antagonist d(CH_2)$_5$-Tyr(Me-[Orn^8]vasotocin delayed ejaculation (Argiolas *et al.*, 1988; Arletti *et al.*, 1992), indicating that central oxytocin plays a more important role in ejaculation than peripheral oxytocin.

In addition, co-administration of L-368899 with paroxetine did not significantly affect sexual behaviour. Although a trend towards a delayed second and third ejaculation was visible, large individual differences between the rats prevented statistical signifi-

cance. Taken together, these results suggest that delayed ejaculation induced by acute paroxetine treatment is not prevented by increased oxytocin release in the bloodstream through 5-HT_{1A} receptor activation.L-368899 was administered systemically

Conclusion

The present study confirms that acute co-administration of the SSRI paroxetine with the selective 5-HT_{1A} receptor agonist WAY-100635 strongly inhibits ejaculation, while

Table 2: The effects of vehicle or paroxetine (10 mg/kg p.o.), co-administered with WAY-100635 (0.1 mg/kg s.c.) or L-368899 (3 mg/kg s.c.) on the first, second and third mount frequency (MF) and intromission frequency (IF) and the first and second post ejaculatory interval (PEI) of male Wistar rats in a 30 min sexual behaviour test with a receptive female.

Treatment:		**EL 1**	**MF 1**	**IF 1**	**PEI 1**
vehicle	**saline**	213.99 ± 48.06	9.00 ± 1.74	7.00 ± 0.50	248.86 ± 10.03
paroxetine	**saline**	152.25 ± 28.45	4.00 ± 1.52	7.00 ± 0.66	276.03 ± 8.46
vehicle	**UK-427843**	159.40 ± 71.77	5.00 ± 1.89	6.50 ± 1.30	267.82 ± 15.94
paroxetine	**UK-427843**	193.55 ± 64.10	11.00 ± 3.38	8.00 ± 0.68	290.39 ± 17.37
vehicle	**WAY-100635**	337.91 ± 68.31	7.00 ± 1.89	8.00 ± 0.47	250.95 ± 21.87
paroxetine	**WAY-100635**	1800.00 ± 0.00	-	-	-
Treatment:		**EL 2**	**MF 2**	**IF 2**	**PEI 2**
vehicle	**saline**	147.02 ± 29.95	4.00 ± 2.44	3.00 ± 0.86	334.78 ± 14.57
paroxetine	**saline**	138.77 ± 25.85	4.50 ± 0.92	3.00 ± 0.13	335.87 ± 11.23
vehicle	**UK-427843**	120.98 ± 14.12	4.00 ± 1.12	3.50 ± 0.59	342.46 ± 15.18
paroxetine	**UK-427843**	270.94 ± 76.19	10.00 ± 3.60	3.00 ± 0.23	355.48 ± 17.64
vehicle	**WAY-100635**	93.57 ± 13.35	2.50 ± 0.99	3.50 ± 0.53	314.07 ± 25.15
paroxetine	**WAY-100635**	-	-	-	-
Treatment:		**EL 3**	**MF 3**	**IF 3**	**PEI 3**
vehicle	**saline**	131.05 ± 24.42	4.00 ± 1.41	4.00 ± 0.28	-
paroxetine	**saline**	83.85 ± 4.94	3.00 ± 0.93	2.50 ± 0.47	-
vehicle	**UK-427843**	82.96 ± 28.97	3.00 ± 0.46	3.00 ± 0.46	-
paroxetine	**UK-427843**	251.64 ± 55.17	5.50 ± 1.45	3.00 ± 0.40	-
vehicle	**WAY-100635**	107.76 ± 13.18	2.00 ± 0.23	3.00 ± 0.23	-
paroxetine	**WAY-100635**	-	-	-	-

Data are medians ± standard error of the median.

separate injection of each drug does not affect any parameter of sexual behaviour. Apparently, the effects of paroxetine on ejaculation latency are dependent on activation of 5-HT_{1A} receptors, supporting a role for 5-HT_{1A} receptor desensitization in delayed ejaculation induced by chronic SSRI treatment.
Acute co-administration of WAY-100635 and paroxetine does not inhibit ejaculation by a combination of elevated serotonin levels and decreased oxytocin release in the bloodstream, since the peripheral oxytocin receptor antagonist L-368899 does not alter sexual behaviour when serotonin levels are normal or elevated by paroxetine. The activation of 5-HT_{1A} receptors apparently prevents SSRI-induced delayed ejaculation via a different mechanism, for example changes in release or binding of other neurotransmitters involved in sexual behaviour, or the inhibition of serotonergic cell firing via somatodendritic 5-HT_{1A} autoreceptors.

Acknowledgements

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Chapter 7

Individual differences in male rat ejaculatory behaviour: searching for models to study ejaculation disorders

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Abstract

In addition to investigating sexual function in rats that display normal ejaculatory behaviour, studying rats that are either 'hyposexual' or 'hypersexual' may provide important insights into the aetiology of ejaculatory dysfunctions in men, such as premature and retarded ejaculation. To this end, rats were matched into groups of 'sluggish', 'normal' and 'rapid' ejaculators based on their ejaculation frequencies displayed in a series of weekly sexual behaviour tests.

Selecting rats on this parameter revealed large and stable differences in other parameters of sexual behaviour as well, including ejaculation latency and mount frequency, but not intromission frequency and mount latency, putative indices of sexual motivation.

Neuroanatomically, Fos-immunoreactivity as a measure of neuronal activation, was increased in rapid ejaculators compared to sluggish ejaculators in ejaculation-related brain areas, presumably associated with the differences in ejaculatory behaviour. Although the total number of oxytocin neurons within subregions of the hypothalamus did not differ between groups, in the supraoptic nucleus of the hypothalamus more oxytocin neurons were activated in rapid ejaculators compared to the other groups.

Apart from the differences observed in ejaculatory behaviour, groups did not differ with respect to their locomotor activity and approach-avoidance behaviour as measured in the elevated plus maze. Finally, apomorphine-induced stereotypy was comparable in sluggish and rapid ejaculators, suggesting no large differences in dopamine susceptibility. Altogether, the present results suggest stable differences in male rat ejaculatory behaviour. Further exploring the neurobiological mechanisms underlying these differences may be a promising approach to gain insights into the aetiology of sexual dysfunctions such as premature, retarded or an-ejaculation.

Introduction

Lifelong premature ejaculation is probably the most common male sexual disorder, and has been investigated from both a medical and psychological perspective (Schapiro, 1943; St Lawrence and Madakasira, 1992; Waldinger, 2002). A recent survey by Symonds and colleagues (Symonds *et al.*, 2003) has suggested that premature ejaculation has a similar qualitative impact as erectile dysfunction.

The efficacy of various antidepressants to delay ejaculation in laboratory animals (Cantor *et al.*, 1999; Waldinger *et al.*, 2002) and men with premature ejaculation (Waldinger *et al.*, 2004a) suggests a strong neurobiological aetiology of ejaculatory disorders. In contrast to the psychological view that lifelong premature ejaculation results from self-learned behaviour during initial hurried sexual encounters (Masters and Johnson, 1970), it has been postulated that lifelong premature and retarded ejaculation belong to the extremes of a biologically existing variability of Intravaginal Ejaculation Latency Time (IELT) in men (Waldinger *et al.*, 1998a; Waldinger *et al.*, 1994). Recently, a stopwatch-assessed IELT study in a random cohort of 491 men from five countries indeed showed a positively skewed distribution with a median IELT of 5.4 minutes (range: 0.55-44.1 minutes), confirming the variability of IELT in men (Waldinger *et al.*, 2005a).

Increasing understanding of the neurobiology of sexual behaviour has been derived from animal studies in which specific brain areas have been manipulated or animals challenged pharmacologically (for reviews, e.g. Larsson and Ahlenius, 1999; Pfaus, 1999). Nevertheless, most often these studies employ sexually experienced animals that display 'normal' amounts of sexual behaviour.

With regard to certain ejaculatory complaints in men, investigating animals that do not display normal sexual function possibly provides further insights into the aetiology of ejaculatory dysfunctions. For instance, numerous differences exist between sexually naive and experienced rats with regard to certain neurotransmitter levels, such as oxytocin (Hillegaart *et al.*, 1998), responsiveness to pharmacological challenges (Haensel *et al.*, 1991; Mos *et al.*, 1999; Rowland and Houtsmuller, 1998) and their ability to copulate in a novel environment (Pfaus and Wilkins, 1995). In addition, differences have been reported between sexually-inactive and active rats in the hypothalamus with respect to oxytocin production (Arletti *et al.*, 1997) and levels of endogenous opioids (Rodriguez-Manzo *et al.*, 2002a). Interestingly, several studies have demonstrated that sexual behaviour of sexually-inactive rats can be reversed, pharmacologically, into normal sexual function (Drago and Busa, 2000; Gessa *et al.*, 1979; Giuliani *et al.*, 2002; Haensel *et al.*, 1991; Ottani *et al.*, 2002). Together, these findings suggest neurobiological differ-

ences in sexually naive and inactive rats compared to rats that display normal sexual function.
In a previous experiment, we have repeatedly observed stable individual differences in sexual behaviour in large populations of male Wistar rats (unpublished observations). The aim of the present studies was to further explore, quantify and directly compare these differences observed in sexual behaviour as a putative approach to study ejaculation disorders, since –to our knowledge- no previous studies have directly compared these "endophenotypical" differences in sexual behaviour. Subsequently, we studied putative differential activation of the ejaculation-related neural circuitry using c-*fos* as a marker for neuronal activation (Veening and Coolen, 1998) and, moreover, neuronal activation of oxytocin-containing neurons in the hypothalamus. The latter because of the importance of oxytocin in male ejaculatory behaviour and erection (Argiolas, 1999; Carter, 1992). In addition, individual differences in ejaculatory behaviour were manipulated pharmacologically with a serotonin 1A receptor agonist (±)-8-hydroxy-2-(di-n-propyl-amino)tetralin hydrobromide (8-OH-DPAT), which has been shown to potently facilitate ejaculatory behaviour (for instance: Ahlenius and Larsson, 1991a; Ahlenius *et al.*, 1981; Coolen *et al.*, 1997a). Finally, we further investigated whether differences in arousability or other behaviours underlie the observed differences in sexual behaviour as has been shown previously (Kohlert and Bloch, 1996)

Materials and methods

Subjects
Male and female Wistar rats, obtained from the local breeding facilities of the University of Nijmegen, were used in all experiments. At the start of experiments, animals were 3 months old (weights males: 350-450 g; females: 200-300 g) and housed under reversed 12 h dark – 12 h light cycle conditions (lights on from 7 pm – 7 am). Males were housed individually and separated from females; the latter were housed in groups of 2 animals per cage (26x41x18 cm). All animals were housed at controlled room temperature (20 ± 2°C), relative humidity of 60 ± 15% and provided standard rodent food pellets (Ssniff Spezialdiäten GmbH, Soest, Germany) and water available *ad libitum*. Females were ovariectomized bilaterally under isoflurane anaesthesia (Rhodia Organique Fine Ltd., Bristol, UK) and were brought into artificial estrous by a subcutaneous injection of 50 µg oestradiol benzoate (Intervet International BV, Boxmeer, The Netherlands) in 0.1 ml sesame oil, saturated with lecithin at 5°C, 36 h prior to testing. Stimu-

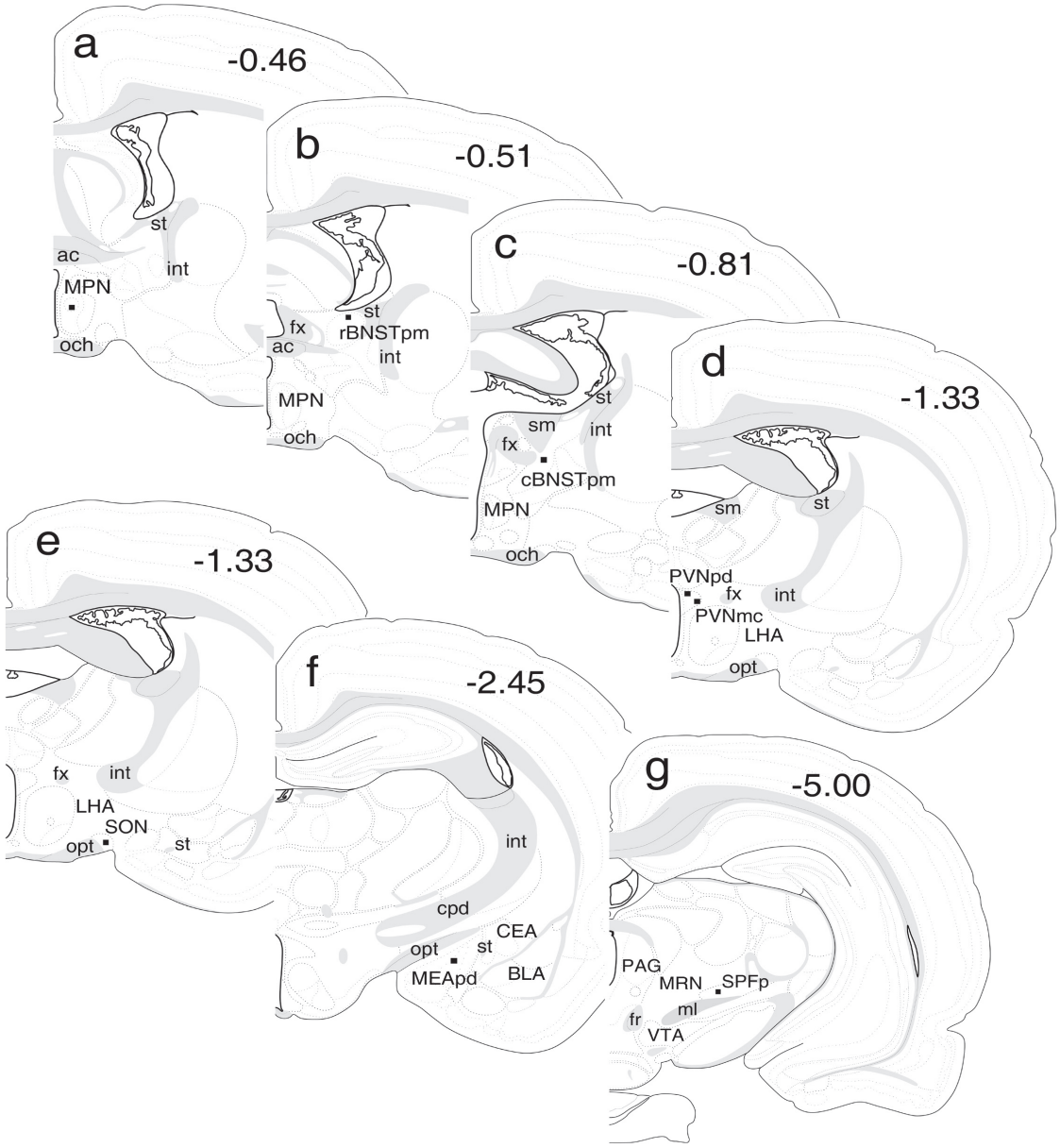

Figure 1: Schematic drawings illustrating the area of Fos/oxytocin analyses as indicated by the black square (100x100 µm) in the brain areas of interest. From rostral to caudal levels: (a) medial preoptic nucleus (MPN); (b) rostral and (c) caudal posterior medial bed nucleus of the stria terminalis (r/cBNSTpm); (d) parvocellular and magnocellular paraventricular hypothalamic nucleus (PVNpd and PVNmc); (e) supraoptic nucleus (SON); (f) posterodorsal medial amygdala (MEApd) and (g) subparafascicular nucleus of the thalamus (SPFp). ac, anterior commissure; cpd, cerebral peduncle; fr, fasciculus retroflexus; fx, columns of the fornix; int, internal capsule; ml, medial lemniscus; och, optic chiasm; opt, optic tract; sm, stria medullaris; st, stria terminalis; BLA, basolateral amygdala; CEA, central amygdala; LHA, lateral hypothalamic area; MRN, mesencephalic reticular nucleus; PAG, periaqueductal grey; VTA, ventral tegmental area. Drawings of coronal sections were adapted from Swanson (1998). Coordinates reflect distance from bregma (in mm).

lus females were used once every two weeks, and females that did not show receptive and proceptive behaviour during testing were replaced by a different female.
Experiments were carried out with the approval of the Animal Ethics Committee of the University of Nijmegen, the Netherlands.

Experiment 1: Behavioural characterization, effects of 8-OH-DPAT and neuronal activation of male rats which differ in sexual behaviour

Procedures
For the behavioural experiments, male rats were tested at least 2 h after onset of the dark period between 10 am and 4 pm with sexually receptive females. Prior to the behavioural experiments, rats were weighed and handled every day for 2 weeks. Rats were tested in weekly sexual behaviour training sessions of 30 min. A sexual behaviour session consisted of transferring male rats to the testing room and placing them in the testing cage. The testing cage was a wooden box (60x40x30 cm) with a Plexiglas front window and sawdust bedding placed in a red lit room. After a habituation period of 10 min, a receptive female was inserted into the testing cage, and male rats were allowed to copulate with the female for 30 min. During this period following elements of male rat sexual behaviour were recorded: mounts (M), the number of mounts without vaginal penetration; intromissions (I), the number of mounts with vaginal penetration; ejaculations (E). These elements of sexual behaviour were scored with the event-recording software The Observer® version 3.0 (Noldus Information Technology, Wageningen, The Netherlands). From the recorded data the following parameters were calculated as previously described (Mos *et al.*, 1999): mount latency (ML), the latency until the first mount; intromission latency (IL), the latency until the first intromission; mount frequency (MF); intromission frequency (IF); ejaculation latency (EL), time from first mount or intromission until ejaculation (in sec); ejaculation frequency (EF); post ejaculatory interval (PEI), time from ejaculation until the first intromission of event of the next cycle i.e. mount or intromission (in sec); intromission ratio (IR), the ratio of IF/(IF+MF). Between various sexual behaviour tests of different males, the cage was not cleaned in order to create a test environment rich with sex-related odours.
All rats received 6 training sessions and the results obtained during these sessions were used to divide the rats into different groups of sexual performance. On the basis of their copulatory behaviour obtained during the sixth training session, rats were matched into sluggish, normal and rapid ejaculators on the basis of their ranking score on EF. Three arbitrary categories were created for EF: sluggish, 0-1 ejaculation; normal,

1-3 ejaculations; rapid, 3 or more ejaculations. Importantly, only animals that fulfilled above criteria in at least four preceding training sessions were appointed to the experimental groups, in order to avoid overlap in EF between groups. The remainder of the animals, a group consisting of animals displaying average/normal ejaculatory behaviour was used for pharmacological experiments not described in the present experiment.

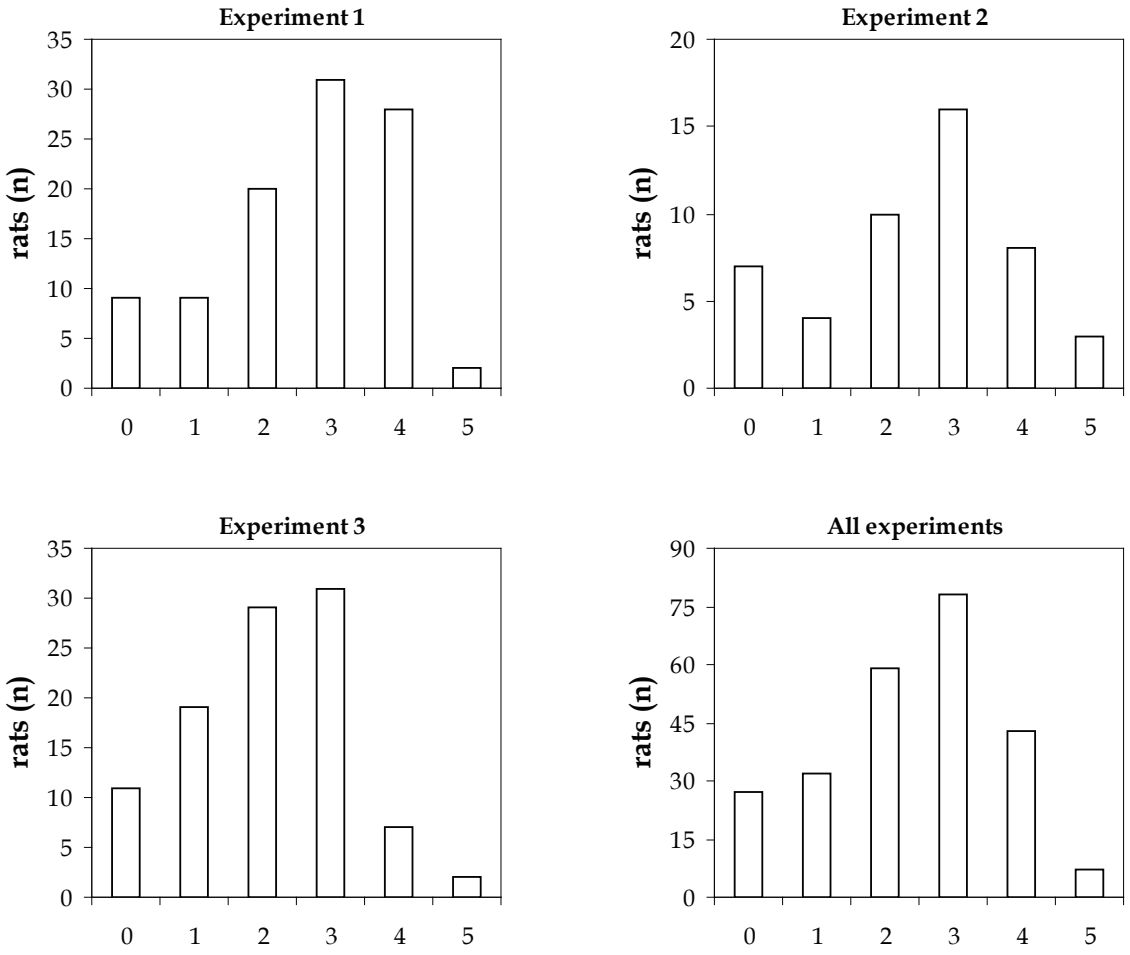

Figure 2. Ejaculation frequency histogram during fourth training session in large populations of maleWistar rats obtained during experiment 1 (n=99; means ± standard error of the mean, 2.7 ± 0.1), experiment 2 (n=48; mean ± standard error of the mean, 2.5 ± 0.2), experiment 3 (n=99; mean ± standard error of the mean, 2.1 ± 0.1) and all experiments combined (n=246; mean ± standard error of the mean, 2.4 ± 0.1).

One week following the sixth training session, animals in the three groups were subcutaneously injected with either 0.8 mg/kg (±)-8-OH-DPAT (Sigma Aldrich, St. Louis, MO, USA) dissolved in 0.9% physiological saline or vehicle (1 ml/kg bodyweight) 30 min prior to testing in a sexual behaviour test.

Immunohistochemistry

Rats were deeply anaesthetized 60 min following the sexual behavioural test, using sodium pentobarbital (50 mg/kg, intraperitoneally; Ceva Sante Animale BV, Maassluis, The Netherlands). Subsequently, animals were perfused transcardially with 100 ml 0.1M phosphate buffered saline (PBS), pH 7.3, followed by 500 ml 4% paraformaldehyde in 0.1M PBS, pH 7.2. Brains were removed and post-fixed overnight for 16-18 h in the same fixative, then stored in 30% sucrose in 0.1M PBS, both at 4°C.

Coronal sections were cut at 50 µm using a freezing microtome (model HM440E, Microm, Walldorf, Germany) and collected in six parallel series, in 0.1 M PBS. All incubations were at room temperature (20°C) under gentle agitation. In total 4 brains were processed per group.

Fos: The free floating sections were washed twice in 0.1M PBS and soaked for one hour in PBS containing 0.1% bovine serum albumin and 0.5% Triton X-100 (PBS-BT). Then the sections were incubated overnight with a primary polyclonal anti-Fos antiserum raised in rabbit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), diluted 1:20000 in PBS-BT. As the next step, the sections were incubated for 60 min in donkey anti-rabbit antibody conjugated to peroxidase (1:1500 in PBS-BT, Jackson Immunoresearch, Westgrove, PA, USA) and for 120 min in ABC-elite (1:800 in PBS; Vectastain, Brunschwig Chemie, Amsterdam, The Netherlands). In between incubations, sections were thoroughly rinsed twice with 0.1M PBS. The Fos-antibody peroxidase complex was visualized by 3,39-diaminobenzidine tetrahydrochloride (DAB; Sigma Aldrich, St. Louis, MO, USA) staining. Sections were incubated for 10 min in a chromogen solution consisting of 0.02% DAB and 0.03% Ni-ammonium sulfate in 0.05 M Tris-buffer (pH 7.6), and subsequently for 10 min in chromogen solution containing hydrogen peroxide (10:1 of a 30% solution). All sections were rinsed several times in 0.1M PBS.

Oxytocin+Fos: The protocol for sections processed for the oxytocin-Fos double staining was comparable to the protocol used for immunocytochemical visualization of Fos except for an additional overnight incubation in a primary anti-oxytocin antiserum raised in rabbit (Peninsula Laboratories Inc., San Carlos, CA, USA), diluted 1:100000 in the incubation medium and visualization by DAB staining without Ni-ammonium sulfate resulting in brown staining for oxytocin. The visualization of Fos was comparable to

the staining for Fos alone, except for the incubation with the primary polyclonal anti-Fos antiserum, which was diluted 1:10000 in PBS-BT. Following immunocytochemical staining for either Fos or oxytocin, sections were mounted on gelatin/chrome aluin-coated glass slides, dried overnight at 37°C, cleared in xylene, embedded with Entellan (Merck KGaA, Darmstadt, Germany) and cover slipped.

Experiment 2: Relapse into sluggish, normal and rapid ejaculations after a challenge with 8-OH-DPAT

In this experiment we recruited sluggish, normal and rapid ejaculators from a new cohort of rats (N=48) as described in experiment 1, and 30 min prior to the sexual behavioural test, rats were subcutaneously injected with a selected dose (0.8 mg/kg) of (R,S)-8-OH-DPAT (Sigma Aldrich Co., St. Louis, MO, USA) dissolved in 0.9% physiological saline in a volume of 1 ml/kg bodyweight. One week following testing with 8-OH-DPAT, animals were again tested for their sexual behaviour in a 30-min sexual behaviour test under drug free conditions.

Table 1. Copulatory behaviour in sluggish, normal and rapid ejaculators.

Behaviour	Sluggish	Normal	Rapid	ANOVA
MF	42 ± 4.0	23 ± 4.0 **a**	8.2 ± 1.8 **ab**	F(2,35)= 23.1; P<0.001
IF	5.6 ± 1.4	7.6 ± 0.9	7.5 ± 1.0	F(2,35)= 1.1; ns
EF	0.2 ± 0.1	1.9 ± 0.3 **a**	3.7 ± 0.2 **ab**	F(2,35)= 70.3; P<0.001
***EL**	1697 ± 80	717 ± 133 **a**	247 ± 45 **ab**	F(2,35)= 62.7; P<0.001
ML	47.6 ± 30.6	6.5 ± 0.8	13.6 ± 7.7	F(2,35)= 1.5; ns
IL	544 ± 197	46.6 ± 12.3	20.8 ± 6.1 **a**	F(2,35)= 6.6; P<0.005
****PEI**	363	298 ± 19	250 ± 14	F(2,24)= 3.3 ; ns
IR	13.7 ± 3.3	31.1 ± 5.1 **a**	56.7 ± 7.8 **ab**	F(2,35)= 14.4; P<0.001

All behaviours were calculated for the first ejaculatory series, except for ejaculation frequency (EF) which was calculated for the entire 30-min period (n=12 rats per group). MF, mount frequency; IF, intromission frequency; EL, ejaculation latency (s); ML, mount latency (s); IL, intromission latency (s); PEI, post-ejaculatory interval (s); IR, intromission ratio (%).
* Note that in sluggish ejaculators that did not achieve ejaculation EL was set at 1800 s; only two of 12 animals achieved one ejaculation with an average EL of 1180 ± 312 s.
** PEI in sluggish ejaculators based on one observation.
Data are means ± standard error of the mean; a=different from sluggish ejaculators, b=different from normal ejaculators; P<0.05

Experiment 3: Further behavioural characterization of sluggish, normal and rapid ejaculators

To further characterize sluggish, normal and rapid ejaculators behaviourally, animals were tested in an open field and elevated plus-maze with a one week interval between the performances of each behavioural paradigm. In addition, susceptibility to the effects of the dopamine agonist apomorphine on locomotor activity and gnawing was determined in sluggish versus rapid ejaculators. To this end, groups of sluggish, normal and rapid ejaculators were recruited by training a new cohort of 100 male rats as previously described in experiment 1.

Open field

Locomotor activity was assessed in a large open field that was placed in a dimly lit, sound attenuating room. To this end animals were placed in the middle of a square open field facing one side of the room. The open field (1.60x1.60 m) was made of a black Perspex floor and had no outer walls. In between tests, the maze was thoroughly cleaned with 70% ethanol. Total distance travelled was monitored for a 30 min period and analysed using a fully automated custom-made tracking system (Cools *et al.*, 1990).

Elevated plus-maze

The elevated plus-maze (black Plexiglas floor and walls) consisted of two closed and two open arms (width: 10 cm; length: 50 cm; height of walls on closed arms: 39 cm) radiating from a common centre platform (10x10 cm) and elevated 50 cm above floor level. At the start of the experiment, animals were placed in the middle of the centre platform facing one of the two open arms and animals were allowed to explore the maze for a 5 min period. In between sessions the maze was thoroughly cleaned with 70% ethanol. Behaviour was monitored and the following parameters were scored and analysed using a fully automated custom-made tracking system: distance and time spent in the open and closed arms; and number of entries into the open arms.

Apomorphine susceptibility

Rats were injected subcutaneously with 1.5 mg/kg (±)-apomorphine (Brocades, ACF) dissolved in sterile water, 30 min prior to testing in a custom-made gnawing box (adapted from the one described by Ljungberg and Ungerstedt, 1978). The box consists of a Perspex hole board (69x69 cm) with a central cubicle (25x25 cm). It contains 32

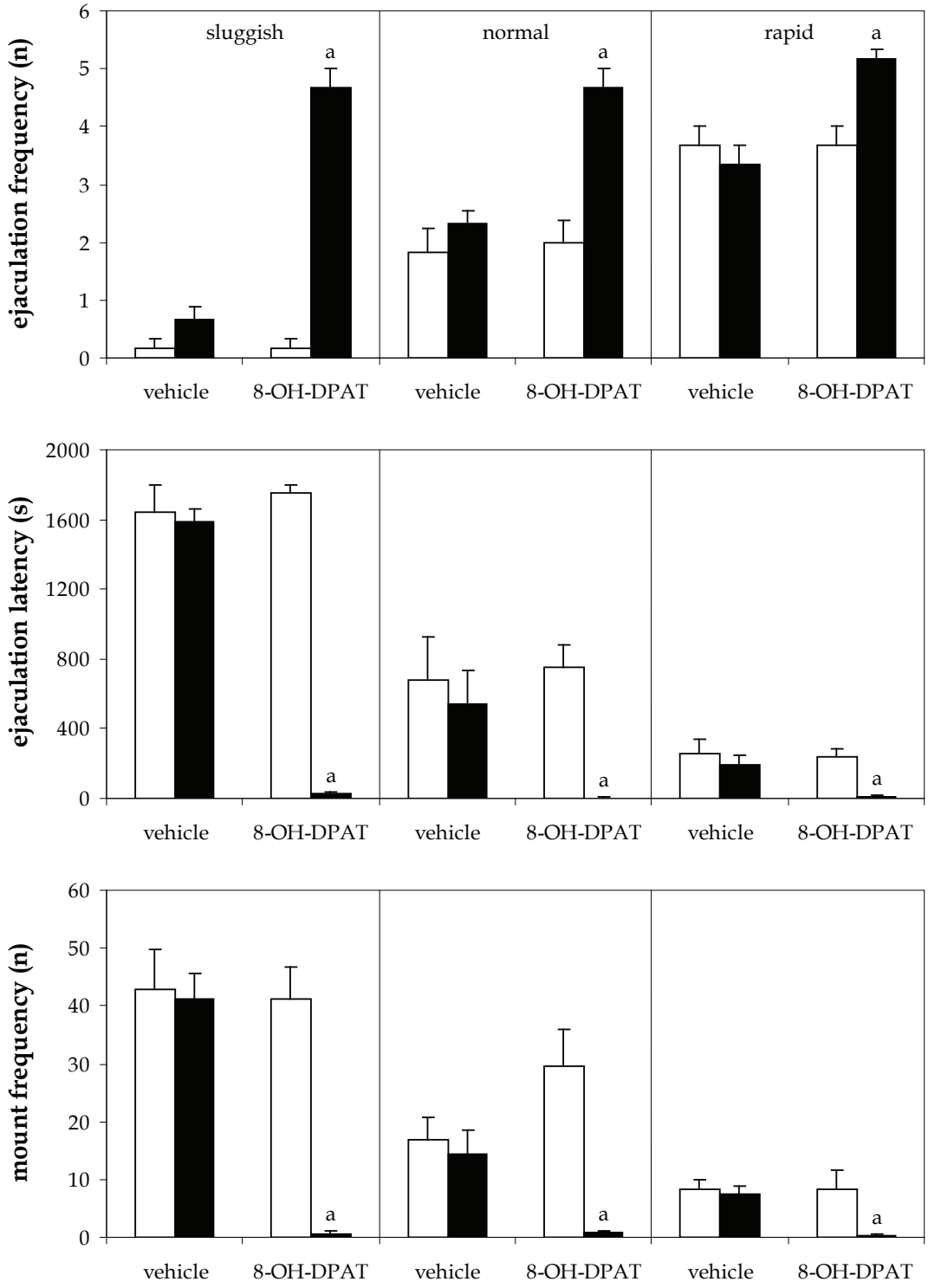

Figure 3. Effects of a selected dose of 8-OH-DPAT (0.8 mg/kg, s.c.) on the ejaculation frequency, ejaculation latency and mount frequency during baseline training (white bars) and testing with either 8-OH-DPAT or vehicle (black bars) in sluggish, normal and rapid ejaculators (n=6 per group). Data are means ± standard error of the mean; a=different from vehicle; $P<0.005$.

holes, each of which is surrounded by five concentric ridges. A microphone is placed underneath the central cubicle to allow registration of sounds. Through this microphone and a large number of infrared beams, a number of behavioural activities can be automatically recorded, like locomotor activity, frequency and duration of hole dipping, etc (Cools *et al.*, 1990). For the present experiments, only the stereotyped gnawing response was determined. The gnawing on the ridges surrounding the holes produces a characteristic sound that is detected by the microphone, fed into the computer and scored as a gnawing count. Animals were placed in the gnawing box for 45 min and a computer registered gnawing score and locomotor activity.

Data analyses

In experiment 1 and experiment 2 all parameters of sexual behaviour and immunohistochemical stainings are presented as means ± standard error of the mean. Analyses of variance (ANOVA) were used to compare each parameter of copulatory behaviour and immunocytochemical stainings. To evaluate the time course of sexual behaviour during the various copulatory sessions and the effects of 8-OH-DPAT on ejaculatory behaviour in the three groups of rats (sluggish, normal and rapid ejaculators) repeated measures ANOVAs were used. Animals that did not ejaculate were included in the analyses; however, their EL was set at 1800 sec.

The quantification of immunoreactive Fos cell nuclei and oxytocin-containing neurons was conducted using a light microscope (model Axiovert 35M, Zeiss, Germany) and imaging software Neurolucida version 2.1 (MicroBrightField Inc., Colchester, VT, USA). Numbers of Fos-IR nuclei and oxytocin-containing IR neurons were counted bilaterally for each region at 20x magnification in homologous square fields (using a grid size 100x100 μm; 0.01 mm^2) in sections displaying the most representative density of stained nuclei/neurons. For Fos both intense black and grey nuclear reaction products were counted immunoreactive. For oxytocin, neurons with clear brown cytoplasmic staining were counted immunoreactive. Figure 1 schematically depicts where Fos-IR and OT-containing IR neurons were counted within the regions of interest.

In experiment 3 the behavioural parameters obtained in the open field and elevated plus-maze are presented as means ± standard error of the mean. Statistical analyses were performed by means of univariate ANOVAs and further post-hoc comparisons were made using Dunnett's *t*-tests, because not under all circumstances equal variances were assumed.

All statistical analyses were performed using the Statistical Package for the Social Sciences version 11.0 (SPSS Inc., Chicago, IL, USA). In case of statistical significant effects

further post-hoc comparisons were made using Dunnett's *t*-tests, because equal variances were assumed not to be the case under all circumstances. A level of P< .05 was considered statistically significant.

Results

Experiment 1: Sexual behaviour in sluggish, normal and rapid ejaculators

To illustrate the variability in ejaculatory behaviour of male Wistar rats, figure 2 depicts the distribution of number of ejaculations in large populations of male rats during the 4th training session in experiments 1, 2 and 3. Consistent with previous unpub-

Table 2. Fos and oxytocin immunoreactivity in sluggish, normal and rapid ejaculators

Fos-IR	**Sluggish**	**Normal**	**Rapid**	**ANOVA**
MPN	33.2 ± 1.3	38.0 ± 1.2	43.6 ± 2.1 **a**	F(2,11)= 9.4, P<0.01
rBNSTpm	11.3 ± 2.1 **b**	28.8 ± 2.5 **a**	36.7 ± 8.0 **a**	F(2,11)= 9.6, P<0.01
cBNSTpm	17.8 ± 3.2	29.5 ± 5.2	30.0 ± 1.1	F(2,11)= 3.7, ns
MeApd	19.8 ± 1.6	27.0 ± 1.6	32.9 ± 2.9 **a**	F(2,11)= 9.6, P<0.01
SPFp	7.1 ± 1.2	14.5 ± 1.7	20.5 ± 4.7 **a**	F(2,11)= 5.1, P<0.05
Oxytocin-IR	**Sluggish**	**Normal**	**Rapid**	**ANOVA**
PVNpd	10.0 ± 1.4	9.8 ± 0.8	9.8 ± 1.1	F(2,11)= 0.2, ns
PVNmc	26.5 ± 3.8	25.5 ± 2.5	22.5 ± 1.8	F(2,11)= 0.6, ns
SON	21.3 ± 1.8	21.8 ± 1.1	22.0 ± 1.7	F(2,11)= 0.1, ns
Oxytocin-Fos double (%)	**Sluggish**	**Normal**	**Rapid**	**ANOVA**
PVNpd	10.5 ± 1.2	21.3 ± 9.8	29.2 ± 11.6	F(2,11)= 1.1, ns
PVNmc	6.3 ±1.0	9.1 ± 3.6	18.3 ± 4.0	F(2,11)= 3.9, ns
SON	11.5 ± 3.4	11.6 ± 4.3	25.0 ± 2.6 **ab**	(F2,11)= 5.1, P<0.05

Fos- and oxytocin-immunoreactive cells of two samples / 0.01 mm^2. MPN, medial preoptic nucleus; r / cBNSTpm, posteromedial division of the bed nucleus of the stria terminalis rostral / caudal parts; PVNmc, magnocellular division of the paraventricular nucleus of the hypothalamus; PVNpd, dorsal parvocellular paraventricular nucleus of the hypothalamus; MEApd, posterodorsal medial amygdala; SON, supraoptic nucleus of the hypothalamus; SPFp, parvocellular subparafascicular nucleus of the thalamus. Data are means ± standard error of the mean; a=different from sluggish ejaculators, b=different from normal ejaculators; P<0.05.

lished observations, figure 2 shows that approximately 10% of each cohort consists of animals that do not achieve ejaculation and 10% that achieve many ejaculations (4 or more ejaculations), with the exception of experiment 1 where a relatively higher proportion achieved 4 or more ejaculations. When animals were matched on the basis of their EF in session 6, a retrospective analysis by means of repeated measures ANOVAs indicated no changes during the course of training on EF (sessions 1-6), but group differences persisted over training sessions [group: $F(2,33)=41.2$, $P<0.001$]. Post hoc comparisons indicated significant differences between all groups. Similarly in a retrospective analysis, also EL in the different groups did not change in the course of training (data not shown), and group differences persisted over training sessions [group: $F(2,33)=45.4$, $P<0.001$]. In addition to EF and EL, group differences also persisted throughout the training sessions with regard to MF, indicating that overall the rapid ejaculators mounted significantly less to achieve ejaculation [group: $F(2,33)=12.9$, $P<0.001$] (data not shown). In contrast to EL and EF, the number of mounts prior to ejaculation did vary over training sessions [session: $F(5,165)=2.9$, $P<0.05$]. IF varied over the course of training as well [session: $F(5,165)=3.0$, $P<0.05$], but there were no significant differences between groups. ML, a putative index of sexual motivation, decreased over training sessions [session: $F(5,140)=13.0$, $P<0.001$], but this decrease was not different between groups (data not shown). The time-course of the parameters PEI and IR was not evaluated over training sessions, because not all rats achieved an ejaculation, particularly during the first training sessions and irrespective of group.
A detailed overview of the other copulatory parameters during the sixth training session in the different groups is presented in table 1. To summarize, when groups were matched on EF, significant group differences were also found on EL, MF and IR. Moreover, rapid ejaculators had a shorter IL compared to sluggish ejaculators. No group differences were detected on the parameters ML and PEI.

8-OH-DPAT

The challenge with a selected and relative high dose of 8-OH-DPAT during the 7th sexual behaviour test induced in all animals symptoms of the '5-HT syndrome', such as lower lip retractions and flat body postures, behaviours that are particularly observed upon activation of 5-HT_{1A} receptors (Berendsen *et al.*, 1989; Green, 1984).
Overall analyses indicated significant groups differences with regard to EF [$F(2,35)=16.7$, $P<0.001$], EL [$F(2,35)=138.8$, $P<0.001$], MF [$F(2,35)=23.5$, $P<0.001$], IF [$F(2,35)=3.8$, $P<0.05$] and IR [$F(2,35)=4.0$, $P<0.05$], but not ML and PEI. 8-OH-DPAT had strong facilitating effects on sexual behaviour and increased EF [$F(1,35)=148.2$, $P<0.001$] and

IR [F(1,35)=24.4, P<0.001], and decreased EL [F(1,35)=341.6, P<0.001], ML [F(1,35)=6.5, P<0.05], IF [F(1,35)=4.6, P<0.05], and MF [F(1,35)=90.4, P<0.001]. No significant overall effects were detected of 8-OH-DPAT on PEI. Moreover, overall significant dose x group interaction effects were detected on EF [F(2,35)=8.6, P<0.005]; EL [F(2,35)=132.1, P<0.001]; and MF [F(2,35)=23.0, P<0.001] indicating group differences in the vehicle-treated animals since 8-OH-DPAT eliminated all differences between groups. Figure 3 shows the effects of 8-OH-DPAT on EF, EL and MF.

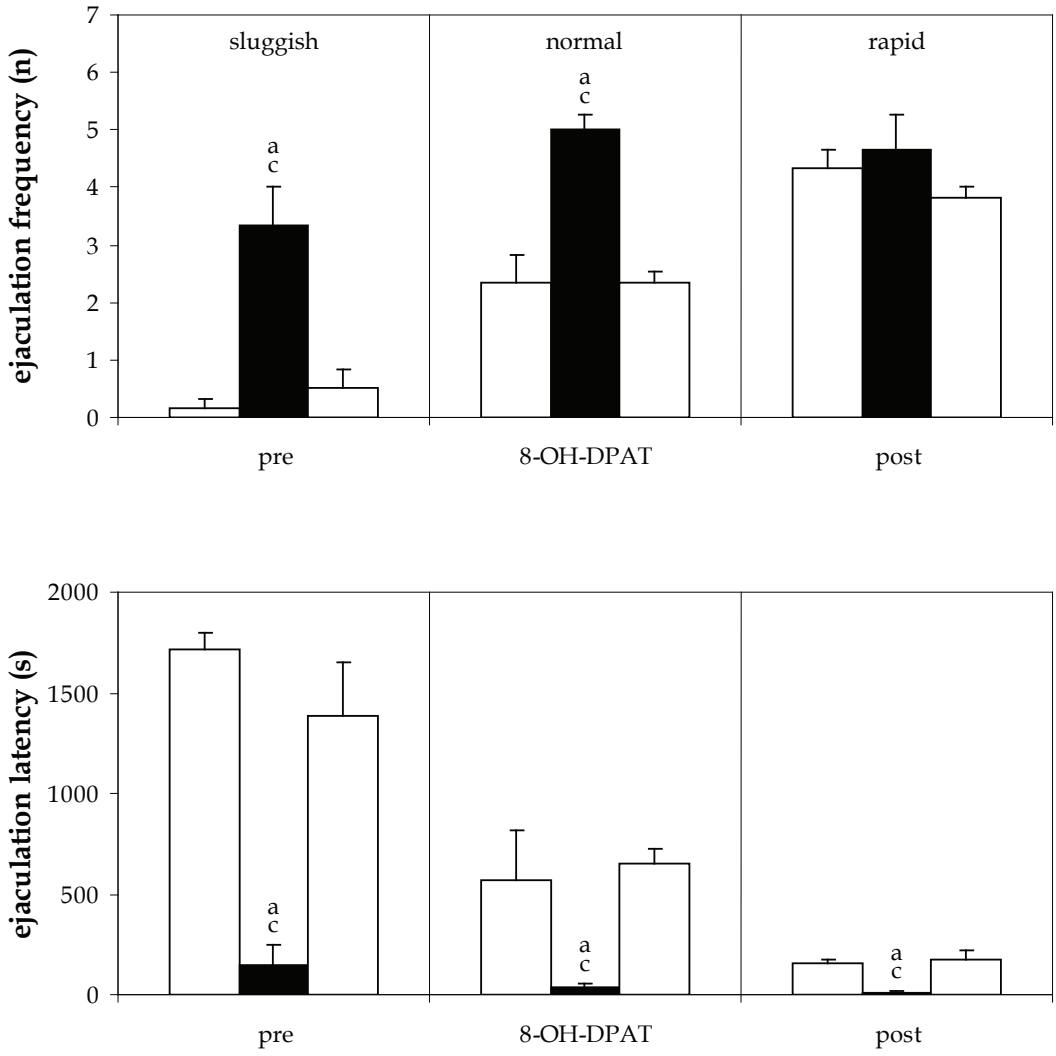

Figure 4. Relapse into 'original' ejaculatory behaviour after facilitatory effects of 8-OH-DPAT (0.8 mg/kg, s.c.). Ejaculation frequency and ejaculation latency during baseline training (pre) and 1 week after (post) test with 8-OH-DPAT (DPAT) in sluggish, normal and rapid ejaculators (n=6 per group). Data are means ± standard error of the mean; a=different from pre and post; P<0.05.

Fos- and oxytocin-immunoreactivity

Table 2 summarizes the Fos-IR and oxytocin-IR results and post hoc comparisons in the various brain regions analysed. In short, in most brain regions activated after ejaculation (see for review Veening and Coolen, 1998) group differences were found between sluggish and rapid ejaculators as indicated in table 2. There were no differences in total numbers of oxytocin-IR neurons in the paraventricular nucleus between groups, neither in the parvocellular nor the magnocellular subdivisions. Also in the supraoptic nucleus of the hypothalamus no differences were found in the numbers of oxytocin-IR neurons. In addition, the percentage of double labeling of oxytocin neurons in the supraoptic nucleus of the hypothalamus did differ between groups with rapid ejaculators displaying the highest proportion of activated oxytocin neurons. Double-labeling in the magnocellular subdivision of the paraventricular nucleus of the hypothalamus did just not reach significance between groups (P=0.06), whereas in the parvocellular subdivision double-labeling was not different between groups.

Experiment 2

Similar to the results obtained in experiment 1, ejaculatory behaviour was potently facilitated by 8-OH-DPAT and in animals symptoms of the '5-HT syndrome' were observed. Overall differences were found between groups on EF and EL [$F(2,15)=34.6$, $P<0.001$ and $F(2,15)=54.8$, $P<0.001$; respectively]. In addition, compared to training sessions before and after 8-OH-DPAT administration, 8-OH-DPAT significantly increased EF [$F(2,30)=25.6$, $P<0.001$] and decreased EL [$F(2,30)=29.6$, $P<0.001$] (figure 4). Moreover, overall significant dose x group interaction effects were detected on EF [$F(4,30)=4.2$, $P<0.05$] and EL [$F(4,30)=7.4$, $P<0.005$]. Ejaculatory behaviour one week after 8-OH-DPAT administration returns to pre 8-OH-DPAT levels in sluggish, normal and rapid ejaculators.

Experiment 3

Out of a cohort of 100 male rats, sluggish, normal and rapid ejaculators were selected (n=9 per group) based on the number of ejaculations achieved within 30 min as described in experiment 1.

Open field

The total distance travelled during the 30 min open field test did not differ between

sluggish (7474 ± 830 cm), normal (8323 ± 1122 cm) and rapid (6923 ± 551 cm) ejaculators, illustrating absence of overall differences in locomotor activity between the various groups [F(2,26)=0.66, NS] (data not shown).

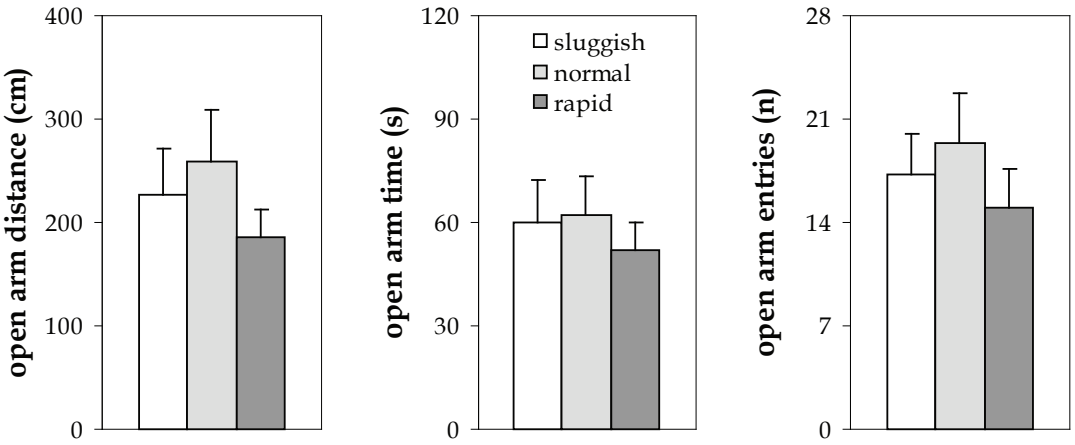

Figure 5. Exploratory behaviour, as measured in distance travelled, time spent and number of entries in the open arm during a 5-min elevated plus-maze test in sluggish, normal and rapid ejaculators (n=9 per group). Data are mean ± standard error of the mean.

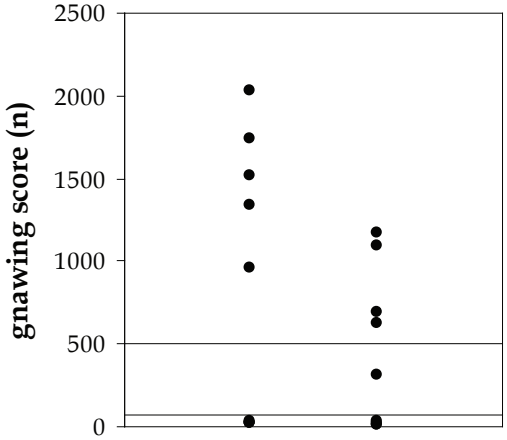

Figure 6. Scatterplot of the effects of apomorphine (1.5 mg/kg, s.c.) on gnawing scores in sluggish and rapid ejaculators (n=9 per group). The horizontal dotted lines indicate the criteria for apomorphine-susceptible (no. of gnaws > 500) and -unsusceptible (no. of gnaws<10) rats.

Elevated plus-maze

The total distance travelled in the open arms and closed arms did not differ between sluggish, normal and rapid ejaculators [$F(2,26)=0.97$, NS and $F(2,26)=1.52$, NS; respectively]. Moreover, there were no significant differences in the percentage time spent in the open and closed arms between groups [$F(2,26)=0.32$, NS and $F(2,26)=0.34$, NS; respectively]. Finally, the number of entries into the open arms did not differ between sluggish, normal and rapid ejaculators [$F(2,26)=0.35$, NS]. Figure 5 depicts the distance travelled, time spent and entries into the open arms during 5 min exposure to the elevated plus-maze.

Apomorphine susceptibility

The apomorphine-induced stereotypic effects on mean gnawing scores were not different between sluggish (855±280 gnaws) and rapid (442±158 gnaws) ejaculators [$F(1,17)=1.65$; NS]. Figure 6 depicts the individual gnawing scores within both groups and indicates that the number of animals susceptible (criterion: >500 gnaws) and not susceptible (criterion: <10 gnaws) to the effects of apomorphine was not different between sluggish and rapid ejaculators.

Discussion

The findings obtained in experiment 1 demonstrate that matching rats in homogenous groups of sluggish, normal and rapid ejaculators on the basis of their ejaculation frequencies revealed differences in a variety of other parameters of copulatory behaviour parameters. Moreover, the difference in ejaculation frequency between the various groups persisted over time, suggesting that in general sluggish ejaculators did not achieve ejaculation from session 1 onwards, whereas rapid ejaculators consistently achieved at least 2 ejaculations throughout the experiment.

In addition to ejaculation frequency, significant differences were found between sluggish, normal and rapid ejaculators in their mean latency to achieve ejaculation. Compared to normal ejaculators, ejaculation latency was shortest in rapid and longest in sluggish ejaculators. Interestingly, also the number of mounts the animals displayed prior to ejaculation varied between groups. Sluggish ejaculators, although the majority did not achieve ejaculation, displayed the highest number of mounts, whereas rapid ejaculators displayed the lowest number of mounts prior to ejaculation. This may suggest differences in penile sensitivity between groups. In humans it also has been shown

that penile sensitivity is altered in men suffering from premature ejaculation compared to controls (Rowland, 1998). Mount latencies, often regarded as a putative index of sexual motivation (see for review (Agmo, 1999)) did not differ between sluggish, normal and rapid ejaculators suggesting no differences in the appetitive components of sexual behaviour. Nonetheless, the observation that intromission ratios in sluggish ejaculators were far below the other groups may also indicate decreased erectile function (Hull *et al.*, 2002; Sachs, 1978) that accounts for the observed inhibited ejaculatory performance and is supported by recent clinical findings indicating a decreased erectile response in men suffering from retarded or an-ejaculation (Rowland *et al.*, 2004).

A relatively high dose of 8-OH-DPAT (0.8 mg/kg, s.c.) potently facilitated ejaculatory behaviour in sluggish, normal and rapid ejaculators, abolishing all pre-existing differences in ejaculatory behaviour by dramatically increasing the number of ejaculations and decreasing ejaculation latencies and mount and intromission frequencies. These findings are in agreement with previous studies showing that 5-HT_{1A} receptor agonists such as 8-OH-DPAT (Ahlenius and Larsson, 1991a; Ahlenius *et al.*, 1981; Coolen *et al.*, 1997a) and flesinoxan (Haensel and Slob, 1997) potently facilitate ejaculations in male rats. Remarkably, these findings strongly indicate that the ejaculatory behaviour of sluggish ejaculators is not due to physiological or physical abnormalities in their reproductive system, since a challenge with 8-OH-DPAT caused all these animals to ejaculate repeatedly within 30 min.

Importantly, however, when we retested sexual behaviour of sluggish, normal and rapid ejaculators in experiment 2 one week following sexual behaviour with a high dose of 8-OH -DPAT, all groups relapsed into their 'original' ejaculatory behaviour. Thus, although ejaculatory behaviour in terms of number of ejaculations improved in all groups, the experience of multiple ejaculations within 30 min and short ejaculation latencies did not change ejaculatory behaviour under drug-free conditions. These findings strongly suggest that the differences in ejaculatory behaviour in the various groups have a neurobiological rather than a psychological origin and do not support the idea that lifelong premature ejaculation results from the experience of early ejaculations during initial hurried sexual contacts (Masters and Johnson, 1970).

In order to study whether the variability in sexual behaviour is reflected by differential activation of the ejaculation-related neural circuitry in sluggish and rapid ejaculators, we studied the induction of Fos, the protein product of the immediate-early gene c-*fos*, as a marker of neuronal activation (for review see, (Morgan and Curran, 1995)) after sexual behaviour. Numerous studies have shown that Fos-immunoreactivity (Fos-IR) is increased in specific brain regions following ejaculation, particularly in areas such

as the posteromedial subdivision of the bed nucleus of the stria terminalis, the medial preoptic area, the posterodorsal subdivision of the medial amygdala and in parvocellular subdivisions of the subparafascicular nucleus of the thalamus (Coolen *et al.*, 1997a; Pfaus and Heeb, 1997; Veening and Coolen, 1998). Activation of this ejaculation-related neuronal circuitry has been suggested to correlate well with visceral sensory stimulation that is relayed via the pelvic nerve (Coolen *et al.*, 1997a).

Possibly, the present findings of differences in ejaculatory behaviour between groups are accompanied by differential activation of components of the ejaculation-related neural circuitry. The neuroanatomical results obtained in experiment 1 indicated differences in Fos-immunoreactivity between sluggish, normal and rapid ejaculators in the aforementioned brain areas well known to be activated after sexual behaviour and ejaculation. Fos-IR was significantly enhanced in all these areas in rapid ejaculators compared to sluggish, but not normal ejaculators. Nevertheless, it should be mentioned that the observed differences in Fos-IR correlate well with the differences in number of ejaculations between groups and therefore are not completely unexpected since earlier findings show that the number of ejaculations influences the amount of Fos-induction in these ejaculation-related areas (Coolen *et al.*, 1996). In future studies, matching animals in the various groups based on their number of ejaculations should resolve these alternative interpretations.

In addition to Fos-IR, we also studied numbers and activation of oxytocin-containing neurons in the hypothalamus, since oxytocin has been shown to play an important role in sexual and ejaculatory behaviour (Argiolas, 1999; Carter, 1992). For instance, oxytocin levels have been shown to be elevated after ejaculation in cerebrospinal fluid of rats (Hughes *et al.*, 1987) and during ejaculation in blood plasma of humans (Carmichael *et al.*, 1987). Oxytocinergic projections have been described from the parvocellular paraventricular hypothalamic nucleus to spinal preganglionic neurons controlling erection (Bancila *et al.*, 2002) and seminal emission (Ackerman *et al.*, 1997), whereas magnocellular oxytocin neurons in the paraventricular hypothalamic nucleus and supraoptic nucleus project to the posterior pituitary where oxytocin is released (Neumann *et al.*, 1993). Interestingly, although total numbers of oxytocin-containing neurons did not differ between groups in neither the paraventricular hypothalamic nucleus nor the supraoptic nucleus, in rapid ejaculators more oxytocin-containing neurons were activated in the supraoptic nucleus. Similarly, although the overall group comparison did not reach significance (P=0.059), a comparison between sluggish and rapid ejaculators indicated a higher number of activated oxytocin neurons in the magnocellular paraventricular hypothalamic nucleus in rapid ejaculators, consistent with previous data

showing that ejaculation increases oxytocin-Fos double-labeling in that area (Witt and Insel, 1994). Together these findings suggest that differential activation of magnocellular paraventricular and supraoptic nuclei in the hypothalamus may underlie the observed differences in ejaculatory behaviour, although similar to the Fos-data we cannot rule out the possibility that multiple ejaculations account for the present results.

The results obtained in experiment 3 indicate that underlying differences in ejaculatory behaviour are not reflected in differences in general, i.e. not sexual, arousability such as locomotor activity or approach-avoidance and stress-related behaviour. These results partly contrast previous data indicating that sexually inactive rats display hyperactive locomotor behaviour in an open field (Kohlert and Bloch, 1996). Nevertheless, some important differences exist between the current study and the one by Kohlert and Bloch (1996). First, their strains of rats, Sprague Dawley and Long-Evans rats, were different from the strain of rats used in the present study. Differences in baseline activity have been shown previously between Sprague Dawley and Wistar rats (Shoaib *et al.*, 1995) and may account for the discrepancy between the present results and the results obtained by Kohlert and Bloch (1996). Second, the selection criteria for sexual inactive behaviour in their study were based on intromission latency and not ejaculation frequency, as in the current study and moreover, these authors reported that only a proportion and not all sexually inactive rats were indeed hyperactive, raising some doubts on the generalization of their findings.

Clinically, still little is known about the comorbidity of ejaculation disorders with other psychiatric disorders, such as anxiety disorders. Although it has been suggested that premature ejaculation is associated with elevated levels of anxiety (Cooper *et al.*, 1993) and social phobias (Figueira *et al.*, 2001), the majority of men suffering from lifelong premature ejaculation appear to function normally in daily life (Waldinger, 2002). To study whether in our populations anxiety-related behaviours were affected we studied approach-avoidance behaviour in the elevated plus-maze. The elevated plus-maze is a widely used animal model to study aspects of anxiety and generally the open arms of the maze induce conflict and avoidance that can be reversed by the classical anxiolytics, although caution should be taken regarding the interpretation of the results (Dawson and Tricklebank, 1995; Rodgers and Dalvi, 1997). We did not find any differences in approach-avoidance behaviour between sluggish, normal and rapid ejaculators with regard to the number of entries, time spent and distance travelled in the open arms parameters that putatively measure conflict and avoidance behaviour, suggesting no differences in anxiety-levels.

Finally, apomorphine-induced stereotypic gnawing (Cools *et al.*, 1990) was similar in

sluggish and rapid ejaculators. In both groups equal amounts of animals were susceptible or resistant to the effects of apomorphine on gnawing scores compared to the criteria (susceptible: >500 gnaws and unsusceptible: <10 gnaws in 45 min) set by Cools and colleagues in their extensive research over the years (see for review, Ellenbroek and Cools, 2002). The present results may suggest that the differences in ejaculatory behaviour are not due to large changes in the brain dopamine receptor complex, although generally dopamine has been shown to facilitate copulation (see for reviews, Bitran and Hull, 1987; Melis and Argiolas, 1995).

Taken together, the results obtained in experiment 3 suggest that the differences in sexual behaviour that we repeatedly observe in a large population of male Wistar rats appear to be restricted to ejaculatory behaviour and are not reflected in differences in other behaviours. This is an important finding, and suggests that possibly neurobiological differences in sluggish, normal and rapid ejaculators may underlie the observed differences in their copulatory behaviour.

To conclude, the present behavioural data suggest stable phenotypic differences in ejaculatory behaviour between sluggish and rapid ejaculators, which appear restricted to ejaculation and not other behaviours. Therefore, studying these phenotypes may be a promising approach to gain more insight into the neural correlates underlying ejaculation dysfunctions. The underlying mechanisms, however, causing these individual differences in ejaculatory behaviour remain to be elucidated and may provide important insights into the aetiology of ejaculation dysfunctions such as premature and retarded ejaculation, and an-ejaculation.

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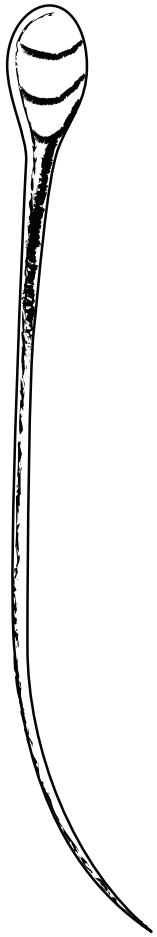
Chapter 8



General Discussion

TR de Jong

Aim of the thesis

The present thesis focused on the psychopharmacology and neuroanatomy of the serotonergic control over ejaculation, in order to gain more knowledge to implement in the treatment of human ejaculatory disorders.

An animal model of ejaculatory dysfunctions?

Similar to humans (Waldinger *et al.*, 2005a), male Wistar rats can be matched in homogenous groups of individuals that persistently display sluggish, normal or rapid ejaculations.
These sluggish, normal and rapid ejaculators putatively model lifelong retarded or premature ejaculation, which can be a substantial step forward in the research on the background and possible treatments of ejaculatory disorders (**chapter 7**).
A second important insight gained from the experiments described in **chapter 7** is that the experience of an abnormally rapid ejaculation, induced by the 5-HT_{1A} receptor agonist 8-OH-DPAT, does not alter future ejaculation latencies. Moreover, rapid or sluggish ejaculators do not display altered levels of anxiety compared to normal ejaculators or each other. These findings strongly suggest that lifelong retarded and premature ejaculation have a neurobiological rather than a psychological cause, indicating that treatment with psychopharmacological drugs might be preferable above psychotherapeutic treatment.
The endophenotypes of sluggish and rapid ejaculating rats provide a useful tool in fundamental research on ejaculatory disorders. However, additional experiments are needed to establish whether sluggish and rapid rats are two extremes of a biological continuum that originates from a single source of neurobiological variation, or rather form two heterogeneous groups that display abnormal ejaculatory behaviour for various reasons.
Therefore, the sensitivity of sluggish and rapid ejaculators to selective receptor agonists or antagonists that affect ejaculatory behaviour might be compared. Apparently, the three endophenotypes do not differ in sensitivity to a high dose of the 5-HT_{1A} receptor agonist 8-OH-DPAT or the D_1/D_2 receptor agonist apomorphine, but their response to lower doses of these drugs as well as (ant)agonists at 5-HT_{1B} and 5-HT_{2C} receptors, peripheral and central acting oxytocin receptors, galanin receptors, noradrenalin receptors and many other receptors should be studied.
Subsequently, neuroanatomical and molecular parameters such as receptor densities and mRNA expression in both endophenotypes can be studied. With the use of real-time Polymerase Chain Reaction, preliminary data indicate that rapid ejaculators express higher levels of galanin mRNA in the paraventricular hypothalamic nucleus (Oosting *et al.*, 2004). This is an interesting starting point for further research, since both galanin and the paraventricular hypothalamic nucleus are implicated in ejaculation.

If clear differences between rapid and sluggish ejaculating rats are found in any of these experiments, this might shed new light on human ejaculatory disorders as well.

Psychopharmacology

Acute and chronic SSRI treatment

Numerous studies (**chapter 2**) have shown that the increase of extracellular serotonin levels throughout the central nervous system, induced by 5-HTP or serotonin releasers, leads to delayed ejaculation (Ahlenius and Larsson, 1984; 1985; 1991b; 1998; Ahlenius *et al.*, 1980; Fernandez-Guasti and Rodriguez-Manzo, 1992; Foreman *et al.*, 1992). The inhibition of ejaculation is putatively mediated by the increased activation of 5-HT_{1B} or 5-HT_{2C} receptors (Ahlenius and Larsson, 1998; Fernandez-Guasti and Rodriguez-Manzo, 1992; Foreman *et al.*, 1992; Hillegaart and Ahlenius, 1998), although it is not yet known in which areas of the central nervous system this is established. The effects of local injections of non-selective 5-HT_{1B} or 5-HT_{2C} agonists in the spinal cord or hypothalamic areas on sexual behaviour have been studied previously (Fernandez-Guasti *et al.*, 1992), but replication and extension of these experiments with currently available selective receptor (ant)agonists is required.

Based on the findings described above, it may be expected that acute treatment with SSRIs inhibits ejaculation as well. However, acute treatment with the SSRIs citalopram (10 mg/kg p.o.) and fluvoxamine (30 mg/kg p.o.) in doses that approximately double or triple serotonin levels in several brain areas (Ago *et al.*, 2005; Bosker *et al.*, 1995a; Ceglia *et al.*, 2004; Denys *et al.*, 2004; Hjorth, 1996; Hjorth and Auerbach, 1999; Romero *et al.*, 1996b; Wegener *et al.*, 2003), did not cause delayed ejaculation (**chapter 3 and 4**) in accordance with previous reports (Ahlenius and Larsson, 1999; Mos *et al.*, 1999; Waldinger *et al.*, 2002). Acute treatment with the SSRI paroxetine (10 or 20 mg/kg p.o.) had more variable effects on ejaculation, despite elevating extracellular serotonin levels similarly to fluvoxamine and citalopram (Felton *et al.*, 2003; Hajos-Korcsok *et al.*, 2000; Nakayama, 2002; Romero *et al.*, 1996a). Paroxetine did not delay ejaculation in one experiment (**chapter 6**), but it affected ejaculation in a minority of rats in another experiment (**chapter 4**). Previously, both normal and delayed ejaculation have been reported in response to acute injection of the same doses of paroxetine (Mos *et al.*, 1999; Waldinger *et al.*, 2002). Apparently, acute paroxetine treatment is under certain circumstances able to delay ejaculation in some individuals, but the prerequisites for this effect are not yet known.

The differences between acute systemic injection with an SSRI versus the serotonin precursor 5-HTP or the serotonin releaser (dex)fenfluramine in their effect on ejaculation latencies might be explained by the degree to which these drugs increase extracellular serotonin levels. Indeed, 5-HTP (50 mg/kg i.p.) combined with an inhibitor of peripheral 5-HTP decarboxylation, a drug treatment that was shown to inhibit ejaculation (Ahlenius *et al.*, 1980), caused a 60-fold increase in 5-HT levels in the striatum (Gudelsky and Nash, 1996). On the other hand, a high dose of dexfenfluramine caused a 4-fold increase in serotonin levels in the hypothalamus (Schwartz *et al.*, 1989), which is similar to the effects of paroxetine and citalopram (Felton *et al.*, 2003). However, the effects of these serotonergic drugs on serotonin levels and ejaculation latencies have not yet been compared directly, which would be necessary to draw reliable conclusions.
In **chapter 3 and 4** it was demonstrated that chronic treatment with paroxetine (10 and 20 mg/kg p.o.), but not citalopram (10 mg/kg p.o.) or fluvoxamine (30 mg/kg p.o.) caused delayed ejaculation, which is consistent with previous findings in rats and humans (Waldinger *et al.*, 1998b; Waldinger *et al.*, 2002; Waldinger *et al.*, 2001). Interestingly, chronic paroxetine treatment delayed ejaculation without increasing the intromission frequency in rats. This is equivalent to the findings in sluggish and rapid ejaculators that show large differences in ejaculation latency but not in intromission frequency (**chapter 7**). Apparently, sluggish ejaculators as well as rats chronically treated with paroxetine display an increased 'inter-intromission interval', or latency time between two intromissions, whereas rapid ejaculators have an abnormally low inter-intromission interval. Since the Intravaginal Ejaculation Latency Time is, most likely, highly correlated to the number of intromissions in humans, further research is needed to determine how the inter-intromission interval in rats can be extrapolated to human sexual behaviour, and how serotonergic neurotransmission influences this parameter.

Role of 5-HT_{1A} receptors
The 5-HT_{1A} receptor agonist 8-OH-DPAT (0.4 or 0.8 mg/kg s.c.) potently facilitated ejaculatory behaviour in sluggish, normal and rapid ejaculating rats by increasing the ejaculation frequency and decreasing the ejaculation latency and the mount and intromission frequencies (**chapter 5 and 7**). 8-OH-DPAT probably lowers the ejaculatory threshold through the activation of presynaptic 5-HT_{1A} autoreceptors, which causes inhibition of serotonin release in projection areas. However, it has been shown that postsynaptic 5-HT_{1A} receptors partly mediate the effects of 8-OH-DPAT as well (Fernandez-Guasti and Escalante, 1991; Fernandez-Guasti *et al.*, 1992; Hillegaart *et al.*,

1991; Lee *et al.*, 1990; Mathes *et al.*, 1990; Svensson and Hansen, 1984). Many areas in the central nervous system that are known to be involved in ejaculation, such as the lumbosacral spinal cord, medial preoptic area, paraventricular hypothalamic nucleus, nucleus accumbens, and arcuate hypothalamic nucleus, contain postsynaptic 5-HT_{1A} receptors (Aznar *et al.*, 2003; Collin *et al.*, 2002; Kia *et al.*, 1996; Li *et al.*, 1997a; Pompeiano *et al.*, 1992; Thor *et al.*, 1993; Wright *et al.*, 1995; Zhang *et al.*, 2004) that might facilitate ejaculation upon activation.

Data in the present thesis indicate that activation of 5-HT_{1A} receptors compensates for the inhibiting effects of elevated serotonin levels on ejaculation in response to acute systemic administration of SSRIs. When acute administration of paroxetine or citalopram was followed by injection of the selective 5-HT_{1A} receptor antagonist WAY-100635 (0.1 mg/kg s.c.), which did not affect ejaculation by itself, around eighty percent of the animals failed to ejaculate (**chapter 3 and 6**).

Systemic administration of 5-HT_{1A} receptor antagonists approximately doubles the increased extracellular serotonin levels induced by acute systemic administration of citalopram (Arborelius *et al.*, 1996; Ceglia *et al.*, 2004; Cremers *et al.*, 2000a; Hjorth, 1996; Invernizzi *et al.*, 1997; Romero *et al.*, 1996b) or paroxetine (Castro *et al.*, 2003; Romero and Artigas, 1997; Romero *et al.*, 1996a). This is putatively mediated by blocking negative feedback loops through somatodendritic 5-HT_{1A} autoreceptors and might underlie the synergistic effects of SSRIs and WAY-100635 on ejaculation. However, blockade of postsynaptic 5-HT_{1A} receptors involved in ejaculation could play a role as well.

Chronic treatment with paroxetine has been found to cause desensitization of pre- and postsynaptic 5-HT_{1A} receptors (Le Poul *et al.*, 1995; Li *et al.*, 1997b), chronic citalopram treatment impaired 5-HT_{1A} receptor functioning in some studies (Ceglia *et al.*, 2004; Cremers *et al.*, 2000b) but not in others (Gundlah *et al.*, 1997; Hjorth and Auerbach, 1994; Invernizzi *et al.*, 1995; Uvnas-Moberg *et al.*, 1999), and chronic fluvoxamine failed to desensitize 5-HT_{1A} autoreceptors (Bosker *et al.*, 1995b). Since SSRI-treatment combined with blockade of 5-HT_{1A} receptors strongly inhibits ejaculation, the degree of SSRI-induced 5-HT_{1A} receptor desensitization might determine the occurrence of delayed ejaculation.

Indeed, the strong facilitation of ejaculation induced by 8-OH-DPAT was significantly attenuated in rats chronically pretreated with paroxetine, which suggests that 5-HT_{1A} receptors involved in ejaculation are desensitized. In contrast, chronic pretreatment with fluvoxamine barely diminished the effects of 8-OH-DPAT on ejaculation (**chapter 4**), indicating a fundamental difference between paroxetine and fluvoxamine in their ability to desensitize 5-HT_{1A} receptors involved in ejaculation. Paroxetine could either

induce delayed ejaculation through desensitization of 5-HT_{1A} autoreceptors, leading to increased availability of serotonin to inhibiting 5-HT_{1B} or 5-HT_{2C} receptors, or through desensitization of postsynaptic 5-HT_{1A} involved in ejaculation, or both. Likewise, a low level of pre- and/or postsynaptic 5-HT_{1A} receptor desensitization could explain the lack of effect of chronic fluvoxamine treatment on ejaculation latency.
The observed differences between paroxetine and fluvoxamine in their ability to change both ejaculation latency and 8-OH-DPAT-induced facilitation of ejaculation are an important finding for the treatment of patients suffering from premature ejaculation. Moreover, they are an indication that individual SSRIs differ fundamentally in their effect on serotonergic neurotransmission, which is involved in many behavioural processes and therefore important for any patient treated with these antidepressants. In future experiments, direct comparisons between SSRIs of their effects on sexual and other behaviour, as well as receptor desensitization, should be made in order to determine the extent of possible differences.

Neuroanatomy: Fos

Sexual behaviour
The immediate early gene *c-fos,* a marker for neuronal activity, was substantially expressed in the prefrontal cortex, core and shell of the nucleus accumbens, ventral lateral septum, medial preoptic area, dorsolateral bed nucleus of the stria terminalis, medial parvocellular paraventricular hypothalamic nucleus, lateral hypothalamic nucleus, medial and central amygdala, arcuate hypothalamic nucleus, ventromedial hypothalamic nucleus, ventral premammillary nucleus, ventrolateral periaqueductal grey, lateral parabrachial nucleus and medial nucleus of the solitary tract, one hour after the sexual behaviour tests, irrespective of the occurrence of an ejaculation (**chapter 3 and 5**). Since control rats that had not performed sexual behaviour prior to perfusion were not included in the experiments in the present thesis, it is possible that the observed Fos-immunoreactivity was induced by experiences before or after the sexual behaviour test. However, consistent with findings from other groups, the Fos-immunoreactive neurons in these areas were probably triggered by pheromonal and genital sensory stimulation as well as the physical exercise, stress, arousal, social interaction or reward during the sexual behaviour test (Balfour *et al.*, 2004; Baum and Everitt, 1992; Coolen *et al.*, 1997b; Iwamoto *et al.*, 1996; Kovacs, 1998; Salchner *et al.*, 2004; Yokosuka *et al.*, 1999).

Ejaculation caused extensive Fos-immunoreactivity in the posteromedial bed nucleus of the stria terminalis, posterodorsal medial amygdaloid nucleus, medial parvocellular subparafascicular thalamic nucleus and sacral parasympathetic nucleus of the spinal cord (**chapter 3, 5 and 7**), consistent with many previous findings (Baum and Everitt, 1992; Coolen *et al.*, 1996; Coolen *et al.*, 1997b; Greco *et al.*, 1996; Greco *et al.*, 1998). In addition, the occurrence of an ejaculation coincided with increased Fos-immunoreactivity in the dorsal parvocellular paraventricular hypothalamic nucleus, in a pattern that strongly correlated with the pattern of Fos-immunoreactive neurons in the other ejaculation-related areas (**chapter 3**). These results suggest that the dorsal parvocellular paraventricular hypothalamic nucleus, which contains oxytocinergic neurons that project to areas in the spinal cord that innervate the genitals (Hallbeck *et al.*, 2001; Veronneau-Longueville *et al.*, 1999), might be part of the neural circuitry activated by ejaculation.

Serotonergic Drugs

Chronic treatment with paroxetine increased Fos-immunoreactivity in the dorsolateral bed nucleus of the stria terminalis, central amygdala and medial nucleus of the solitary tract, similar to other studies using drugs that elevate serotonin levels (**chapter 1**), but the increase was not statistically significant. Chronic treatment with citalopram did not cause any changes in Fos-immunoreactivity in these areas. This concurs with reports that (sub)chronic treatment with serotonergic agents, including SSRIs, attenuate or change the Fos-immunoreactivity compared to acute treatment (Li and Rowland, 1996; Lino-de-Oliveira *et al.*, 2001; Veening *et al.*, 1998).

The different paradigms of the neuroanatomical experiments in this thesis (**chapter 3 and 5**) does not allow a reliable comparison between the effects of chronic paroxetine and citalopram treatments. Nevertheless, chronic treatment with paroxetine but not citalopram strongly reduced the number of Fos-positive cells in the locus coeruleus. This effect of paroxetine has been reported before (Szabo *et al.*, 1999) and might reflect desensitization of 5-HT_{1A} autoreceptors (Haddjeri *et al.*, 1997). Consistently, chronic paroxetine treatment reduced the 8-OH-DPAT-induced increase in Fos-positive numbers in the locus coeruleus.

In contrast, chronic treatment with citalopram, but not paroxetine, reduced Fos-immunoreactivity in the arcuate hypothalamic nucleus. This effect of citalopram was augmented by WAY-100635, suggesting that elevation of serotonin levels suppressed Fos-immunoreactivity in the arcuate hypothalamic nucleus. 8-OH-DPAT increased Fos expression in this area, possibly induced by 5-HT_{1A} autoreceptor-mediated decreased

serotonin levels, which was attenuated by chronic paroxetine pretreatment.
Apparently, chronic treatment with paroxetine but not citalopram impaired the functioning of 5-HT_{1A} autoreceptors mediating Fos-immunoreactivity in the locus coeruleus and the arcuate hypothalamic nucleus.
8-OH-DPAT induced Fos-immunoreactivity in the medial parvocellular and posterior magnocellular paraventricular hypothalamic nucleus, central amygdala and medial nucleus of the solitary tract. These areas have been found to express *c-fos* in response to elevated serotonin levels, as outlined in the introduction of this thesis, suggesting that postsynaptic 5-HT_{1A} receptors in these areas mediate the effects of serotonin on Fos-immunoreactivity. In addition, 8-OH-DPAT decreased the number of Fos-positive neurons in the core of the nucleus accumbens and lateral ventral septum, in contrast to the increased Fos expression in these areas in response to 5-HTP (Moorman *et al.*, 1995) or fluoxetine (Salchner and Singewald, 2002; Torres *et al.*, 1998), indicating that the inhibition of serotonin release through the activation of 5-HT_{1A} autoreceptors might mediate the effects of 8-OH-DPAT in these areas (**chapter 5**).
Chronic treatment with WAY-100635 activated the apical interpeduncular nucleus, which is apparently tonically inhibited by 5-HT_{1A} receptors (**chapter 3**). Both acute challenge with the 5-HT_{1A} receptor agonist 8-OH-DPAT and chronic treatment with the 5-HT_{1A} receptor antagonist WAY-100635 approximately doubled the number of Fos-immunoreactive cells in the medial parvocellular part of the paraventricular hypothalamic area. This brain area is rich in neurons containing corticotropin releasing factor (CRF) that express Fos in response to various stressors (Kovacs, 1998), and this might explain the seemingly contrasting effects of 8-OH-DPAT and WAY-100635 that are both likely to induce stress in rats.
The experiment described in **chapter 5** revealed that many brain areas show a reduced Fos-response to 8-OH-DPAT following chronic paroxetine treatment, indicating that this immunohistochemical tool can be used to detect areas implicated in the antidepressant action of SSRIs. Some of these areas are also possibly involved in the link between serotonin and ejaculation.

Interaction of serotonergic drugs with sexual behaviour
In th epresen thesis, control rats that had received the various drug treatments without performing sexual behaviour prior to perfusion were not included in the experiments. Therefore, it cannot be concluded if the observed changes in Fos-immunoreactivity are induced by the drug treatment alone or by an interaction of the drugs with sexual behaviour. However, based on existing literature, some areas showing increased or

decreased expression of *c-fos* in response to the serotonergic drug treatments are likely to be involved in sexual behaviour.

In the paraventricular hypothalamic nucleus and the locus coeruleus, the 8-OH-DPAT-induced increase in Fos-immunoreactivity was strongly inhibited by chronic pretreatment with paroxetine (**chapter 5**). Since Fos-immunoreactivity in these areas is thought to represent release of respectively oxytocin in the blood plasma (Vicentic *et al.*, 1998; Zhang *et al.*, 2004) and noradrenalin at postsynaptic sites (Hajos-Korcsok and Sharp, 1999), the results suggest that chronic paroxetine-treatment prevents oxytocin and noradrenalin to be released upon 5-HT_{1A} receptor activation. This might affect the ejaculation latency, since increased plasma levels of oxytocin lower the ejaculatory threshold (Stoneham *et al.*, 1985), and stimulation of noradrenergic neurotransmission facilitates copulation and ejaculation (Bitran and Hull, 1987; Carro-Juareza and Rodriguez-Manzo, 2003; Yaici *et al.*, 2002a). In addition, oxytocin reverses fluoxetine-induced inhibition of ejaculation (Cantor *et al.*, 1999) and lesions of the noradrenergic system antagonize the facilitation of ejaculation by the non-selective 5-HT_{1A} receptor agonist 5-Methoxy-N,N-dimethyltryptamine (Fernandez-Guasti *et al.*, 1986), which indicates that release of oxytocin and noradrenalin might enable ejaculation when serotonin levels are elevated.

As discussed before, increased serotonin levels appear to coincide with a reduced number of Fos-positive neurons in the arcuate hypothalamic nucleus and vice versa (**chapter 3 and 5**). Changes in activity of the arcuate hypothalamic nucleus might influence sexual behaviour via its connections with the medial preoptic area, medial amygdala, bed nucleus of the stria terminalis and paraventricular hypothalamic nucleus (Gottsch *et al.*, 2004; Magoul *et al.*, 1994).

A challenge with 8-OH-DPAT induced a significant decrease of Fos-positive neurons in the core of the nucleus accumbens, whereas acute systemic injection of the 5-HT_{1A} receptor antagonist WAY-100635 did the opposite (Jongsma *et al.*, 2002), indicating that 5-HT_{1A} receptor activation inhibits *c-fos* expression in the core of the nucleus accumbens. Altered neurotransmission in this area might affect sexual behaviour. However, Fos-immunoreactivity is increased in the core of the nucleus accumbens in response to copulation, which is probably related to motivational an rewarding aspects of sexual behaviour (Balfour *et al.*, 2004). Therefore, the reduced Fos-expression could reflect the reduced copulatory activity and/or reward experienced by rats injected with 8-OH-DPAT. A similar, non-significant effect was visible in the medial preoptic area (**chapter 5**).

In future experiments, the effects of chronic paroxetine treatment on the expression of

c-fos induced by 8-OH-DPAT should be compared with the effects of chronic treatment with fluvoxamine or citalopram. This might help to locate brain and spinal cord areas, involved in ejaculation or other types of behaviour, where 5-HT_{1A} receptors respond differentially to the individual SSRIs. More knowledge on these subjects would increase the understanding of the neurobiological background of SSRI-induced delayed ejaculation, thereby advancing the development of drugs that specifically alter ejaculation latencies. In addition, it may provide a better insight into the differences between individual SSRIs in their effects on neurotransmission and (sexual) behaviour, which is of major importance to patients treated with these drugs.

Role of oxytocin

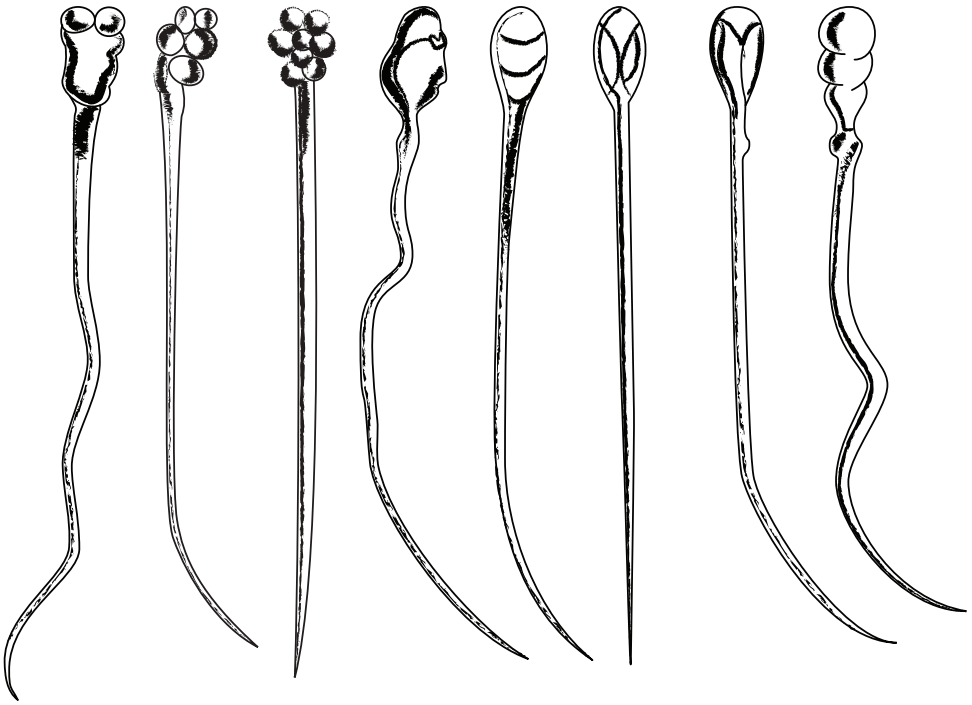
Peripheral release of oxytocin has been implicated in copulation and ejaculation, since magnocellular oxytocinergic cells in the paraventricular hypothalamic nucleus are activated in response to copulatory activity and penile stimulation in rats (Hillegaart *et al.*, 1998; Yanagimoto *et al.*, 1996), systemic injection of oxytocin lowers the ejaculatory threshold in rats (Stoneham *et al.*, 1985) and plasma oxytocin levels are elevated at the time of ejaculation in rabbits and humans (Carmichael *et al.*, 1987; Murphy *et al.*, 1987; Stoneham *et al.*, 1985). These effects are probably mediated by peripheral oxytocin receptors that cause contractions of smooth muscle cells in the testis, epididymis and vas deferens (Filippi *et al.*, 2002; Nicholson *et al.*, 1999; Whittington *et al.*, 2001).
Oxytocinergic neurons in the magnocellular paraventricular hypothalamic nucleus and supraoptic nucleus, which release oxytocin in the blood plasma via the posterior pituitary, showed increased Fos-immunoreactivity in rapid, but not normal or sluggish ejaculators (**chapter 7**). Although Fos may have been expressed in response to the higher number of ejaculations, it is tempting to speculate that in rapid ejaculators ejaculation is accelerated by the release of oxytocin during copulation.
Activation of 5-HT_{1A} receptors located on oxytocinergic neurons in the magnocellular paraventricular hypothalamic nucleus (Zhang *et al.*, 2004), which causes the release of oxytocin in blood plasma (Vicentic *et al.*, 1998), might be a pathway through which ejaculation is enabled when extracellular serotonin levels are elevated by acute treatment with paroxetine. This hypothesis is supported by the finding that the 8-OH-DPAT-induced increase in Fos-immunoreactivity in this brain area was attenuated by chronic paroxetine-pretreatment, which coincided with delayed ejaculation (**chapter 5**), and the finding that systemic injection with oxytocin reversed the inhibition of

ejaculation caused by chronic fluoxetine-treatment (Cantor *et al.*, 1999).
However, co-administration of paroxetine with the peripheral oxytocin receptor antagonist L-368899, as opposed to the 5-HT_{1A} receptor antagonist WAY-100635, did not significantly inhibit ejaculation (**chapter 6**). Apparently, elevated plasma oxytocin levels are not crucial to enable ejaculation following acute SSRI treatment. Further research using acute SSRI treatment or 8-OH-DPAT combined with drugs that block the neurotransmission of, for example, noradrenalin or central oxytocin should be conducted in order to find pathways through which 5-HT_{1A} receptor activation facilitates ejaculation.

Concluding remarks

In the present thesis an attempt was made to review and extent the knowledge on the effects of serotonergic neurotransmission on ejaculation. Elevated serotonin levels are known to increase the ejaculatory threshold, and this effect is generally attributed to the increased activation of inhibiting 5-HT_{1B} and 5-HT_{2C} receptors. In SSRI-induced delayed ejaculation, a possible additional role of impaired 5-HT_{1A} receptor activation, putatively through receptor desensitization, is demonstrated in the present thesis. The degree to which SSRIs impair 5-HT_{1A} receptor functioning might determine the extent to which they delay ejaculation.
The Fos-immunohistochemical data strengthened the theory that the oxytocinergic magnocellular paraventricular hypothalamic nucleus is a likely brain area where postsynaptic 5-HT_{1A} receptors influence ejaculation; however, an additional psychopharmacological experiment did not support this hypothesis.
Future research is needed to determine the brain and spinal cord areas and the postsynaptic neurotransmitters through which the different 5-HT receptor subtypes affect the ejaculatory threshold, using both psychopharmacological and neuroanatomical techniques. In addition, the role of these pathways in ejaculatory disorders can be further investigated using endophenotypic sluggish and rapid ejaculating rats.

Chapter 9



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Frequently used Abbreviations
Nederlandse Samenvatting
Dankwoord
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Frequently used Abbreviations

Sexual behaviour:

EF: ejaculation frequency
EL: ejaculation latency
IF: intromission frequency
IL: intromission latency
IR: intromission ratio
MF: mount frequency
ML: mount latency
PEI: post ejaculatory interval

Psychopharmacology:

5,7-DHT:	5,7-dihydroxytryptamine
5-HT:	5-hydroxytryptamine
5-HTP:	5-hydroxytryptophan
5-MeODMT:	5-Methoxy-N,N-dimethyltryptamine
8-OH-DPAT:	[±]-8-hydroxy-2-(di-n-propylamino)tetralin
DOI:	[±]-2,5-dimethoxy-4-iodoamphetamine
p-CA:	para-chloroamphetamine
p-CPA:	para-chlorophenylalanine
SSRI	selective serotonin reuptake inhibitor
TFMPP:	N-[3-(trifluoromethyl)phenyl] piperazine
WAY 100635:	*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide 3HCL

Neuroanatomy:

Acb:	nucleus accumbens
AcbC:	core
AcbSh:	shell
ARH:	arcuate hypothalamic nucleus
BNST:	bed nucleus of the stria terminalis
rBNSTpm:	rostral posterior medial part
cBNSTpm:	caudal posterior medial part
BNSTdl:	dorsolateral part
CeA:	central amygdala
CeAl:	lateral part

CPu:	caudate putamen
Cx:	cortex
DMH:	dorsomedial hypothalamic nucleus
IML:	intermediolateral cell column
IPA:	interpeduncular nucleus, apical part
LC:	locus coeruleus
LHA:	lateral hypothalamic area
lPB:	lateral parabrachial nucleus
lPBc:	central part
lPBe:	external part
LSt cells:	lumbar spinothalamic cells
LSV:	ventral lateral septum
MeA:	medial amygdala
MeAad:	anterodorsal part
MeApd:	posterodorsal part
MPN:	medial preoptic nucleus
MPOA:	medial preoptic area
MSPFp:	subparafascicular thalamic nucleus, medial parvocellular part
NIc:	nucleus incertus, compact part
nPGi:	nucleus paragigantocellularis
NST:	nucleus of the solitary tract
NSTm:	medial part
PAG:	periaqueductal grey
PAGvl:	ventrolateral part
PFC:	prefrontal cortex
PMV:	ventral premammillary nucleus
PrL:	prelimbic area
PV:	paraventricular thalamic nucleus
PVH:	paraventricular hypothalamic nucleus
PVHdp:	dorsal parvocellular part
PVHmp:	medial parvocellular part
PVHpm:	posterior magnocellular part
SON:	supraoptic nucleus
SPN:	sacral parasympathetic nucleus
VMH:	ventromedial hypothalamic nucleus
VMHdm:	dorsomedial part
VTA:	ventral tegmental area

Nederlandse Samenvatting

Ejaculatiestoornissen, zoals voortijdige of juist vertraagde zaadlozing, zijn aandoeningen die vaak voorkomen en een grote impact kunnen hebben op het leven van patiënten en hun partners. Lange tijd werd gedacht dat ejaculatiestoornissen veroorzaakt worden door psychische problemen, ontstaan door bijvoorbeeld een negatieve ervaring bij de eerste seksuele contacten. Inmiddels zijn er duidelijke aanwijzingen dat ejaculatiestoornissen een neurobiologische achtergrond hebben, zoals het feit dat psychofarmacologische medicijnen die aangrijpen op het serotoninesysteem in het centrale zenuwstelsel de latentietijd tot ejaculatie kunnen veranderen. Dit schept perspectieven voor de ontwikkeling van medicijnen die ejaculatieproblemen aanpakken. Voordat het zover is moet echter meer bekend worden over de neurobiologische achtergrond van ejaculatie, in het bijzonder de rol die serotonine daarin speelt. Dat is dan ook het doel van dit proefschrift.

Seksueel gedrag van ratten

Vanwege de ethische en praktische beperkingen van patiëntenonderzoek werden de experimenten gedaan met Wistar ratten. Dit type rat wordt meestal gebruikt voor psychofarmacologisch en neuroanatomisch onderzoek en vertoont zeer stabiel en gemakkelijk te observeren seksueel gedrag. Bij een standaard seksuele gedragstest wordt een mannetjesrat gedurende een half uur bij een receptief vrouwtje in een observatiekooi gezet en scoort een observator het aantal ejaculaties, de ejaculatielatentietijd, het aantal maal dat het mannetje het vrouwtje beklimt en hoe vaak daarbij een vaginale penetratie plaatsvindt. Tenslotte wordt de lengte van de rustpauze tussen een ejaculatie en de hervatting van de copulatie gemeten. De experimenten in dit proefschrift werden altijd uitgevoerd met seksueel ervaren ratten, die vooraf minstens vier trainingssessies met een receptief vrouwtje hadden ondergaan en daarin stabiel ejaculatiegedrag vertoonden.

Serotonine en ejaculatie

Het is al lange tijd bekend dat de neurotransmitter serotonine (5-hydroxytryptamine of 5-HT) invloed uitoefent op het seksuele gedrag van ratten. Serotonine wordt gemaakt

in speciale clusters van zenuwcellen in de hersenstam. Deze neuronen projecteren hun axonen naar vrijwel alle gebieden in de hersenen en het ruggenmerg, waar ze serotonine uitscheiden in de ruimte tussen de cellen, de synapsspleet. Serotonine bindt zich vervolgens aan serotoninereceptoren in de celmembranen van nabijgelegen neuronen, die hierdoor worden geremd of geactiveerd.
Er bestaan minstens veertien verschillende subtypen serotoninereceptoren die, afhankelijk van de locatie en functie van de cel waar ze zich op bevinden, uiteenlopende effecten op onderandere bloeddruk, eet- en drinkgedrag, voortbeweging, reacties op stress, stemming en pijnwaarneming kunnen hebben. Sommige serotoninereceptoren bevindt zich op de zenuwcellen die zelf serotonine maken. Deze presynaptische receptoren, of autoreceptoren, maken deel uit van een negatief terugkoppelingssysteem: wanneer de hoeveelheid serotonine in de synapsspleet hoger wordt zorgt de versterkte activatie van deze receptoren voor een remming van de uitscheiding van serotonine.
In **hoofdstuk 2** zijn alle experimenten die in de literatuur zijn beschreven over de effecten van serotonine op de ejaculatielatentietijd op een rij gezet. Meerdere studies hebben aangetoond dat een verhoogd serotoninegehalte in de ruimte tussen neuronen de zaadlozing vertraagt, terwijl een verlaagd serotoninegehalte de zaadlozing versnelt. De vertragende werking van serotonine wordt hoogstwaarschijnlijk veroorzaakt door de versterkte activatie van twee subtypen serotoninereceptoren, 5-HT_{1B} en 5-HT_{2C} receptoren. Farmaca die specifiek deze receptoren activeren (agonisten) vertragen namelijk de ejaculatie, terwijl farmaca die 5-HT_{1B} en 5-HT_{2C} receptoren blokkeren (antagonisten) kunnen voorkomen dat serotonine de zaadlozing vertraagd.

SSRIs en ejaculatie

Mannen die lijden aan premature ejaculatie worden momenteel vaak behandeld met selectieve serotonine heropname remmers (SSRIs). Deze medicijnen worden voornamelijk voorgeschreven tegen depressies, maar kunnen ook zeer effectief zijn in het vertragen van de zaadlozing.
Alle SSRIs, waaronder fluoxetine (Prozac), paroxetine, citalopram en fluvoxamine, blokkeren serotoninetransporters. Deze eiwitten zorgen er normaal gesproken voor dat serotonine, nadat het is uitgescheiden en serotoninereceptoren heeft geactiveerd, weer wordt opgenomen door de serotoninecel en dus verwijderd uit de synapsspleet. Het blokkeren van de transporters tijdens behandeling met een SSRI resulteert in verhoogd serotoninegehalte in de synapsspleet en extra activatie van serotoninerecepto-

ren, wat in geval van depressie waarschijnlijk een positief effect op de stemming heeft. In eerste instantie is het effect klein, omdat de negatieve terugkoppelingssystemen voluit werken en daardoor de uitscheiding van serotonine beperkt wordt. Na verloop van tijd worden de autoreceptoren echter ongevoelig voor serotonine, een proces dat desensitisatie wordt genoemd, waardoor de negatieve terugkoppeling afzwakt en het serotoninegehalte continu verhoogd blijft. Dit is waarschijnlijk één van de redenen waarom SSRIs de zaadlozing vertragen. Met name desensitisatie van 5-HT_{1A} receptoren, het meest bekende type autoreceptor, zou belangrijk kunnen zijn.

5-HT_{1A} receptoren

In tegenstelling tot 5-HT_{1B} en 5-HT_{2C} agonisten, die de zaadlozing vertragen, zorgen selectieve 5-HT_{1A} receptor agonisten juist voor een sterk versnelde zaadlozing. 5-HT_{1A} receptor agonisten activeren 5-HT_{1A} autoreceptoren, maar ook postsynaptische 5-HT_{1A} receptoren die gelokaliseerd zijn op uiteenlopende typen neuronen en daar allerlei effecten kunnen hebben. Beide varianten spelen waarschijnlijk een rol in de facilitatie van ejaculatie.
SSRIs verhogen het serotoninegehalte overal in het centrale zenuwstelsel en versterken daardoor de activatie van zowel ejaculatie-remmende 5-HT_{1B} en 5-HT_{2C} receptoren als ejaculatie-versnellende 5-HT_{1A} receptoren. In de experimenten beschreven in **hoofdstuk 3 en 6** van dit proefschrift werd getest of activatie van 5-HT_{1A} receptoren ervoor zorgt dat ratten niet vertraagd ejaculeren na eenmalige toediening van een SSRI. Daartoe werd het seksueel gedrag van ratten bekeken na een eenmalige injectie met de SSRIs citalopram of paroxetine, wat inderdaad geen vertraagde zaadlozing opleverde. Wanneer echter simultaan de activatie van 5-HT_{1A} receptoren werd geblokkeerd door de selectieve antagonist WAY-100635 werd de ejaculatie sterk geremd. WAY-100635 had zelf geen enkel effect op de ejaculatielatentietijd, waaruit blijkt dat 5-HT_{1A} receptoren niet cruciaal zijn voor de ejaculatie onder normale omstandigheden, maar wel tijdens behandeling met een SSRI.
Vervolgens werd er met behulp van de experimenten beschreven in **hoofdstuk 4** bekeken of desensitisatie van 5-HT_{1A} receptoren een rol zou kunnen spelen bij het ontstaan van vertraagde ejaculatie tijdens chronische behandeling met SSRIs. Daartoe werden de effecten bestudeerd van een selectieve 5-HT_{1A} receptor agonist, 8-OH-DPAT, op de ejaculatielatentietijd van ratten die chronisch waren voorbehandeld met de SSRIs paroxetine of fluvoxamine. Normaal gesproken veroorzaakt 8-OH-DPAT een sterk

versnelde ejaculatie, maar chronische voorbehandeling met paroxetine voorkwam dit effect. Hoewel hiermee niet is aangetoond dat 5-HT_{1A} receptoren daadwerkelijk gedesensitiseerd zijn, is het wel duidelijk dat chronische toediening van paroxetine de werking van 5-HT_{1A} receptoren die betrokken zijn bij ejaculatie vermindert. Helaas maakt 8-OH-PDAT geen onderscheid tussen pre- en postsynaptische 5-HT_{1A} receptoren, zodat het nog onduidelijk is welke variant de belangrijkste rol speelt in deze resultaten.
Een interessante vinding in de experimenten in **hoofdstuk 3 en 4** is het verschil tussen de SSRIs in hun effect op de ejaculatielatentietijd. Chronische behandeling met paroxetine vertraagde de ejaculatie significant, terwijl chronische behandeling met fluvoxamine of citalopram zeer weinig effect op de ejaculatielatentietijd had. Dit verschil is ook aangetoond in mannen die voor premature ejaculatie werden behandeld met deze SSRIs. Daarnaast was het effect van 8-OH-PDAT op de ejaculatielatentietijd nauwelijks verminderd door voorbehandeling met fluvoxamine, waaruit blijkt dat fluvoxamine weinig invloed heeft op de werking van 5-HT_{1A} receptoren die betrokken zijn bij ejaculatie. De mate waarin paroxetine, fluvoxamine en wellicht citalopram desensitisatie van pre- en/of postsynaptische 5-HT_{1A} receptoren veroorzaken, zou dus bepalend kunnen zijn voor de mate van vertraging van de zaadlozing.

Neuroanatomie: Fos

Er zijn veel delen van het centrale zenuwstelsel betrokken bij de uitvoer van seksueel gedrag en ejaculatie. Daarnaast wordt serotonine op talloze plaatsen in het brein en ruggenmerg uitgescheiden. Het is daarom moeilijk te bepalen via welke gebieden of paden serotonine de zaadlozing beïnvloedt, maar toch werd in dit proefschrift een poging gewaagd.
Het eiwit Fos wordt in veel zenuwcellen tot expressie gebracht als reactie op verscheidene activerende of, in sommige gevallen, inhiberende stimuli. Wanneer het brein en het ruggenmerg van een rat een uur na het uitvoeren van seksueel gedrag, al dan niet onder invloed van serotonerge farmaca, worden gefixeerd, kan met een immunohistochemische kleuring worden aangetoond welke neuronen als gevolg van deze handelingen Fos hebben aangemaakt en dus blijkbaar zijn 'gebruikt'.
Uit de experimenten beschreven in **hoofdstuk 3, 5 en 7** blijkt dat seksueel gedrag de Fos-immunoractiviteit verhoogt in een groot aantal gebieden, waaronder de mediale preoptische kern (MPOA). Van dit gebied is bekend dat het een cruciale rol speelt bij

de copulatie. De MPOA verwerkt waarschijnlijk sensorische informatie uit het olfactorische systeem (feromonen van receptieve vrouwtjes) en de genitaliën (met name penetraties) en stimuleert daarop het copulatiegedrag. De andere geactiveerde gebieden zijn waarschijnlijk betrokken geweest bij het verwerken van motiverende en belonende stimuli, lichamelijke activiteit en stress die door de ratten werd ervaren tijdens de seksuele gedragstest. Wanneer een rat één of meerdere ejaculaties had, was bovendien een verhoogde Fos-immunoreactiviteit te zien in kleine clusters van cellen in de posteromediale bed nucleus van de stria terminalis (BNSTpm), posterodorsale mediale amygdala (MeApd) en de mediale parvocellulaire subparafasciculaire thalamische kern (mSPFp). Dit resultaat was reeds bekend uit voorgaande onderzoeken en is waarschijnlijk representatief voor het signaal dat een ejaculatie heeft plaatsgevonden. Een vierde gebied, het dorsale parvocellulaire deel van de paraventriculaire hypothalamische kern (PVHdp), werd eveneens Fos-immunoreactief na een ejaculatie. Dit gebied bevat oxytocine-aanmakende neuronen die contact maken met de motorneuronen in het ruggenmerg die de genitaliën aansturen. De PVHdp stimuleert de erectie, maar speelt misschien dus ook een rol bij de (verwerking van een) ejaculatie.
Toediening van SSRIs en/of 5-HT_{1A} receptor (ant)agonisten voor de seksuele gedragstest veranderde het patroon van Fos-positieve celkernen. Dit kan betekenen dat de serotonerge farmaca het activatieniveau van deze cellen hebben veranderd zonder dat het invloed heeft gehad op de ejaculatielatentietijd, of dat het aantal Fos-kernen is veranderd door wijzigingen in het seksuele gedrag. In enkele gevallen zou het zo kunnen zijn dat de serotonerge farmaca het activatieniveau in een hersengebied heeft veranderd waardoor vervolgens het seksuele gedrag is aangepast, en dat zou een schakel tussen serotonine en ejaculatielatentietijd kunnen betekenen.
Eén zo'n gebied is de locus coeruleus. Deze groep cellen, die de neurotransmitter noradrenaline aanmaakt, vertoonde verminderde Fos-immunoreactiviteit na chronische behandeling met paroxetine, maar niet met citalopram. Daarnaast veroorzaakte de 5-HT_{1A} receptor agonist 8-OH-PDAT een sterke toename in Fos-positieve cellen, wat werd afgezwakt door chronische voorbehandeling met paroxetine. Daarmee is het Fos-patroon omgekeerd evenredig met de ejaculatielatentietijd. Noradrenaline heeft uiteenlopende functies in het centrale zenuwstelsel, maar er zijn aanwijzingen dat het de ejaculatie kan versnellen.
Hoewel chronische behandeling met paroxetine geen effect had op het aantal Fos-positieve celkernen in de posteriore magnocellulaire paraventriculaire hypothalamische kern (PVHpm), verminderde het de verhoogde Fos-immunoreactiviteit na toediening van 8-OH-DPAT. Het Fos-patroon in de PVHpm is daarmee omgekeerd evenredig met

het aantal vaginale penetraties dat voorafging aan een ejaculatie. De PVHpm bestaat uit cellen die de neuropeptide oxytocine uitscheiden in de bloedbaan, onderandere na activatie van 5-HT_{1A} receptoren. Omdat injectie van oxytocine in het bloed de ejaculatielatentietijd verkort, het aantal penetraties voor een ejaculatie vermindert en de remming op de zaadlozing na chronische behandeling met de SSRI fluoxetine herstelt, zou het kunnen dat activatie van 5-HT_{1A} receptoren op oxytocinecellen de ejaculatie versnelt. Om te testen of dit een rol speelt na éénmalige toediening van paroxetine, werd deze SSRI in een vervolgexperiment gecombineerd met de perifeer-werkende oxytocinereceptor antagonist L-368899 (**hoofdstuk 6**). In tegenstelling tot WAY-100635 had L-368899 gecombineerd met paroxetine geen effect op de ejaculatielatentietijd. Dit resultaat suggereert weliswaar dat de perifere uitscheiding van oxytocine niet van cruciaal belang is voor ejaculatie tijdens SSRI-behandeling, maar het neemt niet weg dat de uitscheiding van oxytocine in het centrale zenuwstelsel, bijvoorbeeld via het hersenvocht, een rol zou kunnen spelen.

Het patroon van Fos-positieve neuronen in de hypothalamische arcuatus kern (ARH) na toediening van SSRIs en 5-HT_{1A} receptor (ant)agonisten suggereert dat activatie van de ARH wordt geremd door serotonine. De ARH is vooral betrokken bij de regulatie van voedselopname en energiebalans, maar lijkt door contacten met de MPOA, MeA, BNST en PVH ook een schakel tussen metabolisme en copulatiegedrag te vormen. Via deze connecties zou een verandering in activatie van de ARH, bijvoorbeeld door een verhoogd serotoninegehalte, de ejaculatielatentietijd kunnen beïnvloeden.

Een vierde gebied waar serotonine seksueel gedrag kan veranderen is de kern van de nucleus accumbens. In het experiment in **hoofdstuk 5** veroorzaakte 8-OH-DPAT een vermindering van Fos-immunoreactiviteit in dit gebied. Uit voorgaand onderzoek is gebleken dat lokale injectie van 8-OH-DPAT in de nucleus accumbens de ejaculatielatentietijd verkort, hoewel de verantwoordelijke receptoren en neuroanatomische connecties nog niet bekend zijn. Aan de andere kant is het mogelijk dat de nucleus accumbens, die betrokken is bij de verwerking van belonende prikkels, een verlaagde Fos-immunoreactiviteit na injectie met 8-OH-DPAT heeft omdat het aantal vaginale penetraties is verminderd.

Kortom: het aankleuren van Fos is weliswaar een geschikte methode om de zoektocht naar gebieden waar serotonine de ejaculatielatentietijd beïnvloedt mee te beginnen, maar andere methoden zijn nodig om de resultaten te verifiëren en te duiden. Daarnaast zijn er ook gebieden waarvan met andere technieken is vastgesteld dat ze een schakel vormen tussen serotonine en ejaculatie, maar waar het Fos-patroon niet veranderde onder invloed van de serotonerge farmaca en seksueel gedrag. Een belangrijk

voorbeeld hiervan is de nucleus paragigantocellularis (nPGi) in de hersenstam, die serotonine uitscheidt in het ruggenmerg in de nabijheid van motorneuronen en interneuronen die de motorische uitvoer van een ejaculatie aansturen en coördineren. De nPGi zorgt er waarschijnlijk voor dat seksueel gedrag onder normale omstandigheden wordt geïnhibeerd door de continue uitscheiding van serotonine. Wanneer er voldoende seksuele prikkelingen het centrale zenuwstelsel binnenkomen wordt de nPGi zelf geremd, met name door de MPOA. Een ander voorbeeld is het laterale hypothalamische gebied (LHA). In dit gebied stijgt het serotoninegehalte direct na een ejaculatie, wat waarschijnlijk de onderdrukking van copulatie veroorzaakt tijdens de enkele minuten durende post-ejaculatoire rustpauze. Het zou kunnen dat serotonine eveneens een rol speelt bij de effecten van ejaculatie op het activatieniveau van de BNSTpm, MeApd en mSPFp.

Diermodellen van ejaculatiestoornissen

Hoewel het grootste deel van de Wistar ratten in ons laboratorium ongeveer twee of drie ejaculaties heeft in een half uur durende standaardtest, wijkt een klein gedeelte daar opvallend sterk van af (**hoofdstuk 7**). Sommige ratten hebben altijd minstens drie ejaculaties (de 'snelle' ejaculeerders), anderen slechts nul of één (de 'trage' ejaculeerders). Deze ratten blijken eveneens af te wijken wat betreft hun ejaculatielatentietijd en aantal beklimmingen van het vrouwtje voor een ejaculatie. Dit gedrag bleek niet te zijn veranderd na de éénmalige ervaring van een supersnelle ejaculatie, geïnduceerd door 8-OH-DPAT. Bovendien waren abnormale ejaculeerders niet angstiger of minder angstig ten opzichte van normale ejaculeerders.
Deze data suggereren dat het abnormale ejaculatiegedrag van deze Wistar ratten wordt veroorzaakt door neurobiologische variatie en niet door seksuele ervaringen of angstigheid. De trage en snelle ejaculeerders zijn daarom interessant als potentiële diermodellen voor ejaculatiestoornissen en kunnen als zodanig worden gebruikt bij de zoektocht naar psychofarmacologische medicijnen. Daarvoor moet eerst worden bekeken wat precies het neurobiologische verschil is tussen de trage en snelle ejaculeerders. Uit het experiment in **hoofdstuk 7** bleek al dat de dieren verschilden in de hoeveelheid Fos-positieve kernen in magnocellulaire oxytocine cellen na seksueel gedrag, wat variatie in de uitscheiding van oxytocine tijdens de copulatie suggereert. De groepen reageerden daarentegen niet verschillend op apomorphine, een dopamine D_1/D_2 receptor agonist waarvan bekend is dat het de ejaculatie versnelt.

Toekomstig onderzoek

Uit de experimenten in dit proefschrift komt een aantal aanbevelingen voor volgend onderzoek naar voren.
Ten eerste blijkt de combinatie van psychofarmacologische en neuroanatomische experimenten meer dan de som der delen. Bij het onderzoek naar de effecten van serotonine, waar de rol van pre- en postsynaptische receptoren moeilijk te bepalen is, zou meer gebruik gemaakt kunnen worden van lokale injecties. Op die manier kunnen de effecten van bijvoorbeeld 5-HT_{1B} en 5-HT_{2C} (ant)agonisten in het ruggenmerg, of 5-HT_{1A} agonisten in de PVH of locus coeruleus, op de ejaculatielatentietijd beter bestudeerd worden.
Ten tweede worden individuele SSRIs in het gedragsonderzoek vaak op één hoop gegooid, terwijl er duidelijke aanwijzingen zijn dat ze van elkaar verschillen. Deze verschillen kunnen van groot belang zijn voor patiënten die met SSRIs worden behandeld tegen depressies, premature ejaculatie of andere aandoeningen. In een toekomstig experiment zouden bijvoorbeeld de effecten van 8-OH-DPAT op de Fos-immunoreactiviteit na voorbehandeling met fluvoxamine en paroxetine kunnen worden vergeleken, om na te gaan of en waar er verschillen in effecten van 5-HT_{1A} receptor activatie bestaan.
Tenslotte zouden de neurobiologische verschillen tussen trage en snelle ejaculeerders in kaart kunnen worden gebracht. Die verschillen zouden ook de basis kunnen vormen voor ejaculatiestoornissen in mensen, en dat zou een grote stap voorwaarts betekenen in de zoektocht naar medicijnen voor deze patiënten.

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Trynke

About the Author

Trynke Reinouw de Jong was born on June 13th in Grijpskerk, Groningen, the Netherlands. In 1996 she passed her final exams at the Jan van Egmondcollege in Purmerend and proceeded to study biology and journalism at the Rijksuniversiteit Groningen.
In order to receive a masters degree in biology, she investigated the effects of chronic corticosterone treatment on learning and memory, anxiety and hippocampal receptor density in rats under supervision of dr. Alexa Veenema (Rijksuniversiteit Groningen) and the effect of oxidative stress on the fat metabolism of the African Catfish under supervision of dr. Richard van Heeswijk (Rijksuniversiteit Leiden). In addition, as an extension of a three-month internship, she worked one year as a freelance science writer for Het Parool. In august 2001 she graduated in biology and journalism at the Rijksuniversiteit Groningen.
Since 2002, Trynke worked as a PhD-student on the research project "Effects of SSRIs on male sexual behavior" at the department of anatomy of the Radboud University Medical Centre in Nijmegen, under supervision of dr. Jan Veening, prof. dr. Berend Olivier, prof. dr. Alexander Cools and dr. Marcel Waldinger. Most of the results obtained in this project were described and discussed the present thesis.

List of Publications

Articles (First Author)

De Jong TR, Pattij T, Veening JG, Dederen PJ, Waldinger MD, Cools AR and Olivier B (2005) Citalopram combined with WAY 100635 inhibits ejaculation and ejaculation-related Fos immunoreactivity. *Eur J Pharmacol* 509: 49-59

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