"Interactions between oxytocin and α-MSH in the regulation of male sexual behaviour in rats."

Céline Caquineau

P.h.D

The University of Edinburgh

2005

Declaration

I declare that this thesis has been composed by myself, that the experiments described were performed by myself in the laboratory of Neuroendocrinology within the school of Medicine and Veterinary Medicine at the University of Edinburgh, and that any technical contributions that I have received have been specified. Furthermore, this work has not been submitted for any other degree or professional qualification.

Céline Caquineau

March 2005

Acknowledgements

First I would like to express my sincere thanks and gratitude to my supervisors Dr Alison Douglas and Professor Gareth Leng.
Thanks to Alison for her support throughout this PhD and especially for her guidance and help with the surgery, and for her valuable advice during the writing of this thesis. Thanks to Gareth for his expert guidance. His enthusiasm for science and good sense of humour made all the scientific (and less -scientific) discussions inspirational for pursuing my research. I also would like to thank him for his precious comments during the writing of this thesis.

I would like to extend my thanks to the Rotary Club (Nantes, France), the Royal Society and the Medical Research Council who provided financial support and funding for the research.

I also would like to thank the staff from the animal house, especially Carole, Duncan, Graham and Alan for their much-appreciated help in the organization of all behavioral experiments.

Big thank you to everybody in the lab! A special thank to Simone, Louise, and Vicky for their end-less friendly support and encouragements; and my deepest thanks to the girls from A214: Tatie Nancy, Paula and Val whose friendships made the PhD worth doing.

Enfin, je voudrais particulièrement remercier mes parents Moum et Poum, mon frêre Julien et mes grand-parents pour leur soutiens et encouragements pendant ces années de thêse. Un grand merci pour tout! (Sans oublier tous les colis débordants de bonnes choses qui ont rendu l'écriture de cette these beaucoup plus agréable!!)

And at last I would like to thank my parents, my brother and my grandparents for their support throughout this PhD. Thank you for everything and especially for all the food emergency -parcels I received, without which the writing would have been far less enjoyable.

Abstract

Oxytocin is synthesised within the brain mainly in the neurones of the supraoptic nucleus and the paraventricular nucleus in the hypothalamus. Oxytocin is involved in the regulation of several physiological functions and behaviours including male sexual behaviour. Alpha-melanocyte-stimulating hormone (α-MSH) is produced in the brain mainly in the neurones of the arcuate nucleus. α-MSH acts centrally in behaviours including male sexual behaviour via MC3 and MC4 receptors. These central actions are remarkably similar to those mediated by central oxytocin. They both induce penile erection and enhance copulatory behaviours when given centrally. Projections between the arcuate nucleus and the supraoptic nucleus (SON) have been described and MC4 receptor mRNA is expressed in the SON and in the PVN. Oxytocin and α-MSH are both secreted from the pituitary gland into the blood, and peripheral injection of oxytocin or α-MSH enhances male sexual behaviour. Taken together these observations suggest that their actions are not independent and that oxytocin and α-MSH could interact to regulate male sexual behaviour.

Firstly, changes in peripheral secretion of oxytocin and α-MSH, and oxytocin and α-MSH neuronal activity were studied during copulation. Intromission increased plasma concentration of both oxytocin and α-MSH and Fos expression in neurones in the SON, in the PVN and in the arcuate nucleus was increased.

The putative interaction between oxytocin and α-MSH was then investigated by probing at which central or peripheral level this interaction would occur.

Oxytocin antagonist iv-injection had no effect on the intromission –induced secretion of oxytocin and α-MSH, suggesting that peripheral oxytocin does not regulate peripheral α-MSH secretion. However, central administration of oxytocin to non-mating conscious rats decreased plasma α-MSH concentration, suggesting that central oxytocin may regulate the peripheral secretion of α-MSH. These studies therefore confirm an interaction between central oxytocin and α-MSH.

Then, the effects of central α-MSH on oxytocin secretion and neuronal activity were investigated. Intracerebroventricular administration of α-MSH induced Fos expression in magnocellular oxytocin neurones in the SON and in the PVN but had little effect on parvocellular oxytocin neurones. α-MSH directly administered onto the SON induced a strong increase in Fos expression in oxytocin neurones in the SON. These studies indicate that α-MSH modulates directly the activity of selected oxytocin neurones and that consequently, α-MSH may regulate some specific oxytocin effects and not others. However, central administration of α-MSH decreased oxytocin secretion from the pituitary gland. This illustrates that Fos induction is independent from the axon terminal secretion and therefore that it does not necessarily reflect *electrical* excitation of the neurones. Finally, MC4R antagonist centrally administered reduced the Fos expression induced at intromission in magnocellular oxytocin neurones but had no effect on the Fos expression in the parvocellular neurones. These studies confirm the direct modulatory effect of α-MSH on magnocellular oxytocin neurones via MC4 receptors.

Thus, oxytocin and α-MSH interact centrally to enhance male sexual behaviour. Some α-MSH behavioral effects in male sexual behaviour are mediated by magnocellular oxytocin neurones rather than parvocellular neurones. The activation of magnocellular oxytocin neurones by α-MSH via MC4R may result in a central release of oxytocin, which will facilitate the processes of sexual behaviour.

Contents

List of Abbreviations

aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
α-MSH	alpha- melanocyte stimulating hormone
ANOVA	analysis of variance
AGRP	agouti related peptide
apPVN	anterior parvocellular region of the paraventricular nucleus
AOB	accessory olfactory bulbs
Bmax	maximum binding
BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumine
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CNS	central nervous system
CLIP	corticotropin-like intermediate lobe peptide
cpm	counts per minute
DAB	diaminobenzidine
dH_2O	single distilled water
DHT	dihydrotestosterone
dpPVN	dorsal parvocellular region of the paraventricular nucleus
GTP	guanosine triphosphate
GC	guanylate cyclase
h	hour(s)
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.v.	intravenous
lpp	lateral posterior parvocellular region of the paraventricular nucleus
MOB	main olfactory bulbs
MCR	melanocortin receptor
MeA	medial amygdala
min	minute(s)
MPOA	medial preoptic area
mRNA	messenger ribonucleic acid
NCE	noncontact penile erection
NTS	nucleus of the solitary tract
NO	Nitric oxide
NOS	NO synthase
OC	optic chiasm
OTA	oxytocin antagonist
OVLT	organum vasculosum of the lamina terminalis
PB	phosphate buffer
PBS	phosphate buffered saline
POMC	proopiomelanocortin

PVN	paraventricular nucleus
s	second(s)
s.c.	subcutaneous
SEM	standard error of the mean
SON	supraoptic nucleus
TC	total counts
3V	third ventricle
VMN	ventromedial nucleus of the hypothalamus
VNO	vomeronasal organ
vpPVN	ventral parvocellular region of the paraventricular nucleus
VTA	ventral tegmental area

List of Figures

List of Tables

Chapter 1

General introduction

PART 1: Central regulation of male sexual behaviour

1.1. Description of Male Sexual Behaviour:

In rats, mating is orchestrated in bouts where sequences of ordered sexual behaviours alternate with periods of no sexual activity. When a male rat is presented to a receptive female, he follows her and sniffs at her anogenital region (Fig. 1.1). He then starts to mount her, and she responds by adopting the lordosis posture (arched-back posture) (Fig 1.2). After several mounts, the male achieves intromission, during which he palpates the female's flanks with his forepaws and stops pelvic thrusting (Fig. 1.3A). The male may then dismount and display genital grooming behaviour (Fig. 1.3B), and may repeat the sequence several times before he reaches ejaculation. Before and after copulation, the male rat emits vocalizations at frequencies of ≈ 20-22 kHz. After ejaculation, the male falls back and displays genital grooming behaviour followed by a period of sexual inactivity. Another bout of sexual activity may begin after a varied length of time.

Sexual activity in the male rat can be divided into two distinct phases: an appetitive phase, and a consummatory phase, a dichotomy first described by Beach and colleagues in 1956 (Beach, 1967). The first phase is a pre-copulatory or non-consummatory period during which the male seeks sexual contact by investigating the female and presents penile erection. This first period, reflecting sexual motivation, prepares for and leads to the second phase. If during the first phase the stimulation received and given by both male and female are inadequate to maintain sexual excitement, copulation may not occur. The second period is the copulatory phase, or consummatory period, during which the male performs a series of mounts (first without intromission and then with intromission) that lead to ejaculation. The period of sexual inactivity following ejaculation comprises two phases: an absolute refractory period

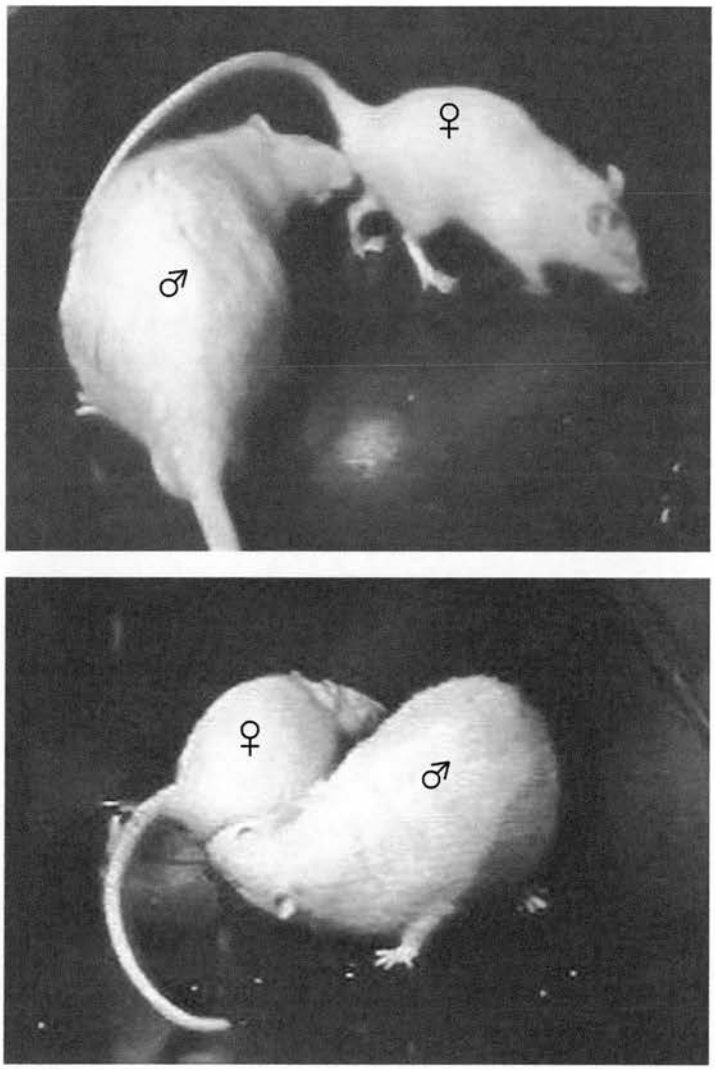

Figure 1.1: Video-extracted pictures illustrating rats sniffing behaviour during the pre-copulatory phase.

Sniffing behaviour is one of the behaviours reflecting rat's sexual motivation. When a male and a receptive female rats are paired, they both investigate each other anogenital regions.

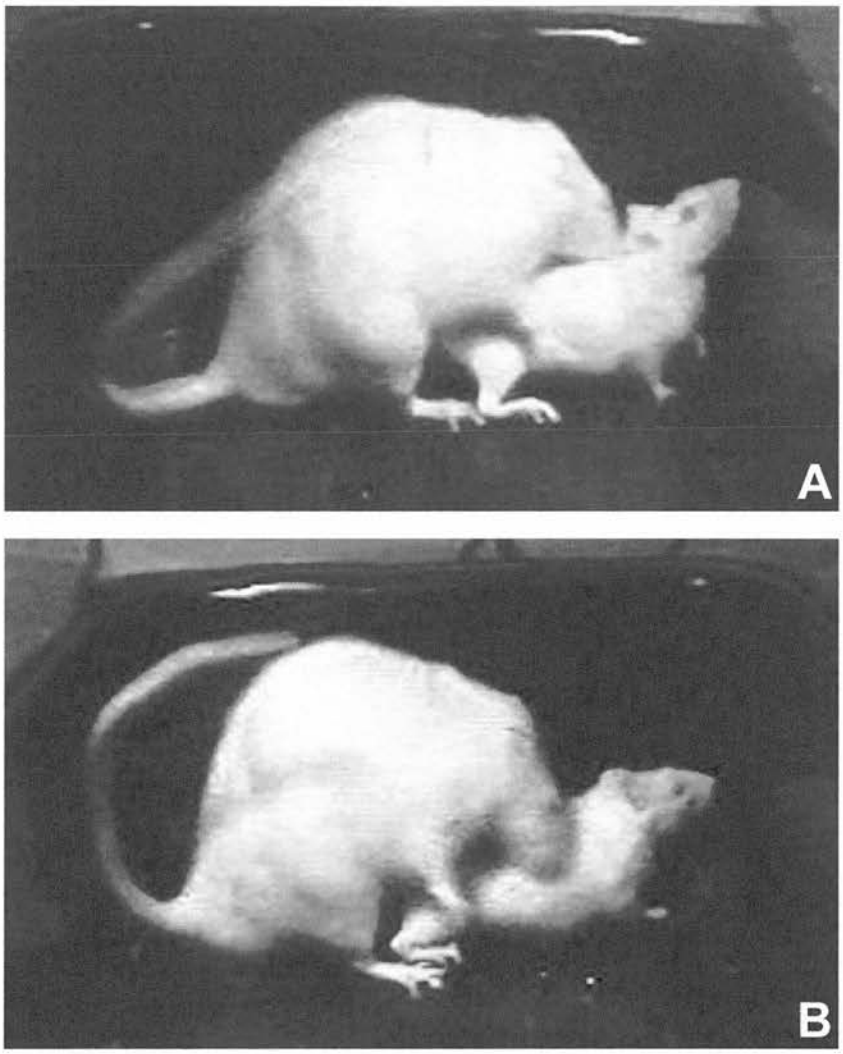

Figure 1.2: Video-extracted pictures illustrating male mounting behaviour (A) and male mounting behaviour with intromission (B) on female in Lordosis posture.

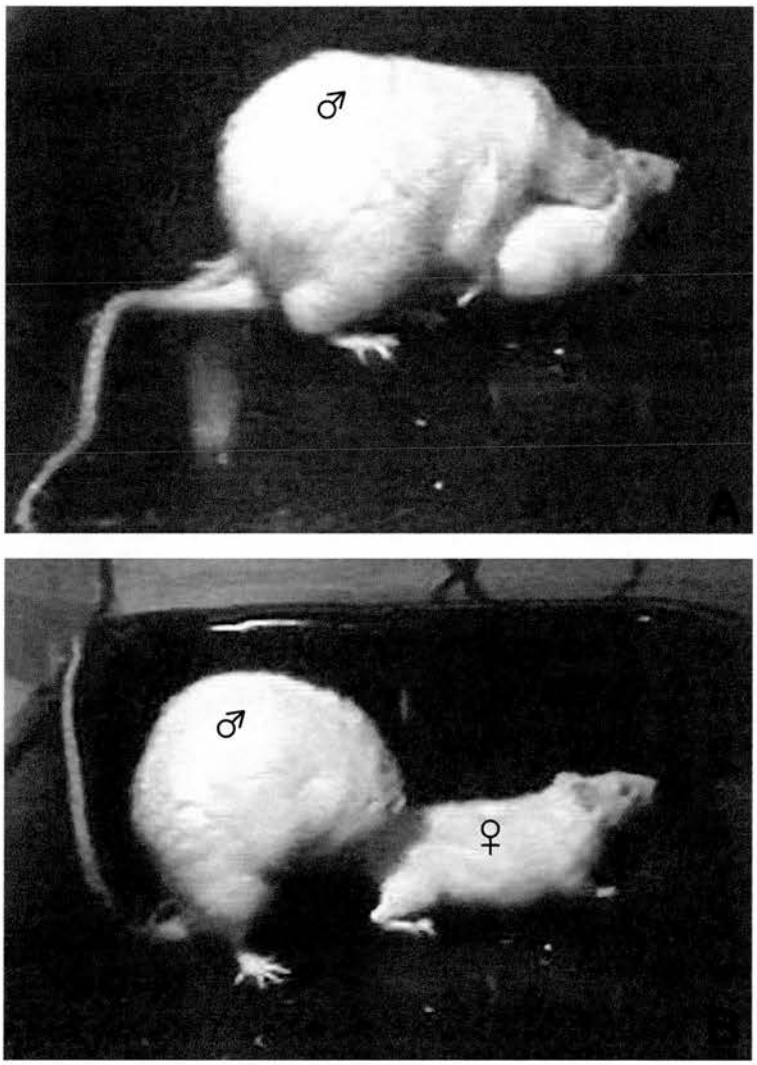

Figure 1.3: Video-extracted pictures illustrating male mounting behaviour with pelvic thrust and putative ejaculation (A) and male grooming behaviour after copulation (B).

The arched-back male posture indicates a deeper thrust leading to a putative ejaculation. (ejaculation can only be confirmed by vaginal samples).

during which the male is insensitive to any sexual stimulation, and a relative refractory period.

Thus male sexual behaviour is a complex behaviour constituted by numerous different components such as partner preference, sexual motivation, erection and non-contact penile erection, performance (copulation and ejaculation), sexual reward and sexual satiety. It can be difficult to distinguish between these components; so, to investigate the neural pathways mediating each of these behaviours, it is important to use appropriate methodological tools (see review Meisel & Sachs, 1994; Agmo, 1997) and to use behavioral terms and parameters consistently to avoid misleading interpretations (Appendix 1 regroups a list of definitions of all major behavioral terms and parameters used throughout this thesis).

1.2 Neural regulation of male sexual behaviour:

Numerous areas of the brain have been reported to be involved in the regulation of male sexual behaviour based on evidence from lesion studies, electrical stimulation, local administration of hormones and peptides or by immunocytochemical staining for Fos, a protein product of the immediate early gene c-*fos* that is used as an indicator of neuronal activation (Meisel & Sachs,1994; Pfaus & Heeb, 1997). Due to the many areas and the complexity of the pathways, this introduction describes examples of some of the major brain structures involved in neuronal pathways that regulate male sexual behaviour in rats. Table 1.1 summarizes the central nervous system (CNS) areas reported to be involved in the regulation of male sexual behaviour.

Brain areas involved in the regulation of male sexual behaviour play different roles: some recognize sensory cues from the female, some areas convey the olfactory information to other brain areas, and some areas control the motor performance. The specificity of each brain area involved in the regulation of sexual activity is illustrated by one major pathway originating from the olfactory system, where sensory cues are

CNS areas	Behavioral phases regulated
Olfactory system:	
Vomeronasal organ	Motivation (detection olfactory cues)
Main olfactory bulbs	Motivation (detection olfactory cues)
Accessory olfactory bulbs	Motivation (detection olfactory cues)
Brain:	
MeA (Medial amygdala)	Motivation
BNST (Bed Nucleus of the Stria Terminalis)	Motivation
Nucleus accumbens	Motivation
VTA (Ventral tegmental area)	Motivation
MPOA (Medial preoptic area)	Motivation/ Performance
MPN (sexually dimorphic preoptic nucleus)	
PVN (Paraventricular nucleus)	Performance
Lateral hypothalamic area	Motivation, Performance
Lateral septum	Motivation, Performance
VMH (Ventromedial nucleus of the hypothalamus)	Performance
Ventral premammillary nucleus	
Subparafascicular thalamic nucleus	Performance
Central tegmental field	
PAG (Periaquaductal gray)	Performance (PE)
Brainstem:	
PGN (Nucleus paragigantocellularis)	Performance (PE)
Medullary raphe pallidus	PE
Locus coerulus	Performance (PE)
Spinal cord:	Performance (PE)

Table 1.1: CNS areas involved in the regulation of male sexual behaviour.
PE: Penile erection.

Adapted from Meisel & Sachs, 1994; Pfaus & Heeb, 1997; Hamson & Watson, 2004; McKenna, 2004.

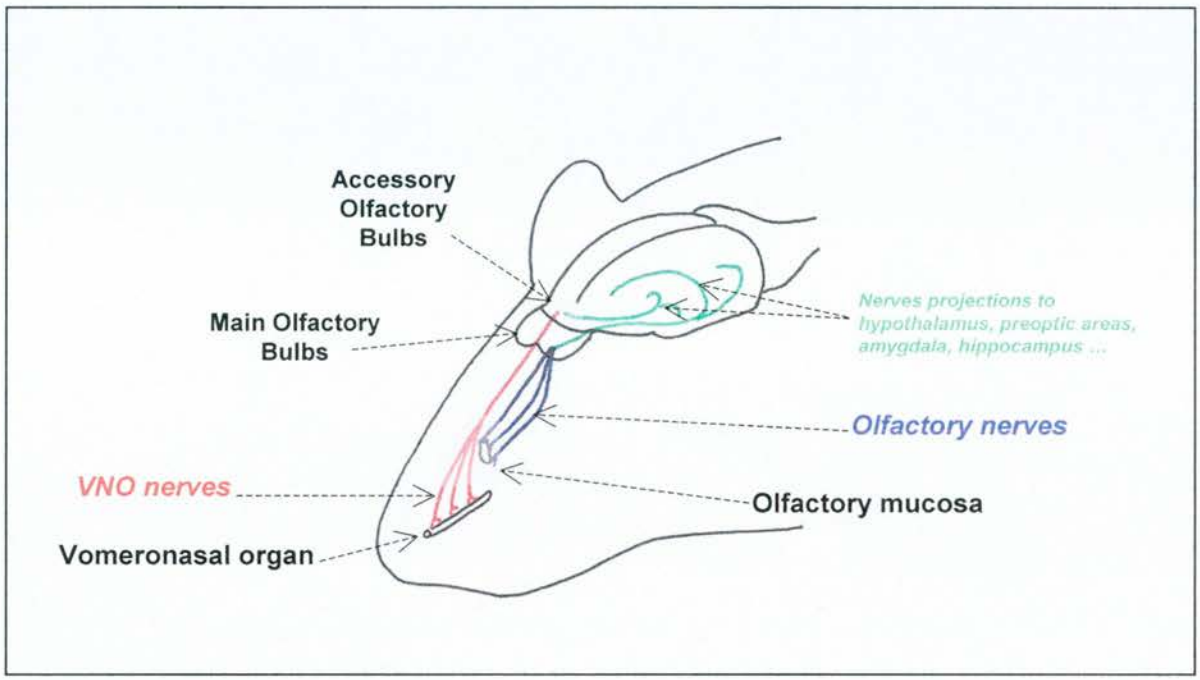

Figure 1.4: The rat olfactory system. *Adapted from Meredith M. website*

collected, to the limbic system and hypothalamus, where motor copulatory patterns are regulated (Fig. 1.4):

1.2.1 The olfactory system:

The olfactory system is especially important in the regulation of sexual behaviour in rodents. It is comprised of the olfactory bulbs (main and accessory) and the vomeronasal organ. Two different pathways perceive olfactory information:

i. Olfactory information travels through the nasopalatine duct to reach the vomeronasal organ (VNO). The vomeronasal nerves project to the accessory olfactory bulbs (AOB).

ii. Olfactory information reaches the main olfactory bulbs (MOB) by the olfactory nerves originating from the olfactory epithelium.

The AOB and MOB project to central structures that include the medial amygdala, the bed nucleus of the stria terminalis (BNST) and the medial preoptic area.

In male hamsters, a selective cutting of the vomeronasal nerve has been reported to disrupt copulation in some studies but not in others (Meredith, 1986; Powers & Winans, 1975; Winans & Powers, 1977). Destruction of receptors in the olfactory epithelium induces similar contradictory effects: it has been reported to disturb copulation in some studies but not in others (Devor, 1973; Lisk et al., 1972; Powers & Winans, 1975; Winans & Powers, 1977). However, interfering simultaneously with the VNO and the olfactory epithelium or bilateral olfactory bulbectomy eliminates copulation (Meisel & Sachs, 1994).

In male rats, Saito *et al.* (1986) described that surgical removal of the VNO does not block copulation in sexually experienced rats, nor impair the non-contact penile erection (NCE) induced by cues from a receptive female, suggesting that desafferrenting the AOB does not block sexual behaviours. By contrast, Kondo *et al.* (1999) reported that lesions of the olfactory epithelium inhibited NCE. This suggests that the

vomeronasal organ is not critical to the development of copulation in male rats, and indicates that the main olfactory bulbs/ olfactory epithelium system is important in the establishment of sexual arousal. Olfactory bulbectomy has been reported to reduce the number of rats copulating to ejaculation (Meisel *et al.,* 1980), and bulbectomized rats that did mate had longer intromission latencies, suggesting a reduction of sexual arousal. It is not clear why some olfactory bulbectomized rats still copulate and achieve ejaculation. No evidence of direct projections from the VNO and from the olfactory epithelium to central structures involved in the regulation of male sexual behavior has been reported. This suggests that rats that have lost olfaction have deficits in sexual behaviour but still have the ability to copulate, illustrating the multi-sensory regulation of male sexual behaviours: although olfaction is predominant in rodent species, other sensory stimuli (e.g.: visual, auditory, tactile and imaginative) can initiate copulation using different neural pathways.

1.2.2. The amygdala:

Manipulations of the amygdala disrupt normal display of sexual activity. The amygdala is comprised of two main regions: the corticomedial amygdala (medial amygdala), and the basolateral amygdala. The major inputs to the medial amygdala derive from the olfactory bulbs. Lesions of the basolateral amygdala do not disrupt copulation and even tend to decrease the onset time to copulation (Harris & Sachs, 1975). By contrast, lesions of the medial amygdala increase ejaculation latencies (Giantonio *et al.*, 1970).

In the medial amygdala (MeA), Fos expression is increased during sexual activity, suggesting that the MeA is not only a relay of information to other brain areas but also that neurones in the MeA are activated to process the information. This increase in neuronal activity follows two distinct patterns: Firstly, Fos expression in the medial MeA is induced by sex-related odours, and secondly, Fos expression in the dorsolateral MeA is increased by ejaculation (Pfaus & Heeb, 1997). This gives evidence that sub-divisions in the amygdala have distinct roles in the regulation of male sexual behaviour.

Thus the MeA receives information from the vomeronasal organ and the olfactory bulbs, processes it and relays it to other brain areas such as the BNST and the medial preoptic area.

1.2.3. The bed nucleus of the stria terminalis:

Lesions of the BNST disrupt the male's ability to copulate to ejaculation by increasing inter-intromission intervals and ejaculation latencies (Emery & Sachs, 1976; Claro *et al.,* 1995). Coolen *et al.* (1996) have shown that Fos expression in the posteromedial part of the BNST (BNSTpm) is increased following sexual activity, and they also described the existence of clusters of Fos immunoreactivity in specific sub-regions of the BNSTpm only after ejaculation. They concluded that specific sub-regions of the BNSTpm were involved in different aspects of sexual activation since different sexual behaviours induced different patterns of Fos expression. Liu *et al.* (1997) reported that, while lesions of the BNST moderately impaired copulation (ejaculation latencies increased), it severely affected non-contact penile erection. Liu and colleagues proposed that BNST lesions might impair penile erection by interfering with the processing of olfactory cues from the receptive female. Pfaus & Heeb (1997) proposed that the role of the BNST cannot be only the relay of chemosensory information from the MeA to the medial preoptic area as suggested by Meisel & Sachs (1994), because lesions of the MeA in sexually experienced rats do not reduce the Fos expression in the BNST induced by sex-related odors.

Thus the BNST plays an important role in penile erection and in copulation. Its role appears to be to process chemosensory information as well as to relay this information to other brain area to maintain sexual motivation and to stimulate copulation.

1.2.4.The medial preoptic area:

The effects of lesions of the MPOA on copulation in rats were first studied by Heimer and Larsson (1964), they reported that rats with MPOA lesions did not copulate

or display any mounting behaviour when paired with a receptive female. Hansen & Gummesson (1982) investigated whether the loss of copulatory behaviours after lesions of MPOA was consequential to interruption of nerve fibres passing through this area to "deliver" information to another brain area, or due to the destruction of neurones that process information that is then passed on to other brain areas. They reported similar effects of electrolytic and neurotoxic lesions, suggesting that neurones within the MPOA play a role in the processing of sensory information leading to copulatory behaviour. This suggestion has been supported by Fos studies revealing that sexual activity increased Fos expression in the MPOA (Pfaus & Heeb, 1997).

It was initially believed that the MPOA was critical in the initiation of sexual activity because lesions in the MPOA eliminated copulation in sexually experienced males. Early studies explained the failure of copulation by a reduction of sexual arousal or motivation (Ginton & Merari, 1977; Edwards & Einhorn, 1986), but Hansen *et al.* (1984) described that MPOA-lesioned male rats still investigated the female and even sometimes mounted her. Everitt *et al.* (1990) suggested that, in MPOA-lesioned rats, the failure to copulate did not result from a reduction of sexual arousal but from a failure to translate signals induced by sexual arousal into copulatory motor patterns.

Thus the MPOA has a critical role in processing the sensory information derived from sexual motivation during the pre-copulatory period to induce sexual performance during the consummatory period. The MPOA also has a role in the regulation of penile erection since MPOA efferents project to the midbrain (Periaqueductal gray) and to the medulla, two areas that project to the spinal cord to regulate penile reflexes (McKenna, 2000). Thus the MPOA is a key area for the regulation of male sexual behaviour, involved in both motivation and regulation of sexual performance.

1.2.5.The paraventricular nucleus:

The paraventricular nucleus of the hypothalamus (PVN) is a very heterogeneous nucleus consisting of several subdivisions where different cell types are located (Fig. 1.6). In the PVN, a large number of neuropeptides are contained in neurones that project to many intra and extra –hypothalamic brain areas, to the brainstem and the spinal cord (Buijs, 1978; Swanson & Sawchenko, 1983; Hallbeck, 2001). Due to this extended network of neuronal projections and the variety of neurotransmitters identified, the PVN is considered as an integration centre involved in the control of numerous functions, including male sexual behaviour. During copulation, Fos expression is increased in the PVN (Pfaus & Heeb, 1997; Coolen *et al.,* 1996) and lesions of the PVN impair copulation by increasing mount and intromission latencies (Hughes, 1987). The identification of projections from the PVN to the lower lumbar regions of the spinal cord involved in penile reflexes suggests an important role for the PVN in the modulation of penile erection. Chen *et al.* (1997) reported that electrical stimulation or chemical stimulation (by glutamate) of the PVN increased the intracavernous pressure (ICP) along with visible erection and ejaculation. The PVN has been recognized to be the site of action of many peptides and neurotransmitters involved in the regulation of male sexual behaviour. Indeed, penile erection can be induced by injection of oxytocin, or apomorphine, or NO donors into the PVN (Melis & Argiolas, 1997).

Thus the PVN is mainly involved in the regulation of sexual performance. However, its role in the regulation of sexual motivation should not be excluded, as, during copulation, the establishment of penile erection also reflects the rat's aroused state. Due to the presence of many neurotransmitters within the nucleus, the PVN is a site of crucial interactions between different systems that modulate copulatory behaviour. To understand the role of the PVN in the regulation of copulatory behaviour in a more detailed manner, the investigation of the role of each of these peptides and neurotransmitters is required. The mechanisms of action of dopamine, oxytocin and nitric oxide and their interactions are described section 1.3 and in section 2.

Taken together, these few examples of brain areas illustrate the high organization of the complex neuronal network involved in the regulation of male sexual behaviour. While some brain areas are more specialized in the detection and the integration of chemosensory cues (e.g.: medial amygdala, BNST), others regulate the motor patterns crucial to sexual performance (e.g.: MPOA, PVN, Brainstem). However, a clear dichotomy is hard to draw as several of these brain areas have roles in both sexual motivation and performance, and even some of these brain areas are also themselves organized in subdivisions that regulate specific aspects of the male sexual behaviour.

1.3. Peripheral regulation of penile erection:

Penile erection is one component of a series of complex physiological events constituting male sexual behaviour and is regulated via both central and peripheral neural pathways (see reviews Steers, 2000); Guiliano & Rampin, 2004). Penile erection depends upon adequate blood inflow to erectile tissues, coordinated dilatation of the penile arteries and relaxation of erectile tissues. As a vascular event, penile erection is therefore controlled by the autonomic nervous system. Parasympathetic and thoracolumbar sympathetic pathways innervate the penis, and while parasympathetic pathways are pro-erectile, sympathetic pathways are anti-erectile and related to detumescence. The penis also receives somatic innervations via the dorsal nerve of the penis, facilitating penile rigidity.
Fig 1.5 represents a schematic illustration of the autonomic pathways controlling penile erection.

1.4 Neurochemical and hormonal regulation of male sexual behaviour:

Numerous neuromodulatory factors including peptides and hormones have been reported to be involved in the central regulation of male sexual behaviour (for review

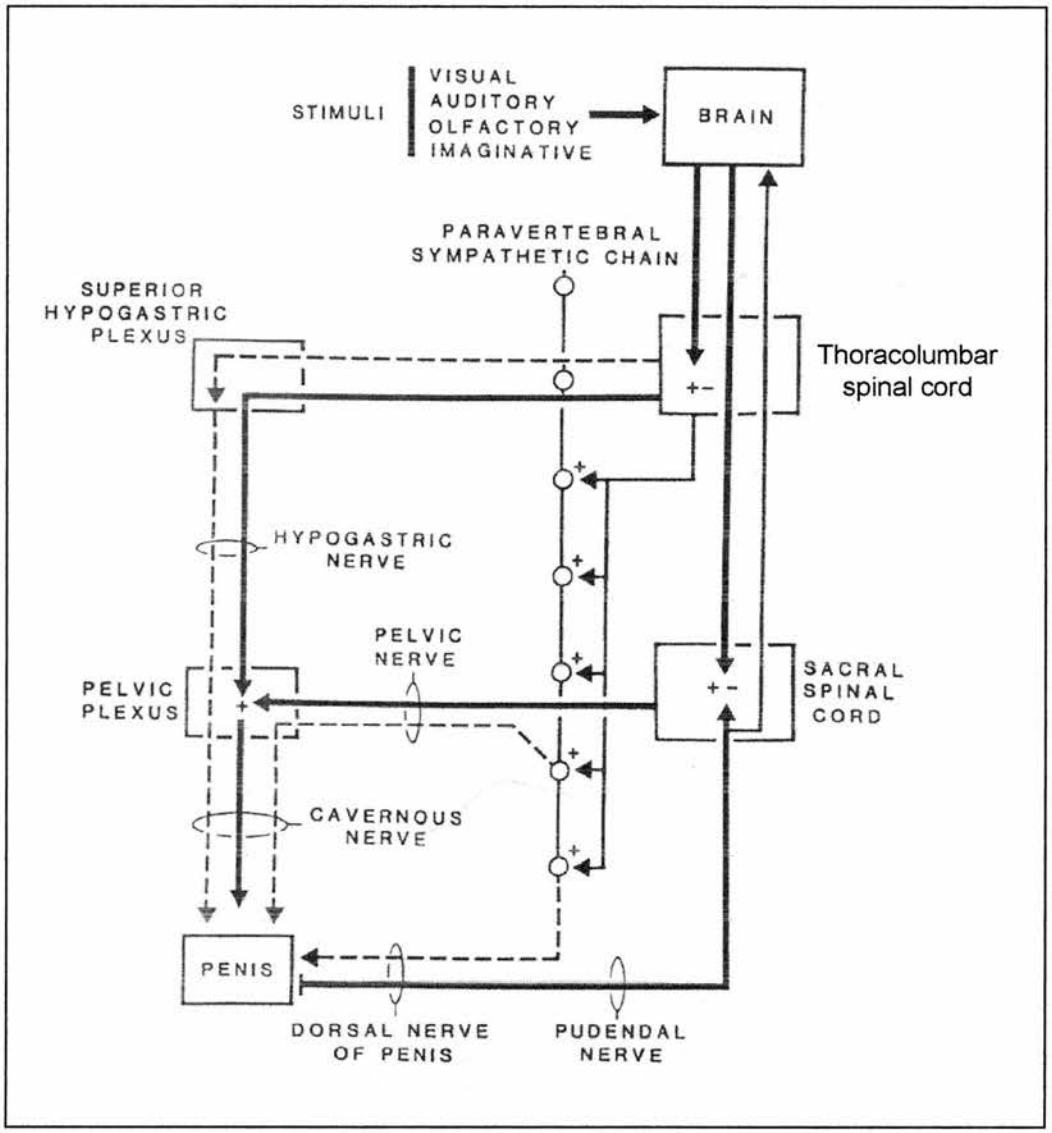

Figure 1. 5: Peripheral and central neural pathways controlling penile erection.

The penis receives innervations from sacral parasympathetic (pelvic), thoracolumbar sympathetic (hypogastric & lumbar sympathetic chain) and somatic (pudental) nerves. The parasympathetic nervous system is the major excitatory input to the penis, inducing vasodilatation of the penile vasculature and therefore erection. Descending brain pathways control lumbar sympathetic and sacral parasympathetic outflow to the penis. The brain also receives sensory information from the penis via ascending spinal pathways.

Adapted from Steers W.D. Neurosc. Biobehav. Rev. (2000)

see Meisel & Sachs 1994; Argiolas, 1999). Table 1.2 summarizes the central effects of some of these neuromodulators. The present introduction mainly focuses on selected factors that have been most studied. The role of oxytocin and ACTH -related peptides such as α-MSH is described in the following parts of the introduction (see Part 2 & 3, Chapter 1).

1.4.1 Dopamine:

Dopamine is the factor that is the most studied in the regulation of male sexual behaviour. In brief, dopamine facilitates male sexual behaviour. In one of the first studies on dopamine and sexual behaviour, Tagliamonte & Gessa (1974) reported that systemic injection of L-DOPA (dopamine precursor) combined with an inhibitor of DOPA carboxylase increased by 50% the number of sluggish male rats that reached ejaculation. However, the role of dopamine in male sexual behaviour is complex: systemic injection of apomorphine (a dopamine agonist) has a biphasic effect on male sexual behaviour as a function of the dose injected. While low doses of apomorphine are facilitative of copulation, high doses tend to inhibit copulation in male rats (Clark & Smith, 1987; Paglietti *et al.,* 1978).

i. Dopamine systems involved in the central regulation of male sexual behaviour:

Dopamine acts on different aspects of the male sexual behaviour via three different dopamine systems:

- The mesolimbic dopamine system:

The mesolimbic system originates in the ventral tegmental area (VTA) and projects to several terminal fields including the nucleus accumbens. The mesolimbic system is critical for appetitive behaviours and plays a major role in the anticipatory, pre-copulatory phase of sexual behaviour (Everitt, 1990; Hull *et al.*, 1999; Argiolas, 1999). In the nucleus accumbens, the concentration of extracellular dopamine is increased in males when they are stimulated by cues from a receptive female (Damsma *et al.*, 1992) but not when they are presented to a non-receptive female (Wenkstern *et al.*, 1993).

Chemical messengers	Effect on sexual behaviour
Dopamine	Facilitatory
Serotonin	Inhibitory/ Facilitatory
Prolactin	Inhibitory (Hyperprolactinemia) ?
ACTH/ MSH peptides	Facilitatory
Opioid peptides	Facilitatory(motivation), Inhibitory (performance),
Oxytocin	Facilitatory
Vasopressin	Inconclusive
LHRH	Facilitatory?
CRF	Inhibitory
NPY	Inhibitory?
Galanin	Inhibitory
CCK	Inconclusive
Substance P	Facilitatory
Neurokinin K	Inhibitory
VIP	Facilitatory
Angiotensin II	Inhibitory
Nitric oxide	Facilitatory

Table 1.2: Central effects of chemical messengers on male sexual behaviour.

Adapted from Meisel & Sachs, 1994; Argiolas, 1999; Hull, 1999; Guiliano & Rampin, 2001.

Injections into the nucleus accumbens of D-amphetamine (that acts by releasing dopamine) reduce mount- and intromission- latencies (Everitt, 1990). Hull *et al.* (1986) reported that injection of apomorphine into the nucleus accumbens slightly decreased intromission latency whereas the injection of apomorphine into the VTA increased intromission latency. Taken together, these results show that the mesolimbic dopamine system is involved in the anticipatory and motivational phase of male sexual behaviour as well as being part of the rewarding process of sexual behaviour, since the VTA and the nucleus accumbens are part of the 'rewarding -related behaviours' pathways.

- The nigrostriatal dopamine system:

The nigrostriatal dopamine system originates in the substantia nigra and projects to the striatum, and is important in the coordination between sensory stimuli and motor performance. Dopamine is released in the striatum during the consummatory phase of sexual behaviour (Damsma *et al.,* 1992), suggesting that the nigrostriatal dopamine system is involved in motor processes related to consummatory behaviours rather than the motivational aspects of copulation (Everitt, 1990; Hull *et al.,* 1999).

- The incertohypothalamic dopamine system:

The two structures the most studied in the incertohypothalamic dopamine system are the medial preoptic area (MPOA) and the paraventricular nucleus (PVN). Injection of apomorphine into the MPOA (0.5μg) decreases the mount latency, the numbers of intromissions required to reach ejaculation and increases the number of ejaculations (Hull *et al.,* 1986). Microdialysis studies revealed that concentrations of dopamine and dopamine metabolites in the MPOA are increased during copulation and decline after ejaculation (Hull *et al.,* 1993). Similar observations have been made for the role of dopamine in the PVN. Injections of apomorphine in the PVN decreased the mount latency and the number of intromission required to reach ejaculation (Melis and Argiolas, 1995). The PVN is also thought to be the site of action of dopamine in regulation of penile reflexes. Melis *et al.* (2003) reported that, during erection, extracellular concentrations of dopamine are increased in the PVN. Moreover, injection

of apomorphine into the PVN stimulates spontaneous erection (Melis *et al.,* 1987) while lesions of the PVN reduce apomorphine-induced erection (Argiolas *et al.,* 1987). Nevertheless, the role of dopamine in the regulation of penile erection is not yet clearly defined.

Undoubtedly the incertohypothalamic dopamine system plays a role in the regulation of consummatory behaviours, but there is growing evidence that dopamine in the MPOA facilitates the anticipatory phase as well as copulatory performance. The MPOA receives sensory inputs from the mesolimbic system (from VTA) and from the olfactory system. Hull *et al.* (1995) found that concentrations of extracellular dopamine in the MPOA in males were increased during pre-copulatory exposure to a receptive female while non-sexual social stimuli did not induce any significant increase, suggesting that the dopamine released in the MPOA could influence sexual motivation.

ii. Dopamine receptors involved in the regulation of male sexual behavior:

Two types of G-protein coupled receptors, distributed throughout the brain, have been identified as mediating the effects of dopamine: D1 and D2 receptors. While the role of D1 receptors is not clearly defined, D2 receptors appear to play a major role in the regulation of copulatory behaviours. Hull *et al.* (1989) reported that in the MPOA, D1 and D2 receptors have opposite roles in copulation: injection in the MPOA of the selective D2 agonist (LY 163502) had the same effect as an injection of a D1 antagonist (SCH 23390): both increased the mount and intromission latencies. In the PVN, D1 receptors have also been reported to be functionally antagonistic to D2 receptor – mediated facilitative actions on penile responses (Eaton *et al.,* 1991).

Through three different dopamine systems and by interacting with other hormones or neuropeptides systems such as testosterone, oxytocin, opioids and nitric oxide (for review see Melis & Argiolas, 1995; Hull *et al.*, 1999), dopamine plays an important role in all aspects of male sexual behaviour during both pre-copulatory

(arousal) and copulatory (performance, erection, seminal emission) phases and possibly in reward too.

1.4.2 Opioids:

Endogenous opioids and opioid receptors are distributed throughout the CNS (Mansour *et al.,* 1988), including in central structures involved in the regulation of male sexual behaviour. Although opioid effects on male sexual behaviour have been generalized as inhibitory, opioids have a dual effect: they facilitate appetitive behaviours but inhibit consummatory behaviours.

Endogenous opioids have been reported to facilitate on sexual motivation (Band & Hull, 1990; Meisel & Sachs, 1994; Argiolas, 1999). However, systemic injection of morphine decreases the number of rats that copulate, and increases the mount and intromission latencies (Agmo & Parades, 1988). Central administration of β-endorphin or met-enkephalin also has inhibitory effects on copulatory behaviours (Argiolas, 1999). When injected systemically or centrally, naloxone, an opioid antagonist, facilitates copulation by reducing mount, intromission, and ejaculation latencies, and by decreasing the number of intromissions to achieve ejaculation (Van Furth *et al.*, 1994); but injection of naloxone also increases the post-ejaculatory interval (Szechtman *et al.*, 1981; Miller & Baum, 1987). This illustrates the complex dual effects of opioids on sexual behaviour and indicates that opioids might be involved in the initiation of a new mating sequence after an ejaculation has occurred.

The different effects of opioids on sexual behaviour might be explained by the numerous sites of action that opioids have in the brain. Opioids have been reported to act in the MPOA, in the MeA, in the ventral tegmental area (VTA), in the nucleus accumbens, and in the PVN. During sexual behaviours, the MPOA has been reported to be the main site of inhibitory opioid actions, which are probably mediated via μ -opioid receptors (Coolen *et al.*, 2004). The μ -opioid agonists, morphine, β-endorphin and met-

enkephalin injected into the MPOA inhibit copulation in a dose-dependent manner (Leyton & Stewart, 1992; Hughes, 1987, 1990). Injection of β-endorphin into the MeA delays copulatory behaviour (McGregor & Herbert, 1992), suggesting that opioids interfere with the processing of the olfactory cues in the MeA. Injection of morphine or dynorphin into the VTA facilitates copulation and repeated injections of morphine increase dopamine transmission in the nucleus accumbens, which has a stimulatory effect on sexual motivation (Mitchell & Stewart, 1990). Injection of morphine into the PVN prevents penile erection induced by oxytocin (Melis *et al.* 1992) or by the proerectile peptide hexarelin (Succu *et al.,* 2003). Finally, opioids, through the VTA and the nucleus accumbens, might also participate to the stimulation of sexual motivation and sexual reward by acting on the mesolimbic dopaminergic system (Van Furth & Van Ree, 1996).

1.4.3 Nitric oxide:

Nitric oxide (NO) has a well-known role in the peripheral mediation of penile erection (Rajfer *et al.,* 1992) but is also implicated in the central regulation of penile erection. NO synthase (NOS) is highly concentrated within the CNS in areas involved in the regulation of sexual behaviours such as the MPOA, the PVN and the spinal cord (Bredt *et al.,* 1990), and NOS mRNA expression in the PVN is twice as abundant in sexually potent rats as in impotent rats (Benelli *et al.*, 1995). Melis *et al.* (1993) showed that i.c.v injection of NOS inhibitors inhibited penile erection induced by dopamine, by oxytocin, or by ACTH. NO donors such as nitroglycerin or isoamyl nitrite injected i.c.v. induced penile erection in a dose-dependent manner (Argiolas, 1994). Melis & Argiolas (1997) suggested that the main site of action of NO in the regulation of penile erection was the PVN. Indeed, injection of NOS inhibitors into the PVN can prevent penile erection induced by oxytocin or by apomorphine, and NO donors injected into the PVN induce penile erection similarly as to when injected i.c.v. To support this hypothesis they reported that injection of oxytocin, or apomorphine at doses that initiate penile erection, increased NO production in the PVN. To clarify the mechanisms of action of NO, they

suggested that NO induces penile erection by activation of central oxytocin transmission as i.c.v. injection of oxytocin antagonists has the same effects as i.c.v. injection of NOS inhibitors: both prevents penile erection induced by oxytocin, apomorphine and by the NO donors nitroglycerin and isoamyl nitrite. Melis & Argiolas (1997) attested that, unlike at the peripheral level, NO does not activate the guanylate cyclase (GC) pathway in the central regulation of penile erection in the PVN. Indeed, i.c.v. injection of a GC inhibitor (methylene blue) at low doses failed to prevent penile erection induced by oxytocin, apomorphine or NO donors.

These results suggest that central NO might be the final mediator of the actions of several neurotransmitters that are involved in the central regulation of penile erection. Nevertheless the mechanisms by which NO interacts with other neural systems remain to be understood. Finally, it is expected that NO facilitates penile erection throughout copulation. Therefore, studying the regulatory role of NO on penile erection in the context of copulation rather than during drug-induced penile erection could give precious information on the real impact of central NO during male sexual activity.

1.4.4 Testosterone:

That normal male sexual behaviour requires testosterone is well–known. Early studies in the 1800s reported that castrated rats were not able to copulate. Davidson *et al.* (1966) described progressive loss of copulatory behaviours within days of castration: castrated rats first lost the ability to ejaculate, then the ability to mount the female with intromission, and, finally, castrated rats lost all mounting behaviour. They also described that the effects of castration could be reversed by s.c. injection of testosterone, confirming that the depletion of testosterone was inducing the disruption in copulation. Davidson *et al.* (1966) also investigated the effect of testosterone implants in different brain areas in castrated rats. They were the first to suggest that the brain was the "primary target" for testosterone action in the regulation of copulation, and reported that

the MPOA and the anterior hypothalamus were the two brain areas most sensitive to testosterone action in the regulation of male sexual behaviour.

In various tissues including the brain, testosterone can be converted into estradiol (by aromatization) or into dihydrotestosterone (DHT) (by reduction). Androgen and estrogen receptors are distributed throughout the brain in areas involved in the regulation of male sexual behaviour (review Meisel & Sachs, 1994). Implants of estradiol in the MPOA restored copulation in castrated rats (Christensen & Clemens, 1974). Injection of an estrogen antagonist or injection of steroids blocking aromatization inhibited the restorative effects of testosterone, confirming that estradiol was necessary to restore copulation in castrated rats (Beyer *et al.*, 1976). Putman *et al.* (2003) studied the effectiveness of the two testosterone metabolites, estradiol and DHT in maintaining copulation in castrated rats. Rats treated with both estradiol and DHT or with testosterone (s.c.) copulated normally, but although rats treated with estradiol-alone or DHT-alone presented intromission behaviours, they failed to ejaculate. This indicates that both androgens and estrogens are required for normal copulation.

The difference between androgens and estrogens in capacity to maintain sexual behaviour may be explained by their different ability to interact with other neural systems or neuropeptides that regulate male sexual behaviour. One major role of testosterone in male sexual behaviour is to facilitate dopamine release in the MPOA during copulation. As described in section 1.4.1, dopamine is important for sexual behaviour, and activation of dopamine receptors in the MPOA facilitates copulation, sexual motivation and genital reflexes (Hull *et al.*, 1995, 1999). Hull *et al.* (1995) showed that the depletion of testosterone by castration inhibited extracellular dopamine release in the MPOA, which is normally induced when a male is presented to a receptive female. Putman *et al.* (2003) reported that s.c. injection of testosterone, or estradiol and DHT, or injection of each metabolite alone, had different effects on basal concentrations of dopamine and on dopamine release within the MPOA during sexual behaviour. While estradiol maintained normal basal concentrations of dopamine in the MPOA that allow

some copulation, androgens were required for the increase in dopamine release, before and during copulation, that facilitates ejaculation. This confirmed the complementary role of testosterone metabolites in the maintenance of copulation, although their mechanisms of action are yet to be understood. Thus, testosterone, by interacting with neuronal systems such as the dopamine system, plays a critical role in the normal mediation of male sexual behaviour.

Taken together, these examples of neuromodulatory factors illustrate the complexity of interactions between different systems involved in the regulation of male sexual behaviour. The following two sections of the general introduction focus on the role of two key peptides involved in the regulation of male sexual behaviour: Oxytocin and α-MSH.

PART 2: Oxytocin and male sexual behaviour

2.1. Oxytocin system:

2.1.1. Structure, localization and oxytocin efferents:

Oxytocin is a nonapeptide whose structure was identified in 1953 by Du Vigneaud and colleagues. Oxytocin is a neurohormone synthesized in the hypothalamus primarily by neurones in the supraoptic nucleus (SON) and in the paraventricular nucleus (PVN):

i. The SON is a nucleus located at the ventral surface of the brain lateral to the optic chiasm. Magnocellular oxytocin neurones are distributed throughout the SON from a ventro-dorsal distribution (anterior SON) to a dorsal distribution (posterior SON). Supraoptic magnocellular oxytocin neurones project to the neural lobe of the pituitary via the median eminence.

ii. The PVN is a heterogeneous nucleus located adjacent to the third ventricle. The PVN is subdivided into several areas where magnocellular and parvocellular oxytocin neurones are distributed (Fig. 1.6). Axons from magnocellular oxytocin neurones project to the posterior pituitary, via the median eminence. Axons from parvocellular oxytocin neurones project to numerous areas within the CNS such as the limbic system, the hypothalamus, the brainstem and the spinal cord (Fig 1.6).

iii. Oxytocin cell bodies have also been reported in the accessory nuclei, in the anterior commissure nuclei, in the anterior and posterior fornical nuclei, in the nucleus circularis and in the nucleus of medial forebrain bundle (Peterson, 1966; Sofroniew, 1983).

Buijs and colleagues (1978, 1979, 1980, 1985) have extensively described the network of oxytocin fibres in the CNS of the rat. They detailed oxytocin pathways

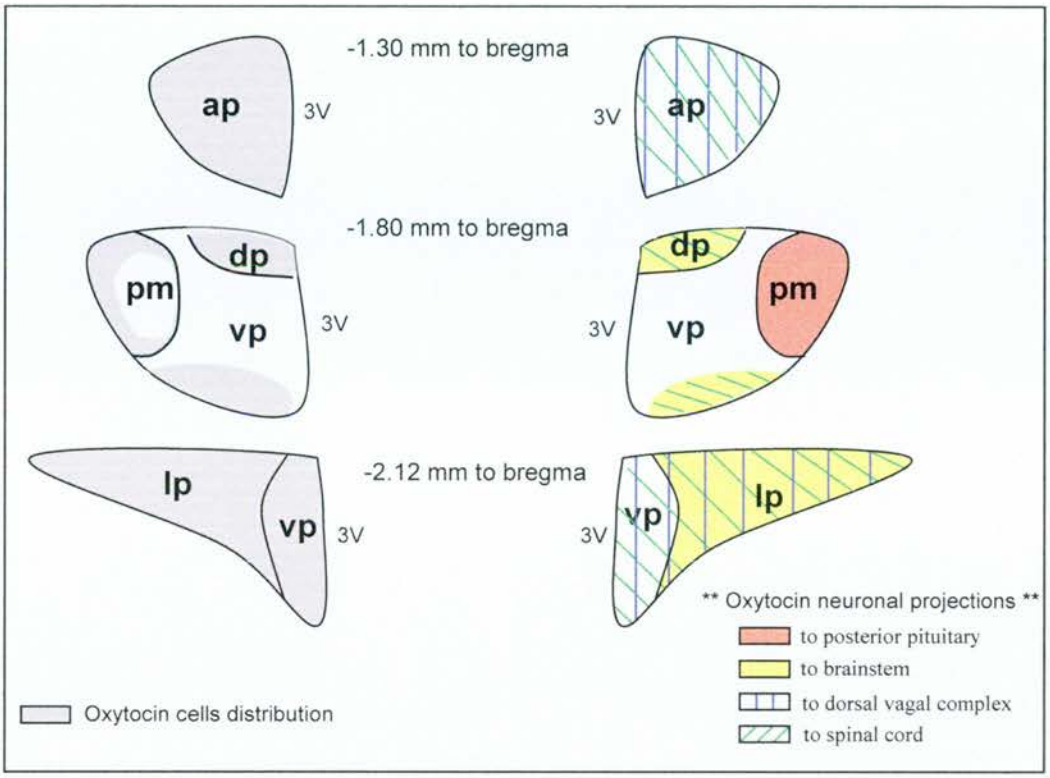

Figure 1. 6: Sub-divisions of the PVN, oxytocin cells distribution and oxytocin neuronal projections within the CNS.

Parvocellular oxytocin neurones also project to areas within the brain.

ap Anterior parvocellular PVN; **pm** Posterior magnocellular PVN; **dp** Dorsal parvocellular PVN; **vp** Ventral parvocellular PVN; **lp** Lateral posterior parvocellular PVN ; **3V** Third ventricle. *Adapted from Swanson & Kuypers, 1980, J. Comp. Neurol., **194**, 555-570.*

within the hypothalamus but also extra-hypothalamic oxytocin pathways leading to the olfactory bulbs, the limbic system, the brainstem and the spinal cord. The distribution of oxytocin fibers is summarized in Table 1.3.

2.1.2. Oxytocin systemic secretion and central release:

At the posterior pituitary, oxytocin is secreted from the magnocellular neurones into the general circulation and blood-borne oxytocin acts at specific high -affinity receptors at distant targets including the uterus, mammary glands, testis and kidneys. Oxytocin is also produced by the Leydig cells in the testis, providing a small additional source of systemic oxytocin.

The blood-brain barrier prevents the oxytocin secreted into the blood from penetrating into the brain, but oxytocin is also released within the CNS from the parvocellular neurones. Although, the secretion of oxytocin from axon terminals is dependent on the neuronal electrical activity, oxytocin can also be released centrally from the soma and dendrites of magnocellular neurones independently from the electrical activity (Pow & Morris, 1989; Ludwig, 1998).

2.1.3. Oxytocin receptors:

Rozen and colleagues (1995) have identified the rat oxytocin receptor gene. The oxytocin receptor is a member of the G- Protein Coupled Receptor family, it has a seven transmembrane α-helices with two potential glycosylation sites. Oxytocin receptors are coupled to $G_{q/11}$ class GTP binding protein that stimulates the activity of phospholipase C to initiate a cascade of intracellular events (For review on gene structure and regulation, receptor characteristics and signal transduction see Barberis & Tribollet, 1996; Gimpl & Fahrenholz, 2001).

OT receptors are widely distributed. In the periphery, oxytocin receptors mRNA or oxytocin binding sites have been identified in a variety of tissues such as the uterus, the Leydig cells in the testis, the mammary glands, the kidney, the heart, the thymus, the adipocytes, the pancreas and the adrenal gland. In the CNS, the distribution of oxytocin

Table 1.3: Distribution of oxytocin receptor mRNA and oxytocin fibres in the central nervous system.

Brain Regions	OT R mRNA	OT binding	OT Fibres
Olfactory system			
Olfactory bulb	+	?	+
Anterior olfactory nucleus	+++	++	+
Olfactory tubercle	+++	++	+
Island of Calleja	ND	+++	?
Piriform cortex	++	?	?
Entorhinal / perirhinal area	+	+	+
Cortical areas			
Peduncular cortex	?	+++	?
Insular cortex	?	+	?
Cingulate cortex	+	ND	?
Retrosplenial cortex	?	ND	?
Frontal cortex	++	(+)	?
Temporal cortex	(+)	+	?
Taenia tecta	+++	(+)	?
Diagonal band of Broca	+	?	?
Basal nucleus of Meynert	ND	ND	?
Basal ganglia			
Caudoputamen	+++	++	?
Ventral pallidum cell groups	++	+++	?
Globus pallidus	ND	ND	?
Nucleus accumbens	+	+	?
Limbic system			
Lateral septal nucleus	+	+	+
Medial septal nucleus	?	?	+
Ventral hippocampus	+	?	+E
Dorsal hippocampus	+	?	+E
Bed nucleus of stria terminalis	+++	+++	+
Amygdaloid-hippocampal area	+++	+	?
Central amygdala	+++	+++	+E
Medial amygdala	++	+	+E
Basolateral amygdala	+++	+	+E
Parasubiculum and presubiculum	ND	++	?
Dorsal subiculum	+++	(+)	+
Ventral subiculum	+++	+++	+
Circumventricular organs			
OVLT	+	?	+E
Subfornical organ	+	?	+E
Thalamus & Hypothalamus			
Anteroventral thalamic nucleus	ND	ND	+
Paraventricular thalamic nucleus	++	+	+
Ventromedial hypothalamic nucleus	+++	++	+
Anterior medial preoptic area	+++	ND	+E
Suprachiasmatic nucleus	+	?	+
Supraoptic nucleus	+++	(+)	+

Paraventricular nucleus	++	(+)	+
Dorsomedial hypothalamic nucleus	?	?	+E
Arcuate nucleus	+	?	+E
Medial tuberal nucleus	ND	++	+
Posterior hypothalamic area	+	ND	+
Supramammillary nucleus	++	+	+
Lateral mammillary nucleus	ND	+	+
Medial mammillary nucleus	ND	ND	+
Brain stem			
Locus ceruleus	?	?	+
Substantia nigra pars compacta	++	ND	++
Medial pars compacta	+	?	?
Ventral and Dorsal tegmental area	++	ND	?
Central gray	+	ND	+
Dorsal raphe nucleus	+	ND	+
Reticular nuclei	+	ND	+E
Medial vestibular nucleus	+	ND	?
Hypoglossus nucleus	++	ND	?
Nucleus of the solitary tract	+	ND	++E
Nucleus ambiguus			+E
Dorsal motor nucleus of the vagus nerve	+++	ND	+E
Inferior olive nucleus	+	ND	?
Spinal cord	+	ND	+E
Pituitary gland	ND	ND	+E

Expression: +++ strong; ++ moderate; + weak; (+) at the detection limit; ND Not Detectable; ? Not reported; E Axonal endings.

adapted from Buijs et al., 1979, 1980, 1985; Gimpl G. & Fahrenholz F., (2001).

receptor mRNA or oxytocin binding sites extends from the olfactory system to the cortex, hypothalamus, limbic system, brainstem and spinal cord, and is summarized in Table 1.3.

2.2. Roles of oxytocin:

Oxytocin has been implicated in a variety of physiological functions and behaviours including regulation of salt and water balance, parturition, milk-ejection reflex, nociception, regulation of cardiovascular functions, thermoregulation, memory, feeding behaviour, maternal behaviour, grooming, yawning and sexual behaviour (see review Richard, 1991). Some of the most well known functions are briefly described below:

2.2.1 Osmolarity:

The structural similarities between oxytocin and the antidiuretic hormone vasopressin, led to the study of the role oxytocin in the regulation of salt and water balance. In response to changes in extracellular fluid osmolarity, oxytocin acts centrally and peripherally to induce physiological and behavioral changes that will restore an adequate osmolarity. Oxytocin actions include the regulation of sodium appetite and thirst (behavioral changes) and the regulation of sodium excretion from the kidneys (physiological changes). In response to osmotic stimulation, such as hypovolemia or hyperosmolarity, magnocellular neurones are activated in the SON and in the PVN, modifying oxytocin and vasopressin secretion. For example, i.p. injection of hypertonic saline increases magnocellular oxytocin neurone activity in the SON (Brimble & Dyball, 1977). Magnocellular neurones from the SON are osmosensitive, as direct administration of hypertonic saline onto the SON induced an increase in neuronal activity (Leng, 1980). Magnocellular neurones also receive afferents from other osmosensitive brain areas including the subfornical organ and the organum vasculosum of the lamina terminalis (OVLT), and from cardiovascular and visceral inputs.

2.2.2 Parturition:

In the rat, oxytocin has two important roles in parturition. Oxytocin plays a role in the expulsive phase and in the timing of parturition. The last days of pregnancy, the local production of prostaglandins by the uterus is increased, leading to a fall in the production of ovarian progesterone. As a result, the expression of oxytocin receptors is increased (Lefebvre *et al.*, 1992). A positive feedback loop between the uterus and the oxytocin cells exits: There are afferent inputs from the cervix and uterus to the brainstem. From the NTS, A2 noradrenegic cells project to oxytocin cells, whose activation, leads to an increase in oxytocin secretion (Douglas *et al.*, 2001). This results in more uterine contractions directly via oxytocin-receptors located in the myometrium and indirectly via stimulation of prostaglandins production in other tissues. During parturition, oxytocin secretion is pulsatile. Each birth is associated with a pulse of oxytocin secretion triggered by a burst of high electrical activity of the oxytocin cells. Neumann *et al.* (1996) showed that centrally released oxytocin stimulates its own release during parturition by a receptor-mediated positive feedback, and suggested that once released, oxytocin might be involved in the coordinated activation of oxytocin neurones.

2.2.3 Lactation and the milk-ejection reflex:

Oxytocin stimulates the contraction of myoepithelial cells surrounding the alveoli and the mammary duct in the mammary gland, inducing milk let down. During lactation, the pups' suckling stimulates pressure receptors in the skin overlying the mammary gland. The suckling stimulus triggers synchronized bursts of activity of oxytocin neurones (Wakerley & Lincoln, 1973). Each burst is sufficient to release oxytocin, which in turns induces the milk-ejection. The milk-ejection reflex involves simultaneous activation of the SON and the PVN (Belin & Moos, 1986). This coordination is under the regulatory influences of other brain areas including the limbic system (Ingram *et al.,* 1995) but is also under the influences of interaction between each oxytocin neurones (Neumann *et al.,* 1996).

The role of oxytocin in lactation is essential. Indeed, in transgenic oxytocin knockout mice, pups will starve although milk is available. In absence of oxytocin, the milk-ejection reflex does not occur, inhibiting the milk expulsion from the mammary gland (Nishimori *et al.*, 1996)

2.2.4. Social behaviours:

Oxytocin is implicated in social behaviours and social recognition mechanisms (see reviews Insel & Young, 2001; Ferguson, 2003; Carter, 2003). Oxytocin plays an important role in two selective social behaviours, the maternal behaviour and the less ubiquitous adult-adult pair bond. Both behaviours require overcoming any avoidance behaviour and stimulating the motivational attraction to a specific individual (infant or partner).

2.2.4.1. Oxytocin & maternal behaviour:

Oxytocin has a dual role in the establishment of maternal care, as it stimulates maternal motivation towards the offspring, and also facilitates the attachment of the infant towards its mother. In rats and sheep, the maternal behaviour is not spontaneous, but is initiated by the process of parturition itself. In rats, oxytocin facilitates the onset of maternal care by overcoming the mother's avoidance behaviours towards the neonates and by stimulating nurturing behaviours (Pedersen *et al.*, 1985). In sheep, the maternal behaviour is selective: an ewe will reject any alien lamb; and oxytocin is responsible for the selectivity of the maternal care that an ewe develops towards her own lamb (Kendrick *et al*, 1987; Keverne & Kendrick, 1992). In mice, the role of oxytocin in developing maternal care is likely to be less critical than in rats and sheep, as oxytocin knockout mice present normal nurturing behaviour; this might be explained by the fact, as the difference of rats and sheep that mice present spontaneous maternal behaviour. Oxytocin also plays a role in the infant-mother attachment. Oxytocin facilitates the learning processes that allow the pups to recognize its mother olfactory cues, and influences the vocalization frequencies that a pup produces to signal its isolation to its mother.

2.2.4.2. Partner preference formation. Pair-bonding:

One model used to investigate the involvement of oxytocin in the pair–bond formation is the prairie voles and the montane voles. Prairie voles are monogamous and forms pair bonds after mating. In female prairie voles, central oxytocin facilitates the development of partner preference formation even in absence of mating (Williams, 1994; Insel & Hulihan, 1995). By contrast, montane voles are polygamous, and central oxytocin fails to induce pair bonding (Winslow *et al.*, 1993). In male prairie voles, oxytocin has no effect on social bonding whereas vasopressin is critical for pair bonds as central injection of vasopressin antagonist blocks partner preference formation (Winslow *et al.*, 1993). The species difference can be explained by the difference in receptors expression. Although the two species share the same oxytocin and vasopressin receptors, their distribution throughout the brain is different. While oxytocin and vasopressin receptors are highly expressed in the prairie voles' brain in regions associated with reward (oxytocin receptors in the nucleus accumbens and the V1a receptor in the ventral pallidum), oxytocin and vasopressin receptors are almost virtually absent from the same areas in the montane vole brain (Lim *et al.*, 2004).

2.3. Oxytocin and sexual behaviour:

2.3.1. Oxytocin and female sexual behaviour:

Central oxytocin facilitates female sexual behaviour. Injection of oxytocin in the MPOA increases sexual receptivity (Cadwell, 1992) and oxytocin injection in the VMN facilitates lordosis behaviour. The effect of oxytocin has been reported to depend on some estrogen and progesterone as infusions of oxytocin in the VMN induces increase in lordosis behaviour in females treated with estrogen and progesterone but not in females treated with estrogen alone (Schumacher *et al.*, 1989).

2.3.2. Oxytocin and male sexual behaviour:

2.3.2.1. Systemic oxytocin and male sexual behaviour:

Melin and Kihlstrom, in 1963, were the first to mention that oxytocin had facilitative effects on male sexual behaviour, reporting that i.v. injection of oxytocin reduced the ejaculation latency in rabbits. In rats, i.v. injection of oxytocin reduces the number of intromissions required to reach ejaculation (Stoneham *et al.*, 1985), and i.p. injection of oxytocin shortens the ejaculation latency and the post-ejaculatory interval (Arletti *et al.*, 1985). Hillegaart *et al.* (1998) reported an increase in plasma oxytocin concentration during copulation in sexually naïve rats and there is good evidence for a pulse of oxytocin secretion at ejaculation (Ivell *et al.* 1997). Although oxytocin receptors have been localized in the male reproductive tract including in the testis and in the prostate (Gimpl & Farenholz, 2001), the mechanisms by which systemic oxytocin acts to facilitate sexual behaviour remain to be identified. Stoneham *et al.* (1985) suggested that systemic oxytocin could 'prime' the male genitalia to respond to subsequent sexual stimuli and therefore facilitate sexual performance.

2.3.2.2. Central oxytocin and male sexual behaviour:

Oxytocin cells are activated during penile erection. Electrical stimulation of the dorsal penile nerve excites 50% of oxytocin cells in the PVN (Yanagimoto *et al.,* 1996) and 60% of oxytocin cells in the SON (Honda *et al.,* 1999), and high concentrations of oxytocin have been measured in the cerebrospinal fluid at ejaculation (Hughes *et al.*,1987). Jirikowski (1992) investigated the effects of three -weeks of sexual activity on oxytocin immunoreactivity in the hypothalamus in male mice. He described that in repeatedly-mated males, oxytocin immunoreactivity was increased in many brain areas including the MPOA, the anterior hypothalamic nucleus, the SON, the PVN and the median eminence. Jirikowski suggested that this increase in oxytocin immunoreactivity was likely to reflect stimulation of synthetic activity in oxytocin neurones, and that sexual behaviour was activating oxytocin production.

Early studies on the central effects of oxytocin on male sexual behaviour had contradictory results. Stoneham *et al.* (1985) reported that i.c.v. injection of oxytocin increased both mount and intromission latencies, suggesting that central oxytocin inhibited copulation. In the other hand, Arletti *et al.* (1985) reported that i.c.v. injection of oxytocin improved copulation by shortening the ejaculation latency and the post-ejaculatory interval. A series of studies by Argiolas and coworkers provided additional information confirming the facilitative role of central oxytocin in the regulation of male sexual behaviour. In 1986, Argiolas *et al.* described oxytocin as "the most potent agent able to induce penile erection so far" in rats, rabbits and monkeys. In rats, they showed that i.c.v. injection of oxytocin (5–90 ng) induced penile erection and yawning behaviour. They also reported that i.c.v. injection of the oxytocin antagonist $d(CH_2)_5$-Tyr(Me)-Orn^8–vasotocin inhibited male copulatory behaviour by decreasing the mount and intromission frequencies and by abolishing ejaculation (Argiolas *et al.*, 1988). Thus, these results illustrate the facilitative role of oxytocin in the central regulation of male sexual behaviour.

It is difficult to say whether oxytocin acts on the anticipatory phase as well as on sexual performance. Most of the studies investigating the role of oxytocin in male sexual behaviour have focused on the ability of oxytocin to induce penile erection. However, penile erection reflects the capacity to copulate, but might not consistently reflect motivation to copulate (Appendix 1). Nevertheless, as described previously, oxytocin plays an important role in socio-sexual behaviour such as partner preference and pair bonding. Melis *et al.* (1999) investigated the effects of oxytocin on sexual arousal. Male rats were injected i.c.v. with the oxytocin antagonist $d(CH_2)_5$-Tyr(Me)-Orn^8–vasotocin and then placed in an apparatus where the access to a receptive female was blocked. The number of non-contact penile erections (NCE, reflecting sexual arousal) was counted. The oxytocin antagonist reduced the number of NCE, suggesting that oxytocin also plays a role in sexual motivation as well as sexual performance. However, more studies clarifying the involvement of oxytocin in the anticipatory phase need to be performed.

2.3.2.3. Central sites of action of oxytocin:

Melis *et al.* (1986) reported that the most sensitive brain area for oxytocin injection to induce penile erection was the PVN. Injection of low dose of oxytocin (3 ng) into the PVN induced penile erection in 60% of the injected -rats; and lesions of the PVN impaired oxytocin –induced penile erection (Argiolas *et al.*, 1987). Witt and Insel (1994) reported that Fos expression was increased in oxytocin neurones in all the parvocellular subdivisions of the PVN during increasing levels of sexual contact. Parvocellular oxytocin neurones project from the PVN to many intra- and extra-hypothalamic areas, including the brainstem and the spinal cord (Fig 1.6), so the sites where oxytocin can act on sexual behaviour might be widely distributed throughout the CNS from the hypothalamus to the spinal cord.

Melis *et al.* (1986) reported that bilateral injections of oxytocin (9 ng) into the CA1 field of the hippocampus induced penile erection. This was supported by a study by Chen & Chang (2001) in which they showed that injection of oxytocin into the hippocampus of male rats increased the intracavernous pressure (ICP: an experimental parameter used to assess penile erection) and that injection of both oxytocin antagonist ($d(CH_2)_5$-Tyr(Me)-Orn^8–vasotocin) and oxytocin into the hippocampus had no effect on the ICP. Thus, these results suggest that oxytocin actions in both the PVN and the hippocampus are involved in the central regulation of penile erection.

A second oxytocin pathway from the PVN has been identified; oxytocin neurones from the PVN project to the spinal cord. Veronneau-Longueville *et al.* (1999) reported the presence of oxytocin binding sites in the thoracolumbar and lumbosacral segments of the spinal cord, areas already described in the 1890s as the "erection – generating center". They showed evidence of synaptic contacts between oxytocin varicosities and lumbosacral parasympathetic pre-ganglionic neurones controlling penile erection.

These studies illustrate that the PVN is at the core of the central actions of oxytocin. Oxytocin is involved in the regulation of copulatory behaviours in different areas within the CNS via oxytocin projections originating from the PVN. For instance, oxytocin regulates penile erection via two pathways originating from the PVN, one reaching the hippocampus, the second one reaching the spinal cord via the ventral medulla.

2.3.2.4. Mechanisms of action of oxytocin:

The mechanisms of action of oxytocin are poorly understood. Oxytocin is part of a complex network of neural systems regulating male sexual behaviour, and while oxytocin mediates the effects of some of these neural systems, some of them regulate the effects of oxytocin.

i. Oxytocin and dopamine incertohypothalamic system:

When injected i.c.v., oxytocin and apomorphine have similar effects on male sexual behaviour: both induce repeated episodes of penile erection and yawning (Argiolas *et al.,* 1986; Melis *et al.*, 1987). Argiolas *et al.* (1987) reported that i.c.v. injection of an oxytocin antagonist prevented penile erection induced by oxytocin and by apomorphine. Although blockade of dopamine receptors impairs penile erection induced by dopamine agonists, it does not disrupt oxytocin -induced penile erection (Argiolas *et al.*, 1988). Melis *et al.* (1989) also studied the effect of concomitant i.c.v. injection of oxytocin and apomorphine on the induction of penile erection; co-injection of oxytocin and apomorphine failed to increase the number of penile erection compared to the number of penile erection induced by the substances given independently. Taken together, these results led Argiolas and colleagues to suggest that dopamine agonists induce penile erection by stimulating the release of oxytocin in the PVN and surrounding structures.

ii. Oxytocin and nitric oxide.

As mentioned in section 1.4.3, the increase in oxytocin transmission facilitating the induction of penile erection is accompanied by an increase in NO production in the PVN and the spinal cord. Indeed, i.c.v. injection of NOS inhibitors inhibit penile erection induced by oxytocin or by apomorphine (Melis & Argiolas, 1993). Melis and Argiolas (1997) measured NO production in the PVN after i.c.v. injection of oxytocin by measuring the concentration of NO_2^- and NO_3^-, the reaction products of newly -formed NO with O_2 and H_2O. They found that i.c.v. injection of oxytocin, at doses that initiate penile erection, increased NO production They also reported that i.c.v. injection of oxytocin antagonists has the same inhibitory effects as i.c.v. injection of NOS inhibitors: both prevent penile erection. Thus, they suggested that NO induces penile erection by activation of central oxytocin transmission.

iii. Oxytocin and testosterone

Melis *et al.* (1994) reported that the facilitative effects of oxytocin on penile erection were prevented by castration and were restored by testosterone replacement or by its metabolites (DHT and estradiol). Tribollet *et al.* (1990) showed that testosterone increased oxytocin binding in the brain in male rats and that castration reduced it. Arletti *et al.* (1992) suggested that a threshold of testosterone was required for oxytocin to improve male sexual behaviour. Although testosterone restored the effects of oxytocin in castrated rats, an increased dose of testosterone did not enhance the effects of oxytocin.

iv. Oxytocin and opioids

As mentioned in section 1.4.2, opioids have inhibitory effects on copulatory behaviours, and hence on penile erection. Melis *et al.* (1992) reported that in the PVN, opioids interacted with oxytocin neurones mediating the penile erection. They suggested that opioids prevented penile erection by inhibiting the oxytocin transmission possibly through μ–receptors located on oxytocin neurones.

Thus, oxytocin has a facilitative role in male sexual behaviour. Although, the effects of oxytocin on copulatory behaviours, especially on penile erection, are well documented, the role of oxytocin in sexual motivation needs further investigation. In the regulation of sexual performance, oxytocin has a crucial role, as it acts as a central mediator of some neurotransmitter-mediated effects (e.g.: dopamine). Oxytocin transmission is controlled by inhibitors (e.g.: opioids), activators (nitric oxide) and permissive compounds (e.g.: gonadal steroids) whose interactions can influence each other's effects.

PART 3. α-MSH and male sexual behaviour:

3.1. α-MSH system:

3.1.1. Structure, secretion and distribution:

The peptide alpha -melanocyte–stimulating–hormone (α-MSH) is part of the melanocortin system, which consists of two subgroups of peptides with structural similarities: melanotropins (α-, β-, γ- MSH), and corticotropin (ACTH). The melanocortins derives from the proopiomelanocortin (POMC) prohormone. Depending of the expression of different cell–specific enzymes, the POMC prohormone is processed by enzymatic cleavage in different melanocortin peptides and in the opiate-like peptide β-endorphin (Fig 1.7). The melanocortins and opiate-like peptides have been detected in a variety of peripheral tissues (e.g.: pituitary gland, blood circulation, kidney, intestine, adrenal glands, pancreas, ovaries, testis, placenta and skin) and in the central nervous system.

α-MSH is produced in the melanotrophs cells in the intermediate lobe of the pituitary gland from which it can be secreted into the general circulation. The secretion of α-MSH from the intermediate lobe is under the influence of intra and extra - hypothalamic regulatory factors such as for instance dopamine or serotonin, which respectively inhibits or stimulates the exocytosis of α-MSH secretory vesicles. Once secreted, α-MSH then behaves as a classic hormone.
α-MSH is also produced within the brain in neurones in the dorsomedial hypothalamus and in the arcuate nucleus of the hypothalamus. α-MSH released within the brain can then act as a neuromodulator. Nerves fibres containing α-MSH project from the arcuate nucleus to numerous areas within the CNS including the hypothalamus, the thalamus, the mesencephalon, the amygdala, the hippocampus, the cortex, the medulla and the spinal cord (O'Donohue & Jacobowitz, 1980).

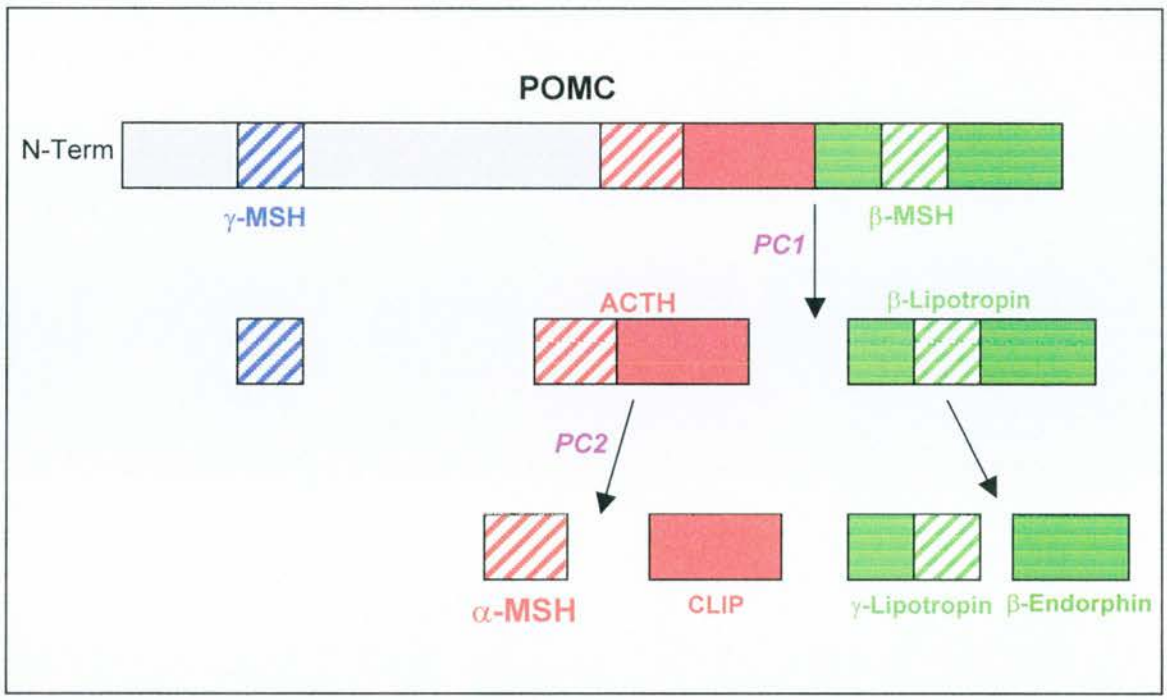

Figure 1.7: Post-translational products of the prohormone proopiomelanocortin.

A first proteolytic cleavage of POMC by enzyme proconvertase 1 (PC1) generates ACTH and b-lipoproteic hormone. The cleavage of ACTH by enzyme proconvertase 2 (PC2) generates α-MSH. The processing of POMC in a tissue dependent manner. *Adapted from Textbook "An introduction to neuroendocrinology" by R.E. Brown. Cambridge University Press.*

3.1.2. Melanocortin receptors:

Five sub-types of melanocortin receptors (MC1 to MC5) have been identified (Adan & Gipsen, 1997; Hadley *et al.*, 1999; Gantz & Fong, 2003) and each sub-type derives from a separate gene (Abdel-Malek, 2001). Melanocortin receptors (MCR) are G-protein coupled receptors. They have seven transmembrane domains with several N-glycosylation sites in their N-terminal domains. MCR are coupled to the Gs protein and adenylate cyclase and therefore mediate their effects by activating the cAMP-dependent signaling pathway. However, the MC3 receptor has also been associated with increases in intracellular Ca^{2+} via the inositol triphosphate pathway and the MC5 receptor has also been associated with the JAK/STAT pathway. MCR may be regulated by phosphorylation as all subtypes contain recognition sites for protein kinase C and protein kinase A. The five subtypes of MCR have different affinities for the melanocortins and the endogenous antagonists agouti and AGRP. The MCR affinities and the MCR distribution and principal identified functions are summarized in Table 1.4.

3.2. Functions of α-MSH:

α-MSH is involved in a variety of physiological functions and behaviours including hair and skin coloration, inflammation, immunomodulation, nociception, nerve regeneration, regulation of cardiovascular functions, energy homeostasis, thermoregulation, avoidance behavior (learning and memory), grooming, yawning and sexual behaviour (see review Hadleyet al, 1999; Gantz & Fong, 2003; Starowicz & Przewlocka, 2003). Some of the most well -known functions are briefly described below:

3.2.1. α-MSH and pigmentation:

α-MSH is involved in skin and hair coloration. In lower vertebrates, α-MSH regulates the rapid color changes. In mammalian species, α-MSH, via MC1R, stimulates the synthesis of the brown pigment eumelanin produced in the melanocytes.

Receptor	Affinity (EC_{50})	Sites of expression	Functions
MC1	α-MSH (2pM)= ACTH (8pM)> β-MSH (10^{-10}M)> γ-MSH	Melanocytes, testis, corpus luteum, placenta, macrophages, neutrophils, endothelial cells, fibroblasts, glioma cells, astrocystes, pituitary, periaqueductal gray.	Pigmentation, immunomodulation, anti-inflammation.
MC2	ACTH	Adrenal cortex, adypocytes.	Stereoidogenesis.
MC3	α-MSH (4nM)= β-MSH= γ-MSH= ACTH	Hypothalamus, medial habenula, lateral septum, ventral tegmental area. Placenta, duodenum, pancreas, stomach.	Functions to be clarified. Cardiovascular functions, anti-inflammation, thermoregulation, feeding behaviour.
MC4	α-MSH (24 nM)= ACTH (18nM) > β-MSH (?) >> γ-MSH (>100nM)	Hypothalamus, olfactory cortex, septum, hippocampus, superior colliculus, brainstem, penis, spinal cord.	Feeding behavior, weight homeostasis, hyperalgesia, pain, grooming and sexual behaviour, stress.
MC5	α-MSH (0.3-1 nM)> ACTH(3-10 nMnM)= β-MSH >> γ-MSH (30-100nM)	Cortex, cerebrellum. Skin, adrenal gland, skeletal muscles, bone marrow, spleen, thymus, testis, ovary, uterus, lung, liver, thyroid, thymus, stomach, kidney, exocrine glands.	Exocrine gland secretion.

Table 1.4: Melanocotin receptors, affinity for agonists, CNS and peripheral major sites of expression and primary functions. *Adapted from Abdel-Malek (2001), Starowicz et al. (2003) and from Adan et al. (1997).*

Conversely, the antagonist agouti peptide induces the synthesis of the yellow pigment phaeomelanin. The final coloration is the result in the competition amongst α-MSH/ ACTH and agouti peptides.

3.2.2. α-MSH and inflammation:

Central and peripheral α-MSH has anti-inflammatory properties. It reduces different types of inflammatory responses such as acute and chronic inflammation and contact hypersensitivity (Starowicz & Przewlocka, 2003). α-MSH injected centrally reduces brain inflammation but also inflammation in peripheral tissues (Rajora *et al.*, 1997; Lotti *et al.*, 2002). The anti- inflammatory effects of α-MSH are though to be mediated via MC1R and via MC3R as these two sub-types are expressed in many cells involved in the regulation of inflammatory processes (macrophages, monocytes, neutrophils, fibroblasts and endothelial cells).

3.2.3. α-MSH and energy homeostasis:

Centrally acting melanocortins are involved in the regulation of food intake and body weight. α-MSH injected i.c.v inhibits food intake (Pogglioli *et al.*, 1986, McMinn *et al.,* 2000), and compared to the other POMC-peptides, α-MSH is the most potent regulator of feeding. Over-expression of agouti peptide in the hypothalamus leads to hyperphagia, reduced energy expenditure and obesity (Lu *et al.*, 1994). The hypotrophic effects of α-MSH are thought to be mainly mediated by MC4R. Injection of the MC4R antagonist, SHU-9119, blocks the inhibitory effect of the melanocortin agonist Melanotan-II (Fan *et al.*, 1997), and mutation in the MC4R gene lead to obesity associated with hyperphagia (Huszar *et al.*, 1997) as described for the over-expression of the MCR antagonist agouti. MC3R knockout mice are obese but with reduced food intake (Chen *et al.,* 2000), MC3R may be implied in the regulation of feeding behaviour too. α–MSH and interact with other systems regulating the energy homeostasis. Indeed, melanocortins have been reported to mediate the effects of leptin and to control the expression of the neuropeptide Y (Vergoni & Bertolini, 2000).

3.3. α-MSH and sexual behaviour:

3.3.1. α-MSH and female sexual behaviour:

In rats, α-MSH injected into the VMN, into the MPOA, or at the median eminence, stimulates sexual activity and lordosis behaviour (Gonzalez *et al*, 1993; Scimonelli *et al.*, 2000). Gonzalez et al. (1993) showed that injection of α-MSH into the VMN induced noradrenaline release while enhancing sexual activity. Scimonelli *et al.* (2000) suggested that α-MSH effects were mediated by noradrenaline acting on β-adrenergic receptors. Indeed, injection of a β- adrenergic antagonist into the median eminence eliminated the facilitative effects of α-MSH. The effects of α-MSH on sexual behaviour are mediated by the central MC3R and/or MC4R, as injection of a MCR antagonist into the VMN eliminates the effects of α-MSH on lordosis in a dose - dependent manner (Cragnolini *et al.*, 2000). Nonetheless, whether it is the MC3R or the MC4R that mediate the central effects of α-MSH in the regulation of female behaviour is still not known.

3.3.2. α-MSH and male sexual behaviour:

3.3.2.1 Systemic α-MSH and male sexual behaviour:

No studies have reported a modification of α-MSH secretion during male sexual behaviour, as no studies have measured plasma α-MSH concentration in males during copulation. However, Wessells *et al.* (1998) reported that s.c injection of the α-MSH analogue melanotan-II induces penile erection in normal men and in men with psychogenic erectile dysfunction, even at very low doses (0.025mg/k.g). Melanocortin receptors have been identified in the male genital tract: MC1R and MC5R can be found in the testis (Abdel-Malek, 2001), and MC4R mRNA has been localized in the penis (Van der Ploeg *et al*, 2002). The role of MC4R in the peripheral regulation of male sexual behaviour has been investigated by studying the effects of the MC4R agonist THIQ (tetrahydroisoquinoline). Martin *et al.* (2002) reported that i.v injection of THIQ

increased the number of erection in a dose-dependent manner, and that these effects were blocked by the iv injection of a MC4R antagonist. Thus, systemic α-MSH facilitates male sexual behaviour but more investigation is required to clarify the role of α-MSH in the peripheral regulation of male sexual behaviour.

3.3.2.2. Central α-MSH and male sexual behaviour:

The involvement of the melanocortin system in the central regulation of the male sexual function has been known since the study by Ferrari and colleagues in 1963. They found that i.c.v injection of ACTH, α-MSH and related peptides in common laboratory animals including rats, induced the "stretching-yawning syndrome" associated with repeated episodes of penile erection and ejaculation. i.c.v injection of α-MSH and ACTH have been since reported to reduce ejaculation latencies and the number of mounts and intromissions required to achieve ejaculation in sexually -experienced rats (Argiolas,1999).

The central mechanisms of action of α-MSH in the regulation of sexual behaviour have yet to be clarified. The effects of α-MSH are likely to be mediated by the MC3R and/ or the MC4R, as they are the MCR the most abundantly expressed within the CNS. Most evidence implicates the MC4R in the regulation of penile erection. Martin *et al.* (2002) reported that central administration of the selective MC4R agonist THIQ increased the intracavernosal pressure and the number of reflexive penile erections in rats, but some contradictory data refute the MC4R involvement in favor of the MC3R. Vergoni *et al.* (1998) showed that injection of the putative specific MC4R antagonist HSO14 into the hypothalamus failed to block penile erection induced by α-MSH at doses that impaired the stretching and yawning reflex.

Although these discrepancies are still not resolved, it is clear that α-MSH effects on male sexual behaviour are modulated by other systems. Indeed, the facilitative effects of ACTH and α-MSH are eliminated by castration but restored by testosterone,

confirming that testosterone has a permissive role in the expression of sexual behaviour (Bertolini, 1975). A pre-treatment with morphine blocks penile erection induced by ACTH, suggesting that opioids interfere with the melanocortins system (Melis & Argiolas, 1995). Finally, NO is likely to be the final mediator of α-MSH effects as NOS inhibitors prevent α-MSH induced penile erection (Benelli *et al.,* 1995; Melis & Argiolas, 1995).

So far, it is difficult to say whether α-MSH plays a role in the regulation of sexual motivation as well as in the modulation of sexual performance. As α-MSH injections have not been reported to facilitate socio-sexual interaction in rats and to stimulate sexual activity in sluggish rats (Argiolas, 1999), it has been assumed that α-MSH and related peptides were involved in the regulation of sexual copulatory performance rather than in sexual motivation. However, Wessells *et al.* (2000) showed that in men, s.c injection of melanotan-II increased sexual desire, suggesting that α-MSH does stimulate sexual motivation. Therefore, additional studies specially designed to investigate the role of α-MSH in sexual motivation are required to clarify these discrepancies.

What is the hypothesis of this PhD thesis?

Oxytocin and α-MSH are involved in the central regulation of a variety of physiological functions and behaviours. Remarkably, the central effects of α-MSH and oxytocin in the regulation of these behaviours are very similar: Oxytocin and α-MSH both reduce food intake when injected centrally (Vergoni *et al.,* 1986; Arletti *et al.,* 1989); they both induce grooming and yawning behaviours (Richard *et al.*, 1991, Argiolas, 2000); and in the regulation of sexual behaviour, oxytocin and α-MSH have similar facilitative effects (sections 2.3.2 & 3.3.2): They enhance female sexual behaviour, induce penile erection, and stimulate sexual performance in males. They both induce penile erection when injected into the PVN, which indicates that the PVN is a shared site of action for both oxytocin and α-MSH in the regulation of male sexual behaviour. Both oxytocin and α-MSH actions are inhibited by opioids and by NOS inhibitors, suggesting that NO is the key mediator of oxytocin and α-MSH effects in the regulation of penile erection. Thus, these observations are persuasive evidence that central actions of oxytocin and α-MSH in the regulation of male sexual behaviour are not independent.

Anatomical evidence also supports the idea of an interaction between oxytocin and α-MSH. α-MSH fibres innervate the SON and the PVN where oxytocin is produced (O'Donohue, 1979); and MC3 receptors and MC4 receptors mRNA expression have been localized in the hypothalamus and high levels of mRNA expression for the MC4R have been found in the SON and in the PVN (Mountjoy, 1994; Adan, 1997).

Finally, previous studies have reported that α-MSH has modulatory effects on oxytocin neuronal activity, as i.c.v injection of α-MSH induces Fos expression in oxytocin neurones in the SON and in the PVN (McMinn, 2000; Olszewski, 2001).

Taken together, these observations lead to the hypothesis that oxytocin and α-MSH interact to enhance male sexual behaviour; in particular,that some effects of oxytocin are mediated by α-MSH, or alternatively, some effects of α-MSH are mediated by oxytocin.

What are the objectives of this PhD thesis?

The objectives of this thesis are to complete the knowledge of the actions of oxytocin and α-MSH in the regulation of male sexual behaviour, and to test whether their actions are not independent. To do so the following questions were asked:

1. Do both peripheral and central α-MSH play a role in the regulation of male sexual behaviour?
   (Although α-MSH given centrally or systemically enhances male sexual behaviour, no studies have yet investigated the changes in α-MSH systemic secretion and in neuronal activity of α-MSH –containing neurones during male sexual behaviour.)

2. At which level (central and/or peripheral) do oxytocin and α-MSH interact?

3. Does oxytocin that modulates the actions of α-MSH or alternatively, does α-MSH modulate the effects of oxytocin? Does blocking the effects of one influence the actions of the other?

4. Does the interaction between oxytocin and α-MSH occur during male sexual behaviour?

5. What is the influence of this interaction on the regulation of male sexual behaviour? Does blocking this interaction impair the behaviour?

What is the interest in studying interactions between oxytocin and α-MSH in the regulation of male sexual behaviour?

Male sexual behaviour is a complex behaviour, whose regulation mechanisms involve numerous systems that are likely to interact to enable the behaviour to occur normally, and most of these regulatory mechanisms remain unclear. Investigating the interaction between oxytocin and α-MSH will allow a better understanding of the physiological role of two of the most potent peptides identified so far to induce penile erection and enhance male sexual behaviour. The clarification of their mechanisms of action could give new ideas and tools to develop new pharmaceutical strategies to solve male sexual disorders such as psychogenic erectile dysfunction.

To a larger extent, if the interaction between oxytocin and α-MSH is underlined, this interaction could be transposed to other behaviours in which oxytocin and α-MSH have similar effects, and could open new areas of research in the comprehension of regulatory mechanisms of these behaviours.

Chapter 2

General methods

2.1. Animals

Sprague-Dawley adult male (350-450g body weight) and female (250g body weight) rats from Bantin & Kingman were used. Following ethical guidance and regulations (Animals (Scientific procedures) Act 1986), animals were housed in the Medical Faculty animal facility at Edinburgh University (MFAA) in stock cages of a maximum of 5 rats. Rats were kept in ambient temperature (22°C) with relative humidity at 55%(± 10%) and with food and water *ad libitum* on a 12 hour light –dark cycle (light: 7:00h – 19:00h). From their day of arrival, rats were allowed a minimum of one week of acclimatisation before any experimental use.

2.2. Non -surgical procedures

2.2.1. Sub-cutaneous injection (s.c.)

The rat was laid on its stomach. The injection was made using a sterile 23-gauge needle inserted into the skin at back of its neck parallel to the body wall.

2.2. Intraperitonal injection (i.p.)

The rat was restrained on its back with the low abdomen extended. The abdominal muscles were gently pushed up. A sterile 23-gauge needle was inserted through these muscles to reach the peritoneal cavity. The needle was slightly withdrawn, without exiting the peritoneal cavity before the drug was injected.

2.3. Anaesthesia & analgesia

In non-recovery experiments, rats were given an i.p. injection of 25% urethane solution (ethyl carbamate, 1.25 mg/ kg rat; Sigma, UK). Urethane induces an increase in Fos expression in the brain, including the hypothalamus (Takayama et al., 1994). Therefore, rats were anaesthetised with an i.p. injection of sodium pentobarbitone (Sagatal, Rhone Merieux, 60mg/kg, 1ml/ kg rat) in all the experiments including Fos expression studies.

In recovery experiments, rats were anaesthetised under halothane (Fluothane, Merial, UK) inhalation: they were first placed in a box with 5% halothane in

600ml/min both oxygen and nitrous oxide and then maintained anaesthetised throughout the surgery with 3% halothane in 1200ml/ min both oxygen and nitrous oxide.

To minimise post-surgery pain, rats were given 0.05ml s.c. of the analgesic Zenecarp (C-Vet/ Products, Grampan Pharmaceuticals Ltd, UK) before surgery.

2.4. Surgery

All procedures were carried out under sterile conditions using autoclaved instruments. The fur was shaved over the incision sites and the skin was disinfected. After each recovery surgery, rats were given 1 ml i.p. or i.v. of 0.9% physiological saline to compensate for any fluid loss. Rats were allowed up to 20 min in a recovery box at 35°C then placed in a clean single cage for a minimum of 3 days to recover before experiment.

2.4.1. Jugular vein cannulation *(performed by Dr A.J. Douglas)*

To withdraw blood samples and to administer drugs intravenously, anaesthetised rats were fitted with a cannula into the jugular vein.

A 1cm incision was made through the skin from the right mandible to the right clavicle to expose the right jugular vein. Two ligatures (5-0 size silk thread, F.S.T., UK) were placed around the vein. The cranial ligature was tightened to avoid blood loss. A silastic catheter (Bore: 0.5mm; Wall: 0.25mm; Altec, UK) containing heparin saline (0.9% saline with heparin 5000 Units/ ml; Sigma, UK) was inserted into the vein for 3 cm reaching the right atrium of the heart. A small amount of blood was withdrawn and then re-infused with heparin saline to check that the blood could be easily withdrawn. The cannula was then secured by tightening the second ligature. Finally, the cannula was exteriorised at the back of the neck, sealed with a blocker (half cut 23-gauge needle sealed with glue), fixed in place with adhesive tape (1 cm width) sutured to the skin with two stitches (2-0 size silk thread) and the ventral incision was sutured closed.

2.4.2. Intracerebroventricular (i.c.v.) cannulation

To infuse drugs into the brain, rats were fitted with an i.c.v. cannula. The anaesthetised rat was placed in a stereotaxic frame (ASI instruments); two ear bars fixed to the frame restrained the rat in position. An incision was made through the scalp to expose the dorsal surface of the skull. Skin and connective tissues were moved apart. To make sure that the brain was horizontal, level of Bregma and Lambda was checked and adjusted if necessary. A hole was drilled in the skull at 0.6mm posterior and 1.6mm lateral to Bregma ("The rat brain in stereotaxic coordinates" G. Paxinos & C. Watson, Academic Press). An i.c.v. guide cannula (22-gauge, 4.5mm length, Bilaney Consultants Lts, USA) was inserted into this hole reaching a depth of 4.5mm from the surface of the skull. Its position was secured onto the surface with two jeweller screws and dental cement (Kemdent, Associated Dental Products Ltd, UK). The skin was replaced above the hard cement and two sutures with 2-0 size silk thread were made around the guide cannula. Finally, a cap rinsed with saline was inserted into the guide cannula.

2.4.3. Preparation & placement of a microdialysis probe onto the supraoptic nucleus (SON)

To infuse drugs onto the SON and to collect dialysates from the same area, sagatal-anaesthetised rats were fitted with a microdialysis probe (membrane 1 mm length, 0.5 mm diameter; CMA-12, CMA/Microdialysis, Stockholm, Sweden) adjacent to the right supraoptic nucleus.

2.4.3.1. Preparation of the microdialysis probe

The probe was placed in a vial filled with artificial cerebrospinal fluid (aCSF: 124mM NaCl, 26mM $NaHCO_3$, 3mM KCL, 1.24mM KH_2PO_4, 2.1mM $CaCl_2$ in distilled water). A 1-ml glass microsyringe (Hamilton, Aldrich) gas-tight and filled with degassed aCSF, was mounted onto an automatic microinjection pump (Stoelting Co, USA) and was connected to a liquid switch with 25 cm of FEP tubing (CMA/ Microdialysis) containing 3µl aCSF. The inlet cannula of the probe was connected to the same liquid switch with 25 cm FEP tubing containing aCSF.

Ten cm of empty FEP tubing were connected to the outlet cannula of the probe in order to facilitate the sample collection into a 100µl eppendorf tube.
The probe contains glycerol while packaged; so to flush out the glycerol, the probe was perfused with ethanol at 40µl/min, in a vial filled with 70% ethanol for 4-5 min. Back in a vial filled with aCSF, the probe was perfused at 40µl/min another 5 min with aCSF to wash out the ethanol and air. To make sure that no air bubbles were trapped inside the membrane, the probe was checked under a microscope.
The probe was then perfused at a 1.5µl /min during the surgery and the experiment.

2.4.3.2. Surgery

Using the same surgical approach as for i.c.v. cannulation (see section 2.4.2.), a perfused microdialysis probe supported by stereotaxic manipulator was inserted into the brain at 0 mm rostrocaudal to Bregma, 1.5mm lateral to Bregma and to a depth of 9.5mm from the surface of the skull.

2.5. Drug administration procedures via surgically fitted cannulae

2.5.1. Intravenous injection (i.v.)

The i.v. cannula was connected to a 1-ml graduated plastic syringe by a silastic tubing filled with 100µl heparin saline. The blood was withdrawn to the tip of the needle; the drug was injected using a second syringe followed by further heparin saline to flush the drug into the bloodstream.

2.5.2. Intracerebroventricular injection (i.c.v.) & injection site examination

2.5.2.1. i.c.v. injection

The tubing containing the drug was connected to the i.c.v. injection cannula inserted into the guide cannula. Using a 10µl Hamilton syringe, the drug was injected at 1µl/ 30 s. A maximum volume of 5µl was injected.

2.5.2.2. Injection site examination

For hormone secretion studies, the injection site was checked by injecting i.c.v. 2µl of a blue dye (5% alcian blue) after the rat was dead. The brain was then

removed and cut with a scalpel blade to check the presence of blue dye in one of the lateral ventricles. For immunocytochemistry studies, the dye was not used, the presence of a cannula tract through the brain to one lateral ventricle was checked under a microscope after the brain had been sectioned with the freezing microtome. Only brains with confirmed correct injection site were analysed.

2.5.3. Drugs infusion onto the SON & infusion site examination

Microdialysis probes were used to deliver drug onto the SON. The drug was chronically infused at 0.75μl/ 30 s. The correct infusion site was checked by looking for a tract through the brain to the vicinity of one SON (anterior) after preparation of brain sections.

2.6. Blood sample collection:

An hour before the first blood sample was collected; the jugular vein cannula was connected to 50cm silastic tubing (Bore: 0.50mm, Wall: 0.50mm; Altec, UK) joined to a 1 ml plastic syringe containing heparin saline. When taking each sample, blood was withdrawn to the tip of this syringe. The blood sample was then collected with a second syringe and placed in a 1.5-ml eppendorf tube containing 10μl of a solution of protease inhibitor (aprotinin 0.039TIU, Sigma, UK) and 60μl of EDTA 5% (anti-coagulant calcium chelating agent, Sigma, UK). Unless specified otherwise, 0.4 ml of blood was withdrawn and, then replaced with the same volume of 0.9% saline to retain a constant volume of blood. Samples were stored in ice (- 4°C) until centrifugation. Plasma was separated from red cells by centrifugation (13,000 r.p.m. for 2-3min) and stored at -20°C until the day of the radioimmunoassays.

2.7. Radioimmunoassays:

2.7.1. General principles

Radioimmunoassay was used to obtain accurate measurements of plasma peptide concentrations. Radioimmunoassay is based upon the competition of ^{125}I-peptide and peptide (in either standard or unknown samples) binding to a limited quantity of antibodies specific against the peptide. As the quantity of peptide in

standards or in unknown samples in the reaction increases, the amount of ^{125}I-peptide able to bind to the antibody is decreased. The complex antibody-bound ^{125}I- peptide can be separated from the free fraction using two different methods:

i. Second antibody method: a second antibody is used to bind the primary antibody to form a high molecular weight complex that can be separated from the free fraction by centrifugation.

ii. Double antibody -polyethylene glycol precipitation method: The polyethylene glycol induces the precipitation of the high molecular weight complex antibodies (primary + secondary)-bound ^{125}I- peptide, which can be separated from the free fraction by centrigugation.

After centrifugation and aspiration of the supernatants, the radioactivity of the precipitates (^{125}I-peptide bound) is measured using an Auto-Gamma Counter (LKB Wallac, Finland). By measuring the amount of ^{125}I-peptide bound as a function of the peptide concentration in the standards, it is possible to construct a standard curve from which the concentration of peptide in unknown samples can be determined using an Ultraterm 2 software programme.

2.7.2. Precision and sensitivity of an assay

Intra-assay coefficient of variation:

To assess variations between samples within an assay, stock solutions of known hormone concentration were measured in duplicated at the beginning and end of each assay. Intra-assay coefficient was calculated using results of one specific standard from the assay using the following equation:

$$\textbf{Intra-assay coefficient of variation} = \frac{SD}{X} \times 100$$

Where X is the mean of hormone concentration from all intra-assay tubes of a specific standard and SD is the standard deviation around that mean.

Inter-assay coefficient of variation:

To assess variations between different assays from comparable experiments, stock solutions of known hormone concentrations from previous assay were placed into the

new assay. The variation was calculated using results from one specific standard for all the assays and using the following equation:

$$\textbf{Inter-assay coefficient of variation} = \frac{\textbf{SD}}{\textbf{X}} \times 100$$

where X is the mean of hormone concentration from all inter-assay tubes of a specific standard for all the assays and SD is the standard deviation around that mean.

Assay sensitivity:

To assess the assay sensitivity, the amount of radioactivity bound by each standard (B) was expressed as a percentage of the maximum binding in the assay (Bmax) using the following equation (A):

Equation(A):

$$\textbf{\%B/Bmax} = \left[\frac{\textbf{(Mean cpm of std- mean cpm of NSB)}}{\textbf{(Mean cpm of Bmax- Mean of NSB)}} \right] \times 100$$

where NBS is the average number of counts for the non-specific binding tubes.

A graph is created by plotting %B/Bmax against the log of the hormone concentration of each standard. The sensitivity of the assay is the concentration of the peptide for which the %B/Bmax obtained is the closest to the sensitivity calculated with the following equation (B):

Equation (B):

$$\textbf{\%Sensitivity} = \left[\frac{\textbf{[Bmax-(2} \times \textbf{SD}_{\textbf{Bmax}}\textbf{)] – NSB}}{\textbf{Bmax - NSB}} \right] \times 100$$

Using the graph, the percentage of sensitivity of the assay is converted a hormone concentration.

2.7.3. Oxytocin radioimmunoassay

The assay procedure was adapted from one elaborated by Prof. T. Higuchi (Higuchi et al., 1985). They developed a sensitive and specific radioimmunoassay to measure oxytocin concentration in the blood. They raised an antibody specific against oxytocin after several injections of an oxytocin –bovine serum albumin conjugate into two female rabbits. They tested the specificity of the antibody and the crossreactivity: The anti oxytocin serum used showed no crossreactivity with arginine-vasopressin, arginine-vasotocin or with any of the products from the degradation of oxytocin.

2.7.3.1.Reagents:

Assay buffer

0.125g sodium phosphate (monobasic)	(Sigma)
0.594g sodium phosphate (dibasic)	(Sigma)
2.55g bovine serum albumin	(Sigma)
0.5g sodium azide	(Sigma)
500ml distilled water	

Antibody buffer

Normal rabbit serum diluted at 1:400 in assay buffer. (SAPU)

Rabbit anti-rat oxytocin, THF3

(Provided by Prof. Higuchi.)

Primary antibody diluted at 1:200,000 in antibody buffer.

Standard oxytocin (4^{th} international standard, National Institute for Biological Standards and Control)

Standards were diluted in assay buffer from an oxytocin stock solution of 50ng/ml. Eleven concentrations of standard were used: 2.4, 4.8, 9.7, 19.5, 39.1, 78.1, 156, 312, 625, 1250 and 2500 pg/ml.

^{125}-I-oxytocin (NEN Life Science Products)

Reconstituted in assay buffer to obtain a concentration between 7,000 and 10,000 cpm/ 50µl.

Donkey anti-rabbit IgG serum (IDS Ltd)

Secondary antibody diluted at 1:25-50 in assay buffer

Pansorbin cells (Novabiochem Ltd)

Diluted at 1:25-50 in assay buffer.

Controls

Inter/intra-assay standards were made from the oxytocin stock solution of 50ng/ml and diluted in assay buffer at 1000, 500, 250, 50 and 10pg/ml.

2.7.3.2. Methods:

Day one

1. 50µl of standards and inter-assay standards were added in triplicates to their respective assay tubes (0.75ml polypropylene tubes, Luckman LP2). 50µl of unknown samples were added in duplicates to their respective assay tubes.
2. 50µl of rabbit anti- oxytocin serum was added to all tubes. To measure non-specific binding, three tubes received 50µl of normal rabbit serum instead of anti-oxytocin serum (NSB tubes).
3.Tubes were vortex-mixed and incubated at 4°C for 24 h.

Day two/ three

1. 50 µl of ^{125}-I-oxytocin were added to each tube. To measure total counts, three tubes only received ^{125}-I-oxytocin (TC tubes).
2. Tubes were vortex-mixed and incubated at 4°C for 48 h.

Day four/ five

1. 50µl of the anti rabbit IgG serum were added to all the tubes except the TC tubes.
2. Tubes were vortex-mixed and incubated at 4°C for 48 h.

Day six

1. 50µl of pansorbin cells were added to all tubes to facilitate the visualisation of the pellet.

2. Tubes were centrifuged at 1700g (3,000 r.p.m.) at 4°C for 30 min.
3. Supernatants were removed by aspiration immediately after centrifugation.
4.Radioactivity in each tube was counted using a Gamma Counter.

A standard curve and a binding curve obtained from an oxytocin radioimmunoassay are shown in Figure 2.1.

2.7.4. α-MSH radioimmunoassays

α-MSH plasma concentrations were determined using two different commercially available kits. The first one (Phoenix pharmaceuticals, INC, Belmont, USA) required extraction of the peptide from the plasma before the radioimmunoassay procedure. The extraction procedure increased the number of manipulations of the samples and therefore the risk of mistakes, to avoid this; a second kit (Euro-diagnostica, Malmö, Sweden) was used to measure the concentration of α-MSH from un-extracted plasma samples.

2.7.4.1. Determination of α-MSH concentration from extracted plasma samples:

i) Extraction of α-MSH from plasma:

Elution solvents:

Buffer A: 1% trifluoroacetic acetic (TFA) in H_2O.
Buffer B: 60% acetonitrile in 1% TFA.

Methods:

Plasma samples were acidified with an equal amount of buffer A and loaded onto a C-18 SEP-COLUMN (Lida, NY, USA). The column was slowly washed with 3ml of buffer A (twice), the wash was discarded. α-MSH peptide was eluted with 3ml of buffer B. The eluant was collected into a tube and evaporated to dryness in a centrifugal concentrator. The final residue was dissolved in 250µl of RIA buffer for radioimmunoassay.

ii) Assay protocol:

Reagents: (Phoenix Pharmaceuticals kit)

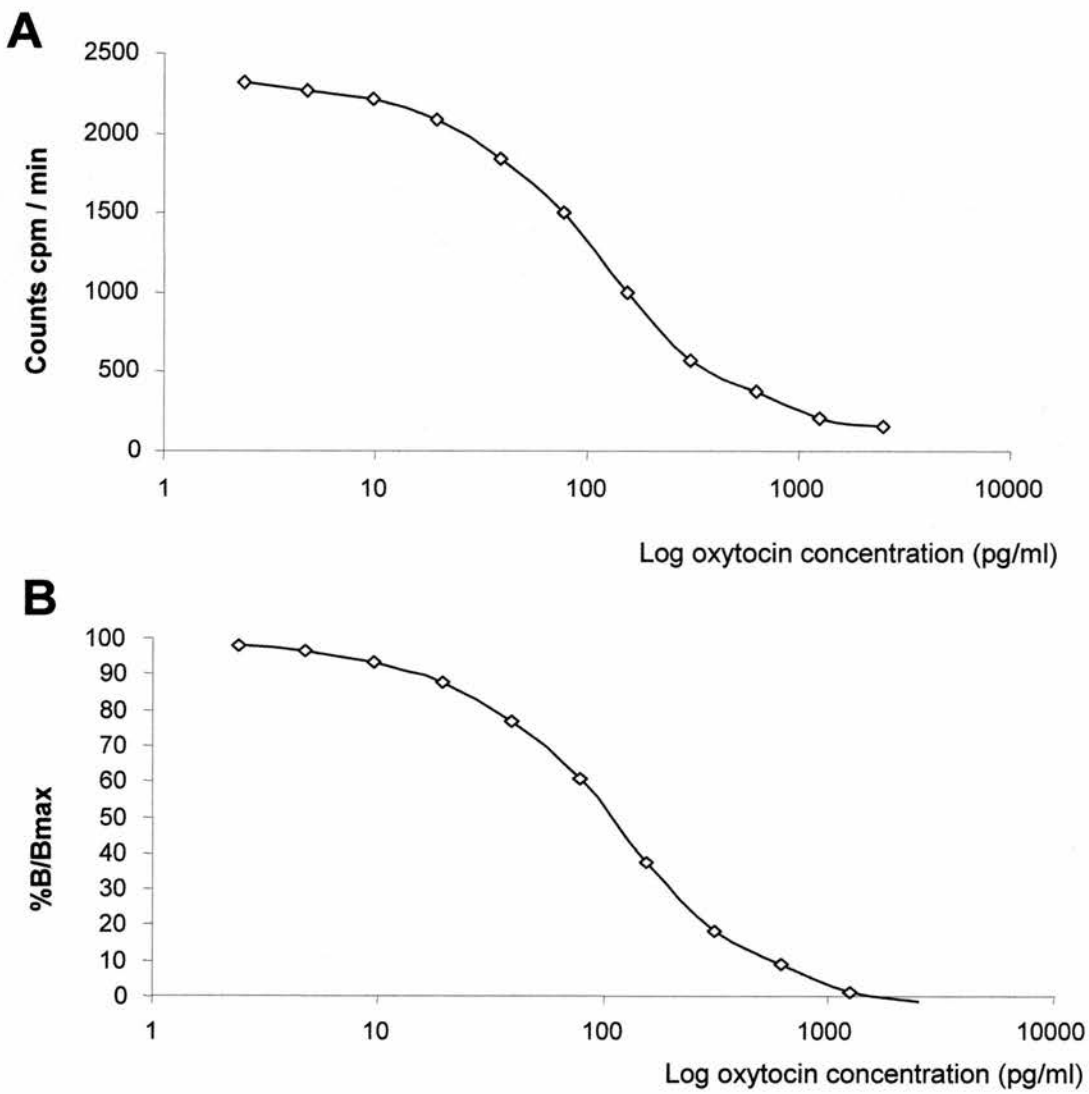

Figure 2.1: Standard curve (A) and binding curve (B) from an example of oxytocin radioimmunoassay.

The sensitivity of the assay is calculated using the equation (B) described in section 2.7.2. The sensitivity of this example of radioimmunoassay is found to be around 5.5pg/ml.

RIA buffer

19nM monobasic sodium phosphate

81nM Bibasic sodium phosphate

0.05 NaCl

0.1% Bovine Serum Albumine

0.1% Triton-X

0.01% NaN_3

α-MSH Standards

Eight concentrations of standard were used: 1, 2, 4, 8,16, 32, 64 and 128pg/tube.

Rabbit anti α-MSH serum

Rabbit antiserum raised against α-MSH conjugated to bovine serum albumine and reconstituted in 13ml RIA buffer.

The anti α-MSH serum used in this kit is directed to the C-terminal part of the α-MSH molecule and shows no crossreactivity with Neuropeptide Y, CART, AGRP and Leptin.

^{125}I-α-MSH

^{125}I-α-MSH was reconstituted with 13ml of RIA buffer to obtain a concentration between 8,000 and 10,000 cpm / 100µl solution.

Goat anti-rabbit IgG serum

Reconstituted with 13ml of RIA buffer.

Normal rabbit serum

Reconstituted with 13ml of RIA buffer.

Methodology:

Day one

1. To measure non-specific binding, 200µl RIA buffer was added to two tubes (NSB tubes). To measure total counts, 100µl RIA buffer was added to two tubes (TC tubes).
2. 100µl of standards and samples were added in duplicates in their respective tubes.

3. 100µl of primary antibody rabbit anti-α-MSH serum was added to all tubes except the TC tubes and the NSB tubes.

4. Tubes were vortex-mixed and incubated at 4°C for 24 h.

Day two

1. 100µl of ^{125}I-α-MSH was added to all tubes.

2. Tubes were vortex-mixed and incubated at 4°C for 24 h.

Day three

1.100µl of goat anti-rabbit IgG serum and 100µl of normal rabbit serum were added to all tubes except the TC tubes.

2.Tubes were vortex-mixed and incubated at room temperature for 90 min.

3.500µl RIA buffer was added to all tubes except the TC tubes.

4. Tubes were vortex-mixed.

5. All the tubes except TC tubes were centrifuged at 1700*g* (3,000 r.p.m.) at 4°C for 20 min.

6. Supernatants from all the tubes except TC tubes were aspirated immediately after centrifugation.

7. Radioactivity of the precipitates was counted using a gamma counter.

Although, we obtained exploitable results using this α-MSH radioimmunoassay kit, we found the step of peptide extraction limiting the accuracy of the results. To facilitate the reproducibility of assays and comparison between assay, we used a second kit were the extraction of the peptide was not required to determine the concentration of α-MSH in the samples.

2.7.4.2 Determination of α-MSH concentration from un-extracted plasma samples.

i) Reagents: (Euro-diagnostica kit)

Anti-α-MSH serum

Rabbit antiserum raised against α-MSH conjugated to bovine serum albumine in 24ml 0.04M Phosphate buffer.

^{125}I-α -MSH

Reconstituted with 25ml distilled water to obtain a concentration between 9,000 and 11,000 cpm/200µl solution.

Double antibody –PEG

Diluted goat anti-rabbit-Ig antiserum and 5.0% w/v polyethylene glycol 6000.

α-MSH Standards

Seven concentrations were used: 0, 4, 7, 9, 18.8, 37.5, 75 and 150 pmol/l

Controls

Two controls with known concentrations from each assay kit.

ii) Methodology

Day one

1. 100 µl of standards and controls were added in duplicates to their respective assay tubes.100 µl of samples were added to their respective assay tubes.
2. 200 µl of anti-α-MSH were added to the tubes. Non-specific binding of the antiserum was calculated from two tubes that received antiserum buffer but no antiserum (NSB tubes).
3. Tubes were vortex-mixed and incubated for 24 h at 4°C.

Day two

1. 200 µl ^{125}I-α -MSH were added to all the tubes. The total radioactivity added (total counts) was determined from two tubes (TC tubes) that only received iodinated α-MSH.
2. Tubes were vortex-mixed and incubated for 24 h at 4°C.

Day three

1. 500 µl double antibody-PEG were added to all the tubes except TC-tubes.
2. Tubes were vortex-mixed and incubated for 60 min at 4°C.
3. Tubes were centrifuged at 1700*g* (3,000 r.p.m.) for 15 min at 4°C.
4. Supernatants were removed by aspiration immediately after centrifugation.

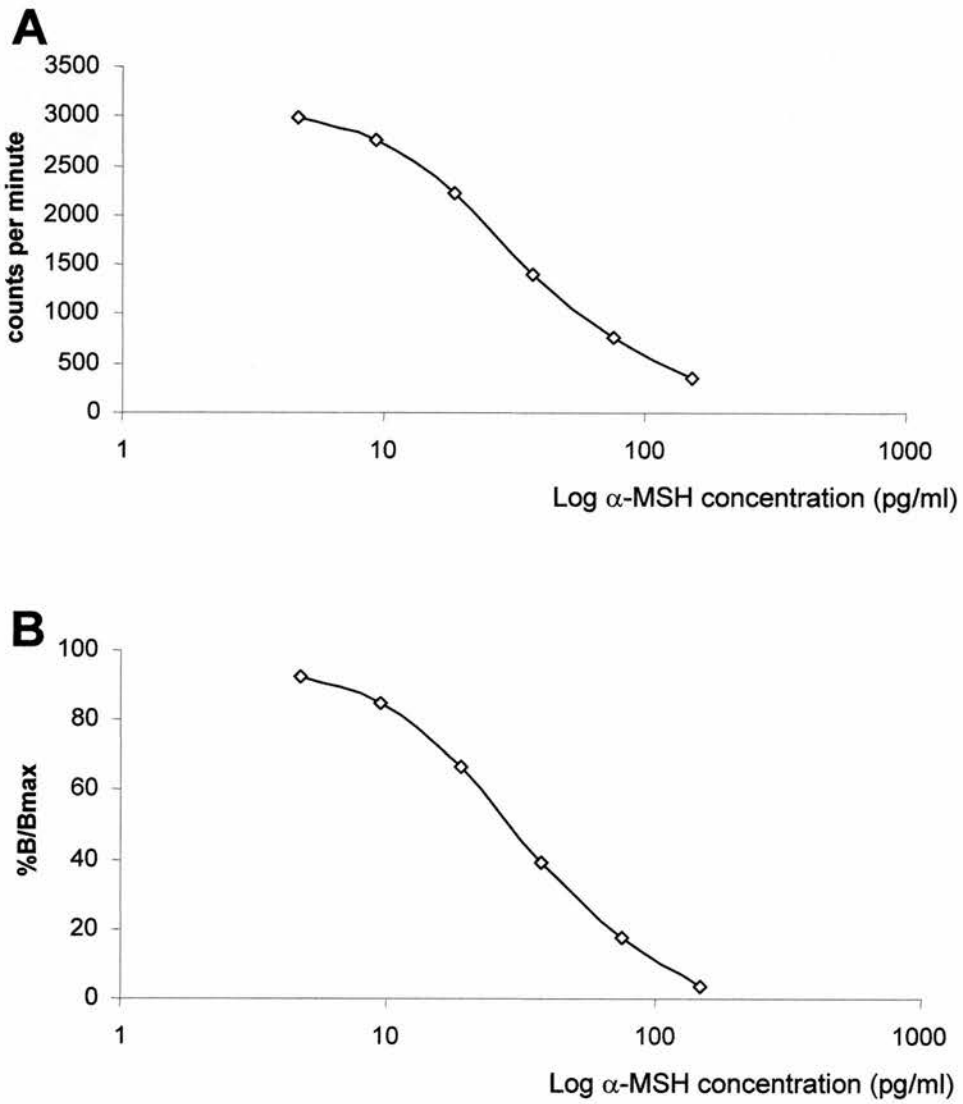

Figure 2.2 : Standard curve (A) and Binding curve (B) of an example of α - MSH radioimmunoassay.

The sensitivity of the assay was then calculated using the equation (B) described in section 2.7.2. The sensitivity of this example of radioimmunoassay was found to be less than 4.7pg/ml.

5. Radioactivity of the precipitates was counted using a gamma counter.

An example of a standard curve and a binding curve obtained from a α-MSH radioimmunoassay are shown in Figure 2.2.

2.8. Brain processing

2.8.1. Transcardial perfusion

Rats, from which the brain was used for immunocytochemistry studies, were transcardially perfused to fix the brain quickly and therefore to stabilise any potential antigens and avoid their degradation.

The rat was deeply anaesthetised with sodium pentobarbitone (Sagatal, 1 ml i.p. or 0.4 ml i.v.) and then placed on its back. An incision in the skin was made to expose the thoracic cage. The chest was opened up and the diaphragm carefully cut to expose the beating heart. After removing the pericardium, a 19-gauge needle was inserted into the apex of the left ventricle and the perfusion started with a rinsing solution of heparin saline (5000 units /ml, 100 ml). A cut into the right atrium was made to allow drainage of the perfusion solutions and body fluids. After 5 min or when the blood had run clear, the rinsing solution was replaced with the fixative solution (4% paraformaldehyde in phosphate buffer (PB) 0.1M, pH=7.4) for another 10 to 15 min (typically 300 ml). Spontaneous movements and stiffening of the limbs should have been observed to indicate a correct perfusion. Upon completion of the perfusion, brains were removed and placed in a post-fix solution (half fixative solution, half 15% sucrose in 0.1M PB) overnight in the fridge at 4°C. Tissues were then placed in a cryoprotective solution of 30% sucrose in 0.1M PB at 4°C and left until they had sunk (typically 48 h). The brain tissues were frozen in dry- ice and stored at -70°C until processing.

2.8.2. Brain sectioning

Brains were cut at 44μm using a freezing microtome. Brains sections were placed in PB 0.1M if used the same day, or stored in cryoprotectant (50% 0.2M PBS, 30% Ethylene glycol, 20% Glycerol, Sigma, UK) at -20°C.

2.8.3. Immunocytochemistry

Immunocytochemistry is a combination of immunology and microscopy. Firstly described by Coons et al. (1941), this technique relies upon the specific binding of an antibody to its antigen in a tissue, and the reaction to localise the antigen-antibody complex. Antigens can be proteins, carbohydrates, lipids, nucleic acids and many other compounds. Antibodies need to have high specificity, affinity and avidity for the antigen. Two types of antibodies can be distinguished: Polyclonal or monoclonal antibodies. Polyclonal antiserum is a mixture of highly specific antibodies, each active against different epitopes on the antigen. Monoclonal antibodies are specific to only one epitope on the antigen. Polyclonal antisera are more sensitive and specific than monoclonal antibodies because they have more paratopes active against the same antigen. Moreover, if the paratope of the monoclonal antibody is destroyed all sensitivity towards the antigen will be lost.

The antigen-antibody complex is then localised using a microscopically dense marker that will highlight the antigen-antibody reaction. This marker possesses an intrinsic property to change a colourless substrate (chromogen) into a coloured product. For light microscopy, the marker can be an enzyme (peroxidase or alkaline phosphatase) or a fluorescent dye.

There are numerous immunolabelling techniques; one of them is the 'three steps' indirect technique where a secondary biotinylated antibody is used between the primary antibody and the dense marker. Here, the avidin-biotin peroxidase complex has been used as the dense marker. The avidin has four high -affinity binding sites for biotin and therefore a preformed avidin-biotin peroxidase complex can bind to the biotinylated secondary antibody, thus amplifying the signal (Fig 2.3). Then, to visualise peroxidase, the chromogen Diaminobenzidine (DAB) is used as substrate for the enzyme reaction during which it turns brown or black when enhanced by nickel ammonium sulfate.

2.8.3.1. Fos immunocytochemistry :

Fos is the protein product of the gene c-*fos* that belongs to the immediate early genes family (IEGs) and is present in many cells including neurones. Once produced, Fos forms a heterodimer with another IEG protein product (Jun for instance), creating

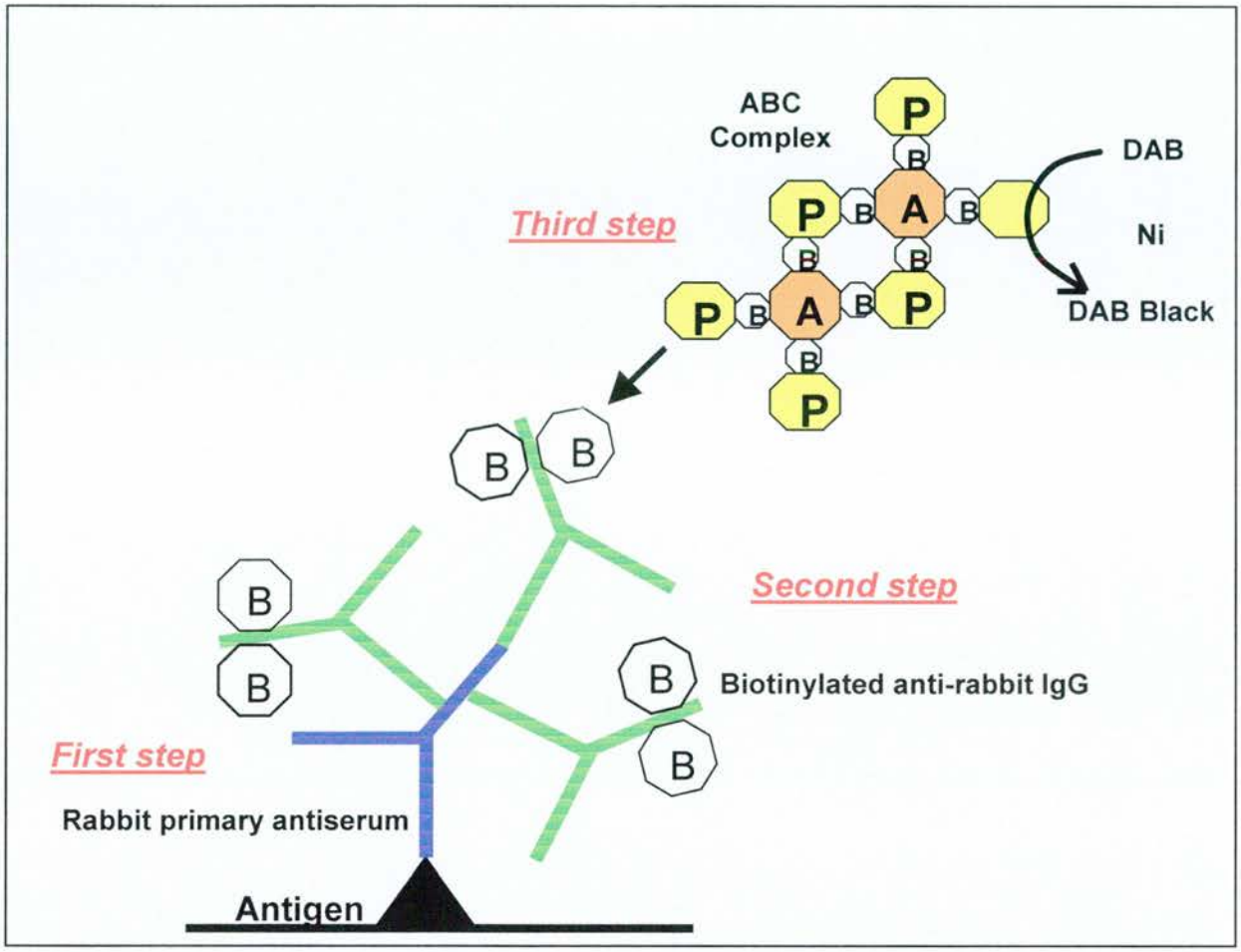

Figure 2.3: Schematic illustration of the "3 steps" indirect technique.

A secondary biotinylated antibody binds to the primary antibody. A preformed avidin-biotin peroxidase complex (ABC) binds to the secondary antibody, thus amplifying the signal. The peroxidase induces the chromagen DAB to turn black. A: Avidin, B: Biotin, P: Peroxidase.

Adapted from Beesley (1993)

a transcription factor AP-1 (activatory protein-1). The binding of AP-1 to DNA regulates the expression of other genes. The mechanisms of activation of c-*fos* transcription remain yet to be clarified.

Fos protein and c-*fos* mRNA levels are very low in basal conditions in many brain regions including the hypothalamus but numerous stimuli can increase these levels. Fos protein and c-*fos* mRNA accumulation within the cell nucleus can be visualised by immunocytochemistry and *in situ* hybridisation. Therefore, Fos is widely used as a marker of neuronal activation.

i) Reagents

Phosphate Buffer 1M (PB), pH=7.4

1 L distilled water

115g Disodium hydrogen orthophosphate (BDH)

27.2g Sodium dihydrogen orthophosphate (BDH)

Phosphate buffer 0.1M with 0.2% Triton X-100 (PB-T), pH=7.4

1 L Phosphate buffer 0.1M (100ml 1M PB + 900ml dH_2O)

2ml Triton X-100 (BDH)

0.3% Hydrogen peroxide in 0.1M PB

99ml 0.1M PB

1ml 30% H_2O_2 (Sigma)

Primary antibody: c-*fos* Ab-2 polyclonal antiserum (Oncogene)

Rabbit antibody raised against the N-terminal amino-acids 4-17 of the protein product of human c-*fos*.

Normal sheep serum (SAPU)

Secondary antibody (Vector Labs PK6101 kit)

Biotinylated anti-rabbit IgG.

Normal goat serum (Vector Labs PK6101 kit)

Sodium acetate buffer 0.2M, pH=6

16.4g sodium acetate (BDH)

1L distilled water

The pH was adjusted to 6.0 using a couple of drops of acetic acid.

ABC solution (Vector Labs PK6101 kit)

For 1ml 0.1M PB-T:

20μl Avidin DH

20μl Biotinylated horseradish peroxidase

This solution was left for 30 min before to use to allow the complex avidin –biotin peroxidase to be formed.

Nickel-DAB solution

i. 25mg DAB (Diaminobenzidine tetrachloride) (Sigma)

50ml distilled water

ii. 2.5g nickel ammonium sulphate (BDH)

50ml 0.2M acetate buffer

When the nickel ammonium was dissolved, 0.4g of glucose (Sigma) and 0.08g of ammonium chloride (BDH) were added to the solution (ii).

The two solutions were then mixed and filtered (filter papers 185 mm diameter; Whatman, UK). Immediately prior to use, 0.003g of glucose oxidase was added to the final solution.

ii) Methodology

First step

To remove the fixative and the cryoprotectant, and to make the cell membrane permeable, sections were rinsed in PB-T 0.1M for 15 min at room temperature (4 times), and then washed 5 min in 0.1M PB.

To block the action of any endogenous peroxidase, sections were placed in a 0.3% H_2O_2 in 0.1M PB solution for 15 min and then rinsed with PB-T 0.1M for 10 min (3 times). To reduce non- specific staining, sections were placed in a 1% normal sheep serum (NSS) solution for 30 min. Sections were incubated for 24 to 48 h with a rabbit

Ab-2 Fos polyclonal antibody (Oncongene science) diluted at 1:1000 in PB-T + 1% NSS at 4°C.

Second step:

To remove the excess of the primary antibody, sections were washed in PB-T 0.1M for 10 min (3 times). To localise the primary antibody, sections were incubated for one hour at room temperature with a secondary antibody, a biotinylated anti-rabbit immunoglobulin diluted at 1:100 in PB-T 0.1M + 3% Normal goat serum (NGS). Sections were then rinsed in PB-T 0.1M for 10 min (3 times).

Third step

To amplify any immunoreactive signal, sections were put in an ABC solution (Avidin DH and Biotinylated horseradish peroxidase in PB- T) for one hour at room temperature. Sections were washed in PB-T for 10 min (twice) and then rinsed with 0.1M acetate buffer for 5 min to be prepared for the visualisation stage.
Visualisation: The glucose oxidase-Ni-DAB solution was poured onto the drained sections. After 30 s, sections were checked under a microscope to detect any immunoreactive signal. To stop the DAB reaction, sections were rinsed in 0.1M acetate buffer for 5 min.
Finally, sections were rinsed in PB 0.1M.

2.8.3.2. Oxytocin immunocytochemistry

The immunocytochemistry procedure for oxytocin labelling was the same as previously described for Fos (section 2.8.3.1.). The primary antibody used was a rabbit Ab-1 oxytocin polyclonal antibody (Oncogene science) diluted at 1:1000. The secondary antibody, the dense marker, the chromagen and the incubation times were the same as for the Fos immunocytochemistry.

2.8.3.3. α-MSH immunocytochemistry

The immunocytochemistry procedure for α-MSH labelling was the same as previously described for Fos (see section 2.8.3.1.). The primary antibody used was a rabbit anti α-MSH polyclonal antibody (Peninsula Lds, USA) diluted at 1:2000 in

0.1M PB-T in 2% NSS. The secondary antibody, the dense marker, the chromagen and the incubation times were as previously described for Fos immunocytochemistry.

8.3.4. Specificity of immunocytochemistry reactions & antibody controls

The immunocytochemistry reaction must be specific for the antigen under investigation. Non-specific immunologic reactions are a major critical point that can invalidate any immunocytochemistry studies. Many factors can lead to non-specific immunologic localization. For instance, if the antigen used during immunization hasn't been highly purified, the antiserum obtained will have contained a small percentage of antibodies not related to the antigen under investigation. The cross reactivity is a second factor. Cross-reactions are immunologic reactions between the antibody and unknown tissue constituents due to stereological configuration similarities between these constituents and the immunizing antigen. These cross-reactions result in an immunolocalisation of wrong antigens/ compounds. Therefore, the specificity of the antibody used needs to be checked to confirm that the results obtained are not due to non-specific immunological reactions (Sternberger, 1979; Beesley, 1993).

i) Rabbit Ab- 2 c-*fos* polyclonal antiserum (Oncogene)

The specificity against the Fos protein of the rabbit Ab-2 c-*fos* polyclonal antiserum is well established. This polyclonal antiserum specifically recognises the residues 4 to 17 of the protein product of the human c-*fos,* a sequence that is immunogenic to human, mouse and rat species. This antibody has been extensively used in our laboratory with results described in many publications, e.g.: Srisawat *et al.*, 2000; Douglas *et al.,* 2000; Johnstone *et al.*, 2000.

ii) Antibody controls in the indirect method:

The antibody controls were as follow:

Control A: Sections were incubated with the primary antiserum to be tested, as previously described.

Control B: A preimmune serum substituted the primary antiserum.

Control C: The primary antiserum was blocked by absorption (it was bound to an excess of the specific antigen before use with the sections)

Control D: Sections were incubated with no antibody.

No immunolabelling should be seen for the controls B, C and D. Control A should present an immunoreactive signal where the cells containing the antigen against which the antibody specificity is tested, are located.

iii) Test of rabbit Ab-1 oxytocin polyclonal antiserum specificity

Using standard immunocytochemistry procedure (see section 2.8.3.2.) a few brain sections throughout the hypothalamus were processed following the 4 controls:
Control A: Sections were incubated with 3μl rabbit anti oxytocin polyclonal antiserum diluted at 1:1000 in 0.1M PB-T + 1% NSS.
Control B: Sections were incubated with neat preimmune serum (normal rabbit serum; 3μl in 3ml of 0.1M PB-T + 1% NSS).
Control C: The antiserum was mixed with 40 or 330μg of oxytocin (control C1 and control C2 respectively) in 0.1M PB-T + 1% NSS for 24 h at 4°C. Then, sections were incubated in the antiserum-oxytocin solution.
Control D: Sections were incubated with 3ml 0.1M PB-T + 1%NSS.
Sections were analysed under the microscope. Sections from control A showed an expected and distinctive labelling from cells in the SON and PVN where oxytocin cells are located (Fig 2.4). Fibres emerging from the PVN and SON and projecting to the median eminence (where numerous oxytocin fibres are located) were detected (results not shown). Sections from controls B and D did not present any labelling. Sections from control C1 presented a pale immumoreactive signal in the SON and PVN. An increase in the quantity of oxytocin peptide in the absorption complex C2 significantly reduced the staining in the nuclei (Fig 2.4)

The distribution of the cells labelled as oxytocin cells with the tested antibody corresponded to the correct distribution of oxytocin cells, no inadequate staining was noticed. The absorption complex significantly suppressed the action of the antibody, confirming the specificity of the rabbit anti oxytocin serum used against the oxytocin peptide.

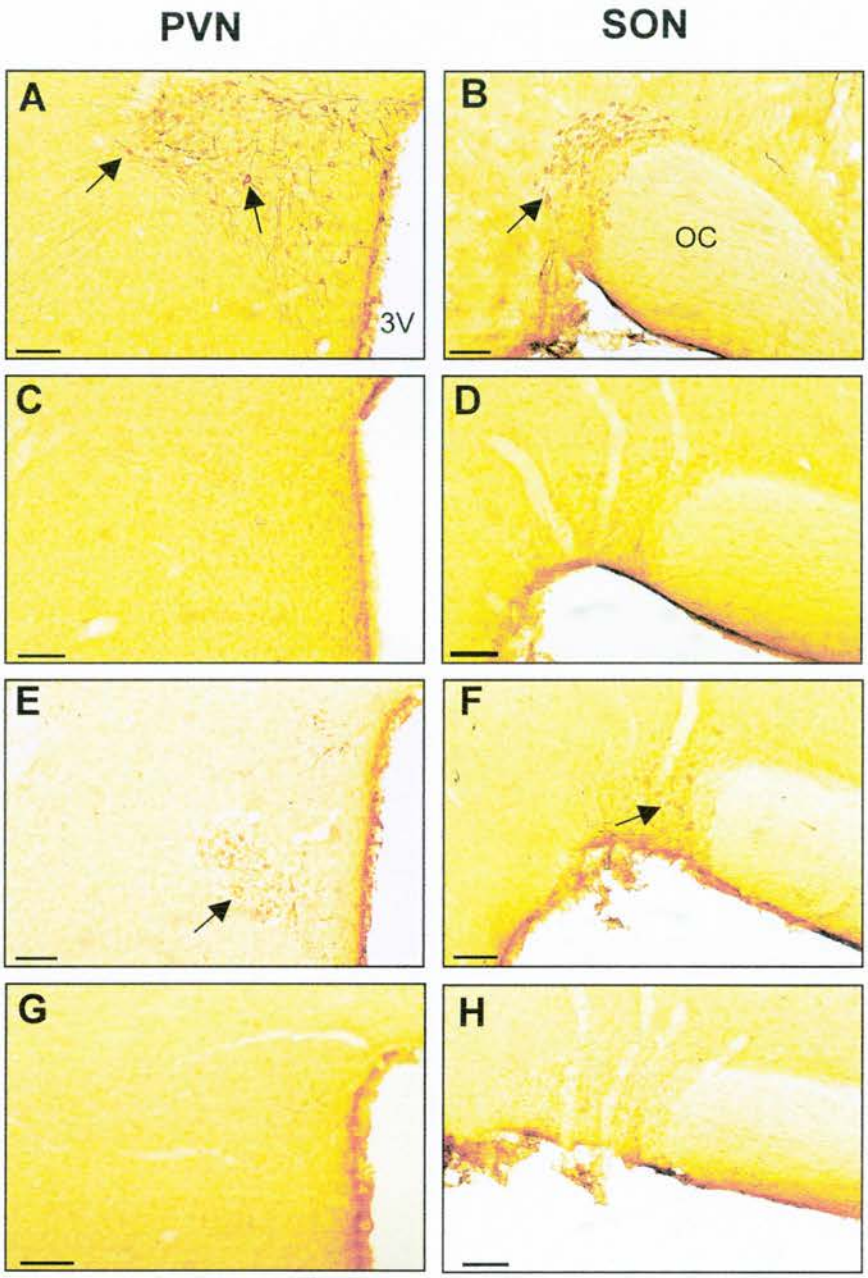

Figure 2.4: Photomicrographs illustrating the specificity test of rabbit Ab-1 oxytocin polyclonal antibody

44 μm coronal sections through the PVN and SON were treated for single immunocytochemistry for oxytocin following 4 antibody controls: Control A: rabbit oxytocin polyclonal serum (A, B); Control B: rabbit pre-immune serum (C, D); Control C1: Absorption complex with 40 μg oxytocin peptide (E, F); Control C2: Absorption complex with 330 μg oxytocin peptide (G, H). Black arrow: Oxytocin cells. 3V: Third ventricle. OC: Optic chiasm. Scale bar: 100 μm.

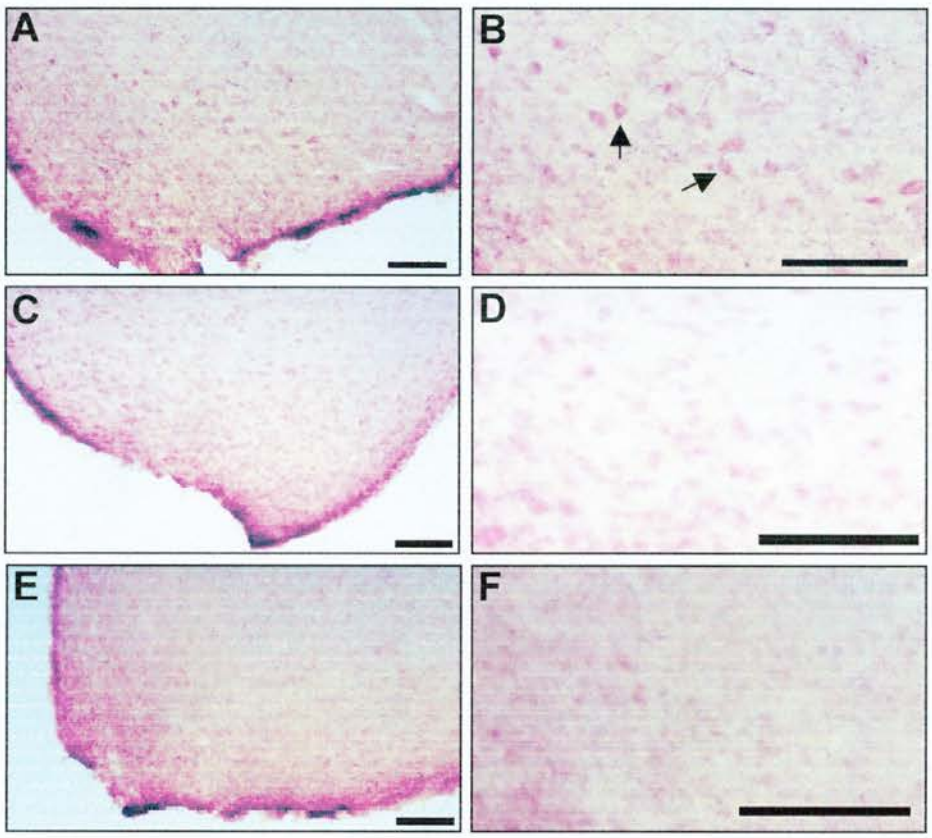

Figure 2.5: Photomicrographs illustrating the specificity test of α-MSH polyclonal antiserum.

44 μm coronal sections through the arcuate nucleus were treated for single immuncytochemistry with A, B: rabbit α-MSH polyclonal antiserum, C, D: rabbit preimmune serum, E, F: absorption complex. Black arrow: α-MSH cells. Scale bar: 100μm

iv) Test of rabbit anti α-MSH polyclonal antiserum specificity

Using standard immunocytochemistry procedure (see section 2.8.3.3.) a few brain sections were processed following the 4 controls:

Control A: Sections were incubated with 3μl rabbit anti α-MSH polyclonal antiserum diluted at 1:1000 in 0.1M PB-T + 1% NSS.

Control B: Sections were incubated with neat preimmune serum (normal rabbit serum; 3μl in 3ml of 0.1M PB-T + 1% NSS).

Control C: The antiserum was mixed with 60μg of α-MSH in 0.1M PB-T + 1% NSS for 24 h at 4°C. Then, sections were incubated in the antiserum α-MSH solution.

Control D: Sections were incubated with 3ml 0.1M PB-T + 1%NSS.

Sections were analysed under the microscope. Sections from control A showed a distinctive labelling from cells in the arcuate nucleus where α-MSH containing cells are expected to be located (Fig 2.5). Sections from controls B, C and D did not present any labelling. No unspecific labelling was observed. Thus, the rabbit anti α-MSH polyclonal antiserum used is therefore specific against the α-MSH peptide.

2.8.3.5 Double immunocytochemistry

To study co-localisation of Fos in individual neurones (e.g.: oxytocin neurones or α-MSH neurones), brain sections were processed for Fos/oxytocin and Fos/α-MSH by double immunocytochemistry. To distinguish between the two immunoreactive signals (Fos protein and peptide), the visualisation step had to be modified during the second immunocytochemistry (peptide labelling). The chromagen DAB was used without the nickel ammonium sulfate in the second immunocytochemistry, and turned brown after the enzyme reaction. Thus, Fos protein was visualised by a black nucleus signal and oxytocin or α-MSH – containing neurones were identified by a brown cytoplasm signal (Fig 2.6.C).

i) Fos/oxytocin double immunocytochemistry

Brain sections were firstly processed for Fos labelling (see section 2.8.3.1.) and then for oxytocin (see section 2.8.3.2.).

ii) Fos/ α-MSH Double immunocytochemistry

Troubleshooting:

We adapted the Fos/ oxytocin double immunocytochemistry procedure for Fos/ α-MSH double immunocytochemistry. Initially, unsatisfactory results were obtained: The background labelling of the sections was high, the immunoreactive signal was weak and the sections were difficult to mount (sticky and easily breakable). To overcome these problems, the protocol was modified:

(1) The number of washes of the sections in 0.1M PB-T was increased.
(2) To make sure that none of endogenous peroxidase was left active, sections were placed again in a 0.3% hydrogen peroxidase solution during the α-MSH immunolabelling process.
(3) To reduce the non-specific staining, the concentration of the normal sheep serum and the time of incubation were increased in the first step of the labelling process of α-MSH (From 30 min in 1% NSS to 90 min in 2% NSS).
(4) To minimise the background staining, the concentration of the biotinylated secondary antibody was decreased (from 1:100 to 1:200 dilution). (Second step)
(5) 100µl hydrogen peroxide was added to the DAB solution. (Third step)
(6) The DAB reaction was stop by a wash in PB 0.1M instead of a wash of acetate buffer to improve the aspect of the sections.

Final protocol:

First step

After labelling for Fos, brain sections were washed in 0.1M PB-T and were then placed in a 0.3% H_2O_2 solution for 15 min. After being rinsed in 0.1M PB-T, sections were placed in a solution of 2% normal sheep serum for 90 min. Finally, sections were incubated with a rabbit anti α-MSH polyclonal antibody diluted at 1:2000 in 0.1M PB-T + 2% NSS, for 24 - 48 h at 4°C.

Second step

After being rinsed in 0.1M PB-T, sections were incubated with the second antibody, a biotinylated anti-rabbit IgG diluted at 1:200 in 0.1M PB-T + 1% normal goat serum for 1 h at room temperature.

Third step

After being rinsed in 0.1M PB-T, sections were placed in the ABC solution diluted at 1:100 in 0.1M PB-T for one hour at room temperature. Sections were rinsed again in PB-T followed by a wash in 0.1M PB for 5min.

Visualisation: 100 μl of 30% H_2O_2, was added to the DAB solution. This solution was then poured onto the sections. When the immunoreactive signal had appeared, usually 60 sec later, the peroxidase-chromagen reaction was stopped by washing the sections in 0.1M PB.

2.8.4. Mounting, dehydratating & coverslipping

After any immunocytochemistry, brain sections were mounted onto gelatinised slides (Super premium microscope slides, 1.0-1.2 mm thick, BDH) following a rostro-causal anatomical order. Slides were cleaned and dehydrated into a series of 70, 90, 95 and 2 x 100 % alcohol baths for respectively 5, 5, 5 and 2 x 10 min. Alcohol was then removed from the sections by dipping the slides for 2 x 10 min into xylene baths. Slides were immediately coverslipped with DPX mounting agent (BDH) and left to dry overnight.

2.8.5. Analysis

All the analysis was done "blind": the identity of each slide was unknown during the counting procedure.

For single immunocytochemistry, sections were analysed using a Wang microscope (Wang Biomedical, UK) with x 20 objective.

For double immunocytochemistry, sections were analysed using a Leica DMR microscope with x 20 and x 40 objectives.

For each brain areas, the same number of sections was counted for each rat and only undamaged sections at the same anatomical level were analysed.

In Fos immunocytochemistry studies, only cells with a black or dark grey nucleus were considered as Fos-positive cells (Fig 2.6.A).

In double immunocytochemistry studies, only cells with a visible nucleus (Fos-negative or positive) were counted (Fig 2.6.B, 2.6.C).

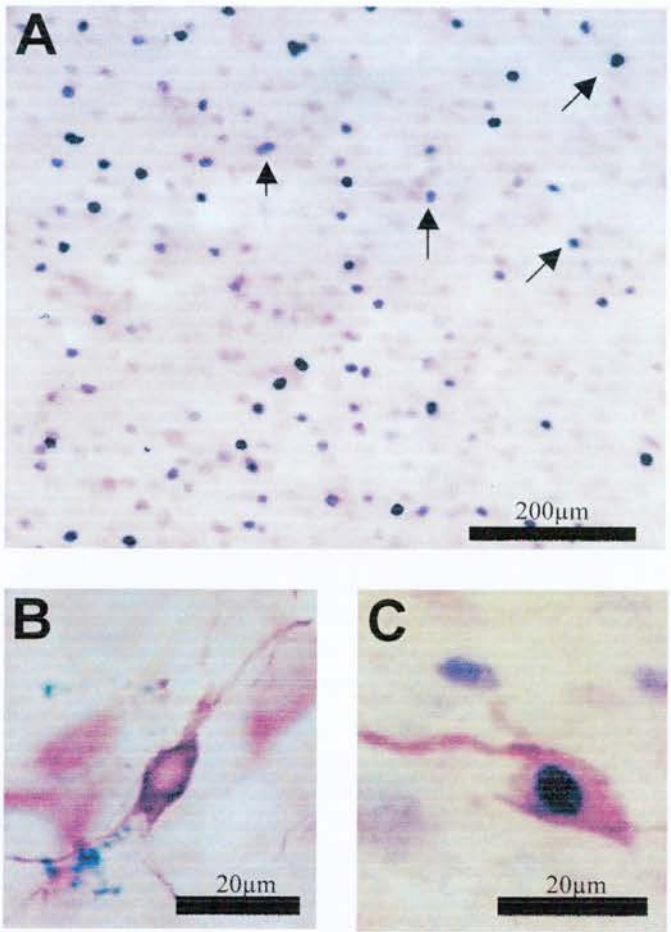

Figure 2.6: Photomicrographs illustrating the controls used for the analysis of the immunocytochemistry.

A: Fos immunocytochemistry: Black arrow: example of Fos-positive cell.
B, C: Double immunocytochemistry Fos / oxytocin. B: Fos -negative oxytocin cell; C: Fos -positive oxytocin cell.

In all experiments studying the Fos distribution, the following whole brain areas were counted:

Analysis of the SON

SON within brain sections from rostro-caudal -1.30 mm to –1.40mm to Bregma (Figures 23, 24; Paxinos & Watson, 1996) were analysed for Fos distribution.

Analysis of the PVN

The subdivisions of the PVN were analysed following the description of the PVN by Swanson and Kuypers, (1980). (Fig 1.5).

Analysis of the Arcuate nucleus

Arcuate nucleus within brain sections from rostro-caudal –2.30 mm to –3.30 mm to Bregma (Figures 27, 31; "The rat brain atlas", Paxinos G. & Watson C.) were analysed for Fos distribution.

2.9. Sexual behaviour experiments

Because rats mate during the night, all sexual behaviour experiments were performed under reverse light cycle (dark: 7:00h –19:00h, light: 19:00h-7:00h) to facilitate the timing of the experiments. At least one week was left before any use to acclimatize the rats to the reverse light cycle. Rats were kept in plastic cages with see-through lids, at 22°C, in a quiet room only designated to the sexual behaviour studies, with food and water *ad libitum.*

2.9.1. Female sexual receptivity

As for all female mammals, female rat reproductive processes are cyclic with fluctuations of sex hormones (e.g. estrogen and progesterone secreted from the ovary). During the cycle, functional and morphological changes occur in the reproductive tract and in sexual receptivity. The period of receptivity or "heat" is called Estrus; the receptive female presents a characteristic posture called Lordosis, when mounted by a vigorous male. Rats are polyestrus mammals, they complete several estrus cycle throughout the year each lasting 4 to 5 days (unless interrupted by

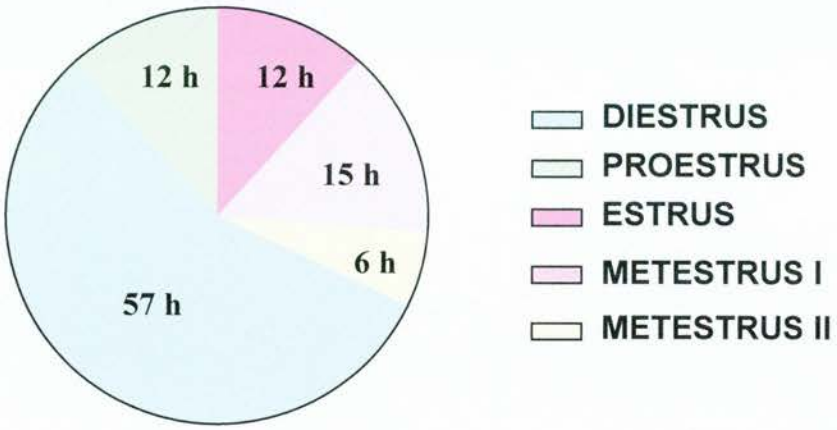

Figure 2.7: Rat estrus cycle.

pregnancy, pseudo-pregnancy or disease) (Fig 2.7). The rat estrus cycle is dependent on diurnal rhythms, with a beginning of the estrus period just after midnight.

2.9.1.1. Estrus cycle study

To establish the sexual status of the female rat, smears prepared from the vaginal fluid were examined every morning at the same time for two weeks before the experiment.

The presence of characteristic cells indicates the stage of the cycle (Fig 2.8; Table 2.1).

STAGES	CHARACTERISTIC CELLS
DI-ESTRUS	Many polymorphonuclear leucocytes. Few nucleated intermediate epithelial cells.
PRO-ESTRUS	Loads of intermediate epithelial cells. Few cornified cells lacking nuclei appear. Leucocytes almost completely absent.
ESTRUS	Hundreds of large cornified superficial cells with degenerate nuclei. Few epithelial intermediate cells.
METESTRUS I	End of the Estrus, masses of adherent cornified cells.
METESTRUS II	Few cornified superficial epithelial cells are left. Loads of polymorphonulear leucocytes.

Table 2.1: Stages of the estrus cycle and characteristic cell types.

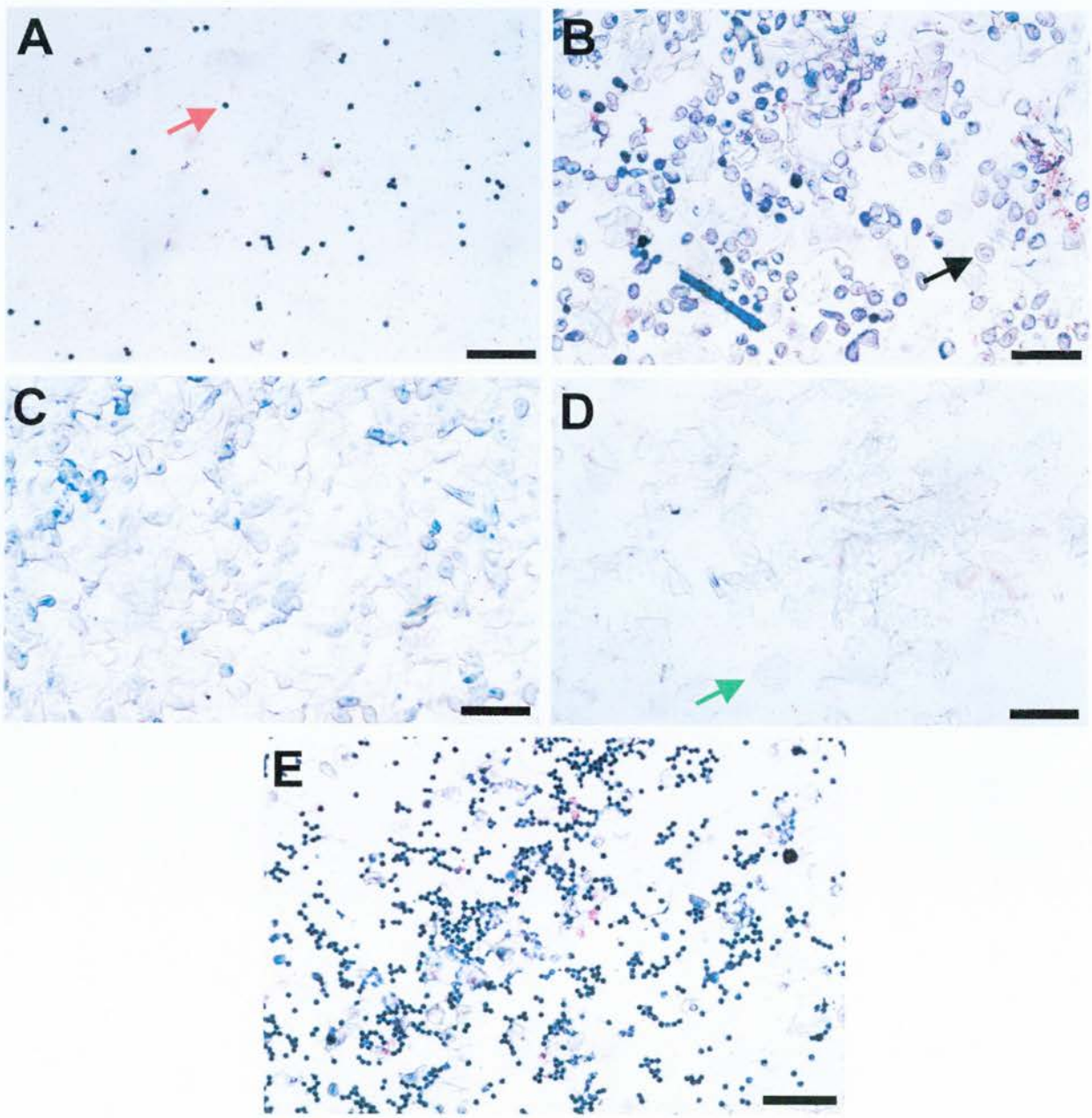

Figure 2.8: Photomicrographs illustrating Estrus cycle smears

A: Diestrus, B: Pro-estrus, C: Estrus, D: Metestrus I, E: Metestrus II

Red arrow: Leucocyte, Black arrow: Intermediate epithelial cell, Green arrow: Cornified cell. Scale bar: 100µm.

During the stages of Estrus and Metestrus I, the female rats are receptive and the mating is permitted, therefore only female rats in these sexual stages were used on the day of experiment.

2.9.1.2 Hormonal induction of receptivity

We noticed several factors limiting the effectiveness of the "smears" technique to study the estrus cycle: Keeping females in groups tended to result in a synchronisation of their estrus cycle; the stress of daily vaginal smearing induced delays in the cycle; and additionally, a couple of females presented a pseudo-pregnancy state (sterile mating). Therefore, to avoid these limiting factors and to be sure to have enough receptive females for each experimental day, sexual receptivity was induced in ovariectomised rats with injection of estrogen and progesterone. Indeed, estrogen is responsible for the onset of the estrus state: 48 h before showing estrus behaviour, the rat is under the influence of a high level of estrogen as well as an increased level of progesterone (Rao and Mahesh, 1986).

i) Ovariectomy:

From a dorsal approach at the lumbar level, two incisions were made through the skin and through the abdominal wall. Some of the adipose tissues were withdrawn to expose the fallopian tubes and the ovaries. A thread was strongly tied at the top of each fallopian tube. The ovaries were cut off and discarded. The fallopian tubes and the adipose tissues were placed back in the abdominal cavity. Finally, the abdominal walls and the skin were sutured with two stitches of 5-0 and 2-0 silk threads.

ii) Hormonal induction:

After being ovariectomised (at least 3 weeks before the experiment) and 48 h before the day of experiment, female rats were injected s.c. with 30µg of 3-beta - estradiol-benzoate (ICN Biomedicals Inc, USA) diluted in 300µL of sesame oil. Six hours before the beginning of the test, 1mg of progesterone (Sigma, UK) diluted in 100µL of sesame oil were injected s.c. into the female rats.

The sexual receptivity of the "induced " female was tested a few minutes before the beginning of the experiment: a vigorous sexually experienced male was placed in the

female cage; only females responding with lordosis posture (within 10 min) were used during the experiment.

2.9.2. Preliminary sexual behaviour study

It was necessary to know, first, whether the rats would mate in our laboratory conditions; and then, whether they would behave following the same range of behavioural parameters and timing that is described in the literature (e.g.: Stoneham *et al.* 1985; Vergoni *et al.* 2000; Ratnasooriya & Jayakody J. 2000). Indeed, slightly different conditions could result in changes in the display and in the timing of the behaviours that could lead to a difficult interpretation of our results. Therefore a preliminary study of mating behaviours of one pair of rats was performed. A receptive female and male were placed in a clean cage. The test started when a short period of exploration of the new cage ended (t0). Both rats showed sexual interest in their partner, they displayed sniffing behaviour. The first mount of the female by the male appeared at +120 s whereas the first lordosis happened at +240 s. The first intromission occurred at +250 s. A series of mounts with pelvic thrusts & dismounts followed by a prolonged session of cleaning of the genitals indicated a putative ejaculation (+ 319 s). The sexual activity was then suspended for 6 min before a new series of mounts started again. The test ended when the mount latency was above 15min. The results obtained were in the range of what was expected from the literature (Table 2.2) and they indicated sufficient laboratory conditions to study mating behaviours.

Parameters	Preliminary Study	Vergoni et al., (2000)	Ratnasooriya et al., (2000)	Stoneham et al., (1985)
Mount latency (s)	**120**	39.7-122	15-195	120
Intromission latency (s)	**250**	48.7-225.5	15-260	-
Ejaculation latency (s)	**319**	360.7-730.2	376-864	30-290
Post-ejaculatory interval (sec)	**600**	325-387.2	-	341-514.26

Table 2.2: Time range of sexual behaviour parameters.
Mount latency: Time to the first mount; Intromission latency: Time to first intromission; Ejaculation latency: Time between the first intromission and ejaculation; Post-ejaculatory interval: Time between the first ejaculation and the next intromission.

2.9.3. Experimental parameters for the study of the male sexual behaviour

To keep constancy between experimental parameters for all the experiments, the following parameters were used:

2.9.3.1. Time limit to onset of mating:

We used a time limit of 15 min for the male to present a mounting behaviour after being paired with a receptive female. Above this time limit, the male was considered as non-mated male.

2.9.3.2. Determination of the intromission behaviour, the reference behaviour:

The mechanisms during the behaviour "intromission" were studied in all the 'sexual behaviour' experiments. Intromission can be recognised when the male performs a series of intensive mounts with slow pelvic thrusts on a female in lordosis, usually followed by a grooming behaviour (Fig 2.9). The third time this behaviour was observed, was considered as our behaviour reference "Intromission". In a series of intensive mounts, several mounts will lead to intromission, but the first mounts do not necessarily mean that a successful intromission happened (male and female can be not perfectly positioned or the female can manage to escape), therefore we used the

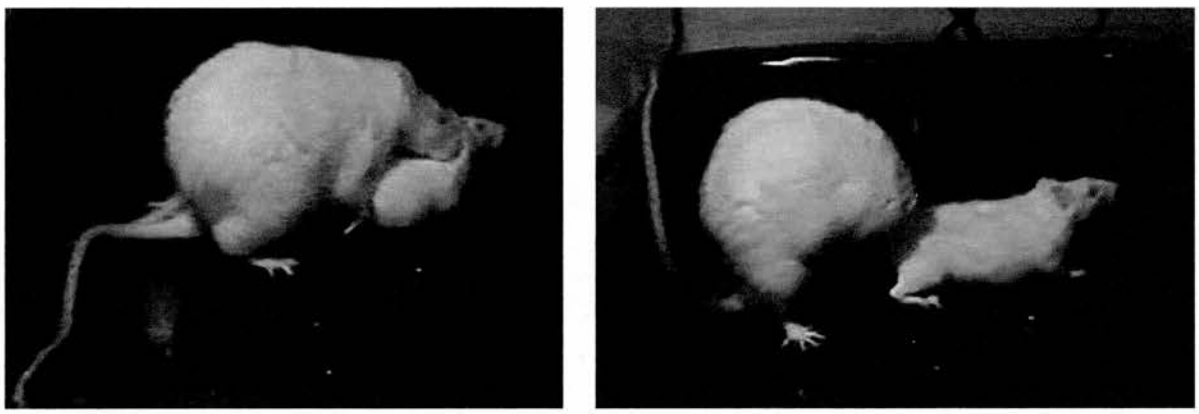

Figure 2.9: Video –extracted photomicrographs illustrating the Intromission Reference behaviour

A: Intromission: Male mounting female (in lordosis) with pelvic thrust. B: Male grooming behaviour indicating a successful intromission.

third time we observed this behaviour to be sure that a intromission had occurred during the mounts.

2.9.4. General experimental design of sexual behaviour experiments

2.9.4.1. For hormone secretion studies:

Two hours before beginning the test, the receptivity of each female was checked using a sexually -experienced male. Only females responding to the male with lordosis postures were used for the experiment. Their cage was removed from the rack onto a table near a red lamp to facilitate behavioural observations.
One hour before beginning the test, catheters for blood sampling and/ or drug injection were connected to the previously implanted cannulae (see section 2.4.1, 2.4.4). Rats were then watched to make sure none of the cannulae or tubes were pulled out.
Thirty minutes before the test, a first basal blood sample was taken, followed by a second sample 30 min later.
At the start of the test, one male was gently removed from its cage and placed in the cage of one of the pre-selected females. This was designated as t0.
If no mounts occurred within 15 min, the male was put back in its own cage and considered as non-mated male. Blood samples were then taken every 10 min.
If mounts occurred within 15 min, behaviours were recorded until the display of the reference behaviour Intromission (tI). Blood samples were taken for each sexual behaviour observed. Five minutes after the reference behaviour occurred (tI +5mins) the male was placed back in its own cage. A last blood sample was taken between tI + 60 and tI +120 min. At the end of the test, rats were killed by an i.v. injection of a lethal dose of Sagatal.

2.9.4.2. For immunocytochemistry studies:

The receptive females were selected as mentioned earlier. At t0, a male was placed in the cage of one of the receptive females and the recording of each behaviour was started. Ninety minutes after the reference behaviour had occurred, the rat was given an overdose of Sagatal (i.p.) and transcardially perfused.

2.9.4.3.Experiments with drug injection:

Tubing for the drug injection was connected to the i.c.v. cannula 1 h before the beginning of the experiment.

The drug or vehicle was injected 2 to 10 min before the mating test depending upon the experiment.

Chapter 3

Systemic oxytocin and α-MSH secretion during male sexual behaviour

3.1. Introduction:

Both oxytocin and α-MSH enhance male sexual behaviour, and both oxytocin and α-MSH are secreted from the pituitary gland into the blood. Although oxytocin is secreted peripherally during male sexual behaviour, no study has yet measured α-MSH secretion (Chapter 1). The present study investigates changes in systemic oxytocin and α-MSH secretion during male sexual behaviour, by studying the effects of copulation on plasma oxytocin and α-MSH concentrations in male rats. As oxytocin and α-MSH have similar effects on male sexual behaviour, we hypothesised that systemic α-MSH secretion would increase during male sexual behaviour; and that this increase would be stimulated by systemic and/or central oxytocin.

In a first experiment, we investigated whether peripheral oxytocin stimulates α-MSH secretion to drive sexual behaviour. To do so, rats were injected i.v. with oxytocin antagonist before being paired with a receptive female, and plasma oxytocin and α-MSH concentrations were measured during male sexual behaviour.

In a parallel experiment, we investigated whether central oxytocin stimulates systemic α-MSH secretion to drive male sexual behaviour. To mimic the central release of oxytocin occurring during male sexual behaviour, conscious rats were injected i.c.v with oxytocin. Plasma α-MSH concentration was then measured over time.

3.2. Methods:

3.2.1. Experiment 1: *Changes in plasma oxytocin and α-MSH concentration during male sexual behaviour. Influence of iv-injection of oxytocin antagonist:*

3.2.1.1. Reverse light cycle:

Three weeks before the beginning of the experiment, rats were placed in reverse light cycle (dark from 7:00h to 19:00h). The following experiment was performed in the dark under red light.

3.2.1.2. Female receptivity:

To predict the sexual receptivity of each female each day of the experiment, the estrus cycle of each female was studied using smears performed every morning at the same time for three weeks before the experiment. In the morning of the experiment, females in estrus and metestrus I stages were paired with a vigorous stud male to check their receptivity. Only females that responded with lordosis behaviour were used for the experiment.

3.2.1.3. Surgery:

Each male was briefly anaesthetized using halothane in the 'reverse-light' room (dark at the time) under red light. Once anaesthetized, the rat was transported to the surgery room where it was fitted with an i.v. cannula while maintained under halothane anaesthesia (for description of the surgery procedure see sections 2.4.1; 2.4.4). The rat was then placed back in the 'reverse-light' room in individual cage for four days of recovery.

3.2.1.4. Experimental design:

i. Oxytocin antagonist injection:

The oxytocin antagonist 'F382' (Ferring Pharmaceuticals inc.) was used. F382, when injected i.v., has been reported to be effective to delay the onset of birth at dose of 60 μg/kg BW (Antonijevic et al., 1995). To ensure the blockade of the oxytocin receptors,

F382 was used at the two doses of 100 μg/kg and 300 μg/kg. 400 μl of the antagonist solutions were therefore injected at the concentration of 40μg/ rat (OTA-40) and at 120μg/ rat (OTA-120).

Even injected i.v. at high doses, it is very unlikely that F382 can cross the blood – brain barrier, since it differs by only one amino acid (Cys to Gly) from the other oxytocin antagonist peptide F792, which has been shown not to cross the blood –brain barrier(Opstad *et al.*, 1996; personal communication from Prof. G. Leng). Thus, the use of this specific antagonist of peripheral oxytocin receptors, guarantees that any effect observed after i.v. injection of F382 are consequential to the blockade of peripheral mechanisms and not central ones.

Because F382 has long-lasting effects that occur rapidly after injection, male rats were injected 2 min before being paired with a receptive female.

ii. Protocol:

Sixty minutes after each i.v cannula was connected to a blood-sampling tube, a first basal blood sample was taken, followed by a second sample 30 min later. Rats were then injected i.v. with 400μl of either vehicle (physiological saline), or OTA-40 or OTA-120. Two minutes after the drug injection, each male was placed into a receptive female's cage and the sexual behaviours were recorded. A blood sample was then taken during the first intense ano-urogenital investigation ('Sniffing' sample) and at intromission (reference behaviour); 5 min after the reference behaviour occurred, the rat was placed back into its own cage, and 60 min later, a final blood sample was taken ('Back cage' sample) before a lethal dose of pentobarbitone (Sagatal, 50mg/kg s.c.) was injected to kill the rat at the end of the test (For description of the blood sample collection procedure see section 2.6). Blood samples were then prepared for radioimmunoassays as described in section 2.6. Plasma samples were processed by radioimmunoassays for determination of plasma oxytocin and plasma α-MSH as described in sections 2.7.3 (oxytocin RIA) and 2.7.4.2 (α-MSH RIA). The percentage of binding and sensitivity of these assays are summarized in Table 3.1:

RIA	% Binding	% Sensitivity	Detection limit of assay
Oxytocin	31	95.8%	Less than 5.5pg/ml
α-MSH	48.14	98.7%	Less than 4.7pg/ml

Table 3.1: Sensitivity of the oxytocin and α-MSH radioimmunoassays for experiment 1.

3.2.2 Experiment 2: *Changes in plasma α-MSH concentration after i.c.v injection of oxytocin or oxytocin agonist in conscious rats:*

3.2.2.1. Surgery:

A week before the experiment, male rats were fitted with i.v. and i.c.v. cannulae under brief halothane anaesthesia (for description of the surgery procedures see section 2.4.1, 2.4.4). Rats were then placed in single cages for recovery.

3.2.2.2. Experimental design:

i. Oxytocin and oxytocin agonist solutions:

Two concentrations of oxytocin were used: OT-10: 10ng/ 2μl and OT-100: 100ng/2μl. The oxytocin agonist Thr4-Gly7-OXT (Sigma, UK) was used at the concentration of 10ng/2μl (OTago-10).

ii. Protocol:

Sixty minutes after each i.v and i.c.v cannulae were connected to blood-sampling and drug-administration catheters, a first basal blood sample was taken followed by a second sample 30 min later. Rats were then injected i.c.v with 2 μl of vehicle (aCSF), OT-10, OT-100 or OT ago-10 at 1μl/ 30s. Blood samples were withdrawn 5, 10, 15, 30,

45 and 60 min after the drug injection. At the end of the test, rats were killed by a lethal injection of Sagatal. Data from rats with a misplaced i.c.v site of injection were discarded (see section 2.5.2 for procedure of injection site checking). Blood samples were then prepared for radioimmunoassay as described in section 2.6. Plasma samples were processed by radioimmunoassay for determination of plasma α-MSH following the procedure described in section 2.7.4.2 (α-MSH RIA; percentage of binding: 48%; assay sensitivity: 98% and sensitivity less than 4.7pg/ml)

3.3. Statistics:

All data were normalised into percentages with basal values equalling 100%. Data presented in the graphs are means ± S.E.M. To compare the effect of i.v. injections of oxytocin antagonist on the onset to sexual behaviour, a one -way ANOVA followed by a pairwise multiple comparison procedure (Student-Newmann-Keuls method) was used. For the two experiments, a two -way repeated measures ANOVA test was used to compare oxytocin and α-MSH secretion during different phases of sexual behaviour, followed by a pairwise multiple comparison procedure (Student-Newmann-Keuls method). A P value < 0.05 was considered as statistically significant.

3.4. Results:

3.4.1 Experiment 1:

i.) Effects of i.v. injection of oxytocin antagonist on the display of male sexual behaviour:

While all rats injected with vehicle mated when paired with a receptive female, 2 out of 6 rats and 1 out of 7 rats injected with respectively 40 mg and 120 mg of oxytocin antagonist did not mate. In rats that did mate, the injection of oxytocin antagonist slowed down the sexual performance since the intromission latency significantly increased in rats injected with 120 mg of oxytocin antagonist (P=0.002, one -way ANOVA; Fig 3.1).

ii.) Changes in plasma oxytocin and α-MSH concentration during sexual behaviour:

During sexual behaviour, in all the three groups, plasma oxytocin increased to a maximal level at intromission (Fig 3.2). In the vehicle-treated rats, the plasma concentration of oxytocin rose by 65% at intromission compared to basal. In each of the OTA-injected groups, the plasma concentration of oxytocin was significantly higher at intromission than at basal (OTA-40: P=0.009; OTA-120: P=0.003; Two -way RM ANOVA). At intromission, the plasma concentration of oxytocin in the OTA-40 –treated group was also significantly higher compared to vehicle-treated animals (P=0.038, Two -way RM ANOVA), but there was no significant difference in the increase in plasma oxytocin concentration seen at intromission between the OTA-120 –treated group and the vehicle –treated group (P=0.058) and between the two OTA-treated groups (P=0.938).

During sexual behaviour, in all the three groups, the plasma concentration of α-MSH increased to a maximal level at intromission and decreased to values similar to basal values when the rat was returned to its own cage (Fig 3.3). In each of the three groups, the plasma concentration of α-MSH was significantly higher at intromission than at basal (Vehicle: P=0.006; OTA-100: P=0.048; OTA-300: P=0.015; Two -way RM ANOVA). However, there was no significant difference in the increase in plasma α-MSH concentration seen at intromission between the oxytocin antagonist –treated groups and the vehicle –treated group. In the vehicle –treated group, the plasma concentration of α-MSH at intromission was also significantly higher than during sniffing behaviour (P=0.033).

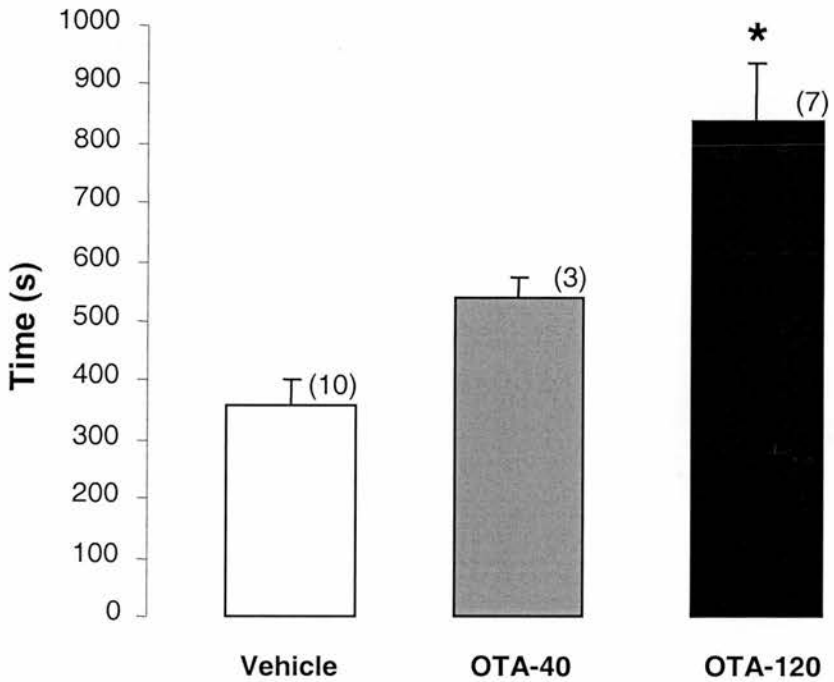

Figure 3.1: Effects of i.v. injection of oxytocin antagonist on intromission latency.

Injection of 40μg (OTA-40) or 120μg (OTA-120) of oxytocin antagonist. *P<0.05 versus Vehicle group (One way ANOVA). The number of rats per group is in parentheses.

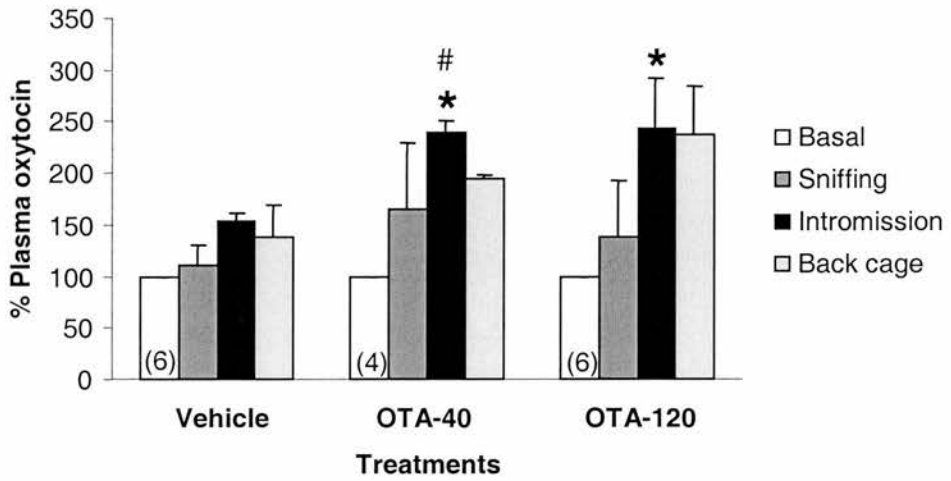

Figure 3.2: Effects of i.v.-injection of oxytocin antagonist on plasma oxytocin concentration during male sexual behaviour.

Injection of 40μg (OTA-40) or 120μg (OTA-120) of oxytocin antagonist. *P<0.05 versus Basal within each treatment (Two-way RM ANOVA). #P<0.05 versus vehicle at intromission (Two-way RM ANOVA). The number of rats per group is in parentheses.

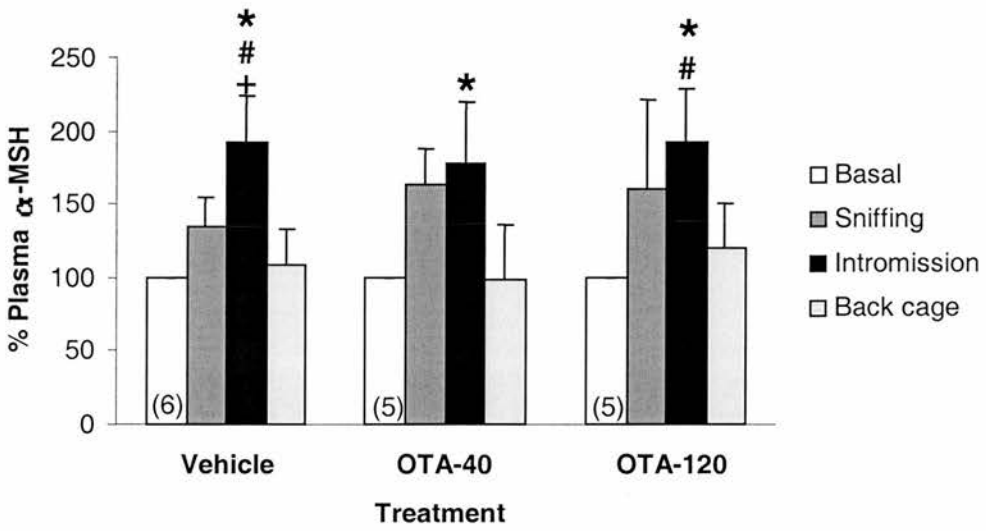

Figure 3.3: Effects of i.v.-injection of oxytocin antagonist on plasma α-MSH concentration during male sexual behaviour.

Injection of 40μg (OTA-40), or 120μg (OTA-120) of oxytocin antagonist. *P<0.05 versus Basal, +*P*<0.05 versus Sniffing, #*P*<0.05 versus Back cage within each treatment (Two -way RM ANOVA). The number of rats per group is in parentheses.

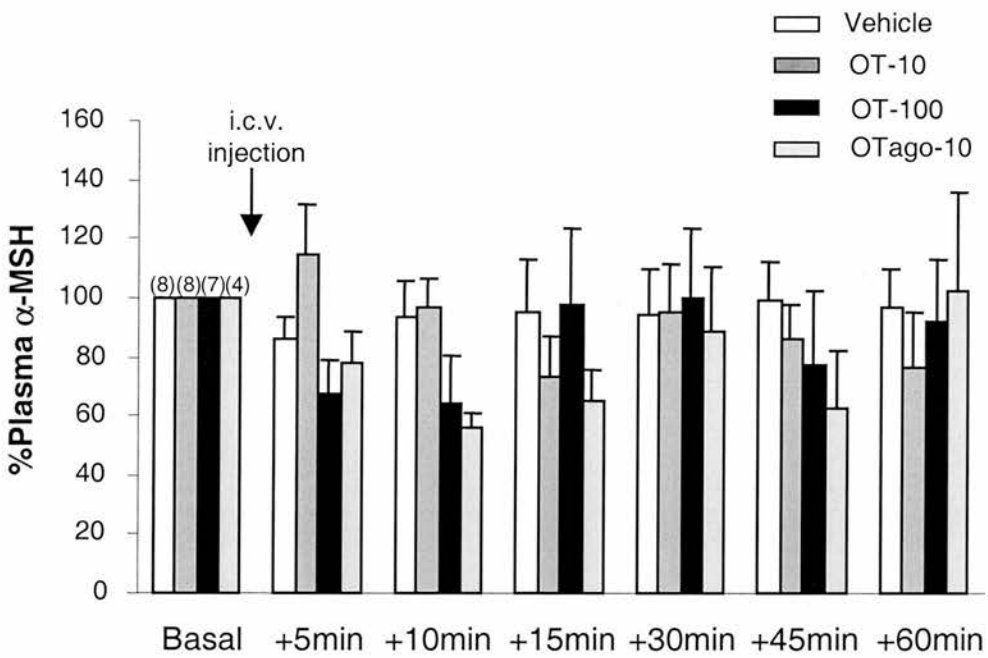

Figure 3.4: Changes in plasma α-MSH concentration after i.c.v injection of vehicle, oxytocin or oxytocin agonist in conscious male rats.

OT-10: oxytocin 10ng/2μl; OT-100: oxytocin 100ng/2μl; OTago-10: oxytocin agonist 10ng/2μl. The number of rats per group is in parentheses.

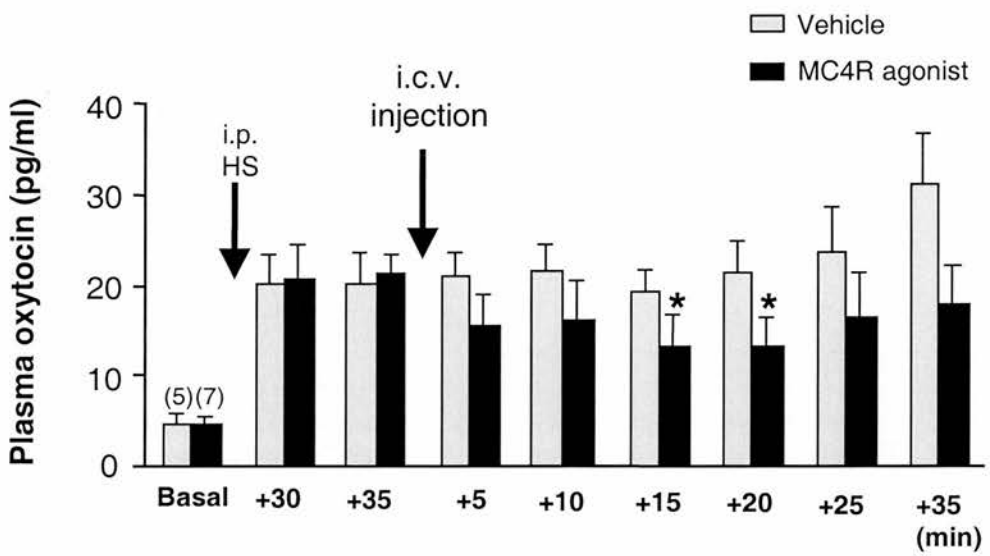

Figure 3.5: Decrease in systemic oxytocin secretion after i.c.v injection of MC4 receptor agonist.

One -way RM ANOVA followed by post- hoc t test. *P<0.05 versus basal within MC4 R antagonist treatment. The number of rats per group is in parentheses. HS: hypertonic saline.

Experiment performed by Dr P.Bull; Figure adapted from Sabatier et al., (2003) J. Neuroscience 23(32): 10351-10358.

3.4.2. Experiment 2:

The injection of 10 or 100 ng of oxytocin i.c.v had no significant effect on plasma α-MSH concentration at any time point (Fig 3.4). A reduction of 44 % in plasma α-MSH concentration can be noticed 10 min after the i.c.v injection of 10ng of oxytocin agonist, but this reduction was not statistically significant (Two -way RM ANOVA, P=0.051).

3.5. Discussion:

Systemic oxytocin and the display of male sexual behaviour:

Only one behavioral parameter, the intromission latency, was recorded in the present study and therefore the interpretation of the effects of the i.v. injection of oxytocin antagonist on sexual behaviour is limited. Nonetheless, we found that an i.v injection of oxytocin antagonist slowed down sexual performance: Some rats injected with oxytocin antagonist did not mate when presented with a receptive female within the observation period and within the rats that did mate the intromission latency was significantly increased when the higher dose of oxytocin antagonist was given. These results suggest that systemic oxytocin is important in copulatory behaviour: when peripheral actions of oxytocin are blocked, copulation is delayed. Systemic oxytocin could be involved in the motor and physical aspects of the stimulation of penile erection although no oxytocin receptors have yet been localized in the penis and on corpora cavernosa smooth muscles.

Systemic oxytocin and α-MSH secretions are increased at intromission:

The present results show for the first time, that plasma α-MSH concentration is increased during male sexual behaviour, especially at intromission. We also noticed an

increase in plasma oxytocin concentration at intromission, this increase was not significantly different from basal values in the vehicle-treated rats, but this observation is consistent with the study of Hillegaart et al. (1998), in which they reported that oxytocin secretion is increased during copulation in male rats. Thus, these studies illustrate that both oxytocin and α-MSH are peripherally secreted during male sexual behaviour. However, how systemic oxytocin and α-MSH are involved in the mediation of male sexual behaviour remains to be understood. There are indications about how systemic oxytocin and α-MSH could intervene: Oxytocin receptors have been identified in the testis and prostate (Gimpl & Fahrenholz, 2001), and evidences report a role of systemic oxytocin in the spermatogenesis by influencing stereoidogenesis and the contractibility of the seminiferous tubuli (Nicholson, 1991). Systemic oxytocin could also act on the release of prostate secretion during ejaculation as suggested by Lippert *et al*, (2003). MC1R and MC5R have been identified in the testis, and MC4R have been localized in the penis. Martin *et al,* (2002) have reported the importance of the peripheral MC4R in male sexual behaviour as an i.v –injection of MC4R agonist increased the number of penile erection in rats. Thus, once secreted into the blood, both oxytocin and α-MSH may act on distant targets (e.g.: testis and penis) to maintain sexual performance and facilitate ejaculation, but they may also act on other physiological systems (e.g.: cardiovascular system) that require modulations to answer the physiological demands of sexual exercise.

Peripheral oxytocin does not modulate peripheral a-MSH secretion during male sexual behaviour:

In the first experiment, we found that i.v. injection of two doses of the oxytocin antagonist 'F382' had no effect on the increases in plasma α-MSH concentration observed at intromission. 'F382', which even injected i.v at high doses does not cross the blood-brain barrier, only acts by blocking the peripheral oxytocin receptors. Therefore, these results suggest that peripheral oxytocin has no modulatory effects on α-

MSH secretion. This is supported by the lack of evidence of oxytocin receptors in the intermediate lobe of the pituitary gland from where α-MSH is secreted into the blood. It is difficult to interprete the significant increase in oxytocin plasma concentration observed at intromission in the rats injected with the lower dose of oxytocin antagonist compared to vehicle-injected rats. As mentioned previously, the oxytocin antagonist injection disrupted the display of male sexual behaviour: Intromission was delayed in OTA-injected rats compared to vehicle injected rats. The increase of plasma oxytocin concentration seen once intromission had occured could be related to stress mechanisms (induced by male's impossibility to perform) or could reflect a negative retrocontrole of oxytocin on its own secretion.

These results suggest that oxytocin and α-MSH do not interact peripherally to facilitate male sexual behaviour. If an interaction between oxytocin and α-MSH exists, it must occur centrally rather than peripherally.

Central oxytocin does not stimulate peripheral α-MSH secretion:

We have hypothesized that oxytocin released centrally during male sexual behaviour stimulates peripheral α-MSH secretion to facilitate male sexual behaviour. In the second experiment, to mimic the central release of oxytocin during male sexual behaviour, conscious rats were injected i.c.v with oxytocin (or oxytocin agonist). We found that i.c.v. injection of oxytocin did not increase plasma α-MSH concentration, suggesting that central oxytocin does not stimulate systemic α-MSH secretion. These results illustrate that central oxytocin is unlikely to control systemic α-MSH secretion during male sexual behaviour.

Taken together, these results illustrate a new similarity between oxytocin and α-MSH involvement in male sexual behaviour, as both systemic oxytocin and α-MSH secretion are stimulated to facilitate copulation. Although systemic oxytocin is likely to be involved in the regulation of sexual performance, as a systemic injection of oxytocin antagonist delayed intromission, the mechanisms by which systemic oxytocin acts to facilitate copulation remain unclear.

Systemic oxytocin does not regulate α-MSH secretion, as systemic injection of oxytocin antagonist had no effect on α-MSH secretion. This suggests that the interaction between oxytocin and α-MSH during male sexual behaviour must occur centrally rather than peripherally.

The putative interaction between central oxytocin and α-MSH during male sexual behaviour needs further investigation. Although we found that central oxytocin did not stimulate systemic α-MSH secretion, central α-MSH might modulate oxytocin secretion during male sexual behaviour. This is supported by findings from our laboratory. Dr P. Bull investigated the effects of an i.c.v. injection of MC4 receptor agonist on the systemic secretion of oxytocin. He measured the plasma oxytocin concentration in anaesthetized rats after i.c.v injection of 400 ng of MC4 receptor agonist. In a first experiment, i.cv. injection of MC4 receptor agonist did not stimulate plasma oxytocin concentration (results not shown). In a second experiment, he investigated whether the i.c.v. injection of MC4 receptor agonist could induce an decrease in plasma oxytocin concentration. Because oxytocin concentration is low in basal conditions, oxytocin secretion was pre-stimulated with i.p injection of hypertonic saline to obtain plasma level on which an eventual inhibition by i.c.v. injection of MC4 receptor agonist could be distinguishable. He found that i.c.v. injection of MC4 receptor agonist did significantly decreased plasma oxytocin concentration, suggesting that central α-MSH can inhibit systemic oxytocin secretion (Fig 3.5). These results illustrate that central α-MSH can interact with the oxytocin system to modulate systemic oxytocin secretion and therefore, that this interaction could occur during male sexual behaviour.

Chapter 4

Neuronal activation in the paraventricular nucleus, supraoptic nucleus and arcuate nucleus at intromission: Influence of previous sexual experience.

4.1. Introduction:

Oxytocin is secreted into the general circulation from magnocellular neurones projecting from the PVN and the SON to the posterior pituitary. In Chapter 3, we confirmed that oxytocin was secreted into the blood during male sexual behaviour, especially at intromission. Witt & Insel (1994) reported that the number of cells activated in the magnocellular regions of the PVN increased following intromission and ejaculation. *Ex copula* electrophysiological recordings of the activity of magnocellular oxytocin neurones in the SON and in the PVN reported that electrical stimulation of the dorsal penile nerve excitated oxytocin cells in the PVN (Yanagimoto, 1996) and in the SON (Honda, 1999). Thus, these studies suggest that magnocellular oxytocin neurones in the PVN and in the SON are activated during copulatory behaviour. Using Fos as an indicator of neuronal activation, we investigated whether magnocellular oxytocin neurones are activated during male sexual behaviour by studying Fos expression in oxytocin neurones in the SON and in the PVN at intromission.

Both α-MSH and oxytocin are involved in the central regulation of male sexual behaviour since they both facilitate sexual behaviours when given centrally (see Chapter 1 sections 2.3.2.2 & 3.3.2.2) Centrally -projecting parvocellular oxytocin neurones in the PVN are activated during male sexual behaviour (Witt & Insel, 1994). However, the activation of α-MSH –containing cells in the arcuate nucleus during male sexual behaviour has not been described yet. In the present study, we investigated whether α-MSH –containing cells are activated during male sexual behaviour by studying the Fos expression in α-MSH –containing cells in the arcuate nucleus at intromission. As a positive control, we also studied the Fos expression in parvocellular oxytocin neurones in the PVN to check that the experimental conditions used were adequate to investigate neuronal activity related to male sexual behaviour.

Sexually -experienced rats are usually used in any study investigating mechanisms related to sexual behaviour to ensure that the rats used are physically able

to mate. We hypothesized that a previous sexual experience could induce long-term changes in the neuronal activity involved in the regulation of male sexual behaviour, thus facilitating the copulation process. Investigating changes in the neuronal activity after one copulation or after several, could give an indication of the role of different brain areas involved in the regulation of the sexual behaviour. Therefore, in the present study, we also compared Fos expression observed at intromission in the PVN, the SON and the arcuate nucleus between sexually -naive and sexually -experienced rats.

4.2. Methods:

4.2.1. Preparation of the males:

Sexually -naive and sexually -experienced rats were used. Two weeks before the experiment, male rats gained sexual experience by being placed twice with a female for a period of four days (length of one estrus cycle) until a plug was found. Only males that successfully mated were considered as sexually experienced rats.

4.2.2. Reverse light cycle:

Two weeks before the beginning of the experiment, male and female rats were placed in reverse light cycle (dark from 7:00h to 19:00h). The following experiment was performed in the dark under red light.

4.2.3. Female receptivity:

To predict the sexual receptivity of each female each day of the experiment, the estrus cycle of each female was studied using smears performed every morning at the same time for two weeks before the experiment. In the morning of the experiment, females in estrus and metestrus I stages were paired with a vigorous stud male to test their receptivity. Only females that responded with lordosis behaviour were used for the experiment.

4.2.4. Experimental design:

Four groups of animals were used: Sexually –naïve control or mated and sexually –experienced control or mated. Two males were removed from their own cages at the same time and either placed in an empty cage (control group) or in a receptive female's cage (experimental group). Sexual behaviour was recorded until the reference behaviour (intromission) occurred, and 10 min later, each rat was returned into its cage. Ninety minutes after the reference behaviour, rats received a lethal dose of pentobarbitone (Sagatal, 50mg/kg, s.c.), and were then perfused transcardially with heparinised physiological saline (0.9%) followed by 4% paraformaldehyde in 0.1M phosphate buffer (see section 2.8.1). The brains were removed, post-fixed overnight, cryoprotected in 30% sucrose and stored at -70°C until processed for immunocytochemistry. Coronal sections of the hypothalamus were cut at 44μm using a freezing microtome. Free-floating sections were processed for single immunocytochemistry for Fos (see section 2.8.3.1), and for double immunocytochemistry for Fos and oxytocin (see section 2.8.3.2) or for Fos and α-MSH (see section 2.8.3.3). Fos-positive nuclei and the percentage of Fos positive oxytocin or α -MSH cells were counted blind throughout the PVN and the SON or throughout the arcuate. Six SON/ rat were counted for the dorsal SON; two profiles/ rat were counted for each subdivisions of the PVN and Fos-positive nuclei in six arcuate nuclei/ rat were analysed throughout the arcuate nucleus. To establish the percentage of oxytocin or α-MSH cells activated, only cells with a clear positive or negative nucleus were counted, and between 20 and 30 oxytocin cells were counted per SON or PVN and between 20 and 35 α-MSH cells were counted per arcuate nucleus.

4.3. Statistics:

A t-test was used to compare behavioral parameters (success of mating and intromission latency) between sexually -naive and sexually -experienced rats.
A two -way ANOVA followed by a pairwise multiple comparison procedure (Student – Newmann –Keuls method) was used to compare Fos expression between the four groups

of rats (sexually -naïve/ control, sexually -naïve/ intromission and sexually -experienced/ control, sexually -experienced/ intromission). When only data for the sexually experienced group were available, a t-test was used to compare Fos expression at intromission with Fos expression in control conditions. A P value < 0.05 was considered as statistically significant.

4.4. Results:

i) Behavioral recordings:

Previous sexual experience facilitated the display of sexual performance, as it increased the percentage of rats that successfully mated and reduced the intromission latency. While 88% of sexually experienced rats (7 out of 8) mated when presented to a receptive female, only 71% of naïve rats (5 out of 7) mated in the same conditions. The intromission latency was 324 ± 30s in naïve rats (n= 5) significantly greater than in experienced rats (180 ± 18s, n= 7; P=0.002, t-test) (Fig 4.1).

ii) Changes in Fos expression in the SON at intromission:

While the level of Fos expression in the SON at intromission was not significantly different from control level in sexually -naïve rats, Fos expression was significantly greater in the SON at intromission in sexually -experienced rats. In sexually -experienced rats, the number of Fos –positive cells increased from 1 ± 1 cell in control rats to 8 ± 3 cells in mated rats (P=0.040, t-test) (Fig 4.2, Fig 4.3). Of these five rats, two rats showed a mean of 2 Fos –positive cells/ section, a mean that overlaps the levels of Fos expression in the control group, and three rats had between 9 and 15 Fos –positive cells /section.

Sections from all rats were processed for double immunocytochemistry for Fos and oxytocin. Within the sexually –experienced group, in the three mated rats in which clear elevated levels of Fos expression existed (Fig 4.4), we estimated what proportion of oxytocin cells were activated. Estimates in the three rats were 7, 8 and 22% of oxytocin cells activated during male sexual behaviour. Those may be underestimates because of

the method of analysis. Sections were analyzed by only counting oxytocin cells with a distinct nucleus Fos-positive or Fos-negative; as the SON is packed with oxytocin cells, the high density of cells made difficult to be able to distinguish individual cells. Therefore, from the double immunocytochemistry it was very difficult to be sure that any cells in which Fos was expressed were oxytocin negative, but equally it was not possible to be certain that all Fos -positive cells were oxytocin positive.

iii) Changes in Fos expression in oxytocin neurones in the PVN at intromission:

Due to technical problems, brain sections processed for Fos single immunocytochemistry were damaged and therefore not analyzable. A few sections were processed for Fos and oxytocin double immunocytochemistry and analyzed, but no sections of the anterior parvocellular PVN or lateral posterior PVN from sexually naive rats were usable.

In the parvocellular PVN subdivisions:

In all the parvocellular subdivisions of the PVN except in the lateral posterior PVN, a higher percentage of oxytocin neurones that were Fos –positive at intromission was observed in both sexually -naïve and -experienced rats. However, these increases were not statistically significant (Fig 4.5)

In the magnocellular PVN subdivisions:

In the PVN, in both sexually -naïve and sexually -experienced rats, the percentage of magnocellular oxytocin neurones that were Fos –positive increased at intromission (Fig 4.6). Indeed, in sexually -experienced rats, no oxytocin cells were Fos –positive in control rats but 3 ± 1 % of oxytocin cells were Fos –positive at intromission (n= 5, P=0.025, Two -way ANOVA followed by Student –Newman -Keuls method). In naïve rats, the increase in the percentage of magnocellular oxytocin neurones Fos –positive at intromission was not statistically significant (P= 0.079). In control conditions or at intromission, there was no statistical difference in the percentage of magnocellular oxytocin neurones Fos –positive between sexually –naïve and –experienced rats.

iv) Changes in Fos expression in the arcuate nucleus at intromission:

Fos expression was significantly increased in the arcuate nucleus at intromission in both sexually -naive and sexually -experienced rats (Fig, 4.7, 4.8A). Indeed, in sexually -naïve rats, the number of Fos –positive cells rose from 20 ± 2 cells in the control group to 28 ± 2 cells in the mated group (n= 5, P= 0.006, Two -way ANOVA, followed by Student –Newman -Keuls method). In sexually -experienced rats, the number of Fos –positive cells was 23 ± 2 cells in the control group, not significantly different from the naive rats, and 30 ± 2 cells in the mated group (n=5, P= 0.013, Two -way ANOVA, followed by Student –Newman -Keuls method). However, there was no significant difference in the Fos expression at intromission between the sexually -naïve and the sexually -experienced rats, suggesting that previous sexual experience had no influence on the Fos expression in the arcuate nucleus observed at intromission.

After double immunocytochemistry for Fos and α-MSH, no significant difference in the percentage of α-MSH–containing cells that were Fos –positive was noticed between intromission and control group for either sexually –naïve rats or sexually -experienced rats (Fig 4.7, 4.8B).

v) Observations concerning the morphological arrangement of α-MSH fibres:

During the analysis of brain sections processed for Fos and α-MSH double immunocytochemistry, the α-MSH fibres distribution throughout the hypothalamus especially in the PVN could clearly be seen. Remarkably, in the PVN of rats that mated, numerous Fos-positive cells were surrounded by α-MSH fibres (Fig 4.9). Although this observation needs to be confirmed by further detailed analysis, this nonetheless indicates that α-MSH fibres projecting from the arcuate nucleus to the PVN could have contacts with cells involved in the regulation of male sexual behaviour. This anatomical evidence could therefore support the hypothesis that α-MSH can be released within the PVN and SON, and then can interact with the oxytocin system to regulate male sexual behaviour.

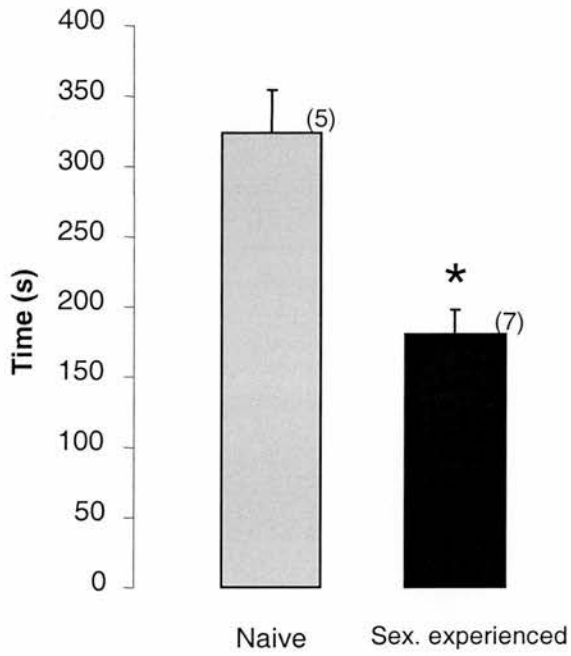

Figure 4.1: Intromission latency in sexually naïve and experienced rats.

Values are mean ± SEM. *p= 0.002, t-test. The number of rats per group is in parentheses.

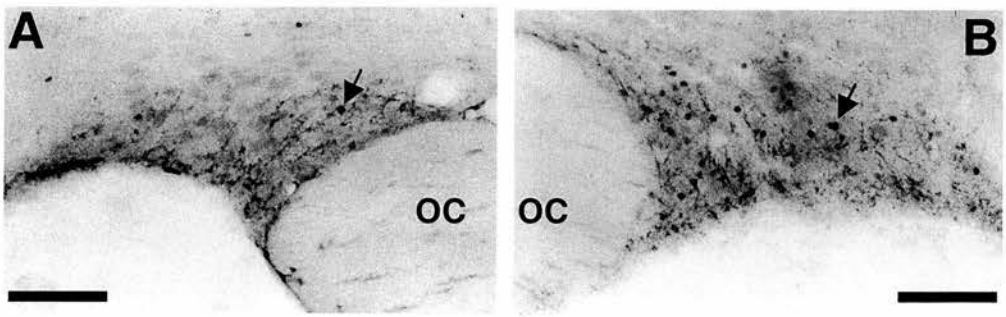

Figure 4.2: Photomicrographs illustrating Fos immunoreactivity (arrow) in the SON of sexually -experienced rats in control conditions (A) or at intromission (B).

OC: Optic chiasm. Scale bar: 100μm.

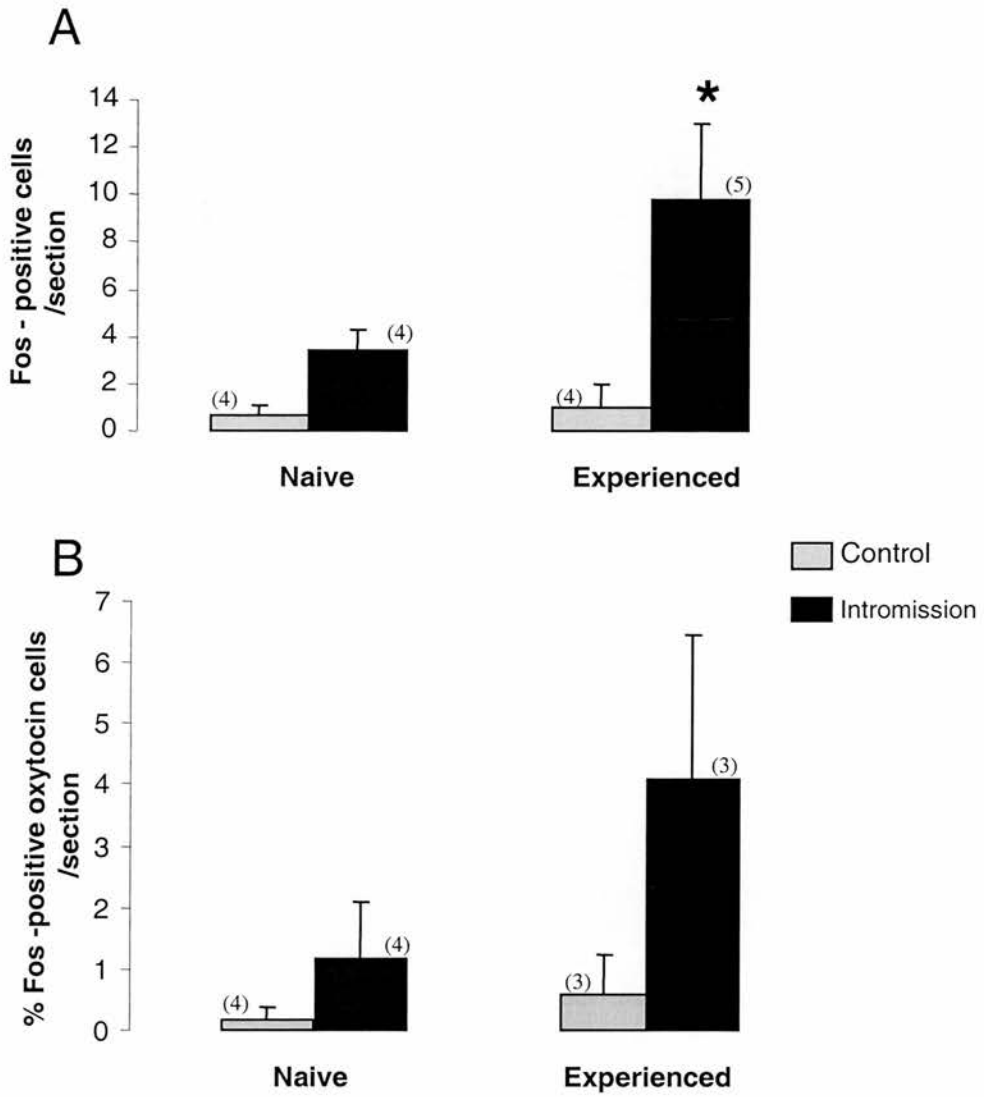

Figure 4.3: Fos expression at intromission in the SON (A) and in oxytocin cells in the SON (B).

Values are mean Fos-positive/ section ± SEM (A) or mean % of oxytocin cells Fos –positive / section ± SEM (B). *p=0.02 versus control (2 way ANOVA , Student-Newmann-Keuls method). The number of rats per group is in parentheses.

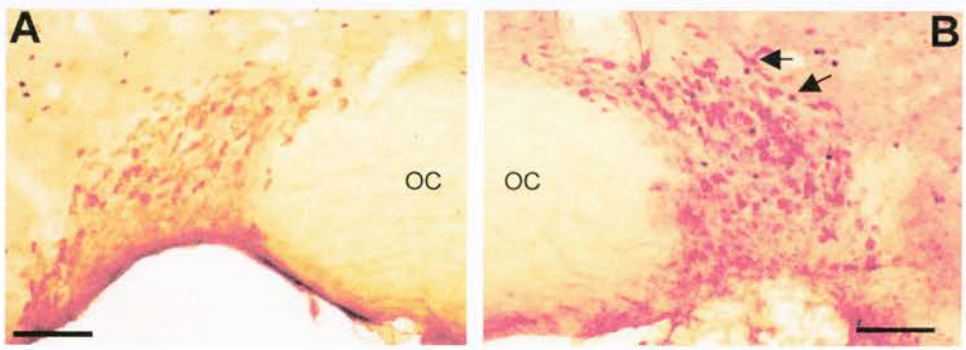

Figure 4.4: Photomicrographs illustrating Fos immunoreactivity in oxytocin neurones (arrow) in the SON of sexually -experienced rats in control conditions (A) and at intromission (B).

OC: Optic chiasm. Scale bar: 100μm.

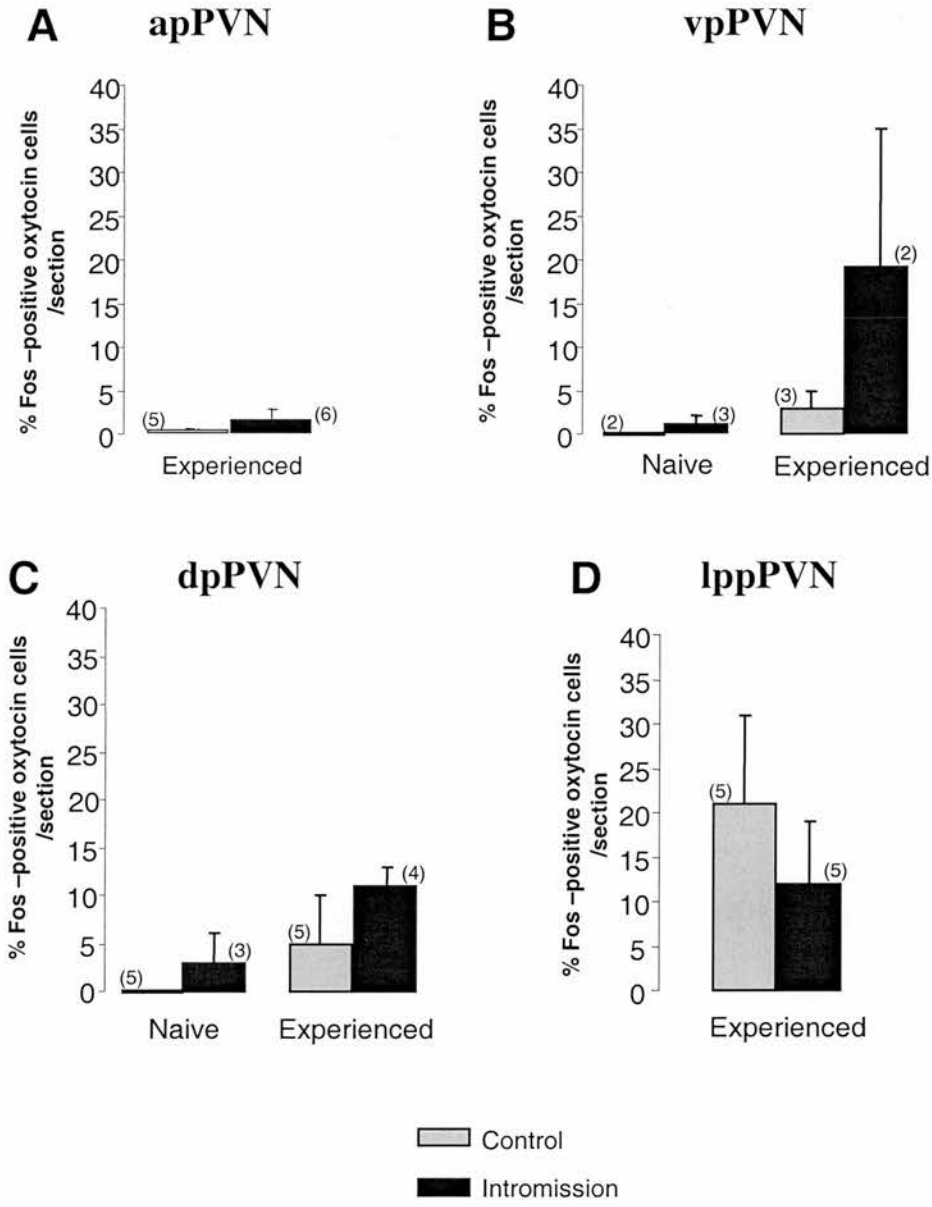

Figure 4.5: Fos expression in oxytocin neurones in the parvocellular subdivisions of the PVN at intromission in sexually - naive and -experienced rats.

Values are mean of percentage of oxytocin neurones Fos –positive/ section ± SEM. The number of rat per group is in parentheses. A: apPVN: anterior parvocellular PVN. B: vpPVN: ventral parvocellular PVN. C: dpPVN: dorsal parvocellular PVN. D: Lpp: Lateral posterior parvocellular PVN.

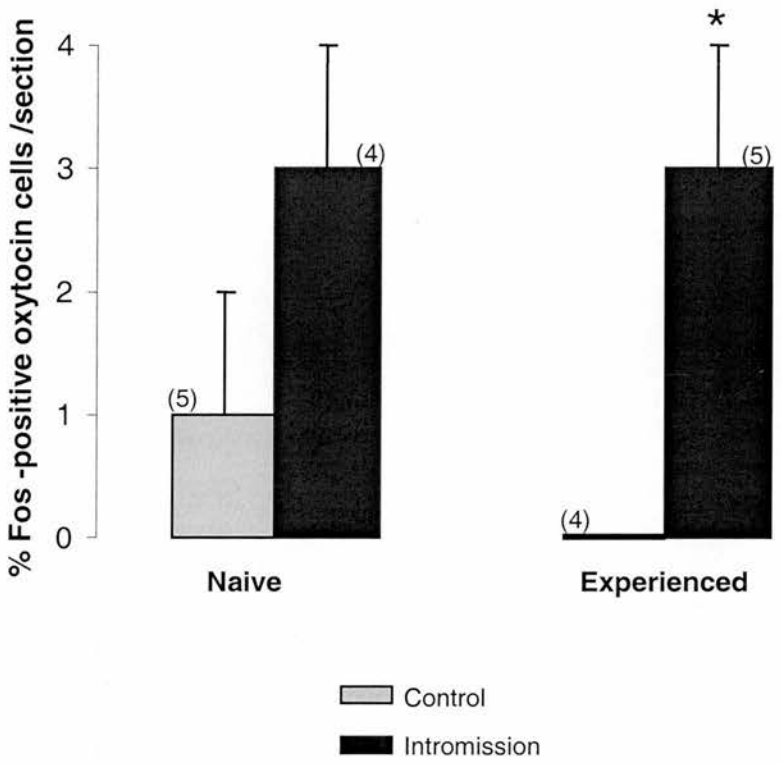

Figure 4.6: Fos expression in oxytocin neurones in the magnocellular subdivisions of the PVN at intromission in sexually -naïve and -experienced rats.

Values are mean of percentage of oxytocin neurones Fos –positive/ section ± SEM. *P= 0.025, Two -way ANOVA followed by Student- Newman- Keuls method. The number of rat per group is in parentheses.

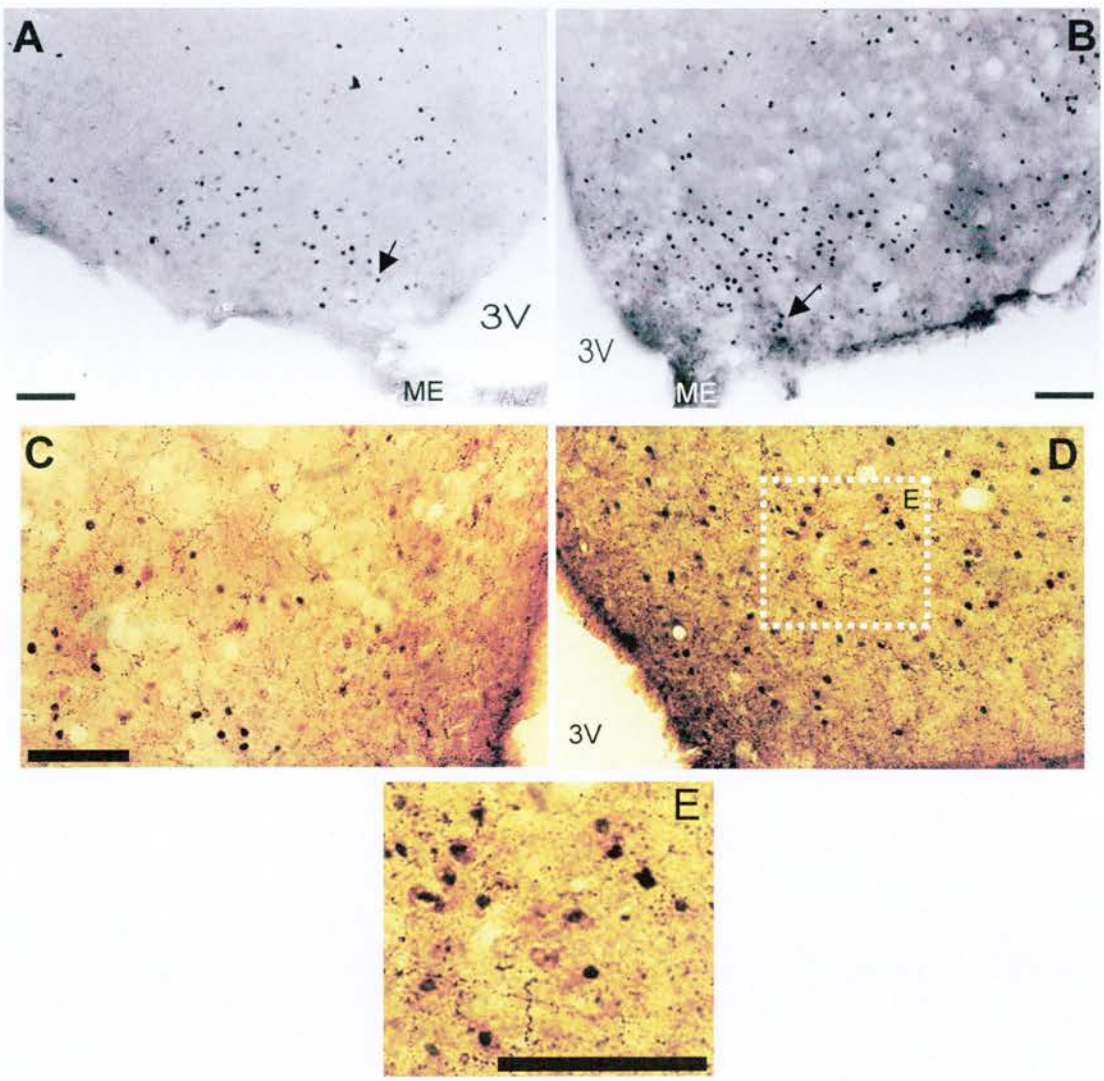

Figure 4.7: Photomicrographs illustrating Fos immunoreactivity (black arrow) in α-MSH –containing cells (blue arrow) in the arcuate nucleus of sexually -experienced rats in control conditions (A, C) and at intromission (B, D,E).

3V: Third ventricle; ME: Median eminence. Scale bar: 100μm.

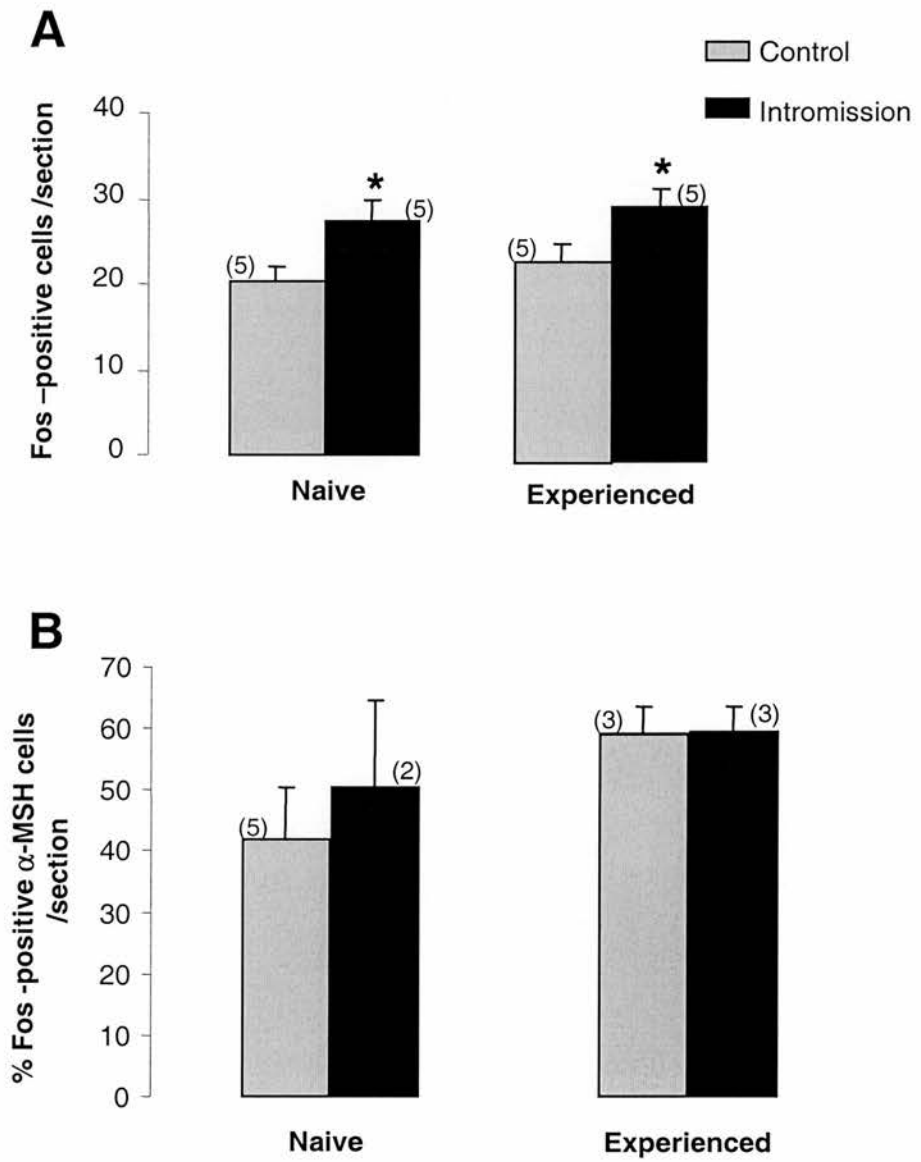

Figure 4.8: Fos expression at intromission in the arcuate nucleus (A) and in α-MSH containing cells in the arcuate nucleus (B).

Values are mean Fos-positive/ section ± SEM (A) or mean % of α-MSH cells Fos –positive / section ± SEM (B). *P<0.05 versus control (Two -way ANOVA , Student-Newmann-Keuls method). The number of rats per group is in parentheses.

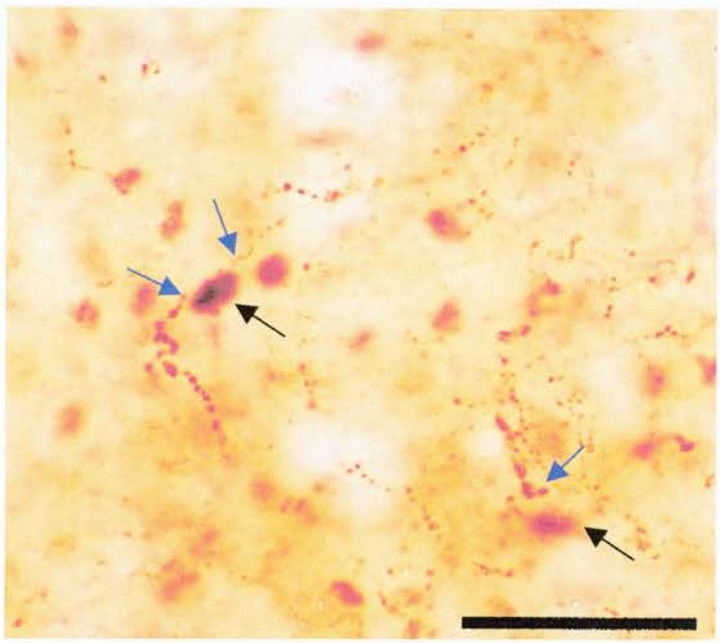

Figure 4.9: Photomicrographs illustrating α-MSH –containing fibres (blue arrow) at the vicinity of Fos –positive nuclei (black arrow) in the PVN at intromission.

Scale bar: 50μm.

4.5. Discussion:

Methodology and experimental design limits:

The control group chosen was male rats placed in a new cage when experimental rats were placed with a receptive female. Control rats had no sexual stimulation and no physical contact with a receptive female, and the Fos expression at intromission was compared with the Fos expression at the same time point in control rats. By using the control group chosen here, we cannot distinguish the Fos expression induced by intromission itself as the Fos expression observed at intromission results from other physical aspects occurring at the same time including mounting behaviour and penile erection and results as well from sexual motivation. Therefore, we investigated Fos expression induced by male sexual behaviour as a whole and not just induced by intromission. To investigate the effect of intromission itself on Fos expression, the control group should have physical or at least chemosensory contacts with a receptive female but without being able to display intromission behaviour.

Within the same experiment, the influence of previous sexual experience on Fos expression during male sexual behaviour was investigated, and to do so rats were divided into sexually -naive and sexually -experienced rats, reducing the number of rats per group. Technical problems with the brain sections and immunocytochemistry procedures reduced the number of section available for analysis, and at the end, the volume of data available was considerably reduced to just two rats in some groups. Consequently, the data was not sufficient to compensate for the variability amongst rats in each group and thus in some cases, a statistical difference between groups could not be perceived.

Parvocellular oxytocin neurones are activated during male sexual behaviour:

Intromission increased the percentage of oxytocin neurones activated in all the parvocellular subdivisions of the PVN except in the lateral posterior PVN. The number of sections available for analysis was low and high variability between rats could be seen. Nonetheless, overall these results suggest that parvocellular oxytocin neurones are activated during sexual behaviour, supporting the study of Witt & Insel (1994) who reported that oxytocin neurones in all parvocellular subdivisions are activated following intromission and ejaculation.

Are magnocellular oxytocin neurones activated during male sexual behaviour?

We investigated whether magnocellular oxytocin neurones were activated during male sexual behaviour by studying Fos expression in magnocellular oxytocin neurones in the PVN and in the SON at intromission. In the PVN, we found that magnocellular oxytocin neurones were activated during male sexual behaviour and that the activation was significantly increased compared to control, supporting the study by Witt & Insel (1994) who reported an increase in neuronal activity in the magnocellular subdivisions of the PVN during male sexual behaviour.

In the SON, the neuronal activity was increased during male sexual behaviour since the levels of Fos expression in the SON significantly increased at intromission. This result importantly shows that the magnocellular system from the SON is activated during male sexual behaviour. After double immunocytochemistry for Fos and oxytocin, we also found magnocellular oxytocin neurones activated during male sexual behaviour. However, whether this activation concerned exclusively magnocellular oxytocin neurones or also involved magnocellular vasopressin neurones requires further investigation.

The arcuate nucleus is activated during male sexual behaviour, are α-MSH – containing cells activated?

When injected i.c.v α-MSH modulates male sexual behaviour, suggesting that during male sexual behaviour α-MSH is released centrally. Once released α-MSH, might act in different brain areas including the SON and the PVN, and interact with different neuronal populations to regulate copulation. We hypothesized that during male sexual behaviour, α-MSH is centrally released from axonal projections from neurones located in the arcuate nucleus. We showed for the first time that the neuronal activity in the arcuate nucleus was increased during male sexual behaviour since Fos expression in the arcuate nucleus significantly increased at intromission. Using double immunocytochemistry for Fos and α-MSH, we found many α-MSH –containing cells activated during male sexual behaviour. However, the neuronal activity of α-MSH – containing cells at intromission was not significantly increased compared to control in both sexually -naive and -experienced rats. This result is hard to interpret, firstly because the level of Fos expression in control animals was high and therefore it limited the visualization of a putative increase of activation at intromission, and secondly because only a few cell bodies in the arcuate nucleus were immunostained for α-MSH. Thus, the activation of α-MSH –containing cells during male sexual behaviour requires further investigation.

Influence of previous sexual experience on male sexual behaviour and on neuronal activation during male sexual behaviour:

Rats with previous sexual experience were more successful at mating than naïve rats, and presented significantly lower intromission latency than naïve rats. We hypothesized that previous sexual experience might facilitate sexual behaviour by inducing long-term changes in the neuronal organization of specific brain areas. In the present study, we investigated the neuronal activity in the PVN, SON and arcuate nucleus between rats mating for the first time and rats with previous sexual experience.

Although the activation of the arcuate nucleus was similar at intromission between experienced and naive rats, the neuronal activation was greater in the SON and in oxytocin neurones in the PVN of rats with previous sexual experience than in rats with no sexual experience. This illustrates that sexual experience influences the neuronal activity in brain areas involved in the regulation of male sexual behaviour but also that these brain areas are differently influenced by previous sexual experience. Oxytocin neurones appear to be sensitive to previous sexual experience as their activity increased in experienced rats compared to naïve rats. The facilitation of sexual behaviour could result from the facilitation of the motivation period or /and the sexual performance. Indeed, several sexual experiences might enhance the responsiveness of the cells towards the signals induced by sexual stimulation and might also lead to an increase in neuronal networking improving, for instance, the rat's ability to perceive sensory information from the receptive female or to develop the physical aptitudes required to perform.

The present study firstly confirmed that the experimental conditions used were adequate to undergo behavioral studies to investigate the neuronal regulation of sexual behaviour. Secondly, we have shown that previous sexual experience facilitated the display of male sexual behaviour. However, further investigation is required to extent this study to other brain areas and other neuronal populations, and to understand why some areas or neuronal populations are influenced by previous sexual behaviour and some areas are not. More importantly, we confirmed that parvocellular oxytocin neurones in the PVN were activated during male sexual behaviour and described that during male sexual behaviour magnocellular oxytocin neurones from the PVN and from the SON were activated during male sexual behaviour. The increase in neuronal activity in magnocellular oxytocin neurones from the PVN and the SON might reflect the stimulation of oxytocin secretion into the blood occurring at intromission (Chapter 3).

Finally, we have shown for the first time that, as well as the PVN and the SON, the arcuate nucleus was also activated during male sexual behaviour. Although, the activation of α-MSH –containing cells in the arcuate nucleus during male sexual behaviour needs further investigation, we reported, anatomical evidences in the PVN, of α-MSH fibres at the vicinity of cells activated during male sexual behaviour. This indicates that α-MSH can interact with another neuronal system to regulate male sexual behaviour, and this system could be the oxytocin system.

Chapter 5

α-MSH modulation of neuronal activity in the supraoptic nucleus, paraventricular nucleus and arcuate nucleus.

5.1. Introduction:

Previous studies have shown that Fos expression is increased in the SON and in the PVN after central administration of α-MSH or agonists, suggesting that α-MSH has excitatory effects on oxytocin neurones (Thiele *et al.,* 1998; McMinn *et al.*, 2000; Olszewski *et al.,* 2001). Thus, it may be expected that a central administration of α-MSH or agonists will induce secretion of oxytocin into plasma. However, as described in Chapter 3, injection of an MC4 receptor agonist led to a reduction in oxytocin secretion, suggesting inhibitory effects of α-MSH. To understand the action of α-MSH on oxytocin neurones, these discrepancies needed to be clarified. In a first experiment, we studied the effects of i.c.v. administration of α-MSH on oxytocin secretion into blood, and in the same rats on Fos expression in oxytocin neurones in the SON. We compared the results to those obtained from a positive control group: rats i.v.-injected with CCK, known to induce oxytocin secretion into blood and to induce Fos expression in oxytocin neurones (Verbalis *et al.*, 1986; Renaud *et al.*, 1987).

The PVN is a very heterogeneous nucleus, with different subdivisions where specific populations of oxytocin neurones are located. Previous studies of Fos expression in the PVN after α-MSH administration did not distinguish between the parvocellular oxytocin neurones and the magnocellular oxytocin neurones. These two types of oxytocin neurones project to different areas, and are involved in different regulatory functions. Because parvocellular oxytocin neurones project within the brain, they are the only oxytocin system that is generally recognized to be involved in central regulation of oxytocin-dependent behaviours. Thus, if α-MSH interacts with oxytocin neurones to mediate its central effects, we would expect to see an increase in Fos expression in parvocellular oxytocin neurones after i.c.v. injection of α-MSH. To investigate if α-MSH modulates specific populations of oxytocin neurones, especially parvocellular neurones, and so to see if α-MSH has a

specific role in the regulation of oxytocin neuronal activity, Fos expression in oxytocin neurones in each subdivisions of the PVN was studied.

In Chapter 4, we found that the arcuate nucleus was activated at intromission. To investigate whether the increase in neuronal activity in the arcuate nucleus observed during male sexual behaviour was also stimulated by α-MSH, Fos expression in the arcuate nucleus was studied.

Finally, in a second experiment, to identify if the effect of α-MSH on Fos expression in oxytocin neurones was direct or via other type of neurone, Fos expression in the SON was investigated after local administration of α-MSH.

5.2. Methods:

5.2.1. Experiment 1: *Fos expression in the SON, PVN and arcuate nucleus in conscious rats after i.c.v injection of α-MSH:*

To clarify the apparent discrepancies observed between the effects of α-MSH on Fos expression and on oxytocin secretion, any experimental artefacts or faults needed to be excluded:

a. Previous Fos expression studies used conscious rats, whereas our previous study of oxytocin secretion used urethane-anaesthetised rats (Chapter 3). Although urethane anaesthetic does not impair the physiological reflexes of oxytocin neurones, we used conscious rats throughout this experiment to ensure that the anaesthesia process could not influence the actions of α-MSH.
b. Rats injected intravenously with CCK were used as a positive control group. CCK induces Fos expression in oxytocin neurones and induces oxytocin secretion into the general circulation (Verbalis *et al.*, 1986; Renaud *et al.*, 1987).

5.2.1.1) Surgery:

Male rats were fitted with an i.v. and an i.c.v. cannulae under brief halothane anaesthesia (sections 2.4.1; 2.4.4). They were then placed in individual cages for two days of recovery.

5.2.1.2.) Experimental design:

Sixty minutes after the cannulae (i.v. and i.c.v.) were connected to drug-administration and blood-sampling tubes, rats were divided into 4 groups: Rats from the first two groups received a single i.c.v. 5 µl-injection of α-MSH (1µg / 5µl) or isotonic saline. Rats that had not been equipped with an i.c.v. cannula were injected intravenously with CCK (20µg/kg/ml) or isotonic saline. Four blood samples (300 µl) were taken for each rat (a basal sample 10 min before the drug injection and 3 samples 10, 15 and 80 min after). Blood samples were kept in ice and centrifuged 3 min at 6500 r.p.m.; plasma samples were aliquoted and kept in freezer -20°C until they could be processed for radioimmunoassay for oxytocin (section 2.7.3; the oxytocin radioimmunoassay in this experiment were performed by Dr P. Bull). Ninety minutes after drug injection, the rats were deeply anaesthetised with an overdose of sodium pentobarbitone (50mg/kg, s.c.) and perfused transcardially with heparinised physiological saline (0.9%) followed by 4% paraformaldehyde in 0.1M phosphate buffer (section 2.8.1). The brains were removed, post-fixed overnight, cryoprotected in 30% sucrose and stored at -70°C until processed for immunocytochemistry. Coronal sections of the hypothalamus were cut at 44µm using a freezing microtome. Only brains with a correct injection site were processed for immunocyochemistry. Free-floating sections were processed for double immunocytochemistry for Fos and oxytocin using standard procedures, described in section 2.8.3. Fos-positive nuclei and the percentage of activation of oxytocin neurones throughout the PVN and the SON were counted blind. Three sections /rat were counted for each subdivisions of the PVN and for the dorsal SON. Fos-positive nuclei in six arcuate nuclei/ rat were analysed throughout the arcuate nucleus.

5.2.2 Experiment 2: *Fos expression in the SON after local administration of α-MSH:*

5.2.2.1 Surgery:

Rats, anaesthesised with sodium pentobarbitone (Sagatal, 60mg/kg, i.p.), were fitted with an microdialysis probe adjacent to the right SON at the coordinates: 0 mm

rostrocaudal to Bregma, 1.5mm lateral to Bregma and to a depth of 9.5mm from the surface of the skull. (complete description of the procedure section 2.4.3.).

5.2.2.2. Experimental design:

Two hours after the end of surgery, probes in the SON were microdialysed with aCSF at 1.5 µl/min. Thirty minutes later, the drug administration started: rats were were dialysed with either vehicle aCSF or aCSF containing α-MSH (1.7 µg/µl) for 30 min and then with aCSF for the last 30 min. Sixty minutes after beginning the drug injection, rats were killed with an overdose of pentobarbitone and transcardially perfused as previously described (section 2.8.1). The brains were removed and processed for Fos immunocytochemistry using standard procedures as detailed in section 2.8.3. Three sections per rat through the SON were counted blind at the level of maximal cross-sectional area.

5.3. Statistics:

Data presented are mean Fos –positive cells/ section or % of oxytocin neurones Fos-positive/ section ± S.E.M.

5.3.1. Experiment 1:

A one -way repeated measures ANOVA test, followed by a multiple pairwise comparion Student- Newman-Keuls ad hoc test was used to compare oxytocin concentration at each time point with basal oxytocin concentration within each of the four groups (i.v. vehicle, i.v. CCK, i.c.v. vehicle, i.c.v. α-MSH).

A one -way ANOVA test followed by a Student- Newman-Keuls ad hoc test test was used to compare Fos expression in the SON between the 4 groups.

A t-test or a Mann Whitney rank sum test was used to compare Fos expression in the PVN and in the arcuate nucleus between α-MSH -treated rats and vehicle -treated rats.

5.3.2. Experiment 2:

A paired t-test was used to compare the Fos-positive cells in the ipsilateral nucleus with its contralateral nucleus within the same coronal section for the vehicle -treated group and for the α-MSH -treated group.

A t-test was used to compare the ipsilateral- contralateral difference in Fos-positive cells between the vehicle -treated group and the α-MSH -treated group.

5.4. Results:

5.4.1. Experiment 1: *Fos expression in the SON, PVN and arcuate nucleus in conscious rats after i.c.v injection of α-MSH:*

i) Changes in oxytocin secretion and Fos expression in the SON after i.c.v. injection of α-MSH:

In the same conscious rats we tested the effect of an i.c.v. injection of α-MSH on oxytocin secretion and on Fos expression in oxytocin neurones. We compared the results obtained with α-MSH to results obtained in conscious rats injected i.v. with CCK.

As expected, rats i.v.-injected with CCK presented a significant increase in plasma oxytocin concentration 10 min after injection ($P<0.001$, One -way RM ANOVA; Fig 5.1), as well as a significant increase in Fos expression in the SON (55 ± 11 Fos-positive cells/section) compared to rats i.v -injected with vehicle (18 ± 6 Fos-positive cells/section) (Fig 5.2; 5.3). In the SON of rats injected with CCK, Fos expression was predominantly dorsally distributed, and double immunocytochemistry confirmed that this increase concerned oxytocin cells.

In rats injected with α-MSH, no significant changes in oxytocin concentration were perceived (Fig 5.1). Note that the low circulating concentration of oxytocin in conscious rats makes the inhibition of secretion by α-MSH undetectable in this study. However, Fos expression was significantly higher in the SON of rats injected with α-MSH (110 ± 19 Fos-positive cells/section) than in the SON of rats injected with vehicle (42 ± 18 Fos-positive cells/section; $P=0.004$, One way ANOVA, Student-Newman-Keuls) or than in the SON of rats injected with CCK ($P=0.005$) (Fig 5.2; 5.3).

After double immunocytochemistry for Fos and oxytocin, the increase of Fos expression in the SON induced by α-MSH proved to concern mainly oxytocin neurones (Fig 5.4; 5.5). There was a significant increase in Fos expression in oxytocin neurones after i.c.v. –injection of α-MSH (52 ± 5% Fos- positive oxytocin cells) compared to vehicle (9 ± 3 %, $P<0.001$, One -way ANOVA), and this increase in Fos expression in oxytocin neurones after α-MSH injection was even significantly higher than after the CCK-injection (37 ± 6%, $P= 0.025$).

ii)Changes in Fos expression in the paraventricular nucleus after i.c.v. injection of α-MSH:

In the rats injected with α-MSH, Fos immunoreactivity was widespread in the PVN. As expected, α-MSH injection increased the number of Fos-positive cells in the whole PVN, supporting the study by Thiele *et al.*, 1998; McMinn *et al.*, 2000; Olszewski *et al.* 2001. To investigate whether α-MSH specifically modulates different populations of oxytocin neurones, we studied Fos expression in oxytocin neurones in all the subdivisions of the PVN:

In the parvocellular subdivisions of the PVN:

No significant changes between the rats injected with vehicle and the rats injected with α-MSH were observed in the four parvocellular subdivisions of the PVN (Figs 5.6; 5.7; 5.8.). In the anterior parvocellular PVN (Fig 5.8), the increase in Fos expression in rats injected with α-MSH was noticeable although not significant (P=0.122, t-test). Due to the small number of rats per group (4 for vehicle and 3 for α-MSH), the non-significant values from the lateral posterior PVN should be interpreted cautiously (Fig 5.8).

After double immunocytochemistry for Fos and oxytocin, activation of oxytocin neurones by α-MSH was perceptible in the selected subdivisions:
In the anterior parvocellular PVN, rats injected with α-MSH presented 11 ± 3% of oxytocin neurones expressing Fos, while rats injected with vehicle had 1 ± 3% of oxytocin neurones which were Fos-positive (Fig 5.9; 5.11). Thus, α-MSH produced a small but significant increase in Fos expression in oxytocin neurones in the anterior

parvocellular PVN (P=0.041, t-test). In the ventral parvocellular PVN (Fig 5.9; 5.11), there was no significant difference in the percentage of oxytocin neurones that express Fos between rats injected with vehicle (19 ± 7%) and rats injected with α-MSH (33 ± 6%). In the dorsal parvocellular PVN (Fig 5.10; 5.11), the percentage of oxytocin neurones that contain Fos was not significantly different in the rats injected with α-MSH (10 ± 4%) than in the rats injected with vehicle (4 ± 3%). In the lateral posterior parvocellular PVN(Fig 5.10; 5.11), there was no significant difference in the percentage of oxytocin neurones that expressed Fos between the rats injected with α-MSH rats (13 ± 8%) and the rats injected vehicle (12 ± 4%).

In the magnocellular subdivisions of the PVN:

The injection of α-MSH significantly increased the number of Fos-positive cells in the magnocellular subdivisions of the PVN (Fig 5.12; 5.13): from 12 ± 2 cells in rats injected with vehicle to 28 ± 3 cells in rats injected with α-MSH (P<0.001, t-test).

Double immunocytochemistry for Fos and oxytocin, confirmed that magnocellular oxytocin neurones were activated after i.c.v injection of α-MSH:
In the posterior magnocellular PVN (Fig 5.14; 5.15), no oxytocin neurones expressed Fos in the vehicle-injected rats. By contrast, in α-MSH- treated rats, the percentage of oxytocin neurones that expressed Fos was 21± 5%. Thus, the injection of α-MSH significantly increased Fos expression in oxytocin neurones in the posterior magnocellular PVN (P<0.001, Mann -Whitney rank sum test).

iii) Changes in Fos expression in the arcuate nucleus after i.c.v. injection of α-MSH:

In rats injected with α-MSH, 38 ± 3 cells/ arcuate nucleus were Fos-positive, whereas in rats i.c.v.-injected with vehicle 30 ± 2 cells/ arcuate nucleus were Fos-positive (Fig 5.16; 5.17). Thus, α-MSH injection significantly increased Fos expression in the arcuate nucleus (P=0.020, t-test).

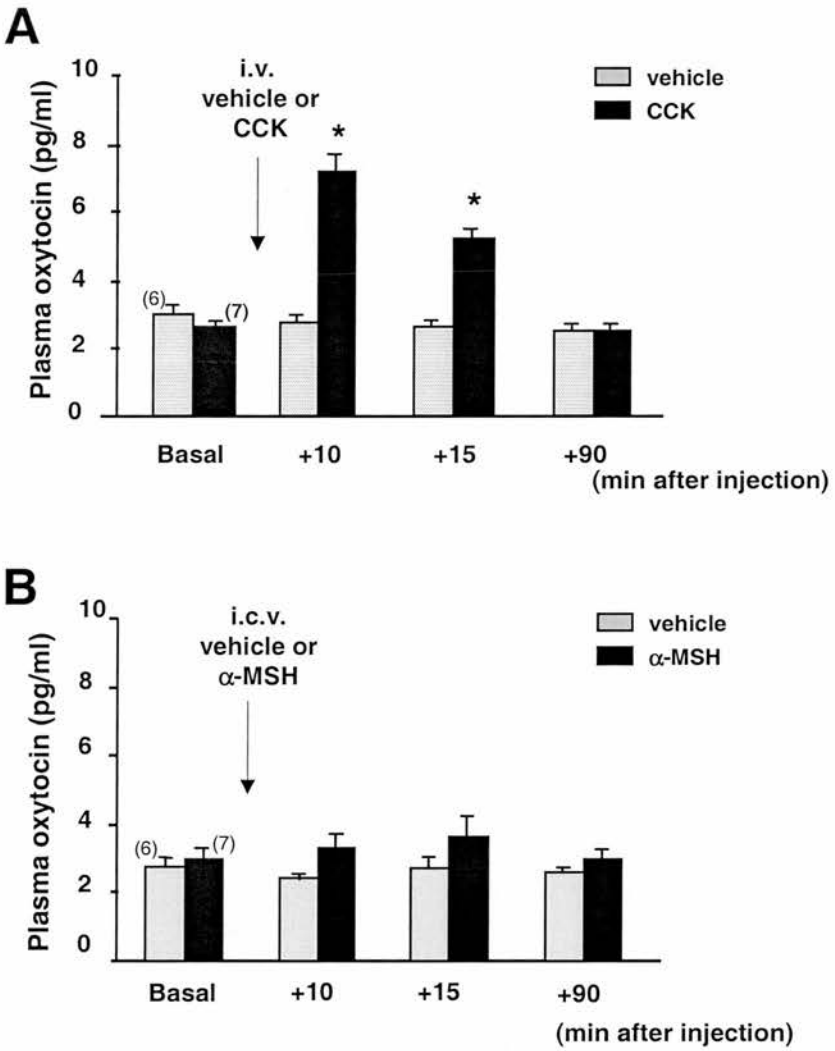

Figure 5.1: Effect of α-MSH i.c.v injection on plasma oxytocin secretion in conscious rats.

A: Changes in plasma concentration of oxytocin after i.v. injection of CCK (20μg/kg) or vehicle. B: Changes in plasma oxytocin after i.c.v. injection of α-MSH (1μg/5μl) or vehicle. CCK induced an 180% increase in oxytocin secretion whereas α-MSH had no significant effect (*P<0.05, One way RM ANOVA). The number of rats per group is in parentheses.

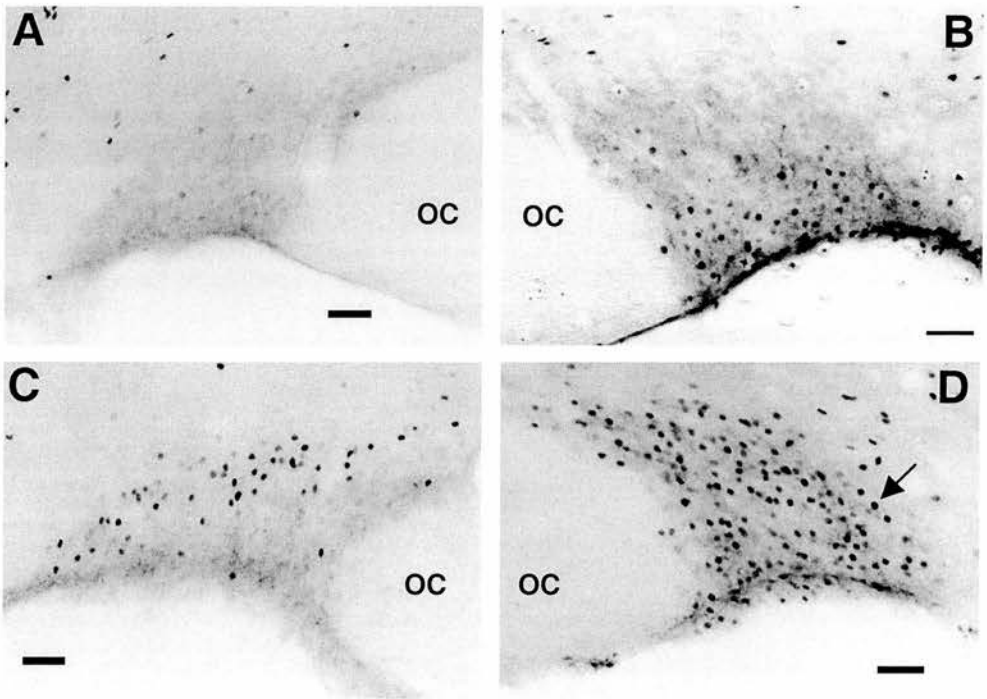

Figure 5.2: Photomicrographs illustrating Fos immunoreactivity in the SON after i.v. injection of vehicle (A) or CCK(C) or after i.c.v. injection of vehicle (B) or α-MSH (D).

OC: Optic chiasm. (arrow: Fos protein: black nuclear stain). Scale bar: 50μm.

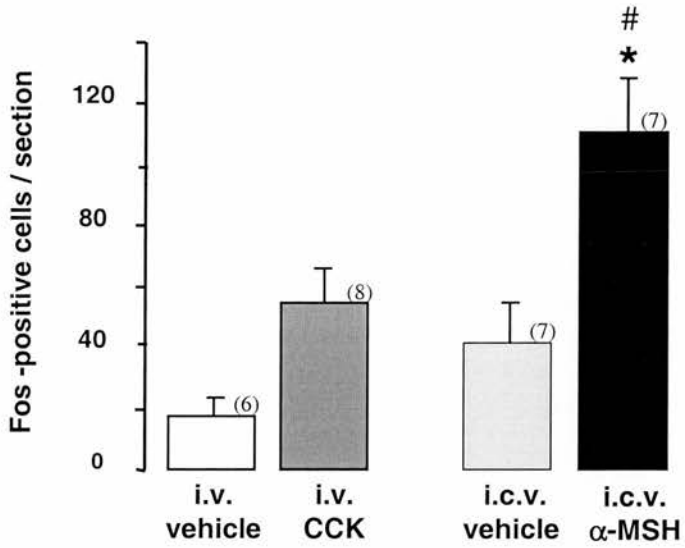

Figure 5.3: Fos immunocytochemistry in the SON after i.v. injection of vehicle or CCK or after i.c.v. injection of vehicle or α-MSH.

Values are means ± SEM. The number of rats per group is in parentheses. *P=0.004 versus i.v. vehicle, #P=0.005 versus i.v. CCK (one way ANOVA followed by Student-Newman-Keuls method).

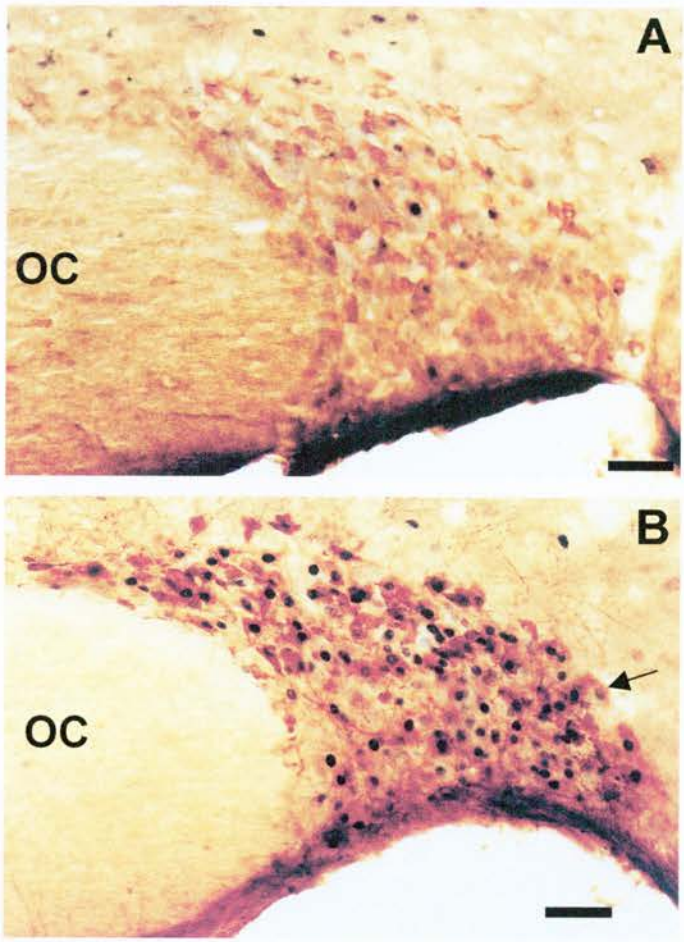

Figure 5.4: Photomicrographs illustrating Fos immunoreactivity (Black nuclear stain) in oxytocin neurones (Brown cytoplasmic stain, arrow) in the SON after i.c.v. injection of vehicle (A) or α-MSH (B).

OC: Optic chiasm. Scale bar: 50μm.

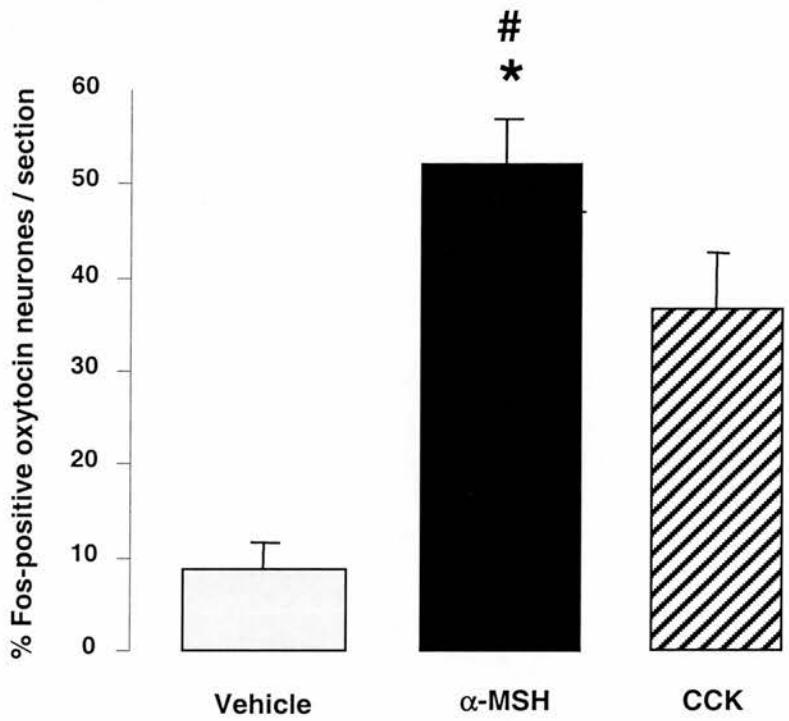

Figure 5.5: Fos expression in oxytocin neurones in the SON after i.c.v administration of α-MSH.

Values are mean % oxytocin neurones Fos-positive/ section ± SEM. n=8. *P<0.001 versus vehicle treated group, #P=0.025 versus CCK treated group (One way ANOVA foolwed by Student-Newman-Keuls method).

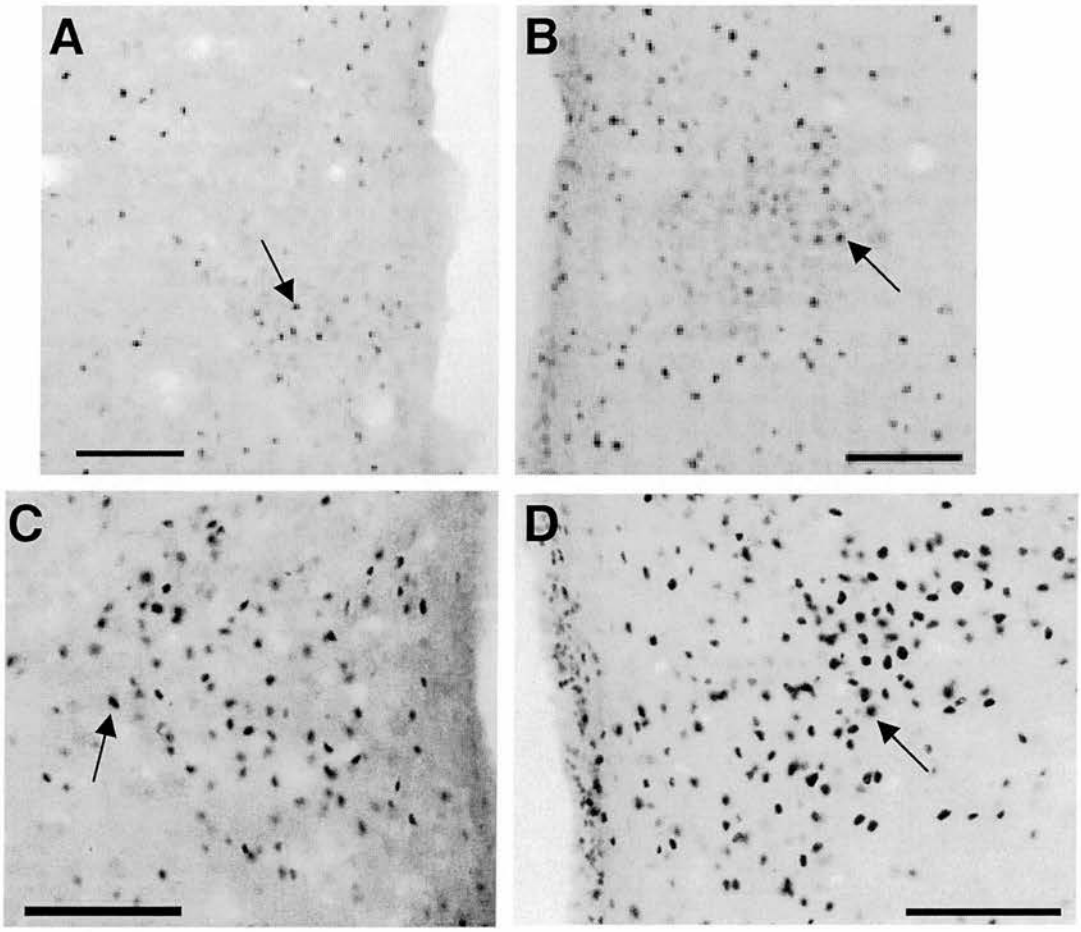

Figure 5.6: Photomicrographs illustrating Fos immunoreactivity (arrow) in the anterior parvocellular PVN (A, B) and in the ventral parvocellular PVN (C, D) after i.c.v injection of vehicle (A, C) or α-MSH (B, D).

Scale bar: 100μm.

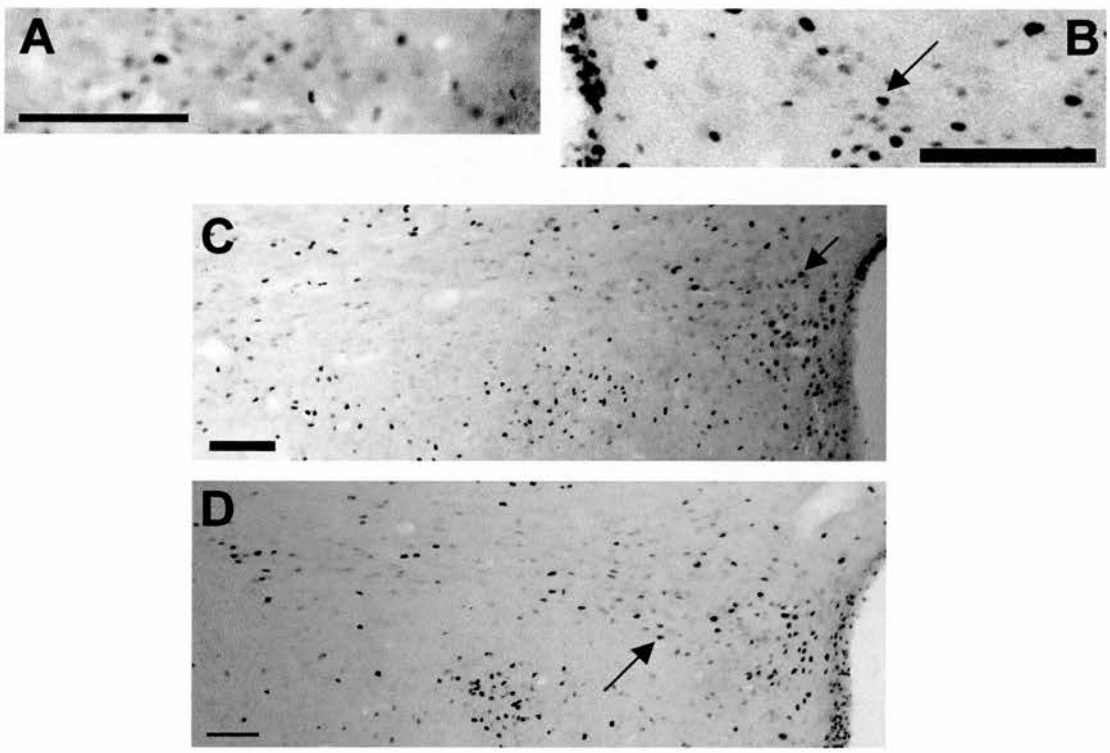

Figure 5.7: Photomicrographs illustrating Fos immunoreactivity (arrow) in the dorsa parvocellular PVN (A, B) and in the lateral posterior parvocellular PVN (C, D) after i.c.v injection of vehicle (A, C) or α-MSH (B, D).

Scale bar: 100μm.

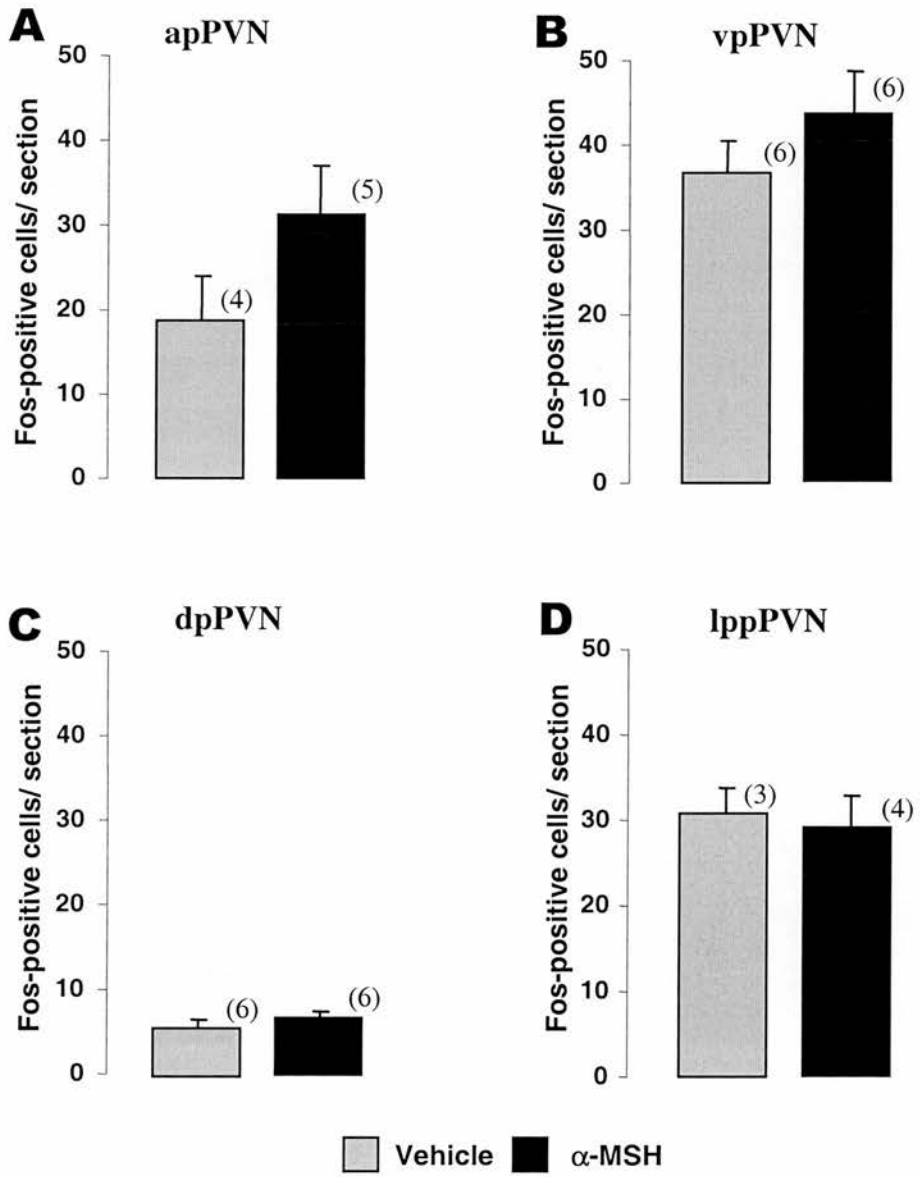

Figure 5.8: Fos expression in the parvocellular subdivisions of the PVN after i.c.v. injection of α-MSH.

A: apPVN: anterior parvocellular PVN; B: vpPVN: ventral parvocellular PVN; C: dpPVN: dorsal parvocellular PVN; D: lppPVN: lateral posterior parvocellular PVN. Values are means ± SEM. The numbers of rats per group is in parentheses.

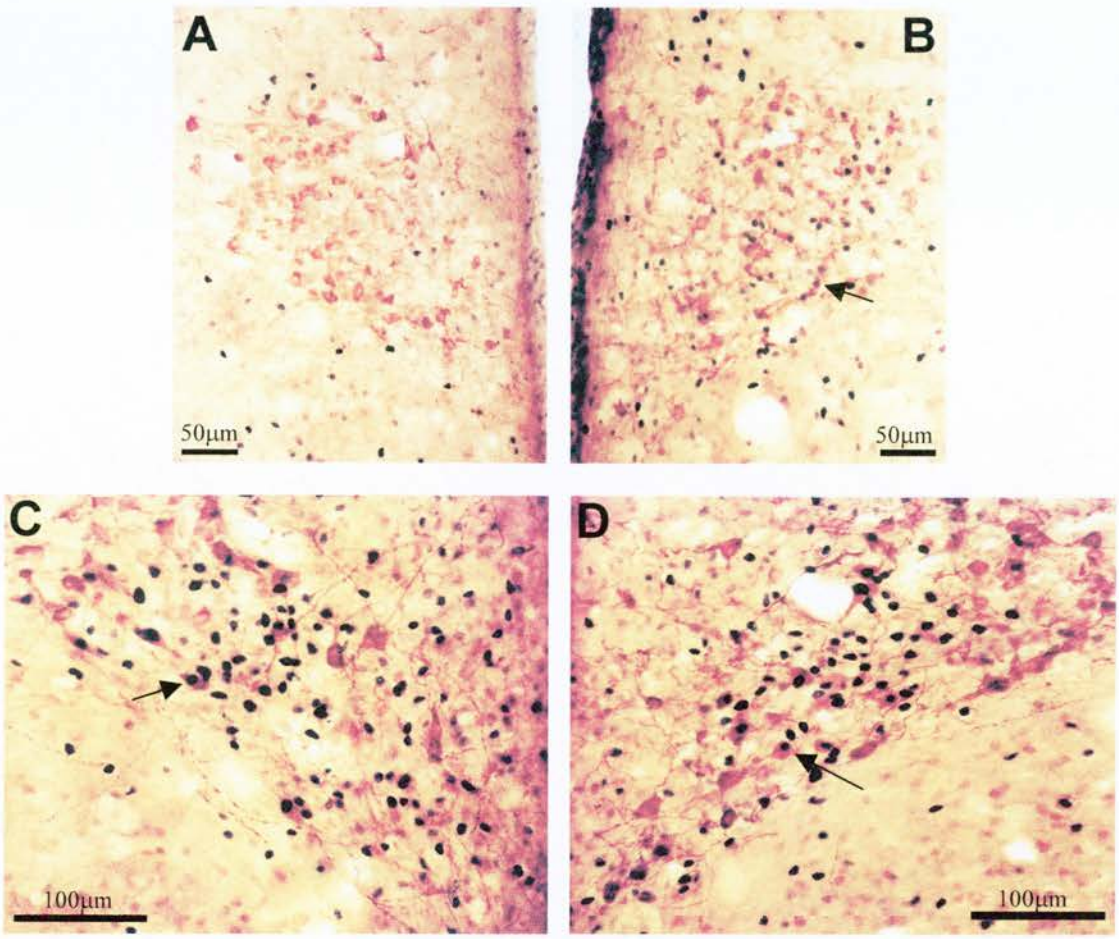

Figure 5.9: Photomicrographs illustrating Fos-immunoreactivity (Black nuclar stain) in oxytocin neurones (Brown cytoplasmic stain, arrows) in the anterior parvocellular PVN after i.c.v. injection of vehicle (A) or α-MSH (B) and in the ventral parvocellular PVN after i.c.v. injection of vehicle (C) or α-MSH (D).

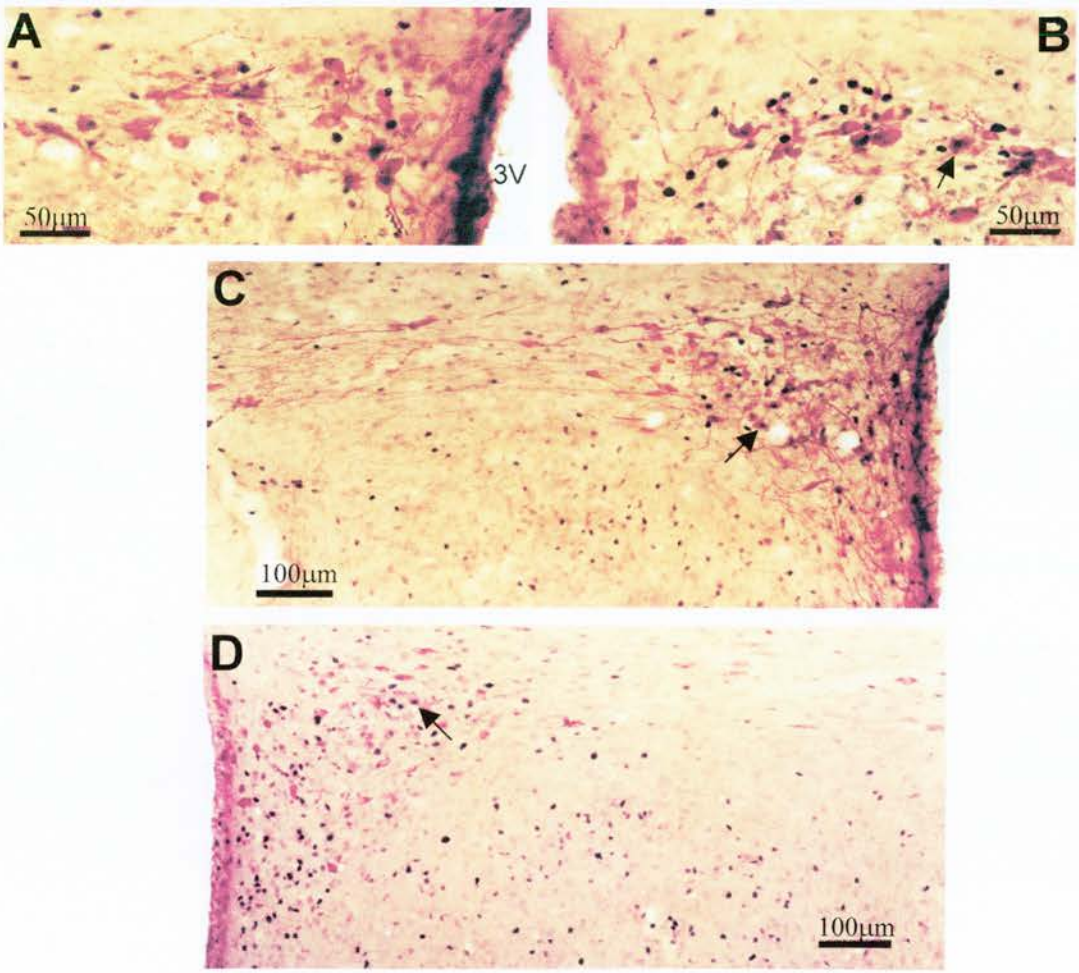

Figure 5.10: Photomicrographs illustrating Fos immunoreactivity (Black nuclear stain) in oxytocin neurones (Brown cytoplasmic stain, arrows) in the dorsal parvocellular PVN after i.c.v. injection of vehicle (A) or α-MSH (B) and in the lateral posterior parvocellular PVN after i.c.v. injection of vehicle (C) or α-MSH (D). 3V: Third ventricle.

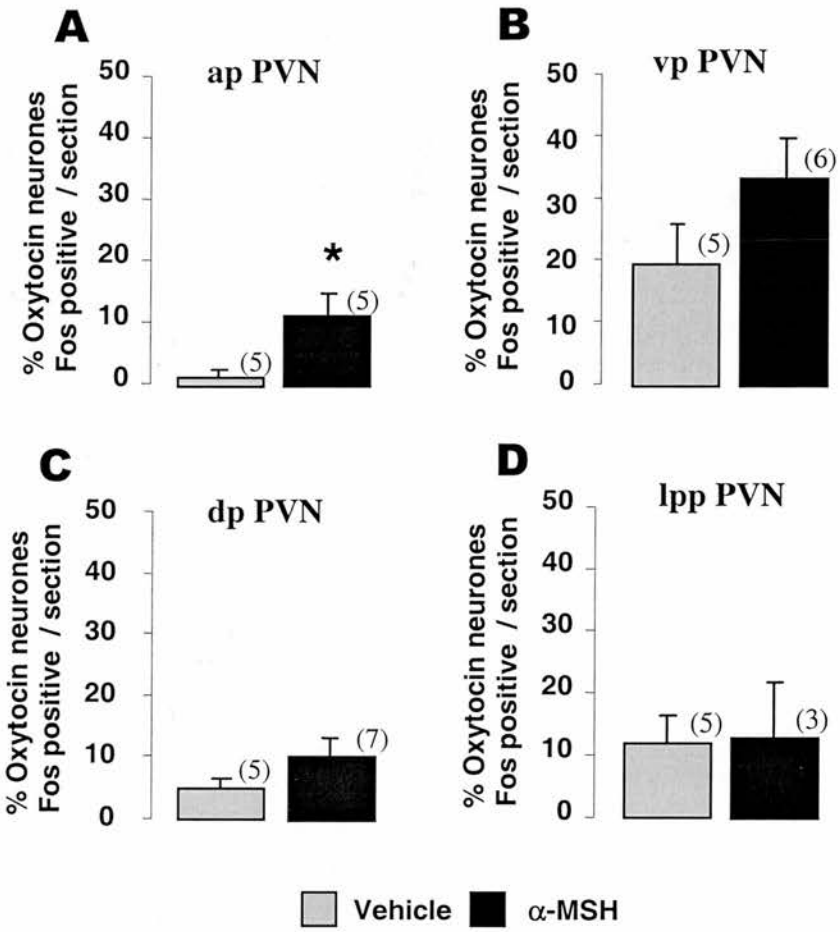

Figure 5.11: Fos expression in oxytocin neurones in the parvocellular PVN after i.c.v. administration of α-MSH.

A: apPVN: anterior parvocellular PVN; B: vpPVN: ventral parvocellular PVN; C: dpPVN: dorsal parvocellular PVN; D: lppPVN: lateral posterior parvocellular PVN. Values are mean % oxytocin neurones Fos-positive/ PVN ± s.e.m. The number of rats per group is in parentheses. *P=0.041 versus vehicle treated group, t-test.

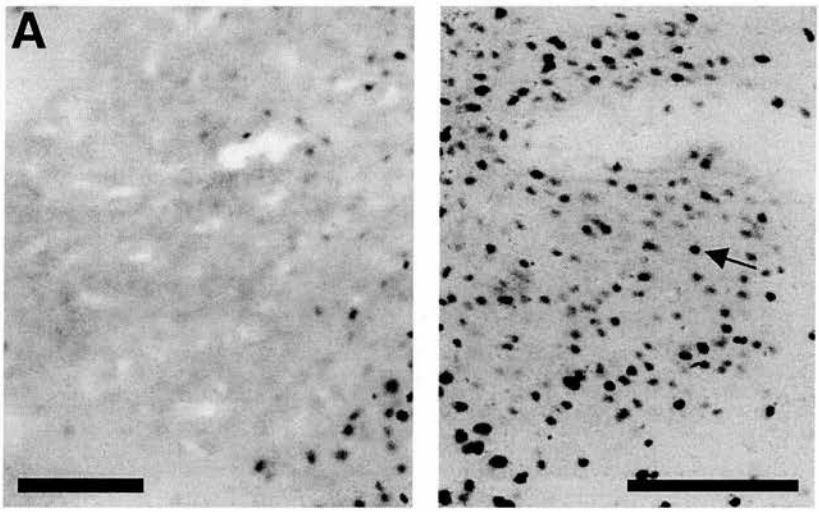

Figure 5.12: Photomicrographs illustrating Fos immunoreactivity (arrow) in the magnocellular PVN after i.c.v injection of vehicle (A) or after i.c.v injection of α-MSH (B).

Scale bar: 100μm.

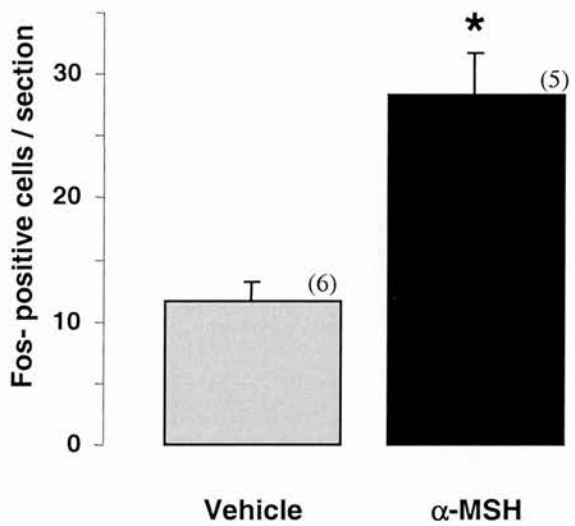

Figure 5.13: Fos expression in the magnocellular subdivisions of the PVN after i.c.v.injection of α-MSH.

Values are means ± SEM. *p<0.001 versus 'vehicle' treated group, t-test. The number of rats per group is in parentheses.

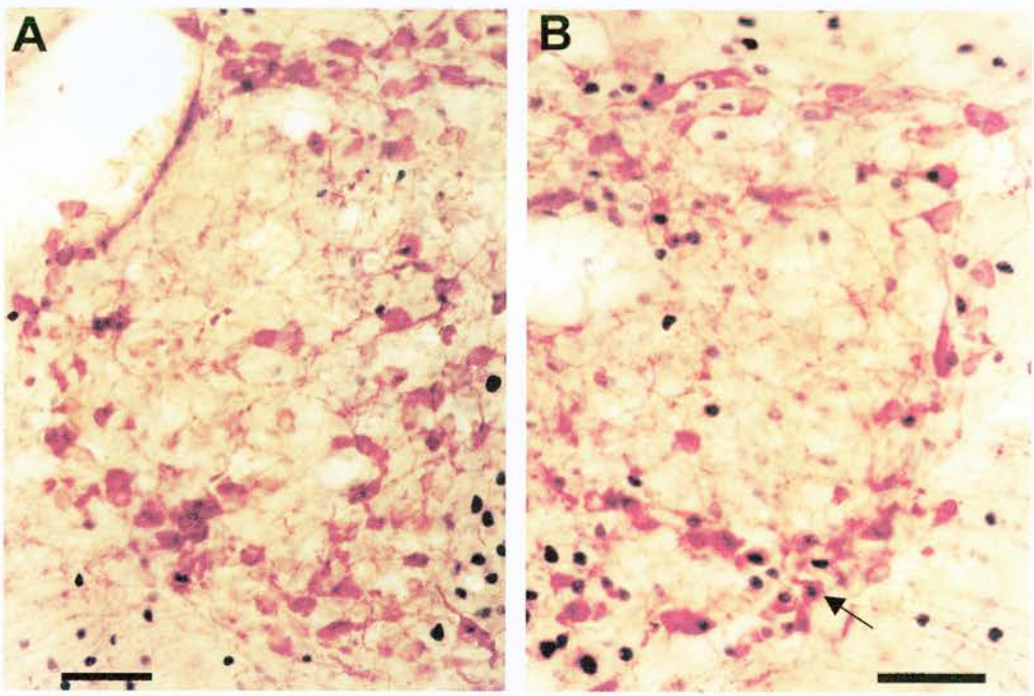

Figure 5.14: Photomicrographs illustrating Fos immunoreactivity (Black nuclear stain) in oxytocin neurones (Brown cytoplasmic stain, arrow) in the posterior magnocellular PVN after i.c.v. injection of vehicle (A) or α-MSH (B).

Scale bar: 100μm

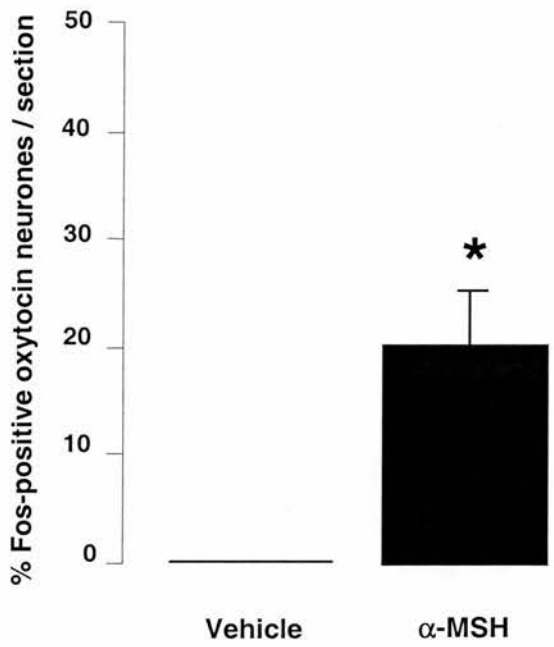

Figure 5.15: Fos expression in oxytocin magnocellular neurones in the PVN after i.c.v administration of α-MSH.

Values are mean % oxytocin neurones Fos-positive/ section ± SEM. n=7. *P<0.001 versus vehicle treated group, Mann-Whitney rank sum test.

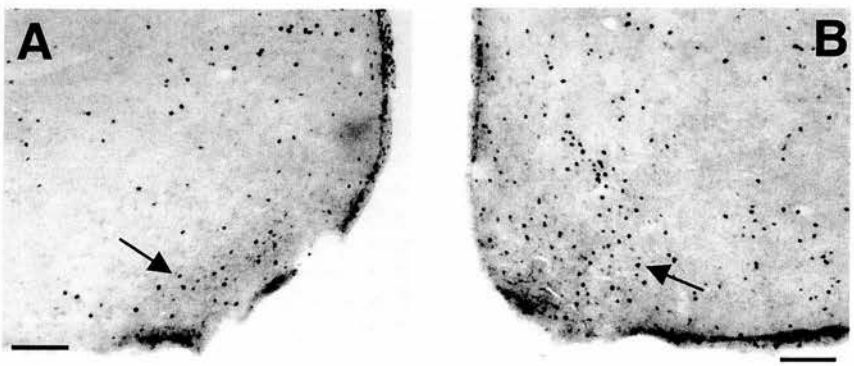

Figure 5.16: Photomicrographs illustrating Fos immunoreactivity (arrow) in the arcuate nucleus after i.c.v injection of vehicle (A) or after i.c.v injection of α-MSH (B).

Scale bar: 100μm.

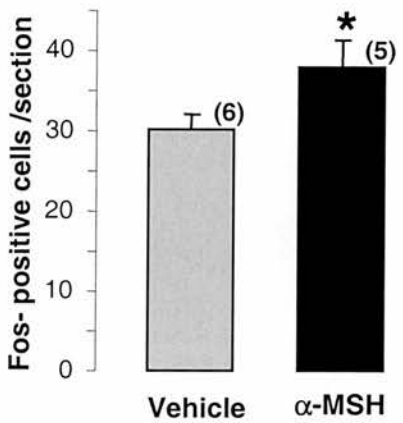

Figure 5.17: Fos expression in the arcuate nucleus after i.c.v.injection of α-MSH.

Values are means ± SEM. *P=0.020 versus vehicle treated group, t-test. The number of rats per group is in parentheses.

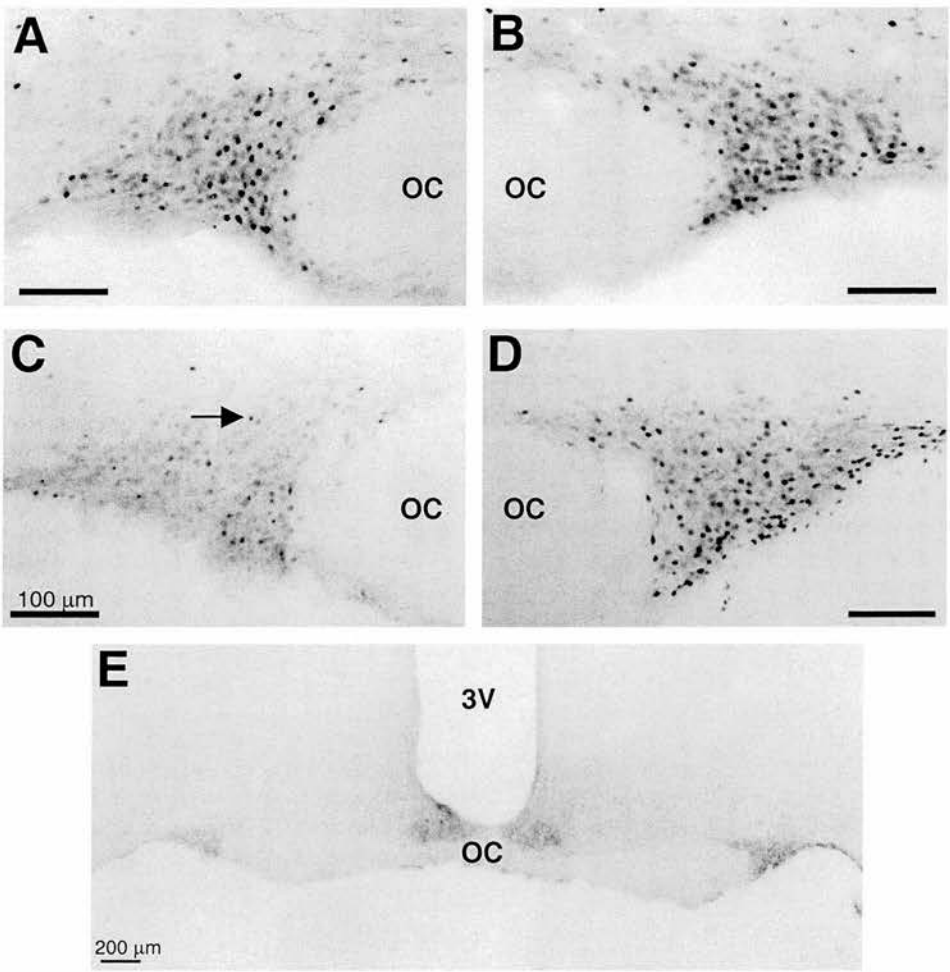

Figure 5.18: Photomicrographs illustrating Fos immunoreactivity (arrow) in the SON after local retrodialysis of isotonic saline (A: contralateral side, B: ipsilateral side) or α-MSH (C: contralateral side, D: ipsilateral side, E: Coronal hypothalamic section with the two SON).

OC: Optic chiasm. 3V: Third ventricle.

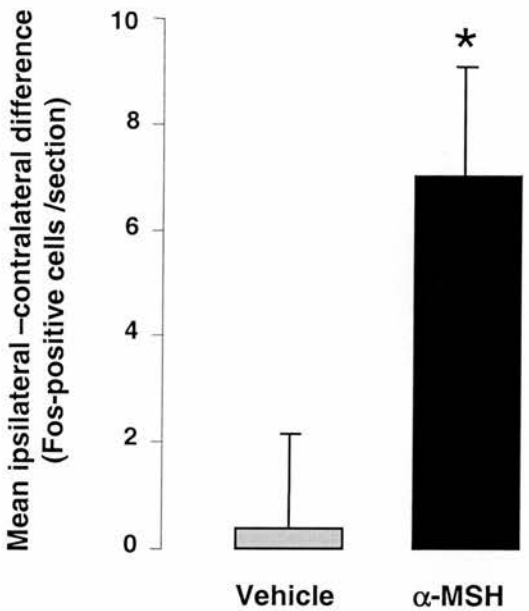

Figure 5.19: Fos expression in the SON after local retrodialysis of α-MSH.

Values are mean ipsilateral-contralateral difference in Fos-positive cells ± SEM. n=6. *P=0.01, t-test.

5.4.2. Experiment 2: *Changes in Fos expression in the SON after local administration of α-MSH:*

In rats dialysed with vehicle aCSF, there was no significant difference between the two supraoptic nuclei: the supraoptic nucleus adjacent to the microdialysis probe (ipsilateral nucleus) contained 10 ± 7 Fos-positive cells/ SON while the control supraoptic nucleus (contralateral nucleus) contained 10 ± 7 Fos-positive cells/ SON (Fig 5.18). In rats dialysed with aCSF containing α-MSH, the ipsilateral nucleus contained 14 ± 5 Fos-positive cells/ SON while the contralateral nucleus contained 8 ± 3 Fos-positive cells/ SON. Thus, Fos expression in the ipsilateral nucleus was significantly higher than in the contralateral nucleus (P=0.005, paired t-test). The mean ipsilateral-contralateral difference in rats dialysed with vehicle was 0.4 ± 2 Fos-positive cells/ section but was significantly higher in rats dialysed with α-MSH (7 ± 2 Fos-positive cells/ section; P=0.011, t-test) (Fig 5.19). This indicates that the increase in Fos expression observed in the ipsilateral supraoptic nucleus is not related to the insertion of the microdialysis probe, but is induced by administration of α-MSH.

5.4.Discussion

The present study illustrates the modulatory effects of α-MSH on oxytocin neurones in the PVN and SON. Surprisingly, we found that the modulation of oxytocin neurones by α-MSH was remarkably selective as α-MSH injection activated especially magnocellular oxytocin neurones and had little effect on parvocellular oxytocin neurones. We also found that α-MSH had modulatory effects on neurones from the arcuate nucleus. These two main findings are discussed in the following sections:

I. α-MSH and Fos expression in the arcuate nucleus:

i.c.v. administration of α-MSH significantly increased Fos expression in the arcuate nucleus. The arcuate nucleus is a heterogeneous nucleus that contains different neuronal populations with specific neurotransmitters that mediate particular

effects. So, the increase of Fos expression by α-MSH could reflect the modulation of activity of POMC neurones such as endorphin-, corticotropin- or melanotropic peptide -containing neurones. α-MSH could also interact directly or indirectly with α-MSH –containing neurones as an auto-control of its own release. Although MC3 and MC4 receptors are expressed in the arcuate nucleus, the presence of melanocortin receptors on α-MSH neurones still needs to be investigated. Thus, this result could reflect the role of α-MSH in the regulation of a vast number of biological functions and behaviours. For instance, the arcuate nucleus is one of the regulation centres of feeding behaviour and because α-MSH is a potent anorexic peptide, an increase of Fos expression in the arcuate nucleus after i.c.v. α-MSH could reflect neuronal interactions involved in the regulation of feeding. Another possible candidate with whom α-MSH could interact is the neuropeptide Y (NPY, from the pancreatic polypeptide family). The arcuate nucleus is the major source of NPY expression in the hypothalamus, and amongst other physiological functions, NPY is known to stimulate feeding. Growing evidence suggests that α-MSH and NPY systems interact to regulate each other's activity. Hansen *et al.* (2002) have shown that α-MSH i.c.v administration (1 - 4nmol) attenuates NPY-induced food intake.

II. a-MSH modulates directly and selectively magnocellular oxytocin neurones:

We found that α-MSH modulates directly and selectively the activity of oxytocin neurones. Unexpectedly α-MSH had no or little effect on parvocellular oxytocin neurones, but predominantly activated magnocellular oxytocin neurones. Nonetheless, α-MSH had no effect on oxytocin secretion into the plasma. These results place the relationship between Fos and neuronal electrical activity, and the interaction between α-MSH and the magnocellular oxytocin system into question:

- ***Relationship between Fos and neuronal depolarization:***

Since its discovery in stimulated neurones by Sagar and colleagues (1988), Fos, the protein product of the immediate early gene c-*fos,* has been widely used as

an anatomical marker of neuronal activation. It has been generally accepted that the presence of Fos in a neurone reflects the electrical neuronal excitation, and is consequential to a stimulus -induced depolarization. However, the present study of the effects of α-MSH on the oxytocin system contradicts this general assumption. To try to clarify the real meaning of Fos, it needs to be understood why the presence of Fos in a stimulated neurone led to the assumption that this neurone was excited, and some evidence countering this general assumption, including a brief description of the main signaling pathways that lead to the transcription of the c-*fos* gene, needs to be examined.

1. Why has Fos been assumed to reflect neuronal excitation?

In numerous neuronal systems, the presence of Fos is associated with an increase in the neuronal electrical activity in various conditions:

Parvocellular and magnocellular oxytocin neurones when stimulated generally express the protein Fos. Numerous stimuli, for instance stress (Sharp *et al.*, 1991), osmotic stimulation (Giovannelli *et al.*, 1990), peripheral injection of CCK (Verbalis *et al.*, 1991) and behaviours like parturition (Douglas *et al.*, 1995) induce Fos expression in magnocellular oxytocin neurones. In each of these examples, Fos expression is associated with an increase of the neuronal firing rate and/ or with an increase in plasma oxytocin secretion. For instance, osmotic stimuli such as a systemic injection of hypertonic saline and water deprivation induce strong Fos expression in magnocellular oxytocin neurones (Giovannelli *et al.,* 1990), and increase oxytocin secretion in the general circulation (Stricker & Verbalis, 1986). Shibuki et al., (1988) showed that hypertonic saline injection also induced an increase in the firing rate of oxytocin neurones. Another example of stimulus inducing Fos expression is a peripheral injection of CCK. CCK induces secretion of oxytocin but has no effect on vasopressin secretion (Verbalis *et al.*,1986), increases the electrical activity of oxytocin neurones but has no effect on vasopressin neuronal activity (Renaud *et al.*, 1987), and induces Fos expression in oxytocin neurones but not in vasopressin neurones (Verbalis *et al.,* 1991). These studies suggested that Fos expression reflects an increase in neuronal activity in magnocellular neurones. Compared to an injection of hypertonic saline, systemic injection of CCK induces a

mild and transient increase in electrical activity of magnocellular oxytocin neurones, but nonetheless, it also induces Fos expression in magnocellular oxytocin neurones, suggesting that Fos expression does not only reflect a strong and sustained neuronal activation. This was illustrated in a study by Hamamura *et al.* (1991), in which they compared the degree of c-*fos* mRNA expression with the electrical activation of oxytocin neurones after systemic injection of CCK using an i.p injection of hypertonic saline as a positive control. Injection of hypertonic saline induced an intense increase in neuronal activity and strong c-*fos* mRNA expression in magnocellular oxytocin neurones. Although, CCK injection induced a brief increase in neuronal activity of magnocellular oxytocin neurones, it induced a detectable increase in c-*fos* mRNA expression. This illustrated that a brief and small electrical activation was sufficient to induce c-*fos* mRNA expression in magnocellular oxytocin neurones. Thus, these results suggested that the Fos was a specific and precise marker of increased neuronal electrical activity associated with stimulated secretion.

Taking these observations together, it has been deduced that a neurone containing Fos was an excited neurone and led to the general assumption that Fos was a marker of neuronal excitation.

2. Contradicting evidence

Firstly, Fos expression is not always associated with increased neuronal activity. Indeed, previous studies have shown that α-MSH induced Fos expression in vasopressin neurones (Olszewski *et al.*, 2001). If Fos reflects neuronal depolarization, α-MSH should also induce an increase in firing rate of vasopressin neurones. However, Sabatier *et al.* (2003) showed that α-MSH i.c.v. had no effect on the electric activity of vasopressin neurones.

Conversely, there are examples were depolarization (and/ or secretion) is not associated with Fos expression:

i). LHRH neurones contain NMDA (N-methyl-D-aspartate) receptors. An injection of a NMDA receptor agonist increases the secretion of GnRH and hence the

secretion of LH, but fails to induce Fos expression in LHRH neurones (Hoffman, 1993).

ii). Excitatory efferents from the cerebral cortex project to regions in the basal ganglia including the striatum. Fu & Beckstead (1992) reported that electrical stimulation of the cerebral cortex induces Fos expression in striatal neurones. However, Sgambato *et al.* (1997) reported that electrophysiological activation of striatal cells was not systematically accompanied by Fos expression. They electrically stimulated different cortical areas in the rat and looked at the Fos induction in the striatum and measured the electrical activity of striatal cells. Whereas electrical stimulation of the motor or auditory cortex induced increase in neuronal activity and Fos expression in the striatum, electrical stimulation of the somatosensory facial area (visual and orbital cortices) increased electrical activity in the striatum but failed to induce Fos expression in the striatal areas receiving these cortical excitatory inputs. They therefore suggested that Fos induction does not accurately reflect neuronal activity.

The present study of α-MSH effect on the Fos expression in oxytocin neurones and on oxytocin secretion, also illustrates dissociation between Fos expression and neuronal electrical activity. While i.c.v. injection of α-MSH increased Fos expression in magnocellular oxytocin neurones in the SON and in the PVN, it did not stimulate the secretion of oxytocin into plasma. In addition, we previously described that an i.c.v. injection of MC4 receptor agonist inhibited plasma oxytocin secretion (Chapter 3). Positive control CCK-injected rats presented, as expected, an increase of Fos expression in the SON as well as an increase in oxytocin secretion, demonstrating that the apparent contradictory results obtained for α-MSH –injected rats were not due to an experimental artifact. This would suggest that α-MSH increases Fos expression and inhibits oxytocin secretion from the nerve terminals in the posterior pituitary and therefore inhibits oxytocin electrical activity. An inhibition of secretion might result either from a reduction in electrical activity of the neurones, or from an inhibitory action at the nerve terminals analogous to the actions of opioid peptides (Bicknell and Leng, 1982). Sabatier *et al.* (2003) showed an inhibition of electrical activity of oxytocin neurones by α-MSH. They tested the

effect of α-MSH and MC4 receptor agonist on oxytocin neurone electrical activity. α-MSH produced a rapid inhibition of oxytocin cells and MC4 receptor agonist inhibited oxytocin neurones in a dose-dependant manner; Thus, these results strikingly suggest that Fos expression does not necessarily reflect excitation of the neurones, and can be associated with the inhibition of electrical activity; whether α-MSH also inhibits oxytocin secretion by an action at the nerve terminals is not established.

3. Intracellular signaling pathways regulating c-fos transcription:

c-*fos* gene transcription is controlled by two major promoter sequences CaCRE (Ca^{2+}/ cAMP responsive element) occupied by CREB (CRE -binding proteins) regulated by phosphorylation, and SRE (serum responsive element) to which TCF (ternary complex factors) and SRF (SRE –binding factors) binds (Fig 5.20). Various complex signaling pathways lead to the activation of these two promoter sequences. CREB, for instance, can be phosphorylated by several kinases, which are themselves activated by changes in intracellular calcium or cAMP concentration.

The fact that calcium is a major second messenger in the c-*fos* transcription signaling pathways and that an increase in intracellular calcium concentration can induce Fos expression confirmed the assumption that Fos induction was related to depolarization, as the opening of voltage –dependent Ca^{2+} -channels by depolarization would result in an entry of calcium. However, an entry of calcium by depolarization is not sufficient to induce Fos expression. Indeed, Luckman *et al.* (1994) demonstrated that an increase in spike activity of suproptic neurones was not sufficient to induce Fos expression in the same neurones. They compared the effect of i.c.v. carbachol (a muscarinic receptor agonist) and antidromic stimulation on Fos expression and firing rate of magnocellular neurones in the SON. Carbachol increased the firing rate of both oxytocin and vasopressin neurones as well as increased the Fos –immunoreactivity in the SON. However, Fos expression in the SON after antidromic stimulation was not significantly different from the control.

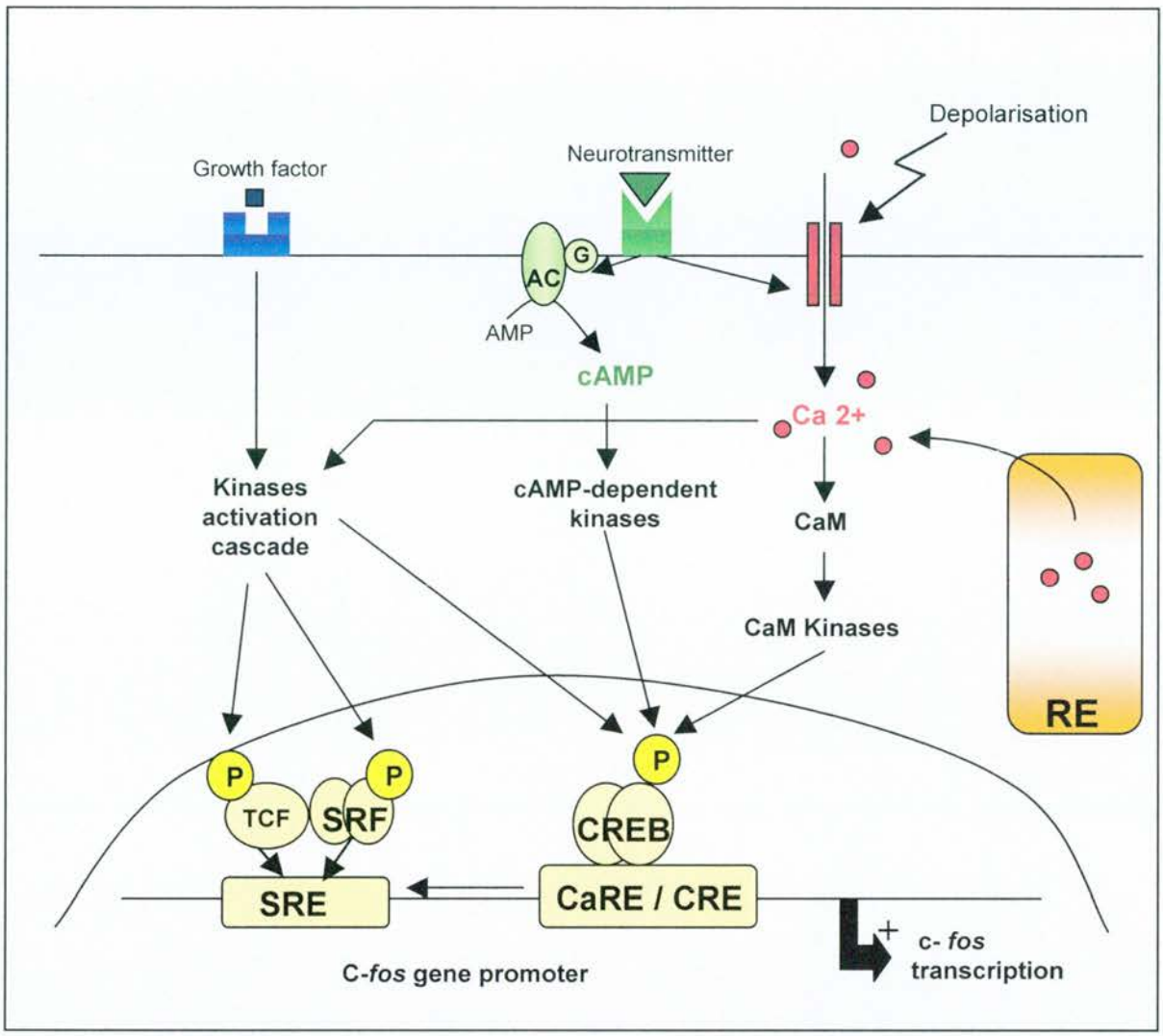

Figure 5.20: Schematic illustration of the main signalling pathways leading to Fos expression. *(adapted from Hoffman & Lyo 2002)*

There are two major promoter sequences controlling c-*fos* gene transcription: CaRE/ CRE (calcium / cAMP responsive element) and SRE (serum responsive element). In the first main pathway, an increase of either intracellular calcium or cAMP induces, by activation of specific kinases, the phosphorylation of transcription factor CREB (CRE binding proteins). The binding of numerous neurotransmitters to their receptors can induce an increase of intracellular cAMP or intracellular calcium. Depolarisation, via activation of voltage-dependent Ca2+ channels or relargage of Ca2+ from the reticulum endoplasmic store (RE) can also increase the level of intracellular calcium. In a second pathway, the binding of growth factors or mitogens on their receptors induces the activation of kinases (e.g.: MAPkinases) which induces the phosphorylation of the transcription factors SRF (serum response factors) and TCF (ternary complex factor) which bind to SRE.

AC: Adenylate cyclase; cAMP: cyclic adenosine monophosphate; CaM: Calmodulin; G: G-protein.

This suggests that Fos is not induced by the influx of Ca^{2+} resulting from the spike activity but requires some additional intracellular signal.

In addition, changes in intracellular Ca^{2+} can result from a release of Ca^{2+} from the endoplasmic reticulum stores (ER). Gissel *et al.* (1997) used thapsigargin (an irreversible inhibitor of Ca^{2+}-ATPase of the ER) to deplete the Ca^{2+} ER store of primary neurones in culture, and measured changes of c-*fos* mRNA. Thapsigargin induced an increase in c-*fos* mRNA expression. Thus, a surplus of Ca^{2+} ions from depolarization is not the only source of changes of intracellular Ca^{2+} that induce c-*fos* transcription, Ca^{2+} from ER store intervenes too.

As indicated by its name, CaCRE (Ca^{2+}/ cAMP responsive element) is also under the regulation of intracellular cAMP levels. Indeed, an increase in intracellular cAMP concentration activates various kinases that then phosphorylate CREB, which in turn activates CaCRE. The production of cAMP results from activation of metabotropic receptors by a variety of neuropeptides and neurotransmitters (Hoffman & Lyo, 2002).

Thus, the presence of Fos in a neurone can result from the activation of various signaling pathways that can be independent from any changes in intracellular Ca^{2+}. This illustrates that considering Fos expression as a reflection of depolarization and neuronal excitation is inadequate. The complex cellular mechanisms and signaling cascades that induce Fos still need to be clarified and until then, no assumption about Fos significance should be made. The presence of Fos in a neurone after stimulation indicates that some changes in the intracellular signaling pathways have been induced by the stimulus used: the neurone is '*activated*'; but these changes can be associated either with an excitation, an inhibition or with no modification of the neuronal electrical activity.

- ***α-MSH and the magnocellular oxytocin system:***

1. Direct action of α-MSH onto magnocellular oxytocin neurones:

In the second experiment, we found that a local infusion of α-MSH onto the SON induced an activation of oxytocin magnocellular neurones, and so confirmed

that this modulation was direct on the oxytocin neurones and not through other types of neurones. This result corroborates the study of Sabatier *et al.* (2003) who tested the effect of α-MSH directly administrated onto the SON by retrodialysis. They measured the changes in the firing rate of five oxytocin neurones during retrodialysis of α-MSH (concentrations from 1 to 3.4μg/μl) and found that the oxytocin neurones were inhibited by α-MSH. Two types of melanocortin receptors MC3 and MC4 receptors, are involved in the central effects of α-MSH. No MC3 receptors or MC3 receptor mRNA has been located in the SON or PVN but high levels of MC4 receptor mRNA have been found (Mountjoy *et al.,* 1994). Furthermore, Sabatier *et al.* (2003) demonstrated that i.c.v. administration of a MC4 receptor agonist significantly decreased oxytocin firing rate. Taken together, these results indicate that the direct effect of α-MSH on oxytocin neurones is mediated via MC4 receptors.

2. Magnocellular oxytocin system and central regulation of oxytocin-dependent behaviours:

Generally, oxytocin is limited in its ability to cross the blood -brain barrier and so very little of the oxytocin that is secreted in the blood can reenter in the brain. Because magnocellular oxytocin neurones project to the pituitary gland (where oxytocin is released in the blood) and have very few axon collaterals terminating within the central nervous system, it has been assumed that the magnocellular oxytocin system was not involved in central regulation of behaviours. Instead, it has been assumed that the parvocellular oxytocin neurones, which project to different brain areas where functional oxytocin receptors have been located, were the only oxytocin system involved in the central regulation of oxytocin-dependent behaviours.

Since α-MSH and oxytocin are thought to interact centrally to modulate their central effects, an i.c.v injection of α-MSH was expected to activate parvocellular oxytocin neurones with no major effect on magnocellular oxytocin neurones. However, we found the opposite: although α-MSH had little effect on parvocellular oxytocin neurones, as it activated only oxytocin neurones from the anterior parvocellular PVN; α-MSH predominantly activated magnocellular oxytocin neurones in both the PVN and the SON. We also found that α-MSH administration

induced no significant changes in oxytocin secretion. These results place the role of magnocellular oxytocin neurones into question:

If the only role of the magnocellular oxytocin system is to control the secretion of oxytocin into the systemic circulation, why does α-MSH, which has no effect on oxytocin secretion, induce changes in the intracellular pathways of magnocellular oxytocin neurones (reflected by the presence of Fos)?

Could magnocellular neurones be involved in the central regulation of oxytocin-dependent behaviours? Perhaps, oxytocin released from the dendrites of magnocellular neurones could act as a neurohormone by diffusing to distant brain areas and interact with other neuronal systems to regulate various functions and behaviours. Several lines of evidence support this hypothesis:

i). As mentioned in chapter 1, oxytocin fibres and oxytocin receptors have been located in many areas throughout the CNS. Their distribution is well documented (see Buijs *et al.,* 1978, 1979, 1985; Yoshimura *et al.,* 1993; Elands *et al.,* 1998; Vaccari *et al.*, 1998; Gimpl & Fahreholz, 2001; Table 1.3). Going through the literature, discrepancies within the distribution of oxytocin fibres and receptors become apparent. Although, several authors mentioned the existence of mismatches between the localization of oxytocin fibres and the localization of oxytocin receptors (Yoshimura *et al.* 1993), no study ever precisely detailed or listed those mismatches. In brief, by comparing areas where both oxytocin innervation and oxytocin receptors distribution have been investigated, it is noticeable that the distribution of oxytocin axon terminals and oxytocin receptors does not follow any 'rules of organisation' (Table 1.3): Some areas are highly innervated with oxytocin fibers and possess numerous oxytocin receptors (e.g.: hypothalamus); conversely, some areas are weakly innervated and possess a small number of oxytocin receptors (e.g.: SFO, hippocampus). But interestingly, in some areas, while oxytocin innervation is weak, oxytocin receptors are disproportionately abundant (e.g.: olfactory system, subiculum, amygdala, BNST).

The functionality of these receptors has been confirmed by electrophysiological studies (DeKloet *et al.*, 1990). It seems unlikely that the existence of receptors independent from any oxytocin axon terminals is due to a redundant expression of oxytocin receptors in these brain areas.

ii) In many brain areas, the content of oxytocin in extracellular fluid is much higher than what should be expected by local release from axon terminals only. Oxytocin release from different micro-dissected brain areas has been measured in basal and 'stimulated' (high levels of K^+ induced depolarization) (Moos *et al.*, 1984; Buijs *et al.*, 1985). Even in conditions where the release is stimulated, the levels of oxytocin released represent only around 3% of the local tissue content. Landgraf *et al.* (1992) performed push-pull perfusion studies *in vivo* and measured the amount of oxytocin release from different brain areas in basal conditions. They found that the quantity of oxytocin release from the axon terminals was not substantial enough to explain the large amount of oxytocin found in many brain areas. The cerebrospinal fluid (CSF) also contains oxytocin (from 0.5 to 0.7pg/ml at basal levels), of which the source is unclear. The oxytocin present in both CSF and extracellular fluid in many brain areas could derive from the diffusion from magnocellular neurones as the major source of oxytocin in the brain is the magnocellular system and dendrites from the magnocellular neurones are packed with oxytocin neurosecretory granules.

iii) Pow and Morris (1989) have demonstrated that oxytocin can be released from the dendrites of magnocellular neurones by exocytocis. Numerous stimuli are known to induce dendritic release of oxytocin (Ludwig, 1998). Central dendritic release of oxytocin and peripheral secretion of oxytocin from axons terminals are regulated by different mechanisms and dendritic release is semi- independent from the electrical activity. Oxytocin itself has been reported to induce dendritic release of oxytocin without any oxytocin secretion into the plasma (Ludwig, 1998). In a similar way, α-MSH could trigger oxytocin release from magnocellular neurones without inducing peripheral secretion.

Thus, some central effects of α-MSH could be mediated via dendritic release of oxytocin from magnocellular neurones rather than via oxytocin release from the axon terminals of the parvocellular neurones.

Taken together, these results illustrate the fact that central α-MSH could interact with the oxytocin system to regulate some physiological functions and behaviours. Indeed, α-MSH modulates the activity of specific subpopulations of oxytocin neurones: it predominantly modulates the activity of oxytocin magnocellular neurones and does not modulate the activity of oxytocin parvocellular neurones, except the anterior parvocellular neurones. α-MSH may act directly on oxytocin magnocellular neurones via MC4 receptors to trigger oxytocin release from the dendrites. Thus, some behavioural effects of α-MSH that are mediated by oxytocin may reflect central dendritic release of oxytocin from magnocellular neurones, rather than from parvocellular neurones.

Chapter 6

Central interaction between oxytocin and α-MSH during male sexual behaviour.

6.1. Introduction:

Magnocellular oxytocin neurones in the PVN and in the SON are activated at intromission (Chapter 4). In conscious rats, i.c.v. administration of α-MSH increased Fos expression in magnocellular oxytocin neurones but had no effect on parvocellular oxytocin neurones (Chapter 5). We concluded that α-MSH modulated magnocellular oxytocin neurones specifically, probably via MC4 receptors. In the present study, we investigated whether the activation of magnocellular oxytocin neurones observed at intromission is induced by α-MSH via MC4 receptors.

We studied whether blocking the central effects of α-MSH that are mediated by MC4 receptors would attenuate the Fos expression in magnocellular oxytocin neurones observed at intromission. The goal of this study was to confirm a plausible interaction between α-MSH and oxytocin in the regulation of their central effects and, at the same time, to clarify the involvement of the MC4 receptors in the mediation of male sexual behaviour. Rats were injected i.c.v. with a MC4 receptor antagonist or vehicle. The Fos expression induced at intromission was then analyzed in all the subdivisions of the PVN and in the SON.

Finally, to clarify the previous results that α-MSH induced Fos expression in the arcuate nucleus (Chapter 5); a nucleus that is activated at intromission (Chapter 4), we measured changes in Fos expression in the arcuate nucleus after i.c.v administration of MC4 receptor antagonist. This study contributes to investigate a putative action of α-MSH on the arcuate nucleus and to discuss the involvement of the arcuate nucleus in the regulation of male sexual behaviour.

6.2. Methods:

6.2.1. Preparation of the females: *Induction of sexual receptivity:*

A month before the beginning of the experiment, 12 female rats were ovariectomised under brief halothane anesthesia (section 2.9.1.2) and then placed into single cages for recovery. Sexual receptivity was induced by s.c. injection of 3-β-estradiol monobenzoate (30μg/ 300μl; 48h before the test or experiment) and progesterone (1mg/ 100μl; 5 to 6h before the test or experiment) (section 2.9.1.2). A week before the experiment, each female was tested with a sexually-experienced male (stud male) to ensure that the doses used were sufficient to induce receptivity in all the females injected; and the timing of injection was adjusted to ensure the females were receptive at the starting time of the experiment i.e.: 13:00h.

6.2.2. Reverse light cycle:

Two weeks before the beginning of the experiment, rats were placed in reverse light cycle (dark from 10:00h to 22:00h). All the following tests and experiments were performed in the dark phase under dim red light illumination.

6.2.3. Preparation of the males:

6.2.3.1.) Test of male sexual vigor:

Sexually-experienced rats were used in this experiment. To ensure that males were physically-capable of mating, male sexual vigor was tested one week before the experiment. Each male was placed with a receptive female for 15 min. Only males that successfully mated were used for the experiment.

6.2.3.2) Surgery:

Three days before the experiment, males were fitted with an i.c.v. cannula under brief halothane anaesthesia (section 2.4.4) and then placed in a single cage for recovery until the beginning of the experiment.

6.2.4. Experimental design:

6.2.4.1. Injection of MC4 receptor antagonist:

The selective MC4 receptor antagonist L-863224 (Merck Laboratories, USA) (also named MBP10) was used.

i.c.v injections of MC4 receptor antagonists have been reported to have effects on sexual behaviour at doses from 5µg/ rat to 10µg/ rat (Vergoni *et al.,* 1998 & 2000; Martin *et al.*, 2002). It was important to ensure that the injection of MC4 receptor antagonist would not block male sexual performance, as the objective was to compare neuronal activation during intromission with vehicle-injected rats. Therefore, a low dose of 5.5µg/ 2µl/ rat of MC4 receptor antagonist was used, as used by Martin *et al.* (2002).

Dilution for 1 ml of MC4 receptor antagonist solution:

2.75 mg MC4 R antagonist (Merck)

400 µl Polyethylene glycol 6000 (BDH)

100 µl EtOH (BDH)

500 µl saline

The timing of the drug injection was important, as the effect of the antagonist needed to be synchronized with the rat behaviours. If the injection was too early before the test, then the drug effects would have disappeared long before the rat become aroused, and so the mating would then happen as normal. If the injection was too late, again the injection would have no effect on the mating process. In previously published studies related to male sexual behaviour, melanocortin receptor antagonists were injected before pairing the male and the female together (Vergoni *et al.,* 1998 & 2000; Martin *et al.*, 2002). Unfortunately, no more precise time of injection was described. In one study about the role of melanocortin receptors in sexual behaviour in female rats, sexual activity was tested 10 min after the i.c.v. administration of a central melanocortin receptor antagonist (Cragnolini, 2000). As described in chapter 3, i.c.v. injection of MC4 receptor agonist induced a significant decrease in plasma oxytocin concentration 15 min after the injection. In our laboratory conditions, intromission, the behaviour reference chosen, happened around 4 min after pairing a male with a receptive female.

The timing of injection chosen was 10 min before pairing to ensure that the expected duration of drug action would cover the time period during which rats would have mated in normal conditions (injection time + 14 min).

6.2.4.2. Protocol:

Ten minutes after connecting the drug administration tubes to the i.c.v. cannula, rats were injected with 2 µl of MC4 receptor antagonist (5.5 µg) or vehicle (saline). Ten min later, each injected-rat was paired with a receptive female in the female's cage. Sexual behaviours were recorded for 15 min or until the intromission reference behaviour had occurred. Males were then put back in their own cage. Rats were killed with an overdose of pentobarbitone (50mg/kg, s.c.), 95 min after being paired with a female for the control non-mating rats, or 90 min after intromission for the mating rats. Rats were perfused transcardially with heparinised physiological saline (0.9%) followed by 4% paraformaldehyde in 0.1M phosphate buffer (section 2.8.1). The brains were removed, post-fixed overnight, cryoprotected in 30% sucrose and stored at -70°C until processed for immunocytochemistry. Coronal sections of the hypothalamus were cut at 44µm using a freezing microtome. Only brains with a i.c.v.-injection site confirmed were processed for immunocyochemistry. Free-floating sections were processed for double immunocytochemistry for Fos and oxytocin as described in section 2.8.3. Fos-positive nuclei and the percentage of activation of oxytocin neurones throughout the PVN and the SON were counted blind. Four SON/ rat were counted for the dorsal SON; two profiles/ rat were counted for each subdivisions of the PVN. Fos-positive nuclei in six arcuate nuclei/ rat were analysed throughout the arcuate nucleus (posterior arcuate nucleus excluded).

6.3. Statistics:

A t-test was used to compare the mount and intromission latencies between vehicle-treated rats and MC4 receptor antagonist–treated rats.

Against expectations, a few rats injected with the MC4 receptor antagonist did not mate, creating a third experimental group with a small *n* number. A one-way ANOVA test was performed to compare Fos expression between the three groups (vehicle-treated mating rats, MC4 receptor antagonist-treated mating rats, MC4 receptor antagonist–treated control (non-mating) rats), followed by a pairwise multiple-comparison post hoc test (Student-Newman-Keuls Method). In a case of a failure of the equal test of variance, a one-way ANOVA on ranks followed by a pairwise multiple-comparison procedure (Dunn's Method) was performed instead. A P value < 0.05 was considered as statistically significant.

Statistical differences between data from the vehicle-injected mating rats and the MC4 receptor antagonist-treated non-mating rats were not taken into account, as the rats from these two groups have different treatment and experimental status.

6.4. Results:

i)Changes in sexual behaviour parameters after i.c.v. injection of MC4 receptor antagonist:

While all rats injected with vehicle mated when paired with a receptive female, only 5 out of 8 rats injected with MC4 receptor antagonist mated in the same conditions (Fig. 6.1.A). In the rats that did mate, injection of the MC4 receptor antagonist slowed down the mating process (Fig. 6.1.B):

After 78 ± 24s since pairing with a female, vehicle-injected rats displayed the first intensive mount whereas an average of 188 ± 94s were required for the MC4 receptor antagonist-injected rats (P=0.059, t-test). The reference behaviour (intromission) occurred after 127 ± 29s in vehicle-treated rats and after 274 ± 137s in rats treated with MC4 receptor antagonist (P=0.052, t-test). The time between the first intensive mount observed and intromission also increased in the MC4 receptor antagonist-treated group (87 ± 43s against 49 ± 9s for vehicle-treated rats).

ii)Changes in Fos expression in the PVN after i.c.v injection of MC4 receptor antagonist:

In the parvocellular subdivisions of the PVN:

Due to technical issues no sections with the lateral posterior parvocellular PVN were available for analysis for Fos alone.

In all the other subdivisions of the parvocellular PVN, no significant changes in the Fos expression were measured between the vehicle-treated rats and the MC4R antagonist-treated rats (Fig. 6.2; 6.3; 6.4). Nonetheless, in all parvocellular subdivisions, Fos expression in MC4R antagonist-treated rats that did not mate was generally lower than in the mating rats, verifying that the injection of MC4 receptor antagonist itself did not induce Fos expression.

The same observations were obtained after double immunocytochemistry for Fos and oxytocin: no significant changes in the Fos expression in parvocellular oxytocin neurones were noticed between the vehicle-treated rats and the MC4R antagonist-treated mating rats (Fig. 6.5, 6.6, 6.7).

In magnocellular subdivisions of the PVN:

No significant changes in Fos expression at intromission were noticed between the vehicle-treated rats and the MC4R antagonist-treated rats (Fig. 6.8; 6.9). However, after double immunocytochemistry for Fos and oxytocin, attenuation in Fos expression in magnocellular oxytocin neurones in MC4R antagonist-treated group was significant (Fig. 6.10; 6.11). Indeed, at intromission, 14 ± 6% of magnocellular oxytocin neurones/ PVN were Fos-positive in vehicle-treated rats against 5 ± 3% of magnocellular oxytocin neurones/ PVN in MC4R antagonist-treated rats (P=0.015, One-way ANOVA). No oxytocin neurones were Fos-positive in the MC4R antagonist-treated rats that did not mate, verifying that the injection itself did not induce any Fos (Fig. 6.11).

iii)Changes in Fos expression in the SON after i.c.v injection of MC4 receptor antagonist:

In the SON (Fig. 6.12; 6.13), i.c.v injection of MC4R antagonist had no effect on Fos expression induced at intromission. The MC4R antagonist–treated rats that didn't mate showed a significantly lower level of Fos expression in the SON, verifying that the injection of MC4R antagonist itself did not induce Fos expression.

Double immunocytochemistry for Fos and oxytocin confirmed that intromission increases Fos expression in oxytocin neurones in the SON as Fos expression in oxytocin neurones was significantly higher in MC4 receptor antagonist–treated rats that mate than in MC 4 receptor antagonist –treated non-mating rats (P=0.036, One-way ANOVA on ranks, Dunn's method). Moreover, Fos expression induced at intromission in oxytocin neurones was significantly attenuated by injection of MC4 receptor antagonist (Fig. 6.14; 6.15). Indeed, in vehicle–treated rats, 26 ± 4% oxytocin neurones/ SON were Fos – positive at intromission compared with 11 ± 3% oxytocin neurones Fos-positive / SON in MC4R antagonist -treated rats (P<0.05).

iv)Changes in Fos expression in the arcuate nucleus after i.c.v injection of MC4 receptor antagonist:

In the arcuate nucleus (Fig. 6.16; 6.17), i.c.v injection of MC4 receptor antagonist had no significant effect on Fos expression induced at intromission. The MC4 receptor antagonist-treated/ Control group presented a similar level of Fos expression to the two mating groups.

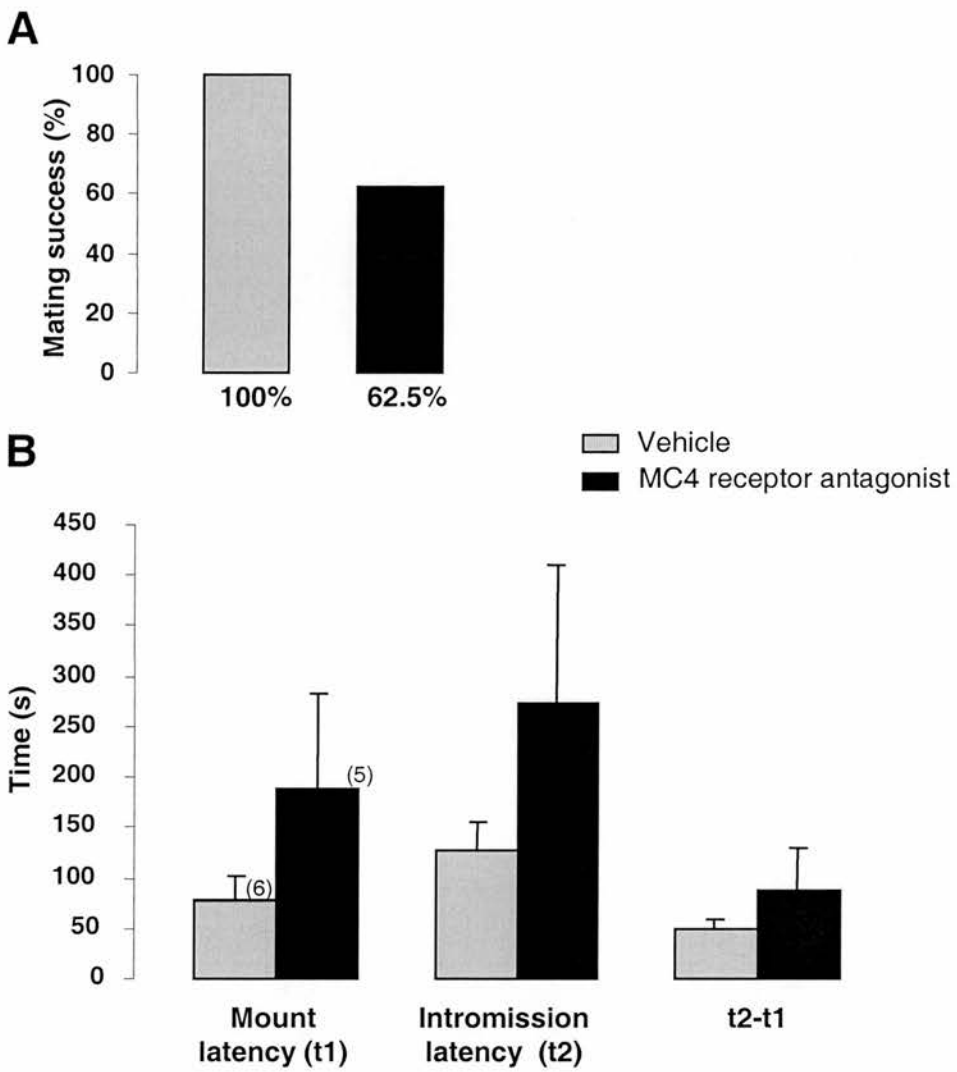

Figure 6.1: Changes in behavioural parameters after i.c.v. injection of MC4 receptor antagonist

A: Success of mating. Values are percentage of rats that mated. B: Time to onset of mating behaviours after i.c.v injection of MC4 receptor antagonist.Values are means ± SEM. Mount latency: Time (s) to first intensive mount; Intromission latency: Time (s) to intromission; t2-t1: Time between the first mount and the first intromission.

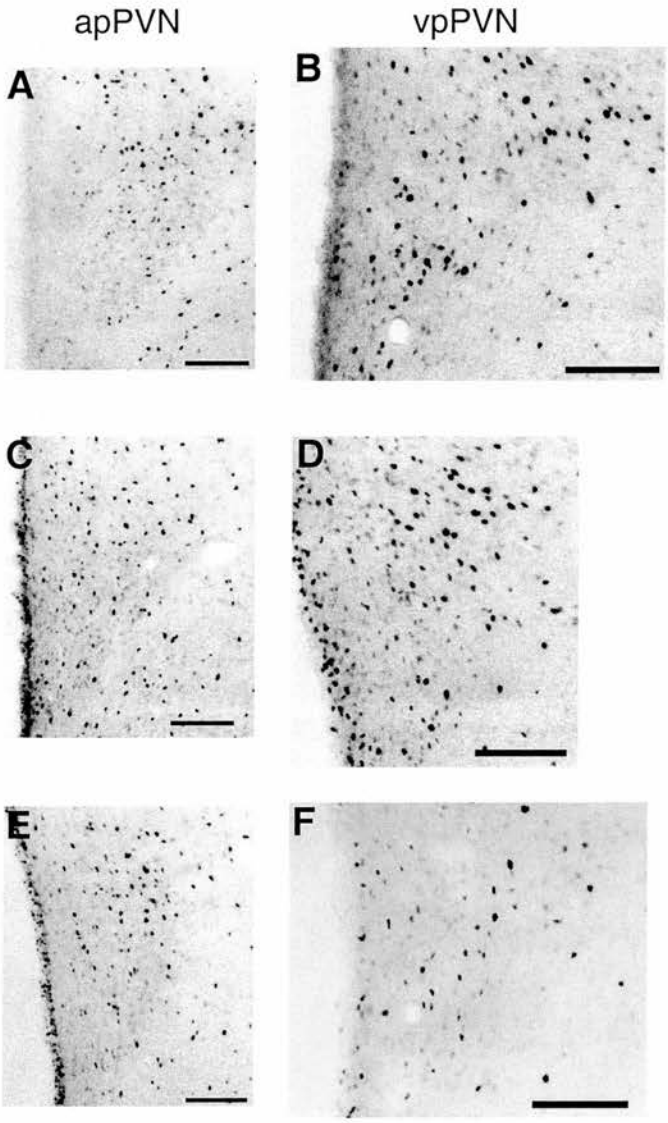

Figure 6.2: Photomicrographs illustrating Fos immunoreactivity (arrow) in the anterior parvocellular PVN (A, C, E) and in the ventral parvocellular PVN (B, D, F) at intromission after i.c.v injection of vehicle (A, B) or i.c.v injection of MC4 receptor antagonist (C, D) or in control rats after i.c.v injection of MC4 receptor antagonist (E, F).

Photomicrographs illustrating the increase in Fos expression in the apPVN and in the vpPVN at intromission (A, B, C, D) compared to control conditions (E, F). However, there is no difference in Fos expression present at intromission between vehicle –injected rats (A, B) and MC4 receptor antagonist –injected rats (C, D).

3V: Third ventricle. Scale bar: 100μm.

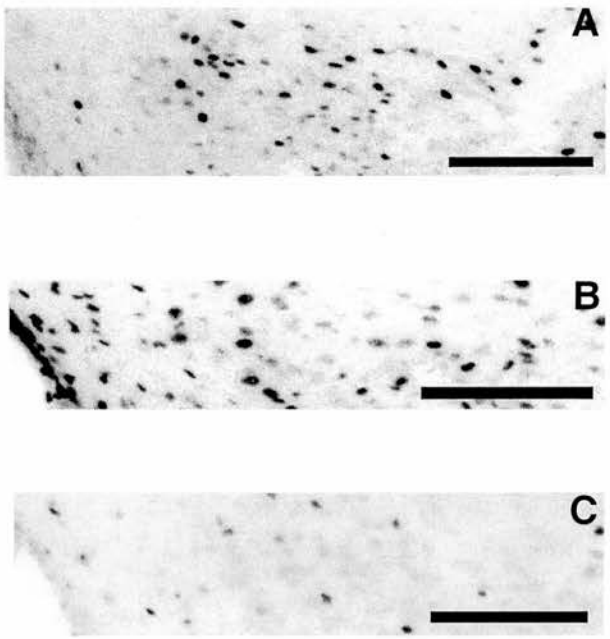

Figure 6.3: Photomicrographs illustrating Fos immunoreactivity (arrow) in the dorsal parvocellular PVN at intromission after i.c.v injection of vehicle (A) or i.c.v injection of MC4 receptor antagonist (B) or in control rats after i.c.v injection of MC4 receptor antagonist (C).

Photomicrographs illustrating the increase in Fos expression in the dpPVN at intromission (A, B) compared to control conditions (C). However, there is no difference in Fos expression present at intromission between vehicle –injected rats (A) and MC4 receptor antagonist –injected rats (B).

3V: Third ventricle. Scale bar: 100μm.

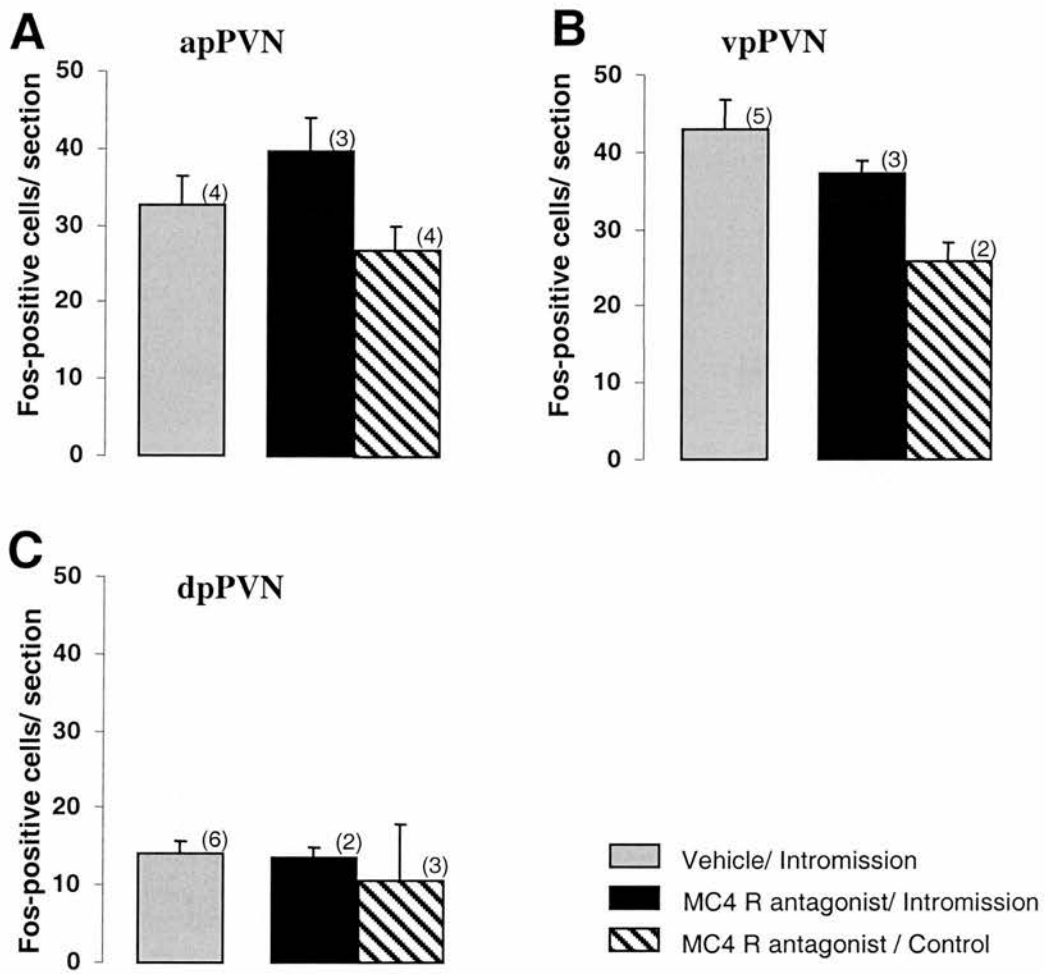

Figure 6.4: Fos expression in the parvocellular subdivisions of the PVN after i.c.v injection of MC4 receptor antagonist.

Values are means Fos-positive cells/ section ± SEM. A: apPVN: anterior parvocellular PVN. B: vpPVN: ventral parvocellular PVN. C: dpPVN: dorsal parvocellular PVN. The number of rats per group is in parentheses.

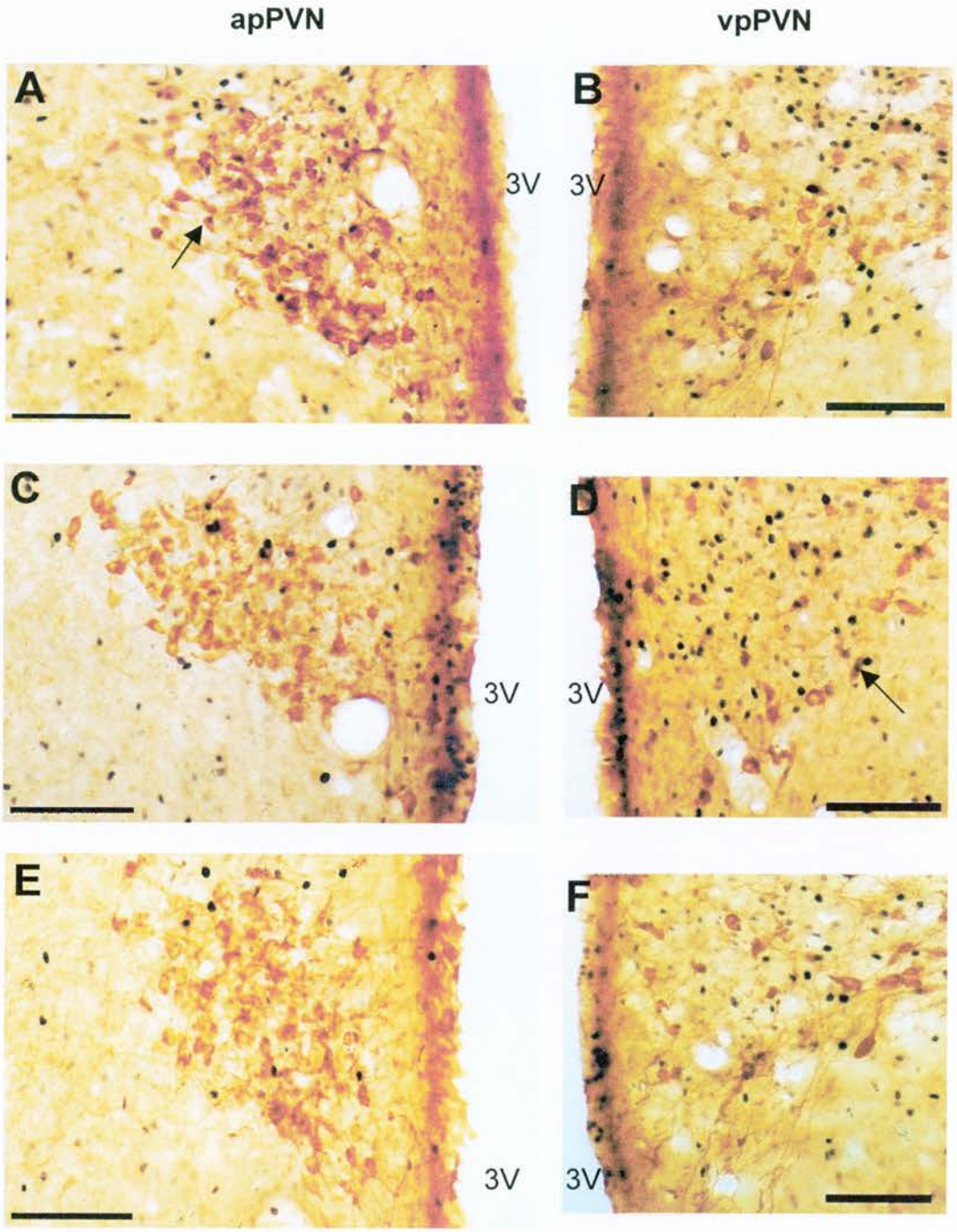

Figure 6.5: Photomicrographs illustrating Fos in oxytocin neurones (arrow) in the anterior parvocellular PVN (apPVN; A, C, E) and in the ventral parvocellular PVN (vpPVN; B, D, F) at intromission after i.c.v injection of vehicle (A, B) or i.c.v injection of MC4 receptor antagonist (C, D) or in control rats after i.c.v injection of MC4 receptor antagonist (E, F).
3V: Third ventricle. Scale bar: 100μm.

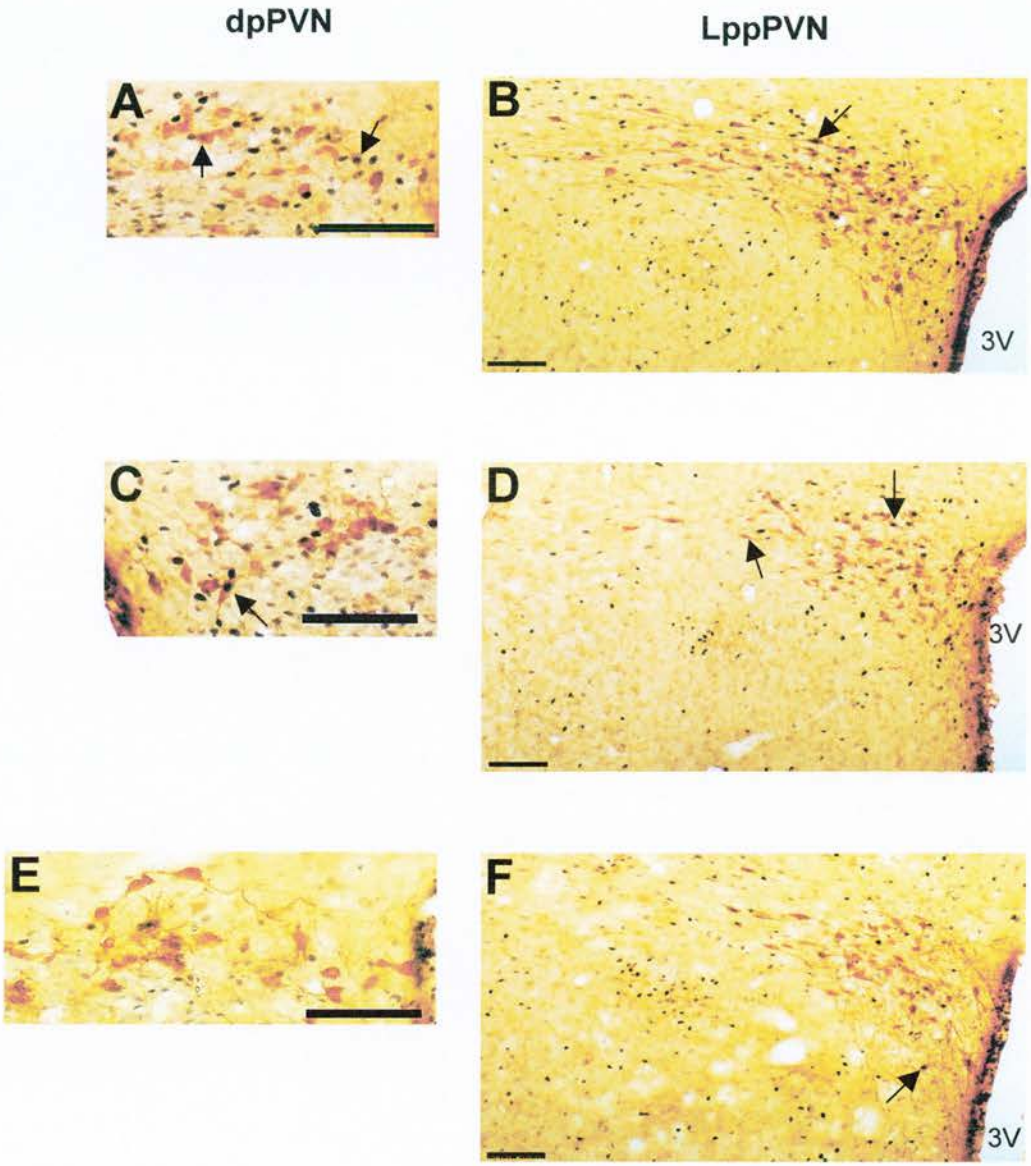

Figure 6.6: Photomicrographs illustrating Fos in oxytocin neurones (arrow) in the dorsal parvocellular PVN (dpPVN; A, C, E) and in the lateral posterior parvocellular PVN (LppPVN; B, D, F) at intromission after i.c.v injection of vehicle (A, B) or i.c.v injection of MC4 receptor antagonist (C, D) or in control rats after i.c.v injection of MC4 receptor antagonist (E, F).

3V: Third ventricle. Scale bar: 100μm.

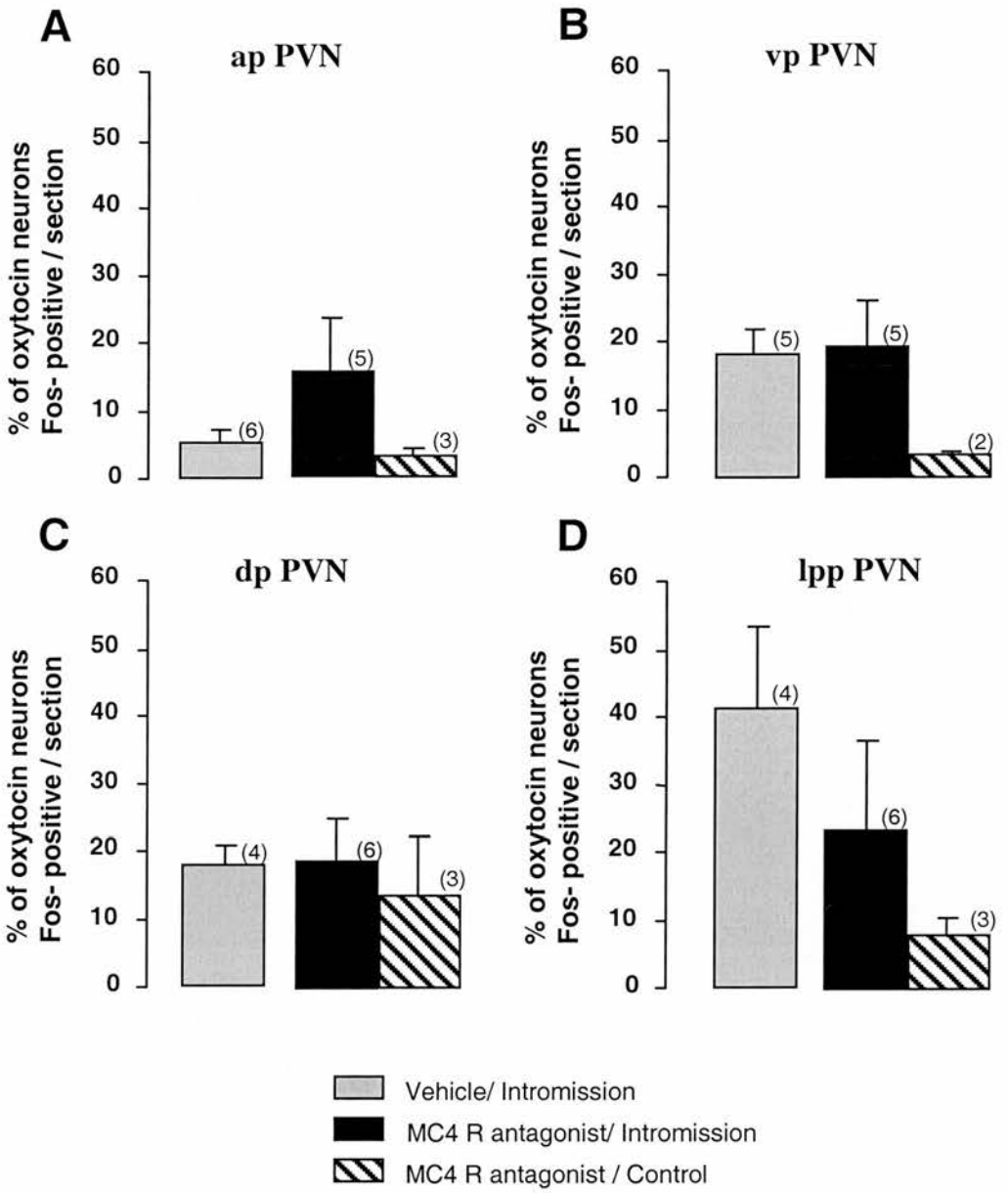

Figure 6.7: Changes in Fos expression in oxytocin neurones in the parvocellular subdivisions of the PVN after i.c.v. injection of MC4 receptor antagonist.

Values are means % of oxytocin neurones Fos-positive/ section ± SEM. The number of rats per group is in parentheses. A: apPVN: anterior parvocellular PVN. B: vpPVN: ventral parvocellular PVN. C: dpPVN: dorsal parvocellular PVN. D: Lpp: Lateral posterior parvocellular PVN.

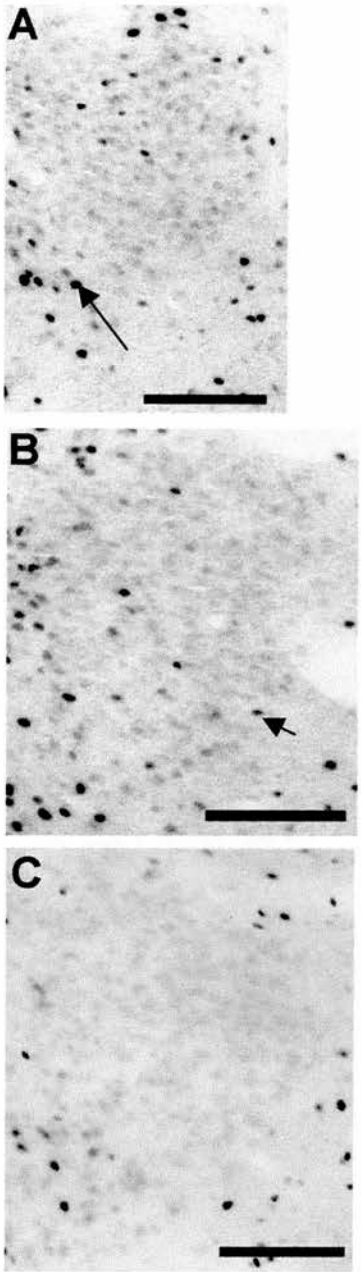

Figure 6.8: Photomicrographs illustrating Fos immunoreactivity (arrow) in the magnocellular PVN at intromission after i.c.v injection of vehicle (A) or MC4receptor antagonist (B) or in non-mating rats after i.c.v injection of MC4 receptor antagonist (C).

Scale bar: 100μm.

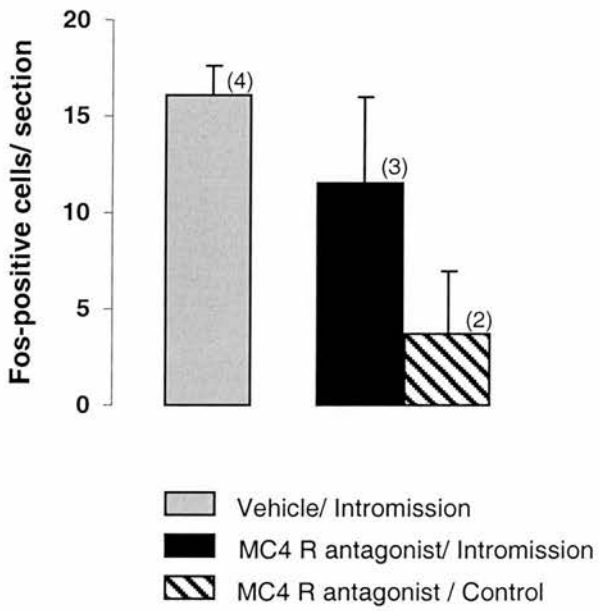

Figure 6.9: Fos expression in magnocellular subdivisions in the PVN at intromission after i.c.v. injection of MC4 receptor antagonist.

Values are mean Fos-positive/ section ± SEM. The number of rats per group is in parentheses.

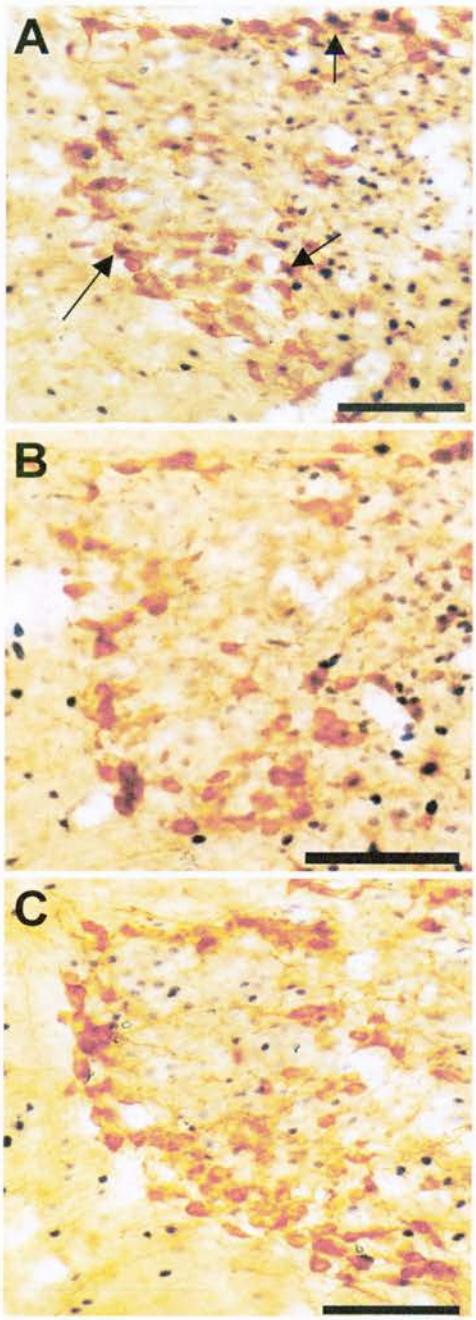

Figure 6.10: Photomicrographs illustrating Fos immunoreactivity in oxytocin neurones (arrow) in the magnocellular PVN at intromission after i.c.v injection of vehicle (A) or MC4receptor antagonist (B) or in control rats after i.c.v injection of MC4 receptor antagonist (C).

Scale bar: 100μm.

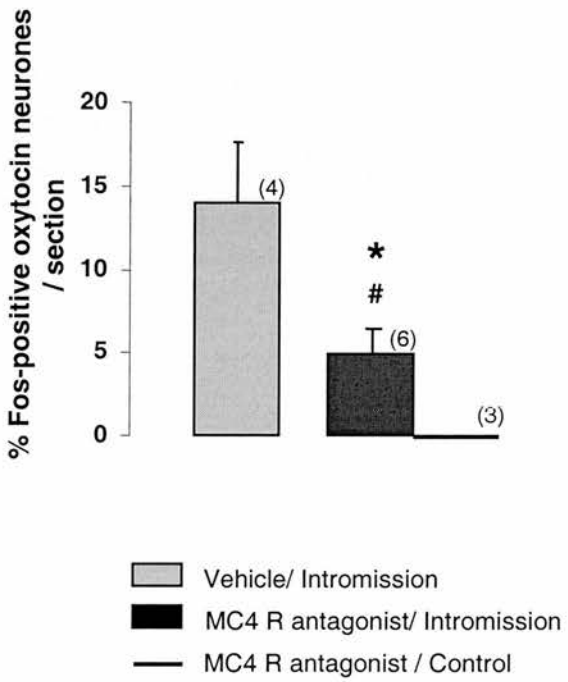

Figure 6.11: Fos expression in magnocellular oxytocin neurones in the PVN at intromission after i.c.v. injection of MC4 receptor antagonist.

Values are mean % oxytocin neurones Fos-positive/ section ± SEM. *P=0.015 versus vehicle-treated group, #P=0.038 versus MC4 R antagonist / Control group (One way ANOVA, Student-Newman-Keuls method). The number of rats per group is in parentheses.

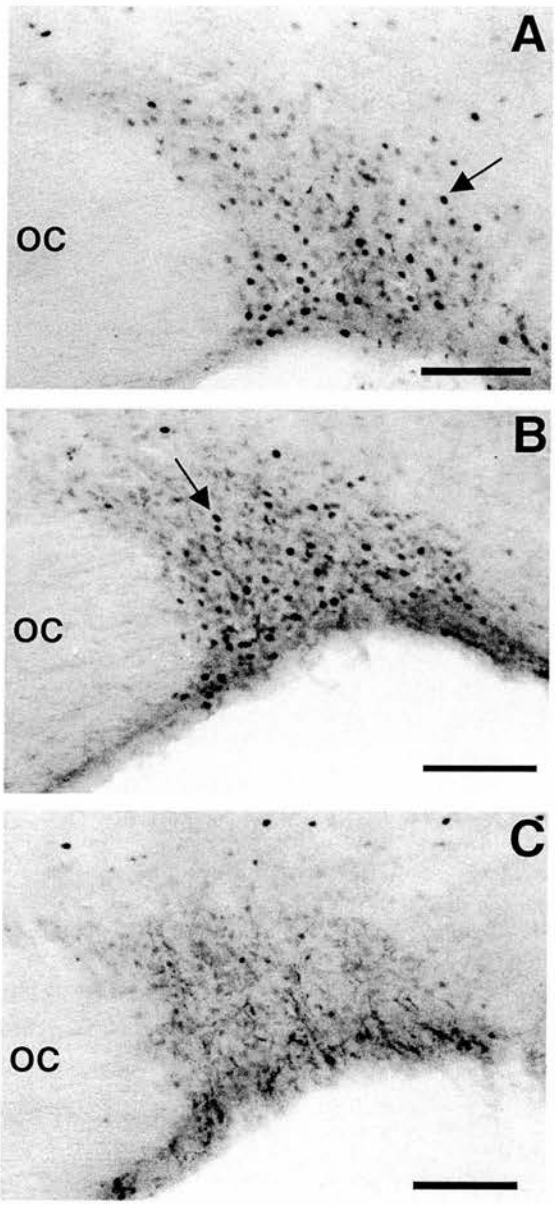

Figure 6.12: Photomicrographs illustrating Fos immunoreactivity (arrow) in the SON at intromission after i.c.v injection of vehicle (A) or MC4 receptor antagonist (B) or in control rats after i.c.v injection of MC4 receptor antagonist (C).

OC: Optic chiasm. Scale bar: 100μm.

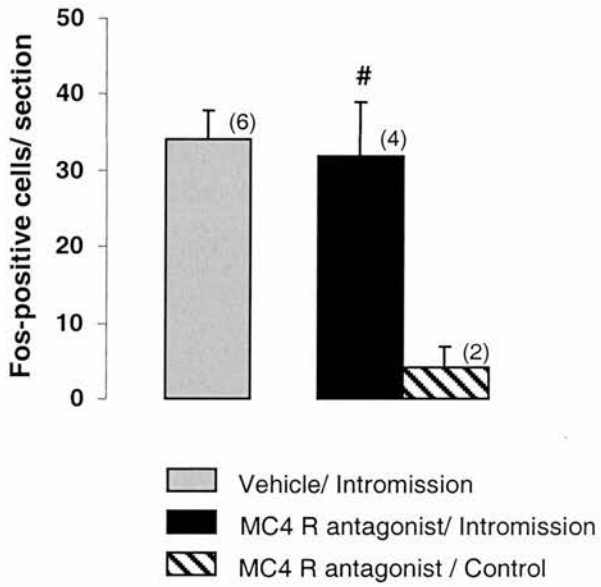

Figure 6.13: Changes in Fos expression in the SON after i.c.v. injection of MC4 receptor antagonist.

Values are means Fos-positive cells/ section ± SEM. The number of rats per group is in parentheses. #*P*=0.034 versus MC4 R antagonist / Control group (One way ANOVA, Student-Newman-Keuls method).

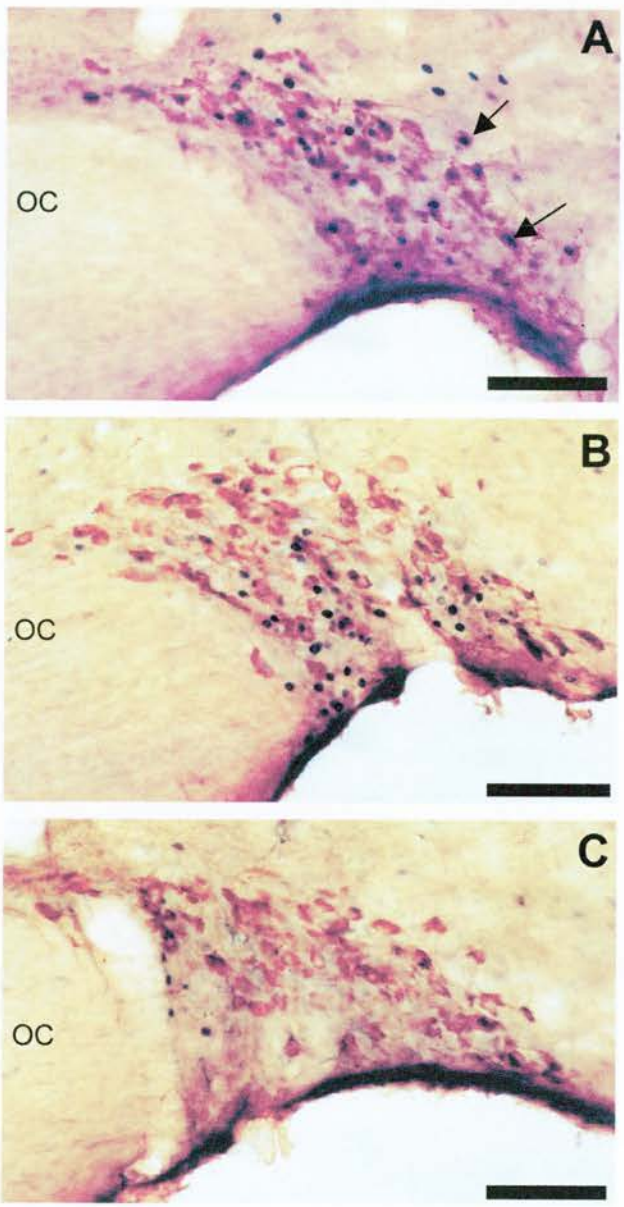

Figure 6.14: Photomicrographs illustrating Fos immunoreactivity in oxytocin neurones (arrow) in the SON at intromission after i.c.v injection of vehicle (A) or MC4 receptor antagonist (B) or in control rats after i.c.v injection of MC4 receptor antagonist (C).

OC: Optic chiasm. Scale bar: 100μm.

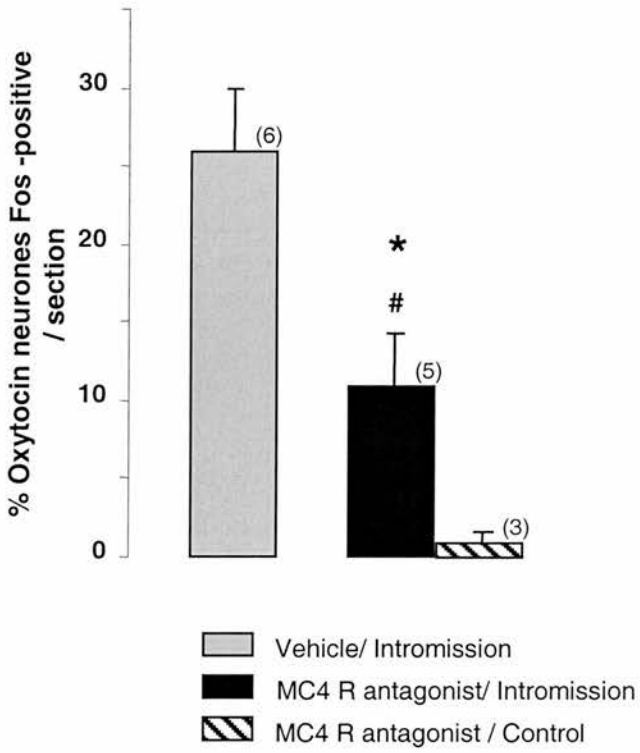

Figure 6.15: Fos expression in oxytocin neurones in the SON at intromission after i.c.v injection of MC4 receptor antagonist.

Values are mean % oxytocin neurones Fos-positive/ section ± SEM. *P=0.014 versus vehicle-treated group (One way ANOVA, Dunn's Method), #P=0.014 versus MC4 R antagonist / Control group (One way ANOVA, Student-Newman-Keuls method). The number of rats per group is in parentheses.

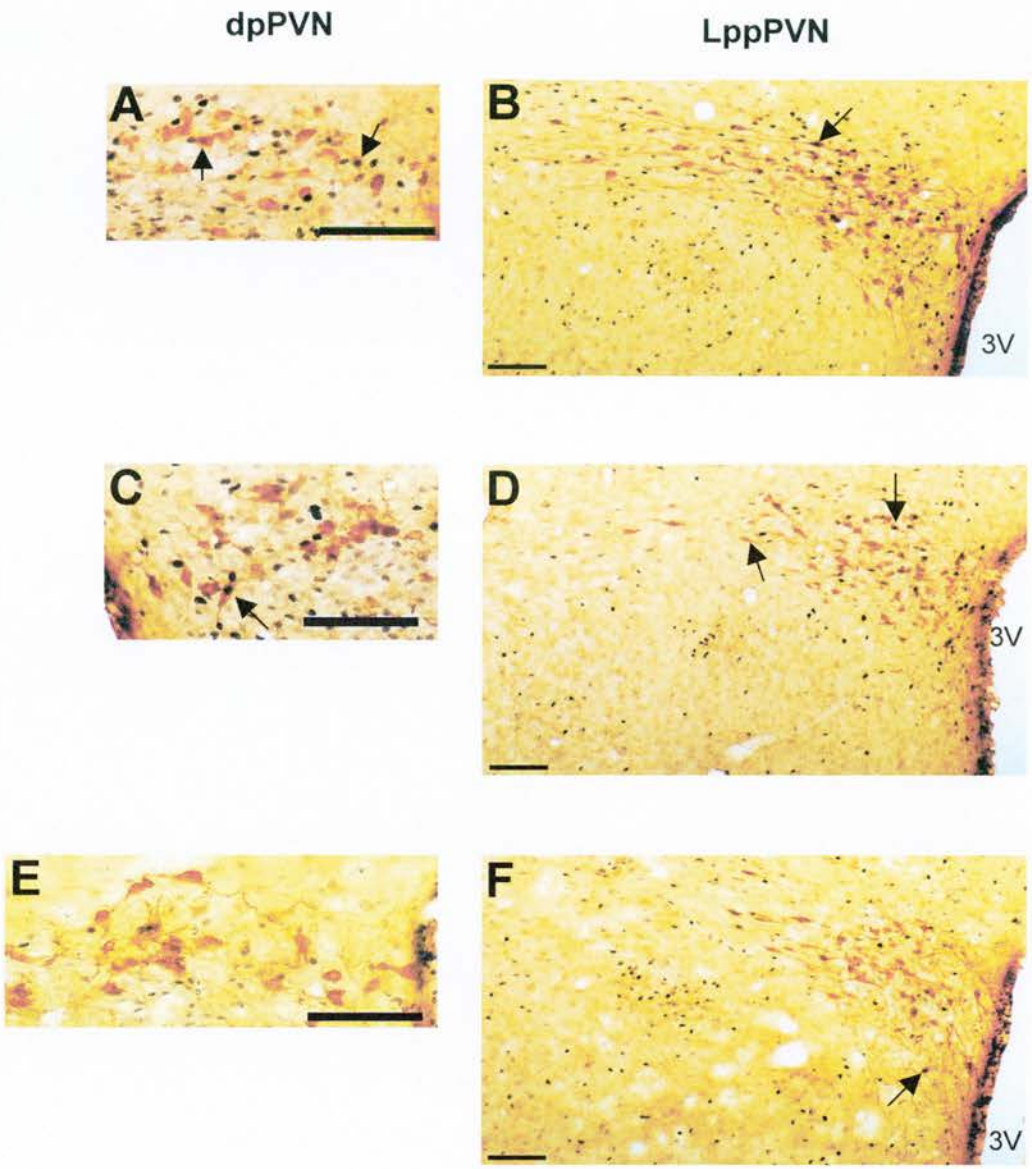

Figure 6.6: Photomicrographs illustrating Fos in oxytocin neurones (arrow) in the dorsal parvocellular PVN (dpPVN; A, C, E) and in the lateral posterior parvocellular PVN (LppPVN; B, D, F) at intromission after i.c.v injection of vehicle (A, B) or i.c.v injection of MC4 receptor antagonist (C, D) or in control rats after i.c.v injection of MC4 receptor antagonist (E, F).

3V: Third ventricle. Scale bar: 100μm.

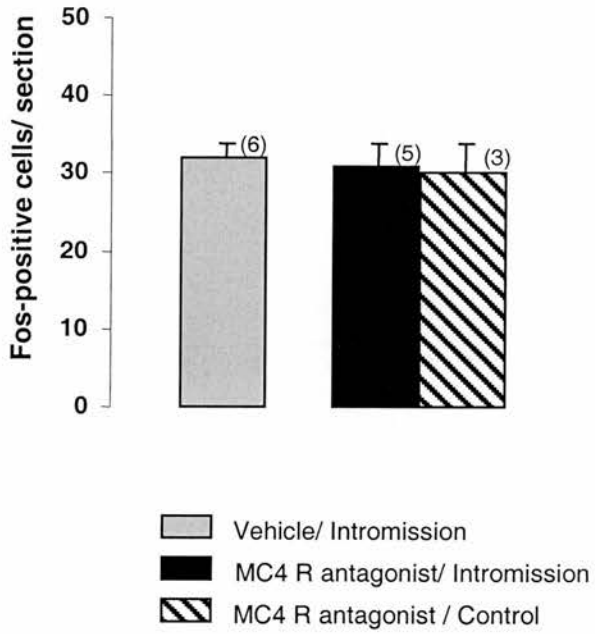

Figure 6.17: Fos expression at intromission in the arcuate nucleus after i.c.v injection of MC4 receptor antagonist.

Values are means ± SEM. The number of rats per group is in parentheses.

6.5. Discussion:

The present study investigated whether blocking the central effects of α-MSH mediated by MC4 receptors would attenuate the Fos expression in oxytocin neurones induced at intromission. The data indicate that i.c.v. MC4 receptor antagonist injection specifically reduced Fos expression in magnocellular oxytocin neurones, but had no effect on the parvocellular oxytocin neurones. This study highlights the controversial role of MC4 receptors in the regulation of male sexual behaviour, and provides evidence of an interaction between α-MSH and the magnocellular oxytocin system during male sexual behaviour. Finally, the lack of effect of MC4 receptor antagonist on the neuronal activity in the arcuate nucleus during sexual behaviour will be discussed.

Methodology and experimental issues:

A few rats injected with MC4 receptor antagonist did not mate within the 15 min period of behavioural recording; this created a third experimental group of rats. This was not expected, as the dose of MC4 receptor antagonist used was small and not previously reported to block mating behaviours. Splitting the MC4 receptor antagonist –treated rats in two groups (i.e.: non-mating and intromission), reduced the number of rats in the MC4 receptor antagonist/ intromission group. Consequently, the statistical comparisons between the three groups were limited and inconclusive for some brain areas. However, the MC4 receptor antagonist/ control group gave precious information about the effect of the drug itself on the Fos expression. In all subdivisions of the PVN, in the SON, Fos expression in the control group was lower than in the MC4 receptor antagonist/ intromission group. Thus, the MC4 receptor antagonist injection did not induce Fos; and the Fos expression observed was linked to intromission.

Are MC4 receptors involved in the modulation of male sexual behaviour?

In the present study, we found that i.c.v - injection of MC4 receptor antagonist delays mating. Indeed, the rats injected with the antagonist took more time to mate than the vehicle –injected rats, the mounting and intromission latencies increased, and even three rats did not mate within the observation period. These results support the involvement of the MC4 receptors in the mediation of sexual behaviour and are in line with previous findings:

Martin *et al.* (2002) investigated the erectogenic role of the MC4 receptor by studying changes in intracavernosal pressure (ICP) during penile erection 'ex copula' (out of the context of copulation). They tested the effect of systemic and central injections of the MC4 receptor agonist THIQ, and observed that THIQ increased ICP and the number of penile erections. They suggested that the activation of MC4 receptors was sufficient for pro-erectile activity. Studies in mice showed that i.v. injection of THIQ enhanced penile erection initiated by stimulation of the cavernous nerve in wild type but not in MC4 receptor knock -out mice (Van der Ploeg *et al.*, 2002). They also reported that i.v. –injection of THIQ reduced mounting and intromission latencies. These results suggest that MC4 receptors participate in the modulation of the erectile activity and thus in the regulation of sexual behaviour. However, these results conflict with other finding in which MC4 receptors were excluded from the regulation of sexual activity. Vergoni (1998) reported that in rats, i.c.v. -injection of a MC4 receptor antagonist (HSO14), could block the yawning-stretching reflex and the grooming behaviour induced by α-MSH; but failed to block penile erection induced by α-MSH (Vergoni 1998, Argiolas *et al.*, 2000). Even if stretching, yawning and grooming behaviours have often been associated with penile erection, they concluded that MC4 receptors were mediating stretching, yawning and grooming behaviours but that, in contrast, the penile erection was mediated by another MC receptor type. They suggested that MC3 receptors were likely to be the main MC receptors involved in the regulation of penile erection, as MC 3 receptors are located in brain areas that modulate sexual behaviours including the hypothalamus (Roselli-Rehfuss *et al.*, 1993).

The interpretation of the results from previous studies is difficult: most of the MC4 receptor antagonists or agonists that have been used (e.g.: HSO14, THIQ), bind to MC3 receptors as well. Indeed, although THIQ is more selective for MC4 receptors over MC3 receptors by ~3000 fold, HSO14 selectivity for MC4 receptors over MC3 receptors is only modestly stronger (~ 17 fold) (Schiöth *et al.*, 1998; Martin *et al.*, 2002 Van der Ploeg *et al.*, 2002). As the doses of drugs used in these studies are large (range from 5 to 20μg/rat), it is likely that they also have effects on the MC3 receptors. Thus, no definitive conclusion refuting the involvement of one type of MC receptor in the regulation of male sexual behaviour should have been made.

Are two independent α-MSH-related pathways involved in the regulation of male sexual behaviour?

MC3 and MC4 receptors are the two types of MC receptors present in the brain, and therefore the only types of MC receptors that can mediate central effects of α-MSH. No studies have directly demonstrated the role of MC3 receptors in male sexual behaviour. It has been speculated that MC3 receptors could mediate sexual behaviour because some studies refuted the involvement of MC4 receptors (Vergoni 1998, Argiolas *et al.*, 2000). But growing evidence, as well as the present study, demonstrates that MC4 receptors are part of the mediation of the sexual behaviour.

Perhaps these discrepancies cannot be reconciled, and trying to distinguish which one of the two MC receptors is involved is a mistake. Involvement of both MC3 and MC4 receptors in the mediation of male sexual behaviour is plausible. Martin and colleagues (2002) suggested that the induction of penile erection by α-MSH could require activation of both MC3 and MC4 receptors. Indeed, both MC3 and MC4 receptors could be activated during sexual behaviour: α-MSH could activate two independent pathways, one mediated by MC3 receptors and the other one mediated by MC4 receptors, whose activations would lead to increased erectile activity and enhanced sexual behaviour, and each pathway could be sufficient to facilitate and enhance sexual activity as shown for the MC4 receptor-related pathway. The MC4 receptor-related

pathway may interact with the oxytocin system while the MC3 receptor-related pathway may involve different independent systems other than oxytocin system. This suggestion is supported by the study by Vemulapalli *et al.*, (2001), in which SHU 9119, a nonselective MC3 and MC4 receptor antagonist, abolished the increase in ICP induced by Melanotan-II (melanocortin receptor agonist) in anaesthetized rabbits.
Whether this suggestion is correct needs to be investigated. Testing the effects of true selective agonist or antagonist towards either MC4 or MC3 receptors on sexual activity could clarify the discrepancies observed.

α-MSH and oxytocin interact to drive male sexual behaviour:

We found that injection of a MC4 receptor antagonist did not affect Fos expression in parvocellular oxytocin neurones at intromission but significantly reduced Fos expression in magnocellular oxytocin neurones. It is unlikely that the attenuation of Fos expression in the magnocellular oxytocin neurones in MC4R antagonist –injected mated rats is an indirect result from the disruption of the mating, as it would had been expected to see the same effect on the parvocellular oxytocin neurones.
Nonetheless, these results conflict with the study by Mizusawa *et al.* (2002), who reported that central administration of α-MSH induced penile erections in anaesthetized rats but that these effects were not blocked by a central injection of the oxytocin antagonist CAP.
However, Martin *et al.* (2002) mentioned the importance of oxytocin pathways in the modulation of penile erection mediated by MC4 receptors in '*ex copula*' studies. They found that central administration of another oxytocin receptor antagonist (L-368899) blocked penile erections induced by MC4 receptor agonist THIQ. Therefore, these results illustrate the possibility that some of the central effects of α-MSH involved in the regulation of male sexual behaviour and mediated via MC4 receptors could be modulated by magnocellular oxytocin neurones. Thus, activation of magnocellular oxytocin neurones by α-MSH would then induce changes in sexual behaviour.

α-MSH and magnocellular vasopressin neurones at intromission:

In the SON, i.c.v injection of the MC4 receptor antagonist had no effect on the overall Fos expression induced at intromission. However, double immunocytochemistry for Fos and oxytocin showed that i.c.v. injection of MC4R antagonist attenuated the Fos expression in oxytocin neurones at intromission. These results could suggest that Fos expression in magnocellular vasopressin neurones was increased at intromission in MC4R antagonist –injected rats. It is unlikely that the injection of MC4R antagonist itself induced Fos expression in vasopressin neurones as the level of Fos expression in MC4R antagonist rats that did not mate was significantly lower than for rats that did mate (Fig 6.13; P=0.034). The increase in Fos expression in magnocellular vasopressin neurones could be related to intromission. However, since vasopressin has strong vasoconstrictor effects, it is difficult to distinguish whether the activation seen in vasopressin neurones is correlated to vascular events indirectly related to the behaviour, or whether the activation is associated with sexual behaviour itself.

This also leads to the discussion of whether α-MSH may also modulate vasopressin neurones activity. i.c.v injection of α-MSH induces Fos expression in vasopressin neurones in the SON and in the PVN (Abdel-Malek, 2001; Starowicz *et al*, 2003; Adan *et al*, 1997). Sabatier *et al.* (2003) showed that i.c.v MC4R agonist had no effect on the firing rate of vasopressin neurones, and thus on vasopressin secretion. They also reported that neither MC4R agonist nor α-MSH had any effect on $[Ca^{2+}]i$ in vasopressin neurones *in vitro*. These results could suggest that α-MSH modulates vasopressin neural activity via MC3R rather than MC4R; however, no evidence so far has shown MC3R localized in the SON and PVN. Taken together, these results suggest that the increase in Fos expression in vasopressin neurones seen after i.c.v injection of α-MSH is likely to be an indirect effect.

α-MSH and the arcuate nucleus at intromission:

In the arcuate nucleus, i.c.v injection of the MC4 receptor antagonist had no effect on Fos expression induced at intromission. However, the Fos expression in non-mated rats injected with MC4 receptor antagonist was as large as the Fos expression induced at intromission. It is difficult to distinguish whether this Fos expression was induced by the injection of the drug, or whether this Fos expression is the basal level of Fos expression in normal conditions. Therefore, the activation of the arcuate nucleus during intromission should be discussed. In Chapter 4, 30 ± 2 cells were Fos-positive in the arcuate nucleus from mating rats, significantly higher than in the non-mating rats. In the present study, the two mating rats groups showed between 28 and 33 cells Fos-positive/ section. The level of Fos expression in the arcuate nucleus at intromission observed in the present study coincides with our previous results. In that case, it could be said that the injection may have induced the increase of Fos expression in the non-mating rats. However, it is difficult to determinate whether the antagonist itself or the vehicle, in which the antagonist has been dissolved, induced Fos expression. The MC4 receptor antagonist used has been prepared in 50% saline / 50% polyethylene glycol/ ethanol solution (as described in section 6.2.4.1). In our laboratory Dr Louise Johnstone have noticed large levels of Fos expression in the arcuate nucleus in rats injected with a vehicle solution containing 50% saline / 50% polyethylene glycol/ ethanol. She reported that modifying the vehicle solution to 25% polyethylene glycol/ ethanol / 75% saline, significantly reduced the level of Fos expression in the arcuate nucleus (data not published, personal communication). Thus, the large Fos expression observed in the arcuate nucleus in MC4 receptor antagonist/ non-mating rats may have been induced by the vehicle rather than by the antagonist itself. Obviously, this suggestion needs further experimental investigations to be validated or refuted.

Numerous findings still need to be reconciled to better understand the regulatory mechanisms of male sexual behaviour in which α-MSH and oxytocin are involved. In

the present study, we have given further evidence that MC4 receptors are involved in the regulation of sexual activity, and we have demonstrated that α-MSH and oxytocin interact to drive male sexual behaviour. As hypothesized in the previous chapter, magnocellular oxytocin neurones activated by α-MSH via MC4 receptors, could release oxytocin. Thus, this central (dendritic) release of oxytocin could induce changes in the behaviours, such as the drive facilitation of male sexual behaviour.

Chapter 7

General discussion

The present study aimed to investigate a putative interaction between oxytocin and α-MSH during male sexual behaviour to lead to a better understanding of how these two peptides facilitate copulation, and in particular, to establish whether one mediates the central effects of the other. The following sections will summarize the main conclusions of the experimental studies and critically review their interpretation. Consequently, a new hypothesis, as well as suggestions for its investigation, will be proposed.

- ***Oxytocin and a-MSH characteristics during copulation:***

During male sexual behaviour, oxytocin is secreted into the general circulation especially during the consummatory phase; and centrally, oxytocin neuronal activity is increased. Both parvocellular and magnocellular oxytocin neurones are activated during male sexual behaviour (Chapters 3 & 4). Although, these findings are supported by numerous studies that have reported an increase in oxytocin secretion, and an increase in neuronal activity of parvocellular oxytocin neurones in all subdivisions of the PVN, this is the first description of the activation of magnocellular oxytocin neurones in both the PVN and the SON at intromission. This major finding highlights the underestimated role of the magnocellular neurones in the central regulation of sexual behaviour, or even of any behaviour.

In parallel to the study of the oxytocin system during copulation, changes in α-MSH secretion and neuronal activity were investigated. The plasma concentration of α-MSH was measured before, during and after copulation, and the activity of α-MSH – containing neurones was studied using immunocytochemistry for Fos. We found that, during male sexual behaviour, α-MSH is secreted into the blood with a large increase at intromission, and evidence suggests that α-MSH –containing neurones in the arcuate nucleus are also activated. These findings demonstrate that both central and peripheral

α-MSH might play a role in the regulation of male sexual behaviour. Centrally, modulatory effects of α-MSH on male sexual behaviour are likely to be mediated via MC4 receptors, as central injection of a MC4 receptor antagonist delayed the onset of copulation (Chapter 6). Thus, oxytocin and α-MSH not only induce similar effects when injected centrally, they are both secreted peripherally during copulation, and both oxytocin neurones and α-MSH –containing neurones are activated at intromission. These similarities reinforced the initial hypothesis that oxytocin and α-MSH interact to regulate male sexual behaviour, and that oxytocin could mediate the central effects of α-MSH or vice versa.

- ***Peripheral or central interaction?***

Sexual behaviour, like any complex behaviour, depends upon the modulation of many physiological systems, which are orchestrated at both central and peripheral levels. As central and systemic oxytocin and α-MSH are involved in the regulation of male sexual behaviour, the two peptides could interact peripherally and/ or centrally. Oxytocin might modulate the α-MSH secretion or alternatively α-MSH might modulate oxytocin secretion. We firstly hypothesized that central and peripheral oxytocin modulate α-MSH secretion during copulation, but, as reported in chapter 3, systemic or central oxytocin had no effect on the peripheral secretion of α-MSH during copulation, as a central injection of oxytocin or a systemic injection of oxytocin antagonist had no effect on the α-MSH plasma concentration. Therefore, the alternative was tested: that central α-MSH modulates oxytocin systemic secretion and central release during copulation.

Previous studies have reported that MC4 receptor mRNA is expressed in the SON and in the PVN, and that central injection of α-MSH stimulates neuronal activity in the SON and in the PVN. It has thus been assumed that α-MSH has excitatory effects on oxytocin neurones. It was therefore surprising that, as shown in chapter 3, central α-MSH did not stimulate systemic secretion of oxytocin as expected, but reduced it.

Investigating these discrepancies confirmed, that while α-MSH modulates oxytocin neuronal activity (as α-MSH induces Fos expression in oxytocin neurones), it does not stimulate peripheral secretion of oxytocin (chapter 5). These findings lead to two main conclusions: Firstly, Fos is a marker of neuronal activation rather than neuronal excitation: although the presence of Fos is, in some cases, associated with an excitation of the neurone, the presence of Fos is independent of the electrical activity, and so is independent of the secretion from the axon terminal. The second conclusion is that the interaction between oxytocin and α-MSH is central rather than peripheral: central α-MSH might regulate central release of oxytocin as it modulates oxytocin neuronal activity but it has no effect on (or inhibits) oxytocin systemic secretion.

- ***What are the characteristics of the interaction between oxytocin and α-MSH?***

  The modulation of oxytocin neurones by α-MSH has three features:

  - This modulation is direct, and does not involve any other populations of neurones. In chapter 5, infusion of α-MSH onto the SON by retrodialysis induced a widespread distribution of Fos in the SON neurones.

  - This modulation is specific to the type of oxytocin neurone. α-MSH modulates selectively magnocellular oxytocin neurones rather than parvocellular oxytocin neurones, as central injection of α-MSH has little effect on oxytocin parvocellular neuronal activity but strikingly increases the neuronal activity of magnocellular oxytocin neurones in both the SON and PVN in conscious rats (Chapter 5).

  - This modulation occurs during male sexual behaviour. In chapter 6, central α-MSH selectively modulates the oxytocin magnocellular neurones via the MC4 receptors, as central injection of MC4 receptor antagonist selectively attenuated magnocellular oxytocin neuronal activity in the SON and in the PVN at intromission while delaying the onset of copulation.

These results, taken together, demonstrate that, during male sexual behaviour, central oxytocin and α-MSH interact to drive male sexual behaviour. α-MSH centrally released during sexual behaviour, stimulates magnocellular oxytocin neurones directly via MC4 receptors without influencing the parvocellular oxytocin neurones. This is an exciting conclusion as it not only confirms our initial hypothesis that α-MSH and oxytocin interact during male sexual behaviour, but it also highlights the underestimated role of the magnocellular neurones in the central regulation of sexual behaviour and probably of any behaviour. If the only role of magnocellular oxytocin neurones was the secretion of oxytocin into the systemic circulation, why would α-MSH modulate magnocellular oxytocin neuronal activity without stimulating oxytocin secretion from the axon terminals? As discussed in chapter 5, oxytocin can be released from the dendrites of the magnocellular neurones. Therefore, magnocellular oxytocin neurones could intervene in the central regulation of the male sexual behaviour via the dendritic release of oxytocin stimulated by α-MSH.

However, a few points in the present study need to be clarified or require further investigations:

Firstly, the activation of α-MSH- containing neurones in the arcuate nucleus or the dorsomedial hypothalamus during male sexual behaviour has not been directly proved, nor has it been shown that α-MSH is released centrally. Nonetheless, strong evidence was given supporting that α-MSH –containing neurones are activated at intromission in the arcuate nucleus.

Secondly, the role of systemic oxytocin and α-MSH has not been clarified. Although, oxytocin and α-MSH are secreted into the general circulation during male sexual behaviour, with maximal secretion during the consummatory phase, the exact role of peripheral oxytocin and α-MSH and their physiological consequences remain unclear. Once secreted, oxytocin and α-MSH could act on distant targets directly involved in the male sexual behaviour (oxytocin receptors have been found in the testis and in the prostate, and MC4 receptors have been located in the penis) but could also act on other

physiological systems, whose activity need to be adapted to enable the sexual behaviour to occur.

Finally, a degree of uncertainty exists: direct evidence that α-MSH stimulates dendritic release of oxytocin from the magnocellular neurones during male sexual behaviour has not been given. However, previous findings support this postulation:

- Sabatier *et al.* (2003), reported that α-MSH can induce an increase in intracellular calcium concentration, as infusion of α-MSH onto isolated supraoptic neurones induced a transient increase in $[Ca^{2+}]i$. They suggested that α-MSH induced a release of calcium from intracellular stores rather than via an entry of Ca^{2+} through voltage- gated channels, as even in absence of extracellular calcium, α-MSH still induced an increase in $[Ca^{2+}]i$. in supraoptic neurones. They also reported that α-MSH was acting on oxytocin neurones via MC4 receptors, as the effects of α-MSH on $[Ca^{2+}]i$ were blocked by a MC4 receptor antagonist.

- Ludwig *et al.* (2002), reported that changes in $[Ca^{2+}]i$ trigger dendritic release of oxytocin and this dendritic release is independent from the secretion of oxytocin from the axon terminals (Ludwig, 1998).

-Sabatier *et al.* (2003), showed that α-MSH stimulates, *in vitro,* the dendritic release of oxytocin in isolated supraoptic nuclei, and that the effect of α-MSH can be blocked by an MC4 receptor antagonist. They also reported that the stimulatory effect of α-MSH on dendritic release of oxytocin was not blocked by a lower concentration of extracellular calcium, confirming that the stimulation of oxytocin dendritic release by α-MSH is induced by changes in $[Ca^{2+}]i$ caused by mobilization of calcium from intracellular store rather than via depolarization.

Thus, α-MSH can stimulate the release of oxytocin from the dendrites of magnocellular neurones *in vitro*, supporting the hypothesis that α-MSH could have the same effect *in vivo* during male sexual behaviour.

In conclusion, from the present findings, a new hypothesis can be proposed: During male sexual behaviour, α-MSH released centrally stimulates, via MC4 receptors,

the dendritic release of oxytocin from magnocellular neurones without influencing the release of oxytocin from the axon terminals of either magnocellular or parvocellular oxytocin neurones. Once released oxytocin modulates other neuronal systems to enhance male sexual behaviour. Thus, some of the central effects of α-MSH in the regulation of male sexual behaviour are mediated via oxytocin (Fig 7.1; 7.2).

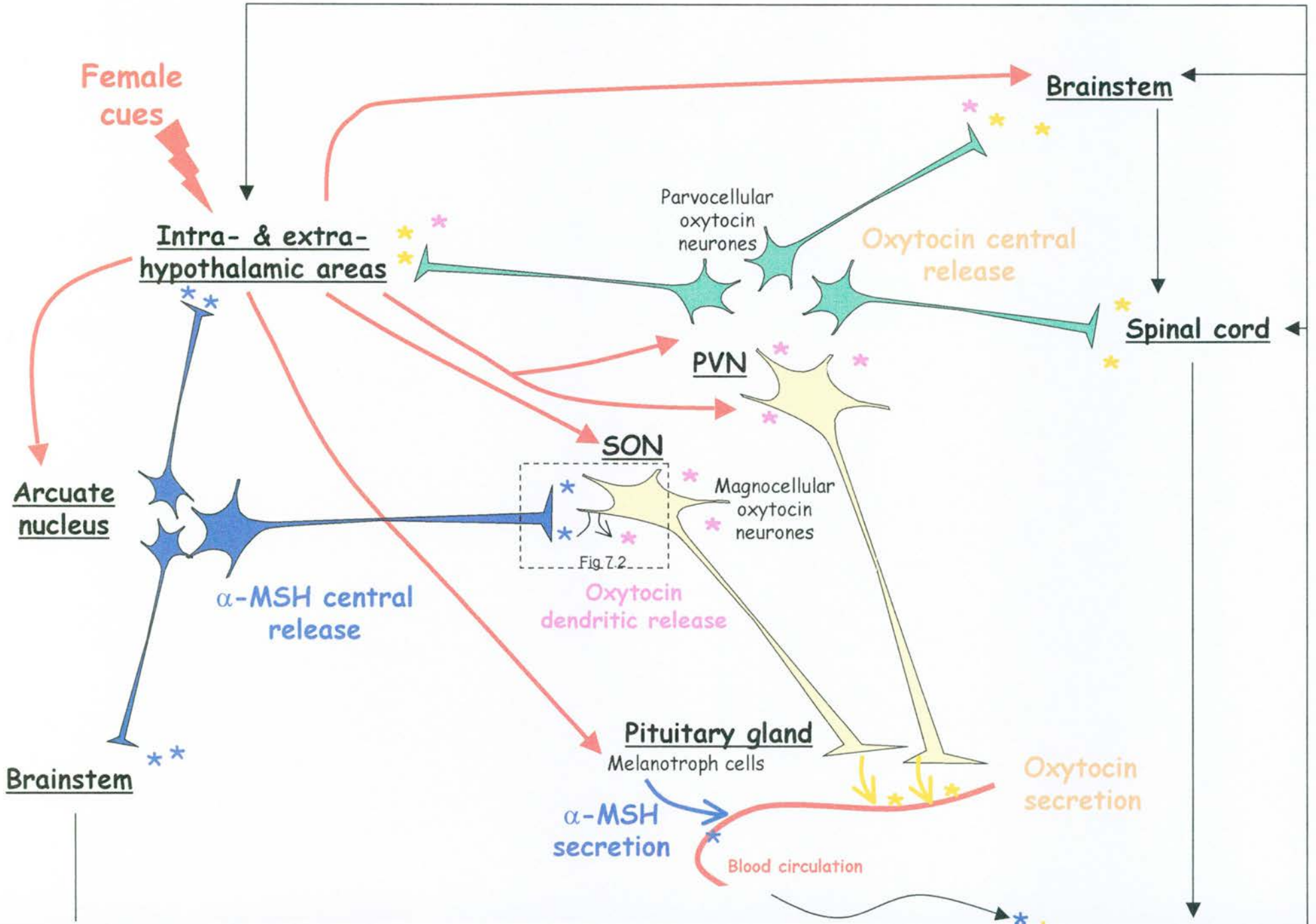
Female cues
Brainstem
Intra- & extra-hypothalamic areas
Parvocellular oxytocin neurones
Oxytocin central release
Spinal cord
PVN
SON
Arcuate nucleus
Magnocellular oxytocin neurones
α-MSH central release
Oxytocin dendritic release
Brainstem
Pituitary gland
Melanotroph cells
Oxytocin secretion
α-MSH secretion
Blood circulation

Fig 7.2

Figure 7.1: α-MSH and oxytocin actions during male sexual behaviour.

During male sexual behaviour, female cues, via intra- and extra- hypothalamic neuronal pathways, activate oxytocin neurones in the SON and in the PVN leading to central release of oxytocin from the parvocellular neurones and peripheral secretion of oxytocin from the magnocellular neurones into the blood circulation. Central and peripheral α-MSH releases are also stimulated respectively from neurones from the arcuate nucleus and from the melanotroph cells in the intermediate lobe of the pituitary gland . Once secreted into the blood, oxytocin and α-MSH can act on peripheral organs including the penis and testis to facilitate the behaviour's display. Centrally, α-MSH and oxytocin act via neuronal projections or diffuse to any CNS areas where MC receptors and oxytocin receptors are located to induce changes in the behaviours. In parallel, central α-MSH stimulates the dendritic release of oxytocin from magnocellular oxytocin neurones in the PVN and in the SON. Oxytocin released from the dendrites also diffuse to any brain & brainstem areas containing oxytocin receptors to modulate sexual behaviours.

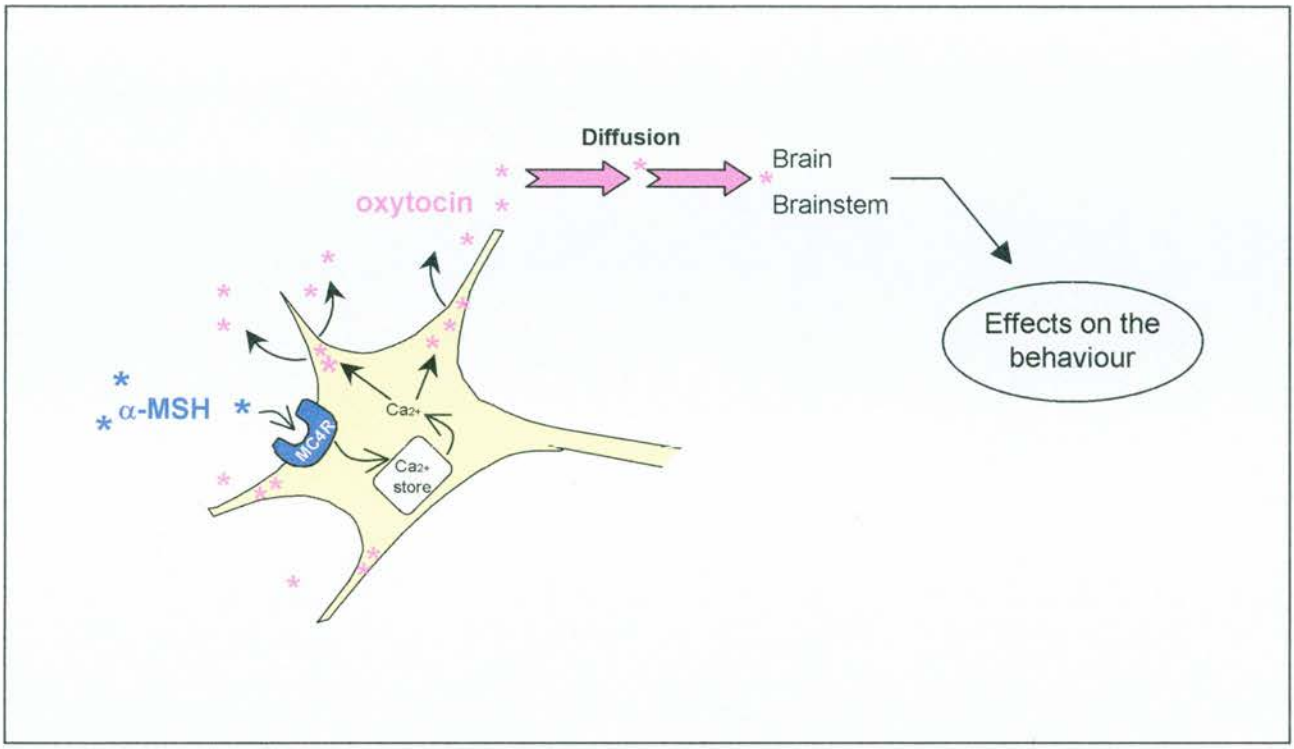

Figure7. 2: Stimulation of oxytocin dendritic release from magnocellular oxytocin neurones by α-MSH during male sexual behaviour *(New hypothesis)*

Once centrally released, α-MSH acts via MC4R to activate the dendritic release of oxytocin by increasing intracellular Ca2+ concentration. Oxytocin then diffuse to distant areas where oxytocin receptors are located to induce changes in the behaviours.

Further studies:

The present study was a starting point in the investigation of the interaction between oxytocin and α-MSH during male sexual behaviour. The new hypothesis that some of the central effects of α-MSH are mediated via oxytocin released from the dendrites of magnocellular neurones will require further investigation.
New experiments would have to be designed to be able to answer the main questions raised by the new hypothesis, and to test whether oxytocin released from the dendrites plays an important role in the regulation of male sexual behaviour. However, it is necessary to keep in mind that oxytocin knockout mice, which both lack of oxytocin, successfully reproduce and mate. This suggests that although oxytocin is important to facilitate the behaviour, oxytocin itself is not necessary to induce the behaviour; thus, blocking oxytocin released from the dendrites to expect to block the behaviour might be an inappropriate approach to prove the role dendritically released oxytocin.

1. Is oxytocin dendritically released during male sexual behaviour?

There is evidence of central release of oxytocin during copulation, but no studies have yet investigated whether some of this central oxytocin comes from dendrites of magnocellular neurones. This could be studied by measuring the release of oxytocin from magnocellular neurones during male sexual behaviour. Using a microdialysis probe placed onto the SON, samples of extracellular fluid would be collected before, during and after copulation. These samples would be then assayed for oxytocin concentration. This experiment could be extended to the PVN, keeping in mind that the release of oxytocin could reflect the release of oxytocin from axon terminals of parvocellular neurones.

2. Does α-MSH trigger dendritic release of oxytocin during male sexual behaviour?

Using a similar experimental design, rats could be centrally injected with a MC4 receptor antagonist or vehicle before being placed with a receptive female. Dialysates would be then collected via the probe throughout the sexual behaviours, and assayed for oxytocin concentration. We would expect to see an increase in oxytocin concentration in the copulating control rats, reflecting the dendritic release of oxytocin; and no significant changes or an attenuated response in MC4 receptor antagonist -injected rats. Another possibility could be to infuse the antagonist directly onto the SON via the microdialysis probe. To minimize technical issues, the effect of α-MSH on dendritic release could be firstly tested in conscious rats but without mating (i.e.: Infusions of α-MSH or aCSF onto the SON and measure oxytocin concentration in dialysates).

3. What is the role of dendritically released oxytocin in male sexual behaviour?

Investigating the role of oxytocin released from the dendrites will require overcoming the problem that magnocellular oxytocin neurones are also involved in the peripheral regulation of male sexual behaviour by the systemic secretion of oxytocin. The dendritic release from magnocellular oxytocin neurones from both the SON and the PVN will have to be selectively blocked without influencing the systemic secretion of oxytocin, which also plays a role in the behaviour and when blocked, would interfere with the behavioral process. Thus, to be able to study the behavioral changes induced by a lack of oxytocin released from the dendrites, experiments should be selective of the magnocellular neurones without interfering with the parvocellular neurones and be selective of the oxytocin release from the dendrites without disturbing the oxytocin secretion from the axon terminals.

For now, it is difficult to obtain an adequate experimental design, but the development of research on the difference in exocytosis mechanisms at the dendrites and at the axon terminals should produce new tools for investigating selectively the release from the dendrites. Hopefully, understanding what these differences are will allow to selectively

block the exocytosis mechanisms at the dendrites without interfering with the axonal secretion.

4. When does the interaction between α-MSH and magnocellular oxytocin neurones occur during male sexual behaviour?

Investigating when the interaction occurs will give precious information about the role of oxytocin released from the dendrites in the regulation of male sexual behavior. If α-MSH stimulates the release of oxytocin at the beginning of the sexual process, during or at the result of the sexual motivation phase, this could indicate that the release oxytocin facilitates the establishment of the following consummatory behaviour; whereas if α-MSH stimulates oxytocin release during the second phase, the role of oxytocin could be to maintain the behaviour. This could be tested by measuring the concentration of α-MSH and oxytocin in extracellular fluid in the SON and in the PVN, in parallel with a precise recording of the behaviour.

5. How does α-MSH stimulate the dendritic release of oxytocin while at the same time, inhibiting the electrical activity of the same magnocellular neurones?

The cellular mechanisms of the activation of the dendritic release of oxytocin and the inhibition of the electrical activity by α-MSH will have to be clarified.
It could be possible that the increase in $[Ca^{2+}]i$ induced by α-MSH, could activate, in parallel to the release of oxytocin from the dendrites, some Ca^{2+}-dependent K^+ – channels, whose activation leads to a hyperpolarisation and so to the inhibition of the electrical activity.
Another possibility is that the increase in $[Ca^{2+}]i$ also stimulate the release of endogenous cannabinoids from the phospholipid membrane (Wilson & Nicoll, 2001). Endogenous cannabinoids could then act on the pre-synaptic terminals to stimulate the liberation of GABA or to inhibit the liberation of glutamate, which will lead to the

electrical inhibition of the oxytocin neurone. Studies have reported that dendritically released oxytocin stimulates the release of endogenous cannabinoids which can inhibits the glutamate release from pre-synaptic terminals (Hirasawa *et al.*, 2004).

How does dendritic release of oxytocin influence male sexual behaviour or indeed any behaviour?

Answering this question relies upon a new concept of neuronal communication: The diffusion of neuromodulators from their sites of release to distant sites of action within the brain. In our example, oxytocin once secreted from the dendrites could act locally, or diffuse unselectively to distant brain areas where it could interact with any neuronal populations that express the oxytocin receptor. The mechanisms of diffusion of oxytocin itself would be not orientated towards a brain area or neuronal population; but the pattern of distribution of oxytocin receptors in some areas and the presence of oxytocin receptors on some neuronal populations that would determine the selectivity of action. Once bound to its receptors, oxytocin might induce a cascade of intracellular events leading to a modulation of the neuronal activity. The primed neurones would be then prepared to be activated (excitated or inhibited) by the subsequent stimuli. Consequently, this increase in responsiveness would facilitate the neuronal communication and therefore enhances behavioral processes.

For now, it is difficult to demonstrate how oxytocin, or neuromodulators in general, can diffuse within the brain; but as already mentioned in chapter 5, there is evidence supporting this concept. These include, for instance, the mismatch between the distribution of oxytocin receptors and the terminals endings of oxytocin fibres; the fact that in the SON, oxytocin is predominantly located within neurosecretory granules in the dendrites rather than in the axons; and especially the fact that, when injected centrally, without reproducing any spatio-temporal pattern of any endogenous stimulus, oxytocin

(or peptides in general) induces an organized behavioral response rather than a disorientated and random response. Investigating the diffusion mechanisms will require the possibility to switch on demand and selectively the dendritic release of oxytocin, and measure precisely the quantity of oxytocin released in selected brain areas (where the density of oxytocin receptors is high, for instance), or be able to selectively tag the oxytocin release from the dendrites at a precise time and visualize the distribution of the tagged protein within the brain.

The present study not only gives precious information about how two of the most potent erectogenic peptides interact to drive male sexual behaviour, it also gives persuasive evidence for an important role of the magnocellular oxytocin neurones in the central regulation of behaviours via dendritic release of oxytocin. These findings not only open new areas of research for a better understanding of male sexual behaviour with a hope of obtaining results that will lead to therapeutic schemes; they are also evidence for a new concept in neuronal communication, whose investigation will lead to a better understanding of the central mechanisms that regulate any behaviours.

References

Abdel-Malek Z.A. (2001). "Melanocortin receptors: their functions and regulation by physiological agonists and antagonists." *Cellular and molecular life sciences.* **58**: 434-441.

Adan R.A.H. and Gipsen W.H. (1997). "Brain melanocortin receptors: From cloning to function." *Peptides.* **18(8):** 1279-1287.

Ågmo A. (1997). "Male sexual behavior". *Brain. Res. Protocols.* **1:** 203-209.

Agmo A. and Parades R. (1988). "Opioids and sexual behavior in the male rat." *Pharmacol. Biochem. Behav.* **30(4):** 1021-34.

Antonijevic I.A., Douglas A.J., Dye S., Bicknell R.J., Leng, G., Russell, J.A. (1995). Oxytocin antagonists delay the initiation of parturition and prolong its active phase in rats. *J. Endocrinol.* **145**: 97-103.

Argiolas A., Melis M.R. and Gessa G.L. (1986). "Oxytocin: an extremely potent inducer of penile erection and yawning in male rats". *Europ. J. Pharmacol.* **130**: 265-272.

Argiolas A., Melis M.R., Mauri A. and Gessa L. (1987). "Paraventricular nucleus lesion prevents yawning and penile erection induced by apomorphine and oxytocin but not by ACTH in rats." *Brain. Res.* **421(1-2):** 349-352.

Argiolas A., Melis M.R., Vargiu L. and Gessa L. (1987). "d$(CH_2)_5$Tyr(Me)-Orn^8-vasotocin, a potent oxytocin antagonist, antagonizes penile erection and yawning induced by oxytocin and apomorphine, but not by ACTH-(1-24)". *Europ. J. Pharmacol.* **134(2)**: 221-224.

Argiolas A., Collu M., Gessa G.L., Melis M.R. and Serra G. (1988). "The oxytocin antagonist $d(CH_2)_5Tyr(Me)$-Orn^8-vasotocin inhibits male copulatory behaviour in rats". *Europ. J. Pharmacol.* **149**:3 89-392.

Argiolas A., Melis M.R. and Gessa G.L. (1988). "Yawning and penile erection: Central dopamine-oxytocin-adrenocorticotropin connection." *Ann. NY. Acad. Sci.* **525**: 330-337.

Argiolas A., Melis M.R. Stancampiano R. and Gessa G.L. (1989). "Penile erection and yawning induced by oxytocin and related peptides: structure-activity relationship." *Peptides.* **10(3)**: 559-563.

Argiolas A. (1994). "Nitric oxide is a central mediator of penile erection." *Neuropharmacol.* **33(11):** 1339-1344.

Argiolas A. and Melis M.R. (1995). "Neuromodulation of penile erection: an overview of the role of neurotransmitters and neuropeptides." *Prog. Neurobiol.* **47:** 235-255.

Argiolas A. (1999). " Neuropeptides and sexual behaviour". *Neurosci. Biobehav. Rev.* **23**: 1127-1142.

Argiolas A., Melis M.R., Murgia S. and Schiöth H. B. (2000). "ACTH- and α-MSH-induced grooming, stretching, yawning and penile erection in male rats: Site of action in the brain and role of melanocortins receptors." *Brain Res. Bull.* **51(3)**: 425-431.

Arletti R., Bazzani C., Castelli M. and Bertolini A. (1985). "Oxytocin improves male copulatory performance in rats". *Horm Behav.* **19(1):** 14- 20.

Arletti A., Benelli A. and Bertolini A. (1992). "Oxytocin involvement in male and female sexual behavior". *Ann. NY. Acad. Sci.* **12:** 180-193.

Band L.C. and Hull E.M. (1990). "Morphine and dynorphin (1-13) microinjected into the medial preoptic area and nucleus accumbens: effects on sexual behavior in male rats". *Brain Res.* **524(1):** 77-84.

Barberis C. and Tribollet E. (1996). "Vasopressin and oxytocin receptors in the central nervous system". *Critical Rev. Neurobiol.* **10(1):** 119-154.

Beach F.A. (1967). "Cerebral and hormonal control of reflexive mechanisms involved in copulatory behavior". Physiol Rev. 47(2) 289-316.

Bednarek M.A., MavNeil T., Kalyani R.N., Tang R., Van der Ploeg L.H.T. and Weinberg D.H. (2001). "Selective, High affinity peptide antagonists of α-Melanotropin action at human melanocortin receptor 4: Their synthesis and biological evaluation in vitro". *J. Med. Chem.* **44**: 3665-3672.

Beesley J.E. 1993. "Immunocytochemical avenues." In: *Immunocytochemistry: A practical approach.* IRL Press, UK. pp 6-13.

Belin V. and Moos F. (1986). "Paired recordings from the supraoptic and paraventricular oxytocin cells in suckled rats: recruitment and synchronization." *J. Physiol.* **377:** 369-390.

Benelli A., Bertolini A., Poggioli R., Cavazuti E., Calza L., Giardino L. and Arletti R. (1995). "Nitric oxide is involved in male sexual behavior of rats." *Europ. J. Pharmacology.* **294**: 505-510.

Bertolini A. and Baraldi M. (1975). "Anabolic steroids: permissive agents of ACTH-induced penile erections in rats." *Life Sci.* **17(2):** 263-266.

Beyer C., morali G., Larsson K. and Soderstein P. (1976). "Steroid regulation of sexual behavior". *J. Steroid Biochem.* **7(11-12):** 1171-1176.

Bloch G.J., Babcock A.M., Gorski R.A., Micevych P.E. (1988). "Effects of cholescystokinin on male copulatory behavior and lordosis behavior in male rats". *Physiol. Behav.* **43:** 351-357.

Bohus B. (1977). "Effect of desglycinamide-lysine vasopressin (DG-LVP) on sexually motivated t-maze behavior of the male rat." *Horm. Behav.* **8:** 52-61.

Bredt D.S, Hwang P.M. and Snyder S.H. (1990). "Localization of nitric oxide synthase indicating a neural role for nitric oxide." *Nature.* **347:** 768-770.

Brimble M.J. and Dyball R.E. (1977). "Characterization of the responses of oxytocin- and vasopressin- secreting neurones in the supraoptic nucleus to osmotic stimulation." *J. Physiol.* **271:** 253-271.

Buijs R.M., Swaab K.F., Dogterom J. and van Leeuwen F.W. (1978). "Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat." *Cell Tissue Res.* **186:** 423-433.

Buijs R.M. and Swaab D.F. (1979). "Immuno-electron miscroscopial demonstration of vasopressin and oxytocin synapses in the limbic system of the rat." *Cell Tissue Res.* **204:** 355-365.

Buijs R.M. (1980). "Immunocytochemical demonstration of vasopressin and oxytocin in the rat brain by light and electron microscopy." *J. Histochem. & Cytochem.* 28(4): 357-360.

Buijs R.M., De Vries G.J. and van Leeuwen F.W. (1985). "The distribution and synaptic release of oxytocin in the central nervous system." In: *Oxytocin: Clinical and laboratory studies.* J.A. Amico and A.G. Robinson, Eds.: 77-8. Elservier Science Publishers. UK.

Cadwell J.D., (1992). "Central oxytocin and female sexual behavior." *Ann. NY. Acad. Sci.* **12:** 166-179.

Carter C.S. (2003). "Developmental consequences of oxytocin". *Physiol. Behav.* **79:** 383-397.

Chen K.K., Chan S.H., Chang L.S. and Chan J.Y. (1997). "Participation of paraventricular nucleus of hypothalamus in central regulation of penile erection in the rat." *J. Urol.* **158(1):** 238-244.

Chen A.S., Marsh D.J., Trumbauer M.E., Frazier E.G., Guan X.M., Yu. H.,Rosenblum C.I., et al. (2000). "Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass." *Nature Genet.* **26(1):** 8-9.

Chen K. and Chang L.S. (2001). "Oxytocinergic neurotransmission at the hippocampus in the central neural regulation of penile erection in the rat." *Urology.* **58(1)**: 107-112.

Christensen L.W. and Clemens L.G. (1974). "Intrahypothalamic implants of testosterone or estradiol and resumption of masculine sexual behavior in long-term castrated male rats." *Endocrinol.* **95(4):** 984-990.

Clark J.T., Kalra P.S., Kalra S.P. (1985). "Neuropeptide Y stimulates feeding but inhibits sexual behavior in rats". *Endocrinology.* **117:** 2435-2442.

Clark J.T. and Smith E.R. (1987). "Effects of apomorphine on sexual behavior in young and middle-aged rats." *Neurobiol. Aging.* **8(2):** 153-157.

Clark J.T. (1989). "A possible role for Angiotensine II in the regulation of male sexual behavior in rats." *Physiol. Behav.* **45:** 221-246.

Claro F., Segovia S., Guilamon A. and Del Abril A. (1995). "Lesions in the medial posterior region of the BST impair sexual behavior in sexually experienced and inexperienced male rats." *Brain. Res. Bull.* **36(1):** 1-10.

Coolen L.M., Peters H.J. and Veening J.G. (1996). "Fos immunoreactivity in the rat brain following consummatory elements of sexual behavior: a sex comparison." *Brain. Res.* **738(1):** 67-82.

Coolen L.M., Fitzgerald M.E., Yu L. and Lehman M.N. (2004). "Activation of μ opioid receptors in the medial preoptic area following copulation in male rats". *Neurosc.* 124(1): 11-21.

Cragnolini A., Scimonelli T., Ester Celis M. and Schioth H.B. (2000). "The role of melanocortin receptors in sexual behavior in female rats". *Neuropeptides.* **34(3&4)**: 211-215.

Damsma G., Pfaus J.G., Wenkstern D., Phillips A.G. and Fibiger H.C. (1992). " Sexual behavior increases dopamine transmission in the nucleus accumbens and striatum of male rats: comparison with novelty and locomotion". *Behav. Neurosci.* **106(1):** 181-91.

Davidson J.M. (1966). "Characteristics of sex behaviour in male rats following castration." *Am. Behav.* **14(2):** 266-272.

Dekloet E.R., Rotteveel F., Voorhuis T. and Terlou M. (1990). " Topography of binding sites for neurohypophyseal hormones in rat brain". *Europ. J. Pharmacol.* **110:** 113-119.

Devor M. (1973). "Components of mating dissociated by lateral olfactory tract transection in male hamsters". *Brain. Res.* 64: 437- 441.

Dornan W.A. and Malsbury C.W. (1989). "Peptidergic control of male sexual behavior: the effects of intracerebral injections of substance P and cholecystokinin". *Physiol. Behav.* **46:** 547-556.

Dorsa D.M. and Smith E.R. (1980). "Facilitation of mounting behavior in male rats by intracranial injections of luteinizing hormone-releasing hormone." *Peptides.* **1:** 147-155.

Douglas A.J., Bicknell R.J. and Russell J.A. (1995). "Pathways to parturition". *Adv. Exp. Med. Biol.* **395:** 381-394.

Douglas A.J., Johnstone H., Brunton P. and Russell J.A. (2000). "Sex-steroid induction of endogenous opioid inhibition on oxytocin secretory responses to stress". *J. Neuroendocrinol.* **12:** 343-350.

Douglas A. J., Scullion S, Antonijevic I. A. , Brown D, Russell J. A. and Leng G. (2001). "Uterine contractile activity stimulates supraoptic neurons in term pregnant rats via a noradrenergic pathway." *Endocrinology.* **142(2):** 633-644.

Dunbar J.C. and Lu H. (2000). "Proopiomelanocortin (POMC) products in the central regulation of sympathetic and cardiovascular dynamics: studies on melanocortin and opioids interactions." *Peptides.* **21**:211-217.

Du Vigneaud V., Ressler C. and Trippett S. (1953). "the sequence of amino acids in oxytocin, with a proposal for the structure of oxytocin." *J. Biol. Chem.* **205(2):** 949-957.

Eaton R.C., Markowski V.P., Lumley L.A., Thompson J.T., Moses J. and Hull E.M. (1991). "D2 receptors in the paraventricular nucleus regulate genital responses and copulation in male rats." *Pharmacol. Biochem. Behav.* **39(1):** 1771-181.

Edwards D.A. and Einhorn L.C. (1986). "Preoptic and midbrain control of sexual motivation". *Physiol. Behav.* **37(2):** 329-335.

Elands J., Beetsma A., Barberis C. and De kloet E.R. (1998). "Topography of the oxytocin receptor system in rat brain: an autoradiographical study with a selective radioiodinated oxytocin antagonist." *J. Chem. Neuroanat.* **1:** 293-302.

Emery D.E., Sachs B.D. (1976). "Copulatory behavior in male rats with lesions in the bed nucleus of the stria terminalis." *Physiol. Behav.* **17**: 803-806.

Everitt B.J. (1990). "Sexual motivation: a neural and behavioural analysis of the mechanisms underlying appetitive and copulatory responses of male rats". *Neurosci. Biobehav. Rev.* **14(2):** 217-32.

Fan W., Boston B.A., Kesterson R.A., Hruby V.J. and Cone R.D. (1997). "Role of melanocortinergic neurons in feeding and the agouti obesity syndrome". *Nature.* **385**: 165-168.

Ferguson J.N., Young L.J. and Insel T.R. (2002). "The neuroendocrine basis of social recognition". *Front.Neuroendocrinol.* **23:** 200-224.

Ferrari W., Gessa G.L. and Vargui L. (1963). "Behavioral effects induced by intracisternally injected ACTH and MSH". *Ann. N.Y. Acad. Sci.* **104:** 330-45.

Fu L. and Beckstead R.M. (1992). "Cortical stimulation induces Fos expression in the striatal neurons." *Neuroscience.* **46:** 329-334.

Gantz I. and Fong T.M. (2003). "The melanocortin system". *Am J. Physiol. Endocrinol. Metab.* **284**: E468-E474.

Giantonio G.W., Lund N.L. and Gerall A.A. (1970). "Effect of diencephalic and rhinencephalic lesions on the male rat's sexual behavior". *J. Comp. Physiol. Psychol.* **73(1):** 38-46.

Gimpl G. and Fahrenholz F. (2001). " The oxytocin receptor system: Structure, Function, and Regulation." *Physiol. Rev.* **81(2):** 629-683.

Ginton A. and Merari A. (1977). Long range effects of MPOA lesion on mating behavior in the male rat." *Brain. Res.* **120(1):** 158-163.

Giovannelli L.P., Shiromani P.J., Jirikowski G.F. and Bloom F.E. (1990). "Oxytocin neurons in the rat hypothalamus exhibit c-*fos* immunoreactivity upon osmotic stress." *Brain Res.* **531:** 299-303.

Gissel C., Doutheil J. and Paschen W. (1997). "Temporal analysis of changes in neuronal c-fos mRNA levels induced by depletion of endoplasmic reticulum calcium stores: Effect of clamping cytoplasmic calcium activity at resting levels." *J. Neurochem.* **69:** 2538-2545.

Gonzalez M.I., Celis M.E., Hole D.R. and Wilson C.A. (1993). "Interaction of oestradiol, alpha-melanotrophin and noraderenaline within the ventromedial nucleus in the control of female sexual behaviour." *Neuroendocrinol.* **58(2):** 218-226.

Giuliano F. and Rampin O. (2000). "Central neural regulation of penile erection." *Neurosci. Biobehav. Rev.* **24:** 517-533.

Hadley M.E. and Haskell-Luevano C. (1999). "The proopiomelanocortin system". *Ann. NY Acad. Sci.* **885**: 1-21.

Hallbeck M, Larhammar D, Blomqvist A. (2001). "Neuropeptide expression in rat paraventricular hypothalamic neurons that project to the spinal cord." *J Comp Neurol.* **433(2):** 222-238.

Hamamura M., Leng G., Emson P.C. and Kiyama H. (1991). "Electrical activation and c-*fos* mRNA expression in rat neurosecretory neurones after systemic administration of cholecystokinin." *J. Physiol.* **444:** 51-63.

Hamson D.K. and Watson N.V. (2004). "Regional brainstem expression of Fos associated with sexual behavior in male rats." *Brain. Res.* 233-240.

Hansen S and Gummesson BM. (1982). "Participation of the lateral midbrain tegmentum in the neuroendocrine control of sexual behavior and lactation in the rat." *Brain Res.* 251(2): 319-325

Hansen S. and af Hagelsrum L.J. (1984). "Emergence of displacement activities in the male rat following thwarting of sexual behavior". *Behav. Neurosc.* **98(5):** 868-883.

Hansen M.J. and Morris M.J. (2002). "Evidence for an interaction between neuropeptide Y and the melanocortin –4 receptor on feeding in the rat." *Neuropharmacology.* **42:** 792-797.

Harris V.S. and Sachs B.D. (1975). "Copulatory behavior in male rats following amygdaloid lesions." *Brain. Res.* **86(3):** 514-518.

Higuchi T., Honda K., Fukuoka T., Negoro H., Wakabayashi K. (1985). "Release of oxytocin during suckling and parturition in the rat." *J. Endocrinol.* **105:** 339-346.

Hillegaart V., Alster P., Uvnäs-Moberg K. and Ahlenius S. (1998). "Sexual motivation promotes oxytocin secretion in male rats." *Peptides.* **19(1):** 39-45.

Hirasawa M., Schwab Y., Natah S., Hillard C.J., Mackie K., Sharkey K.A. and Pittman Q.J. (2004). "Dendritically released transmitters cooperate via autocrine and retrograde actions to inhibit afferent excitation in rat brain." *J. Physiol.* **559(2):** 611-624.

Hoffman G.E., Smith M.S. and Verbalis J.G. (1993). "c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems." *Frontiers in Neuroendocrinology.* **14(3):** 173-213.

Hoffman G.E. and Lyo D. (2002). "Anatomical markers of activity in neuroendocrine systems: are we all 'Fos-ed out'?" *J. Neuroendocrinol.* **14(4):** 259-268.

Honda K., Yanagimoto M., Negoro H., Narita K., Murata T. and Higuchi T. (1999). "Excitation of oxytocin cells in the hypothalamic supraoptic nucleus by electrical stimulation of the dorsal penile nerve and tactile stimulation of the penis in the rat". *Brain Res. Bull.* **48(3)**: 309-313.

Hughes A.M., Everitt B.J. Lightman S.L. and Todd K. (1987). "Oxytocin in the central nervous system and sexual behaviour in male rats". *Brain Res.* **414**: 133-137.

Hughes A.M., Everitt B.J. and Herbert J. (1990). "Comparative effects of preoptic area infusions of opioid peptides, lesions and castration on sexual behaviour in male rats: studies of instrumental behaviour, conditioned place preference and partner preference." *Psychopharmacol.* **102(2):**243-256

Hull E.M., Bitran D., Pehec E.A., Warner R.K., Band L.C. and Holmes G.M. (1986). "Dopaminergic control of male sex behavior in rats: effects of an intracerbrally-infused agonist". *Brain Res.* **370(1):** 73-81.

Hull E.M., Warner R.K., Bazzet T.J., Eaton R.C., Thompson J.T. and Scaletta L.L. (1989). "D2/D1 ratio in the medial preoptic area affects copulation of male rats." J. *Pharmacol. Exp. Ther.* **251(2):** 422-427.

Hull E.M., Eaton R.C., Moses J. and LorrainD. (1993). " Copulation increases dopamine activity in the medial preoptic area of male rats". *Life Sci.* **52** (11): 935-40.

Hull E.M., Du J., Lorrain D.S. and Matuszewich L. (1995). "Extracellular dopamine in the medial preoptic area: Implications for sexual motivation and hormonal control of copulation". *J. Neurosc.* **15** (11): 7465-7471.

Hull E.M., Lorrain D.S., Du J., Matuszewich L., Lumley A.L., Putnam S.K. and Moses J. (1999). "Hormone-neurotransmitter interactions in the control of sexual behavior". *Behav. Brain. Res.* **105**: 105-116.

Huszar D., Lynch C.A., Fairchild-Huntress V., Dunmore J.H., Fang Q., Berkemeier L.R., Gu.W. et al. (1997). "Targeted disruption of the melanocortin-4 receptor results in obesity in mice." *Cell.* **88(1):** 131-141.

Ingram CD, Adams TS, Jiang QB, Terenzi MG, Lambert RC, Wakerley JB, and Moos F. (1995). "Mortyn Jones Memorial Lecture: Limbic regions mediating central actions of oxytocin on the milk-ejection reflex in the rat." *J Neuroendocrinol.* **7(1):**1-13.

Insel T.R. and Hulihan T.J. (1995). A gender-specific mechanism for pair-bonding: oxytocin and partner preference formation in monogamous voles." *Behav. Neurosci.* **109:** 782-789.

Insel T.R. and Young L. (2001). "The neurobiology of attachment". *Nature reviews.* **2**: 129- 136.

Ivell R., Balvers M., Bathgate R. and Einspanier A. (1997). "Oxytocin and male reproductive function". *Adv. Exp. Med. Biol.* **424:** 253-264.

Jirikowski G.F. (1992). "Oxytocinergic neuronal systems during mating, pregnancy, parturition, and lactation". *Ann. NY. Acad. Sci.* **12:** 253- 270.

Johnstone L. E., Brown C.H., Meeren H.K., Vuijst C.L., Brooks C.L., Leng G. and Leng G. (2000). " Local morphine withdrawal increases c-fos gene, Fos protein, and oxytocin

gene expression in hypothalamic magnocellular neurosecretory cells." *J. Neurosci.* **20(3):** 1272-1280.

Kendrick K.M., Keverne E.B. and Baldwin B.A. (1987). "Intracerebroventricular oxytocin stimulates maternal behavior in the sheep". *Neuroendocrinology.* **46:** 56-1.

Keverne E.B. and Kendrick K.M. (1992). "Oxytocin facilitation of maternal behavior in sheep". *Ann. NY. Acad.Sci.* **652:** 83-101.

Kondo Y., Tomihara K. and Sakuma Y. (1999). "Sensory requirements for noncontact penile erection in the rat." *Behav. Neyrosci.* **113(5):** 1062-1070.

Landgraf R., Neumann I., Russell J.A. and Pittman Q. (1992). "Push-pull perfusion and microdialysis studies on central oxytocin and vasopressin release in freely moving rats during pregnancy, parturition, and lactation." *Ann. N.Y. Acad. Sci.* **652:** 326-339.

Farsson K. and Heimer L. (1966). " Mating behaviour of male rats after lesiosn in the preoptic area." *Nature.* **202:** 413-414.

Lee L.R., Smith M.S., Hoffman G.E. (1990). " Luteinizing hormone releasing hormone (LHRH) neurons express c-Fos during the proestrus LH surge." *Proc. Natl. Acad. Sci.* **87:** 5163-517.

Lefebvre D.L. Giaid A., Bennett H., Lariviere R. and Zingg H.H. (1992). "Oxytocin gene expressionin rat uterus." *Science.* **256:** 1553-1555.

Leng G. (1980). "Rat supraoptic neurones: The effects of locally applied hypertonicsaline." *J. Physiol.* **304:** 405-414.

Leyton M. and Stewart J. (1992). "The stimulation of central kappa opioid receptors decreases male sexual behavior and locomotor activity". *Brain Res.* **594(1):** 56-74.

Li S., Varga K., Archer P., Hruby V.J., Sharma S.D., Kesterson R.A. Cone R. D. and Kunos G. (1996). "Melanocortin antagonists define two distinct pathways of cardiovascular control by α- and γ-melanocyte-stimulating hormones." *J. Neurosc.* **16(16)**: 5182-5188.

Lippert T.H., Mueck A.O., Seeger H. and Pfaff A. (2003). "Effects of oxytocin outside pregnancy". *Horm. Res.* **60:** 262-271.

Lisk R.D., Zeiss J. and Ciaccio L.A. (1972). " The influence of olfaction on sexual behavior in the male golden hamster (Mesocricetus auratus)." *J. Exp. Zool.* **181(1):** 69-78.

Lim M.M., wang Z., Olazabal D.E., Ren X., Terwilliger and Young L.J. (2004). "Enhanced partner preference in a promiscuous species by manipulating the expression of a single gene". *Nature.* **429:** 754- 757.

Linden A., Hansen S., Bednar I., Forsberg G., Sodersten P. and Uvnas-Moberg K. (1987). "Sexual activity increases plasma concentrations of cholescystokinin octapeptide and offsets hunger in male rats." *J. Endocrinol.* **115:** 91-95.

Liu Y.C., Salamone J.D. and Sachs B.D. (1997). "Lesions in medial preoptic area and bed nucleus of stria terminalis: differential effects on copulatory behavior and noncontact erection in rats." *J.Neurosci.* **17(13):** 5245-5253.

Lotti T., Bianchi B., Ghersetich I., Brazzini B. and Hercogova J. (2002). "Can the brain inhibit inflammation generated in the skin? The lesson of α-melanocyte-stimulating hormone." *Intern. J. Dermatology.* **41**: 311-318.

Lu D., Willard D., Patel I.R., Kadwell S., Overton L., Kost T., Luther M., Chen W., Woychik R.P., Wilkison W.O. *et al.* (1994). "Agouti protein is an antagonist of the melanocytes-stimulating-hormone receptor." *Nature.* **371(6500):** 799-802.

Luckman S.M., Dyball R.E. and Leng G. (1994). " Induction of c-fos expression in hypothalamic magnocellular neurons requires synaptic activation and not simply increased spike activity." *J. Neurosc.* **14:** 4825-4830.

Ludwig M. (1998). " Dendritic release of vasopressin and oxytocin." *J. Neuroendocrinol.* **10:** 881-895.

Ludwig M., Sabatier N., Bull P.M., Landgraf R., Dayanithi G. and Leng G. (2002). "Intracellular calcium stores regulate activity-dependent neuropeptide release from the dendrites." *Nature.* **418:** 85-89.

Mansour A., Khachaturian H., Lewis M.E., Akil H. and Watson S.J. (1988). "Anatomy of CNS opioid receptors." *Trends. Neurosci.* **11(7):** 308-314.

Martin W.J., McGowan E., Cashen D.E., Gantert L.T., Drisko J.E., Hom G.J., Nargund R., Sebhat I., Howard A.D. Van der Ploeg L.H.T. and MacIntyre D.E. (2002). "Activation of melanocortin MC4 receptors increases erectile activity in rats *ex copula*." *Europ. J. Pharmaco.* **454**: 71-79.

McGregor A. and Herbert J. (1992). " The effects of beta-endorphin infusions into the amygdala on visual and olfactory sensory processing during sexual behaviour in the male rat". *Neurosci.* **46(1):** 173-9.

McKenna K.E. (2000). "Some proposals regarding the organization of the central nervous system control of penile erection". *Neurosc. Biobehav. Rev.* **24**: 535-540.

McMinn J.E., Wilkinson C.W., Havel P.J., Woods S.C. and Schwartz M.W. (2000). "Effect of intracerebroventricular α-MSH on food intake, adiposity, c-Fos induction, and neuropeptide expression." *Am. J. Physiol. Regulatory Integrative Comp. Physiol.* **279:** R95-R703.

Meisel R.L. and Sachs B.D. (1994). "The physiology of male sexual behavior". In: *The Physiology of Reproduction.* Ed by E. Knobil & J.D Neill. Raven Press Ltd, NY. 3- 105.

Meisel R.L., Lumia A.R., Sachs B.D. (1980). "Effects of olfactory bulb removal and flank shock on copulation in male rats." *Physiol. Behav.* **25:** 383-387.

Melin P. and Kihlstrom J. E. (1963). "Influence of oxytocin on sexual behavior in male rabbits". *Endocrinology.* **73**: 433-435.

Melis M.R., Argiolas A. and Gessa G.L. (1986). "Oxytocin-induced penile erection and yawning: site of action in the brain." *Brain Res.* **398(2)**: 259-265.

Melis M.R., Argiolas A. and Gessa G.L. (1987). "Apomorphine-induced penile erection and yawning: site of action in the brain." *Brain Res.* **415(1)**: 98-104.

Melis M.R., Argiolas A. and Gessa G.L. (1989). "Evidence that apomorphine induces penile erection and yawning by releasing oxytocin in the central nervous system." *Europ. J. Pharmacol.* **164**: 565-570.

Melis M.R., Stancampiano R. and Argiolas A. (1992). "Hippocampal oxytocin mediates apomorphine-induced penile erection and yawning." *Pharmacol. Biochem. Behav.* **42(1)**: 61-66.

Melis M.R., Stancampiano R., Gessa G.L. and Argiolas A. (1992). "Prevention by morphine of apomorphine- and oxytocin-induced penile erection and yawning: site of action in the brain." *Neuropsychopharmacology.* **6(1)**: 17-21.

Melis M.R. and Argiolas A. (1993). "Nitric oxide synthase inhibitors prevent apomorphine- and oxytocin-induced penile erection and yawning in male rats." *Brain Res. Bull.* **32(1)**: 71-74.

Melis M.R., Mauri A. and Argiolas A. (1994). "Apomorphine- and oxytocin-induced penile erection and yawning in intact and castrated male rats: effect of sexual steroids." *Neuroendocrinology.* **59(4)**: 349-354.

Melis M.R. and Argiolas A. (1995). "Dopamine and sexual behavior". *Neurosc. Biobehav. Rev.* **19** (1): 19-38.

Melis M.R. and Argiolas A. (1997). "Role of central nitric oxide in the control of penile erection and yawning." *Prog. Neuropsychopharmacology.* **21**: 899-922.

Melis M.R., Spano M.S., Succu S. and Argiolas A. (1999). "The oxytocin antagonist d(CH2)5-Tyr-(Me)2-Orn8-vasotocin reduces noncontact penile erections in male rats." *Neurosci. Lett.* **265(3):** 171-174.

Melis M.R., Succu S., Mascia M.S., Cortis L. and Argiolas A. (2003). " Extracellular dopamine increases in the paraventricular nucleus of male rats during sexual activity". *Europ. J. Neurosc.* **17**: 1266-1272.

Meredith M. (1986). "Vomeronasal organ removal before sexual experience impairs male hamster mating behavior." *Physiol. Behav.* **36:** 737-743.

Miller R.L. and Baum M.J. (1987). "Naloxone inhibits mating and conditioned place preference for an estrous female in male rats soon after castration." *Pharmacol. Biochem. Behav.* **26(4):** 781-789.

Mitchell J.B. and Stewart J. (1990). " Facilitation of sexual behaviors in the male rat associated with intra-VTA injections of opioates." *Pharmacol. Biochem .Behav.* **35** (3): 643-50.

Mizusawa H., Hedlund P. and Andersson K-E. (2002). "α-Melanocyte stimulating hormone and oxytocin induced penile erections, and intracavernous pressure increases in the rat." *J. Urology.* **167**: 757-760.

Moos, F., Freund-Mercier M.-J., Guerne Y., Guerne J.M., Stoeckel M.E. and Richard P. (1984). "Release of oxytocin and vasopressin by magnocellular nuclei *in vitro*, specific facilitatory effect of oxytocin on its own release." *J. Endocrinol.* **102:** 63-72.

Mountjoy, K.G., M.T. Mortrud, M.J. Low, Simerly R.B. and Cone R.D. (1994). "Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain." *Mol. Endocrinol.* **8:** 1298-1308.

Neumann I., Douglas A.J., Pittman Q.J., Russell J.A. and Landgraf R. (1996). "Oxytocin released within the supraoptic nucleus of the rat brain by feedback action is involved in parturition-related events." *J. Neuroendocrinol.* **8(3):** 227-233.

Nicholson H.D., Guldenaar S.E., Boer G.J. and Pickering B.T. (1991). "Testicular oxytocin: Effects of intratestitular oxytocin in the rat." *J. Endocrinol.* **130:** 231-238.

Nishimori K. Young L.J., Guo Q., Wang Z., Insel T. R. and Matzuk M.M. (1996). "Oxytocin is required for nursing but is not essential for parturition or reproductive behavior." *Proc. National. Acad. Sci. USA.* **93:** 11699-11704.

O'Donohue T.L. and Jacobowitz D.M. (1980). "studies of alpha-MSH-containing nerves in the brain." *Prog. Biochem. Pharmacol.* **16:** 69-83.

Olszewski P.K., Wirth M.M., Shaw T.J., Grace M.K., Billington C.J., Giraudo S.Q. and Levine A.S. (2001). "Role of α-MSH in the regulation of consummatory behavior: immunohistochemical evidence." *Am. J. Physiol. Integrative Comp Physiol.* **281**: 673-680.

Opstad K., Bull P., Leng G., Melin P. and Russell J.A. (1996). " Central and peripheral inhibition of the milk-ejection reflex by a peptide oxytocin antagonist in anaesthetized lactating rats." Proceedings J. Physiol. **495P**.

Paglietti E., Quarantotti B.P., Mereu G. and Gessa G.L. (1978). "Apomorphine and L-DOPA lower ejaculation threshold in the male rat." *Physiol. Behav.* **20(5):** 559-52.

Paxinos G. and Watson C. (1996) "The rat brain in stereotaxic coordinates, 3rd edition". Academic Press, USA

Pederson C.A., Asher J.A., Monroe Y.L. and Prange A.J. (1982). "Oxytocin induces maternal behavior in virgin female rats." *Science.* **216:** 648-650.

Peterson R.P. (1966). "Magnocellular neurosecretory centers in the rat hypothalamus." *J. Comp. Neurol.* **128:** 181-190.

Pfaus J. G. and Heeb M.M. (1997). "Implications of immediate-early gene induction in the brain following sexual stimulation of female and male rodents". *Brain Res. Bull.* **44(4):** 397-407.

Poggioli R., Vergoni A.V. and Bertolini A. (1986). "ACTH-(1-24) and alpha-MSH antagonize feeding behavior stimulated by kappa opiate agonists." *Peptides.* **7(5):** 843-848.

Pow D.V. and Morris J.F. (1989). "Dendrites of hypothalamic magnocellular neurons release neurohypophysial peptides by exocytosis." *Neuroscience.* **32(2)**: 435-439.

Powers J.B. and Winans S.S. (1975). "Vomeronasal organ: Critical role in mediating sexual behavior of the male hamster." *Science.* **187:** 961-963.

Putnam S.K., Sato S and Hull E.M. (2003)."Effects of testosterone metabolites on copulation and medial preoptic dopamine release in castrated male rats." *Horm Behav.* **44(5):** 419-426.

Rajfer J., Aronson W.J., Bush P.A., Dorey F.J. and Ignarro L.J. (1992). "Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission." *N. Engl. J. Med.* **326(2):** 90-94.

Rajora N., Boccoli G., Burns D., Sharma S., Catania A.P. and Lipton J.M. (1997). "α-MSH modulates local and circulating tumor necrosis factor-a in experimental brain inflammation". *J. Neurosc.* **17(6)**: 2181-2186.

Rao I.M. and Mahesh V.B. (1986). "Role of progesterone in the modulation of the preovulatory surge of gonadotropins and ovulation in the pregnant mare's serum gonadotropin-primed immature rat and the adult rat." *Biol. Reprod.* **35(5):** 1154-1161.

Ratnasooriya W.D. and Jayakody J.R.A.C. (2000). "Long-term administration of large doses of paracetamol impairs the reproductive competence of male rats." *Asian.J. Androl.* **2:** 247-255.

Renaud L.P., Tang M., McCann M.J., Stricker E.M. and Verbalis J. G. (1987). "Cholecystokinin and gastric distension activate oxytocinergic cells in the rat hypothalamus". *Am. J. Physiol.* **253:** R661-665.

Richard P., Moos F. and Freund-Mercier M.J. (1991). "Central effects of oxytocin". *Physiol. Re.* **71:** 331-370.

Roselli-Rehfuss L., Mountjoy K.G., Robbins L.S., Mortrud M.T., Low M.J., Tatro J.B., Entwistle M.L., Simerly R.B. and Cone R.D. (1993). " Identification of a receptor for γ melanotropin and other proopiomelanocortin peptides in the hypothalamus and the limbic system". *Proc. Natl. Acad. Sci.* **90**: 8856-8860.

Sabatier N., Caquineau C., Dayanithi G., Bull P., Douglas A.J., Guan X., Jiang M., Van der Ploeg L. and Leng G. (2003). *J. Neurosc.* **23 (32)**: 10351-10358.

Saito T.R. and Moltz H. (1986). "Copulatory behavior of sexually naïve and sexually experienced male rats following removal of the vomeronasal organ." *Physiol. Behav.* **37:** 507-510.

Sagar S.M. Sharp F.R. and Curran T. (1988). " Expression of c-fos protein in the brain: Metabolic mapping at the cellular level." *Science.* **240:** 1328-1331.

Schumacher M, Coirini H, Frankfurt M, and McEwen BS. (1989). "Localized actions of progesterone in hypothalamus involve oxytocin." *Proc Natl Acad Sci* U S A.. **86(17):** 6798-801.

Schioth H.B., Mutulis F., Muceniece R., Prusis P. and Wikberg J. E.S. (1998). "Discovery of novel melanocortin 4 receptor selective MSH analogues". *Bri. J. Pharmaco.* **124**: 75-82.

Scimonelli T., Medina F., Wilson C. and Ester Celis M. (2000). "Interaction of α-melanotropin (α-MSH) and noradrenaline in the median eminence in the control of female sexual behavior". *Peptides.* **21**: 219-223.

Sgambato V., Abo V., Rogard M., Besson M.J. and Deniau J.M. (1997). "Effect of electrical stimulation of the cerebral cortex on the expression of the Fos protein in the basal ganglia." *Neuroscience.* **81:** 93-112.

Sharp F.R., Sagar S.M., Hicks K., Lowenstein D. and Hisanaga K. (1991). "c-*fos* mRNA, and Fos-related antigen induction by hypertonic saline and stress." *J. Neurosci.* **11(8):** 2321-2331.

Shibuki K., Leng G. and Way S. (1988). " Effects of naloxone and of intraperitoneal hypertonic saline upon oxytocin release and upon supraoptic neuronal activity." *Neurosci. Lett.* **88(1):** 75-80.

Sofroniew M.V. (1983). "Morphology of vasopressin and oxytocin neurones and their central and vascular projections." *Prog. Brain Res.* **60:** 101-114.

Srisawat R., Ludwig M., Bull P.M., Douglas A.J., Russell J.A. and Leng G. (2000). "Nitric oxide and the oxytocin system in pregnancy". *J. Neurosci.* **20(17):** 6721-6727.

Starowicz K. and Przewlocka B. (2003). "The role of melanocortins and their receptors in inflammatory processes, nerve regeneration and nociception." *Life Sciences.* **73**: 823-847.

Steers W.D. (2000). "Neural pathways and central sites involved in penile erection: neuroanatomy and clinical implications." *Neurosc. Biobehav. Rev.* 507-516.

Sternberger L.A. (1979). "Immunocytochemistry, 2nd Edition". *Wiley Medical Publication, USA.*

Stoneham M.D., Everiit B.J., Hansen S., Jightman S.L. and Todd K. (1985). "Oxytocin and sexual behaviour in the male rat and rabbit". *J. Endocr.* **107**: 97-106.

Stricker E.M. and Verbalis J.G. (1986). "Interaction of osmotic and volume stimuli in regulation of neurohypophyseal secretion in rats." *Am J. Physiol.* **250(2):** R267-275.

Succu S., Mascia M. S., Tiziana M., Melis M.R., Deghenghi R. and Argiolas A. (2003). " Activation of $GABA_A$ and opioid receptors reduce penile erection induced by hexarelin peptides." *Pharmacol. Biochem. Behav.* **76:** 563-570

Swanson L.W. & Kuypers H.G.J.M. (1980). "The paraventricular nucleus of the hypothalamus: Cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods." *J. Comp. Neurol.* **194:** 555-570.

Swanson L.W. and Sawchenko P.E. (1983). "Hypothalamic integration: Organization of the paraventricular and supraoptic nuclei". *Ann. Rev. Neurosc.* **6**: 269-324.

Szechtman H., Hershkowitz M. and Simantov R. (1981). "Sexual behavior decreases pain sensitivity and stimulated endogenous opioids in male rats." *Eur. J. Pharmacol.* **70(3):** 279-285.

Tagliamonte A., Fratta W., Del FiaccoM. And Gessa G.L. (1974). "Possible stimulatory role of brain dopamine in the copulatory behavior of male rats." Pharmacol. Biochem. Behav. **2(2):** 257-20.

Tribollet E., Audigier S., Dubois-Dauphin M. and Dreifuss J.J. (1990). "Gonadal steroids regulate oxytocin receptors but not vasopressin receptors in the brain of male and female rats. An autoradiographical study." *Brain Res.* **511(1)**: 129-140.

Thiele T.E., van Dijk G., Yagaloff K.A., FisherS.L., Schawrtz M., Burn P. and Seeley R.J. (1998). "Central infusion of melanocortin agonist MTII in rats: Assessment of c-Fos expression and taste aversion." *Am. J. Physiol.* **274**: R248-R254.

Vaccari C., Lolait S.J. and Ostrowski N.L. (1998). "Comparative distribution of vasopressin V1b and oxytocin receptor messenger ribonucleic acids in brain." *Endocrinology.* **139(12):** 5015-5033.

Van Furth W.R., Wolterink-Donselaar I.G. and Van Ree. J.M. (1994). "Endogenous opioids are differentially in appetitive and consummatory aspects of sexual behavior of male rats." *Am. J. Physiol.* **266(2):** R606-R613.

Van der Ploeg L.H.T., Martin W.J., Howard A.D., Nargund R.P., Austin C.P., Guan X., Drisko J., *et al.* (2002). "A role for the melanocortin 4 receptor in sexual function". *PNAS* **99(17):** 11381-11386.

Vemulapalli R. Kurowski S., Salisbury B., Parker E. and Davis H. (2001). "Activation of central melanocortin receptors by MT-II increases cavernosal pressure in rabbits by the neuronal release of NO". *Brit. J. Pharmaco.* **134**: 1705-1710.

Verbalis J.G., McCann M.J., McHale c.M. and Stricker E.M. (1986). "Oxytocin secretion in response to cholecystokinin and food: differentiation of nausea from satiety." *Science.* **232:** 1417-1419.

Verbalis J.G., Stricker E.M., Robinson E.M. and Hoffman G.E. (1991). "Cholescystokinin activates c-*fos* expression in hypothalamic oxytocin and corticotrophin-releasing hormone neurons." *J. Neuroendocrinol.* **3:** 205-214.

Vergoni A.V., Bertolini A., Mutulis F., Wikberg J.E.S., and Schioth H.B. (1998). "Differential influence of a selective melanocortin MC4 receptor antagonist (HS014) on melanocortin-induced behavioral effects in rats." *Europ. J. Pharmaco.* **362**: 95-101.

Vergoni A. and Bertolini A. (2000). "Role of melanocortins in the central control of feeding". *Europe. J. Pharmacology.* **405**: 25-32.

Vergoni A.V., Bertolini A., Guidetti G., Karefilakis V., Filaferro M., Wikberg J.E.S. and Schioth H.B. (2000). "Chronic melanocortin 4 receptor blockage causes obesity without influencing sexual behavior in male rats". *J. Endocrinology.* **166**: 419-426.

Veronneau-Longueville F., Rampin O., Freund-Mercier M.-J. Tang Y., Calas A., Marson L., McKenna K.E., Stoeckel M.-E., Benoit G. and Guiliano F. (1999). "Oxytocinergic innervation of autonomic nuclei controlling penile erection in the rat." *Neuroscience.* **93(4)**: 1437-1447.

Wakerley J.B. and Lincoln D.W. (1973). " The milk-ejection reflex of the rat: a 20- to 40-fold acceleration in the firing of paraventricular neurones during oxytocin release." *J. Endocrinol.* **57:** 477-493.

Wenkstern D., Pfaus J.G. and Fibiger H.C. (1993). "Dopamine transmission increases in the nucleus accumbens of male rats during their first exposure to sexually receptive female rats". *Brain Res.* **618(1):** 41-6.

Wessells H., Fuciarelli K., Hansen J., Hadley M.E., Hruby V.J., Dorr R. and Levine N. (1998). "Synthetic melanotropic peptide initiates erections in men with psychogenic erectile dysfunction: Double- blind, placebo controlled crossover study." *J. Urology.* **160:** 389-393.

Wessells H., Gralnek D., Dorr R., Hruby V.J., Hadley M.E. and Levine N. (2000). "Effect of an alpha-melanocyte stimulating hormone analog on penile erection and sexual desire in men with organic erectile dysfunction." *Urology.* **56(4)**: 641-646.

Williams J., Insel T., Harbaugh C. and Carter C. (1994). "Oxytocin administrated centrally facilitates formation of a partner preference in female prairie voles." *J. Neuroenocrinol.* **6:** 247-250.

Wilson R.I, and Nicoll R.A. (2001). "Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses." *Nature.* **410:** 588-592.

Winans S.S. and Powers J.B. (1977). "Olfactory and vomeronasal deafferentation of male hamsters: Histological and behavioral analyses". *Brain. Res.* **126**: 325-344.

Winslow J.T., Hastings N., Caret C.S., Harbaugh C.R. and T.R. Insel. (1993). "A role for central vasopressin in pair bonding in monogamous prairie voles." *Nature.* **365:** 545-548.

Winslow J.T., Shapiro L.E., Carte C.S. and Insel .T.R. (1993). "Oxytocin and complex social behaviors: species comparisons." *Psychopharmacol. Bull.* **29:** 409-414.

Witt D.M. and Insel T.R. (1994). "Increased Fos expression in oxytocin neurons following masculine sexual behavior". *J. Neuroendo.* **6**: 13-18.

Yanagimoto M., Honda K., Goto Y. and Negoro H. (1996). " Afferents originating from the dorsal penile nerve excite oxytocin cells in the hypothalamic paraventricular nucleus of the rat." *Brain Res.* **733**: 292-296.

Yoshimura R., Kiyama H., Kimura T., Araki T., Maeno H., Tanizawa O. and Tohyama M. (1993). "Localisation of oxytocin receptor messenger ribonucleic acid in the rat brain." *Endocrinology.* **133:** 1239-1246.

Appendix 1:

Behavioral terms and parameters definitions

Terms:

Pre-copulatory, appetitive or non-consummatory behaviours: behaviours reflecting rat's sexual motivation (sniffing, vocalizations, penile erection).

Motivation: search for sexual contact.

Arousal: motivational state during which rat seeks sexual contact.

Copulatory, consummatory behaviours: behaviours reflecting rat's sexual performance (penile erection, mounts, intromission, ejaculation).

Performance: ability to complete copulation.

Penile erection: Penile erection that occurs in the context of copulation, results from two aspects: the rat being sexually aroused and the rat having the physical abilities to have an erection. Therefore, penile erection does not just reflect sexual motivation but also sexual performance. It is important to make a distinction with the following terms:

- **Non -contact penile erection (NCE):** Spontaneous erection in isolated rats, ex copula. A male and a receptive female are kept separated in a special apparatus; the male can see and detects cues from the female but cannot have any contact with her. NCE is defined as being initiated by chemosensory, visual, auditory or imaginative stimuli.
- **Reflexive erection:** Penile erection induced ex copula by the "erection generating center" in the spinal cord, independently from any environmental sexual stimulation or brain influences.
- **Drugs-induced penile erection:** it is not possible to distinguish whether the drug induces penile erection by mimicking a arousal state or by directly activating motor pathways leading to the erection.

Parameters:

Mount latency (ML): Time (s) from the introduction of the female until the first mount. ML reflects rat's sexual motivation.

Intromission latency (IL): Time (s) from the introduction of the female until the first intromission.

Ejaculation latency (EL): Time (s) from the beginning of the first intromission to the beginning of the first ejaculation.

Inter-intromission intervals (III): Time (s) from the end of one mount with intromission until the beginning of the next.

IL, EL and III reflect rat's motivation and ability to perform.

Appendix 2

Abstract presented at conferences:

"Secretion of oxytocin and α-MSH during male sexual behaviour". *(Poster presentation)*
Celine Caquineau & A.J. Douglas.
'Centre for Neuroscience day', 2002. Edinburgh, UK

"Oxytocin and alpha-MSH interaction in male sexual behaviour" ***(Poster presentation)***
Celine Caquineau & Alison Douglas.
International Conference of Neuroendocrinology (ICN). 2002. Bristol, UK.

"αMSH induces Fos expression in oxytocin neurones but decreases systemic oxytocin secretion in the rat"
Celine Caquineau, Nancy Sabatier, Philip Bull, Alison Douglas & Gareth Leng.
Oral communication: XXII Congress of the Spanish society for physiological sciences joint meeting with The Physiological Society. 2003. Tenerife, Spain.
Poster presentation: 'Centre for Neuroscience day', 2003. Edinburgh, UK

"Alpha-Melanocyte-stimulating hormone (αMSH) induces Fos expression in selected oxytocin neurones in the paraventricular nucleus" ***(Poster presentation)***
Celine Caquineau, Nancy Sabatier, Alison J. Douglas & Gareth Leng.
World Congress of Neurohypophyseal Hormones (WCNH). 2003. Kyoto, Japan.

"Oxytocin and alpha-Melanocyte Stimulating Hormone (αMSH) interaction during male sexual behaviour" ***(Poster presentation)***
Celine Caquineau, Nancy Sabatier, Philip Bull, Gareth Leng & Alison J. Douglas.
Society for Neuroscience. 2003. New Orleans, USA.

"Magnocellular oxytocin neurones mediate the effects of alpha-Melanocyte Stimulating Hormone (αMSH) during male sexual behaviour." ***(Poster presentation)***
Celine Caquineau, Nancy Sabatier, G.Leng & A.J. Douglas
British Society for Neuroendocrinology (BSN). 2004. Glasgow, UK.

Publications:

Sabatier N, Caquineau C, Douglas A.J. and Leng G. (2003). "Oxytocin released from magnocellular dendrites. A potential modulator of α-melanocyte –stimulating hormone behavioral actions?" *Ann. NY. Acad. Sci.* **994**: 218-224.

Sabatier N, Caquineau C, Dayanithi G, Bull P, Douglas A.J., Guan X-M. M., Jiang M., Van der Ploeg L. and Leng G. (2003). "α-melanocyte-stimulating hormones stimulates oxytocin release from the dendrites of hypothalamic neurons while inhibiting oxytocin release from their terminals in the neurohypophysis". *J. Neuroscience.* **23 (32):** 10351-10358.