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Oxytocin neurone activity and release following administration of Melanotan-II in anaesthetised rats

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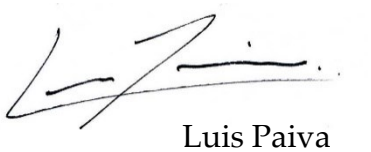
Declaration

I declare that this thesis has been composed by myself, and that the experiments described here were performed by myself at the Centre for Integrative Physiology, University of Edinburgh, and that any technical contribution made by others have been clearly indicated. I also declare that this work has not been submitted for any other degree or professional qualification.



Luis Paiva
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Abstract

Oxytocin release within the brain modulates several social behaviours in animals and humans. Moreover, low central oxytocin content has been linked to neuropsychiatric disorders, such as anxiety and autism. The exogenous administration of oxytocin has been proposed for therapeutic treatment, but oxytocin does not cross the blood-brain barrier (BBB) in physiologically significant amounts. An alternative approach to oxytocin administration is to stimulate central oxytocin release using melanocortins.

Central administration of the naturally occurring melanocortin, α-MSH, has been shown to trigger somatodendritic oxytocin release *in vitro*. Unfortunately, endogenous melanocortins also do not penetrate the BBB in neuroactive amounts. In this study, I investigated whether systemic administration of synthetic melanocortin receptor 3/4 ($MC_{3/4}$) agonist, Melanotan-II (MT-II), affects oxytocin neuronal activity and secretion in anaesthetised rats. I hypothesised that systemic administration of MT-II directly (centrally) acts on magnocellular oxytocin neurones to trigger somatodendritic oxytocin release from neurones of the supraoptic nucleus (SON) of the hypothalamus *in vivo*.

Firstly, using double immunohistochemistry against Fos protein, a widely used marker for neural activity, and oxytocin, I showed that intravenous (i.v.; 1 mg/kg), but not intranasal (1 and 30 μg rat), administration of MT-II markedly induced Fos expression in magnocellular oxytocin neurones of the SON and paraventricular nuclei (PVN) of the hypothalamus, and this response was prevented by prior intracerebroventricular (i.c.v.) administration of the melanocortin antagonist, SHU-9119 (1 μg rat). In addition, brain areas receiving peripheral inputs which are involved in the regulation of oxytocin and vasopressin release

were also analysed, showing that i.v. MT-II significantly increased Fos expression in the nucleus tractus solitarii (NTS), but not in circumventricular organs of the anteroventral third ventricle (AV3V) region. MT-II-induced Fos in the NTS was not prevented by the i.c.v. melanocortin antagonist.

Then, using *in vivo* electrophysiology, I investigated whether i.v. administration of MT-II affects the electrical activity of SON neurones. Extracellular single-unit recordings from identified magnocellular neurones of the SON showed that MT-II significantly increased the firing rate in oxytocin neurones, however, no significant changes in firing rate were detected in vasopressin neurones.

Finally, *in vivo* oxytocin release experiments showed that i.v. administration of MT-II did not trigger somatodendritic oxytocin release within the SON as measured by microdialysis and subsequent radioimmunoassay. Interestingly, the i.c.v. administration of MT-II (1 μg rat) also failed to trigger oxytocin release within the SON. The analysis of oxytocin content in plasma revealed that the change in oxytocin concentration was significantly greater in i.v. MT-II injected rats compared to vehicle-injected rats.

Taken together, these results show that after i.v., but not intranasal, administration of MT-II, the activity of magnocellular neurones of the SON is increased. As previous studies showed that SON oxytocin neurones are inhibited in response to direct application of melanocortin agonists, the actions of i.v. MT-II are likely to be mediated, at least in part, indirectly by activation of inputs from the caudal brainstem.

Lay summary

Oxytocin is a hormone produced in the brain and transported to the posterior pituitary gland from where is release into the blood to act on peripheral organs. However, oxytocin is also released within the brain, where it modulates several social behaviours in animals and humans. Interestingly, low levels of oxytocin within the brain have been linked to neuropsychiatric disorders, including anxiety and autism. Thus administration of oxytocin has been proposed for therapeutic treatment, but oxytocin cannot penetrate through layers protecting the brain (blood-brain barrier). An alternative approach is to stimulate the release of oxytocin within the brain using a group of hormones (melanocortins), which have been shown to activate the oxytocin system. However, the naturally occurring melanocortin α-MSH, which has been shown to activate oxytocin neurones, also does not penetrate the blood-brain barrier.

Here, I study whether injection of the synthetic melanocortin Melanotan-II (MT-II) acts on the brain affecting the activity of oxytocin-producing neurones resulting in oxytocin release within the brain in rats. Using a gene product activated by changes in neuronal activity, I showed that intravenous MT-II, affected the activity of oxytocin neurones, and this effect was prevented by the administration of an MT-II blocker (antagonist). Then, I recorded the firing pattern of oxytocin neurones, showing that MT-II enhanced their spontaneous electrical activity. To determine whether MT-II induced release of oxytocin within the brain, the content of oxytocin was measured in a brain region where a group of these neurones are clustered, the supraoptic nucleus of the hypothalamus.

All our findings indicate that after intravenous administration of MT-II the activity of oxytocin neurones is increased, but this does not result in

oxytocin released within the brain. Hence, MT-II is not an appropriate drug for the treatment of neuropsychiatric disorders associated with low levels of oxytocin within the brain.

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List of abbreviations

$[Ca^{2+}]_I$	Intracellular calcium concentration
α-MSH	Alpha-melanocyte stimulating hormone
aCSF	Artificial cerebrospinal fluid
ACTH	Adrenocorticotropic hormone
AgRP	Agouti-related protein
AOB	Accessory olfactory bulb
AP	Area postrema
AV3V	Anteroventral third ventricle region of the hypothalamus
BBB	Blood-brain barrier
cAMG	Central amygdala
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CNS	Central nervous system
CRH	Corticotrophin releasing hormone
CSF	Cerebrospinal fluid
EPL	External plexiform layer
EPSCs	Excitatory post-synaptic currents
ERα	Oestrogen receptor type alpha
Fos	Finkel-Biskis-Jenkins osteosarcoma
GPCR	G-protein coupled receptor
GPF	Green fluorescent protein
G_s	G stimulatory protein
i.c.v.	Intracerebroventricular(ly)
i.p.	Intraperitoneal(ly)

i.v.	Intravenous(ly)
IPSCs	Post-synaptic inhibitory currents
LDCVs	Large dense-cored vesicles
MC_3	Melanocortin receptor isotype 3
MC_4	Melanocortin receptor isotype 4
MCL	Mitral cell layer
mRNA	Messenger ribonucleic acid
MT-II	Melanotan-II
NaCl	Sodium chloride
NTS	Nucleus tractus solitarii
OTR	Oxytocin receptors
OVLT	Organum vasculosum lamina terminalis
PB	Phosphate buffer
PFA	Paraformaldehyde
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus of the hypothalamus
RIA	Radioimmunoassay
ROI	Region of interest
S.E.M.	Standard error of the mean
SFO	Subfornical organ
SON	Supraoptic nucleus of the hypothalamus
TRPV1	Transient receptor potential vanilloid-1 channel

Chapter 1

General introduction

1.1. BACKGROUND

Oxytocin, firstly sequenced by Du Vigneaud *et al.* (1953b), was originally described as a hormone by its uterotonic actions following intravenous (i.v.) administration of pituitary extracts (Dale, 1906). Nowadays, it is well established that oxytocin also acts within the brain: central (i.e. synaptic and somatodendritic) oxytocin release modulates pro-social behaviours (Fahrbach *et al.*, 1984; Insel *et al.*, 1998) which are elicited by activation of oxytocin receptors (OTR) expressed in structures involved in cognitive and emotional processing (Ferguson *et al.*, 2001; Keebaugh & Young, 2011).

Several reports have linked neuropsychiatric disorders to alterations of oxytocin systems, these alterations include mutation in peptide forms and receptors, and changes in peripheral and central oxytocin content. Interestingly, low content of oxytocin in the cerebrospinal fluid (CSF) has been associated with autism, anxiety, depression, and schizophrenia (Linkowski *et al.*, 1984; Modahl *et al.*, 1992; Insel *et al.*, 1999; Heim *et al.*, 2009).

The exogenous administration of oxytocin has been proposed for the treatment of neuropsychiatric disorders, but systemically administered oxytocin is not able to penetrate the brain in physiologically significant amounts (Zaidi & Heller, 1974; Mens *et al.*, 1983; Ermisch *et al.*, 1985). Therefore, alternative approaches are required for the treatment of mental disorders associated with low central oxytocin levels. The endogenous melanocortin alpha-melanocyte stimulating hormone (α-MSH) stimulates somatodendritic oxytocin release *in vitro* while inhibiting peripheral release from nerve endings by decreasing electrical activity *in vivo* (Sabatier *et al.*, 2003a). Unfortunately, like oxytocin, α-MSH does not penetrate the brain in neuroactive amounts (Wilson *et al.*, 1984; Wilson, 1988).

Melanotan-II (MT-II) is a synthetic α-MSH analogue acting on melanocortin receptors isotype 3 (MC_3) and 4 (MC_4). These receptors are expressed in the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus (Kishi *et al.*, 2003) which contain large populations of oxytocin (and vasopressin) neurones. It is thought that, at least some, MT-II might penetrate the blood-brain barrier (BBB) following peripheral administration, as suggested by behavioural actions (i.e. stretch and yawning) observed in animals and humans (Dorr *et al.*, 1996). Recently, Modi *et al.* (2015) reported that systemic injection of MT-II stimulates pair-bonding in prairie voles by an

oxytocin-dependent mechanism, supporting the notion that peripheral administration of MT-II might modulate central oxytocin systems.

Whether MT-II acts centrally (directly) on oxytocin neurones or through peripheral pathways following i.v. injections of MT-II is unknown. Furthermore, there is no evidence about the effects of i.v. administered MT-II on electrical activity and release of SON oxytocin neurones *in vivo*. In this thesis, I investigate these questions which are key to validate new therapeutic strategies for the treatment of neuropsychiatric disorders related to low central oxytocin levels.

1.2. LITERATURE REVIEW

1.2.1. Oxytocin system

1.2.1.1. Oxytocin and vasopressin synthesis

Oxytocin is a nonapeptide synthesised as part of a large precursor containing the carrier peptide neurophysin-I. The structure and sequence of oxytocin is closely related to those of the peptide vasopressin, differing at the amino-acid residues 3 and 8 (Du Vigneaud *et al.*, 1953a; Du Vigneaud *et al.*, 1953b); vasopressin is associated to neurophysin-II and the glycopeptide copeptin. Oxytocin and vasopressin are synthesised and packaged in vesicles in the soma of separate neurones. Then, these peptide-containing large dense-cored vesicles (LDCVs) are transported to different neuronal compartments to be released. The primary sites of synthesis of oxytocin are the SON, and PVN of the hypothalamus (Brownstein *et al.*, 1980).

The SON contains neurones exclusively with a large cell body, called *magnocellular neurones*. The soma of SON magnocellular neurones are densely clustered on the ventral surface of the brain, along the optic chiasm (Swanson

& Sawchenko, 1983). These neurones have between one and three dendrites (Pow & Morris, 1989) laying parallel to the ventral surface, and intermingled within a layer of glial cells, the *glia ventral limitans* (Yulis *et al.*, 1984). The neurones of the SON mainly express either oxytocin or vasopressin, and are organised in a distinctive pattern, in which oxytocin neurones are distributed in the anterodorsal SON (Sawchenko & Swanson, 1983; Swanson & Sawchenko, 1983). Additionally, oxytocin neurones can be found lying on the ventral surface of the brain, called the *retrochiasmatic* part of the SON. Virtually all SON neurones project their axons to the posterior pituitary (neurohypophysis) (Swanson & Sawchenko, 1983).

The PVN contains magnocellular neurones, and also neurones with a smaller cell body (parvocellular neurones). Oxytocin (and also vasopressin) is synthesised in magnocellular and parvocellular neurones of the PVN; within these two subpopulations, several anatomical subdivisions have been described (Swanson & Kuypers, 1980). As in the SON, magnocellular neurones of PVN project to the posterior pituitary, whereas parvocellular populations project to distant targets within the central nervous system (CNS), including the brainstem and spinal cord (Swanson & Kuypers, 1980; Sawchenko & Swanson, 1983), to release their peptides synaptically. The PVN also contains neurones expressing other peptides, including corticotrophin releasing hormone (CRH), thyrotropin releasing hormone (TRH), somatostatin, and enkephalin (Lechan & Jackson, 1982; Sawchenko & Swanson, 1982a; Sawchenko *et al.*, 1984).

In addition to the PVN and SON, oxytocin neurones can be found in small clusters of neurones or accessory nuclei, these include the nucleus circularis located between the PVN and SON, as well as small groups

neurones located in the bed nucleus stria terminalis, and the lateral hypothalamic area (Sofroniew & Glasmann, 1981).

Magnocellular neurones of the SON and the PVN project their axons to the posterior pituitary along the hypothalamic-neurohypophyseal tract which passes through the internal zone of the median eminence towards the posterior pituitary, where the nerve endings release oxytocin and vasopressin into the bloodstream (Sawchenko & Swanson, 1983; Swanson & Sawchenko, 1983). Parvocellular neurones project their axons to several targets within the brain, including the external zone of median eminence, amygdala, hippocampus, olfactory bulb, and brainstem (Sawchenko & Swanson, 1982a; Yulis & Rodriguez, 1982; Sawchenko & Swanson, 1983; Castel & Morris, 1988).

1.2.1.2. Oxytocin receptor

The human OTR complementary deoxyribonucleic acid (cDNA) was first sequenced by Kimura *et al.* (1992), encoding a 388- amino acid protein receptor with seven transmembrane domains. The receptor is highly conserved among the mammals, showing a 93 % of the sequence homology with the rat OTR (Rozen *et al.*, 1995).

The OTR is a member of the G-protein coupled receptor (GPCR) family, and its seven transmembrane domains are highly conserved among the GPCR family. For the OTR, it has been proposed that changes in the orientation of transmembrane domains 3 and 6 command the switch between the active to inactive conformation, consequently exposing G-protein binding sites (Gimpl & Fahrenholz, 2001). In uterine myometrial cells, the OTR is

associated with a $G_{q/11}$ protein that mediates its actions through the phospholipase C pathway, leading to an increase in intracellular calcium concentration ($[Ca^{2+}]_I$) (Ku *et al.*, 1995; Sanborn *et al.*, 1998).

So far, just a single OTR isoform has been reported. However, the OTR shows 40 % to 50 % of sequence homology compared to the vasopressin V_1 and V_2 receptors. As a consequence, there are low levels of cross reaction between oxytocin and vasopressin. It is believed that between OTR and vasopressin receptors there are common and specific contact sites for the ligand binding interaction (Gimpl & Fahrenholz, 2001). However, the OTR has a 10-fold greater affinity for oxytocin than vasopressin (Baribeau & Anagnostou, 2015).

Oxytocin receptor distribution

In the brain, the OTR is expressed in several regions, including the amygdala, brainstem, hippocampus, hypothalamus, and the olfactory system (Table 1.1.). The expression and distribution of OTR vary according to the species, sex, and age (Insel & Shapiro, 1992; Vaccari *et al.*, 1998; Young, 1999; Bale *et al.*, 2001). Peripherally, the receptor is expressed in several tissues, including the uterus, mammary gland, ovary, kidney, and heart (Gimpl & Fahrenholz, 2001).

Several factors regulate the expression of the OTR, but oestrogen is probably one of the most relevant regulators. In the brain and uterus, activation of the oestrogen receptor type alpha (ERα) upregulates cell expression of OTR (Alves *et al.*, 1998; Young *et al.*, 1998). Although basal synthesis of the OTR has been detected in ERα knock-out mice, ERα is

indispensable for the induction of OTR binding in the brain by oestrogen (Young *et al.*, 1998).

1.2.1.3. Oxytocin release

Oxytocin- and vasopressin-containing vesicles can be released from all compartments of the magnocellular neurones (i.e. nerve endings, non-dilated segments of the axon, axon swellings, soma, and dendrites) (Pow & Morris, 1989; Morris & Pow, 1991). The release from these compartments is differentially regulated in magnocellular neurones.

Peripheral release

The posterior pituitary stores a large amount of oxytocin and vasopressin, which are able to maintain, in steady-state conditions, basal plasmatic concentrations for 3-4 weeks. It is estimated that approximately 5 % of the neuropeptide content is released to the bloodstream in 24 h (Jones & Pickering, 1972). However, high demand conditions can dramatically increase oxytocin (and vasopressin) release, for instance, 3 days of water deprivation can deplete the posterior pituitary stores to only 10 % of its normal level. In steady-state conditions, newly-made peptides arrive at the posterior pituitary at a rate that closely matches the release and destruction of old peptide-containing vesicles, keeping the peptide content roughly constant. Nevertheless, under high-demand conditions the increase in release is not coupled to the newly-synthesised peptide due to a lag in the peptide synthesis, leading to the depletion of the posterior pituitary (Leng *et al.*, 2012).

The release of peptide-containing vesicles from the nerve endings is determined by action potentials arriving from the soma of magnocellular neurones, which invade the nerve endings, opening voltage-gated Ca^{2+} channels that allows Ca^{2+} entry, and consequently, leading to the fusion of vesicles with the cell membrane (Nordmann, 1983). The amount of oxytocin, and also vasopressin, released with each action potential (spike activity) increases with the frequency and pattern of activity, and in oxytocin neurones, increasing frequencies of action potentials sustain the release of larger amounts of peptide into the bloodstream (Bicknell, 1988). In contrast to low spike frequencies (which do not invade all the nerve endings, and induce a small increase in $[Ca^{2+}]_I$ because the voltage-gated Ca^{2+} channels are opened for a very brief period), high spike frequencies result in a greater number of nerve endings depolarized, and also induce a larger Ca^{2+} entry which is essential to trigger and facilitate the exocytosis of vesicles (Bourque, 1991).

Central release

In magnocellular neurones, oxytocin and vasopressin release from the dendritic compartment can occur independently or semi-independently of peripheral release. The release from dendrites in the hypothalamus and nerve endings located in the posterior pituitary can be triggered on different time points in response to the same stimulus or can be regulated completely independently. For instance, in lactating rats, suckling stimulus evokes oxytocin release within the SON that precedes peripheral oxytocin release (Moos *et al.*, 1989). On the other hand, systemic osmotic stimulation evokes peripheral oxytocin release, but the release within the SON is delayed approximately by 1 h (Ludwig *et al.*, 1994).

Oxytocin, and vasopressin released from dendrites have autocrine and paracrine effects which are mediated by activation of autoreceptors (Freund-Mercier *et al.*, 1994; Hurbin *et al.*, 2002). Oxytocin itself (and also other peptides, such as α-MSH) can elicit dendritic oxytocin release without inducing release from nerve endings, and conversely, electrical activity in the soma can trigger release from nerve endings without evoking dendritic release (Ludwig *et al.*, 2002). Dendritic peptide release has a role in autoregulation of the electrical activity of the same neurone from which the peptide is released, as shown by direct application of vasopressin which results in inhibition of phasic vasopressin neurones (Leng & Mason, 1982; Abe *et al.*, 1983; Ludwig & Leng, 1997).

As described above, dendritic release can occur independently of electrical activity. In oxytocin neurones, oxytocin itself stimulates dendritic release by mobilising intracellular Ca^{2+} from thapsigargin-sensitive stores, and consequently, rising $[Ca^{2+}]_i$ (Lambert *et al.*, 1994) which result in oxytocin release without electrical activity (Ludwig *et al.*, 2002). In vasopressin neurones, the rise in $[Ca^{2+}]_I$ is mediated by combined extracellular Ca^{2+} entry and mobilisation of intracellular Ca^{2+} from thapsigargin-sensitive stores (Ludwig *et al.*, 2005).

In addition, mobilisation of intracellular Ca^{2+} stores make dendritic (but not nerve endings) oxytocin stores available (priming) for activity-dependant release (Ludwig *et al.*, 2002). Priming increases the pool of readily-releasable vesicles by relocating vesicles in close proximity to the dendritic plasma membrane (Tobin *et al.*, 2004), which results, for example, in a larger amount (potentiation) of oxytocin dendritically released in response to hypertonic stimulation in thapsigargin-pretreated rats *in vivo* (Ludwig *et*

al., 2002). Nevertheless, priming effects are not immediate because it requires relocation of vesicles, and its effects can be detected 30 min after priming stimulation, however, these are long-lasting (+ 90 min), as observed *in vitro* (Ludwig *et al.*, 2002). Hence, priming modifies the level of dendro-dendritic communication, increasing the coordination of magnocellular neurones, and increasing dramatically the amount of peptide released.

Finally, oxytocin and vasopressin are also released synaptically from parvocellular neurones of the PVN which project to central targets (Sawchenko & Swanson, 1982a). However, the parvocellular system contain much less peptide than the magnocellular system, and this content does not account for CSF concentrations, but because virtually all magnocellular neurones project to the posterior pituitary it has been assumed that central actions are driven by release from parvocellular neurones (Ludwig & Leng, 2006).

1.2.1.4. Physiological roles of oxytocin

1.2.1.4.1. Central roles of oxytocin

Oxytocin has several behavioural effects when it is centrally released. Since peripherally released oxytocin cannot re-enter to the brain in significant amounts due to the BBB (Zaidi & Heller, 1974; Mens *et al.*, 1983; Ermisch *et al.*, 1985), these behavioural actions are evoked by oxytocin released from dendrites of magnocellular neurones and oxytocin synaptically released from parvocellular neurones (Neumann, 2007; Stoop, 2012). Interestingly, several oxytocin pathways projecting from the PVN to different brain areas have been reported, including to the amygdala, lateral septum and hippocampus (Buijs, 1978; Buijs & Swaab, 1979).

Maternal behaviour

The role of oxytocin as a modulator of maternal behaviour was firstly reported by Pedersen and Prange (1979), showing that intracerebroventricular (i.c.v.) administration of small doses of oxytocin elicited full maternal behaviour in virgin rats. Subsequent studies using pharmacological blockage of OTR, and optogenetic stimulation of oxytocin neurones of the PVN have confirmed that central oxytocin release is crucial to evoke maternal behaviour (van Leengoed *et al.*, 1987; Marlin *et al.*, 2015). At the end of pregnancy, high levels of prostaglandins increase the expression of OTR which combined with large amounts of central (and peripheral) oxytocin released during labour (Pedersen *et al.*, 1982) are the key factors to initiate recognition and nurturing of the offspring, two key components of maternal care.

The maternal behaviour is mainly regulated by OTR expressed on the medial preoptic area and bed nucleus of the stria terminalis, as it has been shown by lesioning of these areas (Numan & Young, 2016). Oxytocin released from PVN projections during labour, and then during lactation are thought to be responsible for activation of neurones located in the medial preoptic area (Numan *et al.*, 2006). In addition, Marlin *et al.* (2015) have shown that optogenetically-evoked PVN oxytocin release acting on OTR of the left auditory cortex stimulates pup retrieval in virgin mice.

Pair Bonding

The evidence of the involvement of oxytocin in partner preference has been obtained from pharmacological, behavioural, and neuroanatomical

studies of non-monogamous and monogamous species of voles (genus *Microtus*). The monogamous prairie voles establish pair bond following mating.

The i.c.v. administration of oxytocin, but not vehicle, strongly induces partner preference in virgin female prairie voles cohabiting with a male for 6 h without mating (Williams *et al.*, 1994). Moreover, partner preference induced by mating can be prevented by the administration of an OTR antagonist into the nucleus accumbens or into the prefrontal cortex (Young *et al.*, 2001).

Neuroanatomical studies of the distribution of the OTR in monogamous and non-monogamous species of voles (i.e. prairie, montane and meadow voles) have shown differences in the expression of OTR in brain structures involved in cognitive processing. In the monogamous prairie vole, high densities of OTR are expressed in the prefrontal cortex, lateral amygdala, and nucleus accumbens, whereas in the non-monogamous montane vole the expression of OTR is almost absent in the nucleus accumbens (Insel & Shapiro, 1992). This indicates that activation of OTR expressed in neurones of the nucleus accumbens is responsible for monogamous behaviour in voles.

Genital stimulation during mating has been proposed as the mechanism responsible for the release of oxytocin into the nucleus accumbens (Ross *et al.*, 2009). In addition to oxytocin, other peptides and neurotransmitters are also involved in the establishment of partner preference in both sexes, including vasopressin and the neurotransmitter dopamine (Young & Wang, 2004).

Sexual behaviour

Central and systemic oxytocin facilitate sexual behaviour in male and female rats, and also others species. Oxytocin exerts its actions on sexual behaviour by different pathways that comprises direct innervations of genitals and endocrine actions following peripheral and central release (Carmichael *et al.*, 1987; Hughes *et al.*, 1987). Oxytocin participates in several phases of the sexual behaviour, including sex arousal and sexual satiety (Mahalati *et al.*, 1991; Blaicher *et al.*, 1999).

In male rats, sexual behaviour stimulates oxytocin neurone activity and release as part of the facilitation of intromission. Tracing studies have shown projections from the penis and sexual accessory glands to parvocellular and magnocellular neurones of the PVN (Marson & McKenna, 1996; Normandin & Murphy, 2011). During mating, increased Fos expression in both parvocellular and magnocellular neurones of the PVN, and the SON have been reported (Witt & Insel, 1994; Caquineau *et al.*, 2006), as well as increased oxytocin release within the PVN as measured by *in vivo* microdialysis (Waldherr & Neumann, 2007). Furthermore, electrical stimulation of penile nerves has been shown to excite approximately 50 % of the SON and PVN oxytocin neurones (Yanagimoto *et al.*, 1996; Honda *et al.*, 1999).

Peripheral oxytocin also participates in sexual behaviour, as shown by reduced numbers of erections in hypophysectomized rats. However, systemic oxytocin release from the posterior pituitary is not essential to induce this behaviour (Argiolas *et al.*, 1989). I.c.v. administration, and direct injection of oxytocin into the PVN have been shown to induce penile erections in a dose-dependent manner (Argiolas, 1992). Moreover, i.c.v.

administration of oxytocin increases intromission latencies and ejaculatory behaviour (Arletti *et al.*, 1985; Stoneham *et al.*, 1985).

In females, central oxytocin also participates in sexual behaviours. I.c.v. administration of oxytocin stimulates lordosis postures in female rodents, as part of the pro-receptive behaviour. The lordosis posture is triggered by activation of OTR located in the ventromedial hypothalamic nucleus (Bale *et al.*, 2001), however, oestrogen and progesterone are also required to elicit this behaviour (Veening *et al.*, 2015).

1.2.1.4.2. Peripheral roles of oxytocin

Parturition

Before the onset of labour, the ratio of progesterone and oestrogen in plasma, and their uterine receptors increase, leading to an upregulation of OTR expression in myometrial cells, and consequently increasing the sensitivity of myoepithelial cells to oxytocin (reviewed by Kota *et al.*, 2013). Then, myoepithelial contractions are initiated by locally produced prostaglandins which weakly stimulate the contraction of the uterus. These initial uterine contractions stimulate sensory afferents pathways projecting via the vagus nerve to noradrenergic neurones of the nucleus tractus solitarii (NTS) which, in turn, send excitatory projections to magnocellular oxytocin neurones, activating them (Douglas *et al.*, 2001). The oxytocin secreted enhances the uterotonic actions of prostaglandins by a direct action on the myometrium, and also by increasing the synthesis of prostaglandins by the endometrium. As delivery progresses, a positive feedback is established

(Ferguson reflex) between neuronal afferents and more frequent and strong contractions (Ferguson (1941) reviewed by Russell *et al.* (2003)).

At the level of the hypothalamic nuclei, magnocellular oxytocin neurones fire in intermittent synchronised bursts that release large pulses of oxytocin, stimulating the foetus expulsion (Summerlee, 1981). This pulsatile release pattern allows myometrial cells to avoid receptor desensitisation caused by the sustained increase of oxytocin in plasma (Engstrom *et al.*, 1988; Willets *et al.*, 2009). In addition, peripheral oxytocin release is accompanied by an increase in the dendritic release of oxytocin which has a role in synchronising the intermittent bursts of oxytocin neurones. This dendritic release plays a crucial role during delivery, as shown by intra-SON administration of an OTR antagonist which attenuated the rise of oxytocin release within the SON associated with parturition, resulting in a reduced number of pups delivered during the antagonist application (Neumann *et al.*, 1996).

Interestingly, transgenic mice null for oxytocin or OTR can give birth normally, indicating redundant and compensatory mechanism involved in parturition (Nishimori *et al.*, 1996; Young *et al.*, 1996).

Lactation

Despite the fact that oxytocin is not involved in milk synthesis, it plays a crucial role allowing the milk let-down in response to suckling. Oxytocin is the only hormone able to trigger the milk ejection reflex, as shown in mice null for oxytocin (Young *et al.*, 1996).

At the end of pregnancy, OTR expression increases in alveolar myoepithelial cells enhancing their sensitivity to oxytocin which causes the contraction of these cells, and consequently the ejection of milk due to the increased pressure within the mammary alveoli. In lactating rats, when pups suckle, milk is ejected every 10 – 20 min (Wakerley & Lincoln, 1971), these milk ejections are preceded (~ 10 s) by a very short, and synchronised high-frequency burst of activity of magnocellular SON and PVN neurones that lasts 1 – 3 s, this leads to a large pulse release of oxytocin which is followed by a silent period of activity (Wakerley & Lincoln, 1973; Lincoln & Wakerley, 1974).

These bursts of activity are synchronised among all the magnocellular oxytocin neurones located in the SON and PVN. It is thought that communication between magnocellular nuclei have an important role in the burst synchronisation (Belin & Moos, 1986), in addition to priming of dendritic oxytocin release (Lambert *et al.*, 1993; Rossoni *et al.*, 2008). Oxytocin neurones release more oxytocin when spikes occur in bursts than at lower frequency (Leng *et al.*, 2012).

Similarly to uterine contraction during labour, the mammary gland responds to high concentrations of oxytocin, but its continuous exposure to oxytocin can lead to receptor desensitisation (Smith *et al.*, 2006; Willets *et al.*, 2009), therefore, pulsatile release allows the cells maintain their responsiveness to oxytocin.

1.2.1.5. Oxytocin and neuropsychiatric disorders

Based on neuroanatomical studies showing projections of oxytocin neurones to limbic areas and the prosocial effects observed following central administration of oxytocin in animals, several studies have investigated the roles of oxytocin and vasopressin in anxiety, stress-coping, and sociality. It is commonly accepted that central oxytocin exerts anxiolytic and antidepressive effects in rodents (Neumann, 2008; Neumann & Landgraf, 2012). Moreover, in humans, reports have linked neuropsychiatric disorders to alteration of oxytocin system, including changes in CSF and plasma oxytocin content. In recent years, an increasing number of publications have reported changes in plasma oxytocin content in patients with neuropsychiatric disorders, however, as in animals, peripheral oxytocin release does not reflect its central release (Kagerbauer *et al.*, 2013). The measurement of plasma oxytocin as a biomarker has been used, probably, based on the lesser invasiveness of blood sampling compared to CSF sampling, however, dissimilar results, and more importantly, inconsistent values have been reported (Leng & Sabatier, 2016).

In CSF, lower oxytocin content has been found in suicide attempters compared to healthy volunteers, as well as a significant inverse correlation between CSF oxytocin content and suicide intent (Jokinen *et al.*, 2012). Women exposed to childhood abuse had lower CSF oxytocin concentrations compared to non-exposed women, and an inverse correlation between oxytocin content and anxiety was also found (Heim *et al.*, 2009). Similarly, Lee *et al.* (2009) reported an inverse correlation between CSF oxytocin content and a life story of aggression in women. In schizophrenic subjects, lower levels of neurophysin-I, the oxytocin associated carrier protein, has been

detected when compared to control subjects (Linkowski *et al.*, 1984). In contrast, other studies have not found significant changes in CSF oxytocin content in schizophrenic subjects (Glovinsky *et al.*, 1994; Sasayama *et al.*, 2012), but the severity of symptoms, measured by the positive and negative syndrome scale (PANSS) for schizophrenia, were inversely correlated with the oxytocin concentrations in the CSF (Sasayama *et al.*, 2012). Furthermore, women suffering depression showed no differences in CSF oxytocin content compared to controls (Pitts *et al.*, 1995), but plasma oxytocin content was higher in depressed women, probably, due to alteration of oxytocin release patterns (Cyranowski *et al.*, 2008).

Finally, alterations of oxytocin content in plasma have been reported in depression (Scantamburlo *et al.*, 2007; Parker *et al.*, 2010), schizophrenia (Legros *et al.*, 1992; Goldman *et al.*, 2008; Strauss *et al.*, 2015), borderline personality disorder (Jobst *et al.*, 2016), anxiety (Scantamburlo *et al.*, 2007; Cyranowski *et al.*, 2008), and autism spectrum disorder (ASD) (Jansen *et al.*, 2006). Interestingly, in autistic children, low plasma oxytocin content (Modahl *et al.*, 1998) was related to alterations of the enzymatic cleavage of the oxytocin precursor, leading to an increase of the oxytocin precursor, and consequently to the ratio oxytocin precursor/oxytocin (Green *et al.*, 2001).

Since the exogenous administration of oxytocin is not able to cross the BBB in neuroactive amounts (Zaidi & Heller, 1974; Mens *et al.*, 1983; Ermisch *et al.*, 1985; Ludwig *et al.*, 2013), an alternative approach for the treatment of neuropsychiatric disorders is to stimulate the endogenous oxytocin systems using melanocortins which have been shown to induce central oxytocin release (Sabatier *et al.*, 2003a).

1.2.1.5.1 Melanocortins as a therapeutic tool targeting central oxytocin systems

The melanocortin α-MSH is a potent stimulus to induce dendritic oxytocin release. In SON explants, application of α-MSH mobilised intracellular Ca^{2+} stores, triggering dendritic oxytocin release, and when injected i.c.v., it stimulates the expression of Fos protein (the product of the immediate early gene *c-fos*) in magnocellular oxytocin neurones of the SON while inhibiting peripheral oxytocin release by decreasing the electrical activity of these neurones (Sabatier *et al.*, 2003a). The actions of α-MSH are mediated through MC_4 which are expressed in the SON and PVN (Mountjoy *et al.*, 1994; Kishi *et al.*, 2003). However, like oxytocin, α-MSH is not able to penetrate the BBB in neuroactive amounts (Wilson *et al.*, 1984; Wilson, 1988), and therefore itself is not useful for systemic administration to stimulate the central oxytocin systems. However, in the last years, several synthetic melanocortin agonists have been synthesised, some of these molecules (e.g. MT-II) are thought to have better brain penetration properties due to their shorter amino acid sequence and cyclic structure.

1.2.2. The central melanocortin system

1.2.2.1. Melanocortin structure and synthesis

The melanocortins are a group of related peptides derived from the proopiomelanocortin (POMC) precursor, which is post-translationally processed by successive enzymatic cleavages into the adrenocorticotropic hormone (ACTH), and the melanotropins alpha-, beta-, and gamma-melanocyte-stimulating-hormone (α-, β-, γ-MSH). α-MSH is a 13-amino acid peptide cleaved from the N-terminal region of the 39-amino acid ACTH.

Additionally, the opioid peptide β-endorphin, and the lipid-mobilizing peptides beta-, and gamma-lipotropin (β-, γ-LPH) are also derived from the cleavage processing (Cawley *et al.*, 2016). POMC is differentially cleaved by tissue and cell-specific processing enzymes to yield different end products (Figure 1.1.).

In the brain, α-MSH and β-endorphin are the main products synthesised by the hypothalamic neurones. α-MSH is synthesised in two neuronal populations located in the arcuate nuclei and dorsolateral to the third ventricle of the hypothalamus (Watson & Akil, 1979; Guy *et al.*, 1980). A third population of neurones expressing POMC-related peptides (i.e. ACTH, α-MSH, and β-endorphin) have also been identified in the NTS (Joseph *et al.*, 1983; Palkovits *et al.*, 1987). α-MSH fibres projecting from hypothalamic populations have been detected in several brain structures, including the amygdala, cortex, hippocampus, hypothalamus, and preoptic and septal areas (Jacobowitz & O'Donohue, 1978; Guy *et al.*, 1981). Within the hypothalamus, a moderate number of α-MSH, and its precursor ACTH nerve fibres have been found in the PVN and SON (Sawchenko *et al.*, 1982).

Peripherally, melanotropins are synthesised by cells of the intermediate pituitary gland from which they are released into the bloodstream. Similarly, to the hypothalamus, α-MSH and β-endorphin are the end peptide products of the intermediate pituitary. The release of melanotropins from the intermediate pituitary is regulated by several hypothalamic factors including serotonin and dopamine which stimulate and inhibit, respectively, the release of secretory granules containing MSH peptides into the bloodstream (Cawley *et al.*, 2016).

1.2.2.2. Melanocortin receptors

Five melanocortin receptor genes have been cloned, encoding the melanocortin receptors: MC_1, MC_2, MC_3, MC_4, and MC_5 (Dores *et al.*, 2016). Separate genes encode each receptor isotype; the sequence homologies among human melanocortin receptors range from 60 % to 38 % identity. The melanocortin receptors belong to the seven-transmembrane domain GPCR family. Compared to other G-protein receptors the melanocortin receptors are the smallest G-protein receptors, with short amino- and carboxyl-terminal ends, and a short extracellular loop. The melanocortin receptors are coupled to a G_s protein that activates the cyclic adenosine monophosphate (cAMP) signalling pathway (Abdel-Malek, 2001; Yang, 2011).

The five receptor subtypes are differentially expressed in the several tissues, including peripheral organs and the CNS; they display different affinities for their endogenous agonist, and antagonist melanocortin ligands (i.e. the agouti-related protein (AgRP), and agouti signalling protein (ASIP)) (Dores *et al.*, 2014; Dores *et al.*, 2016).

Central expression of melanocortin receptors

In the brain, melanocortins exert their actions through activation of the MC_3 and MC_4, which are expressed in different subsets of neurones of the hypothalamus and limbic system. MC_3 expression has been detected mainly in the hypothalamus, whereas MC_4 expression has been reported in several brain areas, including the olfactory bulb, hypothalamus, hippocampus, and brainstem (Roselli-Rehfuss *et al.*, 1993; Kishi *et al.*, 2003) (Table 1.2.). Both

receptors participate in the regulation of several functions, including energy balance, thermogenesis, and cardiovascular actions (Rowland *et al.*, 2010; do Carmo *et al.*, 2013; Monge-Roffarello *et al.*, 2014).

1.2.2.3. Central actions of melanocortins

Sexual behaviour

Early studies have shown that i.c.v. administration of ACTH in dogs, cats, and rabbits induces penile erection and ejaculation (Bertolini *et al.*, 1969). Similarly, i.c.v. injections of ACTH or α-MSH elicit penile erections in rodents. Furthermore, injections of the synthetic α-MSH analogue MT-II into the PVN significantly increases spontaneous erections in urethane anaesthetized rats (Giuliano *et al.*, 2006). The actions of melanocortins on sexual behaviour are thought to be mediated by MC_3 and MC_4, however, the involvement of the MC_3 still remains unclear. Knock-out mice for MC_4 display increased mounting and intromission latency compared to wild-type mice, as well as a reduced number of ejaculations over 1 h observation period (Van der Ploeg *et al.*, 2002).

In female rats, i.c.v. administration of α-MSH or $ACTH_{4-10}$ increased lordosis behaviour in non-receptive rats primed with oestradiol and progesterone (Thody *et al.*, 1981). Moreover, i.v. injection of MT-II facilitates solicitation behaviours, including hop and darts, and ear wiggling, in oestradiol and progesterone-primed ovariectomized rats (Rossler *et al.*, 2006). A brain site of action for melanocortins has been reported by Cragnolini *et al.* (2000), showing that injections of α-MSH into the ventromedial nucleus of the hypothalamus increases lordosis behaviour in rats.

Grooming, and stretch and yawning syndrome

Increased grooming and repeated episodes of stretch and yawning were the first behavioural effects observed following central administration of melanocortins (Ferrari *et al.*, 1963). Melanocortins elicit all the components of grooming behaviour which includes: body and genital grooming, scratching, body shakes, paw and tail licking, and face washing. These behaviours have been observed in several mammals, including laboratory rodents, rabbits, dogs, and cats. According to Bertolini *et al.* (2009), "grooming and scratching usually start after about 10 min from the peptide injection and are maximally displayed during the first hour". This suggests the involvement of long-lasting chemical messengers, and interestingly, the half-life of α-MSH, and also oxytocin –a suggested mediator of melanocortin actions (Sabatier *et al.*, 2003b)– have been reported to be 30 min approximately in the CSF (De Rotte *et al.*, 1980; Jones & Robinson, 1982; Mens *et al.*, 1983).

In rats, i.c.v. injections of low doses of $ACTH_{1-24}$ increases grooming behaviour. Similarly, the injection of α-MSH or $ACTH_{1-24}$ (1 μg) into hypothalamic nuclei of the periventricular region of the third ventricle induce grooming, yawning, stretching (Argiolas *et al.*, 2000). The induction of grooming behaviour is dose-dependent (Bertolini *et al.*, 1988). Stretch and yawning syndrome can also be observed in rats and mice following central administration of melanocortins; however, stretching is up to 5 times more prevalent than yawning in these animals (Bertolini *et al.*, 1988).

Food intake and energy homeostasis

An early study showed that i.c.v. administration or direct injection into the ventromedial hypothalamus of $ACTH_{1–24}$ in rats fasted for 24 h markedly inhibits food intake. In these rats, the central administration of $ACTH_{1–24}$ reduced food intake by approximately 75 % during the first hour after injection (Vergoni *et al.*, 1986). Similarly, injections of MT-II into the PVN of fasted rats reduced food intake in a dose-dependent manner for up to 24 h after the injection. On the other hand, injections of a melanocortin antagonist into the PVN increased food intake compared to the control group (Giraudo *et al.*, 1998).

The melanocortin actions on food intake are mediated, at least in part, by activation of MC_4. In mice, targeted disruption of MC_4 leads to develop a maturity-onset obesity syndrome in which 15-weeks old homozygous male mutants were 50 % heavier than wild-type mice. Hyperphagia, hyperglycaemia, and hyperinsulinemia were also observed in these animals (Huszar *et al.*, 1997). Furthermore, chronic i.c.v. administration of the selective MC_4 antagonist HS014, potently increases food intake and body weight after 2 weeks, increasing body weight by 20 % when compared to saline treated rats (Kask *et al.*, 1999). The role of MC_3 in food intake and energy balance is still not fully understood. Sutton *et al.* (2008) reported impairment in anticipatory patterns of activity during restricted feeding in MC_3 null mice.

1.2.2.4. The synthetic melanocortin agonist Melanotan-II (MT-II)

MT-II, Ac-Nle-c[Asp,DPhe,Arg,Trp,Lys]-NH_2, is a synthetic cyclic heptapeptide analogue of α-MSH (Al-Obeidi *et al.*, 1989b). MT-II has been described as "a superpotent, prolonged acting, enzyme resistant melanotropic peptide" (Hadley *et al.*, 1989) when compared to α-MSH (Hadley & Haskell-Luevano, 1999); showing 1 and 90 fold higher potency in frog and lizard skin bioassays, respectively, relative to α-MSH (Al-Obeidi *et al.*, 1989a; Al-Obeidi *et al.*, 1989b).

The cyclic structure of MT-II is believed to confer better brain penetration properties than linear melanocortin agonists (e.g. α-MSH, MT-I), as it has been suggested by behavioural actions (i.e. yawning and stretching) following subcutaneous administration in human trials (Dorr *et al.*, 1996; Wessells *et al.*, 1998; Wessells *et al.*, 2000), and i.v. administration in rats (Adan *et al.*, 1999). In mice, after intraperitoneal (i.p.) administration of MT-II (1 mg/kg), high plasma concentrations were detected at 30 min after injection, to then suffer fast plasmatic clearance at 1 h to 2 h; in brain homogenates, low concentrations of MT-II were found, suggesting restricted capacity to penetrate the brain (Hatziieremia *et al.*, 2007).

Trivedi *et al.* (2003) studied the sites of actions of MT-II, showing that systemic injection of (125)Iodo-MT-II penetrated the brain parenchyma in low amounts, however, a high concentration of radiolabelled MT-II was bound to the circumventricular organs, which are located outside of the BBB, and are known to modulate the activity of oxytocin and vasopressin neurones of the PVN and SON (Cottrell & Ferguson, 2004; Ferguson, 2014).

1.2.3. Circulating melanocortins as potential modulators of oxytocin and vasopressin neurone activity

The BBB controls the passage of blood-borne molecules into the brain, including oxytocin (Zaidi & Heller, 1974; Ermisch *et al.*, 1985), and endogenous melanocortins, such as α-MSH (Wilson *et al.*, 1984; Wilson, 1988). Expression of tight junctions between endothelial cells of the brain's capillary system restrict the passage of water-soluble molecules, peptides, amino acids, and drugs through the intercellular space into the brain parenchyma. Only lipid-soluble molecules (e.g. sexual steroids), water, and some gases can cross the BBB by passive diffusion. The passage of restricted molecules into the brain is determined by the expression of specific transport systems (e.g. amino acids) (Engelhardt & Sorokin, 2009; Banks, 2012).

In addition, specialised areas of the BBB have been described:

(i) the blood-CSF barrier which prevents the passage of molecules from the vascular system of the choroid plexuses to the ventricular system (CSF). Tight junctions expressed on the choroidal epithelial cells completely seal the intercellular space, whereas fenestrated capillaries are located in the inner structure of the choroid plexuses. Similarly to the BBB, specific transport systems mediate the entry of compounds, including glucose, prolactin, and others molecules (Rodriguez *et al.*, 2010).

(ii) the blood-circumventricular organs barrier which prevent the diffusion of blood-borne molecules from discrete brain areas into the –deep– brain parenchyma. Tight junction proteins are expressed in the ependymal cells surrounding these discrete brain areas, whereas fenestrated capillaries lay in the inner structure (Langlet *et al.*, 2013). The circumventricular organs allow neurones

located in these regions to monitor and sense blood-borne molecules and to modulate the activity of brain areas protected by the BBB (Miyata, 2015).

1.2.3.1. Circumventricular organs

The subfornical organ (SFO), and organum vasculosum lamina terminalis (OVLT) are sensory circumventricular organs located in the rostral wall, or *lamina terminalis*, of the third ventricle. Both brain structures play a pivotal role in the regulation of vasopressin and oxytocin neurone activity, as exemplified by studies of their role in osmoregulation. This regulation is achieved by neurones expressing receptors for several hormones (including the MC_4; Kishi *et al.* (2003)), which are present in the bloodstream and diffuse into the circumventricular organ milieu. Tracing studies have shown retrogradely labelled neurones in both circumventricular organs projecting to the PVN and SON (Sawchenko & Swanson, 1983).

The area postrema (AP) is a sensory circumventricular organ located in the brainstem that also expresses receptors for circulating hormones, including melanocortins (Kishi *et al.*, 2003; Rowland *et al.*, 2010). The AP has extensive connections with the NTS which is known to modulate vasopressin and oxytocin neurones (Renaud *et al.*, 1987; Leng *et al.*, 1991; Verbalis *et al.*, 1991).

<u>The subfornical organ (SFO)</u>

The SFO is an unpaired structure located in the midline of the dorsal third ventricle. Morphologically, an outer shell and ventromedial core can be

distinguished in the SFO, the latter displays a high degree of vascularization. The SFO regulates cardiovascular, behavioural (i.e. water intake, and salt consumption), metabolic, and neuroendocrine functions (Fry & Ferguson, 2007). Receptors for several hormones, including angiotensin II, and relaxin are expressed in the SFO (McKinley *et al.*, 1998). I.v. administration of these peptides (e.g. relaxin) have been shown to induce activation of SFO (and also OVLT) neurones projecting to the SON and PVN, as shown by expression of Fos protein in fluorogold retrolabelled neurones (Sunn *et al.*, 2001). Although the expression of MC_4 messenger ribonucleic acid (mRNA) has also been detected in the SFO (Kishi *et al.*, 2003), its role is unknown.

The SFO sends direct excitatory projections to oxytocin and vasopressin magnocellular neurones of SON and PVN, as well as to parvocellular populations of the PVN (Fry & Ferguson, 2007). Contrariwise, scarce oxytocin-containing fibres have been detected in the SFO (Buijs, 1978; Yulis & Rodriguez, 1982). Neurones of the SFO are intrinsically osmosensitive (Anderson *et al.*, 2000), and electrolytic lesioning of SFO has been shown to impair the osmoresponsiveness of the oxytocin system (Leng *et al.*, 1989).

The organum vasculosum lamina terminalis (OVLT)

The OVLT is an unpaired structure located in the midline of the rostroventral wall of the third ventricle, and dorsal to the optic chiasm. Two anatomical areas can be distinguished in the OVLT: a lateral area, and a dorsal cap (Fry & Ferguson, 2007). Similarly to the SFO, the OVLT neurones express receptors for several circulating hormones, including angiotensin II,

relaxin, as well as the MC_4 mRNA (Kishi *et al.*, 2003). Interestingly, in addition to the MC_4, high densities of POMC- and AgRP-immunoreactive fibres have been found in the OVLT (Bagnol *et al.*, 1999), but their functional role has not been established.

The OVLT exerts its regulatory actions through direct afferent projections to the PVN and SON (Fry & Ferguson, 2007), which are crucial for the regulation of vasopressin and oxytocin release. Experimental electrolytic lesion of the anteroventral third ventricle region of the hypothalamus (AV3V; which includes the OVLT and median preoptic nucleus) decreases the basal spontaneous activity of SON neurones, and reduces peripheral oxytocin and vasopressin release in response to hyperosmotic stimulation (Leng *et al.*, 1989). The osmotic response is commanded, at least in part, by activation of OVLT osmosensitive neurones expressing the transient receptor potential vanilloid-1 (TRPV1) channels (Ciura & Bourque, 2006). Similarly, lesion of the AV3V impairs peripheral vasopressin release in response to i.v. administration of angiotensin II.

The area postrema (AP)

In rats, the AP is a single structure located in the midline of the fourth ventricle. It is highly vascularized, displaying similar vascular features than the SFO. The AP is well known for its role as chemosensor, detecting toxins and triggering emesis. As with other circumventricular organs, the AP express receptors for several hormones (McKinley *et al.*, 1998). The AP send direct projections to the NTS. In addition, afferent projections from the PVN, and NTS to the AP have been described (Shapiro & Miselis, 1985).

Experimental lesion of the AP has been shown to decrease basal vasopressin concentration in plasma, and attenuate the peripheral vasopressin release in response to hypovolemia and hyperosmolality (Arima *et al.*, 1998). Moreover, Huang *et al.* (2000) have reported impaired peripheral oxytocin (and vasopressin) release in response to i.v. administration of 1M NaCl in AP-lesioned rats, indicating that the AP could be involved in the release response of magnocellular neurones to circulating compounds.

1.2.3.2. Nucleus tractus solitarii (NTS)

The NTS is a complex brain nucleus formed by heterogeneous groups of neurones located in the brainstem. The NTS receives visceral afferent projections from peripheral organs through the vagus nerve, and also from brain structures, such as the AP. Peripherally, changes in parameters of cardiovascular, respiratory, or gastrointestinal systems lead to modifications in neural activity of different subsets of NTS neurones which, in turn, modify other neural systems for an appropriate physiological response. For oxytocin-vasopressin systems, most of the current knowledge has been obtained from stimuli acting on aortic baro- or chemo-receptors or blood-borne molecules, like the gastric peptide cholecystokinin (CCK), which activate NTS neurones that in turn activate magnocellular neurones of the SON (Zoccal *et al.*, 2014).

The SON, and both magnocellular and parvocellular populations of the PVN receive projections from some neurones of the NTS (A2 cells), and the ventrolateral medulla (A1 cells) which are mostly noradrenergic (Sawchenko & Swanson, 1982b). Oxytocin and vasopressin neurones are equally innervated by noradrenergic afferents in virgin rats, however, in

lactating rats the innervation of oxytocin neurones is increased. Remarkably, projections from A2 cells of the NTS have been found in the posterior pituitary, which are thought to facilitate the release of oxytocin and vasopressin. Subsets of noradrenergic neurones also co-express other peptides including neuropeptide Y (NPY), and substance P. Moreover, several peptides have also been found in non-noradrenergic neurones projecting to the magnocellular system, such as inhibin B, somatostatin and enkephalin (Leng *et al.*, 1999).

The excitatory actions of noradrenaline cells on magnocellular neurones is potentiated by NPY, as shown by enhanced excitation of SON neurones *in vitro* (Sibbald *et al.*, 1989), and oxytocin release in lactating rats.

1.4. HYPOTHESIS

Briefly, morphological evidence supports the regulation of central oxytocin systems by POMC neurones which synthesise the POMC product α-MSH. The direct application of α-MSH to SON explants has been shown to trigger somatodendritic oxytocin release *in vitro*, while i.c.v. administration of α-MSH inhibited peripheral oxytocin release by decreasing electrical activity of SON oxytocin neurones *in vivo* (Sabatier *et al.*, 2003a). MT-II is a synthetic cyclic analogue of α-MSH that is thought to have better brain penetration properties than α-MSH, as suggested by behavioural actions following peripheral administration (Dorr *et al.*, 1996; Modi *et al.*, 2015).

The main hypothesis of this thesis is that: **intravenous (i.v.) administration of MT-II directly (centrally) acts on oxytocin neurones to trigger somatodendritic oxytocin release from SON neurones *in vivo*.**

1.5. OBJECTIVES

To test this hypothesis, my objectives were as follows:

1. To determine whether i.v. injection of MT-II induces activation of PVN and SON oxytocin neurones, using the product of the immediate early gene *c-fos*, Fos protein, as a marker for neural activity by immunohistochemistry.
2. To determine whether i.v. injection of MT-II activates brain regions that are known to modulate oxytocin and vasopressin neurone activity, using immunohistochemistry for Fos protein.
3. To study the electrophysiological responses of oxytocin and vasopressin neurones to i.v. administration of MT-II in urethane anaesthetized rats.
4. To evaluate whether i.v. administration of MT-II triggers somatodendritic oxytocin within the SON in urethane anaesthetized rats, as measured by microdialysis and highly sensitive radioimmunoassay (RIA).

I also set an additional objective:

5. To study the effect of intranasally administered MT-II on neural activity of PVN and SON oxytocin neurones, and layers of the olfactory bulb, using immunohistochemistry for Fos protein.

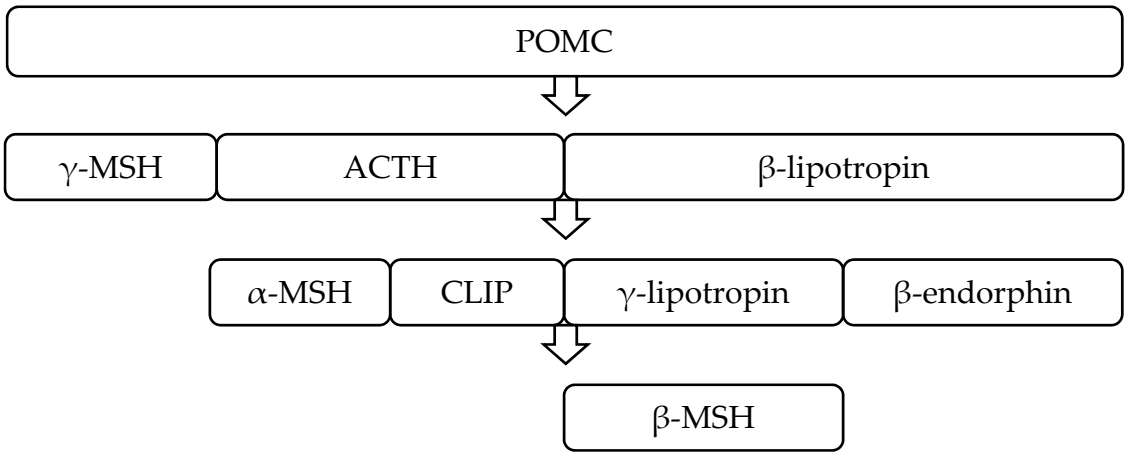

Figure 1.1. Schematic representation of the processing of the POMC precursor protein and its related subproducts. Neurones of the arcuate nucleus and cells of the adenohypophysis synthesize POMC which is differentially processed by tissue-specific enzymes leading to different subproducts. POMC - proopiomelanocortin, ACTH - adrenocorticopic hormone, α- β- γ-MSH - alpha- beta- gamma-melanocyte stimulating hormone, CLIP - corticotropin-like intermediate lobe peptide.

Table 1.1. Detection of oxytocin fibres and oxytocin receptor (OTR) mRNA in the central nervous system of the adult rat

Brain regions	Oxytocin fibres	OTR mRNA	Oxytocin binding
Olfactory system			
Anterior olfactory nucleus	+	+++	++
Olfactory bulb	+	+	?
Olfactory tubercle	+	+++	+ +
Islands of Calleja		ND	+ + +
Entorhinal/perirhinal area	+	+	+
Piriform cortex		++	?
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	++	++	+ + +
Globus pallidus		+	ND
Nucleus accumbens	+	+	+
Circumventricular organs			
Subfornical organ	+	+	
Organum vasculosum of the lamina terminalis	+	+	
Thalamus and hypothalamus			
Anteroventral thalamic nucleus	+	ND	ND
Paraventricular thalamic nucleus	+	++	+
Anterior medial preoptic area	+	+++	ND
Dorsomedial hypothalamic nucleus	+		
Ventromedial hypothalamic nucleus	+	+++	+ +
Arcuate nucleus	+	+	
Paraventricular nucleus (PVN)	+	++	(+)
Supraoptic nucleus (SON)	+	+++	(+)
Suprachiasmatic nucleus (SCN)	+	++	

Posterior hypothalamic area	+	+	ND
Medial tuberal nucleus	+	ND	+ +
Supramammillary nucleus	+	++	+
Lateral mammillary nucleus	+	ND	+
Medial mammillary nucleus	+	ND	ND
Limbic system			
Dorsal hyppocampus	+	+	
Ventral hyppocampus	+	+	
Lateral septal nucleus	+	+	+
Medial septal nucleus	+		
Bed nucleus of stria terminalis (BNST)	+	+++	+ + +
Amygdaloid-hippocampal area		+++	+
Central amygdala	+	+++	+ + +
Medial amygdala	+	++	+
Basolateral amygdala	+	+++	+
Parasubiculum and presubiculum		ND	+ +
Dorsal subiculum	+	+++	(+)
Ventral subiculum	+	+++	+ + +
Brainstem			
Substantia nigra pars compacta	++	++	ND
Ventral and dorsal tegmental area		++	ND
Locus ceruleus	+		
Central gray	+	+	ND
Dorsal raphe nucleus	+	+	ND
Reticular nuclei	+	+	ND
Medial vestibular nucleus		+	ND
Hypoglossus nucleus		++	ND
Nucleus of the solitary tract (NTS)	++	ND	(+)
Dorsal motor nucleus of the vagus nerve	+	+++	+
Nucleus ambiguus	+		
Inferior olive nucleus		ND	+
Substantia gelatinosa of trigeminal nucleus		+	+
Pituitary gland			
Anterior lobe (lactotrophs)		+	+
Posterior lobe		+ (IHC)	
Spinal cord	+	+	

Adapted from Bujis *et al., (*1979, 1980, 1985); Adan *et al*., (1995); Vaccari *et al*., (1998); Gimpl and Fahrenholz (2001). Expression: +++ strong; ++ moderate; + weak; ND = no detection, blank space = not reported, IHC = immunohistochemistry.

Table 1.2. Detection of melanocortin receptors mRNA and autoradiographic binding in the central nervous system of the adult rat.

Brain regions	MC3R mRNA	MC4R mRNA	MC3/4R binding
Olfactory system			
Olfactory tubercle		+++	++
Olfactory bulb		++	
Anterior olfactory nucleus		+	
Olfactory tubercle		++	
Islands of Calleja		+	
Piriform cortex	+	++	
Entorhinal/perirhinal area		++	
Cortical areas			
Peduncular cortex		++	
Insular cortex		+	
Cingulate cortex		+	
Retrosplenial cortex		+	
Auditory cortex		+	
Visual cortex		++	
Taenia tecta		+	
Diagonal band of Broca		+	
Basal ganglia			
Caudoputamen		+++	
Ventral pallidum		+	
Nucleus accumbens		++	++
Circumventricular organs			
Subfornical organ		++	+++
Organum vasculosum of the lamina terminalis			++
Area postrema		+	+++
Thalamus and hypothalamus			
Parafascicular nucleus		+	
Habenular nucleus	++	+	
Zona incerta		+	
Anterior hypothalamic area		++	+++
Dorsal hypothalamic area		+++	
Anteroventral preoptic nucleus	++	+	

Anteroventral periventricular nucleus	+	+++	
Dorsomedial hypothalamic nucleus		++	
Ventromedial hypothalamic nucleus	+++	+	++
Arcuate nucleus	++	+	
Paraventricular nucleus (PVN)		+++	+++
Supraoptic nucleus (SON)		++	
Suprachiasmatic nucleus (SCN)		ND	
Posterior hypothalamic area	++	+	
Medial preoptic area			++
Medial preoptic nucleus	++	+++	++
Dorsal tuberomammillary nucleus		+	
Limbic system			
Hippocampus	+	+	
Lateral septal nucleus	++	+++	+
Medial septal nucleus		++	
Bed nucleus of stria terminalis (BNST)	+	++	++
Amygdaloid-hippocampal area		+	
Central amygdala		++	
Medial amygdala	+	+++	
Basolateral amygdala		++	
Ventral subiculum		+	
Brainstem			
Substantia nigra pars compacta	+	+	
Dorsal tegmental nucleus		++	
Locus ceruleus		+	
Periaqueductal gray	+	+	+
Dorsal raphe nucleus	++	+	
Reticular nuclei		+	
Rostral ventrolateral medulla		+	
Nucleus of the solitary tract (NTS)		+++	++
Dorsal motor nucleus of the vagus nerve		+++	+++
Nucleus ambiguous		+	
Inferior olive nucleus		+	
Red nucleus		+++	
Pituitary gland			
Spinal cord		+++	

Adapted from Roselli-Rehfuss *et al., (*1993); Xia and Wikberg (1996); Lindblom *et al., (*1998); Kishi *et al., (*2003); Tivedi *et al., (*2003); Siljee-Wong (2011). Expression: +++ strong; ++ moderate; + weak; ND no detection; blank space = not reported.

Chapter 2

General material and methods

2.1. ANIMALS

Adult male Sprague-Dawley rats weighing 250-350 g, bred in the animal unit of the Hugh Robson Building at The University of Edinburgh, were used in the studies. The animals were housed in stock cages of 6 rats maximum, with *ad libitum* access to rodent food and water. The animals were maintained on a 12-hour light/dark cycle (lights on: 7 h – 19 h), and a room temperature of 20 ± 1 °C. All the experiments started at 9:30 h, and lasted a maximum of 7 hours (i.e. *in vivo* microdialysis). Housing, handling, care and experimental procedures of animals were conducted in accordance with UK Home Office Animals Scientific Procedures Act 1986, and were approved by the Ethical Committee of the University of Edinburgh.

2.2. ANAESTHESIA AND ANALGESIA

For non-recovery procedures, rats were briefly anaesthetized under isoflurane inhalation (IsoFlo, Abbott Laboratories Ltd., Berkshire, UK) in a glass chamber. Then, rats were i.p. injected with 25 % urethane solution (ethyl carbamate; SigmaAldrich, Dorset, UK) at a dose 1.25 g/kg. Urethane provides a long-lasting stable anaesthesia whereas maintaining the physiological functions of SON neurones (Leng & Sabatier, 2014). It has been the anaesthetic of choice for *in vivo* electrophysiology, and microdialysis studies, however, urethane is known to induce a mild increase in plasma osmolality, and consequently, the expression of Fos protein is increased in magnocellular neurones (Leng & Sabatier, 2014). In Fos expression studies, rats were anaesthetized with an i.p. injection of sodium pentobarbital (Ceva Santé Animale, Libourne, France) at a dose 50 mg/kg, then anaesthesia was maintained by i.v. injections of sodium pentobarbital at a dose 25 mg/kg every 45 min using an i.v. cannula implanted as described below.

For recovery experiments, rats were anaesthetised using inhalation isoflurane anaesthesia delivered by a continuous-flow anaesthetic machine. Briefly, rats were placed in an induction chamber, and 5 % isoflurane in 1.5 l / min of oxygen was delivered into the chamber. Anaesthesia was maintained using 2.5 % isoflurane in 1.5 l / min of oxygen delivered via an anaesthetic mask. For postoperative analgesia, rats were given carprofen (Rimadyl, Pfizer Animal Health; Surrey, UK) 5 mg/kg s.c. every 24 h for 2 days.

For euthanasia, rats were given an i.p. injection of sodium pentobarbital (Euthenal, Merial Animal Health Ltd., Harlow, UK) at a dose 160 mg/kg.

2.3. NON-SURGICAL PROCEDURES

2.3.1. Subcutaneous injections (s.c.)

The rat was restrained in prone position, then using the thumb and index fingers the skin over the flank was gently tented, and a sterile 26-gauge needle was inserted into the base of the loose skin and the drug injected. Only carprofen for postoperative pain control was injected using this route of administration.

2.3.2. Intraperitoneal injections (i.p.)

The rats were restrained on their back and the abdomen exposed. Then, a sterile 23-gauge needle was inserted through the abdominal wall at the lower right quadrant of the abdomen in an angle of 45° to the body. The syringe was slightly withdrawn to check the correct placement (i.e. outside of blood vessels) before injecting the drugs into the peritoneal cavity.

2.4. SURGICAL PROCEDURES

All procedures were conducted under aseptic conditions, and tools and instruments were autoclaved. The incision sites were shaved, and the skin disinfected using a chlorhexidine gluconate 4 % w/v solution (Hibiscrub; Regent Medical Ltd, Manchester, UK). During recovery procedures, body temperature was maintained using a heatmat at 37 °C. In non-recovery procedures, body temperature was maintained using a light bulb and a cotton cover. Rats were given 1 ml i.v. or i.p. of 0.9 % saline solution for any fluid loss associated with the surgical procedures.

2.4.1. I.v. cannula implantation

Under non-recovery anaesthesia, rats were placed in supine position and the left leg extended and secured to the table surface with tape, then using a sterile disposable scalpel a 1.5 cm incision was made in the skin over an imaginary line between the leg and abdomen. Under a stereomicroscope, the connective tissue was gently separated, and the femoral vein, artery, and nerve were carefully dissected. Two ligatures (4-0 size silk thread; Fine Science Tools GmbH, Heidelberg, Germany) were tied around the vein; the ligature located in the distal end of the vein was immediately tightened, the proximal end of the vein, close to the abdominal wall, was clamped using a stainless steel clamp. A very small incision was made and a polyethene cannula (0.5 mm internal diameter; 0.25 mm wall) was inserted into the femoral vein. The clamp was removed, the cannula gently pushed further into the vein, and the proximal ligature firmly tightened. Finally, the cannula was secured to the leg with tape.

2.4.2. I.c.v. cannula implantation

Under inhalation isoflurane anaesthesia the rats were placed in a digital stereotaxic frame (Stoelting Co., Wood Dale, IL, USA); two non-puncture ear bars fixed the head in position to the frame. The head was shaved, and a skin incision was made along the midline of the head to expose the dorsal surface of the skull. The connective tissue was removed, and any bleeding stopped to clearly visualise the bregma and lambda points. The level of the skull was adjusted and corrected when necessary using the bregma and lambda points. Then a burr hole was drilled through the skull (coordinates: 0.6 mm caudal to bregma, 1.5 mm lateral to midline) and a 4.5 mm long guide cannula (cat. C315G; Plastics One Inc., VA, USA) was

positioned into the right cerebral ventricle. The cannula was secured in place using two stainless screws fixed to the skull and dental cement (Kemdent, Associated Dental Products Ltd., Swindon Wiltshire, UK). A dummy cap was inserted into the guide cannula (cat. C315DC; Plastics One Inc., VA, USA). Rats were allowed to recover for 4 days.

2.5. DRUGS

For i.v. injections, the melanocortin agonists MT-II (American Peptide Company, Inc., CA, USA), and TCMC-A01 (kindly provided by Dr Michael Callahan; Tensive Controls, Inc., MO, USA) were dissolved at a concentration 1 mg/ml in sterile physiological 0.9 % saline solution (B. Braun, Melsungen, Germany). For i.c.v. injections, MT-II, and the melanocortin antagonist SHU-9119 (Abcam plc, Cambridge, UK) were dissolved at a concentration of 0.33 μg/μl in artificial cerebrospinal fluid (aCSF, Tocris Bioscience, Bristol, UK). For intranasal MT-II application, 1 μg or 30 μg of MT-II was dissolved in 20 μl of aCSF.

These peptide solutions were prepared in phials. Briefly, the peptides and solvent (i.e. 0.9 % saline or aCSF) were vortexed 3 times 45 s each, then the solutions were sonicated 3 times 30 s each, and allowed to cool down on ice for 1 min between each sonication cycle. Finally, the solutions were aliquoted and stored at -80 °C until they were required. As some peptides are unstable in solution, only fresh peptide aliquots (i.e. 1 month old maximum) were used in each set of experiments in order to avoid peptide degradation.

2.6. DRUG ADMINISTRATION PROCEDURES

2.6.1. I.v. injection

The i.v. cannula was connected to a graduated disposable 1 ml syringe filled with heparinised 0.9 % saline solution. Before injections, 0.1 ml of saline solution was injected to ensure the fluid is able to flow through the cannula, then the syringe was removed and a second syringe containing the drug solution connected to the i.v. cannula, and the drug was slowly injected. Finally, the syringe was removed and the syringe containing heparinised saline reconnected to the cannula and 0.1 ml injected to flush the line.

2.6.2. I.c.v. injection

A 10 μl Hamilton syringe was connected to an injection cannula (cat. C315I; Plastics One Inc., VA, USA) using a short plastic tubing. The dummy cap was removed from the guide cannula previously implanted, and the injection cannula inserted into the guide cannula. The drugs were injected at 1 μl/min rate. Once the injection was completed, the injection cannula was removed 1 min after the injection and the dummy cap refitted.

The correct placement of the i.c.v. cannula into the lateral ventricle was checked by histology using coronal sections of the brain (40 μm).

2.6.3. Intranasal administration

For intranasal administration, rats were anaesthetized with sodium pentobarbital as described above. After a period of 2 h, rats were placed in a supine position, with the head supported at 45° to the body and the drug given intranasally by slowly pipetting a 10 μl volume into each nostril. Inhalation anaesthetics were not used, and all the procedures were conducted under air-controlled conditions in a room in the animal unit

under positive pressure to avoid stimulation of the olfactory system by unspecific odours.

2.7. REAGENTS AND SOLUTIONS

Reagents for solutions used in these studies were purchased from Sigma Aldrich, Dorset, UK unless otherwise stated. Solutions were prepared based on conventional protocols and formulas.

2.8. STATISTICAL ANALYSIS

The data were analysed using Graph Pad Prism 6 (GraphPad Software, CA, USA). Statistical tests used in each experiment are described in the specific results chapters. All data are presented as means ± S.E.M., and statistical significance was set at $P < 0.05$ unless otherwise stated. Significance levels are indicated as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Chapter 3

Fos protein expression in response to administration of Melanotan-II

3.1. INTRODUCTION

Melanocortins regulate the activity of oxytocin neurones when injected centrally. These actions are mediated by the activation of MC_4 which are expressed in neurones of the PVN and SON (Kishi *et al.*, 2003). In virgin rats, α-MSH i.c.v. injected or directly applied to the SON increases the expression of Fos in magnocellular neurones (Sabatier *et al.*, 2003a), however, the α-MSH-induced Fos expression in virgin rats is suppressed during pregnancy (Ladyman *et al.*, 2016). Similarly to α-MSH, central administration of the synthetic $MC_{3/4}$ agonist, MT-II, has been shown to induce the expression of Fos in SON and PVN neurones (Thiele *et al.*, 1998; Trivedi *et al.*, 2003; Rowland *et al.*, 2010).

Activation of oxytocin neurones by melanocortins has been proposed as a putative mediator responsible for a wide range of behavioural and physiological effects (Sabatier *et al.*, 2003b), including sexual behaviour, food intake and energy homoeostasis (see Chapter 1). Interestingly, the MC_4 is also expressed in the amygdala where high mRNA receptor densities have been detected in the central nucleus (cAMG) (Kishi *et al.*, 2003) and i.c.v. administration of the melanocortins ligands, including MT-II, have been shown to increase the neural activity, as shown by the expression of Fos (Thiele *et al.*, 1998; McMinn *et al.*, 2000; Hagan *et al.*, 2001). The amygdala is a complex brain region involved in emotional processing playing a key role in fear and rewarding behavioural responses, and these responses are commanded by a complex interaction of several neurotransmitters and neuropeptides (Janak & Tye, 2015). For example, α-MSH has been shown to modulate stress and anxiety behavioural responses via activation of MC_4 (Kokare *et al.*, 2005; Bhorkar *et al.*, 2014). Moreover, the cAMG also expresses a high density of OTR (Veinante & Freund-Mercier, 1997; Bale *et al.*, 2001; Huber *et al.*, 2005), and evoked release of oxytocin within the cAMG has been shown to attenuate fear response in female rats (Knobloch *et al.*, 2012). Thus, the administration of a melanocortin agonist makes the cAMG a potential site of behavioural actions for melanocortins themselves, or acting indirectly, through melanocortin-stimulated central oxytocin release.

A limitation for drug delivery into the brain is the BBB which restricts the passage of hydrophilic molecules (e.g. peptides) into the brain extracellular fluid by the expression of tight junctions between endothelial cells, and consequently, separating the vascular system from the brain parenchyma (Banks, 2016). Unfortunately, exogenous administration of

naturally occurring melanocortins, including α-MSH, cannot penetrate the BBB in neuroactive amounts (Wilson *et al.*, 1984). α-MSH and other endogenous melanocortins, such as ACTH and β-MSH, are cleaved from the POMC precursor which is largely synthesized in cells of the intermediate and anterior lobe of the pituitary gland, and also in neurones of the arcuate nucleus of the hypothalamus, but their physiological roles are controlled by the BBB (Hadley & Haskell-Luevano, 1999). However, the synthetic α-MSH analogue, MT-II, is thought to have, at least some, brain penetration capacities based on its molecular conformation and physiological effects (Dorr *et al.*, 1996; Hadley & Dorr, 2006). Similarly, the experimental drug TCMC-A01 is a melanocortin ligand with $MC_{3/4}$ agonist properties, and designed with theoretical BBB penetration capacities (Dr M. Callahan, Tensive Controls Inc., MO, USA; personal communication).

In addition, systemic (i.v.) administration of radiolabelled MT-II has been reported to bind to circumventricular organs including the SFO and OVLT (Trivedi *et al.*, 2003) which are located outside of the BBB, and express MC_4 (Kishi *et al.*, 2003). Both the SFO and the OVLT play an important role in the regulation of oxytocin and vasopressin neurones, serving as relay centres that monitor circulating signals (e.g. endogenous peptides like relaxin) related to homeostatic and physiological status, and consequently modifying the activity of magnocellular neurones via activation of afferents pathways projecting to the SON and PVN (Sunn *et al.*, 2002). Another pathway regulating the activity of SON and PVN oxytocin neurones is the vagal-NTS complex, which has been shown to send excitatory noradrenergic afferents inputs from the caudal brainstem to the PVN and SON (Onaka *et al.*, 1995). For example, stimulation of vagal afferents by systemic administration of the

gut-related peptide CCK has also been shown to regulate the activity of oxytocin neurones via brainstem which induces the expression of Fos in PVN and SON neurones (Verbalis *et al.*, 1991).

In recent years, intranasal administration of peptides has been proposed as an alternative route to circumvent the BBB, delivering peptides directly into the brain by intracellular and extracellular pathways (Balin *et al.*, 1986; Thorne *et al.*, 2004; Johnson *et al.*, 2010), and several peptides including oxytocin, $ACTH_{4\text{-}10}$, and vasopressin have been reported to be found in the CSF in variable times following intranasal administration (Born *et al.*, 2002; Striepens *et al.*, 2013). Moreover, it has been reported that intranasal administration of oxytocin exerts prosocial effects in humans (Meyer-Lindenberg *et al.*, 2011), however, whether peptides can reach the brain is still a matter of debate (Leng & Ludwig, 2016; Quintana & Woolley, 2016). Interestingly, the rat olfactory bulb also expresses $MC_{3/4}$ (Roselli-Rehfuss *et al.*, 1993; Kishi *et al.*, 2003), and thus, it may be a potential target for the therapeutic use of intranasal MT-II acting on the olfactory bulb itself, which has been shown to be involved in social behaviour in animals (Sanchez-Andrade & Kendrick, 2009), or acting via afferent projections at distant targets, such as magnocellular neurones of the SON and PVN (Smithson *et al.*, 1989, 1992; Bader *et al.*, 2012).

This study was aimed to investigate whether systemic (i.v.) and intranasal administration of MT-II activates oxytocin neurones using Fos expression as a marker for neural activity. Fos protein is part (together with Jun protein) of the transcription factor AP-1 which is involved in the regulation of target late genes, and it has been widely used as a generic marker for activated neuroendocrine cells, including magnocellular oxytocin

and vasopressin neurones (Kovacs, 2008). Interestingly, the vasopressin, but not oxytocin, gene promoter contains an AP-1 binding site (Yoshida *et al.*, 2006). The expression of Fos is stimulated by several physiological and experimental conditions. However, in osmotically-stimulated SON vasopressin neurones the induction of Fos is not required for vasopressin transcription (Arima *et al.*, 2010). Fos expression is transient and can be immunohistochemically detected 30 min after stimulation, reaching a peak after 90 min (Verbalis *et al.*, 1991; Kovacs, 2008). I analysed changes in the expression of Fos in neurones of the SON and PVN, and also in brain structures involved in the regulation of oxytocin and vasopressin release such as the OVLT and SFO, and the NTS. Then, using a pharmacological approach, I administered (i.c.v.) a non-selective $MC_{3/4}$ antagonist to determine whether i.v. MT-II acts directly (centrally) on oxytocin neurones or through activation of peripheral melanocortin receptors.

3.2. MATERIAL AND METHODS

3.2.1. Drug administration

Rats were briefly anaesthetized using isoflurane and then dosed with sodium pentobarbital as previously described (see Chapter 2). After 2 h, MT-II (1 mg/ml/kg), TCMC-A01 -another synthetic $MC_{3/4}$ agonist- (1 mg/ml/kg), or vehicle (saline 0.9 %; 1 ml/kg) was injected i.v. In the first experiment animals were treated with either MT-II or vehicle (n = 6 for each treatment) and in the second experiment animals were treated with MT-II, TCMC-A01 or vehicle (n = 6 for each treatment). In both experiments the researcher was unaware of which animal had received which treatment until data collection was completed. For intranasal administration, 1 μg or 30 μg

of MT-II dissolved in 20 µl aCSF, or vehicle (aCSF) was given into each nostril (10 µl each). These doses were chosen as equimolar doses of intranasal oxytocin used in a previous study (Ludwig *et al.*, 2013). In another experiment, the melanocortin antagonist SHU-9119 (1 µg/3 µl/rat) or vehicle (aCSF; 3 µl/rat) was given i.c.v. and 10 min later, MT-II (1 mg/ml/kg) was given i.v. After 90 min, the rats were transcardially perfused as described below.

3.2.2. Transcardial perfusion and tissue collection

Rats were given a terminal i.p. injection of sodium pentobarbital (160 mg/kg), and then were placed in supine position. Using scissors the abdominal wall was cut horizontally close to the thoracic cage, and then the diaphragm carefully opened. The ribs were cut using a scissor and the heart exposed, then the pericardium was removed, and a short blunt 23-gauge needle was inserted through the left ventricle reaching the aortic arc. A cut in the right atrium was made to allow the drainage of blood and fixative solutions. The rats were perfused with 300 ml of heparinized (20 U/ml) 0.9 % saline solution followed by at least 300 ml paraformaldehyde (PFA) 4 % in 0.1 M phosphate buffer (PB) at a pH of 7.4. Perfusion with 4 % PFA was stopped once clearly visible sign of fixations were observed (i.e. limbs). Using a guillotine, the head was cut off and the brain carefully removed and immersed in a post-fixative solution containing 2 % PFA and 15 % sucrose in 0.1 M PB at 4 °C overnight. After this, the brains were kept in a solution containing 30 % sucrose for at least 72 h.

3.2.3. Brain slicing

The brains were dissected using razor blades between the rostral margins of the optic chiasm and pons, and coronal sections (40 μm) were cut using a freezing microtome (Leica Microsystems, Wetzlar, Germany). For olfactory bulbs, sagittal sections of 40 μm were obtained. The sections including the main and accessory olfactory bulb (AOB), the SFO, the OVLT, the SON, the PVN, the cAMG, and the NTS were stored in cryoprotectant until they were processed for immunohistochemistry.

3.2.4. Immunohistochemistry

Standard free floating immunochemistry methods were used in this study as previously described (Ludwig *et al.*, 2013). Sections were washed between steps in 0.1 M PB pH 7.4.

For Fos protein immunostaining, the endogenous peroxidase activity was deactivated by incubating the sections for 20 min in 0.1 M PB containing 0.3 % H_2O_2. After this, sections were incubated in a blocking buffer solution containing 3 % normal horse serum + 0.3 % Triton X-100 in 0.1 M PB for 1 h and were then incubated with c-Fos polyclonal antibody raised in rabbit (Ab5, PC38, Calbiochem, EMD Chemicals Inc., CA, USA) diluted at 1 : 20000 in blocking buffer for 48 h at 4 °C. Following this, sections were incubated with biotinylated horse anti-rabbit IgG (Vector Laboratories, Inc., Peterborough, UK) diluted 1 : 500 in 0.1 M PB for 1 h at room temperature. Then sections were incubated in Vectastain Elite ABC Kit (Vector Laboratories, Inc., Peterborough, UK) diluted 1 : 25 in 0.1 M PB for 1 h at room temperature. The antigen-antibody complexes were visualised with a

solution containing 0.025 % diaminobenzidine, 2.5 % nickel II sulphate, 0.08 % ammonium chloride and 0.015 % H_2O_2 in 0.1 M Tris.

For Fos and oxytocin immunostaining, double immunochemistry was performed sequentially. Procedures were conducted as described above. For oxytocin immunostaining, sections were incubated in mouse anti-rat PS38 – oxytocin-neurophysin monoclonal antibody (Ben-Barak *et al.*, 1985) kindly provided by Prof H. Gainer (NIH, Bethesda, MD, USA) diluted 1 : 5000. Then, sections were incubated with biotinylated horse anti-mouse IgG antibody (Vector Laboratories, Inc., Peterborough, UK) diluted 1 : 500 in 0.1 M PB for 1 h at room temperature. To visualise the oxytocin immunoreaction a solution containing 0.025 % diaminobenzidine and 0.015 % H_2O_2 in 0.1 M Tris was used.

The immunostained sections were mounted on gelatinized slides, air-dried for 12 h, and dehydrated in increasing concentrations of ethanol followed by xylene. In some experiments, sections (e.g. brainstem sections) were counterstained with nuclear fast red (Vector Laboratories, Inc., Peterborough, UK) for 3 min before ethanol dehydration. Finally, the slides were cover-slipped using DPX mounting medium (Sigma Aldrich, Dorset, UK).

3.2.5. Quantification of Fos-positive neurones

All quantification was carried out blinded. The number of Fos-positive neurones in the brain regions analysed, include the olfactory bulb, the AV3V, the PVN and SON of the hypothalamus, the cAMG, and the NTS. In the olfactory bulb, Fos-positive neurones were counted in areas of the mitral cell layer (MCL) and external plexiform layer (EPL) of the main olfactory bulb,

and also in the entire accessory olfactory bulb (AOB). In the PVN, parvocellular and magnocellular regions were counted separately by comparing sections with illustrations of a brain atlas (Paxinos & Watson, 1998). At the level counted, the parvocellular PVN includes the dorsal cap and ventromedial subdivisions.

In the cAMG, Fos-positive neurones were counted from sections spanning from rostral (bregma -1.56 mm) to caudal (bregma -2.28 mm). In the NTS, Fos-positive neurones were counted and analysed from sections spanning from rostral (bregma -12.0 mm) to caudal (bregma -14.9 mm) according to Paxinos and Watson (1998). In the olfactory bulb, Fos-positive neurones were counted in dorsal, rostral, and ventral areas (six fields) of each cell layer analysed. Images of the regions were acquired using an upright Leica microscope (10x objective) attached to Leica digital camera and controlled by Leica acquisition software (Leica Microsystems, Wetzlar, Germany). Images from at least six regions from every rat in each treatment group were acquired. Using ImageJ (NIH, Bethesda, MD, USA) the number of Fos-positive neurones was counted using the Count macro, and the surface area of each region of interest (ROI) was measured using the ROI macro, by comparing the section image with the brain atlas (Paxinos & Watson, 1998). The number of Fos-positive neurones within each ROI was normalised by the surface area of that ROI to allow comparison. Thus, the number of Fos-positive neurones in these regions are expressed as the mean ± S.E.M. per 10^4 μm^2 (corresponding to an area of 100 × 100 μm). The mean number of Fos-positive neurones from every rat in each region was averaged and is expressed as the mean ± S.E.M. per region.

Oxytocin neurones are densely packed within the SON and cell structures in the sections overlap making it difficult to distinguish between single neurones which might affect counting Fos-positive nuclei in oxytocin stained neurones. Therefore, I also counted a proportion of clearly distinguishable oxytocin neurones and oxytocin neurones expressing Fos protein directly under the microscope (20x magnification in at least 6 regions in every rat); values are expressed as percentage.

3.2.6. Statistical analysis

Fos expression experiments involving two groups were analysed using a two-tailed Mann-Whitney test. Experiments involving three groups were analysed using Kruskal-Wallis test followed by *post hoc* Dunn's multiple comparison test, comparing the treatment groups with the control (vehicle) group. $P < 0.05$ was considered significant.

3.3. RESULTS

3.3.1. Effect of i.v. MT-II on Fos expression

The SON of rats injected with MT-II (1 mg/kg) showed a significantly higher density of Fos-positive neurones compared with rats injected with vehicle (**P = 0.0016; Fig. 3.1A.; Appendix Table A.). In the PVN, MT-II-treated rats also showed a significantly higher density of Fos-positive neurones (*P = 0.0457; Fig. 3.1B.; Appx. Table A.) compared with rats injected with vehicle. I.v. administration of TCMC-A01 did not significantly change Fos expression either in the SON (P > 0.9999; Fig. 3.1A.), or in the PVN (P = 0.4499; Fig. 3.1B.; Appx. Table A.). The analysis of magnocellular and

parvocellular regions of the PVN (Fig. 3.2A.), showed a higher density of Fos-positive neurones in the magnocellular regions of the PVN in the MT-II group compared to the vehicle group (***P < 0.001; Fig. 3.2B.; Appx. Table A.). I.v. administration of MT-II did not significantly change Fos expression in parvocellular regions (P = 0.1326; Fig. 3.2B.; Appx. Table A.).

Double immunostaining for Fos and oxytocin revealed that both oxytocin neurones and non-labelled, putative vasopressin neurones, displayed more Fos expression in rats injected with MT-II in the SON (oxytocin: *P = 0.0260; non-labelled: *P = 0.0411; Fig. 3.3E.; Appx. Table A.). In the PVN, magnocellular and parvocellular oxytocin neurones also showed significantly more Fos expression in rats injected with MT-II (magnocellular: **P = 0.0087; parvocellular: **P = 0.0043; Fig. 3.3F.; Appx. Table A.). There was also more Fos expression in magnocellular and parvocellular non-oxytocin neurones, but this did not achieve statistical significance (magnocellular: P = 0.1775; parvocellular: P = 0.0823; Fig. 3.3F.; Appx. Table A.).

Oxytocin neurones are densely packed and cells structures overlap making difficult to distinguish between single cells, so a proportion of clearly distinguishable oxytocin neurones was counted under the microscope. The percentage of oxytocin neurones expressing Fos was significantly higher in the SON (**P = 0.0022; Fig. 3.4A.; Appx. Table A.), and magnocellular (**P = 0.0043; Fig. 3.4B.; Appx. Table A.) and parvocellular (**P = 0.0043; Fig. 3.4B.; Appx. Table A.) neurones of PVN of rats injected with MT-II compared to vehicle.

3.3.1.1. Fos expression in neurones of the SFO, OVLT

No significant changes in Fos expression were detected in the circumventricular organs, including the OVLT (MT-II vs vehicle: P = 0.8734, TCMC-A01 vs vehicle: P > 0.9999) and SFO (MT-II vs vehicle: P > 0.9999, TCMC-A01 vs vehicle: P = 0.2623) following i.v. administration of MT-II or TCMC-A01 (Fig. 3.5A,D.; Appx. Table A.).

3.3.1.2. Fos expression in neurones of the cAMG

The i.v. administration of MT-II or TCMC-A01 did not significantly change the expression of Fos in the cAMG (MT-II vs vehicle: P > 0.9999, TCMC-A01 vs vehicle: P > 0.9999; Fig. 3.5F.; Appx. Table A.).

3.3.1.3. Fos expression in the NTS

The analysis of the NTS showed significantly more Fos expression in rats injected with i.v. MT-II than in rats injected with i.v. vehicle (MT-II vs vehicle: *P = 0.0482; Fig. 3.6A.; Appx. Table A.). The distribution of Fos-positive cells within the NTS showed a higher density of Fos expression in the caudal part of the NTS (i.e. bregma -14 mm, Paxinos and Watson (1998)) but this did not achieve statistical significance (P = 0.3477; Fig. 3.6B.). The i.v. administration of TCMC-A01 did not significantly change the expression Fos in the NTS (TCMC-A01 vs vehicle: P > 0.9999; Fig. 3.6A.; Appx. Table A.).

3.3.2. Effect of intranasal MT-II on Fos expression

In rats intranasally administered with 1 μg or 30 μg of MT-II no significant changes in Fos expression were detected in oxytocin neurones

(MT-II 1 μg vs vehicle: P = 0.1875, MT-II 30 μg vs vehicle: P = 0.2338) and non-labelled, putative vasopressin neurones (MT-II 1 μg vs vehicle: P = 0.7740, MT-II 30 μg vs vehicle: P > 0.9999) of the SON (Fig. 3.7A.; Appx. Table B.), and oxytocin neurones (MT-II 1 μg vs vehicle: P > 0.9999, MT-II 30 μg vs vehicle: P = 0.3196) and non-labelled neurones (MT-II 1 μg vs vehicle: P > 0.9999, MT-II 30 μg vs vehicle: P = 0.3196) of the PVN (Fig. 3.7B.; Appx. Table B.).

Intranasal administration of MT-II did not significantly change Fos expression in the AOB (MT-II 1 μg vs vehicle: P = 0.9642, MT-II 30 μg vs vehicle: P > 0.9999; Appx. Table B.) or cell layers of the main olfactory bulb, i.e. the MCL (MT-II 1 μg vs vehicle: P = 0.8981, MT-II 30 μg vs vehicle: P > 0.9999; Appx. Table B.) and EPL (MT-II 1 μg vs vehicle: P > 0.9999, MT-II 30 μg vs vehicle: P > 0.9999; Fig. 3.8A,B.; Appx. Table B.).

3.3.3. Effect of i.c.v. melanocortin antagonist administration on i.v. MT-II-induced Fos expression

To determine whether Fos expression induced by MT-II was facilitated by activation of central melanocortin receptors, the non-selective melanocortin antagonist SHU-9119 was injected centrally (i.c.v.) before systemic injection of MT-II (i.v.). The SON of rats injected with the melanocortin antagonist had significantly less Fos expression in oxytocin (**P = 0.0018), and putative vasopressin (*P = 0.0214) neurones than rats injected with vehicle (Fig. 3.9A.; Appx. Table C.), and there was a lower proportion of Fos-positive oxytocin neurones in the SON (***P = 0.0002; Fig. 3.10A.; Appx. Table C.).

In the PVN, there was significantly less MT-II-induced Fos expression in both magnocellular (**P = 0.0074) and parvocellular (*P = 0.0379) oxytocin neurones in rats injected with i.c.v. antagonist (Fig. 3.9B.; Appx. Table C.). There was also less Fos expression in magnocellular (P = 0.0941) and parvocellular (P = 0.2256) non-oxytocin neurones, however, this did not achieve statistical significance (Fig. 3.9B.; Appx. Table C.). The proportion of oxytocin neurones that expressed Fos in rats injected with the antagonist was significantly lower in both magnocellular (**P = 0.0016; Fig. 3.10B.; Appx. Table C.) and parvocellular neurones of the PVN (**P = 0.0036; Fig. 3.10B.; Appx. Table C.).

3.3.3.1. Fos expression in the NTS

In the NTS, i.c.v. administration of the antagonist did not change Fos expression induced by MT-II (P = 0.3104; Fig. 3.6D.; Appx. Table C.).

3.4. DISCUSSION

This study was designed to test whether i.v. or intranasal administration of the melanocortin agonist MT-II activates oxytocin neurones in pentobarbital anaesthetized rats. I demonstrate that i.v., but not intranasal, administration of MT-II effectively induces the expression of Fos protein in oxytocin neurones of the SON and the PVN. MT-II-induced Fos was attenuated by prior i.c.v. administration of the $MC_{3/4}$ antagonist SHU-9119. No significant changes in Fos expression were observed in the circumventricular organs SFO and OVLT which are known to monitor peripheral signals and modulate oxytocin and vasopressin neurone activity.

In contrast, a significant increase in Fos expression was detected in the NTS which project to the PVN and SON, and modulate oxytocin and also vasopressin neurone activity.

The naturally occurring melanocortin α-MSH has been shown to modulate the activity of magnocellular neurones, as shown by increased expression of Fos in PVN and SON neurones when applied centrally (Olszewski *et al.*, 2001; Sabatier *et al.*, 2003a). Similarly, central administration of the α-MSH analogue, MT-II, increased the expression of Fos in neurones of the SON and PVN (Thiele *et al.*, 1998). This action is mediated, at least in part, by activation of MC_4 which is expressed in the SON and PVN (Kishi *et al.*, 2003). Furthermore, activation of MC_4 by α-MSH has been reported to initiate the ERK-c-fos signalling cascade in hypothalamic rat neurones (Ramirez *et al.*, 2015). Fos protein has been widely used as a marker for neural activity in neuroendocrine cells. In the magnocellular neurones, a large body of evidence has led to the assumption that Fos expression reflects well the neurosecretory activity of these cells. After stimulation, *c-fos* mRNA is rapidly expressed in neurones reaching a peak within 30 min, and its product Fos between 60 to 90 min, remaining stable for 2 – 5 h (Hoffman *et al.*, 1993; Kovacs, 2008).

A limitation for the peptide delivery into the CNS is the BBB which is known to restrict the passage of α-MSH (Wilson *et al.*, 1984; Wilson, 1988). However, the cyclic and short amino acid sequence of MT-II is thought to result in higher brain penetration when compared to α-MSH (King *et al.*, 2007). MT-II has been extensively used to study central and peripheral melanocortin actions, and some physiological effects induced by systemic

administration of MT-II are believed to be, at least in part, mediated by activation of central receptors (Giuliano *et al.*, 2006; Klenerova *et al.*, 2008).

My results show that i.v. injection of MT-II (1 mg/kg) markedly induced the expression of Fos in both oxytocin and non-labelled, putative vasopressin, magnocellular neurones of the SON and PVN. Similarly, a recent study has shown that i.p. injection of MT-II (10 mg/kg) induced the expression of EGR-1, another immediate early gene, in oxytocin neurones of the PVN in prairie voles (Modi *et al.*, 2015). Conversely, Trivedi *et al.* (2003) did not detect changes in Fos expression in the SON and PVN in response to i.v. MT-II. This discrepancy may be related to the different experimental conditions of Trivedi *et al.* (2003) study, namely, use of conscious animals, the time (2 h) between i.v. injection and perfusion, as well as possible differences in cell counting since this information was not provided.

In contrast to the effect of MT-II, the i.v. administration of the melanocortin agonist TCMC-A01 did not significantly change Fos expression in the PVN and SON in this study. TCMC-A01 is an experimental melanocortin with theoretical BBB-penetrating capacities designed to bind $MC_{3/4}$ (Dr M. Callahan, personal communication). So here, I administered TCMC-A01 and MT-II at the same doses in order to compare their effects on Fos expression. Thus, this result suggests that after systemic administration TCMC-A01 cannot penetrate the BBB in neuroactive amounts. I then further analysed Fos expression in brain areas lacking the BBB (and therefore, exposed to high concentration of circulating TCMC-A01), and brain areas receiving peripheral inputs (i.e. NTS). However, TCMC-A01 also failed to induce any significant change in Fos expression in circumventricular organs

of the AV3V and the NTS. Thus, the use of TCMC-A01 in further experiments was discarded.

Since TCMC-A01 is currently under development, additional experiments are required to determine whether TCMC-A01 is able to induce activation of magnocellular neurones, and therefore, validate its systemic use (at higher doses than the used here). These experiments may include *in vitro* receptor binding, and receptor activation studies that will allow estimating physiologically active doses to be administered. The direct (central) administration of different doses of TCMC-A01 should be considered, as this route avoids the restriction of the BBB to reach central receptors, and therefore, it is possible to determine dose-dependent effects, similarly as it has been shown for MT-II after i.c.v. administration (Thiele *et al.*, 1998).

I did not detect changes in Fos expression in the cAMG in response to i.v. administration of MT-II, and TCMC-A01. Conversely, Thiele *et al.* (1998) have shown that i.c.v. administration of 1 μg, but not 0.1 μg, of MT-II induces Fos expression in the cAMG, indicating a dose-dependent effect. Therefore, it is possible that following i.v. administration, MT-II does not reach the cAMG in enough amounts to trigger Fos expression. As Thiele *et al.* (1998) administered MT-II i.c.v., and consequently, reaches other brain structures, it is not possible determine whether the effect reported was mediated by direct activation of MC_4 or indirectly by activation other systems that, in turn, activate cAMG neurones. In this context, for example, central administration of melanocortin agonists could induce dendritic release of oxytocin (Sabatier *et al.*, 2003a) that might act on OTR of the amygdala. Remarkably, oxytocin administration has been reported to induce changes in Fos expression in the amygdala (Ferguson *et al.*, 2001).

Systemic injection of radiolabelled (125)Iodo-MT-II has been reported to penetrate the brain parenchyma in low amounts. However, high densities of (125)Iodo-MT-II was bound to the circumventricular organs (Trivedi *et al.*, 2003). This raised the question whether MT-II-induced Fos in SON and PVN neurones was a direct effect or mediated indirectly by other brain structures. The SFO and OVLT are circumventricular organs in which neurones can monitor and sense compounds, including peptides, present in the bloodstream (Mimee *et al.*, 2013; Ufnal & Skrzypecki, 2014; Cancelliere *et al.*, 2015) to, in turn, modulate systemic and central vasopressin and oxytocin release by neuronal pathways that connect them with the SON and PVN (Miselis *et al.*, 1979; Boudaba *et al.*, 1995; Leng *et al.*, 1999). Interestingly, both the SFO and the OVLT express MC_4 (Kishi *et al.*, 2003).

The analysis of the OVLT and SFO did not show any significant change in Fos expression induced by systemic MT-II. However, there was a significant increase in Fos expression in neurones of the NTS (protected by the BBB), which also expresses MC_4 (Kishi *et al.*, 2003). *In vitro* electrophysiology studies have shown that direct application of α-MSH has excitatory and inhibitory effects on different subsets of NTS neurones (Mimee *et al.*, 2014). However, the NTS receives visceral inputs through the vagus nerve to modulate gastrointestinal and cardiorespiratory functions, and melanocortins have been shown to be involved in modulating these functions (Rinne *et al.*, 2012; Campos *et al.*, 2014), so it may be that activation of peripheral receptors in response to i.v. MT-II administration contributed to the increased Fos expression in the NTS.

The analysis of sub-regions of the NTS showed a higher density of Fos in the caudal part of the NTS which is known to contain noradrenaline

neurones that send excitatory projections from the A2 region of the NTS to the SON and PVN (Onaka *et al.*, 1995). Furthermore, it has been shown that Fos expression in magnocellular oxytocin neurones can be indirectly triggered by activation of vagal inputs via brainstem (Verbalis *et al.*, 1991; Antonijevic *et al.*, 1995). Hence, possible contributions from the NTS to the neural activity of magnocellular neurones observed after systemic MT-II should not be dismissed.

In the second experiment, I investigated whether blockade of central melanocortin receptors prevents the central effects of systemically administered MT-II. This showed that i.c.v. administration of the melanocortin antagonist before i.v. administration of MT-II reduced Fos expression induced by MT-II in oxytocin neurones of the SON and PVN, consistent with the conclusion that the increase in Fos expression was mediated by activation of central receptors. However, it has been reported that in a neuronal population of the arcuate nucleus, the antagonist used induces hyperpolarization, and consequently, decrease the excitability of this neuronal phenotype (Smith *et al.*, 2007). The melanocortin antagonist SHU-9119 is structurally related to MT-II, competing for $MC_{3/4}$ (Hruby *et al.*, 1995; Yang *et al.*, 2002), and has been extensively used to reverse MT-II actions (Fan *et al.*, 1997; Wessells *et al.*, 2003; Rinne *et al.*, 2012). The lack of a significant effect on Fos expression in NTS neurones after i.c.v. administration of SHU-9119 indicates that when MT-II is systemically administered, it acts on peripheral targets which are subsequently signalled to the NTS. Hence, the effects of MT-II described in this study appear to be mediated by a combination of central and peripheral effects.

The antagonist also reduced Fos expression in oxytocin-negative neurones in the magnocellular regions of the PVN. These are likely to be magnocellular vasopressin neurones, but the subregions of the PVN are not entirely homogeneous and contain some parvocellular neurones. However, the SON contains only magnocellular neurones, so here, the attenuation of Fos expression in non-oxytocin neurones can be definitively associated with magnocellular vasopressin neurones. Consistently, the expression of MC_4 mRNA has been described predominantly, but not only, in areas densely populated by magnocellular oxytocin neurones in both the PVN and SON (Kishi *et al.*, 2003; Siljee *et al.*, 2013). In addition, MC_4 expression has also been found in parvocellular subregions of the PVN (Kishi *et al.*, 2003; Liu *et al.*, 2003).

The intranasal administration of drugs has been proposed as an alternative route that can bypass the BBB to deliver drugs directly into the brain (Dhuria *et al.*, 2010), however, this is very controversial (Leng & Ludwig, 2016; Quintana & Woolley, 2016; Walum *et al.*, 2016). The BBB bypassing is theoretically achieved by endocytosis of compounds by olfactory or trigeminal neurones, which are then retrogradely transported and released into the brain (Johnson *et al.*, 2010). Consistent with this, changes in gene expression and behaviour following intranasal administration of melanocortin receptor ligands have been reported (Serova *et al.*, 2013, 2014). Here, I found that MT-II administered intranasally at two different doses did not induce any changes in Fos expression either in the olfactory bulbs, or in the PVN and SON. Similarly, Ludwig *et al.* (2013) reported that intranasal administration of equimolar doses of vasopressin and oxytocin failed to trigger Fos expression, suggesting that at the doses

administered these peptides do not reach the CNS in neuroactive amounts. Conversely, it has been reported that low molecular weight compounds, such us lidocaine, can be uptaken by the trigeminal nerve from the nose, and transported into the brain, reaching deep structures, including the brainstem, cerebellum, and spinal cord (Johnson *et al.*, 2010). However, specific transport mechanisms have not been reported. It seems unlikely that indiscriminate substances can use this entry route, as noxious agents present in the air could also get easy access to the brain. Exactly why MT-II failed to induce changes in Fos expression in this experiment is unknown, but it is possible to conjecture that following intranasal administration, MT-II suffered enzymatic degradation, as the nasal mucosa is rich in peptidase and protease enzymes (Sarkar, 1992).

Expression of $MC_{3/4}$ mRNA (Roselli-Rehfuss *et al.*, 1993; Gelez *et al.*, 2010) have been detected in the rat olfactory bulb, and high α-MSH content extracted from olfactory bulb tissue has also been reported (Loh *et al.*, 1979), suggesting that this region is a target for melanocortins. To my knowledge, there are no previous studies about the effect of melanocortins on neural activity in the olfactory bulb. Here, I report that intranasal administration of MT-II failed to induce changes in Fos expression in layers of the main and accessory olfactory bulb, suggesting that the receptor is not associated with Fos expression, or that MT-II does not reach the olfactory bulb in physiologically significant amounts. Consequently, the central (i.c.v. or intraparenchymal) administration of MT-II should be critically considered to elucidate whether activation of (putative) $MC_{3/4}$ effectively trigger Fos (neural activity) in the olfactory bulb.

Since the olfactory bulb is the first neural structure adjacent to the site of administration, I inferred that high concentrations of MT-II could be reached in the olfactory bulb (which project outside of the BBB through the peripheral olfactory nerves). However, whether MT-II can reach this neural structure still remains intriguing. Other compounds, including lidocaine, a low molecular weight anaesthetic, has been detected in high concentrations in the rat olfactory bulb following intranasal administration (Johnson *et al.*, 2010). Since lidocaine acts directly on neurones blocking sodium channels, it seems likely that, at least, some lidocaine may be uptake by peripheral olfactory sensory neurones, and then, transported or diffused into the olfactory bulb. Nevertheless, compounds with higher molecular weights, for example, radiolabeled insulin-like growth factor-I (IGF-I), and IRdye 800 fluorescent dye (Thorne *et al.*, 2004; Johnson *et al.*, 2010) have also been reported to reach the olfactory bulbs, but as for the trigeminal routes, no specific transport mechanisms have been reported. An extracellular route through intercellular clefts of the olfactory epithelium to the subarachnoid space has also been described using horseradish peroxidase administration (Balin *et al.*, 1986). The specific mechanisms by which these compounds (or even peptides) reach the olfactory bulb still remains to be determined.

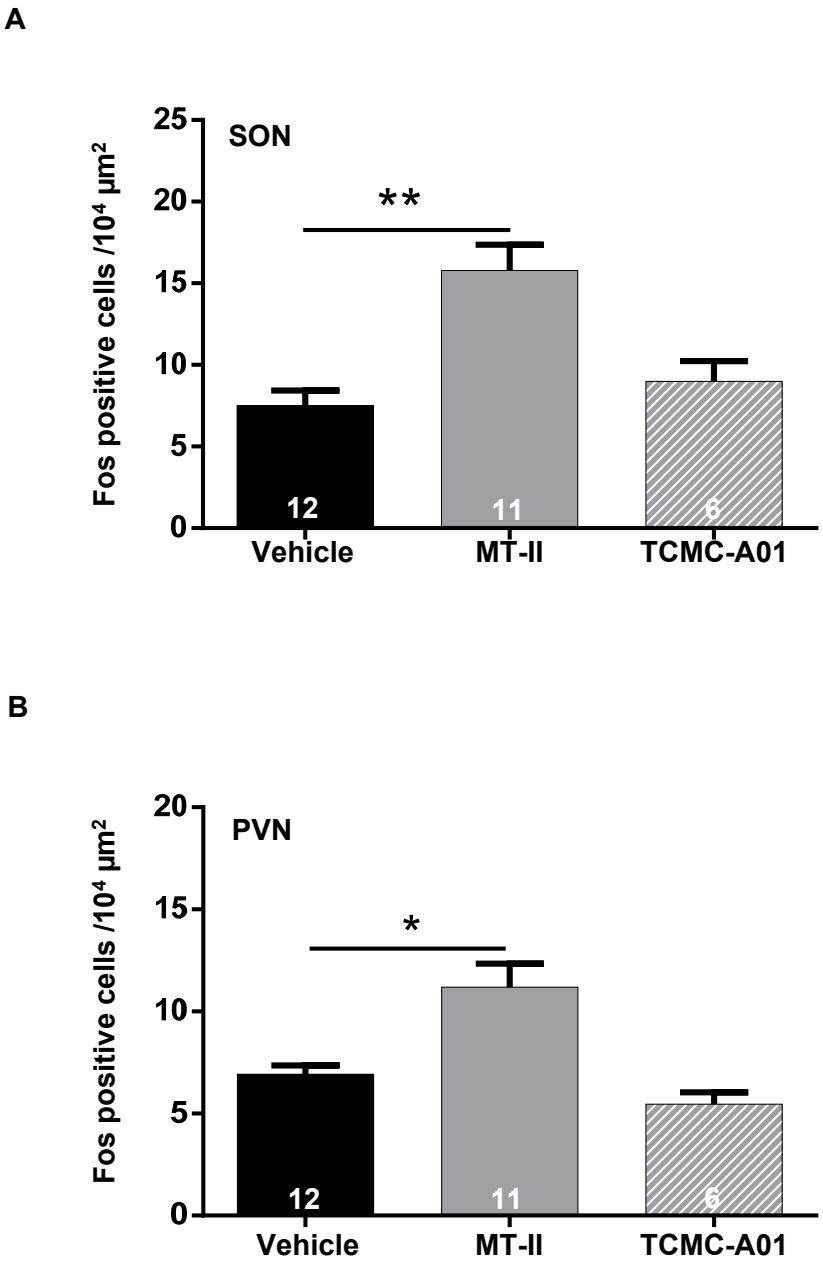

Figure 3.1. Fos expression in the SON and PVN after i.v. melanocortin administration. MT-II, but not TCMC-A01, significantly increased the density of Fos-positive neurones in the **(*A*)** SON, and **(*B*)** PVN. Mean + S.E.M. *P < 0.05, **P < 0.01; Kruskal-Wallis test followed by Dunn's multiple comparison test. Numbers of animals is shown in bars.

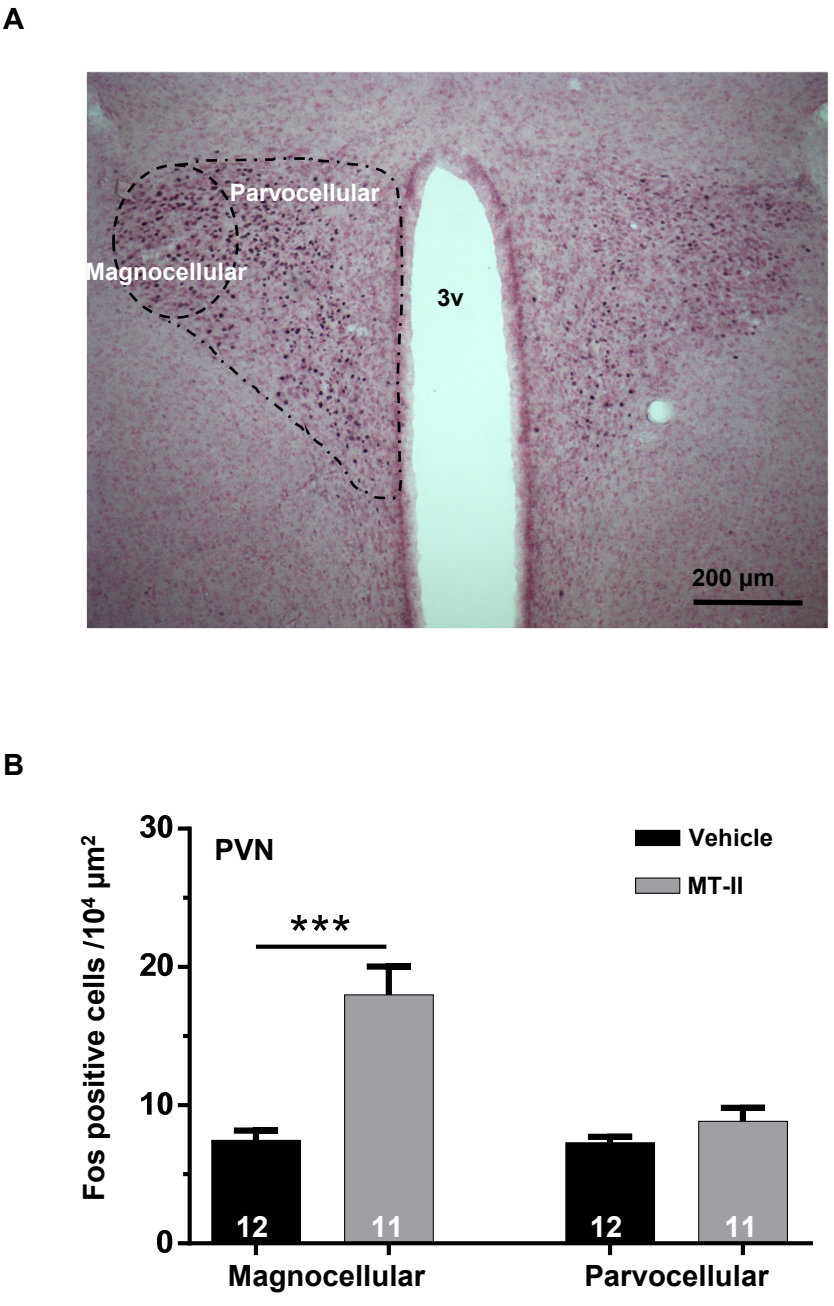

Figure 3.2. Fos expression in subpopulations of the PVN after i.v. MT-II. (*A*) Representative section showing immunohistochemistry for Fos expression in the magnocellular and parvocellular regions of the PVN in a MT-II injected rat. **(*B*)** Density of Fos-positive neurones within the subpopulations of the PVN. Mean + S.E.M. ***P < 0.001; Mann-Whitney test. 3v - third ventricle.

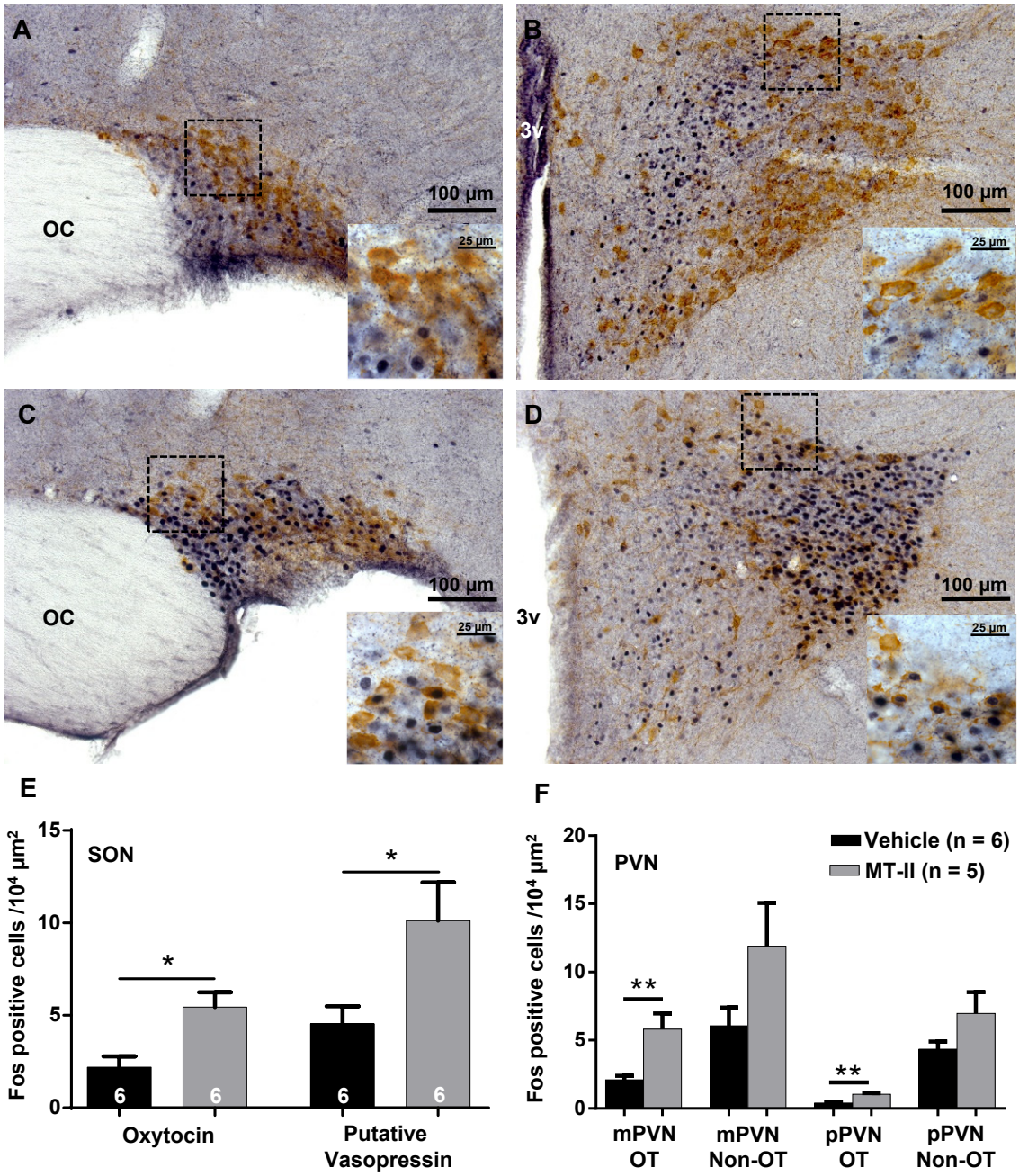

Figure 3.3. Fos expression in oxytocin neurones of the SON and PVN after i.v. MT-II. Representative examples of coronal sections of the hypothalamic (***A,C***) SON and (***B,D***) PVN immunostained for Fos and oxytocin of (***A,B***) vehicle (saline 0.9 %) and (***C,D***) MT-II injected rats. Density of Fos-positive oxytocin neurones and putative vasopressin neurones in the (***E***) SON, and Fos-positive oxytocin neurones and non-oxytocin neurones in subpopulations of the (***F***) PVN. Mean + S.E.M. *P < 0.05, **P < 0.01; Mann-Whitney test. Numbers of animals is shown in bars. OC - optic chiasm, 3v - third ventricle.

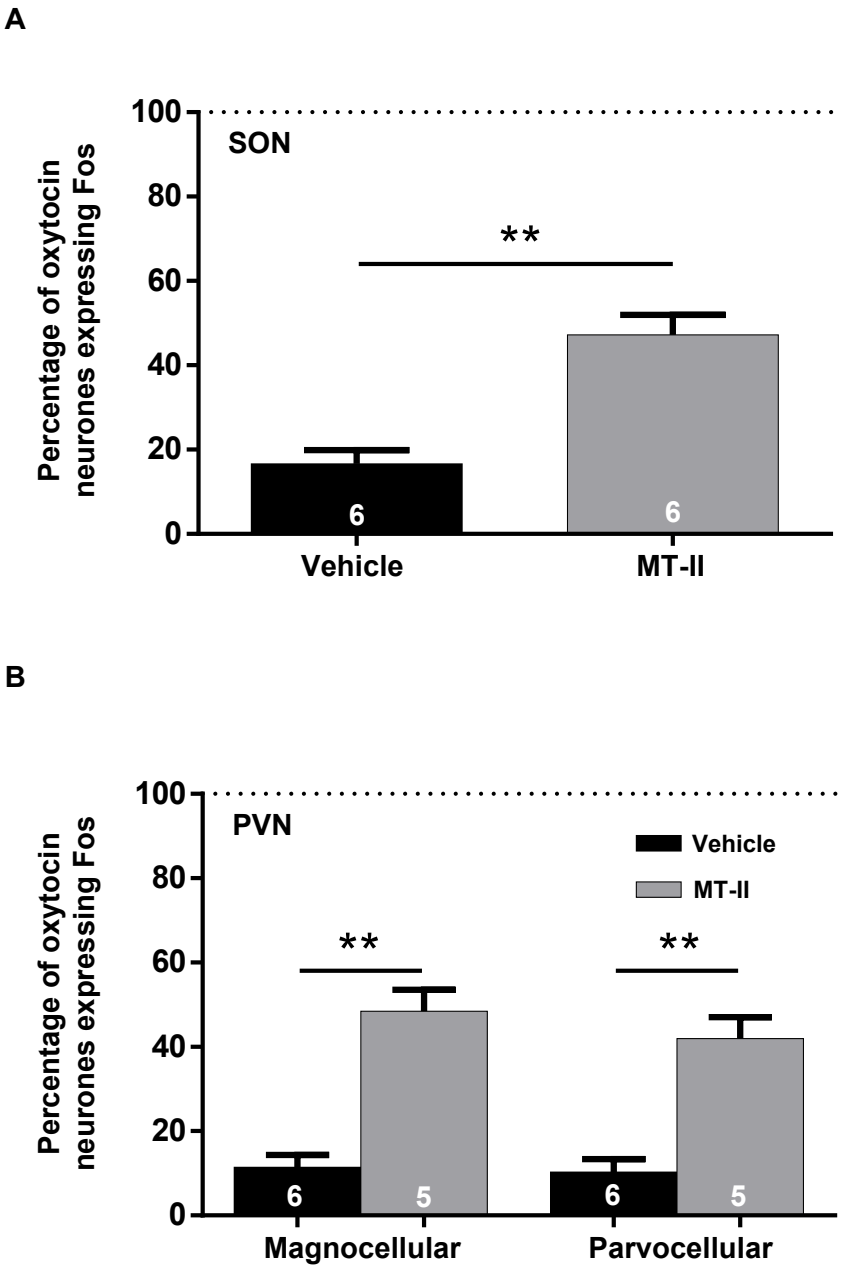

Figure 3.4. Proportion of oxytocin neurones expressing Fos in the SON and PVN after i.v. MT-II. (*A*) MT-II significantly increased the number of Fos-positive neurones within the oxytocin population in the **(*A*)** SON, and in magnocellular and parvocellular regions of the **(*B*)** PVN. Means + S.E.M. **P < 0.01; Mann-Whitney test.

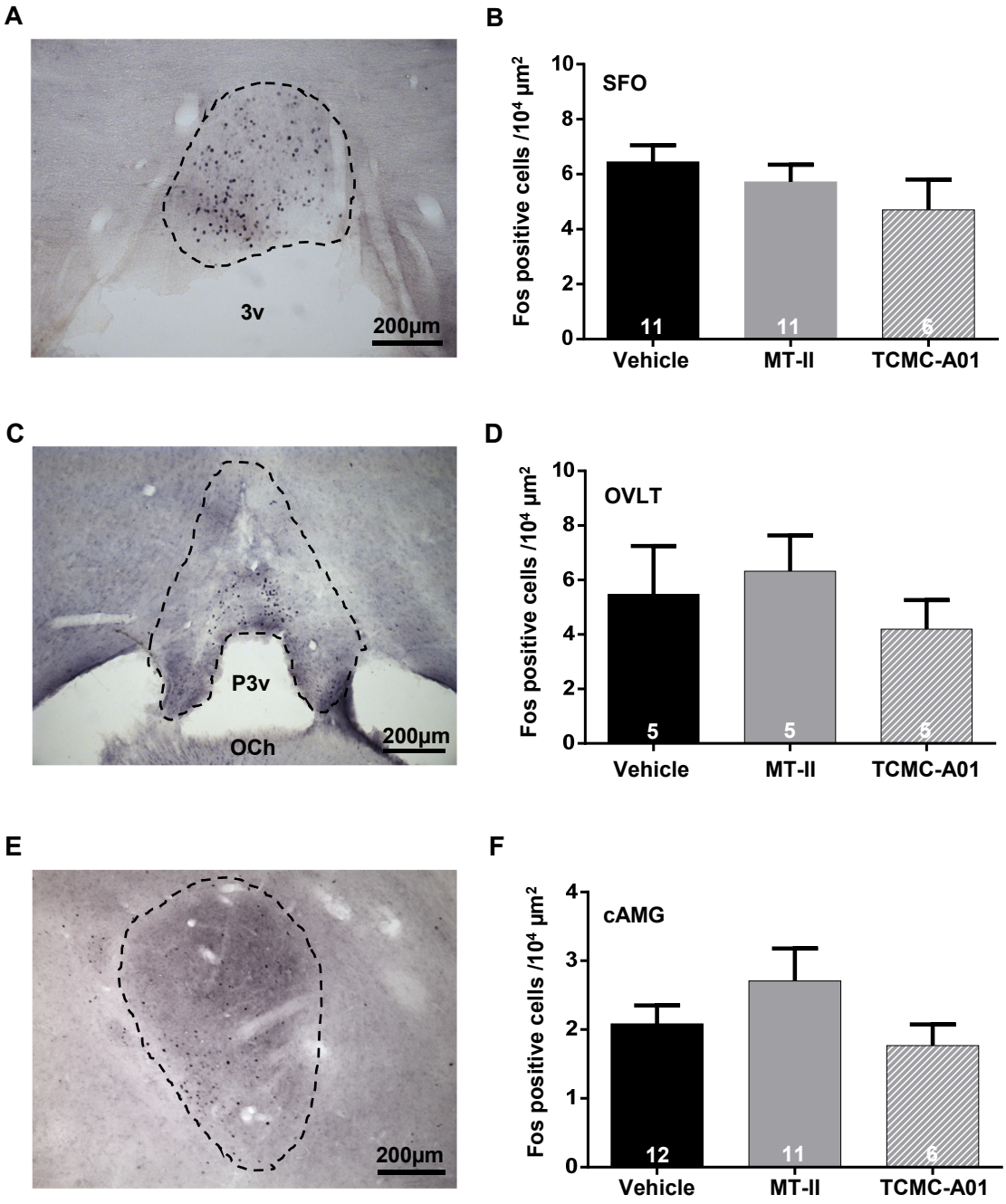

Figure 3.5. Fos expression in the SFO, OVLT and cAMG in response to i.v. melanocortin administration. Representative examples of the areas analysed in the **(*A*)** SFO, **(*C*)** OVLT, and **(*E*)** cAMG in a vehicle-injected rat. No significant changes in Fos expression in the **(*B*)** SFO, **(*C*)** OVLT, and **(*F*)** cAMG were detected in response to MT-II. Mean + S.E.M. Numbers of animals is shown in bars. 3v - third ventricle, P3v - preoptic recess of the third ventricle, OC - optic chiasm.

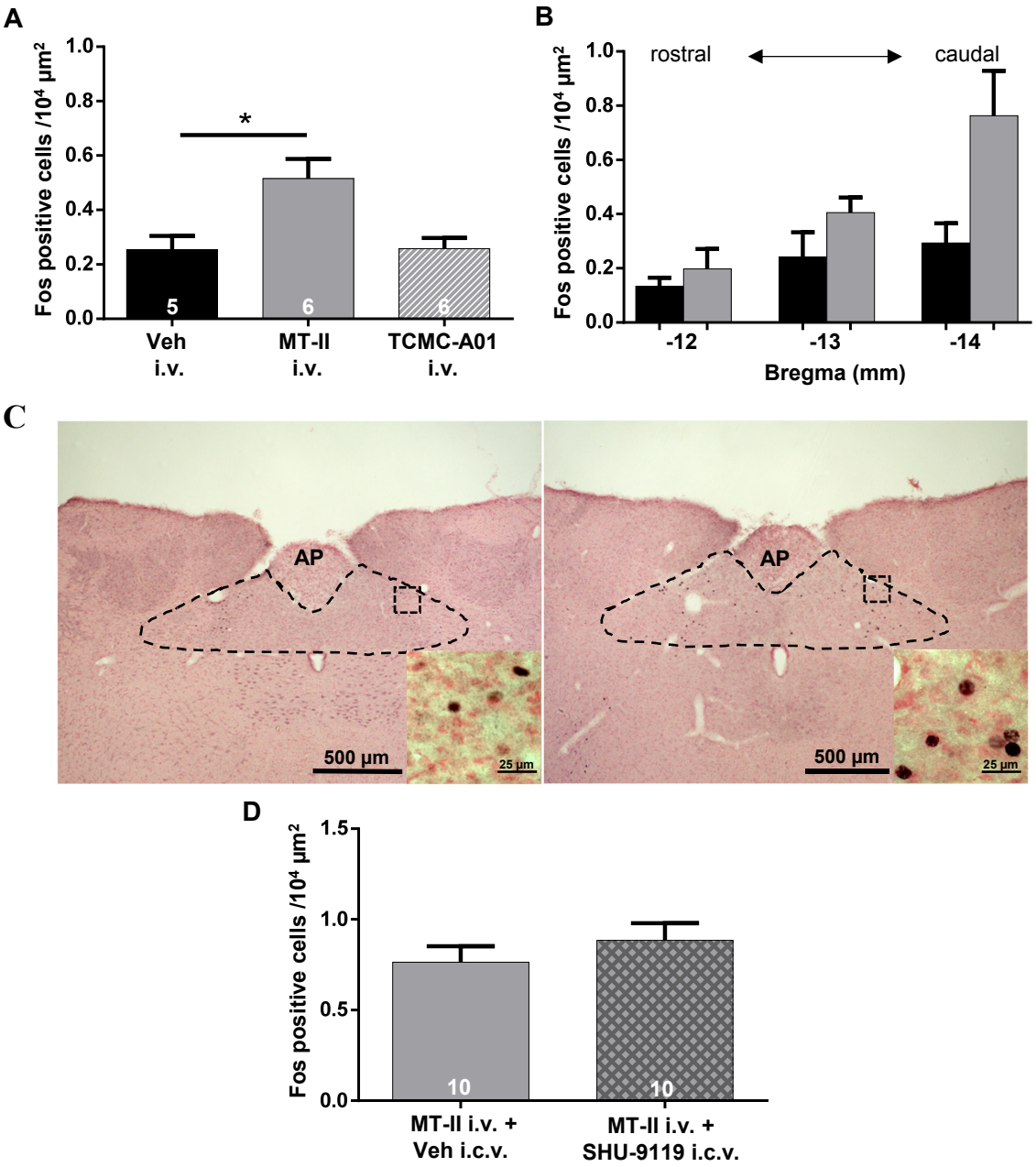

Figure 3.6. Fos expression in the NTS in response to i.v. melanocortin administration. MT-II, but not TCMC-A01, significantly increased the density of Fos expressing neurones in the **(*A*)** NTS, with a higher density of expression (not significant) in the **(*B*)** caudal NTS. **(*C*)** Representative examples of Fos expression in the caudal NTS (bregma -14.0 mm). **(*D*)** MT-II-induced Fos was not reduced by prior i.c.v application of the melanocortin antagonist SHU-9119. Mean + S.E.M. *P < 0.05; Kruskal-Wallis test followed by Dunn's multiple comparison test. Numbers of animals is shown in bars. Veh - vehicle, AP - area postrema.

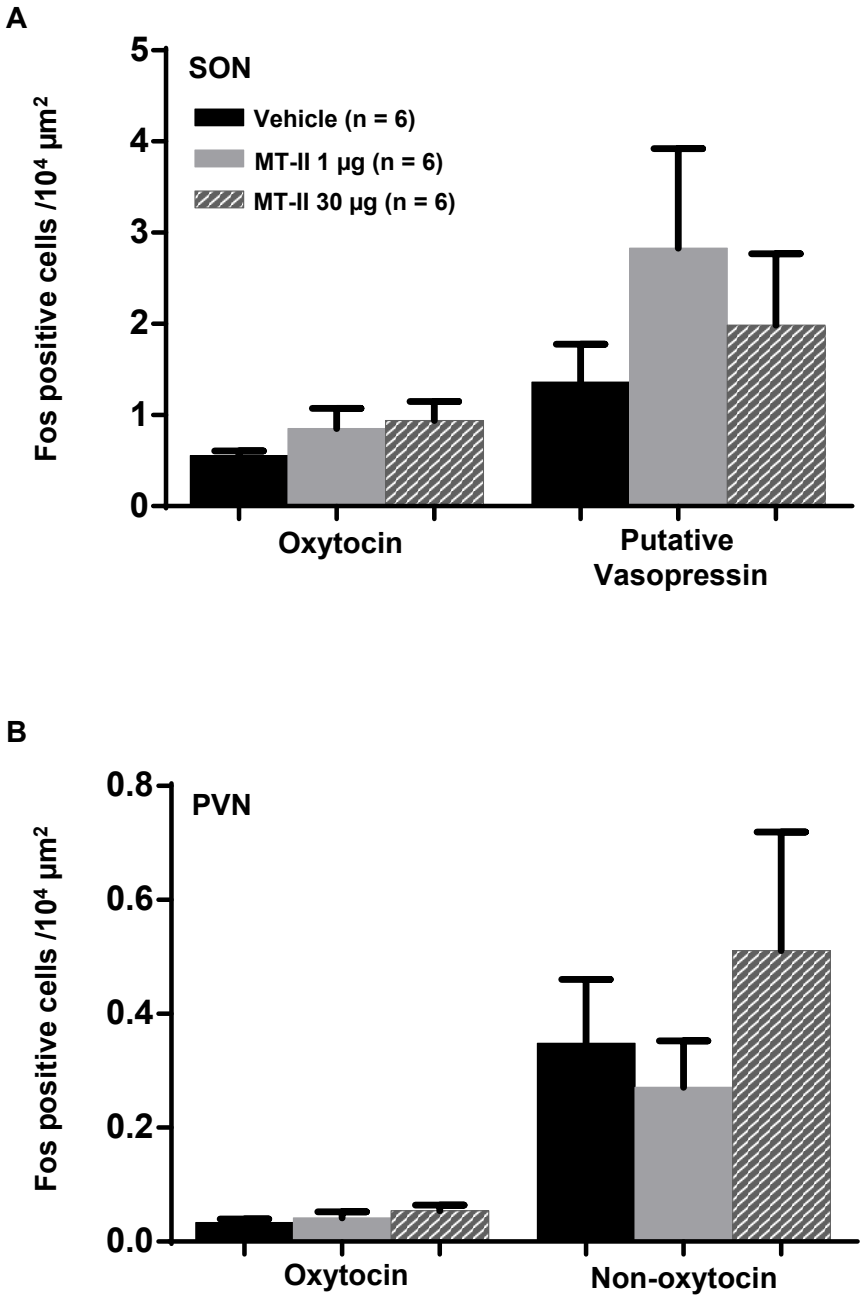

Figure 3.7. Fos expression in the SON and PVN after intranasal MT-II administration. Density of Fos-positive oxytocin neurones and putative vasopressin neurones in the ***(A)*** SON, and Fos-positive oxytocin neurones and non-oxytocin neurones in the ***(B)*** PVN. Means + S.E.M.

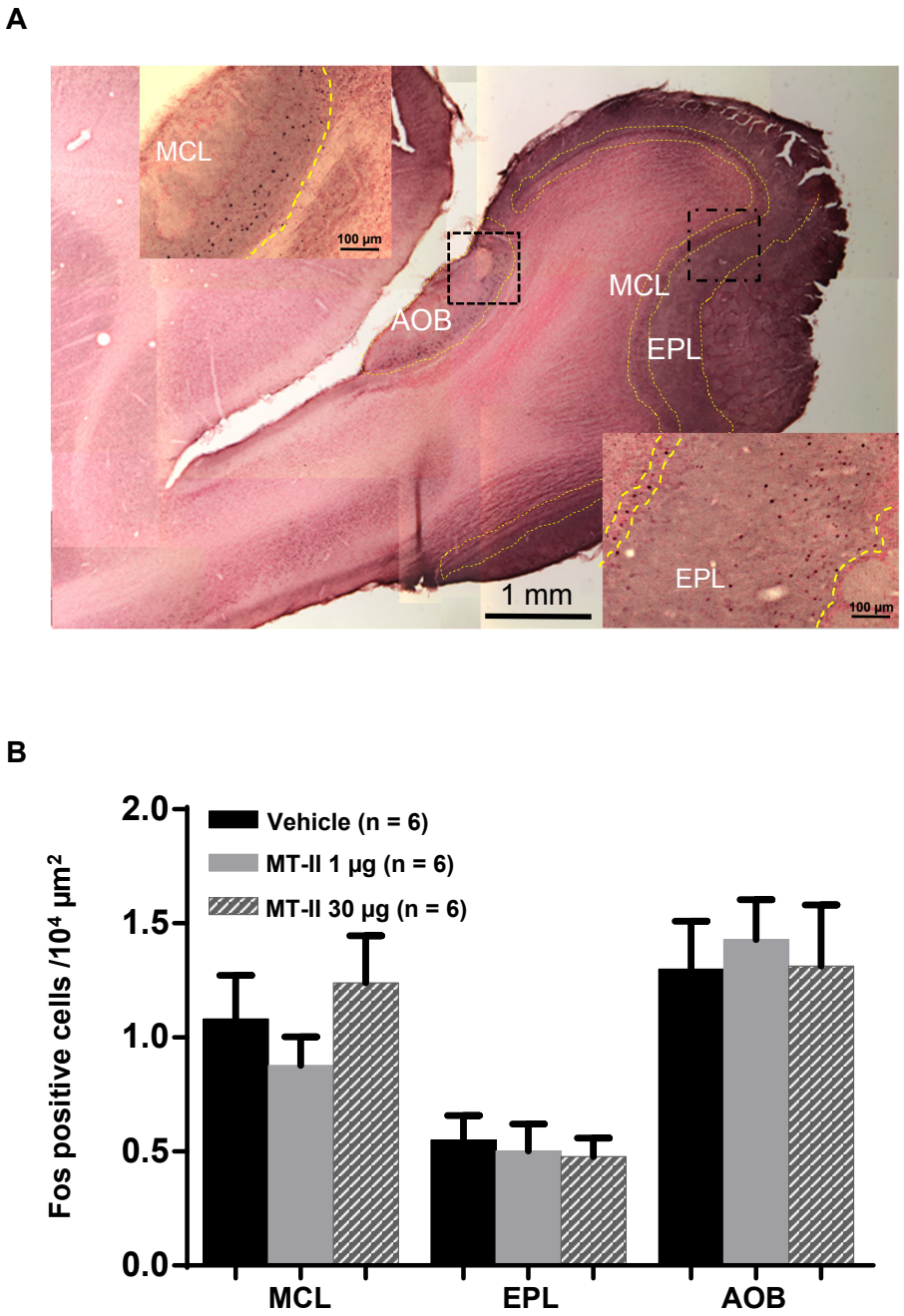

Figure 3.8. Fos expression in the olfactory bulb in response to intranasal MT-II administration. (*A*) Photomontage of a rat olfactory bulb stained for Fos and nuclear fast red in a vehicle-injected rat. **(*B*)** Number of Fos-positive neurones (per 10^4 µm^2) in layers of the olfactory system (per area) following low and high doses of intranasal MT-II administration. MCL - mitral cell layer, EPL - external plexiform layer, AOB - accessory olfactory bulb. Means + S.E.M.

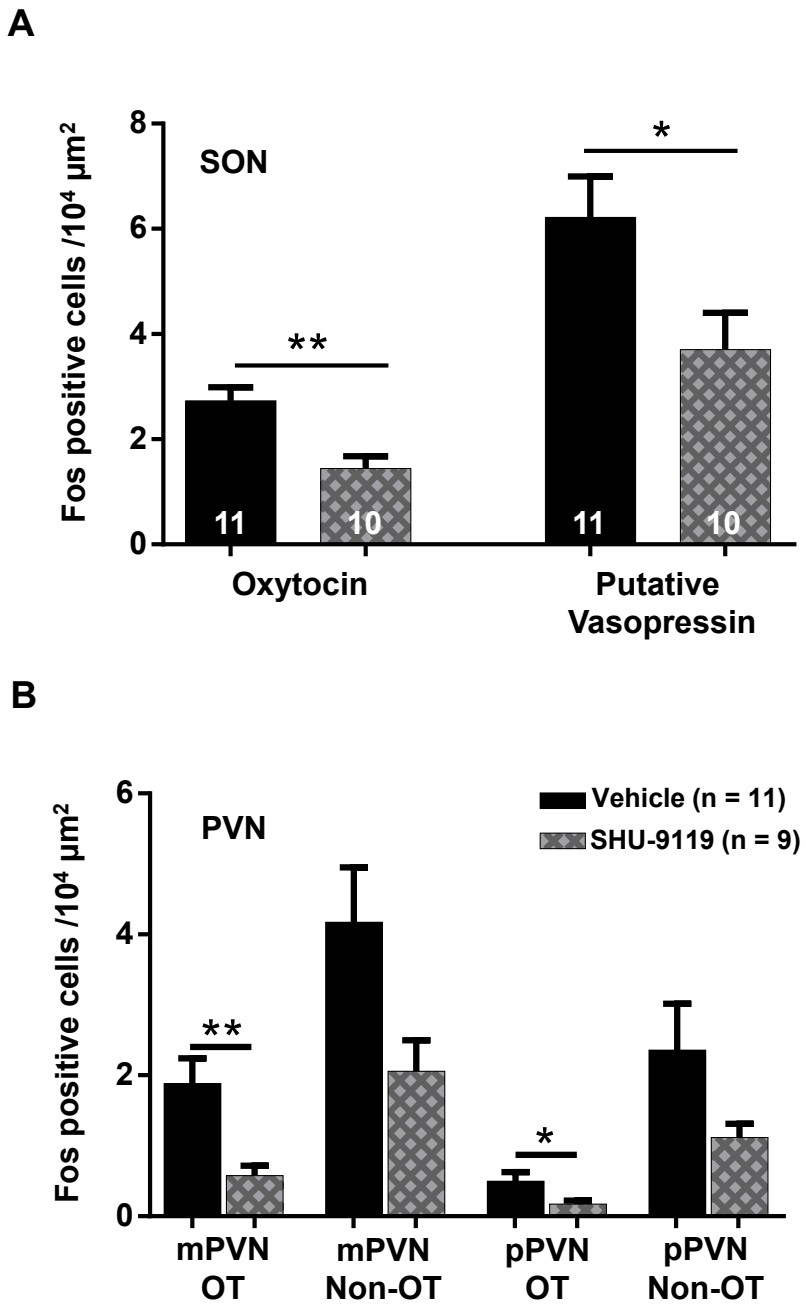

Figure 3.9. Effect of the melanocortin antagonist on MT-II induced Fos expression in the SON and PVN. Intracerebroventricular (i.c.v.) injections of the melanocortin antagonist, SHU-9119, reduced Fos expression induced by i.v. injected MT-II in the (***A***) SON and (***B***) subpopulations of the PVN. Mean + S.E.M. *P < 0.05, ** P < 0.01; Mann-Whitney test. Numbers of animals is shown in bars.

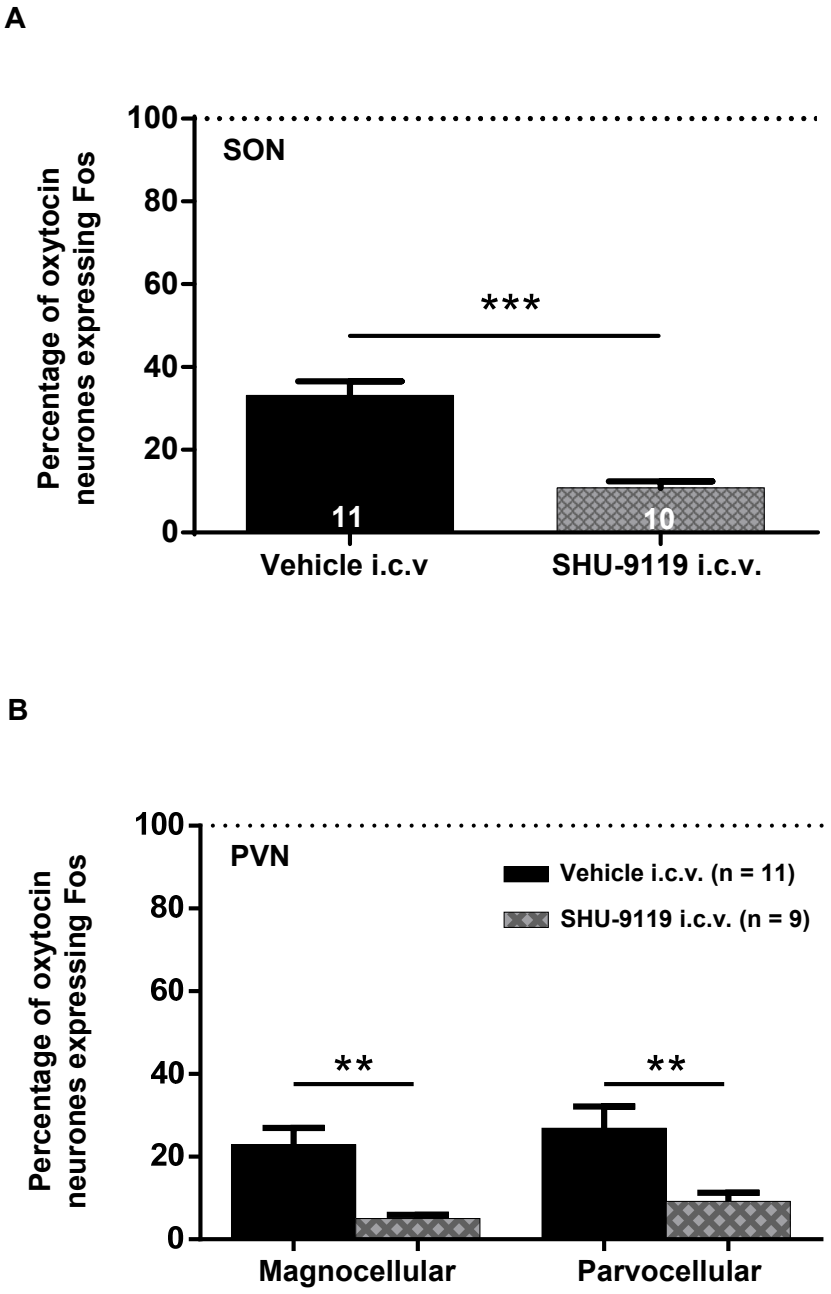

Figure 3.10. Effect of the melanocortin antagonist on the proportion of SON and PVN oxytocin neurones expressing Fos in response to i.v. MT-II. The proportion of oxytocin neurones expressing Fos in the ***(A)*** SON, and in magnocellular and parvocellular parts of the ***(B)*** PVN decreased by prior i.c.v. administration of SHU-9119. Means + S.E.M. **P < 0.01, ***P < 0.001; Mann-Whitney test.

Chapter 4

Changes in the electrical activity of supraoptic oxytocin and vasopressin neurones following intravenous administration of Melanotan-II

4.1. INTRODUCTION

In Chapter 3, I provided evidence showing that i.v. administration of MT-II affects SON oxytocin and vasopressin neurone activity as shown by increased expression of Fos protein, and this effect was attenuated by the prior i.c.v. administration of a non-selective $MC_{3/4}$ antagonist. It is commonly assumed that Fos expression reflects increased neuronal electrical activity (action potentials), but this is not always true (Luckman *et al.*, 1994; Kovacs, 2008), and direct application of the naturally occurring melanocortin, α-MSH, has been shown to inhibit the electrical activity of SON oxytocin neurones yet strongly induce Fos expression (Sabatier *et al.*, 2003a). Moreover, antidromically evoked action potentials do not induce Fos expression in SON neurones (Luckman *et al.*, 1994), showing that "if

depolarization *per se* induced its expression, *c-fos* should have been detected in millions of neurones throughout the brain under basal conditions" (Kovacs, 2008).

The magnocellular neurones of the rat SON, and also the PVN, exhibit spontaneous action potentials (spikes) that are driven by the interaction of intrinsic membrane properties, and afferent excitatory and inhibitory inputs (Li *et al.*, 2007; Brown *et al.*, 2013). These spikes are "stereotypically" fired in two discharge patterns. The continuous firing pattern exhibited by oxytocin and some vasopressin neurones is characterized by slow spike discharge occurring in irregular (slow irregular) or regular (fast continuous) patterns, and fired at a frequency (firing rate) of ~ 2 – 4 spikes/s and ~ 4 – 6 spikes/s, respectively. The phasic firing pattern is only exhibited by vasopressin neurones, and is characterized by periods of intense spike activity, reaching a rate of ~ 15 – 25 spikes/s during the first 10 s then decreasing to ~ 6 spikes/s for the rest of the activity period; these activity periods alternate with silence periods (i.e. non-spike activity) occurring at relatively regular intervals. The intervals of activity and silence periods last a minimum of 15 s and 10 s, respectively, and their duration is stochastically determined (Fig. 4.1.) (Poulain & Wakerley, 1982; Li *et al.*, 2007; Brown *et al.*, 2013).

The basal firing rate and discharge pattern of magnocellular neurones can be modified when a stimulus (e.g. osmolality) is applied to magnocellular neurones. For example, in rats, infusion of hypertonic saline increases the firing rate in both intrinsically osmosensitive magnocellular SON vasopressin and oxytocin neurones (Mason, 1980; Oliet & Bourque, 1993), as well as transiently modifying the firing pattern of phasic vasopressin neurones to a continuous firing pattern (Leng *et al.*, 2001).

Similarly, i.v. administration of peptides, including CCK (Leng *et al.*, 1991) has been shown to transiently increase the firing rate in oxytocin neurones, but has no or an inhibitory effect on vasopressin neurones. CCK is regularly used to identify and to distinguish between SON neurones. Interestingly, the changes in neuronal electrical "behaviour" can be even more marked under certain physiological conditions. During parturition and lactation, oxytocin neurones fire in a discharge pattern characterised by a brief (1 – 2 s) high frequency (firing rates of ~ 50 – 100 spikes/s) of coordinated bursts which are followed by periods of slow irregular background activity lasting ~ 5 min (Belin *et al.*, 1984; Belin & Moos, 1986). These changes in firing rate and pattern allow magnocellular oxytocin and vasopressin neurones to adapt peptide release to sustained physiological activation.

The electrical activity of magnocellular neurones is closely linked with oxytocin and vasopressin release from the nerve endings into the bloodstream, and consequently, the firing rate and discharge pattern are key factors that determine the amount of peptide peripherally released (MacGregor & Leng, 2013). When an action potential reaches the nerve endings, the opening of voltage-gated Ca^{2+} channels results in a Ca^{2+} influx that leads to the *classic* stimulus-secretion coupling (Nordmann, 1983). The spike-associated secretion is, theoretically, not only restricted to the nerve endings, as the characteristics of the somatodendritic cell membrane in some neuronal phenotypes support the propagation of the action potential to the dendritic tree (backpropagation) (Vetter *et al.*, 2001; Casale & McCormick, 2011). Nevertheless, the backpropagation in magnocellular neurones is not completely understood and in addition to action potentials, other factors

including changes in intracellular Ca^{2+} and the actin cytoskeleton, are also involved in the release of peptide-containing vesicles (Tobin *et al.*, 2012).

In this chapter, I study the effect of systemic administration (i.v.) of MT-II on the electrical activity of SON oxytocin and vasopressin neurones *in vivo* in urethane anaesthetized rats by using extracellular single-unit recordings of antidromically identified SON neurones. The use of *in vivo* electrophysiology allows monitoring dynamic changes in the activity of SON neurones accurately whereas preserving the physiological milieu, and therefore, the physiological significance of the responses of magnocellular neurones (Leng & Sabatier, 2014).

4.2. MATERIAL AND METHODS

The following experiments were performed under the direct supervision and assistance of Dr Nancy Sabatier.

4.2.1. *In vivo* preparation and electrophysiological recording

Adult male Sprague-Dawley rats weighing 350 g approximately were anaesthetized with an i.p. injection of urethane (1.25 g/kg); a femoral vein was cannulated for drug administration and an endotracheal tube inserted into the trachea to keep the airway opened. The rats were placed into a stereotaxic frame in supine position, and the right SON and the pituitary stalk were exposed transpharyngeally as previously described (Leng & Dyball, 1991; Leng & Sabatier, 2014). A glass micropipette (filled with 0.15 M NaCl, 20-40 MΩ) was introduced into the SON under direct visual control to record the extracellular activity of single neurones. A bipolar stimulating electrode (SNEX-200X, Clarke Electromedical Instruments, Reading, UK) was

placed on the pituitary stalk and set to deliver single matched biphasic pulses (1 ms, < 1 mA peak to peak) for antidromic identification of SON neurones. The neuronal electrical activity was amplified using an Axonpatch 200B amplifier connected to a CV203BU headstage (Molecular Devices Inc., Sunnyvale, CA, USA) and the signal filtered using a HumBug 50Hz noise eliminator (Quest Scientific Instruments Inc., North Vancouver, BC, Canada). The spikes were processed (Digitimer D130; Digitimer Ltd., Welwyn Garden City, UK) and interfaced (CED1401; Cambridge Electronic Design Ltd., Cambridge, UK) to a personal computer using Spike2 software ver. 7.03 (Cambridge Electronic Design Ltd., Cambridge, UK).

4.2.2. Identification of vasopressin and oxytocin SON neurones

SON neurones were identified by antidromic stimulation of the pituitary stalk which evokes an action potential in the axon that propagates to the cell body. Thus, an antidromic spike occurring at a constant latency (~ 10 ms for SON neurones) serve as a confirmation for a cell as a SON neurone (Fig. 4.2A.). The shape of interspike-interval histogram (which shows the distribution of the observed times between spikes) was also checked for characteristic firing patterns of SON vasopressin and oxytocin neurones; which is highly skewed to the left in vasopressin neurones as previously described (Leng *et al.*, 2001; Sabatier *et al.*, 2004); Fig. 4.2B.). Oxytocin neurones were also distinguished from vasopressin neurones by their discharge pattern (Fig. 4.1.) and by their opposite response to i.v. CCK (20 μg/kg; cholecystokinin-(26-33)-sulphated, Bachem Ltd., Saffron Walden, Essex, UK; Fig. 4.3.), i.e. transient excitatory response of oxytocin neurones and no effect or transient inhibition of vasopressin neurones (Sabatier *et al.*, 2004).

4.2.3. Recording protocol

After a 10 min period of stable baseline recording, CCK was injected i.v. and recordings were made until the electrical activity of the neurones returned to their previous basal condition. Then, after 20 – 30 min of recording under basal conditions, MT-II (1 mg/kg) was given i.v., and in most neurones, recordings were made for 40 min after MT-II injection. A vehicle (0.2 ml of saline 0.9 %) injection was given to determine potential effects of the i.v. injection itself on the SON neurone activity. In the majority of the experiments, only one neurone was tested with MT-II in each rat.

At the end of each experiment, the rats were killed by an overdose of sodium pentobarbital (160 mg/kg, i.v.).

4.2.4. Statistical analysis

The responses to MT-II were analysed by comparing the mean firing rate in 10-min intervals after MT-II injection with the basal firing rate measured in a 10–20-min control period before MT-II injection. A 10 % change in firing rate was set as a threshold to categorise the neurone as a responsive to MT-II. For analysis of individual responsive neurones the firing rate (in 30-s bins) in the 10-min before MT-II injection was compared with the rate in the 10-min after injection for oxytocin neurones, and the firing rate 20-min before MT-II was compared with 40-min after injection for vasopressin neurones because (in phasic neurones) burst of spikes are long and variable when analysed over short periods; these comparisons were tested for significance using a one-tailed paired t-test, accepting $P < 0.01$ as significant.

The mean change in firing rate (spikes/s) in the period 0 to 10 min (in 30-s bins) after the MT-II injection was tested for the entire population of

oxytocin neurones recorded to check whether this change was significantly different from 0 (baseline), using a two-tailed Wilcoxon signed rank test. For vasopressin neurones the period 0 to 40 min (in 10-min bins) after MT-II injection was tested; the longer binwidth was required because many vasopressin neurones fire phasically, so spike counts are very variable over short binwidths. $P < 0.05$ was considered significant.

4.3. RESULTS

4.3.1. Effect of i.v. MT-II on the electrical activity of oxytocin neurones

The electrical activity of oxytocin neurones was recorded in seven rats, only one rat was double tested with i.v. MT-II. Oxytocin neurones showed a consistent excitatory response to MT-II (Fig. 4.4.).

To analyse the effect of MT-II on the oxytocin population, the mean change in firing rate (in 30-s bins) over 10-min after MT-II administration of eight oxytocin neurones was tested. The mean basal firing rate of the eight oxytocin neurones before injection of MT-II was 2.67 ± 0.69 spikes/s. After injection of MT-II, the mean firing rate in these eight neurones was 3.34 ± 0.75 spikes/s, increasing 0.66 ± 0.16 spikes/s ($W = 36.00$, $Z = 2.521$, $^{**}P = 0.0078$, Wilcoxon signed rank test; Fig. 4.5A.) over the 10-min period after MT-II administration. This response reached a plateau between 2.5 and 8 min after injection, to then rapidly return to the mean basal firing rate (change in firing rate > +0.2 spikes/s) in the last 2 min.

The individual analysis of these neurones showed that the change in the firing rate induced by MT-II exceeded the threshold of 10 % in seven of the eight neurones (Fig. 4.5B.). In each of these seven responsive neurones, MT-II induced a significant increase in firing rate over the 10-min period

after injection (**P < 0.01, paired t-test). Two of these responsive neurones showed a strong and long-lasting increase in firing rate over a 40-min period after administration of MT-II (neurone 1 baseline: 0.11 ± 0.01, after MT-II 10 min: 0.19 ± 0.02, after MT-II 40 min: 2.33 ± 0.19 spikes/s; neurone 2 baseline: 0.10 ± 0.02, after MT-II 10 min: 0.94 ± 0.17, after MT-II 40 min: 1.53 ± 0.08 spikes/s). The non-responsive neurone exhibited a high baseline frequency cell which increased 0.27 ± 0.09 spikes/s (baseline: 5.50 ± 0.04, after MT-II 10 min: 5.77 ± 0.09 spikes/s) over the 10-min period after the injection.

4.3.2. Effect of i.v. MT-II on the electrical activity of vasopressin neurones

The electrical activity of vasopressin neurones was recorded in seven rats, only one rat was double tested with i.v. MT-II. A heterogeneous response to MT-II was observed in vasopressin neurones, only three of the neurones tested were responsive to MT-II (Fig. 4.6.).

To analyse the effect of MT-II on the vasopressin population, the mean change in firing rate (in 10-min bins) over 40-min after the administration of MT-II of three continuous- and five phasic-firing neurones was tested. The mean basal firing rate in the eight vasopressin neurones before MT-II injection was 6.61 ± 0.60 spikes/s. After MT-II injection, the mean firing rate in these eight neurones was 7.54 ± 0.51, increasing 0.93 ± 0.59 spikes/s over the 40-min period after injection (W = 20.00, Z = 1.400, P = 0.195, Wilcoxon signed rank test; Fig. 4.7A.).

The individual analysis of vasopressin neurones showed that after i.v. MT-II, the change in firing rate exceeded 10 % in only three of the eight neurones tested (Fig. 4.7B.), and of those three (one continuous- and two phasic-firing) responsive neurones, only one (continuous-firing) neurone

showed a significant increase in firing rate (**P < 0.01, paired t-test). Three phasic-firing neurones showed a transient inhibitory response (~ 2 min) to i.v. injection of MT-II. Two continuous-firing neurones showed a short transient inhibitory response to MT-II (~ 2 min) immediately after injection (Fig. 4.8A.), and also a very brief decrease in spike activity (8 spikes fired in 10 s) following i.v. injection of vehicle (Fig. 4.8B.).

4.4. DISCUSSION

This study was aimed to determine whether systemic administration of the synthetic $MC_{3/4}$ agonist, MT-II, affect the electrical activity of SON oxytocin and vasopressin neurones *in vivo* in urethane anaesthetized rats. I showed that after systemic administration of MT-II, the activity of oxytocin neurones was consistently increased in virtually all the neurones tested. In contrast, vasopressin neurones showed a heterogeneous response to MT-II, only one-third of the neurones tested were classified as responsive, showing an increase in neuronal activity.

The first evidence of the regulation of the electrical activity of SON oxytocin neurones by melanocortins was shown by Sabatier *et al.* (2003a), who reported that, in virgin rats, i.c.v. injection or retrodialysis application of the natural melanocortin, α-MSH, decreased the firing rate of oxytocin neurones, these actions are thought to be mediated by activation of MC_4, as the administration of the high-affinity MC_4 agonist, MRLOB-0005, exerted similar effects in a dose-dependent fashion. Similarly, retrodialysis application of α-MSH (7.5 µg/3 µl/min) decreased the firing rate of SON oxytocin neurones by 50 % in non-pregnant rats, however, it did not affect the response of oxytocin neurones in pregnant rats (Ladyman *et al.*, 2016).

Since i.v. injection of the α-MSH analogue, MT-II, triggered Fos expression in oxytocin neurones which was prevented by the prior central administration of a non-selective melanocortin antagonist (see Chapter 3), I assumed that MT-II directly acts on oxytocin neurones, exerting similar effects on neuronal electrical activity as i.c.v. α-MSH. In contrast, my electrophysiology results show that after i.v. injection of MT-II, the firing rate of the SON oxytocin neurones was transiently increased, suggesting that the effect of MT-II on electrical activity is predominantly driven by indirect pathways. Interestingly, two slow-firing oxytocin neurones showed a long-lasting activation after i.v. MT-II that continued for over 40 min. It is unknown why these neurones displayed this activation pattern, however, I can conjecture that differences in synaptic inputs or intrinsic neurone properties may contribute to this neuronal behaviour.

In this context, afferent inputs projecting to the SON might increase excitatory post-synaptic currents (EPSCs) or decrease post-synaptic inhibitory currents (IPSCs). Glutamate and gamma-aminobutyric acid (GABA) are the main presynaptic inputs to the SON, playing a major role exciting or inhibiting SON neurones, respectively (Brown *et al.*, 2013). The main sources of GABA inputs to the SON are thought to originate from perinuclear neurones located dorsally to the SON (Theodosis *et al.*, 1986), and also from the OVLT (Kolaj *et al.*, 2000). Excitatory glutamate afferents inputs to the SON originate from the OVLT, as shown by pharmacological evidence indicating glutamate release following electrical stimulation of the OVLT (Yang *et al.*, 1994), and this circumventricular organ also expresses MC_4 (Kishi *et al.*, 2003). In addition, noradrenaline inputs from the caudal brainstem have been reported to establish synaptic contacts with both

oxytocin and vasopressin neurones of the SON to induce depolarization of the membrane potential by decreasing the frequency of IPSCs, and consequently, increasing the firing rate of SON neurones (Shibuya *et al.*, 2000).

In other systems, melanocortins have been shown to induce similar, and opposite actions as the described in this preparation. For example, an *in vitro* electrophysiology study has shown that bath application of MT-II (n = 11), or α-MSH (n = 13) increased the firing rate of mice MC_4-green flourescent protein (GFP) PVN neurones which co-express oxytocin/vasopressin, and also CRH, and this effect was blocked by the administration of the $MC_{3/4}$ antagonist SHU-9119 (Ghamari-Langroudi *et al.*, 2011). Consistently with this, i.c.v. administration of MT-II significantly increased the firing rate of PVN leptin-responsive neurones (n = 11 out of 12) *in vivo* (Zhang & Felder, 2004), but the identity of these neurones was not determined. In contrast, Fong and Van der Ploeg (2000) reported that administration of α-MSH inhibited the firing rate of PVN neurones (n = 5) in a dose-dependent manner *in vitro*. Furthermore, in non-peptidergic systems, i.c.v. administration of 3 μg, but not 1 μg, of MT-II has shown to induce opposite *in vivo* electrical responses of serotonin neurones of dorsal raphe nucleus and noradrenaline neurones of locus coeruleus; exiting the former and inhibiting the latter (Kawashima *et al.*, 2003). Similarly, application of α-MSH has been reported to have excitatory and inhibitory effects on different subsets of NTS neurones *in vitro* (Mimee *et al.*, 2014). Thus, these dissimilar effects following melanocortin administration might depend upon different neuronal phenotypes, and therefore, activation of different cellular mechanisms.

In SON oxytocin neurones, the mechanism by which α-MSH inhibits the firing rate has been linked to the release of endocannabinoids which inhibits presynaptic excitatory inputs, as shown by the retrodialysis of a CB1 cannabinoid receptor antagonist which prevented the inhibition of oxytocin neurones *in vivo* (Sabatier & Leng, 2006). Conversely, in CA1 hippocampal neurones, activation of MC_4 by MT-II potentiates the postsynaptic response to glutamate *ex vivo* by increasing the expression of ionotropic AMPA receptors in dendritic spines (Shen *et al.*, 2013).

Enhancement of oxytocin neurone activity by peripheral stimuli, including pharmacological agents, is a pathway well characterised (Renaud *et al.*, 1987; Velmurugan *et al.*, 2010; Scott & Brown, 2011). I.v. injection of the gut-related peptide CCK, has been shown to induce a transient activation of SON oxytocin neurones (Renaud *et al.*, 1987; Leng *et al.*, 1991) via noradrenergic inputs from A2 neurones of the NTS, which in turn, are activated by vagal inputs (Onaka *et al.*, 1995). The stimulation of this pathway has been used in this study for electrophysiological identification of SON neurones *in vivo* (Leng *et al.*, 2001). Similarly to CCK, when MT-II is administered i.v., it is rapidly propelled by the blood flow, reaching virtually all the tissues, and consequently, leading to peripheral (and eventually central) actions by activation of melanocortin receptors expressed in some of these tissues. The expression of $MC_{3/4}$, which are the main MT-II ligands, have been described in several peripheral organs, including the heart and intestine (Gantz *et al.*, 1993; Mountjoy *et al.*, 2003) which are innervated by the vagus nerve. Moreover, using transgenic mice expressing GPF under the control of the MC_4 promoter have shown GFP positive fibres in sensory afferents of the vagus nerve (Gautron *et al.*, 2010), and MT-II has been shown

to modulate spontaneous EPSCs in NTS neurones via presynaptic (glutamate) vagal sensory afferents *in vitro* (Wan *et al.*, 2008). Hence, vagotomy or pharmacological desensitisation of vagal afferents by capsaicin (an agonist of the TRPV1 which is known to desensitise sensory neurones in a dose-dependent fashion (O'Neill *et al.*, 2012)) could be used in further experiments to determine the contribution of vagal inputs on oxytocin neurone activity following systemic administration of MT-II.

My results showed that i.v. administration of MT-II did have different effects on vasopressin neurone activity, however, one-third of the neurones tested in this study exhibited an increase in firing rate over 10 %. A previous study has shown excitation of a larger proportion of SON putative vasopressin neurones following i.v. administration of the $MC_{3/4}$ agonist γ_2-MSH, however, these recorded neurones were identified by histological determination of the placement of the recording electrode (Mitchell *et al.*, 1989). It is known that, in contrast to oxytocin neurones, the population of SON vasopressin neurones are heterogeneous in their electrical "behaviour" (MacGregor & Leng, 2013, 2016), and consequently, the response observed in this study may reflect these phenotypic differences. Interestingly, scattered expression of the MC_4 in areas of the SON populated by vasopressin neurones (i.e. ventromedial SON) has been reported in the rat and human hypothalamus (Kishi *et al.*, 2003; Siljee *et al.*, 2013), supporting a possible role for melanocortins in the regulation of the activity of some vasopressin neurones. Nevertheless, central (i.c.v.) or retrodialysis administration of α-MSH or an MC_4 agonist did not have an effect on the firing rate of any of the SON vasopressin neurones tested in a previous study (Sabatier *et al.*, 2003a). This apparent conflict between studies on the melanocortin actions on

single vasopressin neurones might be related to the administration routes used in both studies. Since I administered MT-II i.v., it is possible to infer that systemic administration leads to activation of other (peripheral) systems, such as the OVLT-glutamate pathway or the vagal-NTS pathway, as described above, which may enhance afferents (excitatory) inputs to SON vasopressin neurones.

Immediately after i.v. administration of MT-II we also observed a transient inhibition of spike discharge in some phasic- and continuous-vasopressin neurones. Vasopressin neurone activity is modulated by afferent pathways originating from low- and high-pressure stretch receptors of the cardiovascular system, and consequently, fluctuations in blood pressure can lead to changes in neurone activity (Leng *et al.*, 1999; Grindstaff & Cunningham, 2001). Interestingly, melanocortins are known to induce cardiovascular effects (do Carmo *et al.*, 2017), and systemic administration of MT-II has been reported to increase the blood pressure and heart rate in a dose-dependent manner in conscious and anaesthetized mice (Rinne *et al.*, 2012), even at lower doses than the dose administered in the present study. Increases in blood pressure have been reported to transiently inhibit phasic-vasopressin neurones in rats (Harris, 1979; Leng & Dyball, 1984). To test whether the spike discharge was affected by the i.v. injection itself, we administered a small volume (0.2 ml) of vehicle showing that, in some neurones, it induced a very short inhibition of spike activity. Hence, the transient inhibition of vasopressin neurones observed here is likely to be associated with transitory changes in blood pressure, as well as other possible cardiovascular effects including increased heart rate (Rinne *et al.*, 2012).

As described previously, oxytocin and vasopressin release from nerve endings is related to spike activity, but the release response is not linear, and this is facilitated at certain firing frequencies. According to Brown *et al.* (2013), "frequency facilitation of oxytocin release is most marked between ~ 5 and 25 Hz, but release continues to increase (albeit with a slower rate of increase) beyond ~ 50 Hz. By contrast, frequency facilitation of vasopressin release is maximal at ~ 13 Hz and sustained higher frequency stimulation actually results in less vasopressin release". Here I reported that after i.v. MT-II administration, half of the oxytocin neurones tested reached a firing frequency over 4 Hz, and in the vasopressin neurones tested the frequencies observed were less than 10 Hz after stimulation. Thus, my results indicate that the increased electrical activity may contribute to changes in peripheral oxytocin release.

Since direct application of α-MSH has been shown to inhibit SON oxytocin neurones (Sabatier *et al.*, 2003a; Ladyman *et al.*, 2016), I cannot establish whether i.v. MT-II acts on central melanocortin receptors on SON neurones. However, the effects observed in this study suggest a possible activation of indirect inputs projecting to SON neurones.

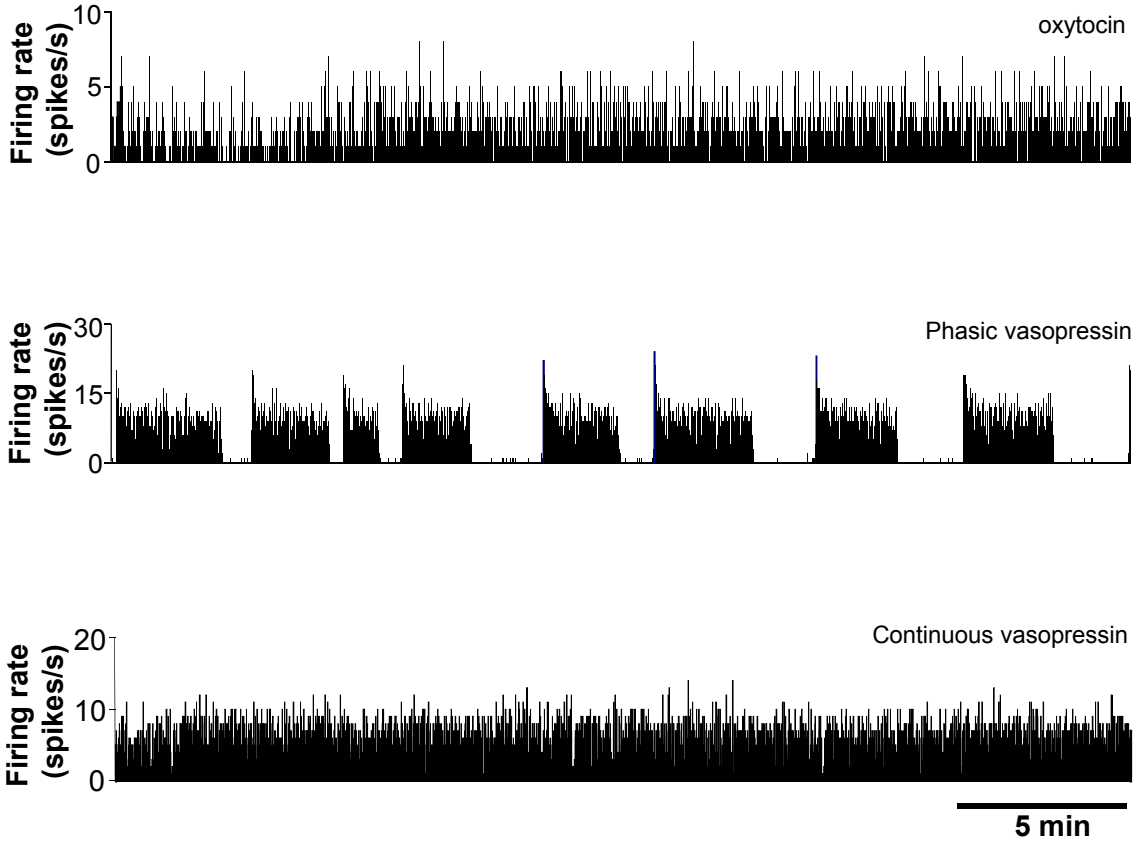

Figure 4.1. Discharge pattern of SON neurones. Representative examples of the firing pattern (spikes/s) in an oxytocin, phasic- and continuous-vasopressin neurones.

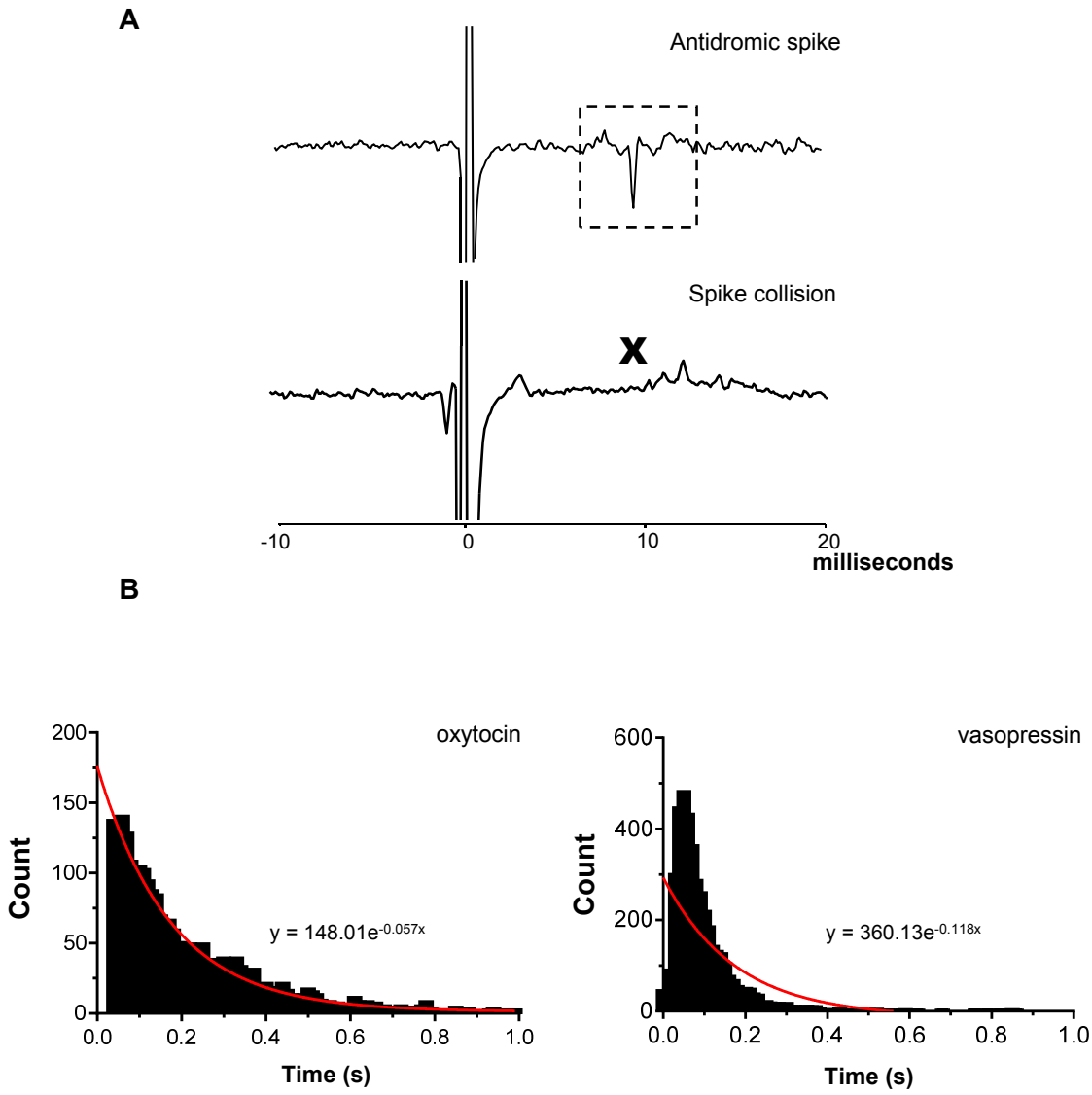

Figure 4.2. Identification of supraoptic neurones. (*A*) SON neurones were identified by antidromic stimulation of the pituitary stalk (0 ms) which evokes an artificial action potential (spike) that propagates to the soma at a constant latency (~ 10 ms; upper panel). The spike collision occurs when an antidromically spike encounters a descending spontaneous orthodromic spike along the axon, and both are extinguished (X; lower panel). **(*B*)** The descending tails of the inter-spike interval distribution can be well fitted using a single negative exponential (red line) in oxytocin, but not in vasopressin neurones which are highly skewed.

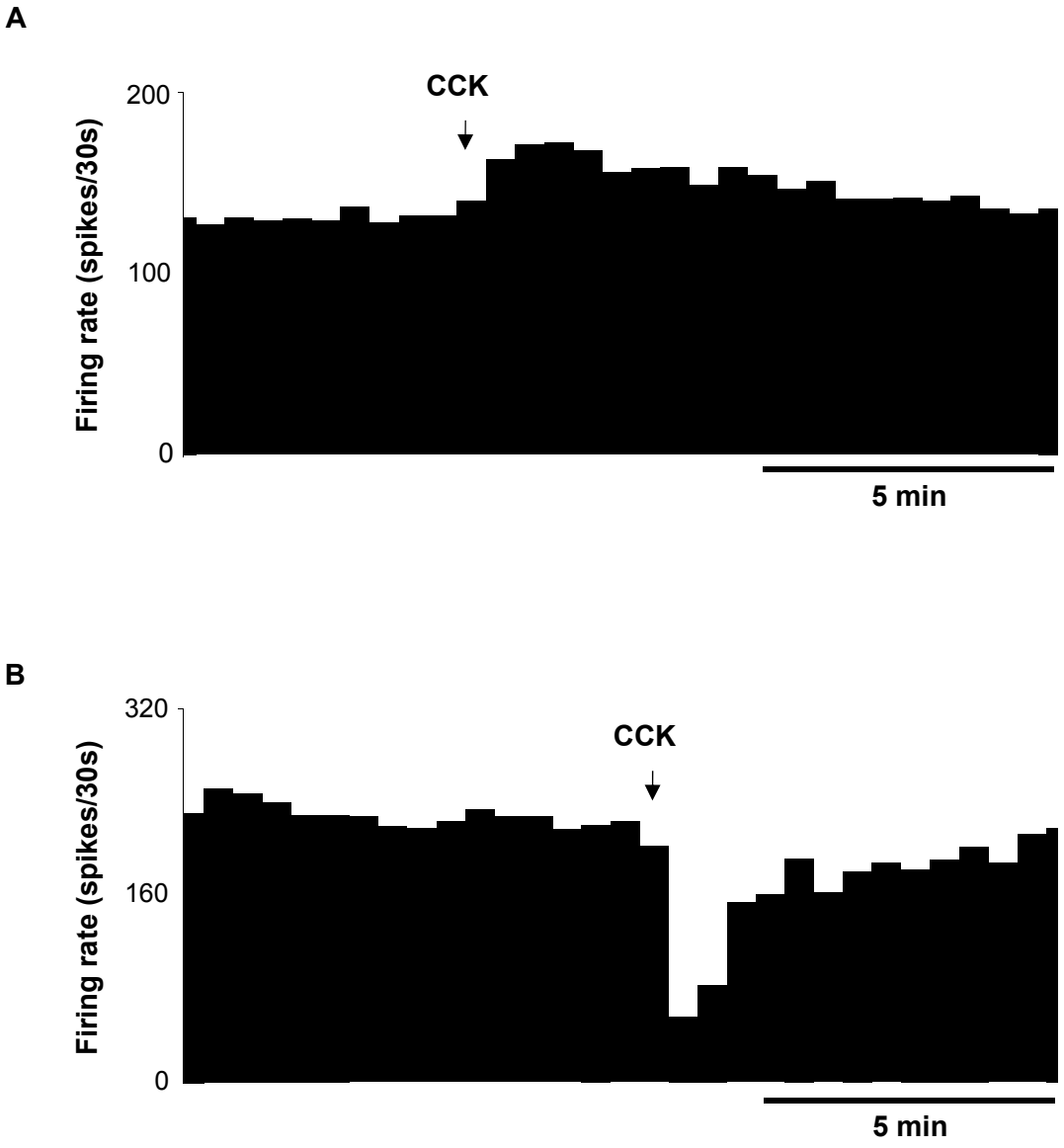

Figure 4.3. Responses of SON neurones to i.v. CCK. I.v. administration of CCK (20 μg/kg) has opposite effect on oxytocin and vasopressin neurones, increasing the firing rate of **(*A*)** oxytocin neurones, and inhibiting **(*B*)** vasopressin neurone activity.

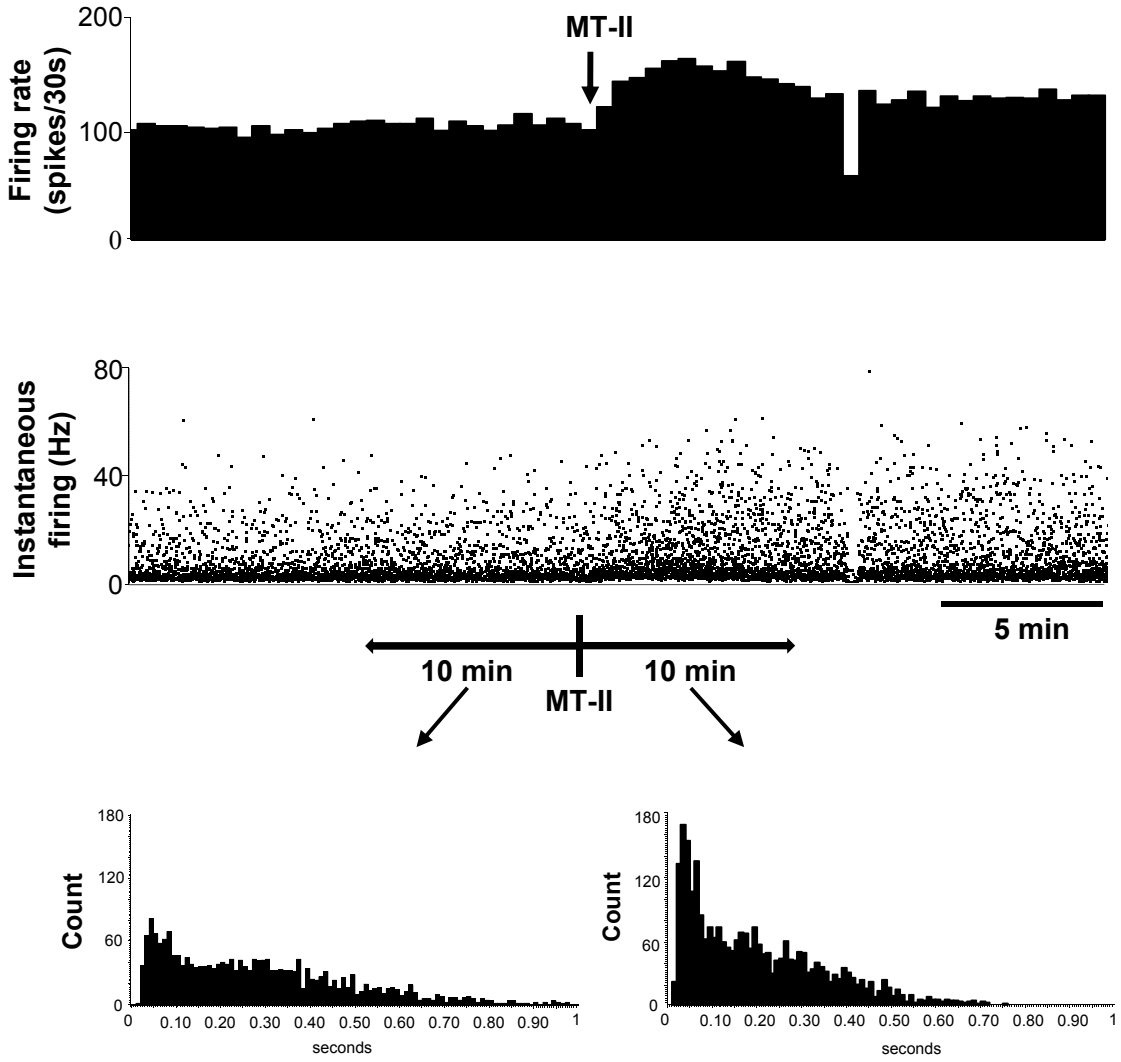

Figure 4.4. Effect of i.v. administration of MT-II on the electrical activity of a SON oxytocin neurone. Representative example showing an increase in the firing rate (in 30 s-bins), the instantaneous firing rate recorded, and the inter-spike interval distributions (before and after stimulation) of an oxytocin neurone in response to MT-II (1 mg/kg).

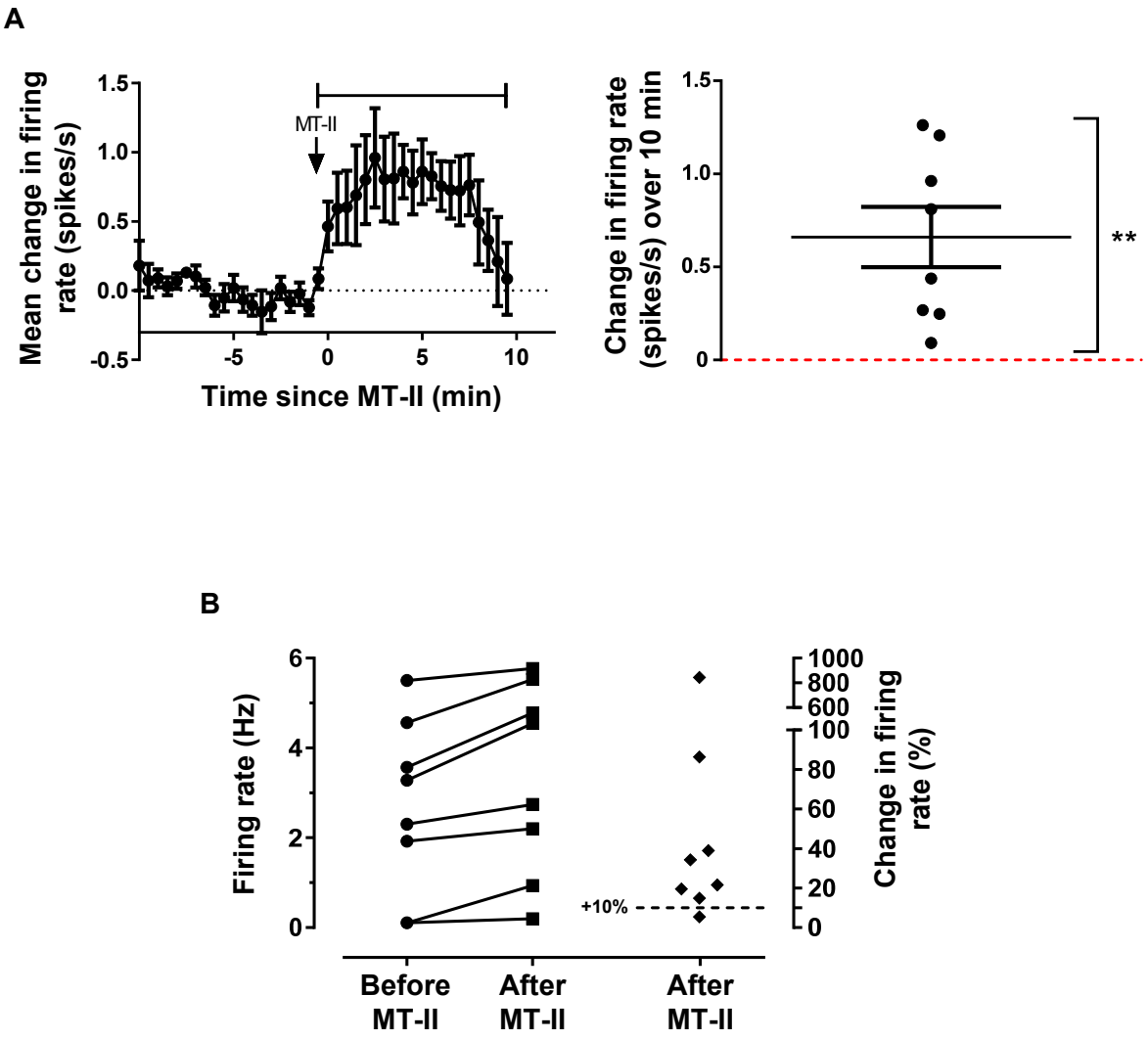

Figure 4.5. Effect of i.v. MT-II on the electrical activity of oxytocin neurones. (*A*) Mean change in firing rate (± S.E.M.) in 30 s-bins from all recorded oxytocin neurones (n = 8), and change in firing rate over the period analysed (0 – 10 min) in each neurone recorded (mean ± S.E.M.; right panel). **(*B*)** Absolute and percentage change in firing rate of individual oxytocin neurones in response to MT-II. **P < 0.01, Wilcoxon signed rank test.

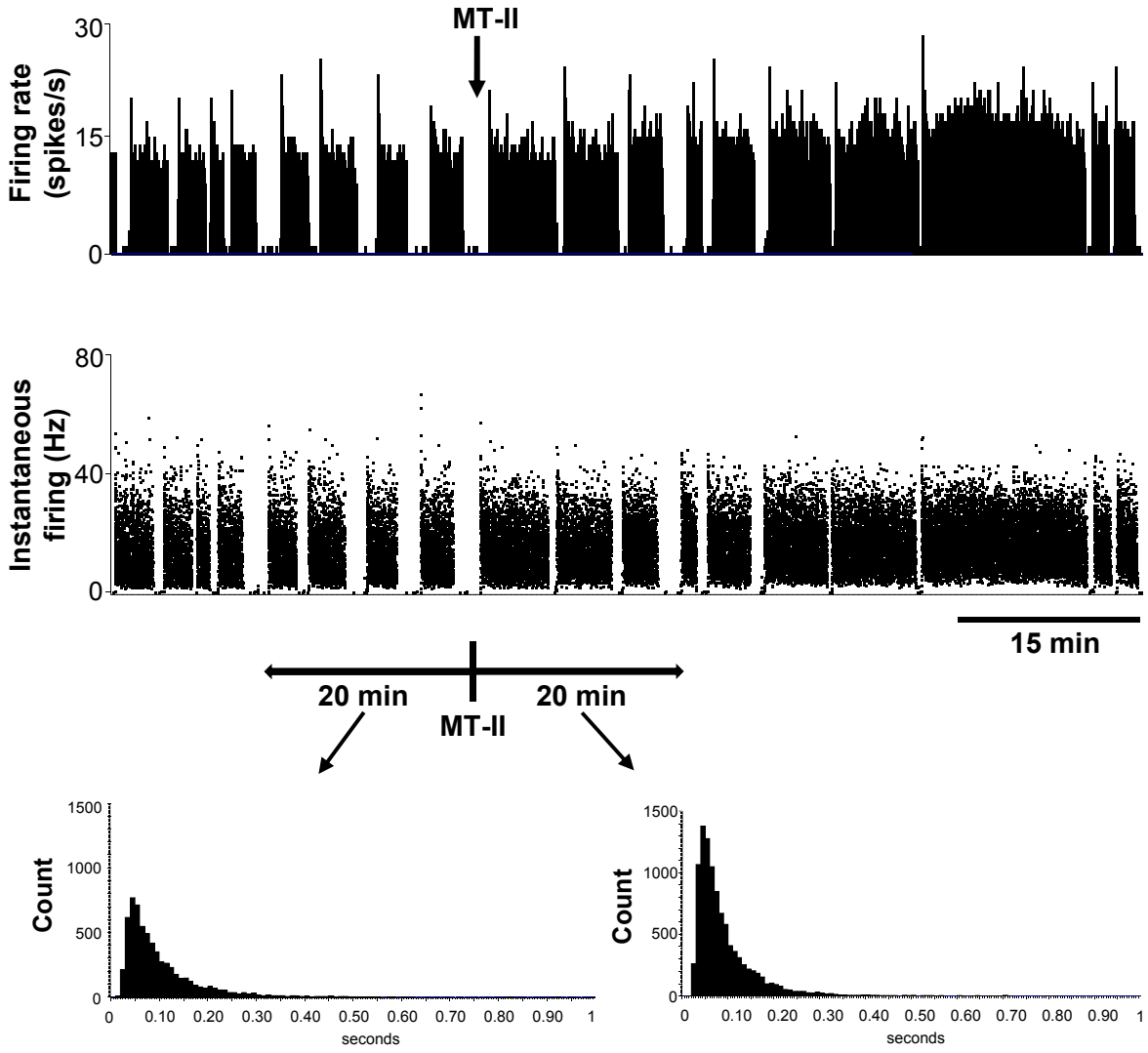

Figure 4.6. Effect of i.v. administration of MT-II on the electrical activity of a responsive SON vasopressin neurone. Example of the increase in firing rate (in 1 s-bins), the instantaneous firing rate recorded, and the inter-spike interval distributions (before and after stimulation) in a vasopressin neurone in response to i.v. administration of MT-II (1 mg/kg).

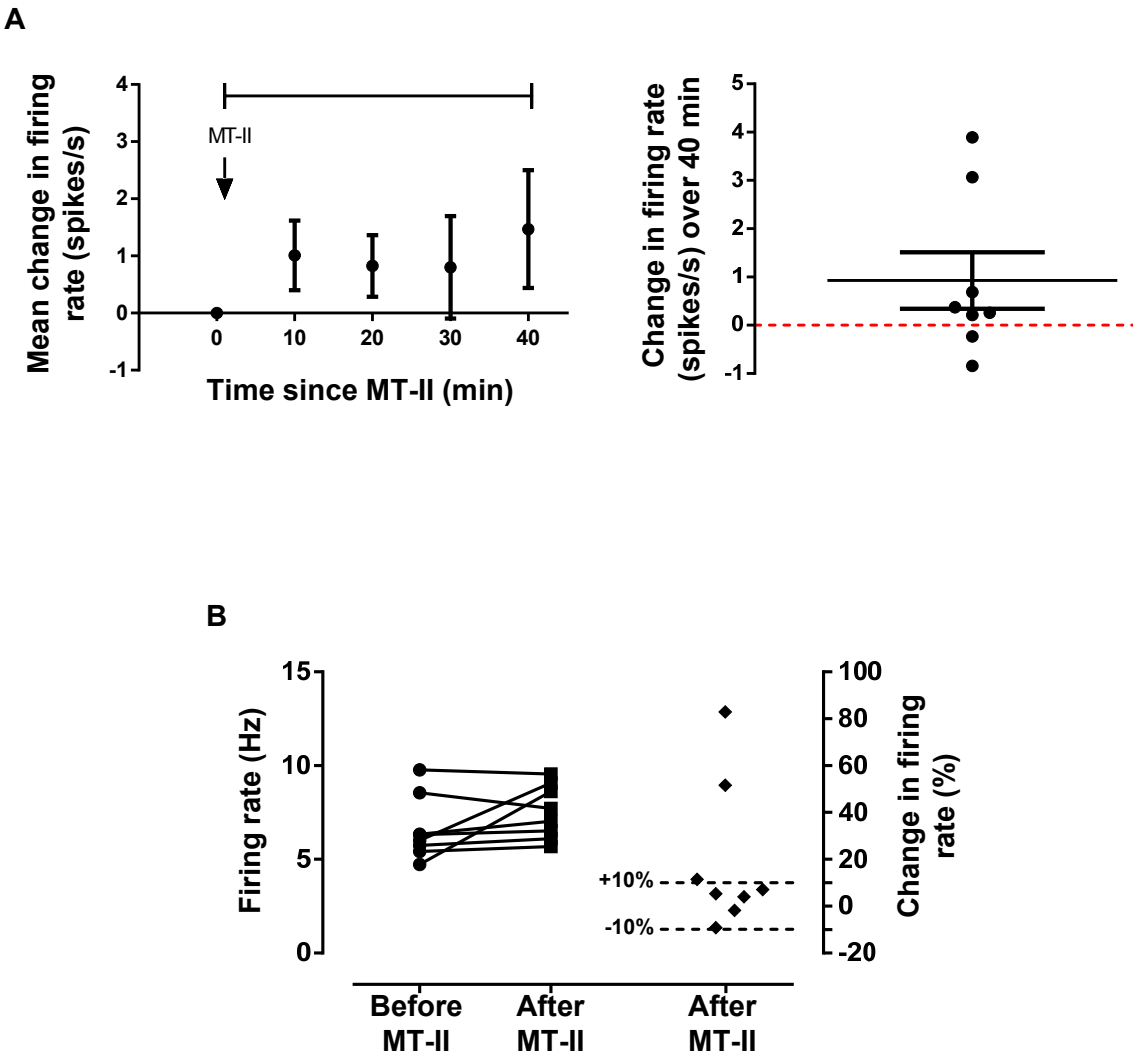

Figure 4.7. Effect of i.v. MT-II on the electrical activity of vasopressin neurones. **(*A*)** Mean change in firing rate (± S.E.M.) in 10 min-bins from all recorded vasopressin neurones (n = 8), and change in firing rate over the period analysed (0 – 40 min) in each neurone recorded (mean ± S.E.M.; right panel). **(*B*)** Absolute and percentage change in firing rate of individual vasopressin neurones in response to MT-II.

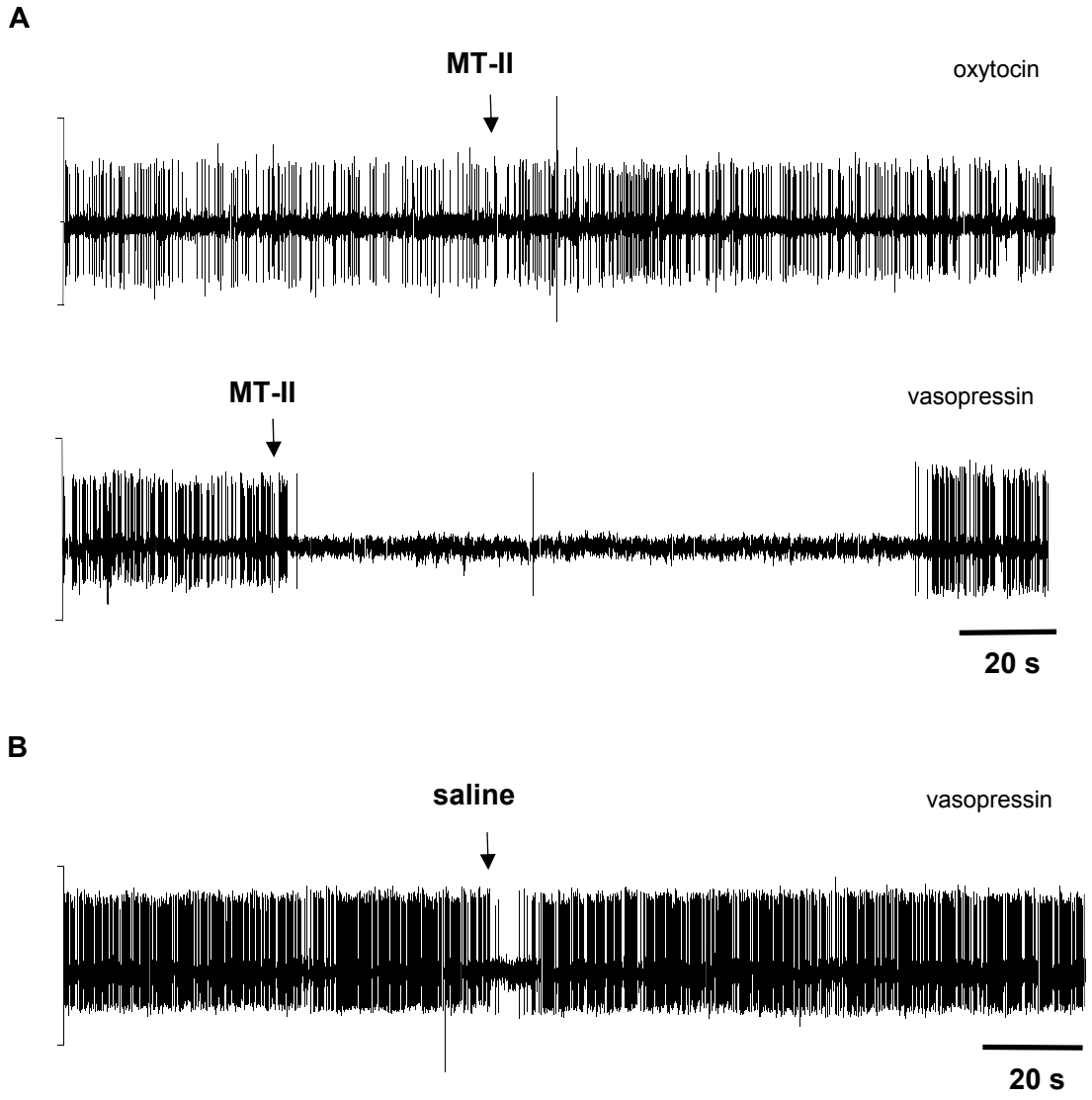

Figure 4.8. Polygraph tracing (raw voltage traces) of transiently inhibition of vasopressin neurones following i.v. injection. (*A*) I.v. administration of MT-II induced an inhibition of spike activity of some vasopressin, but not oxytocin, neurones. **(*B*)** I.v. administration of a small volume (0.2 ml) of vehicle (saline 0.9 %) induced a very short inhibition of spike activity in two vasopressin neurones.

Chapter 5

Central and peripheral oxytocin release following administration of Melanotan-II

5.1. INTRODUCTION

As shown in the previous chapter, i.v. administration of MT-II enhanced electrical activity of SON oxytocin neurones, and this has been linked to oxytocin release into the bloodstream (peripheral release) from the nerve endings located in the posterior pituitary (Bicknell, 1988; Bourque, 1991). A remarkable feature of oxytocin (and also vasopressin) magnocellular neurones is that they can also release oxytocin from other neuronal compartments (central release), including the soma, and dendrites (Pow & Morris, 1989; Morris & Pow, 1991), and this can be regulated independently or semi-independently from peripheral release.

The cellular mechanism governing oxytocin release from somatodendritic and nerve endings compartments are different (see Chapter 1). Briefly, when an action potential reaches the nerve endings, the opening of voltage-gated Ca^{2+} lead to an influx of Ca^{2+} that promotes the release of oxytocin vesicles (Douglas & Poisner, 1964; Lemos & Nowycky, 1989). In contrast, the somatodendritic release of oxytocin can be wholly regulated by mobilisation of intracellular Ca^{2+} from thapsigargin-sensitive stores (Lambert *et al.*, 1994; Ludwig *et al.*, 2002). Interestingly, agents that mobilise Ca^{2+} from intracellular stores can also potentiate the dendritic release of oxytocin in response to activity-induced stimulation (Ludwig *et al.*, 2002). Remarkably, the direct application of α-MSH or an MC_4 agonist (MRLOB-0005) to SON explants have been shown to trigger somatodendritic oxytocin release *in vitro* whereas central (i.c.v.) administration of the MC_4 agonist inhibited peripheral oxytocin release *in vivo*, and both melanocortin agonists have been shown to induce a rise in $[Ca^{2+}]_I$ *in vitro*, even when applied in a medium low in extracellular Ca^{2+} content (Sabatier *et al.*, 2003a)

When released into the blood, oxytocin acts as a neurohormone exerting its classical peripheral effects, which are associated primarily with smooth muscle contraction, and these include milk-ejection and uterine contraction (see Chapter 1). When released centrally, oxytocin participates in the modulation of several behavioural responses (see Chapter 1), as shown by a large body of evidence (Neumann, 2007; Veenema & Neumann, 2008; Marlin & Froemke, 2017). Although these central actions have been classically associated with oxytocin synaptically released from nerve endings of PVN parvocellular neurones, magnocellular neurones contain large amounts of oxytocin in somatodendritic compartments which effectively

account for the high peptide content reported in the CSF (Ludwig & Leng, 2006). Since oxytocin cannot penetrate the BBB (Zaidi & Heller, 1974; Ermisch *et al.*, 1985), and consequently, the BBB prevents the re-entry of peripherally released oxytocin, its central measurement (and actions) reflect well the release from somatodendritic compartments.

The use of simultaneous brain microdialysis and blood sampling have been a very useful tool to study *in vivo* the release patterns of central and peripheral oxytocin under a wide variety of physiological conditions and stimuli (Hattori *et al.*, 1990; Neumann *et al.*, 1993; Bosch *et al.*, 2004; Engelmann *et al.*, 2004). The microdialysis allows to study the composition of the extracellular fluid of discrete brain areas, as analytes (i.e. oxytocin) present in the extracellular fluid pass through a semipermeable membrane into a perfusate collected over a predetermined time and volume (Chefer *et al.*, 2009). Thus, microdialysis together a sensitive quantification method (e.g. RIA) enable studies to determine oxytocin content, as well as monitoring changes in release within distinct brain structures, including the sources of origin (i.e. SON or PVN) or possible sites of action (e.g. limbic areas).

In this chapter, I study the effect of the synthetic $MC_{3/4}$ agonist, MT-II, on central and peripheral oxytocin release in urethane-anaesthetised rats. Using combined *in vivo* microdialysis with blood (plasma) sampling, I determine whether i.v. or i.c.v. administration of MT-II induces changes in oxytocin content within the SON and plasma compartments. The use of *in vivo* microdialysis has been widely used to determine and monitor dynamic changes in somatodendritic release of oxytocin and vasopressin (Landgraf & Ludwig, 1991; Ludwig & Landgraf, 1992; Neumann *et al.*, 1993), as well as

other neuropeptides and neurotransmitters (Sisk *et al.*, 2001; Engelmann *et al.*, 2002; Frost *et al.*, 2008).

5.2. MATERIAL AND METHODS

5.2.1. *In vivo* microdialysis preparation

Rats were anaesthetized with urethane (1.25 g/kg i.p.), and a plastic cannula was implanted into the left femoral vein for blood sampling as described in Chapter 2. Rats were placed into a stereotaxic frame and an in-house design (Horn & Engelmann, 2001) U-shaped microdialysis probe (membrane length 2.0 mm, molecular cut-off of 6 kDa; Ludwig and Landgraf (1992) was implanted aimed at the right SON using the following coordinates: 0.6 mm caudal to bregma, 1.8 mm lateral to midline and 9.4 mm deep from the surface of the skull based on the stereotaxic atlas by Paxinos and Watson (1998). The microdialysis probe was secured to the skull using two jewellers' screws and dental cement.

The probe was perfused with aCSF (NaCl 138 mM, KCl 3.36 mM, $NaHCO_3$ 9.52 mM, Na_2HPO_4 $2H_2O$ 0.49 mM, urea 2.16 mM, $CaCl_2$ 1.26 mM, $MgCl_2$ $6H_2O$ 1.18 mM; pH 7.2) using a microinfusion pump (HumBee; Bioanalytical system Inc., Lafayette, IN, USA) at a flow rate 3 μl/min. The probe was perfused for 2 h without sampling, then consecutive 30-min dialysis samples were collected directly into 1.5 ml microcentrifuge tubes, which were kept on ice and then immediately stored at -80 °C until assayed for oxytocin.

At the end of the experiments, the rats were killed by an overdose of sodium pentobarbital (160 mg/kg) and the brains were removed and stored

until sectioning. Coronal sections of the hypothalamus (40 μm) were used for reconstruction of the placement of the microdialysis probes (Fig. 5.1.). The judgment of successful implantation of the SON was made before analysing microdialysis and plasma samples.

5.2.2. Experiments protocol

5.2.2.1. Microdialysis

In experiments where drugs were given i.v., vehicle (0.9 % saline; 1 ml/kg) or MT-II (1 mg/ml/kg) were injected at the beginning of the fourth sample period, as shown in Figure 5.2. For experiments with i.c.v. injections, MT-II (1 μg/3 μl/rat) and vehicle (aCSF, 3 μl/rat) were administered at the beginning of the third or fifth microdialysis sample period. The sequence of the injections was alternated between the successive trials. In both experiments, aCSF containing 1 M NaCl was retrodialysed directly into the SON during the penultimate microdialysis period and switched back to normal aCSF during the last microdialysis period (Fig. 5.2.). Retrodialysis of aCSF with high NaCl content served as a positive control to confirm the suitability of the microdialysis approach for monitoring dynamic changes in the release of oxytocin as described by Landgraf and Ludwig (1991).

5.2.2.2. Blood collection

Blood samples (0.7 ml) were withdrawn from the femoral vein into heparinized tubes, centrifuged (5 min, 2000 g, 4 °C), and the plasma separated and stored at -80 °C for subsequent oxytocin RIA. Blood cells were

re-suspended in 0.9 % saline and infused via the femoral cannula. Samples were withdrawn as shown in Figure 5.2.

5.2.3. Radioimmunoassay (RIA)

Microdialysates (90 µl) were kept at -80 °C until evaporation and radioimmunological quantification of oxytocin without prior extraction. Plasma samples were kept at -80 °C until extraction and subsequent radioimmunological quantification of oxytocin. Both plasma extraction and RIA were performed by Prof R. Landgraf (RIAgnosis, Sinzing, Germany) as described previously (Landgraf *et al.*, 1995; Paiva *et al.*, 2017). The RIA detection limit is in the 0.1 – 0.5 pg/sample range, depending on the age of the tracer, with typical displacements of 20 – 25 % at 2 pg, 60 – 70 % at 8 pg and 90 % at 32 pg of standard neuropeptide (Sigma Aldrich, Germany). Cross-reactivities with vasopressin, ring moieties and terminal tripeptides of both oxytocin and vasopressin and a wide variety of peptides comprising 13 (α-MSH) up to 41 (CRH) amino acids are < 0.7 % throughout. All evaporated microdialysates and plasma extracts to be compared were treated identically and assayed in the same batch at the same time to avoid inter-assay variability; intra-assay variability is < 8 %. Serial dilutions of plasma samples containing high levels of endogenous oxytocin run strictly parallel to the standard curve indicating immunoidentity.

5.2.4. Statistical analysis

Oxytocin content in microdialysates were analysed using two-way ANOVA with treatment and time as variables, followed by *post hoc*

Bonferroni multiple comparison test. Delta changes in plasma oxytocin content in experiments using i.v. and i.c.v. administration were analysed using two-tailed Mann-Whitney test and two-tailed Wilcoxon matched-pairs signed rank test, respectively. $P < 0.05$ was considered significant.

5.3. RESULTS

Plasma samples and SON microdialysates were collected to determine peripheral and central oxytocin release in response to i.v. or i.c.v. administration of MT-II.

5.3.1 Somatodendritic oxytocin release

Two-way ANOVA analysis of data obtained after systemic injection of MT-II showed a significant effect of factor 'time' ($F_{8, 112} = 24.48$; ***$P < 0.001$), but no effect of factor 'treatment' ($F_{1, 14} = 0.10$; $P = 0.75$), and factor interaction ($F_{8, 112} = 0.42$; $P = 0.91$). No significant changes in the oxytocin content in microdialysates collected from the SON were detected after systemic injection of MT-II (Fig. 5.3.).

Similarly, two-way ANOVA analysis of data obtained after central injection of MT-II showed a significant effect of factor 'time' ($F_{7, 56} = 7.67$; ***$P < 0.001$), but no effect of factor 'treatment' ($F_{1, 8} = 0.07$; $P = 0.81$), and factor interaction ($F_{7, 56} = 0.02$; $P > 0.99$). Central administration of MT-II did not significantly change the oxytocin content in the subsequent microdialysates samples (Fig. 5.4.).

The application of direct osmotic stimulation has been used previously as a positive control to confirm the correct probe position within the SON. The perfusion of the microdialysis probe with hypertonic aCSF (1M NaCl) resulted in oxytocin release within the SON during and immediately after stimulus application in both previously i.v. and i.c.v. injected rats (*P < 0.05, last sample period, *post hoc* test), however, the magnitude of the release response was not different between the MT-II and vehicle-injected rats in both experiments (Fig. 5.3.; Fig. 5.4.).

5.3.2 Peripheral oxytocin release

In rats injected with systemic MT-II (baseline 5.3 ± 1.2 pg/ml, MT-II 8.9 ± 1.3 pg/ml, MT-II$_{60min}$ 8.1 ± 1.2 pg/ml) the change in plasma concentration of oxytocin was significantly greater (***P < 0.001, Mann-Whitney test) than in vehicle-injected rats (baseline 7.3 ± 2.1 pg/ml, saline 6.5 ± 1.9 pg/ml, saline$_{60min}$ 5.5 ± 1.5 pg/ml) (Fig. 5.5.). Central injection of MT-II (MT-II at 120 min: baseline 1.7 ± 0.2 pg/ml, aCSF 2.7 ± 1.2 pg/ml, MT-II 8.5 ± 4.7 pg/ml, MT-II$_{60min}$ 10.2 ± 5.6 pg/ml; vehicle at 120 min: baseline 1.6 ± 0.5 pg/ml, MT-II 5.7 ± 2.8 pg/ml, MT-II$_{60min}$ 5.6 ± 3.1 pg/ml, aCSF 5.3 ± 3.1 pg/ml) also tended to increase peripheral oxytocin concentrations when compared to vehicle injection, but this change did not reach statistical significance (P = 0.06, Wilcoxon matched-pairs signed rank test; Fig. 5.6.).

Retrodialysis of hypertonic aCSF directly into the SON caused an increase in peripheral oxytocin release in i.v. (MT-II 12.2 ± 1.5 pg/ml, vehicle 9.4 ± 1.2 pg/ml) and i.c.v. injected (MT-II at 120 min: 23.4 ± 2.2 pg/ml, vehicle at 120 min: 18.0 ± 3.6 pg/ml) rats, with no differences between MT-II and vehicle (P > 0.05; Fig. 5.5.; Fig. 5.6.).

5.4. DISCUSSION

This study was designed to test whether systemic or central administration of the melanocortin agonist MT-II triggers central and peripheral oxytocin release in urethane anaesthetized rats. I show that neither systemic nor central administration of MT-II induced oxytocin release within the SON, or potentiate (priming) somatodendritic oxytocin release in response to direct osmotic stimulation of the SON. However, systemic administration of MT-II induced a significant increase of plasma oxytocin concentration.

In oxytocin neurones, the somatodendritic release is not invariably coupled with peripheral release. Direct application of the naturally occurring $MC_{3/4}$ agonist, α-MSH, to SON neurones strongly induces oxytocin release from the dendrites *in vitro* whereas it decreases oxytocin release from the nerve endings *in vivo*, and these actions are thought to be mediated by activation of MC_4 which is expressed in SON neurones (Sabatier *et al.*, 2003a). So, based on the –empirical– capacity of the synthetic $MC_{3/4}$ agonist MT-II to penetrate the BBB (see Chapter 3) (Dorr *et al.*, 1996; Adan *et al.*, 1999; Giuliano *et al.*, 2006; Modi *et al.*, 2015), I administered MT-II i.v. However, I found that i.v. administration of MT-II (1 mg/kg) fails to trigger central oxytocin release (within the SON). Similar findings have been reported in female prairie voles in which i.p injection of MT-II (10 mg/kg) did not increase the oxytocin content in microdialysates collected from the nucleus accumbens (Modi *et al.*, 2015), a reported target for central oxytocin actions (Liu & Wang, 2003; Dolen *et al.*, 2013). Hence, it seems that MT-II is not able to trigger somatodendritic oxytocin release from magnocellular neurones in response to systemic administration.

These results raise doubts whether the RIAs were sensitive enough and comparable between these studies. However, in both studies oxytocin content was measured under the same assays conditions, and the detection limit of 0.1 – 0.5 pg/sample seems to be sensitive enough to detect changes induced by other stimuli, as shown by changes in oxytocin content following hypertonic stimulation (Modi *et al.*, 2015; Paiva *et al.*, 2017). The highly sensitive RIA used in this study (Prof Rainer Landgraf; RIAgnosis, Sinzig, Germany) has become the reference quantification method for the detection of changes in central (and peripheral) oxytocin content in a wide range of physiological and experimental conditions, and it has been used in previous studies (Landgraf *et al.*, 1995; Ross *et al.*, 2009). In contrast, other assays and quantification methods have shown inconsistencies in the measurement of oxytocin content (Leng & Sabatier, 2016). Additional limiting factors of the *in vivo* microdialysis are the limited time resolution, and relative recovery of an analyte (which is inversely proportional to the perfusion rate) (Chefer *et al.*, 2009). In this preparation, the microdialysis probe was perfused at 3 μl/min, and samples were collected every 30 min in order to obtain samples containing (basal) oxytocin concentration over the RIA detection limit. However, it seems unlikely that the release of a single, very brief and small pulse of oxytocin could be detected with the current techniques and analytical assay available.

The result obtained in this study raised the question whether MT-II was able to penetrate the BBB, although it is currently accepted that MT-II is a brain-penetrating melanocortin (Hruby *et al.*, 2011; Hruby & Cai, 2013). Thus, to critically determine the effect of MT-II on oxytocin neurones, I directly administered MT-II into the brain (i.c.v.). Unexpectedly, the

administration of 1 μg of MT-II did not trigger somatodendritic oxytocin release from SON neurones. Sabatier *et al.* (2003a) reported that the dose of 1 μg of α-MSH or the synthetic melanocortin agonist MRLOB-0005 triggered somatodendritic oxytocin release *in vitro* from SON explants, and this effect was blocked by the addition of the MC_4 antagonist MRLOB-0004. Consistently with this α-MSH *in vitro* effect, we have recently reported that retrodialysis of α-MSH [167 ng/μl] also induced somatodendritic oxytocin release within the SON (Paiva *et al.*, 2017). This melanocortin-induced oxytocin release is mediated by mobilization of intracellular Ca^{2+} from thapsigargin-sensitive stores as shown in MC_4 transfected HEK293 cells (Mountjoy *et al.*, 2001); the increase in $[Ca^{2+}]_I$ and subsequent *in vitro* dendritic oxytocin release from SON neurones also occurred when the extracellular medium was low in Ca^{2+} content (Sabatier *et al.*, 2003a). Thus, the differences in biological activity observed here might be related to the capabilities of melanocortin ligands to mobilise Ca^{2+} from the intracellular stores.

Previous studies have shown different changes in $[Ca^{2+}]_I$ in response to application of melanocortin ligands, including α-MSH, and MT-II. For example, in the prolactin and growth hormone secreting GH-3 cell line, the application of 0.1 nM, and 1 nM of α-MSH increases $[Ca^{2+}]_I$, whereas MT-II does not significantly affect $[Ca^{2+}]_I$ when administered at the same or even higher dose (Langouche *et al.*, 2001). Furthermore, Ca^{2+} channels from the N-type seem to play a major role in the regulation of somatodendritic oxytocin release from SON explants (Tobin *et al.*, 2011). However, in other systems, such as transfected HEK293 cells and cultured mouse amygdala neurones, activation of MC_4 by MT-II has been reported to inhibit N-type Ca^{2+} channels (Agosti *et al.*, 2014).

The basis of variations in cellular responses to different melanocortin agonist may be related to receptor binding sites bounded by α-MSH, and MT-II, and ultimately, the activation of different intracellular pathways. This theoretical "agonist-specific trafficking of receptor signalling" (Hermans, 2003; Kenakin & Christopoulos, 2013) has been reported to naturally occur, and be induced, for example, by the pituitary adenylyl cyclase-activating polypeptide (PACAP) isoforms 27 and 38 acting on the PACAP type-I receptor, where both isoforms induce adenylyl cyclase pathway (cAMP), and only PACAP-38 stimulates phospholipase C pathway (Spengler *et al.*, 1993). In this context, endogenous melanocortin ligands (i.e. α-MSH) binds GPCRs interacting at specific (native) sites of the receptor known as orthosteric sites, while synthetic ligands, like MT-II, bind these receptors at sites that could potentially be topography distinct (allosteric sites) from the orthosteric sites due to variations in the peptide molecular structure (linear versus cyclic structure in α-MSH, and MT-II, respectively; Hruby (2016). A recent *in vitro* study has shown that, in immortalised hypothalamic neurones, MT-II, but not α-MSH, induces cAMP that persists for at least 1 hour after MT-II removal, and this effect cannot be antagonised by the naturally inverse agonist, AgRP (Molden *et al.*, 2015). Moreover, substitution of amino acid residues in the MT-II molecule has been reported to lead to dramatic changes in potency and function, decreasing the formation of cAMP when compared to the native MT-II (Bednarek *et al.*, 1999). In other systems, the allosteric modulator CPPHA acting on the metabotropic glutamate receptor 5 (mGluR5) potentiates Ca^{2+} mobilisation and ERK1/2 phosphorylation induced by the endogenous mGluR5 receptor ligand in cortical astrocytes *in vitro* (Zhang *et al.*, 2005). Interestingly, melanocortin receptors can activate more than one intracellular signalling pathway depending on the

melanocortin ligand (Breit *et al.*, 2011; Hruby *et al.*, 2011). Thus, *in vivo* studies of activation or enhancement of intracellular pathways, such as immunohistochemistry and western blot analysis, could be performed to compare the effect of MT-II with α-MSH in SON neurones.

The site of injection (i.c.v., right ventricle) for MT-II delivery is another factor that also should be considered. When a drug is delivered i.c.v., high drug concentration, if not virtually all, reaches the adjacent structures, but then, as the drug diffuses and flows into the ventricular system, the concentration decreases. Consequently, there are large concentration gradients within the ventricular system which may result in low amounts of drug delivered to distant targets, and even lower amounts delivered into brain structures located into the brain parenchyma, which are situated at variable distances from the ventricular system (Kuo & Smith, 2014). In rats, the estimation of the total CSF volume is approximately 90 µl (Pardridge, 2011), and consequently, the administration of 1 µg/3 µl ([0.33 µg/µl]) i.c.v. is largely diluted ([0.01075 µg/µl]), resulting in a concentration 30 times lower than the initial concentration delivered (in a non-realistic static system). Other factors, such as diffusion into the brain parenchyma, protein binding, physicochemical properties, as well as peptide loss by melanocortin receptor binding (adsorption) or enzymatic turnover should also be considered (Cook *et al.*, 2009; Kuo & Smith, 2014). Hence, once MT-II completely diffuses into the CSF, it can be predicted a low peptide concentration reaches the end of the ventricular system, and consequently, the SON neurones which are bathed by the CSF of the subarachnoid space. This is particularly relevant for my preparation, as the oxytocin content released from SON neurones was analysed, so the direct application of MT-II by retrodialysis should be

considered to definitely confirm the lack of effect of MT-II on somatodendritic oxytocin release.

A remarkable feature of intracellular Ca^2-mobilizer agents (i.e. thapsigargin and oxytocin itself) acting on oxytocin neurones is that they can dramatically increase the amount of peptide dendritically released (priming) after activity-evoked stimulation. Priming involves an increase in $[Ca^{2+}]_I$, and repositioning of peptide-containing vesicles close to the cell membrane (Tobin *et al.*, 2004) that lead to, for instance, a larger dendritic release of oxytocin in thapsigargin-primed animals compared to non-primed animals in response to hypertonic stimulation (Ludwig *et al.*, 2002). Because α-MSH has also been shown to induce a rise in $[Ca^{2+}]_I$ (Sabatier *et al.*, 2003a), it has been suggested that melanocortins might induce priming of dendritic peptide stores. In female prairie voles, it has been reported that i.p. administration of MT-II (10 mg/kg) potentiates oxytocin release within the nucleus accumbens after hypertonic stimulation (Modi *et al.*, 2015). Conversely, I did not detect any potentiation effect in response to hypertonic stimulation in MT-II-treated (1 mg/kg) male rats. In both studies, the administration of aCSF containing 1M NaCl was applied by retrodialysis, but in contrast to Modi *et al.* (2015), I directly applied the stimulus onto SON neurones, increasing the probability of triggering, and detecting changes in somatodendritic oxytocin release. This apparent conflict between studies in MT-II-induced potentiation of central oxytocin release in response to hypertonic stimulus, could be related to the source of oxytocin (e.g. PVN, or accessory nuclei in Modi *et al.* (2015) or differences in the physiological responses of the animals (rat and prairie voles) used in both studies. Finally, the use of direct evidence, such as electron microscopy to determine

relocation of LDCVs should be considered to elucidate whether MT-II induces priming in oxytocin neurones

The plasma oxytocin RIA showed that after i.v. administration of MT-II the concentration of oxytocin is increased. Thus, this study shows, for first time, that systemic melanocortins may play a role in the regulation of peripheral oxytocin release. Previously, it has been reported that i.c.v. administration of a selective MC_4 agonist decreased plasma oxytocin concentrations *in vivo* (Sabatier *et al.*, 2003a). Since I administered MT-II i.v., these actions are likely to be mediated by activation of peripheral inputs acting on the magnocellular neurones, such as the vagus nerve-NTS (see Chapter 1) or circumventricular organs, or even as a consequence of peripheral effects of melanocortin acting on other systems, for example the cardiovascular system (Rinne *et al.*, 2012). Interestingly, in this preparation, the plasma oxytocin content remained elevated for at least 60 min after MT-II injection. As the estimated half-life of oxytocin in plasma is approximately 5 min (Leng & Ludwig, 2016; Leng & Sabatier, 2016), this result suggests a long-lasting effect on oxytocin release from the nerve endings after MT-II administration.

Interestingly, my results show that i.c.v. administration of MT-II (1 μg) in anaesthetised rats tended to increase peripheral oxytocin release. Similarly, i.c.v. injection of α-MSH (1 μg) in conscious rats also tended (not significant) to increase peripheral oxytocin release, however, a lower dose of an MC_4 agonist (MRLOB-0005; 400 ng) significantly decreased oxytocin release from the nerve endings in hypertonically stimulated anaesthetized rats (Sabatier *et al.*, 2003a). This suggests that, as described previously, activation of MC_4 by a different melanocortin agonist might induce activation

of different intracellular pathways, or act on the MC_4 with dissimilar potencies (Nickolls *et al.*, 2003). Unfortunately, binding or potency parameters of the melanocortins used in these studies, such as the concentration of peptide at 50 % specific binding (IC_{50}) or the concentration of peptide at 50 % maximum cAMP accumulation (EC_{50}), cannot be compared, as these parameter have been provided in different assays using cell lines transfected with human or murine MC_4 (Adan *et al.*, 1999; Mountjoy *et al.*, 2001; Sabatier *et al.*, 2003a).

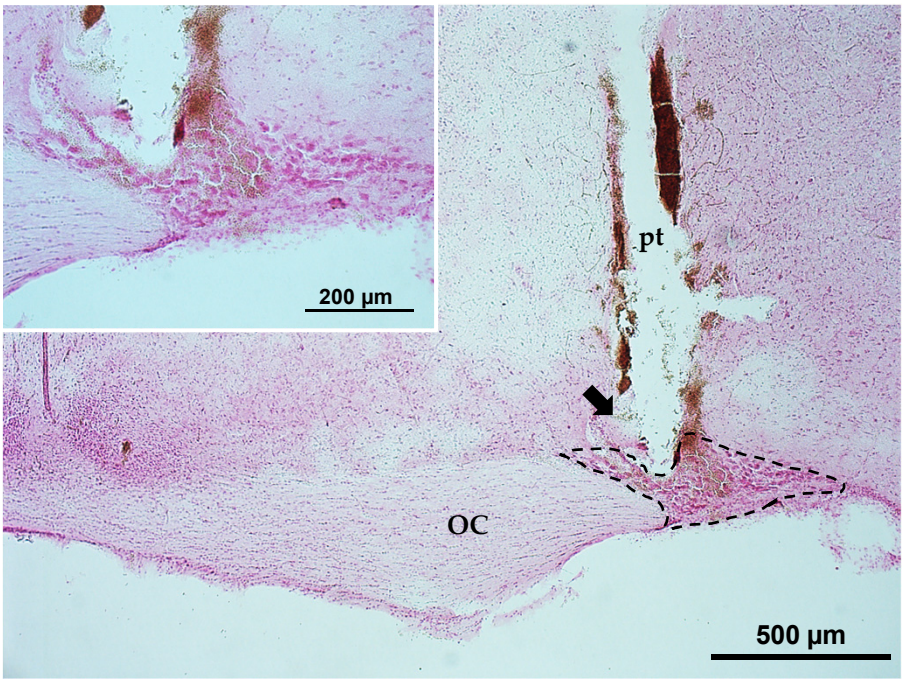

Figure 5.1. Judgment of microdialysis probe implantation. Example of a coronal section counterstained with nuclear fast red, showing the localization of the microdialysis probe in the dorsomedial SON (arrow) of the hypothalamus. OC - optic chiasm, pt - probe track.

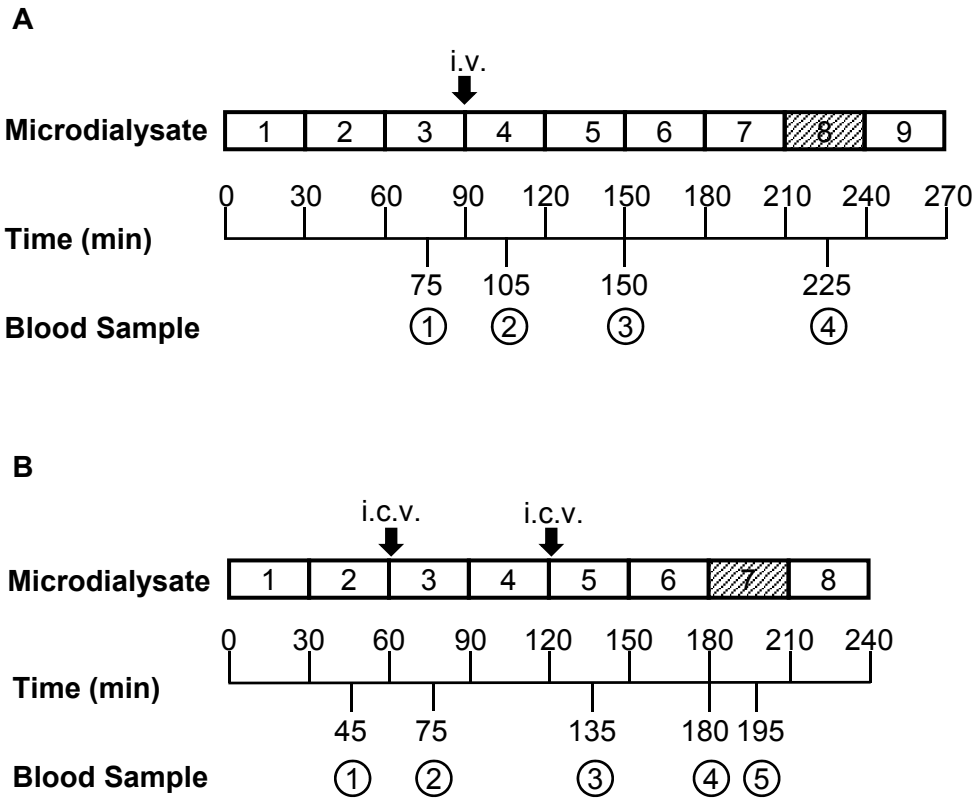

Figure 5.2. Microdialysis experimental design. Flat open rectangles indicate 30-min samples. **(*A*)** MT-II (1 mg/ml/kg) or vehicle (saline 0.9 %, 1 ml/kg) was injected i.v. at the beginning of the 4th dialysis sample (arrow). **(*B*)** Vehicle (aCSF, 3 μl/rat) and MT-II (1 μg/3 μl/rat) were injected i.c.v. at the beginning of the 3rd and 5th dialysis sample (arrows). The sequence of the drug injections was alternated between the successive trials. The microdialysis medium was changed from normal aCSF (isotonic medium) to aCSF containing 1 M NaCl (hatched rectangle) during penultimate dialysis sample. Blood samples were withdrawn at time points as indicated (numbered circles).

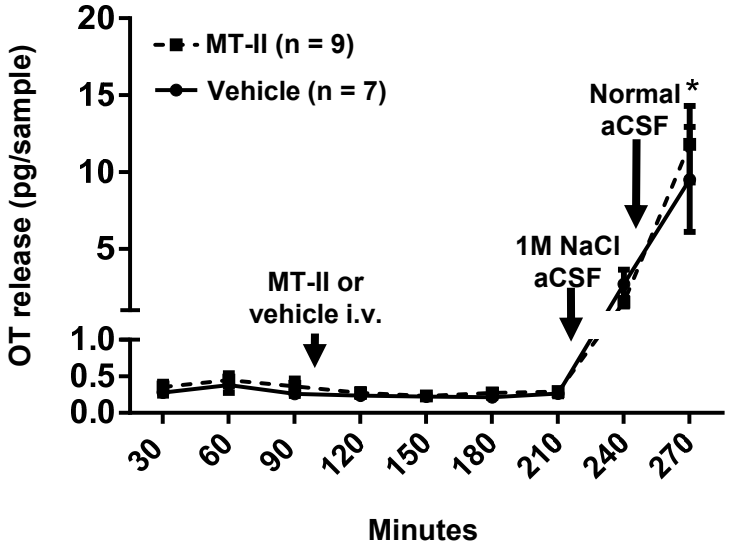

Figure 5.3. Effect of i.v. MT-II on central oxytocin release. MT-II given i.v. did not trigger somatodendritic oxytocin release from SON neurones measured by microdialysis. However, oxytocin release was increased in the vehicle and MT-II treated rats in response to microdialysis administration (retrodialysis) of hypertonic (1 M NaCl) aCSF directly into the SON. Means ± S.E.M. *P < 0.05; Two-way repeated measures ANOVA, followed by Tukey's multiple comparison tests.

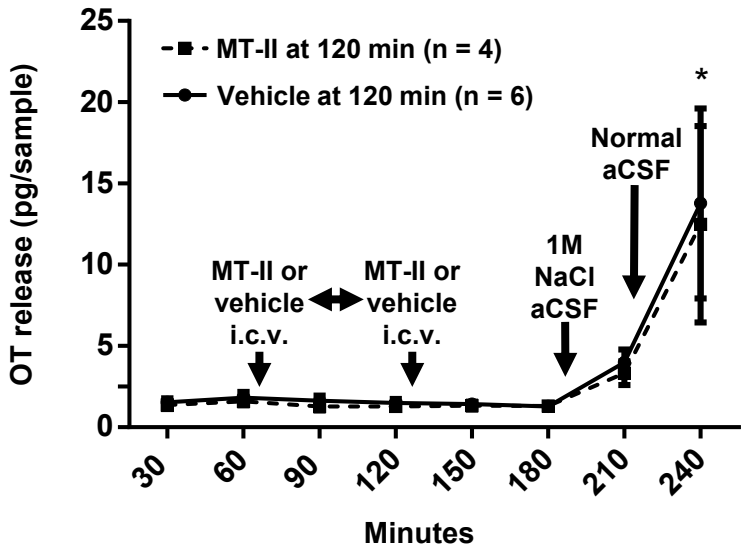

Figure 5.4. Effect of i.c.v. MT-II on central oxytocin release. I.c.v. administration of MT-II did not trigger somatodendritic oxytocin release from SON neurones measured by microdialysis. However, oxytocin release was increased in the vehicle and MT-II treated rats in response to microdialysis administration (retrodialysis) of hypertonic (1 M NaCl) aCSF directly into the SON. Means ± S.E.M. *$P < 0.05$; Two-way repeated measures ANOVA, followed by Tukey's multiple comparison tests.

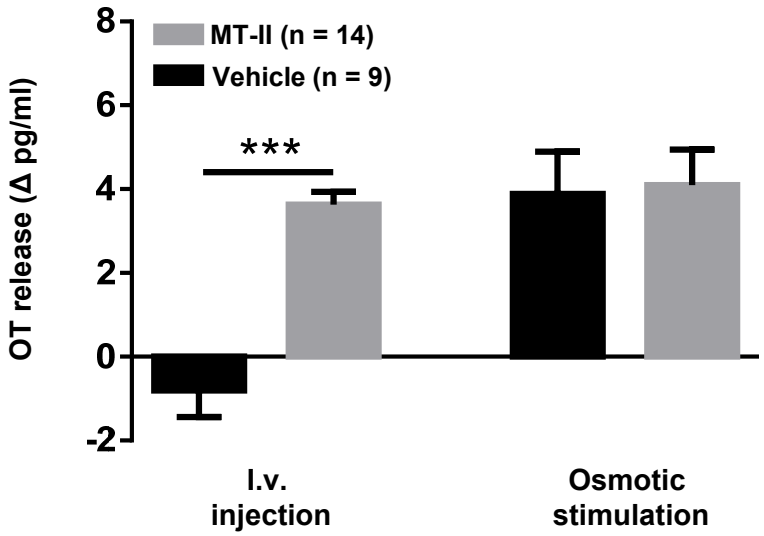

Figure 5.5. Effect of i.v. MT-II on peripheral oxytocin release. Delta change in plasma oxytocin concentration following i.c.v. MT-II, vehicle, and direct osmotic stimulation (1 M NaCl aCSF retrodialysis) of the SON. Means ± S.E.M. ***P < 0.001; Mann-Whitney test.

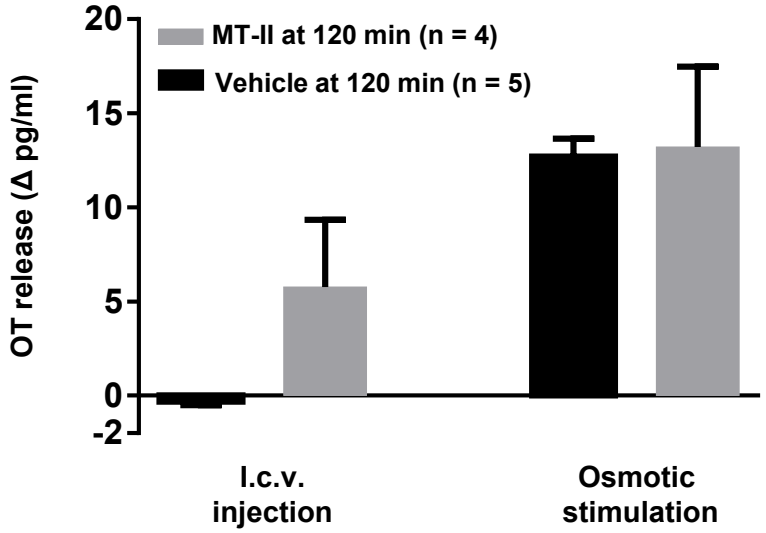

Figure 5.6. Effect of i.c.v. MT-II on peripheral oxytocin release. Delta change in plasma oxytocin concentration following i.c.v. MT-II, vehicle, and direct osmotic stimulation (1 M NaCl aCSF retrodialysis) of the SON. Means ± S.E.M.

Chapter 6

General discussion and conclusion

6.1. DISCUSSION

In this thesis, I proposed that systemic administration of the (theoretical) BBB-penetrating melanocortin agonist MT-II would stimulate central (magnocellular) oxytocin systems resulting in the dendritic release of oxytocin as an alternative approach for the potential treatment of neuropsychiatric disorders associated with low central oxytocin release. To test this, I investigated Fos protein immunoreactivity in the PVN and SON (and also brain areas modulating PVN and SON magnocellular neurones), the electrical activity of SON neurones *in vivo*, and central (within the SON) and peripheral oxytocin release *in vivo* in response to systemic administration of MT-II in anaesthetized adult male rats.

In the first experiment (Chapter 3), evidence was obtained for the efficacy of i.v., but not intranasally, administered MT-II to trigger Fos expression in PVN and SON neurones, similarly to previous studies using i.v. and i.c.v. administration routes (Thiele *et al.*, 1998; Modi *et al.*, 2015). The systemic dose of 1 mg/kg was effective in inducing Fos expression in oxytocin and also non-labelled (putative vasopressin) neurones of the SON and PVN. MT-II also induced Fos in neurones of the NTS, which is known to modulate the activity of PVN and SON magnocellular neurones by relaying peripheral inputs. Although this finding provided evidence supporting the use of MT-II as a starting point for this study, it does not prove that these effects were mediated by activation of central melanocortin receptors. Thus, I then administered the melanocortin antagonist SHU-9119 centrally, to determine whether MT-II was able to cross the BBB to mediate its effects by activation of central receptors (Chapter 3). This experiment showed that prior blockade of central melanocortin receptors prevented systemically MT-II-induced Fos expression in the SON and PVN, but not in the NTS, indicating that, in magnocellular neurones, these actions were mediated by central receptors. As could be expected after systemic administration, peripheral effects were also signalled to the brain (NTS), possibly, via the vagus nerve. Thus, taken together, these pieces of evidence provided support for systemically administered MT-II acting on oxytocin neurones via central $MC_{3/4}$, supporting the initial hypothesis established.

Since Fos expression has been shown to be a marker for *changes* in neural activity rather than an indicator of excitation or inhibition of neurones (Kovacs, 2008) I then investigated the effect of i.v. MT-II on the electrical activity *in vivo*, expecting to observe an inhibition of SON oxytocin neurones,

as found after i.c.v. α-MSH (Sabatier *et al.*, 2003a; Ladyman *et al.*, 2016). However, systemic administration of MT-II resulted in a consistent, strong, and transient *increase* in electrical activity of the oxytocin neurones (Chapter 4), suggesting that this effect might be mediated by indirect pathways, for example, via the NTS (Chapter 3). Systemic administration of other non BBB-penetrating peptides have been shown to activate SON oxytocin neurones via this pathway (Leng *et al.*, 1991; Way & Leng, 1992; Scott & Brown, 2011). Hence, this result should be interpreted as the result of MT-II peripheral actions, for instance, cardiovascular effects (Rinne *et al.*, 2012) as also suggested by observations (i.e. transient inhibition) in some vasopressin neurones tested in this experiment. However, this evidence does not preclude central actions of systemic MT-II on oxytocin neurones that could, for example, evoke simultaneously central and peripheral oxytocin release, or prime the dendritic oxytocin stores for activity-dependant release. It is important to highlight that this result indicates, for first time, a potential role for circulating melanocortins in the regulation of the activity of magnocellular neurones.

To determine whether systemic MT-II triggers somatodendritic oxytocin release, and consequently, validate or reject the hypothesis stated, I analyse the OT content within the SON and also the plasma. I found that i.v. MT-II neither triggered central oxytocin release nor primed dendritic oxytocin stores for activity-evoked release. As predicted (by the electrophysiology results), i.v. MT-II increased peripheral oxytocin release (Chapter 5). In consequence, I have to reject the hypothesis originally stated.

In the light of this outcome, two questions arise which are relevant for an appropriate interpretation of these results: is this lack of effect a

consequence of poor BBB penetration (in physiologically active amounts) of MT-II? And also, does MT-II have similar agonistic effects than the natural α-MSH *in vivo*? Thus, to empirically answer these questions I centrally administered a dose of 1 μg of MT-II which is known to induce changes in neural activity in the SON and PVN, as well as other behavioural effects (Thiele *et al.*, 1998; Rowland *et al.*, 2010). Surprisingly, i.c.v. MT-II did not stimulate central oxytocin release nor potentiate its activity-evoked release, indicating differences in agonistic properties between MT-II and α-MSH that are crucial to exerting similar neuronal effects. In addition, another factor that cannot be excluded is possible sex differences in response to direct application of melanocortin agonists, as here I studied the effect of melanocortin administration on male rats with the expectation to observe similar effects as previous studies using female rats (Sabatier *et al.*, 2003a; Ladyman *et al.*, 2016). Whether i.v. MT-II was able to penetrate the BBB in physiologically active amounts still remains unsolved.

Overall, the effects of systemic MT-II on oxytocin neurones described in this study appear to be mediated by a combination of central and peripheral effects; the apparent predominance of the latter results in an increased peripheral oxytocin release by enhanced spike activity (action potentials). In the next lines, I will expand on the main experimental gaps and interpretation caveats of this study.

A major limitation for the pharmacological targeting of the CNS is the BBB, which prevents the entry of water-soluble substances (e.g. peptides). Only lipophilic molecules or substances having specific transporters can readily cross the BBB (Banks, 2012). To my knowledge, potential BBB transport mechanisms have not been described for MT-II, so far.

Nevertheless, some drugs do penetrate the BBB, and drug transfer can also occur via the blood-CSF barrier (BBB located at the choroid plexuses) at a rate inversely related to the molecular weight of the drugs. This difference in permeability is based on intrinsic properties of epithelial/endothelial barriers, which makes the blood-CSF barrier leakier than the BBB (Pardridge, 2011). This is exemplified by the antiretroviral drug zidovudine (AZT) which has been shown to cross through the choroid plexuses by passive diffusion (Masereeuw *et al.*, 1994).

In this study, i.v. MT-II activated PVN and SON neurones, but not neurones in the cAMG. Interestingly, Thiele *et al.* (1998) have shown a dose-dependent effect of i.c.v. MT-II in the cAMG, but not in the PVN and SON (which were activated by low and high doses). Since this route (i.c.v.) emulates secretion or leakage by choroid plexuses (as the drug is directly placed in the CSF) it is possible that, in my experiments, at least some MT-II penetrated the brain via this route. In this context, the CSF may act as a neuroendocrine signalling pathway (Rodriguez, 1976; Skipor & Thiery, 2008) allowing the diffusion of MT-II into the CSF, reaching the PVN and SON (which are bathed by the CSF) in neuroactive amounts, but not the cAMG which is relatively distant from the ventricular system.

In view of the combined evidence obtained in this study, an intriguing observation is the effect (Fos attenuation) of the melanocortin antagonist SHU-9119. In particular, the electrophysiology results are hard to reconcile with the Fos immunohistochemistry study, where the melanocortin antagonist blocked the i.v. MT-II-induced Fos expression. One possibility that I did not investigate is that SHU-9119 has actions when given alone, either by blocking the effects of endogenous melanocortins or by acting as an

inverse agonist at constitutively active melanocortin receptors. Whether SHU-9119 acted as a competitive antagonist blocking binding sites for MT-II or as an inverse agonist reducing the excitability of SON neurones to, for example, putative noradrenergic inputs arising from the caudal NTS is unknown. This antagonist was originally described as a potent $MC_{3/4}$ competitive antagonist based on the lack of effect on cAMP formation in human MC_4, and a very little agonist activity on human MC_3 (Hruby *et al.*, 1995; Yang *et al.*, 2002). However, an *in vitro* electrophysiology study has shown that, in neurones of the arcuate nucleus identified by the rat insulin 2 promoter (RIPCre) expressing $MC_{3/4}$, the antagonist SHU-9119 acts as an inverse agonist inducing a hyperpolarization of this neuronal phenotype (Smith *et al.*, 2007). This suggests that the biological activity of this antagonist depends upon the cell-specific features or physiological contexts rather than being attributable to the peptide intended design. Consequently, the role of SHU-9119 on SON neurones should be investigated *in vivo* to draw a more precise conclusion about the capacity of MT-II to penetrate the brain. If SHU-9119 acts as competitive antagonist, this strongly indicates that i.v. MT-II triggered Fos by activation of central $MC_{3/4}$, reinforcing the conclusion drawn in Chapter 3. Conversely, an inverse agonist effect of SHU-9119 would not provide evidence for MT-II brain penetration (but would not disprove it either).

Regarding the agonistic properties of MT-II, in Chapter 5 I discussed possible explanations of these differences. Interestingly, it has been recently reported that the MC_3 and MC_4 can heterodimerize *in vitro*, and i.c.v. administration of the bivalent melanocortin agonist ligand CJL-1-87 led to different physiological effects (energy homeostasis) in mice when compared

to a monovalent ligand. However, the co-expression of MC_3 and MC_4 in neurones remains to be shown (Lensing *et al.*, 2017). Thus, it might be that i.c.v. administration of MT-II leads to activation of both MC_3 and MC_4, which results in activation of different molecular mechanism than the mechanism activated by the endogenous melanocortin ligands.

The current evidence indicates that, to elicit dendritic oxytocin release, mobilisation of Ca^{2+} from intracellular stores is crucial (Lambert *et al.*, 1994; Ludwig *et al.*, 2002; Sabatier *et al.*, 2003a). Therefore, any pharmacological agent used to trigger oxytocin release must achieve this neuronal prerequisite. Langouche *et al.* (2001) have reported that α-MSH and MT-II induce different changes in $[Ca^{2+}]_I$ in the prolactin and growth hormone secreting GH-3 cell line: α-MSH induces a clear increase in $[Ca^{2+}]_I$, but MT-II did not change $[Ca^{2+}]_I$. Although this effect could be influenced by the cell phenotype and influenced by the milieu context (*in vitro*), we have recently reported (Paiva *et al.*, 2017) that direct (retrodialysis) application of α-MSH onto the SON triggers somatodendritic oxytocin release *in vivo*, replicating previous *in vitro* results (Sabatier *et al.*, 2003a). Hence, oxytocin neurones seem to preserve their capacity to respond to α-MSH *in vitro* and *in vivo*. The retrodialysis administration of MT-II onto the SON, or even better, the direct application of MT-II to single SON neurones to determine $[Ca^{2+}]_I$ changes by Ca^{2+} imaging should be considered to determine the (lack of) effect of MT-II on intracellular Ca^{2+} mobilisation.

6.1.1. Limitations of this study

One limitation of this study is related to the determination of central oxytocin release, as this was restricted to the SON. This nucleus was selected

based on it homogenous composition (magnocellular neurones only), and to provide direct mechanistic evidence about the potential origin of oxytocin. However, in contrast to neurotransmitters, neuropeptides exert its actions on distant targets that, some cases, are none or scarcely innervated by peptide-containing fibres. Consequently, activation of brain areas containing high densities of OTR could account better for possible behavioural actions. In this line, Modi *et al.* (2015) reported a potentiation of central oxytocin release within the nucleus accumbens induced by i.p. MT-II in response to direct hypertonic stimulation. The nucleus accumbens is mainly innervated by a subset of oxytocin neurones from the PVN. Although this is a supraphysiological stimulus, it suggests possible MT-II effects on other oxytocin sources.

I thus cannot exclude possible differential contributions from other oxytocin sources (i.e. PVN or nucleus circularis), or even, potential differences between PVN and SON magnocellular oxytocin neurones. Supporting this notion, a morphological study of oxytocin afferents innervating the nucleus accumbens has shown similar densities of fibres among rodent species; but, in prairie voles, most of these afferents originated from PVN neurones, and only a few from SON neurones, as shown by fluorogold retrogradely-labelled oxytocin fibres (Ross *et al.*, 2009). Thus, it might be that, regarding behavioural actions, the PVN could be a better site to explore the effects of MT-II. Moreover, although PVN and SON magnocellular neurones seem to share similar morphological and physiological characteristics, evidence suggests that there may be functional differences in somatodendritic oxytocin release responses between the hypothalamic nuclei. For example, in the naturally vasopressin-null

Brattleboro rat, oxytocin release within the PVN, but not in the SON, was detected in response to 10-min forced swimming session, and subsequent osmotic stimulation (Zelena *et al.*, 2009; Zelena *et al.*, 2013). So, further studies await to investigate possible differential effects of MT-II on PVN magnocellular neurones.

Regarding oxytocin released by parvocellular neurones which have been classically described to be responsible for central actions of oxytocin. I cannot neglect the role of targeted synaptic release of oxytocin, as the Fos evidence also supports MT-II actions on this population. However, the amount of oxytocin in these neurones is small, and they do not account for the oxytocin content found in CSF, which is thought to be largely released by magnocellular neurones (Ludwig & Leng, 2006). Whether the synaptic release of oxytocin by these neurones can be detected by current methods is unknown (see Chapter 5). Furthermore, although the vast majority of the axons of magnocellular neurones project to the posterior pituitary, axon collaterals from these neurones have also been reported (Mason *et al.*, 1984; Hatton *et al.*, 1985), and evoked release of oxytocin within the cAMG from collaterals originating mostly from PVN magnocellular neurones have been shown to reduce fear response in rats (Knobloch *et al.*, 2012). Hence, *in vivo* microdialysis of other brain areas should be critically considered, as they might account for possible behavioural effects, as well as reflect release activity of parvocellular, but also magnocellular neurones of the PVN.

Another limitation is the very limited range of melanocortin agents currently available with BBB penetrating properties to validate this approach. Here, I tested MT-II based on its previously reported effects and its commercial availability. I also tested TCMC-A01 (kindly provided by Dr

M. Callahan, Tensive Controls Inc.) with theoretical BBB penetration properties which is currently under development. Experimental data using other melanocortin agonists (e.g. Pf-446687, and PT-141) have also been published (Molinoff *et al.*, 2003; Modi *et al.*, 2015), however, their use is restricted as they are currently under development. Whether these melanocortin compounds can penetrate the BBB has been assumed based on empirical (indirect) evidence, including behavioural effects and pharmacological evidence. The best method would be CSF sampling to detect contents after systemic drug administration. Unfortunately, there are technical limitations related to the amount of CSF sample required, appropriated detection method for the compound, and the lower limit of detection.

6.1.2. Complementary experiments

Other experiments would help to refine and complement the results obtained in this thesis:

In vivo electrophysiology of SON neurones in response to i.v. MT-II after desensitisation of vagal afferents by, for example, capsaicin would confirm that the enhanced electrical activity reported here was mediated by activation of peripheral receptors, which were signalled via NTS. In addition, the use of the natural (non BBB-penetrating) α-MSH could be considered to compare peripherally-induced responses to MT-II responses.

A comparative *in vivo* microdialysis study applying MT-II on female rats could be performed to determine whether the lack of effect on somatodendritic oxytocin release from SON neurones observed in this study

is related to sex differences. In addition, the expression of receptors for sexual steroids –and their possible interactions with the expression of melanocortin receptors– on SON oxytocin neurones could be analysed in both males and females to elucidate the mechanisms involved in this possible sex-related difference.

Brain areas expressing high OTR densities could be analysed by *in vivo* microdialysis to determine changes in oxytocin release that might lead to, or account for behavioural responses. These areas might include, for example, the cAMG (Knobloch *et al.*, 2012), lateral septum (Zoicas *et al.*, 2014), and prefrontal cortex (Young *et al.*, 2014).

Activation patterns and circuits (Gorges *et al.*, 2017) of, for example, limbic areas in response to systemic MT-II in conscious animals could be investigated by functional magnetic resonance imaging (fMRI). These brain areas might be of interest to study the release of oxytocin or even neurotransmitters which could be subsequently analysed using other methods (e.g. Fos activation patterns, simultaneous microdialysis).

In the case of hypothetical oxytocin release in other brain areas, behavioural tests should be performed to elucidate the role of pharmacologically-evoked central oxytocin release in normal animals and models of neuropsychiatric disorders. These tests could, for example include social recognition (Engelmann *et al.*, 2011) and anxiety tests (e.g. elevated plus maze) (Pellow *et al.*, 1985; Walf & Frye, 2007), as well as pharmacological studies (e.g. blockade of OTR) in conscious animals to confirm the effects of endogenous oxytocin release as a strategy to ameliorate neuropsychiatric disorders.

6.1.3. Clinical limitations and perspectives

Since the melanocortin system is widespread throughout the body and is involved in such a wide range of physiological functions, it is possible to predict that systemic routes lead to peripheral effects that, based on my evidence, prevail over central effects. In this study, I selected MT-II based on its theoretical BBB penetrating capacity, and agonist potency (Hruby, 2016). In mammals, after systemic administration of MT-II several effects have been reported, including feeding suppression and weight loss, penile erection, and sexual motivation (Wessells *et al.*, 2000; Pierroz *et al.*, 2002). Moreover, systemic MT-II induces yawning and stretching in humans (Dorr *et al.*, 1996; Wessells *et al.*, 2000), two well-known effects of central administration of melanocortin agonists in other species (Bertolini *et al.*, 2009).

Despite these collateral effects, MT-II has been extensively used for research purposes, and a molecule derivate from MT-II (Bremelanotide, former PT-141) with less side effects is currently under development and testing for use in humans (King *et al.*, 2007; Clayton *et al.*, 2016). A major challenge for the development and use of melanocortin agonists is the limited receptor binding specificity, which leads to activation of non-targeted receptors, and ultimately, non-desired effects (e.g. skin tanning induced by MT-II). For example, MT-II is a potent $MC_{3/4}$ agonist, and also an MC_5 agonist (Grieco *et al.*, 2007), and this receptor is also expressed in several tissues, including exocrine glands and the brain (Fathi *et al.*, 1995; Chen *et al.*, 1997). The consequences of simultaneous actions on other receptors, or tissues expressing these receptors are unknown, but for therapeutic use, the secondary effects should be reduced to the minimum possible. The use of the intranasal route as a "focused" delivery of drugs into the brain, in theory,

avoid to some extent, these limitations, but the feasibility of this route remains to be proven.

Considering all these factors and the evidence obtained in this study, the clinical use of melanocortins seems not to be the best strategy, or at least, is severely restricted (with the current drugs available) for the enhancement of central oxytocin release, and when used the effects must be carefully examined and considered during drug testing. As this strategy requires the manipulation of two systems, i.e. the melanocortin and oxytocin systems, it may be more efficient and precise use an OTR agonist with BBB capacities. For example, the non-peptide OTR agonist WAY-267464 is thought to penetrate the BBB to exert behavioural effects (Ring *et al.*, 2010; Hicks *et al.*, 2012).

To my knowledge, this study shows for first time a potential role of circulating melanocortins in the regulation of magnocellular oxytocin (and also vasopressin) neurone activity. Whether the MT-II-induced peripheral oxytocin release could be used for other clinical applications or health conditions should be considered.

6.2. CONCLUSION

As previous studies have shown that SON oxytocin neurones release oxytocin from somatodendritic compartments *in vitro*, and they are electrically inhibited *in vivo* in response to direct application of the melanocortin agonist α-MSH (Sabatier *et al.*, 2003a; Ladyman *et al.*, 2016), the actions observed in this study following intravenous (i.v.) administration of MT-II are likely to be mediated, at least in part, indirectly, probably via activation of peripheral inputs which are relayed in the NTS, where MT-II also increased Fos expression (Fig. 6.1.).

6.3. SUMMARY

The following findings and results were obtained from this study:

Chapter 3 (Fos expression studies)

- Intravenous (i.v.) administration of MT-II (1 mg/kg) induces the expression of Fos protein in oxytocin, and non-labelled, neurones of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus.
- The expression of Fos in the central amygdala (cAMG) is not changed by intravenous (i.v.) administration of MT-II.
- Intravenous (i.v.) administration of MT-II does not change Fos expression in the circumventricular organs of the anteroventral third ventricle region (i.e. the subfornical organ (SFO)), and the organum vasculosum lamina terminalis (OVLT).
- The expression of Fos in the nucleus tractus solitarii (NTS) is increased in response to intravenous (i.v.) administration of MT-II.
- The prior central (i.c.v.) administration of the melanocortin antagonist SHU-9119 (1 μg/rat) prevents Fos expression induced by intravenous (i.v.) MT-II in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, but not in the nucleus tractus solitarii (NTS).
- Intranasal administration of MT-II (1 μg and 30 μg) fails to induce changes in Fos expression in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, and also in cell layers of the olfactory bulb.

Chapter 4 (*in vivo* electrophysiology study)

- Intravenous (i.v.) administration of MT-II (1 mg/kg) increases the firing rate of supraoptic (SON) oxytocin neurones *in vivo* in urethane-anaesthetised rats.
- The firing rate of supraoptic (SON) vasopressin neurones is not significantly changed in response to intravenous (i.v.) administration of MT-II, however, single vasopressin neurones show heterogeneous responses to MT-II.

Chapter 5 (*in vivo* SON microdialysis and plasma oxytocin studies)

- Neither intravenous (i.v.; 1 mg/kg), nor intracerebroventricular (i.c.v.; 1 μg/rat) administration of MT-II trigger (central) oxytocin release within the supraoptic (SON) nucleus of the hypothalamus.
- Intravenous (i.v.) administration of MT-II stimulates peripheral oxytocin release.

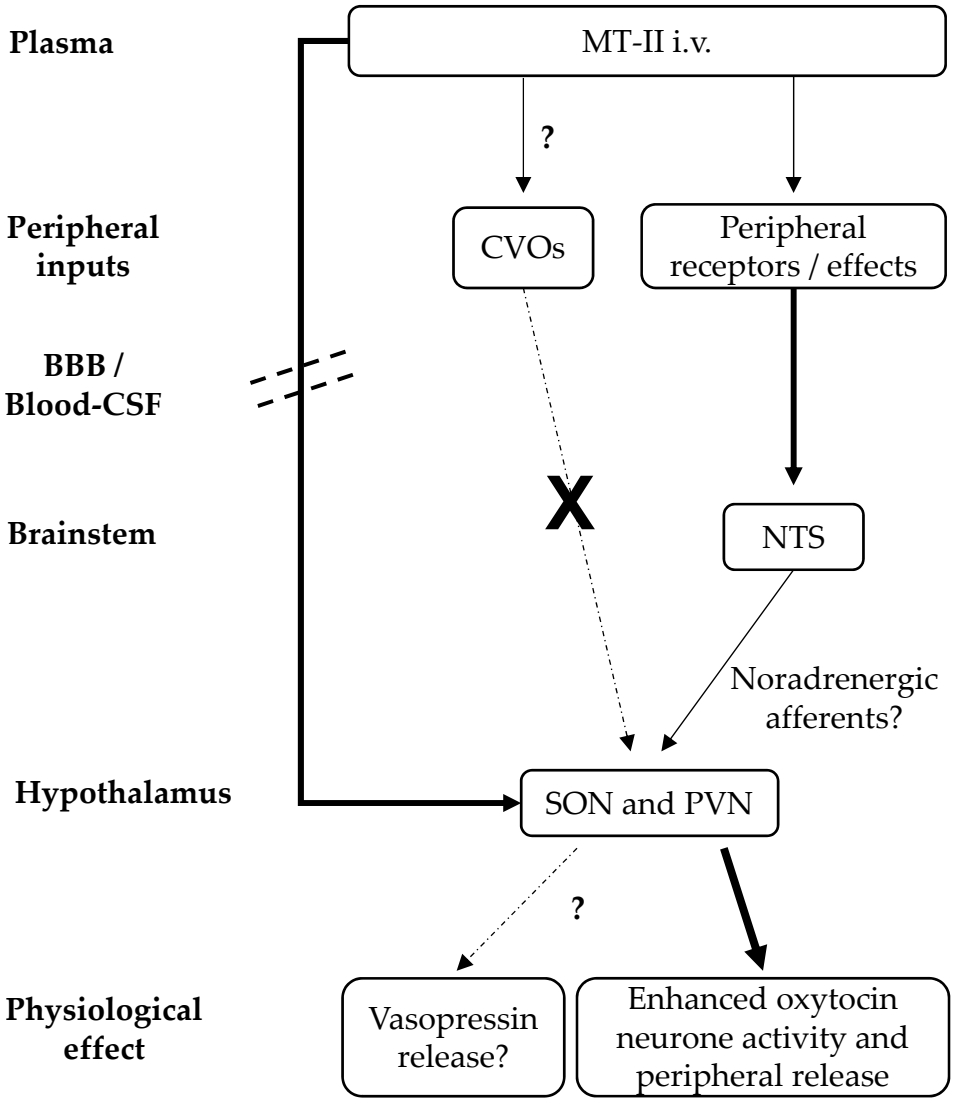

Figure 6.1. Schematic (interpretative) representation of the effects of systemic administered MT-II. I.v. administration of MT-II activates peripheral melanocortin receptors that are subsequently signalled to the NTS (Fos evidence) which, in turn, activates magnocellular oxytocin (and vasopressin; Fos evidence) neurones, as shown by enhanced Fos, electrical activity, and peripheral secretion. The melanocortin antagonist SHU-9119 prevented MT-II-induced Fos, suggesting BBB-penetrant capabilities of MT-II. Segmented arrows = potential pathways/effects based on previous reports, X = no evidence found (Callahan *et al*., 1988; Trivedi *et al*., 2003).

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Appendix

Appendix table A. Fos expression[1] following intravenous (i.v.) administration of melanocortin agonists

Brain area		Treatment			Test statistic	*Post-hoc* analyses	
		Vehicle	MT-II	TCMC-A01		Vehicle vs MT-II	Vehicle vs TCMC-A01
Fos							
SON		7.49 ± 0.95 (*n* = 12)	15.77 ± 1.59 (n = 11)	8.99 ± 1.25 (n = 6)	Kruskall-Wallis *H*=12.71, **P = 0.0017	**P = 0.0016	P > 0.9999
	PVN total	6.92 ± 0.44 (*n* = 12)	11.19 ± 1.15 (*n* = 11)	5.46 ± 0.59 (*n* = 6)	Kruskall-Wallis *H*=10.54, **P=0.0051	*P = 0.0457	P = 0.4499
PVN	mPVN	7.47 ± 0.69	17.99 ± 2.05	n/a	Mann-Whitney *U*=7.00, ***P<0.001	n/a	n/a
	pPVN	8.83 ± 0.99	7.30 ± 0.41	n/a	Mann-Whitney *U*=41.00, P=0.1326	n/a	n/a
SFO		6.44 ± 0.61 (*n* = 11)	5.72 ± 0.62 (*n* = 11)	4.70 ± 1.10 (*n* = 6)	Kruskall-Wallis *H*=2.284, P=0.3191	P > 0.9999	P = 0.2623
OVLT		5.47 ± 1.77 (*n* = 5)	6.32 ± 1.31 (*n* = 5)	4.20 ± 1.07 (*n* = 5)	Kruskall-Wallis *H*=1.340, P=0.5380	P = 0.8734	P > 0.9999
cAMG		2.08 ± 0.27 (*n* = 12)	2.71 ± 0.47 (*n* = 11)	1.77 ± 0.31 (*n* = 6)	Kruskall-Wallis *H*=1.236, P=0.5391	P > 0.9999	P > 0.9999
	NTS whole	0.25 ± 0.05 (*n* = 5)	0.52 ± 0.07 (*n* = 6)	0.26 ± 0.04 (*n* = 6)	Kruskall-Wallis *H*=7.365, *P=0.0176	*P = 0.0482	P > 0.9999
NTS	-12mm	0.13 ± 0.03	0.20 ± 0.07	n/a	Mann-Whitney *U*=12.00, P=0.9444	n/a	n/a
	-13mm	0.24 ± 0.09	0.41 ± 0.06	n/a	Mann-Whitney *U*=8.00, P=0.2424	n/a	n/a
	-14mm	0.29 ± 0.07	0.76 ± 0.17	n/a	Mann-Whitney *U*=2.00, P=0.0952	n/a	n/a
Fos/oxytocin							
	non-OT	4.55 ± 0.94 (*n* = 6)	10.12 ± 2.07 (*n* = 6)	n/a	Mann-Whitney *U*=5.00, *P=0.0411	n/a	n/a
SON	OT	2.20 ± 0.58 (*n* = 6)	5.44 ± 0.81 (*n* = 6)	n/a	Mann-Whitney *U*=4.00, *P=0.0260	n/a	n/a
	OT %	16.59 ± 3.31 (*n* = 6)	47.21 ± 4.75 (*n* = 6)	n/a	Mann-Whitney *U*=0.00, **P=0.0022	n/a	n/a
	non-OT	6.06 ± 1.33 (*n* = 6)	11.89 ± 3.16 (*n* = 5)	n/a	Mann-Whitney *U*=7.00, P=0.1775	n/a	n/a
mPVN	OT	2.09 ± 0.31 (*n* = 6)	5.81 ± 1.15 (*n* = 5)	n/a	Mann-Whitney *U*=1.00, **P=0.0087	n/a	n/a
	OT %	11.43 ± 2.96 (*n* = 6)	48.44 ± 5.07 (*n* = 5)	n/a	Mann-Whitney *U*=0.00, **P=0.0043	n/a	n/a

pPVN	non-OT	4.35 ± 0.57 (*n* = 6)	6.95 ± 1.57 (*n* = 5)	n/a	Mann-Whitney *U*=5.00, P=0.0823	n/a	n/a
	OT	0.40 ± 0.06 (*n* = 6)	1.05 ± 0.07 (*n* = 5)	n/a	Mann-Whitney *U*=0.00, **P=0.0043	n/a	n/a
	OT %	10.33 ± 3.06 (*n* = 6)	41.99 ± 5.07 (*n* = 5)	n/a	Mann-Whitney *U*=0.00, **P=0.0043	n/a	n/a

[1] Values expressed as the mean ± S.E.M. cells per 10^4 μm^2, otherwise stated (OT %, percentage of oxytocin neurones expressing Fos). Number of animals in parentheses. cAMG - central amygdala, NTS - nucleus tractus solitarii, OLVT - organum vasculosum lamina terminalis, OT – oxytocin, PVN - paraventricular nucleus, mPVN - magnocellular PVN, pPVN - parvocellular PVN, SFO - subfornical organ, SON - supraoptic nucleus.

Appendix table B. Fos expression[1] following intranasal administration of MT-II

Brain area		Treatment			Test statistic	*Post-hoc* analyses	
		Vehicle (n = 6)	MT-II 1 μg (n = 6)	MT-II 30 μg (n = 6)		Vehicle vs MT-II (1 μg)	Vehicle vs MT-II (30 μg)
SON	non-OT	1.36 ± 0.41	2.83 ± 1.09	1.98 ± 0.78	Kruskal-Wallis H=0.7836, P=0.6955	P = 0.7740	P > 0.9999
	OT	5.56 ± 0.05	0.85 ± 0.22	0.94 ± 0.21	Kruskal-Wallis H=3.520, P=0.1808	P = 0.1875	P = 0.2338
PVN Total	non-OT	0.35 ± 0.11	0.27 ± 0.08	0.51 ± 0.21	Kruskal-Wallis H=2.012, P=0.3812	P > 0.9999	P = 0.3196
	OT	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	Kruskal-Wallis H=2.012, P=0.3812	P > 0.9999	P = 0.3196
Olfactory system	EPL	0.55 ± 0.11	0.50 ± 0.12	0.48 ± 0.08	Kruskal-Wallis H=0.1871, P=0.9213	P > 0.9999	P > 0.9999
	MCL	1.08 ± 0.19	0.88 ± 0.12	1.24 ± 0.21	Kruskal-Wallis H=1.836, P=0.4154	P = 0.8981	P > 0.9999
	AOB	1.30 ± 0.21	1.43 ± 0.17	1.31 ± 0.27	Kruskal-Wallis H=0.7135, P=0.7281	P = 0.9642	P > 0.9999

[1] Values expressed as the mean ± S.E.M. cells per 10^4 μm^2, otherwise stated (OT %, percentage of oxytocin neurones expressing Fos). number of animals in parentheses. AOB - accessory olfactory bulb, EPL - external plexiform layer, MCL - mitral cell layer, OT - oxytocin, PVN - paraventricular nucleus, mPVN - magnocellular PVN, pPVN - parvocellular PVN, SON - supraoptic nucleus.

Appendix table C. Fos expression[1] prior intracerebroventricular (i.c.v.) administration of the MC3/4R antagonist

Brain area		Treatment		Test statistic
		Vehicle i.c.v. + MT-II i.v.	Antagonist i.c.v. + MT-II i.v.	
SON	non-OT	6.22 ± 0.78 (*n* = 11)	3.70 ± 0.70 (*n* = 10)	Mann-Whitney *U*=26.00, *P=0.0214
	OT	2.73 ± 0.26 (*n* = 11)	1.45 ± 0.23 (*n* = 10)	Mann-Whitney *U*=15.00, **P=0.0018
	OT %	33.1 ± 3.5 (*n* = 11)	10.8 ± 1.6 (*n* = 10)	Mann-Whitney *U*=6.00, ***P=0.0002
mPVN	non-OT	4.17 ± 0.78 (*n* = 11)	2.06 ± 0.44 (*n* = 9)	Mann-Whitney *U*=27.00, P=0.0941
	OT	1.88 ± 0.36 (*n* = 11)	0.58 ± 0.14 (*n* = 9)	Mann-Whitney *U*=15.00, **P=0.0074
	OT %	22.8 ± 4.1 (*n* = 11)	5.1 ± 0.9 (*n* = 9)	Mann-Whitney *U*=10.50, **P=0.0016
pPVN	non-OT	2.36 ± 0.66 (*n* = 11)	1.12 ± 0.20 (*n* = 9)	Mann-Whitney *U*=33.00, P=0.2256
	OT	0.50 ± 0.13 (*n* = 11)	0.17 ± 0.05 (*n* = 9)	Mann-Whitney *U*=22.00, *P=0.0379
	OT %	26.9 ± 5.3 (*n* = 11)	9.2 ± 2.1 (*n* = 9)	Mann-Whitney *U*=13.00, **P=0.0036
NTS		0.76 ± 0.09 (*n* = 10)	0.88 ± 0.10 (*n* = 10)	Mann-Whitney U=36.00, P=0.3104

[1] Values expressed as the mean ± S.E.M. cells per 10^4 μm^2, otherwise stated (OT %, percentage of oxytocin neurones expressing Fos). Number of animals in parentheses. OT - oxytocin, PVN - paraventricular nucleus, mPVN - magnocellular PVN, pPVN - parvocellular PVN, SON - supraoptic nucleus.