Effects of central neuropeptides on social preference and social recognition in male rats

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.) DER FAKULTÄT DER BIOLOGIE UND VORKLINISCHEN MEDIZIN DER UNIVERSITÄT REGENSBURG

vorgelegt von Michael Lukas

aus Roth

im Jahr 2011

Das Promotionsgesuch wurde eingereicht am: 09.08.2011

Die Arbeit wurde angeleitet von: Prof. Dr. Inga. D. Neumann

Unterschrift:

Content

Chapter 1

General Introduction

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1. Neuropeptides

Since first mentioned by David de Wied in the 1970s, the definition of the term neuropeptide underwent several transformations. Therefore, an universal description of the term neuropeptide is hard to find. However, an example for a very clear definition, among many others, was given by Carsten Wotjak (2008): *Neuropeptides are endogenous peptidergic neuromessengers, which are synthesized by and released from nerve cells and involved in nervous system functions.* Throughout neuronal messengers, neuropeptides display effects via a variety of different routes of action (for review see Landgraf and Neumann, 2004; Wotjak et al., 2008). Some may be released as neurohormones in the periphery, but all of them act in the central nervous system (CNS) in the form of neurotransmitters released synaptically at axon terminals or as neuromodulators released from other structures of the neuronal surface, i.e. dendrites and soma (Pow and Morris, 1989). The effects of synaptic release are spatially restricted to the synaptic cleft and directly influence the neuronal activity of the target neurons, e.g. via activation of ion channels at the post-synapse. In contrast, during so called volume transmission high amounts of neuropeptides are released in the areas of their production that spread throughout the CNS to trigger indirect modulatory effects on neurotransmitter action at several target neurons via intracellular signal transduction mechanisms (Agnati et al., 1995; Zoli et al., 1999). The high affinity of neuropeptides to their receptors, which are mainly G protein-coupled receptors, and their inactivation via extracellular degradation or internalization of the receptor-ligand complex speak for a pronounced action of neuropeptides as neuromodulators (Landgraf and Neumann, 2004).

This thesis focuses on the behavioral relevance of the neuropeptides arginine-vasopressin (AVP), oxytocin (OT) and the recently discovered neuropeptide S (NPS).

1.1. OT and AVP

OT and AVP belong to the arginine vasotocin family (Acher et al., 1972) and consist of nine amino acids differing in only two positions. Two cystein residues form a disulfide bridge creating the circular structure of the nonapeptides (Du Vigneaud et al., 1953). Neuropeptides of this family are ubiquitous within vertebrates and evolutionary highly conserved, both in structure and functions (Hoyle, 1999).

OT and AVP are mainly synthesized in a well-defined arrangement of magnocellular neurons located within the supraoptic (SON) and paraventricular nuclei (PVN) of the hypothalamus at the base of the brain. Via axonal projections, OT and AVP reach the neurohypophysis, where they are released into the blood stream in response to appropriate stimuli. In the context of their release into the blood stream, OT has been originally reported as a hormonal key regulator of female reproductive functions in all mammalian species. Thus, OT accelerates the delivery process as it promotes uterine contractions and is essential for the milk ejection during lactation (Freund-Mercier and Richard, 1984; Higuchi et al., 1985). The originally described physiological functions of AVP are the regulation of water resorption in the distal tubulus of the kidney and the constriction of vascular smooth muscle cells (Goldsmith, 1987). However, starting with the fundamental discoveries of David DeWied and Cort Pedersen (De Wied, 1965; Pedersen and Prange, 1979), both OT and AVP emerged as neuromodulators of the brain regulating a broad variety of behaviors.

In the context of their multiple behavioral effects, neuronal release of OT and AVP within the brain is of significant interest. OT and AVP release was shown to occur from dendrites or perikarya of magnocellular neurons described within the hypothalamus (Ludwig and Leng, 2006), i.e. PVN, SON and superchiasmatic nucleus (only AVP), but also from axonal or collateral projections of parvo- or magnocellular neurons targeting, for example, regions of the limbic brain, like the amygdala and the lateral septum (Buijs et al., 1983). Additionally, AVP release within the brain occurs from distinct extra-hypothalamic brain regions synthesizing AVP, namely the bed nucleus of stria terminalis and the medial amygdala (De Vries and Buijs, 1983). AVP cells from the medial amygdala project to the lateral septum and to the ventral hippocampus (Caffe et al., 1987). The bed nucleus of stria terminalis projects to several limbic, prefrontal and hindbrain regions, including the lateral septum, the ventral septal area, the vertical diagonal band of broca, the lateral habenular nucleus, the olfactory tubercle, and the locus coeruleus (van Leeuwen and Caffe, 1983).

The behavioral effects of OT and AVP release in these target regions are mediated by their respective receptors, the OT receptor (OTR), the AVP 1a receptor (V1aR), and the V1bR. These receptors form a phylogenetic group of 7-transmembrane domain G protein-coupled receptors. Owing to its higher and more wide-spread occurrence centrally compared to the V1bR, the V1aR is thought to be the predominant AVP receptor in the brain (Tribollet et al., 1988; Ostrowski et al., 1994; Vaccari et al., 1998). Interestingly, there is a great variation in brain V1aR and OTR expression and binding density, in species that are closely related while showing diverse behavior patterns (Insel et al., 1991; Barberis and Tribollet, 1996; Young et al., 1996; Young et al., 1997; Goodson and Bass, 2001). In a more discrete form, these variations are even noticeable in individuals of the same species (Phelps and Young, 2003;

Olazabal and Young, 2006). These findings suggest that variations in distinct behaviors might be at least to some extent due to variations in V1aR and OTR binding.

1.2 NPS

Compared to the intensively explored neuropeptides OT and AVP, information on NPS is rather sparse. NPS is named due to a serine at the amino-terminal residue of the mature 20 amino acid peptide. The primary structure is highly conserved in birds and mammals, but absent in fish, amphibians, and reptiles (Xu et al., 2007; Leonard and Ring, 2011). The cluster of NPS expressing neurons is very restricted and defines a previously uncharacterized nucleus between the locus coeruleus and the Barrington‘s nucleus (Xu et al., 2007). In contrast, the NPS receptor, an orphan G protein-coupled receptor, is widely expressed within the brain, e.g. in the thalamus, hypothalamus, amygdala, and cortical areas, as well as in the brainstem, regions involved in memory processing and emotionality (Xu et al., 2007; Leonard and Ring, 2011).

2. Involvement of OT, AVP and NPS in emotionality

Among the various behavioral functions of central neuropeptides, the involvement of OT, AVP, and NPS in emotionality takes a very prominent role. In this context it was observed that brain OT functions as an endogenous anxiolytic in rats (Neumann et al., 2000b; Waldherr and Neumann, 2007). In support, acute or chronic administration of synthetic OT reduces anxiety-related behavior in rats and mice (Windle et al., 1997; Ring et al., 2006; Blume et al., 2008; Slattery and Neumann, 2010). The anxiolytic effect of OT could be localized within both the amygdala of female (Bale et al., 2001) and the PVN of male rats

(Blume et al., 2008). Moreover, OT has the potential to inhibit physiological stress responses, such as hypothalamo–pituitary–adrenal (HPA) axis activity in response to psychological stressors (for review see Neumann, 2002; Neumann, 2009).

In contrast, AVP is known for its general anxiogenic properties, firstly demonstrated by an increased anxiety level in AVP-deficient Brattleboro rats (Williams et al., 1983, de Wied, 1993 #242). Furthermore, chronic or acute septal infusions of V1aR antagonist (V1aR-A) decrease anxiety in rats, whereas AVP administration itself has no effect (Liebsch et al., 1996; Everts and Koolhaas, 1999). The anxiogenic effect of endogenous AVP was confirmed by a reduction of anxiety-related behavior on the elevated plus-maze after septal V1aR antisense treatment (antisense sequence: 5'-GGA AAC TCA TGC TGT CCG TAC-3') in rats (Landgraf et al., 1995b). Moreover, viral V1aR upregulation in the septum of mice increases anxiety-related behavior on the plus-maze in mice (Bielsky et al., 2005b). Again, in contrast to OT, central AVP promotes the physiological stress response via increasing adrenocorticotropin (ACTH) release in the median eminence and thereby increasing HPA axis activity (Swanson and Sawchenko, 1980; Swanson et al., 1980; Gillies et al., 1982).

First pharmacological results concerning the involvement of NPS in emotionality include increased general arousal (Reinscheid et al., 2005) and the induction of long lasting wakefulness (Xu et al., 2004). Recent studies also emphasized a role for NPS in the reduction of anxiety-related behavior and fear-related memory processes in rats and mice (Xu et al., 2004; Jüngling et al., 2008; Okamura et al., 2011; Wegener et al., in press).

3. Involvement of OT, AVP and NPS in social behaviors

In addition to their prominent role in the regulation of emotionality, central neuropeptides, especially OT and AVP, are well-known for their involvement in social functions, such as maternal care, inter-male aggression, pair bonding, sexual behavior (only OT), social cognition, and social memory (for review see Bielsky and Young, 2004; Donaldson and Young, 2008; Heinrichs et al., 2009; Neumann, 2009).

3.1 Involvement of OT, AVP, and NPS in rodent social behaviors

As already mentioned, OT and AVP were shown to influence various aspects of rodent social behavior in both males and females. These are mainly reproductive behaviors. Thus, OT was shown to be responsible for the onset of maternal behavior after parturition (Pedersen et al., 1982; van Leengoed et al., 1987). During lactation OT, promotes maternal aggression against intruders (Bosch et al., 2005) and social bonding with the offspring (McCarthy, 1990; Kendrick, 2000), whereas AVP facilitates lactation by increasing arched back nursing (Bosch and Neumann, 2008). In the context of sexual behavior, OT increases sexual receptivity of female rats (Schulze and Gorzalka, 1991) and various aspects of sexual behavior in males, e.g. erectile functions and copulation (Argiolas and Melis, 2004). Moreover, chronic central OT administration in male rats increases social interaction with females (Witt et al., 1992). Both OT and AVP were shown to promote pair bonding in male and female voles (Williams et al., 1994; Cho et al., 1999) and social recognition in rats and mice (Bielsky and Young, 2004).

Additionally, it was shown that AVP increases inter-male aggression in rats (Veenema et al., 2010b).

In contrast to this huge body of literature concerning the involvement of OT and AVP in rodent social behavior, the potential involvement of NPS in social behaviors is completely unexplored until now.

3.2 Neuropeptides and human social behavior

Based on the rich knowledge on the involvement of OT and AVP in rodent social behavior, it is not surprising that these neuropeptides were also shown to be involved in human social behavior. Thus, intranasal and intravenous application of OT was reported to improve a broad variety of social behaviors summarized in various reviews (Meyer-Lindenberg, 2008; Heinrichs et al., 2009; MacDonald and MacDonald, 2010).

Specifically, intranasal OT increases trust in healthy men (Kosfeld et al., 2005) and prevents betrayal-triggered decrease in trust (Baumgartner et al., 2008). In this context, OT increases ratings for trustworthiness and attractiveness of unfamiliar faces (Theodoridou et al., 2009). Moreover, OT-treated subjects were significantly more generous than placebo-treated men during a generosity game (Zak et al., 2007). Thus, in general, OT seems to improve the interpretation of social cues (Kosfeld et al., 2005; Domes et al., 2007a), especially the recognition of fear (Fischer-Shofty et al.). OT also facilitates the recognition of faces (Rimmele et al., 2009) most effectively when there is a positive emotional connection to them (Guastella et al., 2008a; Savaskan et al., 2008). Considering eye contact as a form of initial social approach in humans, it is important to mention that OT promotes a gaze-shift towards the eye region of presented faces (Guastella et al., 2008b) also independent of their

valence as this normally occurred during presentation of fearful faces (Gamer and Buechel, 2009). In the context of OT promoting social bondings it is of interest to mention initial studies demonstrating that OT enhances attachment security (Buchheim et al., 2009). On a more neurophysiological level, human functional imaging studies indicated that OT reduces amygdala responses to threatening, non-social scenes and to angry and fearful faces (Kirsch et al., 2005). More specifically, it could be shown that OT promotes the activity in amygdala regions involved in the processing of positive social stimuli (Gamer et al., 2010), an effect that was shown to generalize to facial expressions, irrespective of their valence (Domes et al., 2007b) .

Compared to OT, only few studies concentrate on the effects of AVP on human social behavior. The behavioral effects found in these studies support the effects found in animal research. Studies investigating acute effects of AVP on human behavior mainly focus on changes in social communication. Thereby it was shown that AVP influences behavioral and physiological responses of test subjects to same-sex facial expressions. Thus, AVP in men shifts the perception of neutral facial expressions in the direction of angry expressions (Thompson et al., 2004), thereby promoting agonistic/aggressive behavior, whereas AVP in women promotes affiliation towards neutral facial expressions (Thompson et al., 2006). Furthermore, intranasal AVP facilitates recognition of happy and angry facial expressions by enhancing the encoding of positive and negative social information (Guastella et al., 2010a). Furthermore, genetic association studies found associations between V1aR promoter length and altruistic behavior in a dictator game (Knafo et al., 2008). The involvement of the AVP system in male pair bonding, which is well-known from rodent studies (Young and Wang,

2004), could also be confirmed in humans as polymorphisms in the V1aR RS3 promoter region were related to the outcome of a partner bonding scale in men (Walum et al., 2008). Together with several association studies that demonstrated a link between the V1aR gene and social deficits in autism spectrum disorders (ASD) (Thibonnier et al., 1996; Kim et al., 2002; Wassink et al., 2004), this further supports the role of the AVP system as a key regulator of social behavior/communication in humans as well.

NPS has only recently been shown to affect rodent behavior. Therefore, there are no clinical studies concerning its properties in humans, so far. However, recent gene association studies in humans identified a single nucleotide polymorphism in the NPS receptor gene that seems to be connected to an increased risk of panic disorder and over-interpretation of fear (Okamura et al., 2007; Domschke et al., 2010; Donner et al., 2010; Raczka et al., 2010). Indications for an involvement of human NPS in social behaviors are not known so far.

3.3 Social behaviors and psychiatric disorders

The regulation of social behaviors by OT and AVP in humans is of specific interest as various psychiatric disorders are associated not only with emotional disturbances, but also with dysfunctions and deficiencies in social interactions (Neumann et al., 2010). Thus, impaired social functions such as social withdrawal, social phobia, aggression and violence, or impaired social cognition are core symptoms for diseases, like major depression, anxiety disorder, posttraumatic stress disorder, borderline syndrome, schizophrenia, and ASD including the Asperger's Syndrome. Deficits in sociability seen in ASD become apparent during standard nonverbal social interactions, e.g. eye contact or affective expression. Reduced empathy like the inability to share enjoyments, interests and achievements with

others, and a lack of social and emotional reciprocity in ASD patients further demonstrate reduced sociability. Moreover, individuals with ASD not only fail to recognize faces, but also facial expressions of emotions caused by impaired social cognitive abilities (Harony and Wagner, 2010).

4. Role of OT, AVP and NPS in social preference and social recognition

To further investigate the neurobiological underpinnings of social deficits found in human psychopathologies, i.e. reduced sociability and impaired social cognition, and to study in more detail the involvement of the central OT and AVP in these deficits, animal studies are needed that test for sociability and social cognition in rodents. Social behaviors, such as social preference and individual social recognition (see Figure 1 and Figure 2) can be tested in rodents and resemble these human behaviors. Furthermore, there are also indications that central neuropeptides, especially OT and AVP, play an important role in the regulation of social preference and social recognition.

4.1 Involvement of neuropeptides in social preference

Based on the pro-social and anxiolytic properties of OT in male rats (see above), it is anticipated that the endogenous OT system would also reduce social anxiety and increase social preference in a non-reproductive context. In support, OT homologues, such as isotocin and mesotocin, have been shown to promote sociability in fish and birds, respectively (Thompson and Walton, 2004; Goodson et al., 2009). A transfer of this concept to rodents is strongly supported by data demonstrating that the prominent pro-social effect of 3, 4 methylenedioxymethamphetamine (“ecstasy”) in rats is, at least partially, mediated via

the endogenous OT system (Thompson et al., 2007). Therefore, it may be of interest to further study the involvement of endogenous OT in natural social preference as well as facilitating effects of synthetic OT on social approach behavior in a non-reproductive context in rodents.

Figure 1: Male rat performing in the social preference paradigm according to Lukas et al. (2011b). Rats are placed in a novel arena (40 × 80 × 40 cm, red light). After 30 sec of habituation, an empty wire-mesh cage (non-social stimulus; 20 × 9 × 9 cm) is placed at one side wall of the arena for 4 min (left). The empty cage is then exchanged by an identical cage containing an unknown adult male Wistar rat (social stimulus) for additional 4 min (right). The percentage of time investigating the non-social versus the social stimulus is calculated, i.e. investigation time / total time [4 min] × 100 %. A significantly higher percentage of investigation of the social versus the non-social stimulus was considered social preference.

Also, AVP is an important modulator of social behaviors, but, in contrast to OT, was shown to elicit rather anxiogenic effects in a non-social context in rodents (see above). In the context of social anxiety, in male goldfish the AVP homologue vasotocin is able to reduce sociability in individuals with high natural sociability (Thompson and Walton, 2004). Furthermore, the septal vasotocin system was shown to modulate flocking behavior in songbirds (Kelly et al.,

2011). Interestingly, effects of AVP on social anxiety have not been investigated in mammals/rodents so far.

As mentioned above, NPS is mostly known for reducing non-social anxiety in both rats and mice (Xu et al., 2004; Wegener et al., in press). However, whether the application of synthetic NPS or its receptor antagonist (NPS-A) is also able to influence social preference or reduce social anxiety is currently not known.

4.2 Involvement of neuropeptides in social recognition

Recognition and discrimination of individuals are basic prerequisites for proper social interactions. In rodents, social recognition depends on detection of olfactory signals by the main and accessory olfactory systems perceived by anogenital investigation of the con-specific. Centrally applied OT facilitates social memory in a dose-dependent manner in male rats. In contrast, infusion of the OTR antagonist (OTR-A) blocks the facilitating effect of OT, but does not impair their social memory *per se* (Benelli et al., 1995). These facilitating effects of OT could be localized in the lateral septum and the medial preoptic area of male rats, again with no effect after administration of the OTR-A alone, indicating no involvement of the endogenous OT system (Popik and van Ree, 1991; Popik et al., 1992). Literature on male mice seems more straightforward with respect to OT and OTR-A effects on social recognition. In male mice lacking the OT gene (Ferguson et al., 2000) or in mice with deficient OT release (Jin et al., 2007) impaired social recognition was consistently found. Importantly, OT bilaterally infused into the medial amygdala is able to restore the cognitive deficits seen in OT knockout mice, whereas OTR-A infusions impair social memory in male wildtype mice (Ferguson et al., 2001). Other regions responsive to synthetic or endogenous

OT in the context of social recognition are the olfactory bulb (Dluzen et al., 1998), and the ventral hippocampus (van Wimersma Greidanus and Maigret, 1996). Also in females, brain OT seems to be important for social recognition. In this context the medial amygdala (Choleris et al., 2007), the septum (Engelmann et al., 1998), and the olfactory bulb (Larrazolo-Lopez et al., 2008) could be identified as sites of action using microdialysis and local pharmacological blockade or downregulation of OTRs.

Figure 2: Rat performing in the social discriminations paradigm according to Engelmann et al. (1995) with some minor modifications. A juvenile rat (black stripes) is introduced into the cage of the experimental rat for 4 min (above). After a defined inter exposure interval, the *same* juvenile rat is re-introduced along with a *novel* juvenile rat (red stripes) for 4 min (below). The percentage of time investigating the *same* and the *novel* juvenile rat (time investigating *same* or *novel* rat / time investigating *same* + *novel* rat × 100 %) is calculated. A significantly lower investigation time directed towards the *same* versus the *novel* juvenile rat was interpreted as social discrimination.

In contrast, involvement of AVP in social memory is exclusively found in male rodents so far (for review see Bielsky and Young, 2004). Septal AVP plays an essential role in male social memory, as local infusions of AVP improve, whereas infusion of V1aR-A impair social

recognition in male rats (Dantzer et al., 1988; Engelmann and Landgraf, 1994; Everts and Koolhaas, 1997, 1999). The crucial involvement of septal AVP in social recognition is confirmed by studies using V1aR knockout mice (Bielsky et al., 2005b). Specifically, these mice demonstrate a total lack of social memory, which can be restored by re-expressing V1aRs in the lateral septum using viral vector-mediated gene transfer (Bielsky et al., 2005b). Furthermore, viral vector-mediated over-expression of septal V1aR leads to a prolongation of social memory in wildtype rats and mice (Landgraf et al., 2003; Bielsky et al., 2005a). However, information regarding the endogenous release of AVP within the lateral septum during the acquisition of social recognition memory is lacking.

Also, synthetic NPS is involved in memory processing as it is able to facilitate the extinction of contextual conditioned fear responses when administered into the amygdaloid fear circuitry in mice (Jüngling et al., 2008; Meis et al., 2008). Furthermore, NPS improves hippocampus-dependent novel object memory after acute icv administration in mice (Okamura et al., 2011). However, whether NPS is able to modulate social memory is currently not known. Therefore, we initially studied the effects of NPS on social memory, specifically on social discrimination abilities of male rats.

5. Rodent models of social stress

In order to investigate not only effects of OT, AVP, and NPS on rodent social preference and social recognition, but also their therapeutic properties concerning social deficits, rodents showing impaired social abilities are needed. An accepted approach to generate social deficits in rats and mice is the use of social stress paradigms, like social defeat and maternal separation (MS).

5.1 Social defeat

The social defeat procedure was initially established by Miczek (1979) and further developed by Heinrichs et al. (1992). The original procedure has been variously adapted, but the general idea is to introduce a test subject into the home cage territory of another male, eliciting an aggressive response followed by the defeat of the experimental rat. Social defeat results in various acute physiological alterations, e.g. increase in heart rate, plasma corticosterone, and plasma testosterone (Koolhaas et al., 1997; Martinez et al., 1998; Buwalda et al., 2005), as well as changes in social and emotional behaviors dependent on the type of defeat. Social defeat paradigms are performed using either single, or repeated defeats by different aggressor animals. Single social defeat, which was mostly done in rats, was induces anxiety-related behavior and social avoidance (Meerlo et al., 1996; Haller and Bakos, 2002; Buwalda et al., 2005). Chronic social defeat additionally induces depressive like and anhedonic behaviors, reduces general locomotion, disturbs sleep patterns and normal circadian rhythmicity (Heinrichs et al., 1992; Tornatzky and Miczek, 1993; Von Frijtag et al., 2000; Avgustinovich et al., 2005; Rygula et al., 2005; Berton et al., 2006; Kinn et al., 2008).

5.2 Maternal separation

Another form of chronic social stress is the MS paradigm, a daily 3 hour separation of pups from their dam for the first 2 weeks of life (Plotsky and Meaney, 1993; Newport et al., 2002). This stress paradigm is based on the observation that abnormal emotional and social behaviors like depression, anxiety disorders or abnormal aggression are often associated with negative experiences during childhood, like child abuse or neglect (Widom, 1989;

Barnow et al., 2001; Heim and Nemeroff, 2001; Heim et al., 2008; Weder et al., 2009). Consequently, MS results in several changes of emotional behaviors in rats and mice, foremost in the form of increased anxiety- and depression-related behaviors (Plotsky and Meaney, 1993; Wigger and Neumann, 1999; Kalinichev et al., 2002; Romeo et al., 2003; Ladd et al., 2004). Furthermore, studies in adult rats and mice as well as juvenile rats discovered first indications for deficits in normal social behavior like increased inter-male aggression (Veenema et al., 2006; Veenema et al., 2007) and juvenile play-fighting (Veenema and Neumann, 2009). In notion with that, rats demonstrating MS-induced high aggression levels and depression-related behavior show increased levels of AVP mRNA and AVP immunoreactivity in the PVN (Veenema et al., 2006). Finally, a human study in females was able to associate low cerebrospinal OT levels with a history of child abuse (Heim et al., 2008). Therefore, potential deficits observed in social behaviors appear to be due to alterations in central neuropeptide systems, especially OT and AVP.

6. Aim of present thesis

Nowadays, human psychopathologies involving deficits in social behaviors, like autism, schizophrenia, or social anxiety disorder, gain more and more attention. First clinical studies indicate an involvement of central neuropeptides like OT and AVP in these pathologies, but the neurobiological underpinnings that are responsible for these social deficits and further details on neuropeptide action on social behaviors in mammals still need to be explored.

Therefore, my initial aim was to explore in more detail the involvement of the social neuropeptides OT and AVP on two basic social behaviors in juvenile and adult rodents, i.e. social preference and social recognition that are prerequisites for many complex social

behaviors, like aggression, sexual behavior, and pair-bonding. Thereby, I adapted previously established tests for social avoidance, in mice, and social discrimination, in rats, for the use in rats and juvenile rats, respectively. Furthermore, I tested the involvement of endogenous OT and AVP in these social behaviors, using specific receptor antagonists. Additionally, I investigated for the first time the potential involvement of the recently characterized NPS in social preference and social recognition abilities of adult male rats.

This thesis also investigated the effects of negative social experiences during infancy (MS) and adulthood (acute social defeat) on social preference and social recognition, respectively. Furthermore, alterations in the central OT and AVP systems of juvenile and adult male rats after MS were investigated, including receptor binding and intracerebral release.

In the following, in order to causally link stress-induced deficits in social behavior with alterations in central neuropeptide systems, I aimed to restore social preference in male rats with social defeat-induced social avoidance and MS-induced social memory deficits, via central administration of synthetic OT and AVP, respectively. Furthermore, I tried to localize these effects in socially relevant brain structures, i.e. the lateral septum and the amygdala.

Lastly, I tested the efficacy of nasal NPS administration, in rats, on non-social anxiety and non-social memory in order to provide a first hint on the potential therapeutic use of this new neuropeptide in clinical research.

7. Outline of the present thesis

Chapter 2 describes the involvement of the endogenous OT and AVP system in natural social preference of male rats and mice. Furthermore, it investigates the effects of acute social

defeat on social preference in male rats and the potential facilitating effects of synthetic OT administration on defeat-induced social avoidance in rats.

Chapter 3 investigates the effects of MS on social recognition of juvenile and adult male rats. Furthermore it investigates septal AVP release during acquisition of social memory in the social discrimination paradigm in control and MS, juvenile and adult rats as well as its causal involvement in social recognition deficits.

Chapter 4 investigates the effects of MS on V1aR binding in brain regions known to be involved in social behaviors, such as the lateral septum, amygdala, and hippocampus.

Chapter 5 investigates the central effects of endogenous and synthetic NPS on social preference and social recognition abilities of male rats. Furthermore, it investigates the efficacy of nasally applied NPS on non-social anxiety and non-social memory of male rats

Chapter 2

The neuropeptide oxytocin facilitates pro-social behavior and prevents social avoidance in rats and mice

Author's contribution:

Lukas: Study design, establishment of behavior tests in rats, performing experiments, analyzing data, writing first draft of the manuscript

Toth: Performing experiments, analyzing data

Reber: Establishment of behavior tests in mice

Slattery: Study design, establishment of behavior tests in mice

Veenema: Study design, writing the manuscript

Neumann: Study design, writing the manuscript

[adapted from: Lukas M, Toth I, Reber SO, Slattery DA, Veenema AH, Neumann ID (2011) The neuropeptide oxytocin facilitates pro-social behavior and prevents social avoidance in rodents. Neuropsychopharmacology. [Epub ahead of print] doi: 10.1038/npp.2011.95.]

ABSTRACT

Social avoidance and social phobia are core symptoms of various psychopathologies but their underlying aetiology remains poorly understood. Therefore, this study aims to reveal pro-social effects of the neuropeptide OT, under both basal and stress-induced social avoidance conditions in rodents using a social-preference paradigm. We initially show that intracerebroventricular (icv) application of an OT receptor antagonist (OTR-A) in naïve male rats (0.75µg/5µl) or mice (20µg/2µl) reduced social exploration of a novel con-specific indicative of attenuated social preference. Prior exposure of male rats to a single social defeat resulted in loss of their social preference and social avoidance, which could be restored by icv infusion of synthetic OT (0.1µg/5µl) 10 min prior to the social preference test. While the amygdala has been implicated in both social and OT-mediated actions, bilateral OTR-A (0.1 µg/1µl) or OT (0.01 µg/1µl) administration into various subnuclei of the amygdala did not affect basal or stress-induced social preference behavior, respectively. Finally, we demonstrate the social specificity of these OT-mediated effects by showing that neither an V1aR-A (0.75µg/5µl, icv) nor the anxiogenic drug PTZ (15mg/kg, i.p.) altered social preference, with OTR-A not affecting state anxiety. Overall, the data indicate that the basal activity of the endogenous brain OT system is sufficient to promote pro-social behavior in rodents while synthetic OT shows potential to reverse stress-induced social avoidance and might thus be of use for treating social phobia and social dysfunction in humans.

INTRODUCTION

Animal and human studies indicate a facilitatory role of the neuropeptide OT in a broad variety of social interactions. Released within the brain (Landgraf and Neumann, 2004; Neumann, 2009), OT promotes various aspects of social behavior in both females and males related to reproduction, including the onset and fine-tuned maintenance of maternal behavior in lactation (Pedersen et al., 1982; van Leengoed et al., 1987; McCarthy, 1990; Kendrick, 2000; Bosch et al., 2005), receptive behavior of female rats (Schulze and Gorzalka, 1991; Witt and Insel, 1991), various aspects of sexual behavior in males (Argiolas and Melis, 2004), as well as pair bonding in female voles (Williams et al., 1994; Cho et al., 1999). Furthermore, chronic central OT administration increases social interactions of male with female rats (Witt et al., 1992). Also, OT plays a role in social recognition in both male (Popik and van Ree, 1991) and female (Engelmann et al., 1998) rats, which has been confirmed in OT and OT receptor knockout mice with impaired social memory (Ferguson et al., 2000; Choleris et al., 2003; Takayanagi et al., 2005).

Intranasal OT administration has been shown to affect many aspects of human sociability ranging from social perception, increased gazing towards the eye region (Guastella et al., 2008b; Gamer et al., 2010), and improved recognition of emotional facial expressions (Domes et al., 2007a; Savaskan et al., 2008) to complex social behaviors like trust, social-risk taking, and empathy (Kosfeld et al., 2005; Baumgartner et al., 2008; Hurlemann et al., 2010). The amygdala, a brain region strongly involved in social perception and emotional

processing, has been implicated as one of the key regions mediating neuronal actions of OT on social behaviors in humans (Kirsch et al., 2005; Baumgartner et al., 2008; Gamer et al., 2010; Hurlemann et al., 2010) as well as in rodents (Ferguson et al., 2001; Choleris et al., 2007; Lee et al., 2007).

In addition to these multiple effects on sociability, brain OT functions as an endogenous anxiolytic neuropeptide in females (Neumann et al., 2000b) and males (Waldherr and Neumann, 2007), and acute or chronic administration of synthetic OT reduces anxiety-related behavior in rodents (Windle et al., 1997; Ring et al., 2006; Blume et al., 2008; Slattery and Neumann, 2010). The anxiolytic effect of OT could be localized within both the amygdala of females (Bale et al., 2001; Neumann, 2002) and the hypothalamic PVN of males (Blume et al., 2008). Based on these behavioral effects, we recently succeeded in showing that the prolonged anxiolysis observed following sexual activity in male rats is mediated by a mating-induced release of OT within the PVN (Waldherr and Neumann, 2007).

Based on the dual pro-social and anxiolytic effects of OT, it is anticipated that the endogenous OT system would also reduce social anxiety and increase social preference in a non-reproductive context. In support of this notion, OT homologues, such as isotocin and mesotocin, have been shown to promote sociability in fish and birds, respectively (Thompson and Walton, 2004; Goodson et al., 2009). A transfer of this concept to rodents is strongly supported by data demonstrating that the prominent pro-social effect of 3,4 methylenedioxymethamphetamine (“ecstasy”) in rats is, at least partially, mediated via the

endogenous OT system (Thompson et al., 2007). Such facilitatory effects of OT on social preference are of particular importance in the context of social dysfunctions in humans, which represent core symptoms of various neuropsychiatric disorders including anxiety- and depression-related diseases (Merikangas and Angst, 1995) and autism spectrum disorders (Caronna et al., 2008). Interestingly, intranasal OT has recently been shown to promote social approach and social comprehension in autistic patients (Hollander et al., 2007; Guastella et al., 2009; Andari et al., 2010; Guastella et al., 2010b).

Here, we hypothesize that brain OT promotes naturally occurring, i.e. normal, social preference and prevents social defeat-induced social avoidance in rodents. Specifically, we predict that blockade of the endogenous OT system by administration of a specific OTR-A, both icv and into various sub-regions of the amygdala, can reduce the preference for a social stimulus over a non-social stimulus. Further, we predict that central administration of synthetic OT will reverse social defeat-induced social avoidance. Finally, in order to exclude any direct interaction between non-social and social anxiety, effects of the anxiogenic compound PTZ on social preference and of the OTR-A on non-social anxiety were tested.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (250-300 g) and adult male C57BL/6 mice (20-24 g) were purchased from Charles River (Sulzfeld, Germany) and group-housed in standard cages (rats: 55 × 22 ×

18 cm, mice: 16 x 22 x 14 cm) for habituation to the laboratory facilities under standard laboratory conditions (12:12 h light:dark cycle, lights on at 6.00 h, 22 ^{o}C, 60 % humidity, food and water ad libitum). After surgery, subjects were transferred to observation cages (rats: 40 x 24 x 36 cm, mice: 16 x 22 x 14 cm) and single housed till the end of the experiment. For social defeat, male rats selectively bred for low anxiety-related behavior, (LAB) with reliable high level of inter-male aggression (Beiderbeck et al., 2007; Neumann et al., 2010), were used as residents. Experiments were performed in the first 3 hours of the light or dark-phase and conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Oberpfalz and the guidelines of the NIH.

Cannulation

For intracerebral drug infusion, guide cannulas were stereotaxically implanted 2 mm above the lateral ventricle or bilaterally 2 mm above the central, medial, or basolateral amygdala to prevent damage to the target region (see Table 1 for stereotaxic coordinates). Rats and mice were anesthetized (rats: isoflurane, Forene®, Abbott GmbH & Co. KG, Wiesbaden, Germany; mice: pentobarbital, 0.08 mg/g body weight, Narcoren®, Merial GmbH, Hallbermoos, Germany), injected with an antibiotic (Baytril®, Bayer Vital GmbH, Leverkusen, Germany), and mounted on a stereotaxic frame. The guide cannula (for icv: 21 G, rats: 12 mm; mice: 8 mm length; for local infusions in rats: 23 G, 12 mm; Injecta GmbH, Germany) was fixed to the skull with two jeweller's screws and dental cement (Kallocryl, Speiko-Dr. Speier GmbH, Muenster, Germany) and closed by a stainless steel stylet (25 G and 27 G, respectively). After

surgery, rats and mice were handled daily (stroking, holding, cleaning of stylets) for five days to minimize non-specific stress responses during the experiment.

Table 1: Stereotaxic coordinates for guide cannula implantation (Paxinos and Watson, 1998). All implantations except icv were performed bilaterally.

Region	caudal	lateral	depth
icv (rat)	+1.0	+1.6	2.0
icv (mouse)	+0.2	+1.0	1.4
blA (rat)	+2.8	±4.8	6.4
ceA (rat)	+2.5	±4.0	6.2
meA (rat)	+2.5	±3.2	7.2

icv, intracerebroventricular; blA, basolateral amygdala; ceA, central amygdala; meA, medial amygdala

Intracerebral infusions

Infusions were performed according to the protocols concerning doses and timepoints that were already established in our lab and proven to have a behavioral effect (Waldherr and Neumann, 2007; Bosch and Neumann, 2008). Briefly, rats received either icv or local infusions of a selective OTR-A (desGly-NH2,d(CH2)5[Tyr(Me)2,Thr4]OVT; icv: 0.75µg/5µl; local: 0.1 µg/1µl and 1.0 µg/1µl), that is 18-fold more selective for OT receptors over V1a receptors (Manning et al., 2008), synthetic OT (icv: 0.1 µg/5 µl; local: 0.01 µg/1 µl), a

selective V1aR-A (d(CH2)5[Tyr(Me)2]AVP; 0.75µg/5µl or vehicle (sterile Ringer solution) via an infusion cannula connected via polyethylene tubing to a Hamilton syringe. After slow infusion the infusion system was left in place for 30 s. Infusions took place either 20 min (icv) or 10 min (local) prior to behavioral testing. Mice underwent the same procedure except that they were given icv infusions with either OTR-A (20µg/2µl) or vehicle.

Administration of PTZ in rats

The anxiogenic PTZ (15mg/kg, i.p., De Souza et al. (1998)) was injected 15 min (Jones et al., 2002) prior to behavioral testing on the elevated plus-maze or in the social preference test in the neutral arena.

Acute social defeat-induced social avoidance

To induce social avoidance, single housed rats were exposed to an aggressive rat for 30 min 2 h prior to the social preference test in the neutral arena. The experimental rat was introduced into the home cage of an unfamiliar trained aggressive rat. This rat usually attacks an intruder within a minute followed by submissive postures of the intruder rat. Physical interactions were terminated by the experimenter as soon as the intruder showed signs of submissive behavior (to prevent physical damage) or after a period of 10 min to the experimental rat by separating the rats by a wire-mesh screen. After a total time of 30 min, the experimental rat was placed back in its home cage.

Social preference paradigm

The social preference paradigm in rats was based on the social approach-avoidance test previously described in mice (Berton et al., 2006). In our study, the test has been modified and used both in rats and mice. The species-dependent durations of habituation, presence of object stimulus, presence of social stimulus and light conditions were determined based on a series of pilot experiments assessing the duration-dependent exploratory behavior in rats (arena and home cage) and mice (arena).

Social preference in novel arena

Rats and mice were placed in a novel arena (rat: 40 x 80 x 40 cm, red light; mouse: 28 x 46 x 27 cm, 100 lux; Figure 3). After 30 sec of habituation an empty wire-mesh cage (object stimulus; rat: 20 x 9 x 9 cm; mouse: 11 x 9 x 6 cm) was placed at one side wall of the arena for 4 min (rats) or 2.5 min (mice). The empty cage was then exchanged by an identical cage containing an unknown male con-specific (social stimulus) for additional 4 min (rats) or 2.5 min (mice). Before each trial, the arena was cleaned with water containing a low concentration of detergent. In rats, all tests took place during the active phase starting one hour after lights off using an unknown Wistar rat as social stimulus. Mice were tested in the early light phase (one hour after lights on) using an unknown male CD1 mouse as social stimulus.

Social preference in home cage

In order to demonstrate that OT-induced changes in social preference are to a large extent independent of the environment, light conditions, and the quality of the social stimulus, rats were additionally tested for social preference in their home cage with slight modifications. Specifically, a red plastic ring (inner diameter 8.3 cm, outer diameter 9.4 cm, 2.8 cm wide) was used as object stimulus during the 4-min object stimulus period. Subsequently, the time investigating a freely moving 3-week-old male Wistar rat - used as social stimulus - was assessed for additional 4 min after removal of the ring. A juvenile rat was chosen as social stimulus because it doesn't elicit aggressive behavior in the experimental rats. The social preference test was performed in the light phase under standard laboratory illumination.

Each test procedure was videotaped and scored afterwards by an observer blind to the treatment using JWatcher behavioral observation software (V 1.0, Macquarie University and UCLA). Object and social stimulus times were scored by measuring the time the rat/mouse spent in active olfactory investigation. As total exposure time differs between rats and mice, data are presented as the percentage of time investigating the object stimulus and the percentage of time investigating the social stimulus, i.e. investigation time / total time [4 min / 2.5 min] × 100 %. A significantly higher percentage of investigation of the social versus the non-social stimulus was considered social preference. Loss of this difference was defined as lack of social preference or accordingly social avoidance.

Elevated plus-maze

The elevated plus-maze was used to assess the effects of the OTR-A or of PTZ on non-social anxiety-related behavior in both rats and mice (Pellow et al., 1985). It consisted of two opposing open arms (rat: 50 x 10 cm; mice: 6 x 30 cm; 100 lux) and two opposing closed arms (rat: 50 x 10 x 40 cm; mice: 6 x 30 x 17 cm; 20 lux) connected by a central area. The apparatus was made of dark grey plastics, elevated to a height of 80 cm (rats) or 35 cm (mice) above the floor, and surrounded by an opaque curtain to avoid external disturbance. Before each trial, the maze was cleaned with water containing a low concentration of detergent. Rats and mice were placed individually in the central area facing a closed arm and were allowed to explore the maze for 5 min. The percentage of time spent on the open arms was assessed as anxiety-related behavior (time on open arms/time on open and closed arms × 100); the number of entries in the closed arms as measurement for locomotion was recorded by means of a video camera mounted above the platform and scored by a trained observer (Plus-maze version 2.0; E. Fricke). The elevated plus-maze testing was performed in the light phase.

Black-white box

To assess non-social anxiety-related behavior in an additional test, rats were monitored in the black-white box for 5 min (Waldherr and Neumann, 2007; Slattery and Neumann, 2010) (white compartment: 40 x 50 cm, 350 lux; black compartment: 40 x 30 cm, 70 lux). The black-white box was surrounded by an opaque curtain to avoid external disturbances. Rats were placed in the dark compartment, and the percentage time spent in the white

compartment (time in white compartement / total time [5 min] × 100 %) was assessed as anxiety-related behavior. Testing was performed during the early-dark phase.

Experimental design

Involvement of endogenous OT in social preference in rats and mice

To reveal the involvement of the brain OT system in basal social preference, the following experiments were carried out in male rats and mice (for group sizes see corresponding figures).

Rats: Rats were icv infused with either OTR-A (0.75µg/5µl) or vehicle 20 min before testing in the social preference paradigm in the neutral arena. A separate group of rats received the same treatments and were tested for social preference in their home cage. In addition, another group of rats was icv infused with the selective V1aR-A (0.75µg/5µl) or vehicle 20 min prior to the social preference paradigm in the arena to test if the closely related brain neuropeptide AVP also influences social preference. Finally, to localize the positive effect of endogenous OT on social preference, additional rats were bilaterally infused with OTR-A at two different doses (0.1µg/µl and 1.0µg/µl) or vehicle into the central, medial, or basolateral amygdaloid nuclei and tested in the social preference paradigm in the arena 10 min later.

Mice: Mice were icv infused with either OTR-A (20µg/2µl or 2µg/2µl) or vehicle 20 min before testing in the social preference paradigm in the arena.

Social defeat-induced social avoidance and reversal by synthetic OT in rats

First, we aimed to verify whether exposure to a single social defeat induces social avoidance. Rats were tested once in the social preference paradigm 2 h after onset of the 30-min social defeat using either the former defeater rat or an unknown male rat as social stimulus. Second, in order to test the hypothesis that social defeat-induced social avoidance can be reversed by OT, male rats were socially defeated and OT (0.1µg/5µl) or vehicle were icv infused 20 min before the social preference test using the former defeater rat as social stimulus. Third, to localize the positive effect of OT on social preference in defeated rats, socially defeated rats were bilaterally infused with either OT (0.01µg/µl) or vehicle into the central or medial nucleus of the amygdala 10 min before being tested in the social preference paradigm (for group sizes see figures).

Involvement of non-social anxiety in the display of social preference in rats and mice

To test if the lack of social preference after blockade of brain OT receptors is specific for social anxiety, additional groups of rats and mice received the following treatments (for group sizes see figures).

Rats: Rats were icv treated with OTR-A (0.75µg/5µl) or vehicle 20 min before testing for non-social anxiety-related behavior in the black-white box.

Mice: Mice were icv treated with OTR-A (20µg/2µl) or vehicle 20 min before testing non-social anxiety-related behavior on the elevated plus-maze.

Rats: To further test for a possible interaction between general state anxiety and social preference, rats were given a single injection of the anxiogenic agent PTZ (15mg/kg, ip) or

vehicle and tested 15 min later on the elevated plus-maze or in the social preference paradigm.

Histology

To verify the infusion site, rats and mice were killed using CO_2 and ink was infused icv or locally (5µl and 2ul, for rats and mice respectively) before removal of the brain. Icv brains were instantly cut coronally and checked for staining of the ventricle, whereas locally infused brains were frozen in pre-chilled *n*-methylbutane on dry ice and cut in 40 µm coronal cryostat sections and stained with cresyl violet. Only those animals with correct infusion site were included in the statistical analysis. Accordingly, a total of 6 rats had to be excluded from statistical analysis due to misplacement of the cannula(s) (local OTR-A: meA (2); local OT: ceA (2), meA (2)).

Statistics

For statistical analysis PASW/SPSS Statistics (Version 17.0) was used. Social preference was statistically analyzed using ANOVA for repeated measures (two-way: factors treatment × stimulus; three-way: factors treatment × stimulus × defeat) followed by a Bonferroni post-hoc test when appropriate. All relevant F-values are provided in Table 1. Non-social anxiety behavior was analyzed using the student's t-test. Significance was accepted at $p<0.05$.

RESULTS

Involvement of endogenous OT in social preference

Rats: Icv OTR-A-treated rats showed a significant decrease in social investigation during the social preference test in the arena ($p<0.01$ versus vehicle; Figure 3). Still, both OTR-A- and vehicle-treated rats investigated the social stimulus significantly longer than the object stimulus ($p<0.05$; Figure 3), thereby demonstrating social preference. Central infusion of the V1aR-A did not change social investigation time, and thus, both vehicle- and V1aR-A-treated groups demonstrated a preference for the social compared to the object stimulus ($p<0.01$, Figure 3).

In line with the results in the novel arena, when tested in their home cage, icv OTR-A-treated rats showed a decrease in social investigation ($p=0.055$ versus vehicle; Figure 3). As a result, OTR-A-treated rats showed no social preference (stimulus versus object: $p=0.673$, Figure 3).

Bilateral infusion of either 0.1µg/µl or 1.0µg/µl OTR-A into either the central, medial, or basolateral amygdala did not result in altered social investigation times compared with respective vehicle-groups (Figure 4). All rat groups independent of treatment showed social preference ($p<0.05$ versus object stimulus, Figure 4).

Mice: Similar to rats, icv OTR-A-treated mice showed a significant decrease in social investigation ($p<0.05$ versus vehicle, Figure 3). In contrast to rats, OTR-A (20µg/2µl) treatment induced social avoidance in mice, thereby completely preventing social preference ($p=0.70$ versus object stimulus, Figure 3). A ten-fold lower dose did not alter any behavior assessed in the social preference paradigm (data not shown).

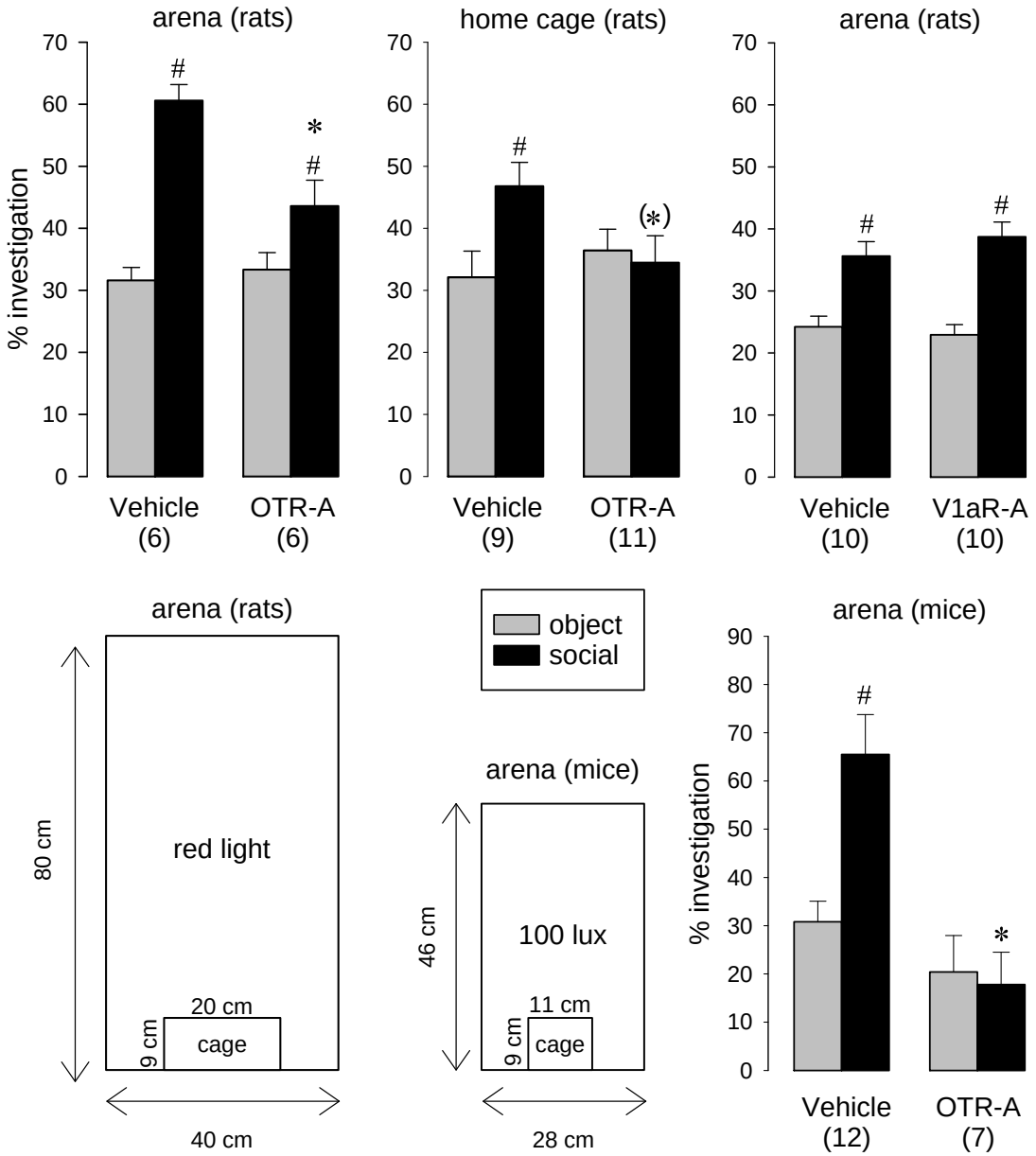

Figure 3: Effects of icv administration of an oxytocin receptor antagonist (OTR-A; rats: 0.75µg/5µl; mice 20µg/2µl) or AVP receptor antagonist (V1aR-A, rats: 0.75µg/5µl) on social preference in a novel arena (rats and mice; see schematic drawing) or in the home cage (rats). Social preference is reflected by the higher percentage of investigation time directed towards the *social (black columns)* versus the *object (grey columns)* stimulus during two sequential exposures. Numbers in parentheses reflect group size. Data are means + SEM, # $p < 0.05$ vs. object stimulus, * $p < 0.05$ vs vehicle, (*) $p = 0.055$ vs. vehicle; two-way ANOVA for repeated measures followed by Bonferroni post-hoc test.

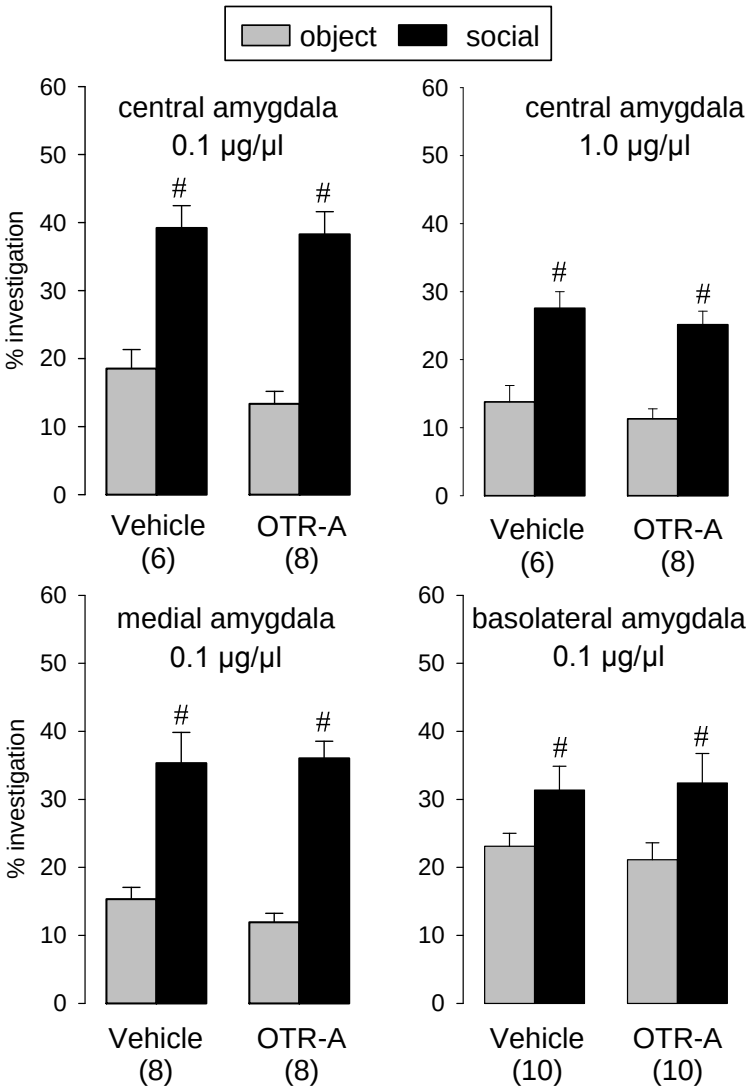

Figure 4: Effects of infusion of an oxytocin receptor antagonist (OTR-A, 0.1µg/1µl and 1.0 µg/µl) bilaterally into the central, medial, or basolateral amygdala on social preference of male rats. Social preference is reflected by the higher percentage of investigation time directed towards the *social (black columns)* versus the *object (grey columns)* stimulus during two sequential exposures. Numbers in parentheses reflect group size. Data are means + SEM, # $p < 0.05$ vs. object stimulus, two-way ANOVA for repeated measures followed by Bonferroni post-hoc test.

Social defeat-induced social avoidance and reversal by icv OT infusion in rats

To demonstrate that a single exposure to social defeat induces social avoidance, male rats were defeated by an aggressive larger male rat and then tested in the social preference paradigm 2 h later. When an unknown male rat was used as social stimulus, both control and defeated rats showed a preference for the unknown social stimulus ($p<0.05$ versus object stimulus, Figure 5A). In contrast, exposure of the defeated males to their former defeater rat

resulted in social avoidance, as demonstrated by a significant decrease in social stimulus investigation ($p<0.05$ vs control rats, Figure 5B) and a lack of social preference.

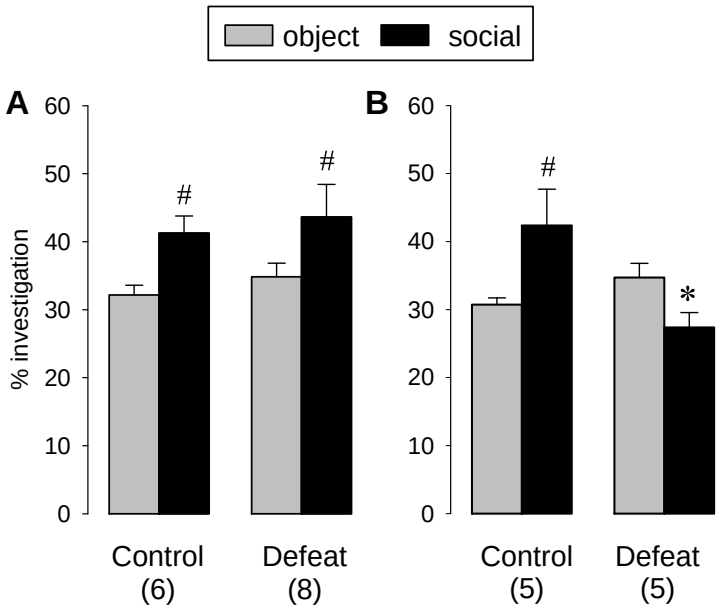

Figure 5: Social preference behavior of socially defeated and control rats. (A) Control and defeated rats were exposed to an unknown male as social stimulus during the social preference test. (B) Socially defeated rats were exposed to their individual defeater rat as social stimulus during the social preference test. Social preference is reflected by a higher percentage of investigation time directed towards the *social (black columns)* versus the *object (grey columns)* stimulus during two sequential exposures. Numbers in parentheses reflect group size. Data are means + SEM, * $p < 0.05$ vs. non-defeated control, # $p < 0.05$ vs. object stimulus; two-way ANOVA for repeated measures followed by Bonferroni post-hoc test.

To test whether OT can reverse defeat-induced social avoidance, defeated rats were icv or locally infused with either synthetic OT or vehicle. Whereas undefeated rats treated with either vehicle or OT showed social preference ($p<0.05$, Figure 6), vehicle-treated defeated rats lost their preference for the social stimulus (former defeater, $p=0.468$ versus object stimulus, Figure 6). The stress-induced social avoidance seen in defeated rats could be prevented by icv OT 20 min prior to the social preference test, resulting in reinstatement of

social preference towards the former defeater ($p<0.05$ social versus object stimulus, Figure 6). Infusion of synthetic OT into either the central or medial amygdala of defeated rats failed to increase social investigation time towards the former defeater resulting in a similar lack of social preference as seen in vehicle-treated defeated rats (Figure 6).

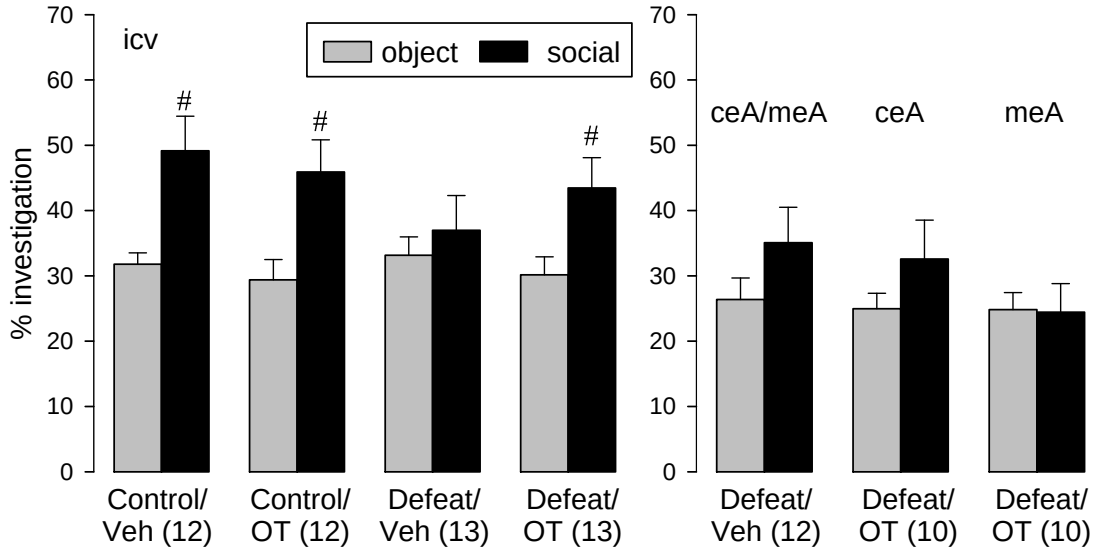

Figure 6: Social preference of socially defeated and control rats after either vehicle (veh) or oxytocin (OT) were applied icv (0.1µg/5µl) or bilaterally into the central (ceA) or medial (meA) amygdala (right) (0.01µg/1µl) 20 min prior to the social preference test. Social preference is reflected by the percentage of investigation time directed towards the *social (black columns)* versus the *object (grey columns)* stimulus during two sequential exposures. Numbers in parentheses reflect group size. Data are means + SEM, # $p < 0.05$ vs. object stimulus; three-way or two-way ANOVA for repeated measures followed by Bonferroni post-hoc test.

Involvement of general anxiety in the display of social preference in rats and mice

Rats: Icv OTR-A infusion in male rats did not change non-social anxiety-related behavior in the black-white box (percentage time spent in the white compartment of the black-white box; $t_{(14)}=1.07$, $p=0.302$) (Figure 7A).

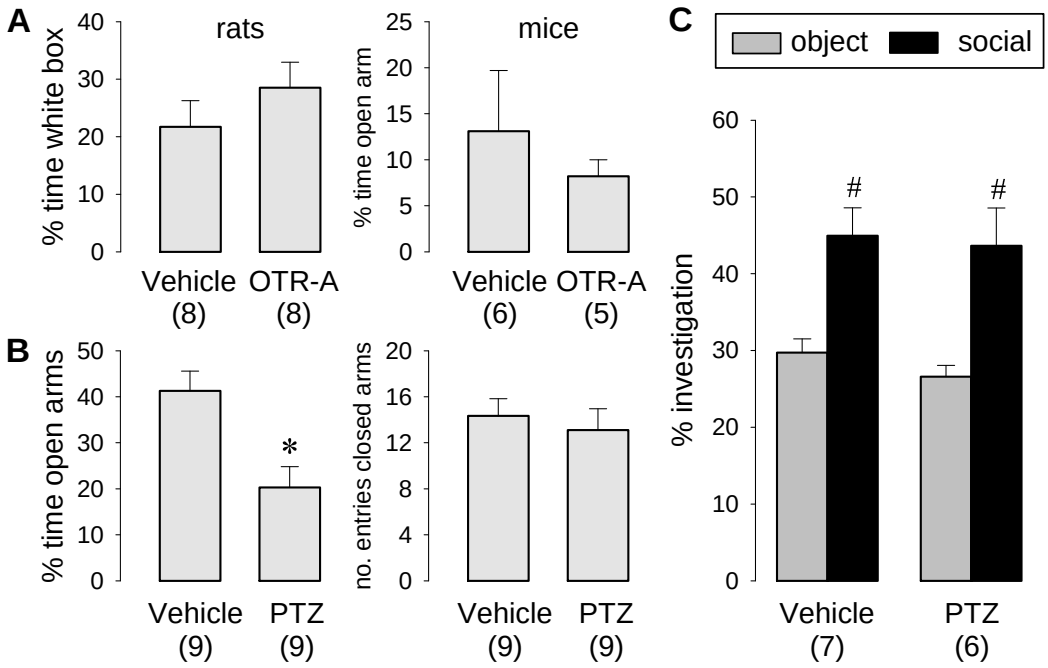

Figure 7: (A) Effects of infusion of an oxytocin receptor antagonist (OTR-A, icv) on non-social anxiety in the black-white-box in rats and on the elevated plus-maze in mice, respectively. Non-social anxiety is reflected by the percentage time from total time [5 min] spent in the white box of the black-white box and the percentage of time spent on the open arms to all arms in the elevated plus-maze. Data are means + SEM, Student's t-test. (B) Effects of PTZ (15 mg/kg, i.p.) on non-social anxiety and locomotion of male rats on the plus-maze. * $p<0.05$ versus vehicle (C) Effects of PTZ on social preference in rats reflected by the higher percentage of investigation time directed towards the *social* versus the *object* stimulus. Data are means + SEM, # $p < 0.05$ vs. object stimulus, two-way ANOVA for repeated measures followed by Bonferroni post-hoc test.

Mice: Similarly, non-social anxiety-related behavior on the elevated plus-maze following icv OTR-A infusion remained unchanged in mice ($t_{(9)}$=-0.65, p=0.531, Figure 7A).

Rats: As expected, injection of the anxiogenic drug PTZ in rats increased anxiety-related behavior on the elevated plus-maze as demonstrated by a reduction in the time spent on the open arms of PTZ-treated rats compared with vehicle-treated rats ($t_{(16)}$=-3.36, $p<0.01$, Figure 7B). Importantly, PTZ did not impair social preference. Here, both vehicle- and PTZ-treated

rats spent more time investigating the social stimulus than the object stimulus ($p<0.05$, Figure 7C).

Table 2: Relevant overall effects for all social preference data (two-way or three-way ANOVA for repeated measures)

Overall effects for Figure 3	factor stimulus	factor stimulus x treatment
Arena (rats)		
icv OTR-A	$F_{(1,10)}=58.5$; $p<0.001$	$F_{(1,10)}=11.9$; $p=0.006$
icv V1aR-A	$F_{(1,18)}=41.8$; $p<0.001$	$F_{(1,18)}=1.09$; $p=0.309$
Home cage (rat)		
icv OTR-A	$F_{(1,18)}=3.42$; $p=0.081$	$F_{(1,18)}=5.88$; $p=0.026$
Arena (mice)		
icv OTR-A	$F_{(1,17)}=15.3$; $p<0.001$	$F_{(1,17)}=20.7$; $p<0.001$
Overall effects for Figure 4	factor stimulus	factor stimulus x treatment
central amygdala (0.1 µg/µl)	$F_{(1,12)}=61.5$; $p<0.001$	$F_{(1,12)}=0.54$; $p=0.476$
central amygdala (1.0 µg/µl)	$F_{(1,12)}=42.3$; $p<0.001$	$F_{(1,12)}<0.001$; $p=0.987$
medial amygdala	$F_{(1,14)}=37.3$; $p<0.001$	$F_{(1,14)}=0.64$; $p=0.439$
basolateral amygdala	$F_{(1,18)}=18.3$; $p<0.001$	$F_{(1,18)}=0.44$; $p=0.515$
Overall effects for Figure 5	factor stimulus	factor stimulus x defeat
Unknown male (A)	$F_{(1,12)}=10.5$; $p=0.007$	$F_{(1,12)}=0.004$; $p=0.950$
Defeater male (B)	$F_{(1,8)}=0.52$; $p=0.490$	$F_{(1,8)}=10.9$; $p=0.013$
Overall effects for Figure 6	factor stimulus	factor stimulus x treatment x defeat
icv OT (left)	$F_{(1,46)}=24.5$; $p<0.001$	$F_{(1,46)}=0.32$; $p=0.324$
	factor stimulus	factor stimulus x treatment
local OT (right)	$F_{(1,29)}=6.37$; $p=0.017$	$F_{(1,29)}=1.96$; $p=0.159$
Overall effects for Figure 7	factor stimulus	factor stimulus x treatment
PTZ (C)	$F_{(1,11)}=23.7$; $p<0.001$	$F_{(1,11)}=0.08$; $p=0.785$

DISCUSSION

The present results provide the first direct evidence that the endogenous OT system facilitates social preference in both male rats and mice. More specifically, central blockade of OT receptors, using an OTR-A, significantly reduced normal social preference towards a novel con-specific in both species. Moreover, centrally-applied synthetic OT was found to rescue normal social preference that had been abolished by prior exposure to an acute social defeat. Furthermore, we were able to dissociate social avoidance from non-social anxiety, as the anxiogenic drug PTZ did not alter social preference in rats, while centrally-infused OTR-A did not alter non-social anxiety. Taken together, these findings highlight a role of the OT system in social preference both under basal conditions and during stress-induced social avoidance.

Most animal research investigating the pro-social properties of neuropeptides, belonging to the OT-AVP family, has been performed either in relation to reproduction or in non-mammalian species (see Introduction). We are now able to demonstrate pro-social effects of the endogenous OT system in two rodent models of social preference as well as of synthetic OT in a rodent model of defeat-induced loss of social preference and social avoidance. Specifically, central application of an OTR-A and, thus, blockade of intracerebral OT receptors attenuated social preference in rats, and dose-dependently caused social avoidance in mice. Together, these results indicate the involvement of the brain OT system

in normal social preference in two different species. Importantly, these results provide the first direct evidence that OT can have a pro-social effect on male-male interactions and not only on reproduction-related behaviors such as pair-bonding, maternal or sexual behaviors. Consequently, basal and/or social stimulus-induced release of OT within the brain (Landgraf and Neumann, 2004; Neumann, 2009) and subsequent OT-receptor interactions within discrete brain regions may underlie the motivation for social approach. Our finding of a facilitatory effect of endogenous OT on rodent social interaction is in line with a study showing that the high levels of sociability observed after 3,4 methylenedioxymethamphetamine ("ecstasy") consumption are prevented by central infusion of an OTR-A (Thompson et al., 2007). We further demonstrate that the promotion of social preference by the OT system is a robust effect as it could be observed both in rats and mice, thus underlining evolutionary conservation. Furthermore, OT-mediated social preference was observed under varying light conditions and environmental settings, i.e. in the novel, unknown arena and in the home cage. Although beyond the scope of the current studies, it would be interesting to determine whether blockade of the endogenous OT system would also result in social avoidance in females in our paradigm. This would seem likely in the light of studies showing alterations in social recognition and anxiety in female OT knockout mice (Crawley et al., 2007) and OTR antisense DNA-treated female mice (Choleris et al., 2007)

Intracerebral release of OT in specific brain regions was found to be increased in response to a variety of social stimuli, including mother-offspring interactions, maternal aggression,

mating in males and females, and exposure to an adult con-specific during social defeat (Veenema and Neumann, 2008; Neumann, 2009). In the context of social stimuli, increased OT release could be identified within the hypothalamic PVN (Neumann et al., 1993; Waldherr and Neumann, 2007), the central amygdala (Bosch et al., 2005), the olfactory bulb (Lévy et al., 1995), the mediolateral septum (Ebner et al., 2000) and the nucleus accumbens (Ross et al., 2009). Together with the present findings, these results support the hypothesis that stimulation of the brain OT system by social cues reinforces appropriate social interactions most likely linked to the particular social cue.

In order to localize the pro-social effects of OT, we chose various subnuclei of the amygdala as the most promising target regions. Within the central amygdala, OT exerts anxiolytic effects and regulates social interactions after stress (Bale et al., 2001; Neumann, 2002; Lee et al., 2007). Moreover, the medial amygdala is prominent for its OT-dependent role in social recognition (Ferguson et al., 2001; Choleris et al., 2007) and social odor approach (Arakawa et al., 2010), and OT receptors were described in relatively high density in the central, but also the medial, and basolateral amygdala (Tribollet et al., 1988; Lukas et al., 2010). Additionally, within the central amygdala, increased OT release has been demonstrated during social stimuli, i.e. during mother-offspring interactions (Bosch et al., 2005). Likewise, human imaging studies suggest an involvement of amygdala subnuclei in oxytocinergic processing of social interactions and fear response (Kirsch et al., 2005; Gamer et al., 2010; Hurlemann et al., 2010; Labuschagne et al., 2010). However, whether OT exerts direct local effects or rather acts via multiple OT-responsive regions upstream of the amygdala after its

intranasal application in humans needs to be shown; a question that could be approached using our rodent model. However, neither an acute effect of the OTR-A (0.1µg/µl and 1.0µg/µl) on social preference, nor of synthetic OT on social defeat-induced social avoidance could be localized within the amygdala. Thus, we suggest that social preference might require OT actions at yet to be identified brain regions where OT receptors are present, including olfactory bulb, prefrontal cortex, nucleus accumbens, lateral septum, and PVN (Tribollet et al., 1988; Barberis and Tribollet, 1996; Young et al., 1996) likely to participate in a complex network to orchestrate social approach behavior. The extent to which diffusion processes after dendritic release, which have recently been discussed, impact on such a network and contribute to the behavioral effects of OT remains to be shown (Ludwig and Leng, 2006).

In accordance with previous studies demonstrating social avoidance after repeated or chronic exposure to social defeat (Meerlo et al., 1996; Haller and Bakos, 2002; Berton et al., 2006; Vidal et al., 2007), we show here that a single social defeat exposure, prior to the social preference test, induces social avoidance in male rats. However, in our hands, loss of social preference could only be induced when the identical defeater rat was used as the social stimulus during the social preference test. This is in line with a study in golden hamsters that also reports specific social avoidance of the former defeater but not of an unfamiliar con-specific (Lai et al., 2005). In our study, the social investigation time of defeated rats was even lower than object investigation time, indicating a high level of social anxiety. Synthetic OT was able to rescue social approach behavior under conditions of stress-

induced social avoidance or social anxiety and, thus, to restore social preference. Despite the role of OT in recognition of familiar con-specifics (Bielsky and Young, 2004), it is unlikely that these effects are due to OT-mediated alterations in social recognition because the dose used in the current study was ineffective in social recognition (Benelli et al., 1995) and, if anything, OT would have facilitated the recognition of the defeater rat, which would have promoted avoidance rather than the rescue of social avoidance as was found in the present study. We further found that exogenous OT did not further increase social preference under basal circumstances. Similarly, in human studies intranasal OT application reduced hyperresponsivity of the amygdala to facial cues in socially anxious patients, while not affecting the response observed in normal patients (Labuschagne et al., 2010). Additionally, in male goldfish (*Carassius auratus*) icv-infused isotocin increased social approach behavior only in individuals with low sociability (Thompson and Walton, 2004). In this respect animal models of low-innate sociability, like the BTBR T+tf/J mice (McFarlane et al., 2008), may be useful in investigating the possible facilitating properties of exogenous OT on social preference.

Remarkably, administration of the V1aR-A was without effect on social preference indicating peptide-specific effects and high specificity of the antagonists used. This is an important finding because AVP is a key modulator of various aspects of social behavior in male rodents such as social memory (Engelmann and Landgraf, 1994), inter-male aggression (Veenema et al., 2010b) and pair bonding (Wang et al., 1998), but exerts rather anxiogenic effects (Landgraf, 2006).

Due to the obvious close link between social and non-social anxiety, we assessed whether OT actions on social preference were in part due to an OT-mediated reduction in general anxiety. Although profound anxiolytic properties of synthetic OT have been described (Windle et al., 1997; Bale et al., 2001; Blume et al., 2008; Slattery and Neumann, 2010), the role of the endogenous brain OT system in the regulation of anxiety-related behavior needs particular attention. It has been repeatedly shown that anxiolytic effects of endogenous OT can only be identified under conditions of high brain OT activity, such as in lactating dams (Neumann et al., 2000b) and after mating in males (Waldherr and Neumann, 2007). In contrast, blockade of OT receptors in virgin females or males under basal conditions failed to alter the level of anxiety (Neumann et al., 2000a). This is in agreement with the present results in naïve male rats or mice demonstrating that icv infused OTR-A did not alter non-social anxiety-related behavior (i) during the social preference test (investigation of the novel object), (ii) in the black-white box (rats) or (iii) on the elevated plus-maze (mice), further substantiating a dissociation between OT effects on social preference and non-social anxiety. In support, the anxiogenic drug PTZ did, as expected, reduce the time rats spent on the open arm of the elevated plus-maze without eliciting social avoidance. These results demonstrate the specificity of the social preference test for social facets of approach behavior, which can be distinguished from parameters mirroring general anxiety. They further demonstrate that the effects of OT on social preference are not due to direct interactions with non-social anxiety.

In conclusion, our results provide direct evidence of an important involvement of the brain OT system in the natural occurrence of social preference in male rats and mice. Further, exposure to a single social defeat is efficient to induce social avoidance, which can be reversed by central administration of synthetic OT. However, these pro-social effects of OT are not likely to be mediated by direct actions of OT on the amygdala, but rather require additional regions of a potential network. Furthermore, these effects of OT are specific to social approach behavior as no alteration in non-social anxiety was observed, while - vice versa - increasing non-social anxiety did not alter social preference. Thus, the brain OT system is a promising therapeutic target for the treatment of social phobia or social avoidance as seen in social anxiety disorder and autism spectrum disorders, respectively.

Chapter 3

Early life stress impairs social recognition due to a blunted response of vasopressin release within the septum of adult male rats

Author's contribution:

Lukas: Study design, performing experiments, analyzing data, writing the manuscript

Bredewold: Study design, performing experiments,

Landgraf: vasopressin radioimmunoassay

Neumann: Study design, writing the manuscript

Veenema: Study design, performing experiments, analyzing data, writing the manuscript

[adapted from: Lukas M, Bredewold R, Landgraf R, Neumann ID, Veenema AH (2011) Early life stress impairs social recognition due to a blunted response of vasopressin release within the septum of adult male rats. Psychoneuroendocrinology 36:843-853.]

ABSTRACT

Early life stress poses a risk for the development of psychopathologies characterized by disturbed emotional, social, and cognitive performance. We used MS (3h daily, postnatal day 1-14) to test whether early life stress impairs social recognition performance in juvenile (5-week-old) and adult (16-week-old) male Wistar rats. Social recognition was tested in the social discrimination test and defined by increased investigation by the experimental rat towards a novel rat compared with a previously encountered rat. Juvenile control and MS rats demonstrated successful social recognition at inter-exposure intervals of 30 and 60 min. However, unlike adult control rats, adult MS rats failed to discriminate between a previously encountered and a novel rat after 60 min. The social recognition impairment of adult MS rats was accompanied by a lack of a rise in AVP release within the lateral septum seen during social memory acquisition in adult control rats. This blunted response of septal AVP release was social stimulus-specific because forced swimming induced a rise in septal AVP release in both control and MS rats. Retrodialysis of AVP (1 µg/ml, 3.3 µl/min, 30 min) into the lateral septum during social memory acquisition restored social recognition in adult MS rats at the 60-min interval. These studies demonstrate that MS impairs social recognition performance in adult rats, which is likely caused by blunted septal AVP activation. Impaired social recognition may be linked to MS-induced changes in other social behaviors like aggression as shown previously.

INTRODUCTION

Child abuse or neglect are severe risk factors for the development of inappropriate and abnormal social and emotional behaviors including excessive aggression, increased anxiety, and depression (Widom, 1989; Barnow et al., 2001; Heim and Nemeroff, 2001; Heim et al., 2008; Weder et al., 2009). To provide insights into underlying brain mechanisms maternal deprivation paradigms have been developed to simulate human child maltreatment conditions in rodents (Ladd et al., 2000; Sanchez et al., 2001; Veenema, 2009). For example, separating pups from their mother daily on postnatal days 1-14 induces long-lasting changes in stress coping and emotional behaviors in rats (Plotsky and Meaney, 1993; Wigger and Neumann, 1999; Kalinichev et al., 2002) and mice (Romeo et al., 2003; Veenema et al., 2007). Recently, we showed that this MS paradigm increases offensive-like behaviors during juvenile play-fighting (Veenema and Neumann, 2009) and increases aggression during adult resident-intruder encounters (Veenema et al., 2006; Veenema and Neumann, 2009) in male rats. These findings suggest that early life stress affects social communication.

Social communication relies on the ability to recognize and discriminate between individuals. In rodents, social recognition depends on detection of olfactory signals by the main and accessory olfactory systems perceived by anogenital investigation of the conspecific. Olfactory information is processed by limbic brain areas including the medial amygdala and the lateral septum (Richter et al., 2005; Baum and Kelliher, 2009; Sanchez-Andrade and Kendrick, 2009). Within the lateral septum, the neuropeptide AVP plays an essential role. For

example, septal infusions of AVP improve social recognition in male rats, whereas infusions of V1aR-A impaired it (Dantzer et al., 1988; Engelmann and Landgraf, 1994; Everts and Koolhaas, 1997, 1999). Likewise, social recognition is impaired in V1aR knockout mice but rescued by restoring V1aR expression using viral vector-mediated gene transfer into the septum (Bielsky et al., 2005a). Correspondingly, viral vector-mediated over-expression of septal V1aR prolongs social recognition in male rats and mice (Landgraf et al., 2003; Bielsky et al., 2005a). These studies suggest that AVP via activation of septal V1aR plays a critical role in social recognition. However, information on the *endogenous* release of AVP within the lateral septum during the acquisition of social recognition memory is lacking.

We hypothesized that MS impairs social recognition and does so by influencing the release of AVP. We demonstrate that MS impairs social recognition memory in adult, but not juvenile, rats. We find that in contrast to non-separated (control) rats, adult MS rats do not show an increase in septal AVP release during the acquisition of social recognition memory. We also show that social recognition can be restored in adult MS rats by administering AVP locally in the lateral septum.

METHODS

Animals

Wistar rats were obtained from Charles River (Sulzfeld, Germany) and maintained under standard laboratory conditions (12 h light/dark cycle, lights on at 0600h, 22 ^{o}C, 60% humidity, food and water ad libitum). After one week of habituation, males and females were mated for five days. Females were subsequently housed in same-sex groups of four to six rats. During the last week of gestation, female rats were individually housed in standard rat cages (42 × 27 × 18 cm). The animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Oberpfalz and the guidelines of the NIH.

MS procedure

MS was performed as described earlier (Veenema et al., 2006). Briefly, on the day after parturition, i.e. on postnatal day 1, each litter was culled to eight to ten pups (in each nest two to four females). Litters were separated from the mother daily between 0900h and noon from postnatal day 1 to 14. To do so, each dam was placed into a separate cage and the litter was, transferred to an adjacent room and kept in a box filled with bedding and placed on a heating pad maintained at 30–33 ^{o}C. After 3 hours, litters were returned to the home cage followed by the dam. Control litters were left undisturbed. Change of bedding occurred on postnatal day 1, 7 and 14 for both control and MS litters. Pups were weaned on postnatal day 21 and housed in sex- and treatment-matched groups of four to five rats until

the start of the experiments. No more than two male pups per litter were used for each of the experiments.

Social discrimination test

The ability of juvenile (5-week-old) and adult (16-week-old) control and MS rats to discriminate between a previously encountered (*same*) and a *novel* 3-week-old male rat was tested according to Engelmann (1995) with some minor modifications. Control and MS rats were individually housed in an experimental cage (40 × 24 × 35 cm) with bedding from their home cage for either 2 h (Exp. 1) or 2 days (Exp. 2 and 3). A 3-week-old rat was introduced into the cage of the experimental rat for 4 min (social memory acquisition period); either 30, 60 or 120 min later, the *same* 3-week-old rat was reintroduced along with a *novel* 3-week-old rat for 4 min (social discrimination period). Repeated testing took place at different days with new sets of 3-week-old rats. Tests were performed between 1300h and 1600h All tests were videotaped and the time spent in investigating the 3-week-old rats (sniffing the anogenital and head/neck regions) was measured by a researcher blinded to the treatment condition using Eventlog (version 1.0, Oct 1986, R. Hedersen). The percentage of time investigating the *same* and the *novel* rat (time investigating *same* or *novel* rat/time investigating *same* + *novel* rat × 100 %) was measured. A significantly lower investigation time directed towards the *same* versus the *novel* rat was interpreted as social recognition. Note that the 3-week-old rats did not elicit play behavior in juvenile control or MS rats nor aggressive behavior in adult control or MS rats. To verify that MS did not alter social approach / social motivation, the absolute time investigating the *same* rat during the

acquisition period and the absolute time investigating the *same* + *novel* rat during the discrimination period were measured.

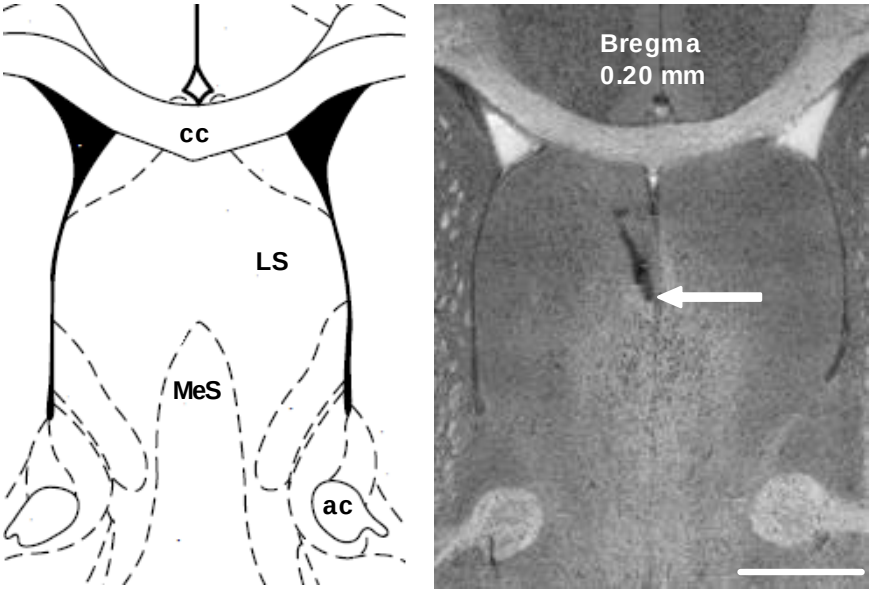

Figure 8: Schematic drawing of the septal area (adapted from Paxinos & Watson; left), and a representative enlargement of the photomicrograph of a cresyl violet-stained coronal section of the rat brain after removal of the microdialysis probe (right). Bracket indicates the size of the semipermeable membrane of the microdialysis probe. White ellipse indicates diffusion range covered by microdialysis probe, containing both sides of the lateral septal area. ac, anterior commissure; cc, corpus callosum; LS, lateral septum; MS, medial septum; LV, lateral ventricle; Scale bar = 1 mm.

Implantation of microdialysis probes

For Exp. 2 and 3, juvenile and adult control and MS rats were anesthetized with isoflurane (Forene®, Abbott GmbH & Co. KG, Wiesbaden, Germany), injected with 0.05 ml of an antibiotic substance (Baytril®, Bayer Vital GmbH, Leverkusen, Germany), and mounted on a stereotaxic frame. The microdialysis probes (self-made, molecular cut-off 10 kDa, for details see Neumann , 1993} were implanted stereotaxically into the lateral septum according to

Beiderbeck (2007). The coordinates relative to bregma were: -0.1 (juvenile) or -0.2 (adult) mm caudal, +2.0 mm lateral to the midline, 5.5 (juvenile) or 6.0 (adult) mm beneath the surface of the skull; angle of 20° to avoid damage to the sagittal sinus; nose: -3.5 mm (Paxinos and Watson, 1998) (see Figure 8). The probes were flushed and filled with sterile Ringer's solution (pH 7.4, B. Braun Melsungen AG, Melsungen, Germany), and were fixed to the skull with two jeweller's screws and dental cement (Kallocryl, Speiko-Dr. Speier GmbH, Muenster, Germany). Two 5 cm long pieces of polyethylene tubing (PE 20, Karmann & Droll, Karlsfeld, Germany) filled with Ringer's solution were connected to the inflow and the outflow of the microdialysis probe and fixed with dental cement. Upon surgery, rats were individually housed in experimental cages (40 × 24 × 35 cm). One day after surgery, rats were handled and familiarized with the experimental procedure to minimize non-specific stress responses during the experiment.

Experimental design

Exp. 1: Effects of MS on social recognition at juvenile and adult age

To measure the effect of MS on social recognition, juvenile (5-week-old) and adult (16-week-old) control and MS male rats (n = 10 in each group, from 6-7 different litters per group) were subjected to the social discrimination test on three consecutive days using inter-exposure intervals of 30, 60 and 120 min, respectively.

Exp. 2: Effects of MS on septal AVP release during the social discrimination test

Another set of juvenile and adult control and MS rats (n = 7 to 8 for each group, from 5-7 different litters per group) were subjected to the social discrimination test (inter-exposure interval of 60 min) one week prior to as well as two days after probe implantation. Repeated testing was performed in order to exclude possible interfering effects of surgery and ongoing microdialysis on social discrimination abilities.

Two days after surgery, the microdialysis probe was connected via polyethylene tubing to a syringe mounted onto a microinfusion pump and perfused with sterile Ringer's solution (3.3 µl/min, pH 7.4) starting at 1000h to establish equilibrium between inside and outside of the microdialysis membrane. Two hours later, five consecutive 30-min dialysates were collected: dialysates 1 and 2 were taken under baseline (undisturbed) conditions, dialysate 3 during the social memory acquisition period starting with the 4-min exposure to the first 3-week-old rat, and dialysates 4 and 5 were taken thereafter. Microdialysates were collected in Eppendorf tubes containing 10 µl 0.1 M HCl, immediately frozen on dry ice, and stored at -20°C until quantification of AVP by radioimmunoassay. After an inter-exposure interval of 60 min, control and MS rats were exposed to the previously encountered (*same*) rat along with a *novel* rat. The behavior of the experimental rats was videotaped during the acquisition and discrimination periods and subsequently scored.

Exp. 3: Effects of MS on septal AVP release during forced swimming in adult rats

To test whether MS causes general deficits in septal AVP release in adulthood or only in a social stimulus-specific context, adult control and MS rats were exposed to forced swimming

for 5 min, which elicits an increase in AVP release within the septum of male Wistar rats {Ebner , 1999). One day after experiment 2, the same control (n = 7) and MS (n = 8) adult rats underwent the microdialysis procedure as described above, except that, after the pre-rinsing period, three consecutive 30-min dialysates were collected: dialysate 1 was taken under baseline (undisturbed) conditions, dialysate 2 includes exposure to 5 minutes of forced swimming (in a plexiglass cylinder, diameter 30 cm, height 50 cm, water 30 cm high, 20 ^{o}C), and dialysate 3 was taken after rats had been returned to the home cage.

Exp. 4: Effects of local application of AVP into the lateral septum on social recognition in adult MS rats

To test whether a blunted response of septal AVP release contributed to the social recognition impairment, another set of adult MS rats (from 7 litters) was exposed to the social discrimination test (inter-exposure interval of 60 min) one week prior to and two days after implantation of the microdialysis probe into the lateral septum. Microdialysis was performed as described above except that after the 2-h pre-rinse period, either Ringer's solution (vehicle, n = 7) or Ringer containing 1 µg/ml synthetic AVP (AVP, n = 10) was perfused during the acquisition period, i.e., vehicle- and AVP-treated MS rats were exposed to the first 3-week-old rat for 4 min during ongoing retrodialysis. During the 30-min retrodialysis approx. 0.5 ng of synthetic AVP was delivered into the lateral septum {Engelmann , 1992}. After an inter-exposure interval of 60 min, vehicle- and AVP-treated MS rats were exposed to the *same* and *novel* rat. The behavior of the MS rats was videotaped and investigation times were subsequently measured.

Histology

Rats were killed using CO_2, brains were removed, quickly frozen in pre-chilled *n*-methylbutane on dry ice, cut into 40-µm coronal cryostat sections, and stained with cresyl violet. All rats showed successful placement of the probes in the lateral septum (see Figure 8). There were no differences in placement of the probes between MS and control groups.

Radioimmunoassay

AVP content was measured in lyophilized dialysates by a highly sensitive and selective radioimmunoassay (detection limit: 0.1 pg per sample; cross-reactivity of the antiserum with other related peptides, including OT, was less than 0.7 %) (Landgraf et al., 1995a). Radioimmunoassays were performed separately for juveniles and adults and are, therefore, not compared. However, to eliminate inter-assay variation, all other samples to be compared were measured in the same assay. Intra-assay variability was in the range of 6 – 9 %.

Statistics

Social discrimination behavior (expressed as percentage time spent investigating the *novel* and the *same* juvenile of total investigation time) within groups was analyzed using the paired samples *t*-test. Social discrimination behavior within groups was also analyzed as time spent investigating the *novel* and the *same* juvenile in sec, but this yielded similar results as when expressed as percentage. In addition, social discrimination between groups was analyzed using the Student's *t*-test. Social investigation behavior (in sec) was analyzed using

a two-way ANOVA (MS × age). Septal AVP release was analyzed using a two-way ANOVA for repeated measures (MS × dialysate), followed by LSD *post hoc* test when appropriate. Correlation analysis of AVP release with the percentage of social investigation time of the *novel* juvenile was carried out using Pearson's correlation analysis. Absolute values of baseline AVP release were analyzed with the Student's *t*-test. For all tests the software package SPSS (version 16) was used. Data are presented as mean ± SEM. Significance was accepted at $p < 0.05$.

RESULTS

Exp. 1: Effects of MS on social recognition at juvenile and adult age

Social discrimination

After an inter-exposure interval of 30 min, *all* groups (i.e. control and MS juvenile and adult rats) spent more time investigating the *novel* than the *same* rat ($p < 0.05$) (Figure 9, B). After an interval of 60 min, control rats at both ages as well as juvenile MS rats spent more time investigating the *novel* than the *same* rat ($p < 0.05$) (Figure 9A, B). In contrast, adult MS rats failed to discriminate between the *same* and *novel* rat at the 60-min interval ($t_{(9)}= -1.08$, $p = 0.31$) (Figure 9B). For complete statistics see Table 3. This suggests that MS impairs social discrimination in adult rats. After an interval of 120 min, none of the rats discriminated between the *same* and *novel* rat (data not shown), which confirms previous findings (Engelmann et al., 1995).

Social approach / social motivation

The total time investigating the *same* rat (during the acquisition period) or the *same* + *novel* rat (during the discrimination period) did not differ between adult or juvenile control and MS rats (Table 3). Thus, the impairment in social recognition memory in adult MS rats was not due to a reduction in social approach or social motivation.

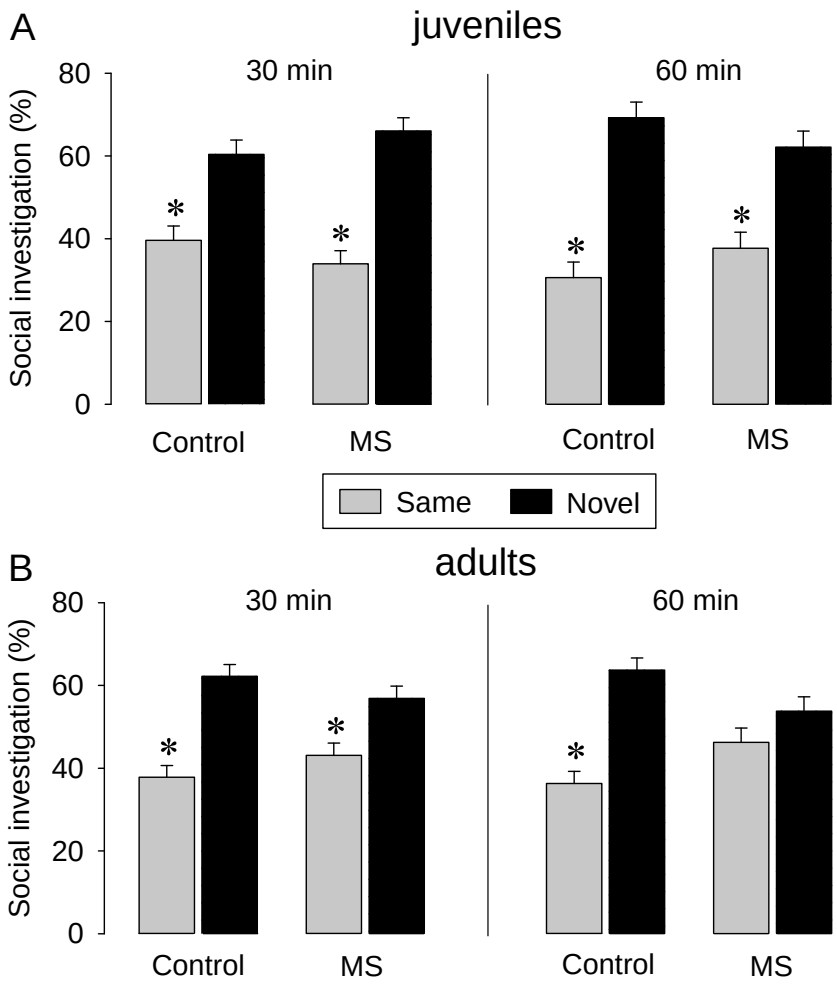

Figure 9: Effects of MS on social recognition in juvenile (A) and adult (B) male rats. Social discrimination abilities are reflected by the percentage of investigation time towards the *novel* and the *same* 3-week-old rat after inter-exposure intervals of 30 and 60 min in control and MS rats. Data are means + SEM (n = 10 for all groups), * p < 0.05 vs. novel rat, paired samples *t*-test (*same* × *novel*), # p < 0.05 vs. control, Student's *t*-test (*same* control × *same* MS). For complete statistics see Table 7.

Table 3: Social approach/social motivation is not altered by MS. The investigation time (in sec) towards the *same* rat (social memory acquisition) and the total investigation time towards the *same* + *novel* rat (social discrimination) was not different between control and MS rats at juvenile and adult age, respectively.

	Juvenile control	Juvenile MS	Adult control	Adult MS
Acquisition	122.6 ± 6.5	116.1 ± 8.4	132.5 ± 9.6	121.6 ± 7.1
Discrimination	99.1 ± 6.1	92.7 ± 9.1	104.8 ± 4.2	116.8 ± 5.3

For all groups, n = 10. Data represent means (investigation time in sec) ± SEM, two-way ANOVA (MS × age).

Exp. 2: Effects of MS on septal AVP release during the social discrimination test

No effect of surgery and ongoing microdialysis on social recognition in rats

Stereotaxic surgery and ongoing microdialysis did not affect social discrimination abilities in juvenile and adult control rats or in juvenile MS rats (Table 4). Confirming the results of Exp. 1, adult MS rats failed to discriminate between the *same* and *novel* rat both before surgery as well as during ongoing microdialysis ($t_{(6)}$ = -1.97, p = 0.096) (Table 4). For complete statistics see Table 7.

Table 4: No effects of surgery and ongoing microdialysis on social recognition. Social recognition is reflected by a significantly lower percentage of investigation time towards the *same* compared with the *novel* rat in the social discrimination test with an inter-exposure interval of 60 min. Juvenile control and MS rats as well as adult control rats demonstrate intact social recognition before surgery and after surgery and ongoing dialysis. Importantly, adult MS rats consistently demonstrate a lack of social recognition.

	Before surgery		During microdialysis	
	Same	Novel	Same	Novel
Juvenile age				
Control	39.0 ± 3.5*	61.0 ± 3.5	39.0 ± 1.3*	61.0 ± 1.3
MS	40.4 ± 3.2*	59.6 ± 3.2	41.3 ± 3.2*	58.7 ± 3.2
Adult age				
Control	41.5 ± 3.2*	58.5 ± 3.2	34.9 ± 2.8*	65.1 ± 2.8
MS	45.5 ± 3.7	54.5 ± 3.7	41.1 ± 4.5	58.9 ± 4.5

For control groups, n = 7-10, for MS groups, n = 7-10. Data represent means (% investigation time) ± SEM. * p < 0.05 vs. novel rat, paired samples *t*-test (*same* × *novel*), Student's *t*-test (*same* control × *same* MS). For complete statistics, see Table 7.

Septal AVP release in juvenile rats exposed to the social discrimination test. Exposure to the 3-week-old rat during the social memory acquisition period induced a significant change in AVP release within the septum of juvenile rats (factor dialysate: $F_{(4,52)} = 10.9$, $p < 0.001$). Juvenile control and MS rats showed an increase in septal AVP release during the acquisition period (control, $p = 0.053$ dialysate 3 vs. dialysate 2, $p < 0.05$ dialysate 3 vs. dialysates 4 and 5; MS, $p < 0.05$ dialysate 3 vs. all other dialysates) (Figure 10A). MS did not alter AVP content in microdialysates sampled under basal conditions (Table 5). No significant correlation was found between septal AVP release and investigation of the novel juvenile in control or MS juveniles($r = 0.07$; $p = 0.80$).

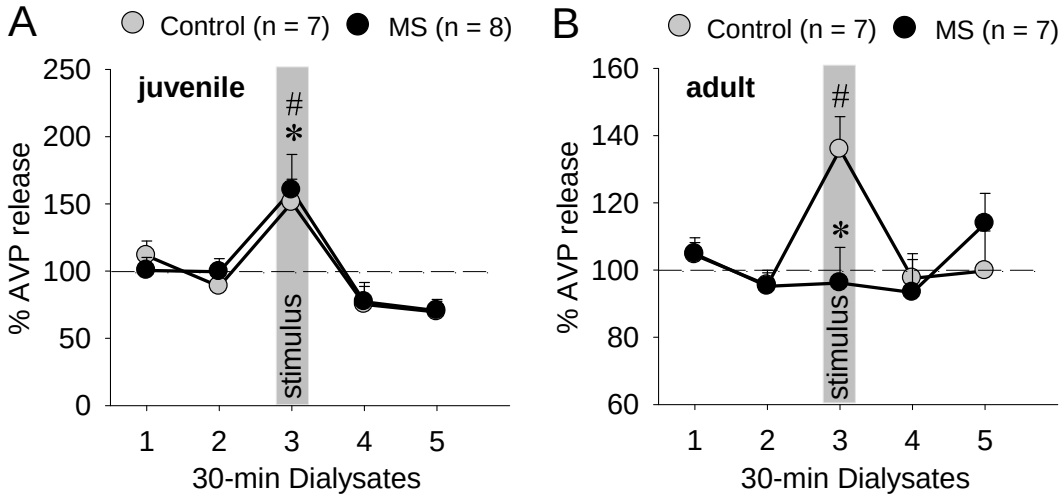

Figure 10: Effects of MS on septal AVP release during social recognition in juvenile (A) and adult (B) male rats. AVP release within the lateral septum of control and MS rats during exposure to the first 3-week-old rat (social memory acquisition period) given as percentage of baseline. Five consecutive 30 min dialysates were taken. Dialysates 1 and 2 were taken under baseline conditions, dialysate 3 included the 4-min exposure to the first 3-week-old rat, dialysates 4 and 5 were taken thereafter. Data are means + SEM. A: * $p < 0.05$ dialysate 3 vs. dialysates 4 and 5 (control) or vs. all other dialysates (MS), # $p = 0.053$ dialysate 3 vs. dialysate 2 (control); B: * $p < 0.05$ MS vs. control (dialysate 3); # $p < 0.05$ dialysate 3 vs. all other dialysates (control), two-way ANOVA followed by LSD *post-hoc* test.

Septal AVP release in adult rats exposed to the social discrimination test. Exposure to the 3-week-old rat during the social memory acquisition period induced a significant change in AVP release within the septum of adult rats (MS × dialysate: $F_{(4,48)} = 3.53$, $p < 0.05$). Control rats showed a significant increase in septal AVP release during the acquisition period ($p < 0.05$ versus all other dialysates). In contrast, MS rats failed to show a rise in septal AVP release (Figure 10B). MS did not alter absolute AVP content in microdialysates sampled under basal conditions (Table 5). No significant correlation was found between septal AVP release and investigation of the novel juvenile in control or MS adults ($r = 0.08$; $p = 0.78$).

Table 5: No effect of MS on baseline septal AVP release. Absolute AVP content per rat was calculated by taken the mean of dialysates 1 and 2 that were sampled under basal conditions, i.e. before exposure to the social discrimination test. AVP content in juveniles and adults result from different radioimmunoassay and was therefore not compared.

	Control	MS
AVP content juveniles	24.5 ± 9.7	10.6 ± 3.9
AVP content adults	68.5 ± 11.4	67.2 ± 9.2

Control, n = 7 (juvenile and adult ages); MS, n = 8 (juvenile and adult ages); Data represent means (pg/dialysate) ± SEM, Student's *t*-test (between control and MS for each age group).

Table 6: No effect of MS on septal AVP release in response to forced swimming. Control (n = 6) and MS (n = 8) adult rats showed a similar rise in septal AVP release in response to 5-min of forced swimming. Three consecutive 30-min dialysates were taken: dialysate 1 was taken under baseline conditions, dialysate 2 includes exposure to 5-min of forced swimming, dialysate 3 was taken during recovery in the home-cage. Absolute AVP content (pg/dialysate, measured in dialysate 1) did not differ between control and MS rats.

	Baseline	Forced swimming	Recovery	AVP content
Control	100 ± 0	131.3 ± 22.9*	79.9 ± 8.9	76.9 ± 11.4
MS	100 ± 0	134.3 ± 13.1*	82.9 ± 5.8	65.6 ± 13.1

Data are presented as means (% AVP release of baseline) ± SEM, * $p < 0.05$ vs. recovery (dialysate 3), two-way ANOVA for repeated measures (MS × dialysate) followed by LSD *post-hoc* test.

Exp. 3: Effects of MS on septal AVP release during forced swimming in adult rats

Exposure to 5-min of forced swimming induced a significant change in AVP release within the lateral septum of adult rats (factor dialysate: $F_{(2,25)} = 14.3$, $p < 0.001$). Both control and MS rats showed a similar increase in AVP release during forced swimming ($p < 0.05$, Table 6).

Exp. 4: Effects of local application of AVP into the lateral septum on social recognition in adult MS rats

To determine whether the blunted response of septal AVP release contributed to the impairment in social recognition memory in adult MS rats, vehicle or synthetic AVP were administered into the lateral septum via retrodialysis for a period of 30 min starting with the 4-min exposure to the first 3-week-old rat. Confirming the results of Exp. 1 and 2, vehicle-treated MS rats failed to discriminate between the *same* and *novel* rat after a 60-min interval ($p = 0.20$). Importantly, local administration of AVP into the lateral septum restored social discrimination abilities in adult MS rats ($p < 0.005$) (Figure 11). Running a separate

Student's *t*-test, this finding was further confirmed by a shorter investigation time towards the *same* rat in AVP-treated MS rats compared with vehicle-treated MS rats ($p < 0.05$) (Figure 11). For complete statistics see Table 7.

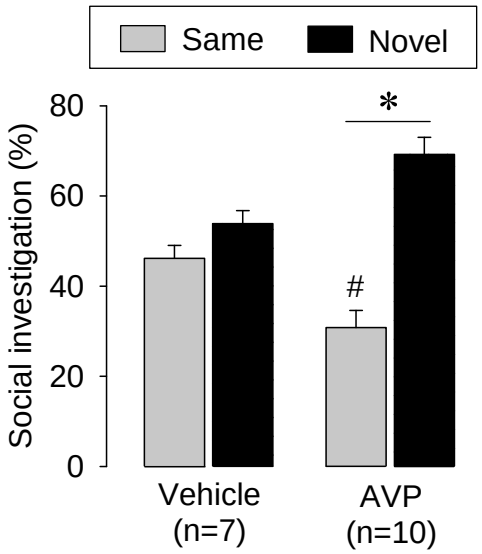

Figure 11: Effects of local application of AVP into the lateral septum on social recognition in adult MS rats. The lateral septum of adult MS rats was perfused with either Ringer's solution (vehicle) or Ringer containing 1 µg/ml synthetic AVP (AVP) via retrodialysis for 30 min starting with the exposure to the first 3-week-old rat (social memory acquisition period) in the social discrimination test. Social discrimination abilities are expressed as percentage of investigation time towards the *novel* and the *same* rat after an inter-exposure interval of 60 min. Data are means + SEM, * $p < 0.005$, paired samples *t*-test (*same* × *novel*), # $p < 0.05$ vs. vehicle, Student's *t*-test (*same* Vehicle × *same* AVP).

Table 7: Complete statistics for all social discrimination data as presented in Figure 9, Table 4, and Figure 11.

Complete statistics for Figure 9	Juveniles		Adults	
	30 min	60 min	30 min	60 min
Paired samples *t*-test				
(*same* control × *novel* control)	$t_{(9)} = -3.021$; $p = 0.014$	$t_{(9)} = -5.146$; $p = 0.001$	$t_{(9)} = -4.257$; $p = 0.002$	$t_{(9)} = -4.690$; $p = 0.001$
(*same* MS × *novel* MS)	$t_{(9)} = -5.080$; $p = 0.001$	$t_{(9)} = -3.162$; $p = 0.012$	$t_{(9)} = -2.330$; $p = 0.045$	$t_{(9)} = -1.081$; $p = 0.308$
Student's *t*-test				
(*same* control × *same* MS)	$t_{(18)} = 1.216$; $p = 0.240$	$t_{(18)} = -1.320$; $p = 0.204$	$t_{(18)} = -1.291$; $p = 0.213$	$t_{(18)} = -2.184$; $p = 0.042$

Complete statistics for Table 4	Juveniles		Adults	
	Before surgery	After surgery	Before surgery	After surgery
Paired samples *t*-test				
(*same* control × *novel* control)	$t_{(9)} = -3{,}089$; $p = 0.013$	$t_{(6)} = -8.136$; $p = 0.000$	$t_{(9)} = -2.692$; $p = 0.025$	$t_{(6)} = -5.049$; $p = 0.002$
(*same* MS × *novel* MS)	$t_{(9)} = -2.985$; $p = 0.015$	$t_{(7)} = -2.690$; $p = 0.031$	$t_{(9)} = -1.238$; $p = 0.247$	$t_{(6)} = -1.973$; $p = 0.096$
Student's *t*-test				
(*same* control × *same* MS)	$t_{(18)} = -0.284$; $p = 0.780$	$t_{(13)} = -0.628$; $p = 0.780$	$t_{(18)} = -0.813$; $p = 0.427$	$t_{(12)} = -1.145$; $p = 0.275$

Complete statistics for Figure 11	Adult MS
Paired samples *t*-test	
(*same* vehicle × *novel* vehicle)	$t_{(6)} = -1.440$; $p = 0.200$
(*same* AVP × *novel* AVP)	$t_{(9)} = -4.763$; $p = 0.001$
Student's *t*-test	
(*same* control × *same* MS)	$t_{(15)} = -2.318$; $p = 0.035$

DISCUSSION

The present study demonstrates that early life stress impairs social recognition in adult male rats. This was accompanied by deficits in the septal AVP system - adult MS rats failed to show a rise in septal AVP release seen during the acquisition/formation of social memory in control rats. Because forced swimming induced a rise in septal AVP release in both control and MS rats, the blunted response in septal AVP release indicates stimulus-specificity. Moreover, the lack of an AVP response likely underlies the impaired social recognition because local application of AVP into the lateral septum rescued social recognition performance at the 60-min interval in adult MS rats. In contrast to adult MS rats, juvenile MS rats showed intact social recognition and a significant rise in septal AVP release during the acquisition/formation of social memory. This indicates that the MS-induced impairment in social recognition and in septal AVP release is established during adult development.

MS alters social behaviors, including sexual behavior and aggression, in adult male rats (Rhees et al., 2001; Greisen et al., 2005; Veenema et al., 2006). We recently showed that alterations in social interactions occur early in development – juvenile male rats exposed to MS showed an increase in nape attacks and biting, and a decrease in submissive behaviors during play-fighting (Veenema and Neumann, 2009). This led us to hypothesize that MS alters important aspects of social communication, including social recognition, already at juvenile age. Here, we show for the first time that social recognition can be tested in 5-week-old juvenile rats using the social discrimination test. Surprisingly, MS impaired social

recognition in adult, but not juvenile, rats. This may point towards MS-induced changes during sexual maturation in neural circuits underlying social behavior. In support, MS altered V1aR and OT receptor binding density in specific brain regions (such as the piriform cortex for V1aR and the lateral septum for OTR) in adult, but not juvenile, rats (Lukas et al., 2010). Age-specific changes in the brain have also been reported in other animal models of early life stress. For example, exposure to paternal deprivation of the biparental rodent Octodon degos suppressed dendritic growth and synapse formation within the orbitofrontal cortex in adults, but not in juveniles (Helmeke et al., 2009). Moreover, early life stress induced by limiting nesting material from postnatal day 1-9 caused late-onset impairments in hippocampal behavioral (spatial memory and object recognition) and cellular (dendritic atrophy, reduced long-term potentiation) function in 12-month-old male rats (Brunson et al., 2005).

Several studies provide compelling evidence that AVP via activation of septal V1aR plays a key role in social recognition in rodents (Dantzer et al., 1988; Landgraf et al., 1995b; Landgraf et al., 2003; Bielsky et al., 2005a). We now demonstrate that the acquisition of social recognition memory is associated with a significant rise in extracellular AVP release within the lateral septum of juvenile (control and MS) and adult (control) male rats. In contrast, adult MS rats failed to respond with an increase in septal AVP release during the acquisition/formation of social memory, which corresponded with a lack of social discrimination at the 60-min interval. Importantly, V1aR binding density in the lateral septum was found to be similar in adult control and MS rats (Lukas et al., 2010), indicating

that the MS-induced impairment of septal AVP release is key to the social recognition impairment. Indeed, we provide evidence for a functional link between impaired social recognition and the lack of septal AVP response to a social stimulus in adult MS rats. Increasing extracellular AVP availability by retrodialysis administration of AVP into the lateral septum restored social recognition in adult MS rats at the 60-min interval. This strongly suggests that the rise in septal AVP release seen during the investigation of the first 3-week-old rat in control rats and juvenile MS rats is critical for successful recognition of that 3-week-old rat at the 60-min interval. This is consistent with the finding that administration of AVP into the lateral septum prolongs social recognition memory in adult male rats for up to 2 h (Dantzer et al., 1988; Engelmann and Landgraf, 1994) or even 24 h (Landgraf et al., 2003).

No correlation was found between AVP release and social discrimination performance (as expressed by investigation time towards the novel juvenile) in any of the control or MS groups. This could be due to a combination of factors, including a low variation in investigation time between individuals, the short (4-min) exposure to the juvenile, and the relatively long (30-min) dialysate sampling. Shorter sampling periods would most likely improve our understanding of the dynamics in AVP release during social investigation. However, detection limits of the radioimmunoassay currently do not allow for shorter sampling periods.

Notably, adult MS rats can discriminate between a previously-encountered and a novel rat at a shorter, i.e., 30-min, interval, but not at a longer, i.e., 60-min, interval. Thus, a more

challenging condition, such as a longer inter-exposure interval, is needed to reveal subtle MS-induced impairments in social memory. In support, male mice lacking the V1b receptor show normal social recognition memory of a familiar female mouse at the typically used short interval of 5-min, but not at the longer 30-min interval (Wersinger et al., 2002). Our finding further suggests that basal septal AVP release, which did not differ between MS and control rats, is sufficient for successful social discrimination at short intervals. This corresponds with the demonstration that a lack of AVP in AVP-deficient male Brattleboro rats (Engelmann and Landgraf, 1994) or blockade of septal V1aR in male Wistar rats (Dantzer et al., 1988) impaired social recognition at the short 30-min interval. Castration is another model to reduce septal AVP signaling as it gradually reduces AVP mRNA expression in the bed nucleus of stria terminalis (BNST) and medial amygdala and their AVP fiber projections to the septum (De Vries et al., 1984b; Miller et al., 1992). Whether the reduction in septal AVP fiber density is accompanied by reduced AVP release in the septum is unknown. Similar to adult MS rats, castrated rats show intact social recognition at the short 30 min interval (Bluthe et al., 1990). Peripheral administration of AVP prolonged social recognition in castrated rats up to 3 h, which could be blocked by co-administration of a V1aR-A as seen in intact male rats (Bluthe et al., 1990). However, whether or not castrated males can still perform social recognition after a 60 min interval has not been investigated.

The absence of a rise in septal AVP release in adult MS rats may reflect MS-induced alterations in brain regions involved in the processing and/or integration of social cues. These can include altered input to the BNST and medial amygdala that send AVP fibers to

the septum (De Vries and Buijs, ; Caffe et al., 1987). AVP cells in the BNST and MeA and AVP fibers in the lateral septum are highly sensitive to gonadal steroids (de Vries et al., 1984a; DeVries et al., 1985; Miller et al., 1992). This opens the possibility that alterations in gonadal steroid production/sensitivity during sexual maturation may affect this extrahypothalamic AVP pathway. In addition, MS-induced alterations in the regulation of the hypothalamic-pituitary-adrenal axis (Plotsky and Meaney, 1993; Wigger and Neumann, 1999; Heim and Nemeroff, 2001) could influence gonadal steroid sensitivity and AVP pathways upon maturation (Viau, 2002). This could serve as a potential mechanism by which MS impaired social recognition in adult, but not in juvenile, rats. It is also possible that social recognition is regulated differently in juveniles and adults. In support, preliminary findings show that blocking V1aR in the septum prevents social discrimination in adult, but not juvenile, male rats (Veenema et al., 2010a). Combined with the demonstration of an intrinsic AVP system in the olfactory bulb involved in social recognition (Tobin et al., 2010), further research is required to fully comprehend the AVP-circuitries involved in normal as well as impaired social recognition in juvenile and adult rats.

Interestingly, a rise in septal AVP release was found in both control and MS rats in response to forced swimming, which agrees with findings in adult male rats (Ebner et al., 1999). This suggests that MS may not have altered AVP synthesis in the BNST and/or MeA. It also suggests that the absence of septal AVP responsiveness in adult MS rats is highly stimulus-specific. In support, intermale aggression correlates with an increase in septal AVP release (Veenema et al., 2010b), while exposure to social defeat does not (Ebner et al., 2000). Yet, if

septal AVP is important for social memory, it is surprising that social defeat is not accompanied by a rise in septal AVP release. On the other hand, septal AVP stimulates active behaviors in the forced swim test (Ebner et al., 1999). This might be a less favorable behavioral strategy when being defeated. An absence of a rise in septal AVP may allow for more passive (such as freezing) behaviors that are considered adaptive in this situation. It would, therefore, be of interest to compare septal AVP response patterns in adult MS rats exposed as either resident (aggression) or intruder (social defeat) to a resident-intruder paradigm.

In addition to its critical role in social recognition, AVP has been shown to regulate other social as well as emotional behaviors (Goodson and Bass, 2001; Engelmann et al., 2004; Landgraf, 2006; Bosch and Neumann, 2008; Veenema and Neumann, 2008; Heinrichs et al., 2009). Accordingly, changes in AVP within the brain, such as AVP release, but also changes in AVP synthesis as repeatedly seen after MS (Veenema et al., 2006; Veenema et al., 2007; Veenema and Neumann, 2009), may have significant consequences for overall social and emotional functioning. MS-induced changes in brain AVP were shown to be due to changes in epigenetic programming resulting in hypomethylation of the methyl CpG-binding protein 2 regulating AVP gene transcription (Murgatroyd et al., 2009). Given that AVP is implicated in social and emotional behaviors in humans as well (Thompson et al., 2006; Yirmiya et al., 2006; Guastella et al., 2010a; Zink et al., 2010), it would be of interest to explore the role of this neuropeptide in humans exposed to child maltreatment. Preliminary data indicate

aberrant AVP levels in urine of orphanage-reared children (Fries et al., 2005). It is not known yet, whether AVP levels in urine reflect AVP levels and release in the brain.

Early life stress in humans enhances the risk to develop social deficits, including excessive aggression, social anxiety, and impaired social communication. Here, we provide first experimental evidence that MS impairs social recognition in adult male rats. Our findings also suggest that aberrant septal AVP release is key to changes in social recognition. We suggest that even modestly impaired social recognition, as seen in adult MS rats, may reflect alterations in the processing and/or integration of social cues. Further research is required to investigate to what extent such alterations would affect other social behaviors, including increased intermale aggression found after MS (Veenema et al., 2006).

Chapter 4

Maternal separation interferes with developmental changes in brain vasopressin and oxytocin receptor binding in male rats

Author's contribution:

Lukas: Study design, performing experiments, analyzing data, writing the manuscript

Bredewold: Performing experiments, analyzing data

Neumann: Study design, writing the manuscript

Veenema: Study design, analyzing data, writing the manuscript

[adapted from: Lukas M, Bredewold R, Neumann ID, Veenema AH (2010) Maternal separation interferes with developmental changes in brain vasopressin and oxytocin receptor binding in male rats. Neuropharmacology 58:78-87.]

ABSTRACT

Brain V1aR and OTR are important modulators of social behaviors. We recently showed that exposure to MS (3 h daily, postnatal days 1-14) induces changes in social behaviors in juvenile and adult male rats. Here, we hypothesize that MS induces brain-region specific changes in V1aR and OTR across development, which in turn, may underlie MS-induced changes in social behaviors. We examined the effects of MS on V1aR and OTR binding in forebrain regions of juvenile (5 wks), adolescent (8 wks), and adult (16 wks) male rats. Robust age-related changes were found for V1aR and OTR binding in several brain regions. For example, in the lateral septum V1aR binding increased with age while OTR binding decreased with age. Most notably, OTR binding in the caudate putamen showed a 2-fold decrease while OTR binding in the ventromedial hypothalamus showed a 4-fold increase with age. Importantly, exposure to MS interfered with these developmental changes in several brain regions. Specifically, MS significantly increased V1aR binding in the piriform cortex (at adolescent and adult ages), the lateral septum (at juvenile age), the hypothalamic attack area (at adolescent age), and the dentate gyrus of the hippocampus (at adolescent age), and decreased V1aR binding in the arcuate nucleus (at juvenile age). Moreover, OTR binding was significantly lower in the agranular cortex (at juvenile and adolescent age), the lateral septum (at adult age) and the caudate putamen (at adult age), but higher in the medial preoptic area (at adolescent age) and ventromedial hypothalamus (at adult age) after exposure to MS. In conclusion, age-dependent changes in V1aR and OTR binding are likely associated with the maturation of behaviors, such as sexual and aggressive behaviors while disruption of these changes by MS might contribute to previously observed changes in social behaviors after MS.

INTRODUCTION

The neuropeptides AVP and OT are key players in the regulation of a wide variety of social behaviors, including aggression, affiliation, sexual behaviors, and social cognition, in both humans and animals (for reviews see Engelmann et al., 1996; Lim and Young, 2006; Caldwell et al., 2008; Goodson, 2008; Heinrichs and Domes, 2008; Veenema and Neumann, 2008). The behavioral effects of AVP and OT are mediated by their respective receptors, the AVP 1a receptor (V1aR), the V1bR, and the OT receptor (OTR). Due to its wide-spread distribution in the brain compared with the more restricted distribution of the V1bR, the V1aR is thought to be the predominant AVP receptor in the brain (Tribollet et al., 1988; Ostrowski et al., 1994; Vaccari et al., 1998). Interestingly, V1aR and OTR undergo brain region-specific changes in expression throughout development (Tribollet et al., 1989; Tribollet et al., 1991), suggesting that these neuropeptide receptors play a role in the maturation of social behaviors. Moreover, large variations in brain V1aR and OTR binding density are observed in closely related species that show distinct social structures (Insel et al., 1991; Barberis and Tribollet, 1996; Young et al., 1996; Young et al., 1997; Goodson and Bass, 2001), and even between individuals of the same species (Phelps and Young, 2003; Olazabal and Young, 2006). These findings suggest that variations in V1aR and OTR binding might contribute to variations in social behaviors.

Negative early life experiences have been associated with robust alterations in social behaviors. Among them, child maltreatment is an acknowledged risk factor for the development of excessive aggression and violence (Widom, 1989; Dodge et al., 1990;

Barnow and Freyberger, 2003; Fonagy, 2004; Lee et al., 2007). Changes in aggressive behaviors were also found in virtually all primate and rodent models of early life stress (reviewed in Veenema, 2009). One of the most frequently used models of early life stress, MS (MS) of rat or mouse pups, induces long-lasting alterations in anxiety- and depression-like behaviors and in neuroendocrine correlates (Plotsky and Meaney, 1993; Wigger and Neumann, 1999; Kalinichev et al., 2002; Romeo et al., 2003; Ladd et al., 2004). We recently demonstrated that exposure to MS alters intermale aggression in rats and mice (Veenema et al., 2006; Veenema et al., 2007). These alterations were already evident early in development, as demonstrated by increased aggressive behaviors during play-fighting in 5-week-old juvenile male rats (Veenema and Neumann, 2009). Based on these findings we proposed that brain systems involved in social behaviors, like the AVP and OT systems, are changed by MS. Indeed, MS increased AVP mRNA expression in the hypothalamic PVN in both juvenile and adult male rats (Veenema et al., 2006; Veenema and Neumann, 2009). This further suggests that MS-induced alterations are consistent across development. However, MS-induced alterations in other parameters were less consistent across development. For example, MS increased corticotropin-releasing hormone mRNA expression in the PVN in response to the resident-intruder test in adolescent and adult, but not in juvenile, rats (Veenema and Neumann, 2009). These findings elucidate the importance of studying early life stress-induced alterations in neurobiological parameters in view of developmental changes.

Given the important role for V1aR and OTR in the regulation of social behaviors, the question arises whether MS alters V1aR and OTR binding density in the brain and if so, whether these changes are stable throughout development. We used receptor autoradiography to measure the effect of MS on V1aR and OTR binding in forebrain regions in juvenile, adolescent, and adult male rats. Identifying changes in V1aR and / or OTR binding density after exposure to MS may generate new hypotheses as to which brain regions are likely involved in the previously observed MS-induced changes in social behaviors.

METHODS

Animals

After one week of habituation in our laboratory facility, female and male Wistar rats (Charles River, Sulzfeld, Germany) were mated for five days. During the last week of gestation, female rats were individually housed in standard rat cages (42 × 27 × 18 cm), and maintained under standard laboratory conditions (12 : 12 light : dark cycle, lights on at 6:00 P.M., 22 ^{o}C, 60 % humidity, food and water ad libitum). The experimental protocols were approved by the Committee on Animal Health Care of the local government and conformed to the international guidelines on the ethical use of animals.

MS procedure

MS was performed as described earlier (Veenema et al., 2006). Briefly, on the day after parturition, i.e. on postnatal day 1, each litter was culled to eight - 10 pups (each litter contained six to eight males and two to four females). Pups were separated from the mother for 3 h daily between 9:00 P.M. and noon from postnatal day 1 to 14. Dams were removed from the home-cage and placed into a separate individual cage until the end of the 3-h separation period. Pups were then removed as complete litters from the nest, transferred to an adjacent room and put into a small box filled with bedding, which was placed on a heating pad maintained at 30–33 ^{o}C. After the 3-h separation period, the pups were returned to the home-cage followed by reunion with the dam. Non-separated control litters were left undisturbed, except for change of bedding on postnatal day 1, 7 and 14. Pups were

weaned on postnatal day 21 and housed in groups of four to five of the same sex and treatment until the start of each experiment. No more than two pups per litter were used per age.

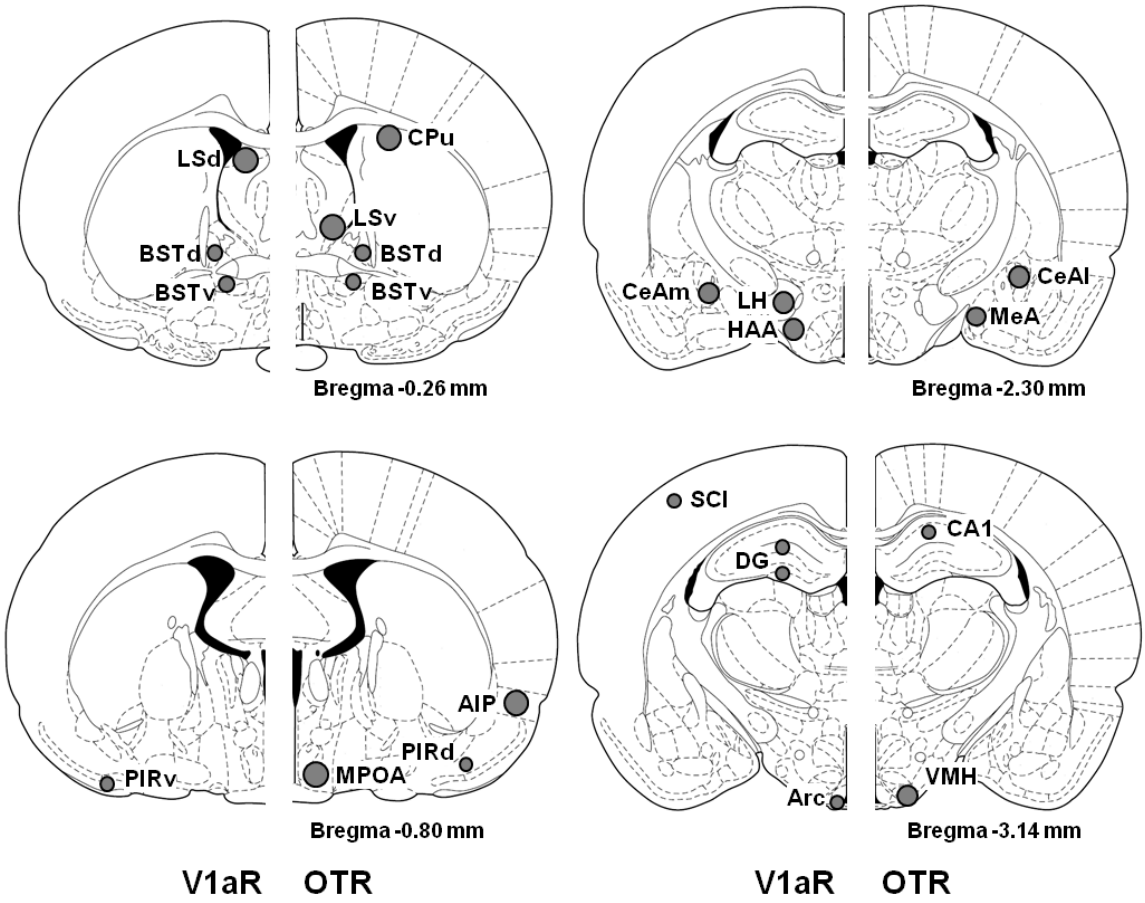

Figure 12: Schematic diagrams adapted from the atlas of Paxinos and Watson (1998) showing the brain regions in which vasopressin V1a receptor (V1aR) and oxytocin receptor (OTR) binding were quantified in 5-, 8-, and 16-week-old male control and maternally separated rats. AI, agranular insular cortex; Arc, arcuate nucleus; BSTd, bed nucleus of the stria terminalis dorsal; BSTv, bed nucleus of the stria terminalis ventral; CA1, CA1 region of the hippocampus; CeAl, central amygdala lateral, CeAm central amygdala medial; CPu, caudate putamen; DG, dentate gyrus of the hippocampus; HAA, hypothalamic attack area, LH, lateral hypothalamus; LSd, lateral septum dorsal; LSv, lateral septum ventral; MeA, medial amygdala; MPOA, medial preoptic area; PirCd, piriform cortex dorsal; PirCv, piriform cortex ventral; SC, primary somatosensory cortex; VMH, ventromedial hypothalamus.

Receptor autoradiography

To measure whether MS altered forebrain V1aR and or OTR binding, 5-week-old (juvenile; control n=17; MS n=15), 8-week-old (adolescent; control n=15; MS n=15), and 16-week-old (adult; control n=17; MS n=16) male rats were decapitated under CO_2 anesthesia, brains were removed, quickly frozen in pre-chilled *n*-methylbutane on dry ice, and stored at -20°C. Brains were cut into 16-µm coronal cryostat sections and mounted on slides. The receptor autoradiography procedure was performed according to Young et al. (1997) using a linear V1aR-A [^{125}I]-d(CH_2)$_5$(Tyr[Me])-AVP (Perkin Elmer, USA) or a linear OTR-A [^{125}I]-d(CH_2)$_5$[Tyr(Me)$_2$-Tyr-Nh_2]$_9$-OVT (Perkin Elmer, USA) as tracers. Briefly, the slides were thawed and dried at room temperature followed by a short fixation in paraformaldehyde (0.1 %). The slides were washed two times in 50 mM Tris (pH 7.4), exposed to tracer buffer (50 pM tracer, 50 mM Tris, 10 mM $MgCl_2$, 0.01 % BSA) for 60 min, and washed four times in Tris + 10 mM $MgCl_2$. The slides were then shortly dipped in pure water, air-dried, and exposed to Biomax MR films (Kodak, Cedex, France). The exposure time varied between 16 h and 21 d depending on the receptor density in the region of interest. The optical density of V1aR and OTR was measured using ImageJ (V1.37i, National Institute of Health, http://rsb.info.nih.gov/ij/). Receptor density was calculated per rat by taking the mean of bilateral measurements of four to six brain sections per region of interest. After subtraction of tissue background, the data was converted to dpm/mg (desintegrated points per minute/milligram tissue) using a [^{125}I] standard microscale (Amersham, Germany). See Figure 12 for location and size of measurements.

Statistics

Receptor density was analyzed with a two-way ANOVA (age × MS) followed by a Bonferroni post-hoc test when appropriate. Due to robust age effects for OTR binding in the caudate putamen and ventromedial hypothalamus, a separate one-way ANOVA was carried out to detect MS effects in adult rats. Data are presented as mean + SEM. Significance was accepted at $p < 0.05$.

RESULTS

Age effects on V1aR and OTR binding. Regardless of MS, V1aR and OTR binding changed significantly across development in several brain regions (see Table 8 – Table 11 for statistical details). In detail, age-related alterations in receptor binding were identified in the piriform cortex (decrease in OTR binding in adult vs. juvenile control and MS rats), lateral septum (increase in V1aR binding in adolescent and adult vs. juvenile control rats; decrease in OTR binding in adolescent and adult vs. juvenile control and MS rats), caudate putamen (decrease in OTR binding in adult vs. juvenile and adolescent control and MS rats), ventromedial hypothalamus (increase in OTR binding in adolescent and adult vs. juvenile control and MS rats), central amygdala (decrease in V1aR binding, increase in OTR binding; no posthoc effects), and hippocampus (decrease in V1aR binding in the dentate gyrus in adolescent and adult vs. juvenile control and MS rats; decrease in OTR binding, no posthoc effects).

MS effects on V1aR binding. MS interfered with the developmental changes in V1aR binding in selected brain regions (see Table 8 and Table 10 for statistical details). In detail, MS increased V1aR binding in the piriform cortex at adolescent and adult age (Figure 13), the lateral septum at juvenile age (Figure 14), and the dentate gyrus of the hippocampus at adolescent age. MS decreased V1aR binding in the arcuate nucleus at juvenile age.

MS effects on OTR binding. MS interfered with the developmental changes in OTR binding in selected brain regions (see Table 9 and Table 11 for statistical details). In detail, MS decreased OTR binding in the agranular cortex at juvenile and adolescent age (Figure 13), the lateral septum at adult age (Figure 14), and the caudate putamen at adult age (Figure 15). MS increased OTR binding in the medial preoptic area at adolescent age and in the ventromedial hypothalamus at adult age (Figure 15).

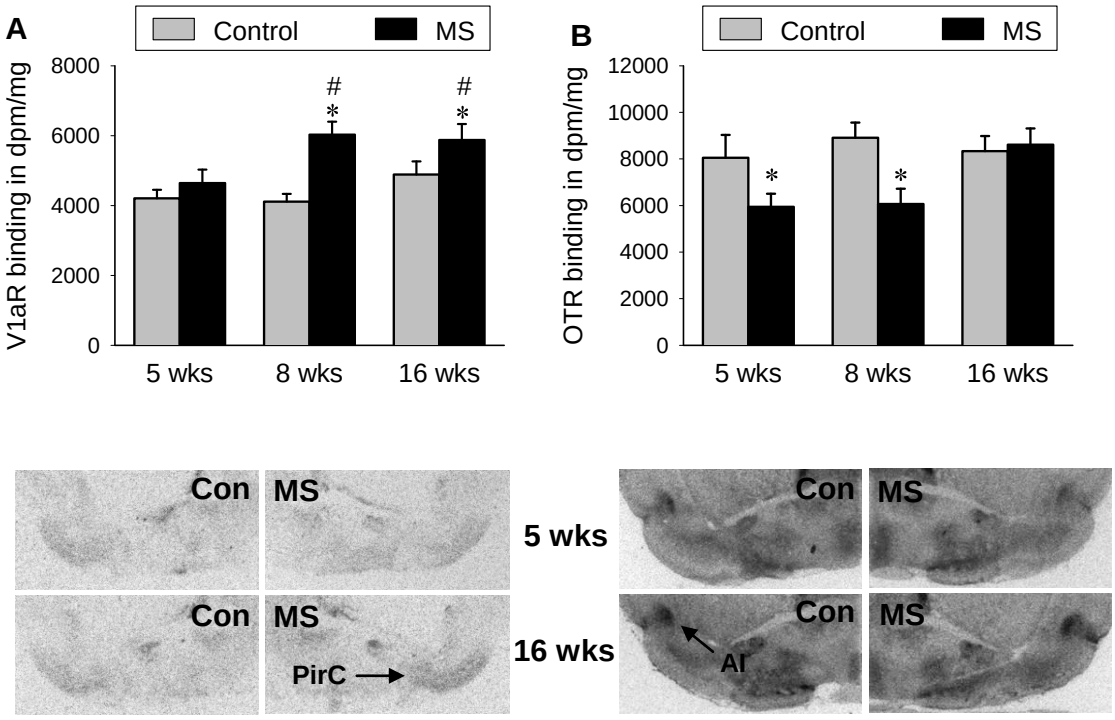

Figure 13: Effect of maternal separation (MS) on vasopressinV1a receptor (V1aR) in the piriform cortex (A) and oxytocin receptor (OTR) binding in the agranular insular cortex (B) of 5-, 8-, and 16-week-old male rats. Images depict V1aR binding in the piriform cortex (PirC) and OTR binding in the agranular insular cortex (AI). Mark the higher V1aR binding in adult MS rats compared with adult control rats and juvenile MS rats, and the lower OTR binding in juvenile MS compared with juvenile control rats. Con, control. Data are means + SEM. * $p < 0.05$ vs. control, # $p < 0.05$ vs. 5 wks, two-way ANOVA followed by Bonferroni post-hoc test.

Table 8: Age, maternal separation (MS), and age × MS effects for vasopressin V1a receptor V1aR) binding in selected forebrain regions.

V1aR binding	age effect	MS effect	age × MS effect
Cortical regions			
Piriform cortex	$F_{(2,87)}$=3.93, p<0.05	$F_{(1,87)}$=15.0, p<0.005	n.s.
Somatosensory cortex	n.s.	n.s.	n.s.
Basal forebrain			
Lateral septum	n.s.	n.s.	$F_{(2,61)}$= 3.76; p<0.05
BST dorsal	n.s.	n.s.	n.s.
BST ventral	$F_{(2,87)}$=6.33; p<0.01	n.s.	n.s.
Hypothalamic regions			
Lateral hypothalamus	$F_{(2,87)}$=8.01; p<0.005	n.s.	n.s.
Hypothalamic attack area	$F_{(2,81)}$=8.81; p<0.001	n.s.	n.s.
Arcuate nucleus	n.s.	n.s.	$F_{(2,51)}$=4.47; p<0.05
Amygdala			
Central amygdala	$F_{(2,78)}$=3.78; p<0.05	n.s.	n.s.
Hippocampus			
Dentate gyrus	n.s.	n.s.	$F_{(2,68)}$=3.88; p<0.05

BST, bed nucleus of the stria terminalis; n.s., not significant.

Table 9: Age, maternal separation (MS), and age × MS effects for oxytocin receptor (OTR) binding in selected forebrain regions.

OTR binding	age effect	MS effect	age × MS effect
Cortical regions			
Piriform cortex	$F_{(2,84)}$=8.02; p<0.005	n.s.	n.s.
Agranular insular cortex	n.s.	$F_{(1,78)}$=7.40; p<0.01	n.s.
Basal forebrain			
Lateral septum	n.s.	n.s.	$F_{(2,85)}$=4.17; p<0.05
BST dorsal	n.s.	n.s.	n.s.
BST ventral	n.s.	n.s.	n.s.
Caudate putamen	$F_{(2,61)}$=30.4; p<0.001	$F_{(2,78)}$=2.81; p=0.068	n.s.
Hypothalamic regions			
Medial preoptic area	$F_{(2,62)}$=5.83; p<0.01	$F_{(2,61)}$=5.78; p<0.05	n.s.
Ventromedial hypothalamus	$F_{(2,78)}$=52.0; p<0.001	$F_{(2,78)}$=2.40; p=0.098	n.s.

Amygdala			
Central amygdala	$F_{(2,84)}$=4.65; p<0.05	n.s.	n.s.
Medial amygdala	n.s.	n.s.	n.s.
Hippocampus			
CA1 region	$F_{(2,81)}$=3.34; p<0.05	n.s.	n.s.

BST, bed nucleus of the stria terminalis; n.s., not significant.

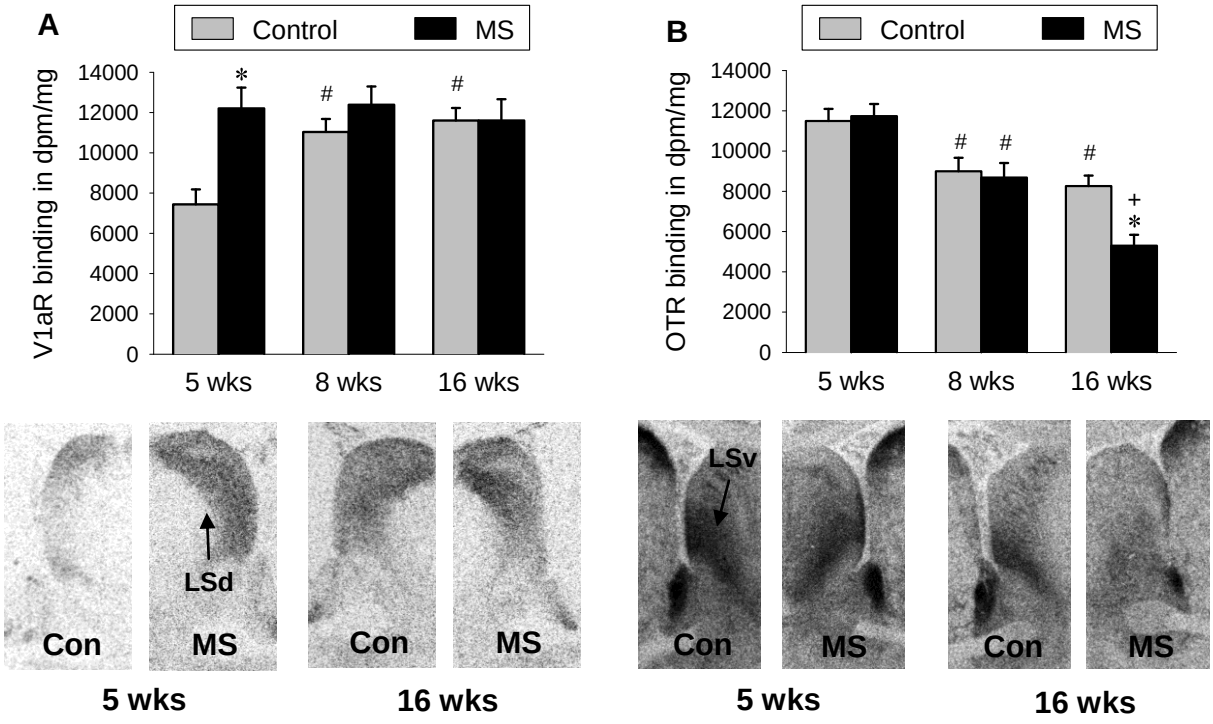

Figure 14: Effect of maternal separation (MS) on vasopressin V1a receptor (V1aR) binding (A) and oxytocin receptor (OTR) binding (B) in the lateral septum of 5-, 8-, and 16-week-old male rats. Images depict V1aR and OTR binding in the lateral septum. Mark the higher V1aR binding in juvenile MS rats compared with juvenile control rats, and the lower OTR binding with age with even lower density levels in adult MS compared with adult control rats. Con, control; LSd, lateral septum dorsal; LSv, lateral septum ventral. Data are means + SEM. * $p < 0.005$ vs. control, # $p < 0.05$ vs. 5 wks, + $p < 0.05$ vs. 5 and 8 wks, two-way ANOVA followed by Bonferroni post-hoc test.

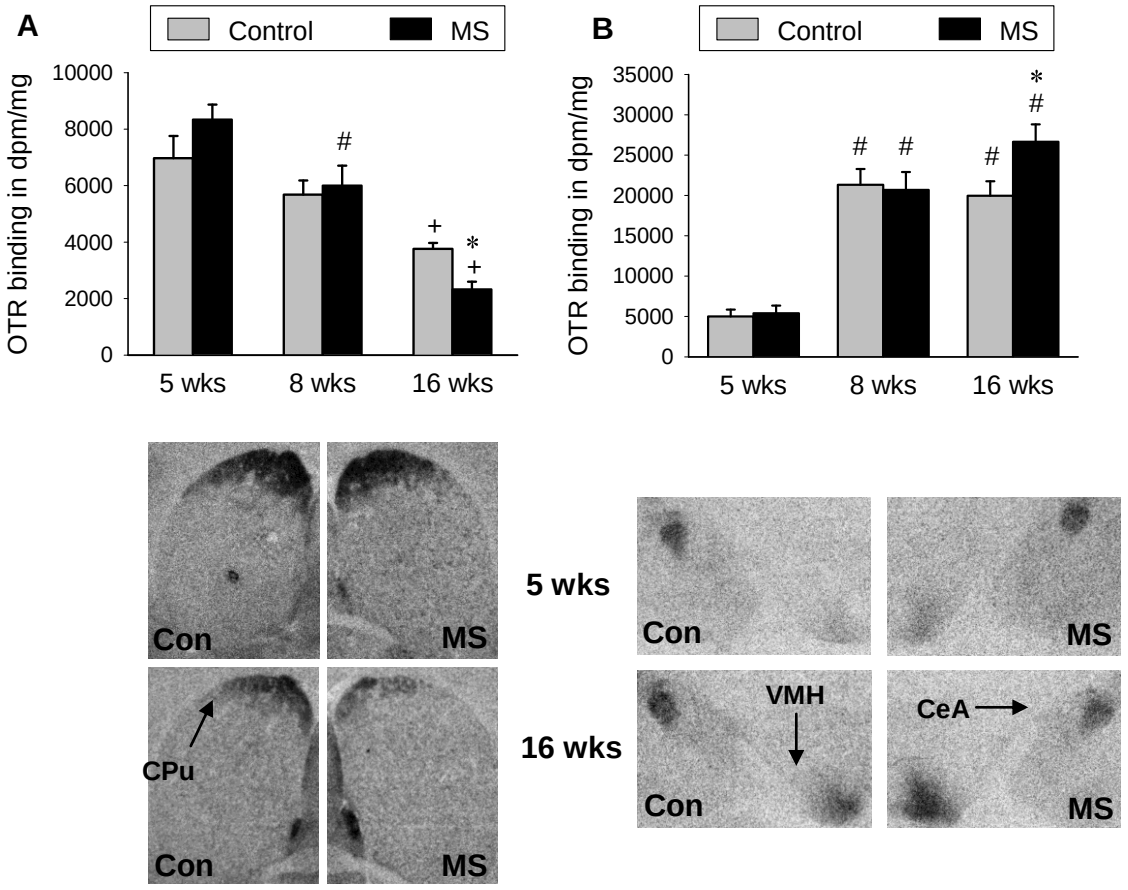

Figure 15 Effect of maternal separation (MS) on oxytocin receptor (OTR) binding in the caudate putamen (A) and in the ventromedial hypothalamus (B) of 5-, 8-, and 16-week-old male rats. Images depict OTR binding in the caudate putamen (Cpu) and in the ventromedial hypothalamus (VMH). Mark the decrease in OTR binding in the caudate putamen with age and the even lower density levels in adult MS compared with adult control rats. Con, control. Data are means + SEM. * $p < 0.05$ vs. control, # $p < 0.01$ vs. 5 wks, two-way ANOVA followed by Bonferroni post-hoc test.

Table 10: Effects of maternal separation (MS) on vaspressin V1a receptor (V1aR) binding density in selected forebrain regions of juvenile (5 weeks), adolescent (8 weeks) and adult (16 weeks) male rats.

V1aR binding	age (weeks)	control	MS	age effect	MS effect
Cortical regions					
Piriform cortex*	5	3089 ± 176	3089 ± 176		n.s.
	8	3321 ± 201	4382 ± 259	p<0.05 vs. 5 (MS)	p<0.05
	16	3734 ± 282	4393 ± 346	p<0.05 vs. 5 (MS)	p<0.05
Somatosensory	5	1137 ± 128	1105 ± 161		n.s.
cortex	8	1237 ± 129	1403 ± 162	n.s.	n.s.
	16	1029 ± 111	1037 ± 108	n.s.	n.s.
Basal forebrain					
Lateral septum*	5	7436 ± 748	2201 ± 1034		p<0.001
	8	11034 ± 647	12381 ± 911	p<0.05 vs. 5 (c)	n.s.
	16	11602 ± 625	11601 ± 1062	p<0.05 vs. 5 (c)	n.s.
BST dorsal	5	1432 ± 158	1439 ± 100		n.s.
	8	1155 ± 96	1584 ± 159	n.s.	n.s.
	16	1649 ± 99	1560 ± 171	n.s.	n.s.
BST ventral	5	2433 ± 173	2373 ± 139		n.s.
	8	2765 ± 144	3005 ± 162	n.s.	n.s.
	16	2906 ± 133	3101 ± 277	p<0.05 vs. 5 (MS)	n.s.
Hypothalamic regions					
Lateral	5	4741 ± 303	5391 ± 504		n.s.
Hypothalamus	8	4270 ± 309	4966 ± 581	n.s.	n.s.
	16	3631 ± 255	3310 ± 234	p<0.001 vs. 5, 8 (MS)	n.s.
Hypothalamic	5	7909 ± 395	7800 ± 386		n.s.
attack area	8	7924 ± 369	9364 ± 255	p<0.05 vs 5, 16 (MS)	n.s.
	16	7144 ± 391	6825 ± 351	n.s.	n.s.
Arcuate nucleus	5	5315 ± 789	2513 ± 458		p<0.001
	8	5420 ± 226	5448 ± 592	p<0.005 vs. 5 (MS)	n.s.
	16	5244 ± 389	4944 ± 396	p<0.005 vs. 5 (MS)	n.s.
Amygdala					
Central amygdala	5	6134 ± 739	5916 ± 408		n.s.
	8	5154 ± 812	5422 ± 518	n.s.	n.s.
	16	4694 ± 323	4515 ± 330	n.s.	n.s.

Hippocampus					
Dentate gyrus	5	3062 ± 172	2879 ± 254		n.s.
	8	2021 ± 149	2876 ± 269	p<0.01 vs. 5 (c)	p<0.01
	16	2026 ± 134	2086 ± 166	p<0.01 vs. 5 (c) p<0.05 vs 5, 8 (MS)	n.s.

Data are presented as means (dpm/mg tissue) ± SEM; Age and MS effects are obtained with Bonferroni post-hoc tests following a two-way ANOVA; * Data is shown in additional figures; BST, bed nucleus of the stria terminalis; n.s., not significant; c, control.

Table 11: Effects of maternal separation (MS) on oxytocin receptor (OTR) binding density in selected forebrain regions of juvenile (5 weeks), adolescent (8 weeks) and adult (16 weeks) male rats.

OTR binding	age (weeks)	control	MS	age effect	MS effect
Cortical regions					
Piriform cortex	5	3873 ± 414	3681 ± 306		n.s.
	8	3012 ± 184	2926 ± 301	n.s.	n.s.
	16	2522 ± 285	2559 ± 168	p<0.05 vs. 5	n.s.
Agranular insular	5	8046 ± 983	5939 ± 565		p<0.05
cortex*	8	8911 ± 645	6066 ± 657	n.s.	p<0.05
	16	8332 ± 652	8612 ± 699	n.s.	n.s.
Basal forebrain					
Lateral septum*	5	11490 ± 600	11786 ± 613		n.s.
	8	8993 ± 674	8677 ± 730	p<0.05 vs. 5	n.s.
	16	8264 ± 521	5301 ± 540	p<0.05 vs. 5, 8 (MS)	p<0.005
BST dorsal	5	5424 ± 464	5160 ± 391		n.s.
	8	5143 ± 455	5238 ± 582	n.s.	n.s.
	16	5292 ± 288	4244 ± 368	n.s.	n.s.
BST ventral	5	3424 ± 428	3388 ± 219		n.s.
	8	3151 ± 201	3095 ± 445	n.s.	n.s.
	16	3189 ± 279	3043 ± 301	n.s.	n.s.
Caudate putamen*	5	6972 ± 783	8339 ± 529		n.s.
	8	5679 ± 505	6001 ± 707	p<0.01 vs. 5 (MS)	n.s.
	16	3764 ± 206	2329 ± 269	p<0.05 vs. 5,8	p<0.05[#]

Hypothalamic regions					
Medial preoptic	5	3081 ± 585	3090 ± 466		n.s.
area	8	3667 ± 419	5696 ± 620	p<0.01 vs. 5 (MS)	p<0.05
	16	4158 ± 402	5189 ± 599	p<0.01 vs. 5 (MS)	n.s.
Ventromedial	5	5003 ± 850	5409 ± 948		n.s.
hypothalamus*	8	21327 ± 1942	20683 ± 2225	p<0.001 vs. 5	n.s.
	16	19953 ± 1801	26653 ± 2153	p<0.001 vs. 5	p<0.05[#]
Amygdala					
Central amygdala	5	22813 ± 3896	24355 ± 3031		n.s.
	8	27798 ± 3961	29702 ± 2811	n.s.	n.s.
	16	31664 ± 2509	35858 ± 4067	n.s.	n.s.
Medial amygdala	5	3504 ± 409	3303 ± 485		n.s.
	8	3434 ± 197	3345 ± 211	n.s.	n.s.
	16	2528 ± 146	3161 ± 328	n.s.	n.s.
Hippocampus					
CA1 region	5	2775 ± 233	2262 ± 321		n.s.
	8	2558 ± 464	2394 ± 307	n.s.	n.s.
	16	1761 ± 199	1745 ± 293	n.s.	n.s.

Data are presented as means (dpm/mg tissue) ± SEM. Age and MS effects are obtained with Bonferroni post-hoc tests following a two-way ANOVA; * Data is shown in additional figures; [#] Obtained with a separate one-way ANOVA; BST, bed nucleus of the stria terminalis; n.s. not significant; c, control.

DISCUSSION

The present study shows brain region-specific developmental changes in V1aR and OTR binding from juvenile over adolescence into adulthood. Most pronounced age effects were found in the lateral septum (increase in V1aR binding, decrease in OTR binding), caudate putamen (decrease in OTR binding), and ventromedial hypothalamus (increase in OTR binding). Exposure to MS significantly interfered with these developmental adaptations resulting in brain region- and age-dependent alterations in V1aR and OTR binding. Most notably, MS increased V1aR binding in the piriform cortex (adolescents and adults) and the lateral septum (juveniles), MS decreased OTR binding in the agranular cortex (juveniles and adolescents), the lateral septum (adults) and the caudate putamen (adults), and MS decreased OTR binding in the ventromedial hypothalamus (adults). Age-related alterations in receptor binding are likely involved in the expression of adult-typical behaviors that emerge over adolescent development while MS-induced alterations in V1aR and OTR binding in forebrain regions might contribute to (previously observed) alterations in social behaviors.

Robust developmental changes in V1aR and OTR binding were observed in several brain regions. These developmental changes are in line with previous findings in the rat (Tribollet et al., 1989; Tribollet et al., 1991) and are likely due to differences in number rather than differences in affinity (Tribollet et al., 1991). Most developmental changes in V1aR and OTR binding were observed around the age of 8 weeks, which corresponds with adolescence in rats. During adolescence, pronounced remodeling of the brain occurs, which is required to

achieve social and sexual maturity (Spear, 2000). The transition of V1aR and OTR binding to adult binding levels in adolescent rats might therefore contribute to sexual maturation and the expression of adult-typical behaviors.

In addition to the developmental changes in V1aR and OTR receptors, developmental changes in central AVP and OTR have also been reported. Here, AVP and OTR mRNA- and protein-expressing cells increase in size and number over the perinatal period in hypothalamic nuclei (AVP and OT), the BST (AVP only), and the medial amygdala (AVP) (Buijs et al., 1980; de Vries et al., 1981; Gainer et al., 1987; Almazan et al., 1989). Adult AVP mRNA levels in the BST and medial amygdala are reached by postnatal day 35 and postnatal day 60, respectively (Szot and Dorsa, 1993). Similarly, AVP fiber projections from the BST and medial amygdala to the lateral septum and lateral habenula emerge over the perinatal period, but reach full maturation only in adulthood (de Vries et al., 1981). Whether the developmental changes in neuropeptide binding co-occur with changes in AVP and OT fiber projections in other brain regions is less well known.

Here, we demonstrate that exposure to MS interferes with the developmental changes in V1aR or OTR binding in specific forebrain regions. Others also reported changes in V1aR and/or OTR in specific brain regions of rats exposed to variations in maternal care (Francis et al., 2002) and of prairie voles exposed neonatally to a single injection of OT or an OTR-A (Bales et al., 2007), suggesting that early environment manipulations can shape the developmental expression of V1aR and OTR in the brain. We previously found higher AVP mRNA expression in the PVN of male MS rats at juvenile and at adult age (Veenema et al.,

2006; Veenema and Neumann, 2009). Whether these changes in mRNA expression correspond with changes in AVP neurotransmission are unknown. It would be of interest to examine to which extent the MS-induced alterations in local neuropeptide receptor binding are associated with changes in dynamic patterns of local neuropeptide release (Landgraf and Neumann, 2004; Neumann, 2008).

Surprisingly, none of the MS-induced alterations in V1aR and OTR binding were stable across the three stages of development (i.e. juvenile, adolescent, adult). Although speculative, changes in V1aR and OTR gene expression and subsequent receptor binding may be induced by epigenetic modulation of respective gene transcriptions after MS. Alterations in the expression of the glucocorticoid receptor gene, which was epigenetically manifested, have recently been demonstrated in male rats receiving low versus high maternal care (Weaver et al., 2004). The epigenetic modulation of the glucocorticoid receptor altered stress responsiveness, but was potentially reversible later in life (Weaver et al., 2005). MS-induced changes in neuropeptide binding that only occurred at juvenile and/or adolescent age (as was seen for V1aR binding in the lateral septum, arcuate nucleus and dentate gyrus, and for OTR binding in the agranular insular cortex and medial preoptic area) could have interfered with the normal development of that particular brain region, and in turn, with the normal development of social or emotional behaviors. The MS effects on V1aR and OTR are discussed below with an emphasis on those brain regions implicated in social behaviors.

Cortical regions. MS significantly increased V1aR binding in the piriform cortex of adolescent and adult rats. OTR binding in this area was not altered by MS. The piriform cortex is part of a network involved in the processing of olfactory cues used for social communication (Richter et al., 2005; Ross and Eichenbaum, 2006; Petrulis, 2009). The piriform cortex receives olfactory information from the main olfactory bulb and has reciprocal connections with orbitofrontal, entorhinal and amygdaloid areas (Haberly, 2001). Activation of the piriform cortex was found to be crucial for the consolidation and for the recall of long-term social memory (Sanchez-Andrade and Kendrick, 2009).

OTR binding in the agranular insular cortex was significantly lower in juvenile and adolescent MS rats compared with same-age control rats, but this difference disappeared at adult age. The agranular insular cortex has reciprocal connections with the piriform cortex and was found to be activated upon stimulation of the olfactory bulb (Cinelli et al., 1987; Datiche and Cattarelli, 1996), suggesting a role in olfactory processing. Moreover, GABAergic inhibitory neurons in the agranular cortex were activated when male rats were confronted with a conspecific and this activation was increased when rats were allowed to display aggression (Halasz et al., 2006). Interestingly, decreased activation of GABAergic inhibitory neurons in the agranular insular cortex correlated with the display of violent aggression in male rats (Halasz et al., 2006).

Thus far, the role of V1aR in the piriform cortex and of OTR in the agranular insular cortex is unclear. Nevertheless, as olfaction is of vital importance in directing behavior in rats, we

propose that MS-induced alterations in V1aR binding in the piriform cortex and OTR binding in the agranular insular cortex may influence the processing of social olfactory cues and hence could result in an altered expression of olfaction-dependent behaviors like social recognition, play-fighting and aggression. Whether these MS-induced changes in V1aR and OTR binding indeed contributed to the previously observed changes in play-fighting and aggression requires further research.

Lateral septum. MS accelerated the maturation of V1aR binding in the lateral septum in such a way that an adult-like V1aR binding pattern was already observed at juvenile age. Interestingly, OTR binding decreased with age, especially in MS rats resulting in significantly lower OTR binding density at adult age than control rats. Accordingly, MS rats had higher V1aR binding at juvenile age and lower OTR binding at adult age. V1aR gene expression in the septum is positively regulated by glucocorticoids (Watters et al., 1996a). In this respect, it is of interest to note that juvenile MS rats have higher baseline plasma corticosterone levels compared with juvenile control rats (Veenema and Neumann, 2009). This suggests that the higher V1aR binding in juvenile MS rats could have been mediated by MS-induced changes in plasma corticosterone concentration. Activation of V1aR in the lateral septum plays a role in the processing of social cues. Overexpression of the V1aR within the lateral septum using viral vector-mediated gene transfer improved social recognition in male rats (Landgraf et al., 2003) and rescued social recognition in V1aR knockout mice (Bielsky et al., 2005b). Moreover, V1aR in the septum mediate the anxiogenic effect of AVP as demonstrated by antisense targeting of V1aR mRNA expression, application of AVP, or

application of V1aR-A into the lateral septum (Landgraf et al., 1995b; Liebsch et al., 1996; Beiderbeck et al., 2007). Finally, activation of V1aR and AVP neurotransmission in the lateral septum have been implicated in the regulation of intermale aggression (Irvin et al., 1990; Koolhaas et al., 1991; Beiderbeck et al., 2007). Less is known about the role of OT or OTR in the septum. One study suggest that local application of OT in the septum improves social recognition (Popik et al., 1992). Furthermore, social defeat caused an increase in OT release within the septum of male rats (Ebner et al., 2000), which may mediate the behavioral response to this emotional stressor, possibly via reducing anxiety (Windle et al., 2004). Considering the effects of V1aR in the septum on social recognition, anxiety and aggression and the potential involvement of OTR in the septum in social recognition and anxiety, it will be of interest to investigate the behavioral significance of the opposite effects of MS on V_{1A} and OTR binding in the lateral septum of male rats in future studies.

BST-Amygdala. The BST and the amygdala regions are involved in the processing of olfactory (rodents) or visual (humans) cues (Bressler and Baum, 1996; Ferguson et al., 2002; Adolphs and Spezio, 2006) and in fundamental emotions like anxiety and fear (LeDoux, 2000; Walker et al., 2003), which have a strong influence on the expression of a variety of social behaviors. However, MS had no effect on V1aR and OTR binding in the BST and amygdala regions. Moreover, only little change across development was observed for V1aR and OTR binding in the BST (ventral part of BST: increase in V1aR) and amygdala regions (central amygdala: decrease in V1aR, increase in OTR), suggesting that the role of V1aR and OTR in these regions is consistent across development.

Caudate putamen. OTR binding in the caudate putamen strongly decreased with age in both control and MS rats. This is in line with previous studies in male rats in which OTR binding was lower in adulthood than in early postnatal life (Tribollet et al., 1989) with an even further reduction during aging (Arsenijevic et al., 1995). The expression of OTR in the caudate putamen did not depend upon gonadal steroid hormones (Tribollet et al., 1990; Arsenijevic and Tribollet, 1998), but might be controlled by dopamine (McGregor et al., 2008). Yet, the function of OTR in this region is unknown so far. However, a recent study found a positive correlation between OTR binding in the caudate putamen and alloparental care in juvenile female prairie voles (Olazabal and Young, 2006). The caudate putamen is generally involved in the control of motor movement and in aspects of learning and memory (Hauber, 1998; Packard and Teather, 1998), functions that might impair during aging. Interestingly, a recent human neuroimaging study reported activation of the dorsal striatum after intranasal OT application and suggested the role of this region in reward feedback information (Baumgartner et al., 2008). Clearly, the precise involvement of local OTR and the implications of lower OTR binding in the caudate putamen of adult male MS rats need to be shown.

Hypothalamic regions. Given the important role of the hypothalamic attack area in eliciting intermale aggression (reviewed in Kruk, 1991) and our own finding of increased intermale aggression in juvenile and adult MS rats (Veenema et al., 2006; Veenema and Neumann, 2009), we speculated that MS persistently altered V1aR binding in this region. However, despite the temporary rise in V1aR binding during adolescence, there was no long-lasting

MS-induced change in V1aR binding in this region. In contrast, long-lasting MS-induced effects were found for OTR binding in the ventromedial hypothalamus. Here, a 4-fold increase in OTR binding in the ventromedial hypothalamus emerged over adolescent development, suggesting a key role for local OT neurotransmission upon sexual maturation, while MS induced an even stronger increase with age resulting in significantly higher OTR binding in adult MS rats compared with adult control rats. Thus far, the role of OTR in the ventromedial hypothalamus is well established in females, but not in males. OTR in this region mediate female sexual behaviors as measured by lordosis responding. Lordosis responding can be enhanced by administration of OT into the ventromedial hypothalamus or abolished by local administration of an OTR-A when given in synchrony with a progesterone injection 4 h before testing (Schulze and Gorzalka, 1991; Witt and Insel, 1991). Considering that MS induced long-term changes in anxiety and neuroendocrine parameters in female rodents (Wigger and Neumann, 1999; Kalinichev et al., 2002; Romeo et al., 2003; Veenema et al., 2007), it would be of interest to study developmental and MS effects on OTR binding in the ventromedial hypothalamus and other regions in females as well. Interestingly, OTR mRNA expression and binding in the ventromedial hypothalamus of male rats is regulated by testosterone and estradiol (De Kloet et al., 1985; Tribollet et al., 1990; Bale and Dorsa, 1995), suggesting the involvement of OTR within this region in male reproductive behaviors. The functional implications of OTR in the ventromedial hypothalamus and that of higher OTR binding in adult MS rats awaits further research.

It is remarkable to notice that age-related changes in brain V1aR and OTR binding were observed three decades ago, but their functional behavioral implications are currently still unknown. Genetic variability in V1aR and OTR has been linked to psychopathologies characterized by severe social deficits, including autism spectrum disorders (Kim et al., 2002; Wassink et al., 2004; Wu et al., 2005; Lerer et al., 2008). Moreover, autism spectrum disorders are identified early in life. These findings necessitate further research into the function of V1aR and OTR and their ligands, AVP and OT, especially across development. Additionally, the utilization of MS and related animal models is important to gain insights into the role of V1aR and OTR in disturbed social behaviors.

In conclusion, exposure to early life stress interferes with the normal development of V1aR and OTR binding in specific forebrain regions. The alterations in V1aR and OTR binding likely contribute to the behavioral alterations found upon MS. Especially the MS-induced alterations in neuropeptide receptor binding in the piriform cortex (V1aR), the agranular insular cortex (OTR), the lateral septum (V1aR and OTR), and the ventromedial hypothalamus (OTR) might contribute to the MS-induced alterations in aggressive (Veenema et al., 2006; Veenema and Neumann, 2009) and other social behaviors, like sexual behaviors or social cognition.

Chapter 5

Nasal application of neuropeptide S prolongs object, but not social memory, and reduces general anxiety

Lukas: Study design, performing experiments, analyzing data, writing first draft of the manuscript

Neumann: Study design, writing the manuscript

[adapted from: Lukas M and Neumann ID; Nasal application of neuropeptide S prolongs object, but not social memory, and reduces general anxiety. Neuropharmacology; In revision]

ABSTRACT

Recent studies described potent behavioral effects of centrally applied NPS in mice and rats. These included increased arousal and wakefulness, facilitation of fear extinction and object memory consolidation and anxiolysis. Here, we compared the effects of NPS on both social and non-social memory, in male rats, and on social preference/social anxiety versus non-social anxiety after either intracerebroventricular (icv) or nasal application. Intranasal application of neuropeptides has been successfully employed to alter behavioral parameters in humans and rodents, but studies concerning nasal application of NPS are lacking so far. First, we confirmed the facilitatory effect of icv NPS (1 nmol) on object discrimination after an inter-exposure interval (IEI) of 240 min. These effects were context-dependent, as icv NPS (1 nmol) did not prolong social memory in a social discrimination paradigm. Second, we confirmed the anxiolytic effect of icv NPS (1 nmol) on the elevated plus-maze, whereas neither icv NPS (1 nmol) nor NPS-A (10 nmol) altered social preference. Third, nasal NPS (4 - 40 nmol applied topically on the rhinarium) facilitated object discrimination in a dose-dependent manner. Also, the anxiolytic effect of NPS on the elevated plus-maze could be confirmed after nasal administration (40 nmol). In contrast, identical doses of subcutaneously injected NPS failed to produce corresponding behavioral effects in both tests.

Our findings provide evidence for memory enhancing and anxiolytic effects of icv NPS in a non-social context. We could further show that these effects are context-specific, as social memory and social preference behavior were unchanged. These effects of icv NPS were replicated by nasal application of the neuropeptide. Thus, nasal application of NPS seems to be a useful method in rodents for screening for behavioral or physiological effects before

more specific and time-consuming, intracerebral methods are employed, and may represent a viable therapeutic approach for NPS treatment of patients with psychiatric illnesses such as anxiety or panic disorders.

INTRODUCTION

NPS has been named after the highly conserved serine at the amino-terminal residue of the mature 20 amino acid peptide in mammals including humans (Xu et al., 2004; Reinscheid, 2007). Neuronal NPS expression has been described to be restricted to distinct brain stem clusters in an area around the locus coeruleus (Xu et al., 2004). In contrast, the NPS receptor seems to be widely expressed within the brain especially within regions involved in emotional and memory processing, such as thalamus, hypothalamus, amygdala, and cortical areas, as well as in regions involved in olfaction (Xu et al., 2007; Leonard and Ring, 2011).

NPS was found to exert a variety of behavioral effects demonstrated after its central administration either into the cerebral ventricles or locally within a selected target region. Thus, it reduced anxiety-related behavior in several relevant tests for non-social anxiety in both mice (Xu et al., 2004; Jüngling et al., 2008; Leonard et al., 2008; Rizzi et al., 2008) and rats (Slattery et al., 2008; Vitale et al., 2008; Wegener et al., in press), mostly accompanied by an increase in locomotor activity. Also NPS modulated sleep patterns (Xu et al., 2004; Okamura and Reinscheid, 2007) and facilitated the extinction of contextual conditioned fear responses. The latter effect was observed when NPS was administered into the amygdaloid fear circuitry in mice with effects on local glutamatergic and GABA-ergic neurotransmission

(Jüngling et al., 2008; Meis et al., 2008). This indicated that NPS is likely to modulate memory responses. Indeed, NPS improved hippocampus-dependent novel object memory after intracerebroventricular (icv) administration in mice (Okamura et al., 2011).

However, whether NPS is able to modulate social memory or other aspects of social behavior such as social anxiety-related behavior is currently not known. Therefore, we initially studied the effects of NPS on social memory, specifically on social discrimination abilities in male rats. Next, we assessed the effects of NPS on social approach behavior in the social preference test recently established in rats (Lukas et al., 2011a).

The ability of NPS to pharmacologically reduce anxiety responses and to promote fear extinction makes the brain NPS system an interesting and promising target for the treatment of various anxiety-related disorders including posttraumatic stress disorders. However, in humans, the central route of administration is impractical, and systemically applied neuropeptides cannot efficiently cross the blood brain barrier (Ermisch et al., 1985). Interestingly, it has been repeatedly shown that intranasal administration of neuropeptides, such as OT, AVP and insulin reliably alters neuronal, behavioral and physiological parameters in humans (Kirsch et al., 2005; Benedict et al., 2006; Thompson et al., 2006; Guastella et al., 2008b; Gamer et al., 2010). AVP concentrations were found to rise in the human cerebrospinal fluid within 10 min after its nasal application and stayed up for at least 70 min (Born et al., 2002) (for review see Hallschmid et al., 2004; Meyer-Lindenberg, 2008; Heinrichs et al., 2009; MacDonald and MacDonald, 2010).

Also, various effects of intranasal administration of neuropeptides have been shown in healthy and pathological rodent models (for review see Hanson and Frey, 2008). For example, intranasal insulin enhanced memory abilities, anxiolysis, and odor discrimination in rats (Marks et al., 2009), and the octapeptide NAP (named after its amino acid sequency: NAPVSIPQ) decreased anxiety-related behavior in aging rats (Alcalay et al., 2004). In addition, the pituitary adenylate cyclase-activating polypeptide (PACAP), and the vasointestinal peptide (VIP) were shown to improve neuropathology, e.g. accumulation of amyloid peptide, and cognitive deficits in different rodent models of Alzheimer's disease after repeated intranasal administration (Gozes et al., 1996; Matsuoka et al., 2007; Rat et al., 2011). However, whether administration of the recently discovered NPS is effective via the nasal route with respect to behavioral functions has never been tested in either humans or rodents.

Therefore, the final aim of the study was to investigate the effects of NPS after its nasal administration on memory functions and anxiety-related behaviors both in a non-social and social context in rats, thus accompanying the icv studies.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (250-300 g) were purchased from Charles River (Sulzfeld, Germany) and group-housed in standard cages (55 × 22 × 18 cm) for habituation to the laboratory facilities under standard laboratory conditions (12:12 h light:dark cycle, lights on at 6.00 h, 22 ^{o}C, 60 % humidity, food and water ad libitum). One week before behavioral testing, rats were single housed in observation cages (40 × 24 × 36 cm). Individual rats were not used twice for the same behavioral experiment, except for the establishment of the social and object discrimination paradigms. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Oberpfalz and the guidelines of the NIH.

Stereotaxic surgery and cannulation

For icv infusion, guide cannulas (21 G, 12 mm; Injecta GmbH, Germany) were stereotaxically implanted 2 mm above the lateral ventricle as described before (stereotaxic coordinates relative to Bregma: 1.0 mm posterior, 1.6 mm lateral, 2.0 mm below the surface of the skull, Paxinos and Watson, 1998; Blume et al., 2008); under isofluran anesthesia (isoflurane, Forene®, Abbott GmbH & Co. KG, Wiesbaden, Germany). The guide cannula was fixed to the skull with two jeweller's screws and dental cement (Kallocryl, Speiko-Dr. Speier GmbH, Muenster, Germany) and closed by a stainless steel stylet (25 G). After surgery, rats were injected with an antibiotic (Baytril®, Bayer Vital GmbH, Leverkusen, Germany), and handled

daily (stroking, holding, cleaning of stylets) for five consecutive days to minimize non-specific stress responses during the experiment.

Drug application

Synthetic rat NPS (H-6164) was purchased from Bachem Holding AG (Bubendorf, Switzerland) and specific NPS-A ([D-Cys(tBu)5]NPS, Guerrini et al., 2010). Both were dissolved to respective concentrations in sterile Ringer solution (mM: 147.1 Na^+, 2.25 Ca^{2+}, 4 K^+, 155.6 Cl^-, pH 7.4; B. Braun Melsungen, Germany).

Intracerebroventricular infusion

In order to study the effects of icv NPS on object memory and non-social anxiety-related behavior in rats, as well as on social memory and social preference, conscious, handled rats received either synthetic NPS (1 nmol / 5 µl; i.e. 2 ug / 5 µl) or vehicle (sterile Ringer solution, 5 µl) via an icv infusion cannula (14 mm) inserted into the icv guide cannula and connected to a Hamilton syringe via polyethylene tubing. In order to investigate the involvement of the endogenous NPS system in social preference, the NPS-A was applied icv (10 nmol / 5µl; i.e. 30 µg / 5 µl). After slow icv infusion the infusion cannula was left in place for 30 s. Icv infusions took place immediately after the acquisition period (social and object discrimination) or 30 min prior to behavioral testing (plus-maze, social preference test).

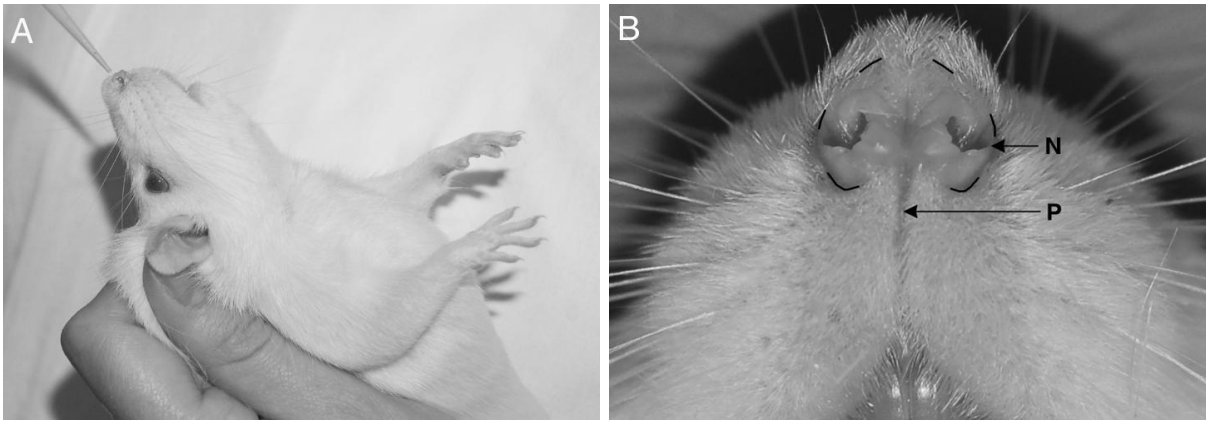

Figure 16: Holding of the conscious rat during nasal application (A). Magnification of the nose region (B). Dotted lines mark the rhinarium – target of nasal application in the present study. N, nostril; P, philtrum.

Nasal application

In order to study NPS effects on object memory and non-social anxiety after nasal administration, rats received either NPS (0.4, 4.0, or 40 nmol / 2 × 10 µl; i.e. 0.8, 8.0, or 80 µg / 2 × 10 µl) or vehicle (sterile Ringer solution, 2 × 10 µl). In detail, the conscious rat was restrained by a trained experimenter (see Fig.1A), and 10 µl of the solution were applied bilaterally on the rhinarium, the area refered to as the glaberous skin around the nostrils which was shown to be highly innervated by free nerve endings (Figure 16B) (Silverman et al., 1986). Application was performed using a 100-µl pipette. The total amount of 20 µl was distributed equally with the tip of the pipette and allowed to diffuse in the squamous epithel of both the left and right rhinarium, whereas direct application into one of the nostrils was avoided, but uptake of fluid and diffusion into the nostril was not prevented. Each of the applications to the left and right rhinarium, respectively, lasted about 1 min. Rats were returned to their home cage thereafter until behavioral testing started 30 min later (object discrimination or plus-maze). In a pilot experiment, careful observation of anesthetized rats

under stereomicroscopic control after nasal administration revealed that the solution (20 µl; Figure 16B) is completely absorbed within 2 min.

Subcutaneous injection

In order to compare the efficacy of nasal versus subcutaneous (sc) application of NPS, conscious rats were injected with sc NPS using identical amounts that were effective after nasal application, i.e. 4.0 or 40 nmol / 500 µl (i.e. 8.0 or 80 µg /500 µl), or vehicle (sterile Ringer solution, 500 µl). Sc treatment was performed in the nape region 30 min before behavioral testing (object discrimination or plus-maze).

Object discrimination paradigm

For studying the effects of NPS applied either icv, nasal or sc on object discrimination abilities the object recognition paradigm (Everts and Koolhaas, 1997) has been adapted to be comparable to the social discrimination test paradigm (Engelmann et al., 1995). An unknown object was placed into one corner of the observation cage for 4 min (object memory acquisition period). After a defined inter-exposure interval (IEI) of either 60, 120, or 240 min, the *same* object was re-introduced at the same position in the cage for 4 min. Simultaneously, a *novel* object was placed in the opposite corner of the cage (object discrimination period). Objects and placements were varied between rats to prevent potential memory-independent preference effects of certain objects or positions. Presented objects were a red plastic ring (height 2.8 cm, outer diameter 9.4 cm, inner diameter 8.3 cm), a red plastic cup (height 9 cm, diameter opening 7.8 cm, diameter bottom 5.2 cm), a

transparent drinking bottle (6.5 × 15 × 6.5 cm) and a black metal block (10 × 3.8 × 10 cm). Objects were cleaned thoroughly with fresh water and a small amount of detergent (Baktolin; Bode Chemie AG, Hamburg, Germany) and dried with a fresh towel between the trials. All tests took place during the late light phase starting between 7 and 8 hours after lights on, i.e. between 1300h and 1800h. All tests were recorded and the time spent investigating the object (sniffing / touching) was measured by a researcher blinded to the treatment condition using JWatcher (Version 1.0, Macquarie University and UCLA). The percentage of time investigating the *same* and the *novel* object (time investigating *same* or *novel* object / time investigating *same* + *novel* object x 100 %) was calculated. A significantly lower investigation time directed towards the *same* versus the *novel* object was interpreted as object discrimination. Rats with less than ten seconds of total investigation time during one of the two exposure periods were excluded from statistical analysis (13 % of all rats).

Social discrimination paradigm

In order to study the effects of icv or nasal NPS on social memory, we used the social discrimination paradigm. The ability of male rats to discriminate between a previously encountered (*same*) and a *novel* 3-week-old (juvenile) male rat was tested according to Engelmann et al. (1995) with some minor modifications. A juvenile rat was introduced into the cage of the experimental rat for 4 min (social memory acquisition period). After a defined IEI, the *same* juvenile rat was re-introduced along with a *novel* juvenile rat for 4 min (social discrimination period). If rats were tested repeatedly, testing took place at different days with new sets of juvenile rats. All tests took place during the late light phase starting

between 7 and 8 hours after lights on, i.e. between 1300h and 1600h. All tests were recorded and the time spent investigating the juvenile rats (sniffing the anogenital and head / neck regions) was measured by a researcher blinded to the treatment condition using JWatcher (Version 1.0, Macquarie University and UCLA). The percentage of time investigating the *same* and the *novel* juvenile rat (time investigating *same* or *novel* rat / time investigating *same* + *novel* rat × 100 %) was calculated. A significantly lower investigation time directed towards the *same* versus the *novel* juvenile rat was interpreted as social discrimination. Note that the 3-week-old juvenile rats did not elicit play or aggressive behaviors in the experimental rats.

Elevated plus-maze

The elevated plus-maze was used to assess the effects of icv, nasal or sc NPS on non-social anxiety-related behavior (Pellow *et al.*, 1985). It consisted of two opposing open arms (50 × 10 cm, 100 lux) and two opposing closed arms (50 × 10 × 40 cm, 20 lux) connected by a central area. The apparatus was made of dark grey plastics, elevated 80 cm above the floor, and surrounded by an opaque curtain to avoid external disturbance. Before each trial, the maze was cleaned with water containing a low concentration of detergent. The rat was placed in the central area facing a closed arm for 5 min. The percentage of time spent on the open arms was assessed as anxiety-related behavior (time on open arms / time on open and closed arms × 100 %); the number of entries in the closed arms as measurement for locomotion was recorded by means of a video camera mounted above the platform and

scored by a trained observer (Plus-maze version 2.0; E. Fricke). The elevated plus-maze testing was performed in the early light phase, i.e. between 0800h and 1200h.

Social preference paradigm

In order to study the effects of icv or nasal NPS on social approach / social avoidance behavior, the social preference paradigm recently established in rats (Lukas et al., 2011b) has been used. Rats were placed in a novel arena (40 × 80 × 40 cm, red light). After 30 sec of habituation an empty wire-mesh cage (non-social stimulus; 20 × 9 × 9 cm) was placed at one side wall of the arena for 4 min. The empty cage was then exchanged by an identical cage containing an unknown adult male Wistar rat (social stimulus) for additional 4 min. Before each trial, the arena was cleaned with water containing a low concentration of detergent. All tests took place during the active phase starting one hour after lights off, i.e. between 1900h and 2200h.

The test procedure was recorded and scored by an observer blind to the treatment using JWatcher behavioral observation software (V 1.0, Macquarie University and UCLA). Exploration times of the non-social and social stimulus were scored by measuring the time the rat spent in active olfactory investigation. Data are presented as the percentage of time investigating the non-social versus the social stimulus, i.e. investigation time / total time [4 min] × 100 %. A significantly higher percentage of investigation of the social versus the non-social stimulus was considered social preference.

Experimental design

Effects of NPS on non-social memory: object discrimination

First, in order to determine the time point at which object discrimination is no longer feasible in our paradigm, male rats were tested in the object discrimination paradigm at IEI of 60, 120, and 240 min.

Next, to investigate the effects of icv NPS on object discrimination, rats received an icv infusion of NPS (1 nmol / 5 µl) or vehicle immediately after the acquisition period (IEI 240 min). To investigate the effects of nasal or sc NPS on object discrimination, rats performed the object discrimination paradigm (IEI 240 min) 30 min after nasal (0.4 nmol / 20µl or 4.0 nmol / 20µl) or sc (4.0 nmol / 500 µl) treatment with NPS or vehicle as described above. Thus, NPS application took place 30 min prior to the initial acquisition period to allow NPS to reach its potential target regions.

Effects of NPS on social memory: social discrimination

First, in order to determine the time point at which social discrimination is no longer feasible in our paradigm, male rats were tested in the social discrimination paradigm at IEI of 60 and 120 min.

To investigate the effects of icv NPS on social discrimination abilities, rats received an icv infusion of NPS (1 nmol / 5 µl) or vehicle immediately after the acquisition period (IEI: 120 min). As effects of icv NPS on social memory were not found, the effect of nasal or sc application of NPS on social discrimination has not been tested.

Effect of NPS on non-social anxiety: elevated plus-maze

In order to confirm the effects of icv NPS on non-social anxiety in rats (Slattery et al., 2008; Wegener et al., in press), and to investigate the effects of nasal or sc NPS on non-social anxiety, rats were treated with either icv (1 nmol / 5 µl), nasal (4 nmol / 20 µl and 40 nmol / 20 µl) or sc (40 nmol / 500 µl) NPS or vehicle 30 min before testing on the elevated plus-maze.

Effects of NPS and NPS-A on social preference / social anxiety

To investigate the effects of NPS on social approach behavior, rats were infused icv with either NPS (1 nmol / 5 µl) or vehicle 30 min before performance in the social preference test. In order to reveal the possible involvement of the endogenous brain NPS system in social anxiety, NPS-A (10 nmol / 5 µl) was applied icv 30 min prior to testing.

As an effect of icv NPS on social preference behavior was not found, the effect of nasal or sc application of NPS has not been tested.

Histology

The experiments were terminated by an overdose of CO_2. The localization of each icv guide cannula was verified after icv infusion of ink (5 µl) before unperfused brains were removed, cut coronally with a razor blade and macroscopic examination of ink distribution. All rats showed stained ventricles and were included in the statistical analysis.

Statistics

For statistical analysis PASW/SPSS Statistics (Version 18.0) was used. Establishment of social and object discrimination without further treatment was analyzed using the paired samples t-test (*same* vs. *novel*). Social and object discrimination behavior as well as social preference with NPS treatment was analyzed using ANOVA for repeated measures (factors treatment × stimulus) followed by a Bonferroni post-hoc test when appropriate. Total investigation times between treatment groups during the acquisition period were compared using the student's t-test. Non-social anxiety behavior was analyzed using the one-way ANOVA or the student's t-test. Significance was accepted at $p<0.05$.

RESULTS

Effects of NPS on object discrimination

After an IEI of 60 min, rats spent significantly less time investigating the *same* than the *novel* object ($t_{(8)} = 5.87$, $p < 0.001$), indicating object memory, whereas after 120 min only a trend in this direction could be recognized ($t_{(6)} = 2.16$, $p = 0.74$). After an interval of 240 min, rats completely failed to discriminate between the *same* and *novel* object ($t_{(5)} = -0.658$, $p = 0.540$) (Figure 17A). Therefore, the IEI of 240 min was chosen in order to test possible memory-enhancing effects of NPS.

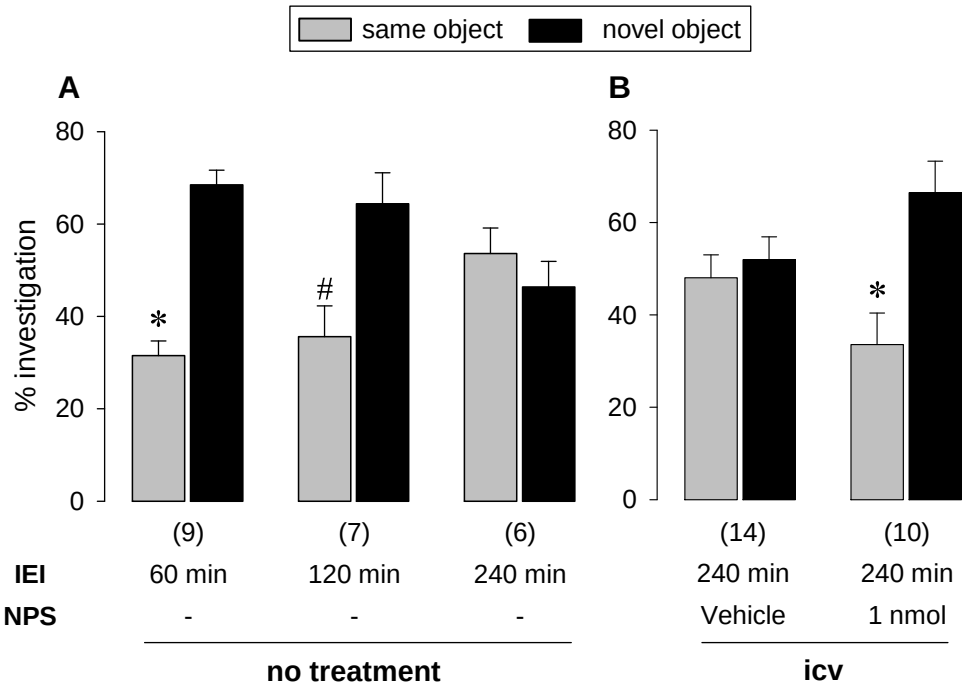

Figure 17: Object discrimination with varying inter-exposure intervals (IEI, A), and effects of icv NPS (1 nmol, B). The object discrimination ability of male rats is reflected by the lower percentage of investigation time towards the *same* versus the *novel* object and was tested after IEI of 60, 120 and 240 min. NPS was applied icv immediately after the acquisition period. Numbers in parentheses indicate group size. Data are means + SEM, * $p < 0.05$ vs. *novel* object, # $p = 0.074$ vs. *novel* object, paired samples t-test (no treatment) and two way ANOVA for repeated measures (factors treatment × stimulus) followed by Bonferroni post-hoc test (icv).

ICV NPS: To determine whether icv NPS facilitates object discrimination at an IEI of 240 min, NPS (1 nmol) or vehicle were administered icv immediately after the acquisition period. NPS-treated rats were still able to discriminate between the *same* and *novel* object after a 240-min interval ($p = 0.016$), whereas vehicle-treated rats failed to discriminate ($p = 0.715$) (Figure 17B).

Nasal NPS: There was a dose-dependent effect of nasal application of NPS. At a dose of 0.4 nmol, NPS-treated rats ($p = 0.430$), as well as vehicle-treated controls ($p = 0.274$), were not able to discriminate between the *same* and *novel* object after an IEI of 240 min. In contrast,

rats treated with 4 nmol of nasal NPS were still able to discriminate ($p < 0.05$). The finding that nasal NPS at the higher dose improved object memory was consistent with the fact that NPS-treated rats displayed significantly less investigation of the *same* object than vehicle-treated rats ($p < 0.05$) (Figure 18A).

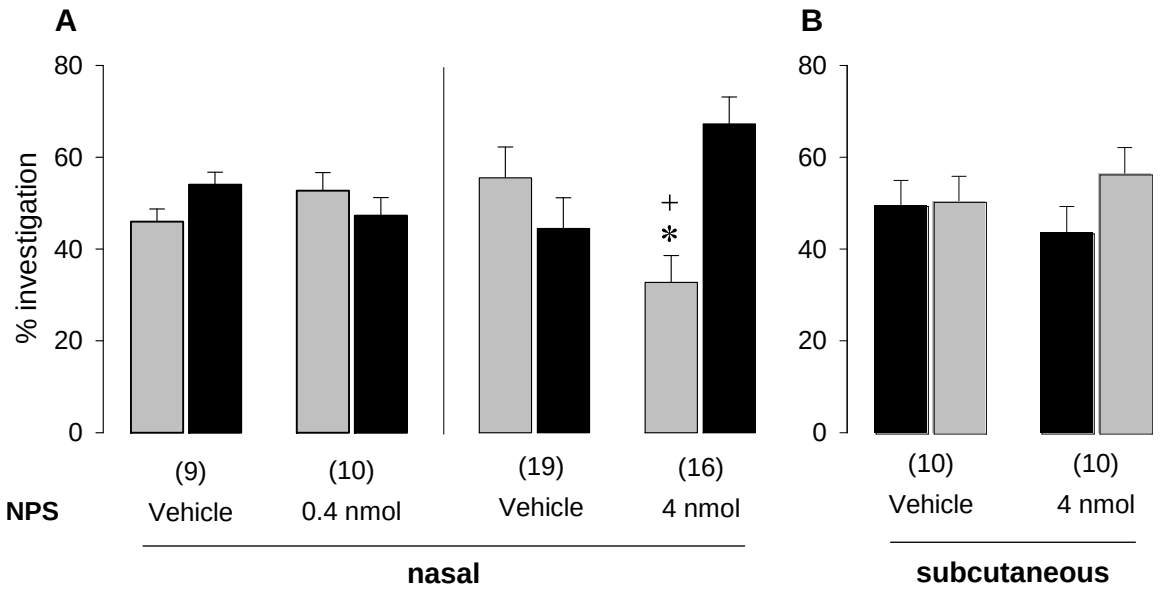

Figure 18: Effects of nasal (0.4 and 4.0 nmol, A) and subcutaneous (sc, 4.0 nmol, B) NPS on object discrimination. Object discrimination abilities of male rats are reflected by the lower percentage of investigation time towards the *same* versus the *novel* object after an inter-exposure interval (IEI) of 240 min. NPS was applied nasally or sc 30 min before the acquisition period. Numbers in parentheses indicate group size. Data are means + SEM, * $p < 0.05$ vs. *novel* object, # $p < 0.05$ vs. vehicle, two way ANOVA for repeated measures (factors treatment × stimulus) followed by Bonferroni post-hoc test.

As object investigation time during the acquisition period did not differ between NPS-treated (64.5 ± 11 s) and vehicle-treated (60.1 ± 9.5 s) rats at the dose of 4.0 nmol ($t_{(33)} = -1.30$; $p = 0.201$), the memory enhancing effects of NPS are not due to a potentially higher general motivation during acquisition caused by the pre-acquisition nasal NPS application.

SC NPS: In contrast to nasal NPS, sc NPS tested at the higher dose of 4 nmol failed to improve object discrimination abilities at an IEI of 240 min (p = 0.392) (Figure 18B). See Table 12 for F-values; see legend to Figs for group sizes.

No effects of NPS on social memory

After an IEI of 60 min, untreated rats spent less time investigating the *same* than the *novel* rat ($t_{(9)}$ = 9.01; p < 0.001) indicating social memory. Social discrimination abilities disappeared after an IEI of 120 min ($t_{(9)}$ = -1.11; p = 0.296) (Figure 19A).

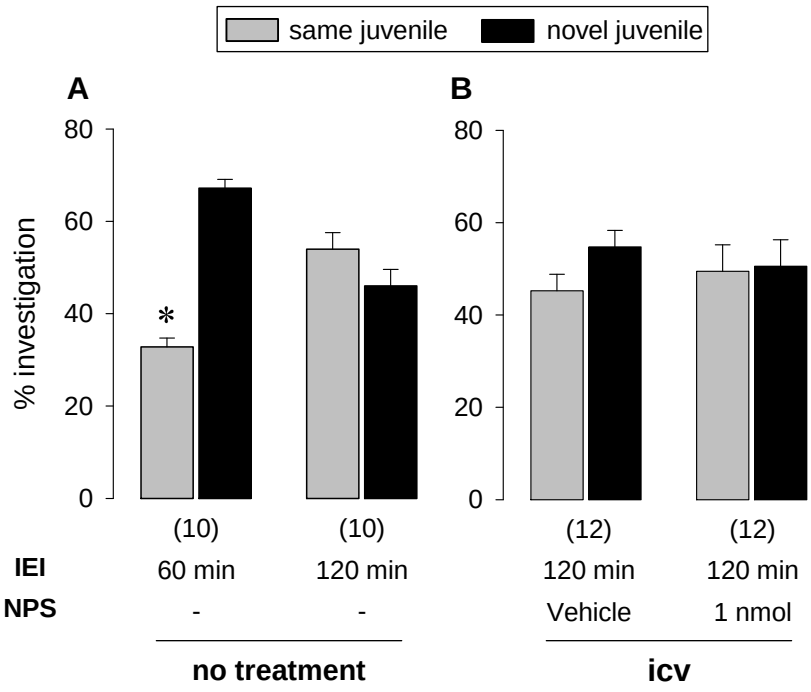

Figure 19: Social discrimination with varying inter-exposure intervals (IEI, A), and effects of icv NPS (1 nmol, B). Social discrimination abilities of adult male rats are reflected by the lower percentage of investigation time towards the *same* versus the *novel* juvenile rat and was tested after IEI of 60 and 120 min. NPS was applied icv immediately after the acquisition period. Numbers in parentheses indicate group size. Data are means + SEM, * p < 0.05 vs. *novel* juvenile rat, paired samples t-test (no treatment) and two way ANOVA for repeated measures (factors treatment × stimulus) followed by Bonferroni post-hoc test (icv).

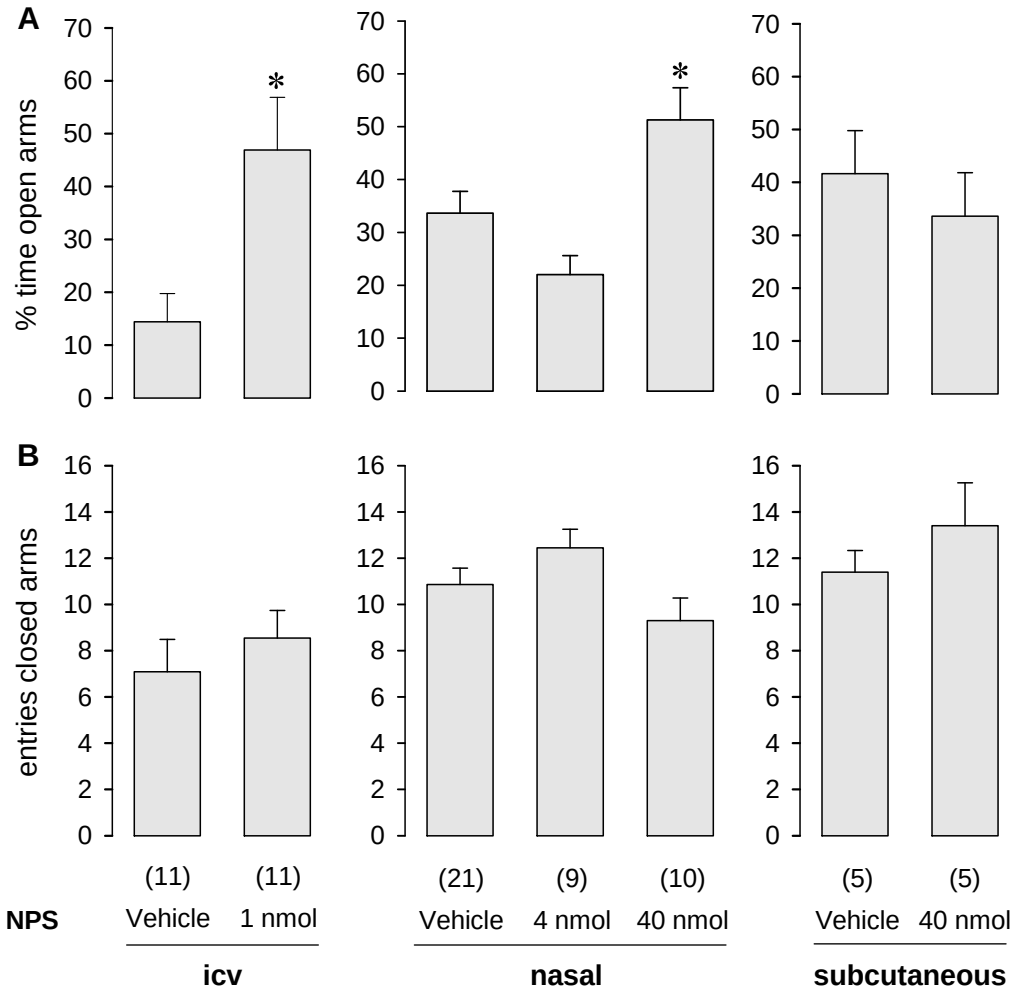

Figure 20: Effects of icv (1 nmol), nasal (4.0 nmol and 40 nmol), and sc (40 nmol) NPS on non-social anxiety. Non-social anxiety-related behavior has been quantified as the percentage of time spent on the open arms of the elevated plus-maze (A). Locomotion was reflected by the number of closed arm entries (B). NPS or vehicle treatments (icv, nasal, and sc) were performed 30 min before start of the 5-min test. Numbers in parentheses indicate group size. Data are means + SEM, * $p < 0.05$ vs. vehicle, one-way ANOVA followed by Bonferroni post-hoc test (nasal) or Student's t-test (icv, sc).

ICV NPS: To determine whether icv NPS facilitates social discrimination at an IEI of 120 min, NPS (1 nmol) or vehicle were administered icv immediately after the acquisition period. Both, vehicle-treated ($p = 0.332$) and NPS-treated ($p = 0.908$) rats failed to discriminate

between the *same* and *novel* rat after a 120-min interval (Figure 19B). See Table 12 for F-values; see legend to Figs for group sizes.

Effects of NPS on non-social anxiety

ICV NPS: Icv infusion of NPS (1 nmol) decreased anxiety-related behavior on the elevated plus-maze as demonstrated by an increase in the percentage of time spent on the open arms ($t_{(20)}$ = -2.88, $p < 0.01$ versus vehicle) (Figure 20A). Importantly, icv NPS did not increase general locomotor activity, as both NPS- and vehicle-treated rats showed a similar number of closed arm entries on the plus-maze (t(20) = -0.791, p = 0.438) (Figure 20B).

Nasal NPS: Application of NPS via the nasal route at a dose of 40 nmol, but not 4 nmol, also resulted in a decrease in anxiety-related behavior indicated by an increase in the percentage of time spent on the open arms of the maze ($p < 0.05$ versus vehicle and 4.0 nmol NPS; $F_{(2,37)}$ = 6.30, $p < 0.005$) (Figure 20A). Again, locomotor activity was found to be unchanged by NPS ($F_{(2,37)}$ = 2.52; p = 0.095) (Figure 20B).

SC NPS: In contrast to the nasal application of NPS, sc NPS (40 nmol) did not alter anxiety-related behavior ($t_{(8)}$ = 0.695; p = 0.507 versus vehicle) (Figure 20A). Also, locomotion was found to be unchanged by sc NPS ($t_{(8)}$ = -0.962; p = 0.364) (Figure 20B). See legend to Figs for group sizes.

No effects of NPS and NPS-A on social preference

ICV NPS: In line with the lack of effect of NPS on social memory, icv infusion of NPS (1 nmol) did not affect social preference behavior. Thus, both vehicle-treated ($p < 0.001$) and NPS-

treated ($p < 0.001$) groups demonstrated a preference for the social compared to the non-social stimulus in the social preference paradigm. Moreover, the investigation time of the social stimulus did not differ between vehicle- and NPS-treated rats ($p = 0.466$) (Figure 21A). ICV NPS-A: Blockade of endogenous NPS with NPS-A (10 nmol) did not affect social preference behavior. Thus, both vehicle-treated ($p < 0.001$) and NPS-A-treated ($p<0.001$) groups demonstrated preference for the social compared to the non-social stimulus in the social preference paradigm. Moreover, the investigation time of the social stimulus did not differ between vehicle- and NPS-treated rats ($p = 0.081$) (Figure 21B). See Table 12 for F-values; see legend to Figs for group sizes.

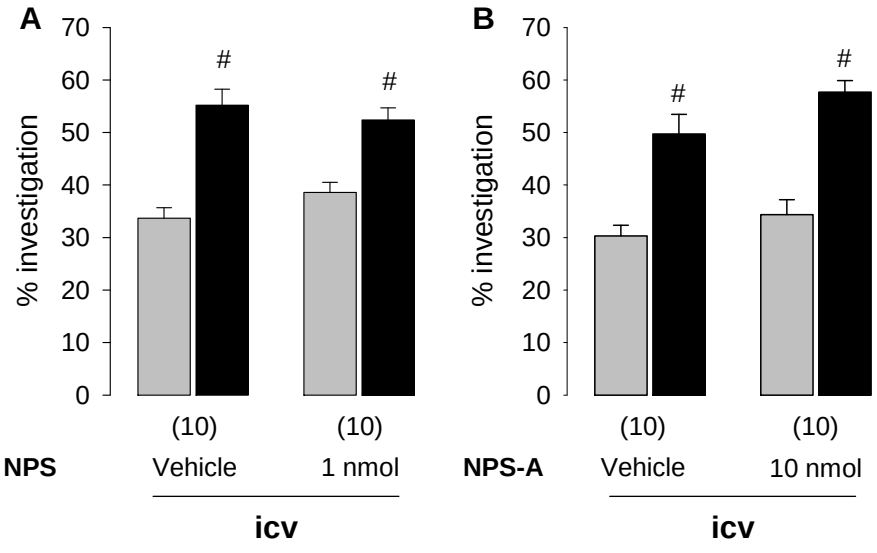

Figure 21: Effects of icv NPS (1 nmol, A) and NPS receptor antagonist (NPS-A; 10 nmol, B) on social preference. Social preference is reflected by a higher percentage of investigation time directed towards the non-social (*grey columns)* versus the social *(black columns)* stimulus during two sequential exposures. NPS, NPS-A or vehicle were applied icv 30 min before the social preference test. Numbers in parentheses indicate group size. Data are means + SEM, # $p < 0.05$ vs. non-social stimulus; two-way ANOVA for repeated measures (factors treatment × stimulus) followed by Bonferroni post-hoc test.

Table 12: F-values and two-way ANOVA for repeated measures

Object discrimination (Figure 17, Figure 18)	stimulus effect	stimulus × treatment effect
Icv	$F_{(1,22)}$=5.02; p<0,05*	$F_{(1,22)}$=3.11; p=0.092
Nasal (0.4 nmol)	$F_{(1,17)}$=0.069; p=0.796	$F_{(1,17)}$=1.89; p=0.187
Nasal (4 nmol)	$F_{(1,33)}$=1.68; p=0.204	$F_{(1,33)}$=6.33; p<0.05*
Subcutaneous	$F_{(1,18)}$=0.768; p=0.392	$F_{(1,18)}$=0.581; p=0.456
Social discrimination (Figure 19)	stimulus effect	stimulus × treatment effect
Icv	$F_{(1,22)}$=0.614; p=0.442	$F_{(1,22)}$=0.382; p=0.543
Social Preference (Figure 21)	stimulus effect	stimulus × treatment effect
Icv (NPS)	$F_{(1,18)}$=49.4; p<0.001*	$F_{(1,18)}$=2.41; p=0.138
Icv (NPS-A)	$F_{(1,18)}$=80.0; p<0.001*	$F_{(1,18)}$=0.677; p=0.421

Stimulus effect: same vs. novel (object/social discrimination) or social vs. non-social (social preference). * indicates a significant p-value.

DISCUSSION

The present study produced three major outcomes. We observed firstly that NPS improved the memory of adult male rats in a context-specific manner. NPS administered icv prolonged object memory, whereas social memory tested in the social discrimination paradigm was not improved by NPS. In line with this, we observed secondly that icv NPS reduced non-social anxiety on the plus-maze, but did not affect social preference / social anxiety. Last, we obtained the first evidence that nasal application of NPS triggers comparable behavioral effects to those observed following icv administration in rats. Thus, nasal NPS also resulted in improved object memory, and in reduced anxiety-related behavior on the elevated plus-maze. In contrast, peripheral (sc) administration of centrally and nasally effective doses of NPS was devoid of behavioral effects.

NPS improves non-social, but not social memory

In our experiments performed in rats icv infusion of NPS prolonged the memory for objects, but seemed to be ineffective in a social memory paradigm. This is in agreement with several recent studies, all performed in mice, indicating a role of NPS in learning and memory processes. Specifically, NPS locally applied into the mouse endopiriform nucleus attenuated the expression of contextual fear (Meis et al., 2008), and acts within the amygdala to facilitate fear extinction (Jüngling et al., 2008). Moreover, icv NPS enhanced aversive contextual memory retention in the inhibitory avoidance paradigm (Okamura et al., 2011) and facilitates spatial memory in the Morris water maze (Han et al., 2009). With respect to novel object recognition it was found that icv NPS (1 nmol) prolonged object memory of C57Bl/6 mice up to 168 h when applied immediately after the acquisition period (Okamura et al., 2011). In our object discrimination paradigm in rats, untreated adult male rats were able to discriminate between the same and the novel object after 60 min and also partly after 120 min, but completely failed to do so after an IEI of 240 min. Central administration of NPS at a dose of 1 nmol immediately after the acquisition period enabled the rats to recognize the novel object even after 240 min.

The memory-enhancing effect of NPS is most likely due to an effect on memory consolidation, as NPS applied at the same dose but either before pre-training or immediately before retrieval was ineffective to alter memory retention in the inhibition avoidance paradigm in mice (Okamura et al., 2011). Recent findings in NPS receptor knockout mice (Okamura et al., 2011) indicated an involvement of the endogenous NPS system in these memory processes. Future studies using specific NPS-A will further prove this hypothesis.

However, our study is the first to test for possible memory-enhancing effects of NPS also in a social context. Male Wistar rats were able to discriminate between the *same* and the *novel* juvenile rat after an IEI of 60, but not 120 min (Figure 19A) (Lukas et al., 2011a). The lack of social discrimination after 120 min also remained after icv NPS indicating that increasing the amount of centrally available NPS is not effective to further prolong memory in a social context.

The anatomical substrates for the memory-enhancing effect of NPS in the object discrimination paradigm still need to be identified, but may include the hippocampus, as object memory is likely to be hippocampus-dependent (Clark et al., 2000) including its various sub-structures (Broadbent et al., 2004; Winters et al., 2004). Moreover, amygdala and cortical regions are also likely to be involved in non-social memory tasks such as object discrimination (Moses et al., 2005). In these regions the expression of the NPS receptor - a former orphan G-protein-coupled receptor - has been localized (Xu et al., 2007; Leonard and Ring, 2011). Consequently, both after icv and nasal (see below) application, NPS should be able to reach these relevant brain sites to allow consequent action at these receptors.

NPS reduces non-social anxiety, but not social preference / social anxiety

The effectiveness of NPS in a non-social context is further supported by the finding that NPS exerts robust anxiolytic effects both in mice (Xu et al., 2004) and rats (Figure 20)(Slattery et al., 2008; Vitale et al., 2008; Wegener et al., in press) in various non-social paradigms such as elevated plus-maze, open field, light dark-box or marble burying. However, the anxiolytic

effect was almost always accompanied by an increase in locomotor activity. In our study performed in rats and using relatively low doses of NPS, we were able to dissociate the anxiolytic and the arousal effects, as icv (and nasal) NPS reduced anxiety-related behavior on the plus-maze without altering the number of entries into the closed arms as indication of general locomotion. In contrast, we could not find any effect of NPS and NPS-A on social preference in an established test for social avoidance and social anxiety (Berton et al., 2006; Lukas et al., 2011b). The lack of an NPS action on social preference was not due to a possible ceiling effect, as the percentage of investigation time of the social stimulus (between 50 and 55% of total exposure time of 4 min) still allowed a possible positive treatment effect and further increase in social investigation. In support of the above findings, blockade of the endogenous NPS system with the specific NPS-A did not impair naturally occuring social preference behavior. Thus, an involvement of the brain NPS system in social behavior seems unlikely.

It is of general interest to note that NPS exerts behavioral effects in non-social, but not social settings. Various other neuropeptides including OT, AVP, neuropeptide Y, or corticotrophin releasing factor were shown to modulate both non-social and social behaviors, including spatial and social memory, non-social and social anxiety and other social behaviors (e.g. sexual, aggressive, maternal) (Argiolas and Melis, 2004; Bielsky and Young, 2004; Engelmann et al., 2004; Lim and Young, 2006; Frank and Landgraf, 2008; Veenema and Neumann, 2008; Bosch, 2011). The lack of NPS effects on social memory and social anxiety is the more remarkable as icv NPS was shown to trigger Fos expression, a marker for neuronal activity, in limbic brain structures involved in both emotional and social behaviors such as the central

and medial amygdala, lateral hypothalamus, arcuate nucleus and ventromedial hypothalamus (Kallupi et al., 2010). However, in order to further substantiate the context–dependent effects of NPS with preferential actions on non-social behaviors, various other tests for social behavior need to be employed including the social interaction test, resident-intruder test for intermale aggression, as well as tests for pair bonding, sexual behavior and maternal behavior.

Behavioral effects of nasal NPS

The intranasal route of administration has become an important and popular tool in human and rodent studies in order to reveal effects of several different neuropeptides on diverse aspects of social and non-social behaviors, emotionality and neuronal activation patterns (for review see Hanson and Frey, 2008; Heinrichs and Domes, 2008; Meyer-Lindenberg, 2008). However, our study is the first to describe behavioral effects of intranasal NPS. Our observation that nasal NPS improves non-social memory and non-social anxiety in rats strongly implies that nasally applied NPS reaches neuronal structures of the brain in significant amounts. In this context it needs to be emphasized that, in our study, NPS was applied not directly into the nostrils, but rather topically on the rhinarium, which is highly vascularized and innervated (Silverman et al., 1986). This ‘nasal’ route of application allowed acute drug application in conscious, although well handled, rats, whereas in many other comparable studies, animals were anesthetized for ‘intranasal’ application (Alcalay et al., 2004; Marks et al., 2009; Rat et al., 2011).

Nasal application of NPS was found to mimic the behavioral effects of NPS seen after its icv administration. Thus, nasal NPS at a dose of 4 nmol was able to prolong object memory even when applied 30 min before memory acquisition and consolidation. This time point has been chosen to allow for substance diffusion into relevant CNS structures. Moreover, in this initial study, we based the choice of application times on human studies, in which behavioral or neuronal effects of OT, for example, appear 30 - 40 min after intranasal application (Kirsch et al., 2005; Gamer et al., 2010). The precise temporal dynamics of nasal uptake and intracerebral presence of synthetic NPS, however, needs to be studied in more detail, also considering differences in chemical properties (linear versus cyclic peptides). In the context of the memory-enhancing effect of NPS it is important to mention that the general motivation for object discrimination was not altered, as object investigation time during the acquisition period did not differ between NPS- and vehicle-treated rats.

Nasal NPS applied at a dose of 40 nmol (but not at 4 nmol) also exerted an anxiolytic effect on the elevated plus-maze, which was not accompanied by an increase in locomotor activity; The finding of comparable behavioral effects after nasal and icv (Figure 20) (Xu et al., 2004; Wegener et al., in press) application of NPS opens the exciting possibility to further test for the therapeutic use of NPS via the intranasal route in humans.

Whereas, nasal application of NPS at a dose of 4 nmol was already efficient to prolong object memory, in contrast, NPS-induced anxiolysis was only observed at a dose of 40 nmol. Thus, the possibility exists that different brain target regions involved in different behavioral functions, vary in their accessibility by nasal NPS. Further, it is possible that different

behavioral effects, i.e. memory-enhancing and anxiolytic effects require different doses of NPS.

In contrast to nasal application, sc administration of NPS at the same dose and time point, i.e. 30 min before either the start of the object discrimination test or the plus-maze test, was without any behavioral effect. Subcutaneous NPS needs to cross the blood brain barrier, which restricts neuropeptide transport in significant amounts (Ermisch et al., 1985). In contrast, it has been hypothesized that intranasally applied neuropeptides are capable of bypassing the bloodstream and directly entering the CNS (Born et al., 2002). Our finding that behavioral effects of NPS were observed after nasal, but not sc, administration, are in line with this hypothesis. However, a follow up study investigating the behavioral effects of intranasal NPS after blockade of central NPS receptors by ICV or local administration of an NPS-A is needed to confirm if the behavioral effects observed in the present study are centrally mediated and to determine their intracerebral localization.

Several human studies suggested an involvement of the brain NPS system in anxiety and anxiety disorders (Laitinen et al., 2004; Domschke et al., 2010; Donner et al., 2010; Raczka et al., 2010). For example, a single nucleotide polymorphism in the human NPS receptor has been associated with an increased risk for panic disorder and overinterpretation of fear (Raczka et al., 2010), and the Ile^{107} receptor variant is associated with enhanced fear acquisition (Domschke et al., 2010). Intriguingly, it was shown that this receptor variant has a 10-fold higher potency for NPS (Reinscheid et al., 2005). Although this seems to argue against a potential therapeutic effect of NPS in humans, studies performed in rat models for hyper-anxiety and depression-like behavior demonstrated that NPS exerts an acute and

robust anxiolytic effect even in these psychopathological animals (Slattery et al., 2008; Wegener et al., in press)

In conclusion, this study confirms the pharmacological effects of synthetic NPS with respect to the improvement of non-social memory and the reduction of non-social anxiety. However, social memory functions and social preference were not found to be altered by NPS indicating a robust context dependency of NPS effects. Importantly, the finding that nasal application of NPS mimics its behavioral effects after central administration should stimulate experiments in both rodents and humans to test for the potential therapeutic use of intranasal NPS specifically in anxiety, panic and posttraumatic stress disorders.

Chapter 6

General Discussion

Content

1. Summary of results

In chapters 2, 3, and 5 of this thesis, I aimed to add to the characterization of the involvement of central neuropeptides such as OT, AVP, and NPS in basic social abilities, i.e. individual social recognition and social approach behavior. Both aspects of social behavior are essentially needed for proper performance of more complex social behaviors, like aggression, sexual behavior or pair bonding.

Additionally, in chapters 2, 3, and 4, I investigated the effects of different social stress paradigms, i.e. social defeat and MS, on social preference and social recognition abilities of male rats. In chapter 2, social stress was induced by an acute social defeat by a male con-specific. In contrast, in chapters 3 and 4, social stress was performed chronically during development, using MS, a deprivation of the pups from the dam during the first two weeks of life.

The main prerequisite of all social interactions in rodents, including social recognition, is the ability to get in close contact with a con-specific. Therefore, in chapter 2, I adapted a social preference paradigm from mouse research (Berton et al., 2006) for rats to characterize social interaction/approach independent of innate anxiety. Using this new paradigm, I demonstrated thatvehicle-treated male rats and mice have a natural preference for a social stimulus over a non-social stimulus, whereas blockade of endogenous OT resulted in social avoidance in male rats and even a total loss of social preference in male mice. Furthermore, I demonstrated specificity of this OT-mediated effect on social anxiety as both, rats injected with the anxiogenic PTZ and rats infused icv with a specific V1aR-A did not show changes in

social preference behavior. Notably, icv infusion of OTR-A in different subnuclei of the amygdala did not result in alterations of social preference behavior. I further confirmed that acute social defeat induced social avoidance in male rats, when tested in the social preference paradigm 2 h later. However, social avoidance was only achieved, when the actual defeater rat was used as social stimulus during the social preference testing. Accordingly, acute icv OT administration prior to behavioral testing restored social preference in these rats - an effect that is also not mediated by the medial or central amygdala.

In Chapter 3, I exposed male Wistar rats to postnatal stress, i.e. MS, and tested social memory, in the social discrimination paradigm, at juvenile (5 weeks) and adult age (16 weeks). Juvenile MS as well as juvenile and adult control rats recognized a juvenile con-specific after 30 and 60 min. In contrast, adult MS rats were able to recognize a juvenile con-specific only after an IEI of 30 min. Thereby, I was able to demonstrate impaired social memory abilities in postnatally-stressed adult male Wistar rats.

Additionally, I measured septal AVP release via intracerebral microdialysis during social discrimination in male juvenile and adult, control and MS rats. While I observed a rise of extracellular septal AVP during acquisition of social memory, both in juvenile control and MS rats as well as in adult control rats, I found a blunted septal AVP release in adult MS rats. Interestingly, forced swimming-induced septal AVP release was not reduced in adult MS rats compared to controls.

In order to counteract this blunted AVP release during social memory acquisition, I infused male MS rats with synthetic AVP using intraseptal retrodialysis. Indeed, synthetic AVP given

during social memory acquisition was able to rescue social recognition abilities of formerly impaired adult MS rats. This indicates a causal connection between the impaired social recognition abilities in adult male MS rats and the blunted AVP release found in these rats.

In chapter 4, we observed brain region-specific developmental changes in V1aR binding from juvenile rats over adolescence into adulthood using receptor autoradiography. The most pronounced age effect, in the form of increased V1aR binding, was found in the lateral septum. Exposure to MS significantly interfered with these developmental adaptations resulting in brain region- and age-dependent alterations in V1aRs. Most notably, MS increased V1aR binding in the piriform cortex (in adolescents and adults) and the lateral septum (in juveniles).

In chapter 5, I was able to demonstrate that neither icv administration of synthetic NPS nor blockade of central NPS receptors was efficient to influence social preference and social recognition in male rats, despite its potent icv effects on non-social memory and anxiety. Moreover, I was able to show for the first time that synthetic NPS, applied nasally to male rats before performing in the object recognition paradigm and on the elevated plus-maze, was able to mimic the memory enhancing and anxiolytic effects of NPS that were already observed after icv administration.

2. Neuropeptides and social behaviors

6.1 Involvement of OT and AVP in social preference

Rats and mice show a natural preference for social stimuli over non-social stimuli. In chapter 2, I showed that blocking the endogenous OT system via infusion of OTR-A induced social avoidance in rats and totally blocked social preference for a social stimulus in mice. Localization of these pro-social abilities of endogenous OT in different subnuclei of the amygdala was not successful. Intriguingly, human fMRI studies report that activation patterns of amygdala subnuclei in social contexts of fear can be reduced via nasal OT application (Kirsch et al., 2005). However, if nasal OT acts directly in the amygdala or in OT receptive regions upstream of the amygdala, e.g. the prefrontal cortex, is not known. Another possibility may be that endogenous OT exerts its effects on multiple brain regions, where OT receptors were shown to be present, including the olfactory bulb, prefrontal cortex, nucleus accumbens, lateral septum, and PVN (Barberis and Tribollet, 1996; Tribollet *et al.*, 1988; Young *et al.*, 1996) that act in concert on promoting social preference. As discussed in more detail later, icv administration of synthetic OT did not increase social preference above the level of control animals.

In contrast, icv administration of the V1aR-A did not affect social preference indicating peptide-specific effects and high specificity of the antagonists used. This finding was rather surprising as AVP is a key modulator of various aspects of social behavior in male rodents such as social memory (Engelmann and Landgraf, 1994), inter-male aggression (Veenema et al., 2010b) and pair bonding (Wang et al., 1998). As icv infusion of AVP is impractical due to the risk of unspecific behavioral responses, e.g. barrel rotations, it was not possible to test

for alterations in social preference after icv administration of AVP itself. However, AVP exerts rather anxiogenic effects (for review see Landgraf, 2006). Due to the high social preference in untreated Wistar rats, it is possible that the anxiolytic effects of the V1aR-A in a social context were not visible in this paradigm. In support of this hypothesis, in a non-mammalian model, using goldfish (Carassius auratus), a social teleost, central infusion of the respective AVP homologue vasotocin decreases social approach behavior, whereas a vasotocin antagonist and the OT homologue isotocin increases it (Thompson and Walton, 2004). However, an increase in social approach is only observed in animals with a low baseline of natural social approach behavior, indicating a comparable ceiling effect in other social animals. In this respect, animal models of low innate sociability, like the BTBR T+tf/J mice (McFarlane *et al.*, 2008) or socially defeated rats (Haller and Bakos, 2002), may be useful for investigating the possible differential effects of exogenous OT and AVP on social preference behavior.

6.2 Involvement of OT and AVP in social recognition

Several studies in male rats report social memory-prolonging effects of synthetic OT, whereas infusion of OTR-A, either icv or locally into the septum and into the medial preoptic area does not affect social recognition (Popik and van Ree, 1991; Popik et al., 1992; Benelli et al., 1995). These studies used an initial paradigm to measure social memory in rodents, called “social recognition paradigm”. In this paradigm social recognition is assessed by comparing social interest directed towards the same social stimulus during repeated exposures.

However, by using the social discrimination paradigm (Engelmann et al., 1995) that simultaneously compares social interaction of a rat with both a novel and a known juvenile stimulus rat, we demonstrated that blocking endogenous OT, indeed impaired social recognition after an IEI of 60 min. This effect was only visible when the OTR-A was infused directly after the first trial (Lukas et al., in preparation). Antagonist infusion at the end of the 60 min IEI did not impair social recognition (Lukas et al., in preparation), indicating that endogenous OT is needed during the consolidation of social memory and is essential for successful social recognition in male rats. In female rats icv blockade of endogenous OT clearly diminishes social memory, when tested in the social discrimination paradigm (Engelmann et al., 1998). Moreover, studies in male OT and OTR knockout mice showed clear deficits in social recognition (Ferguson et al., 2000; Takayanagi et al., 2005), further arguing for the basic necessity of endogenous OT during social memory consolidation.

OTR-A	Social preference	Social recognition (juvenile stimulus)	Social recognition (female stimulus)
Icv	⇩	⇩	⇩
Central amygdala	no effect	no effect	not tested
Medial amygdala	no effect	no effect	⇩
Lateral septum	no effect	⇩	⇩

Figure 22: Schematic summary of icv and local OTR-A effects on social preference and social recognition in male Wistar rats. Arrows indicate impairment in performance of the respective social behavior.

Attempts to localize the involvement of OT in mice revealed the medial amygdala as target for endogenous OT in males (Ferguson et al., 2001) as well as in females (Choleris et al., 2007). However, we were only successful in blocking social recognition in the medial amygdala of male rats, via local infusion of an OTR-A when ovariectomized female rats, instead of male juveniles, were used as social stimuli (Lukas et al., in preparation) (Figure 22). Therefore, OT-dependent olfactory information processing in the medial amygdala seems to be linked to the quality of the olfactory stimulus, as already indicated by studies investigating neuronal activation demonstrating corresponding effects like differences in immediate early gene activation following male and female stimulation (Arakawa et al., 2010; Samuelsen and Meredith, 2011). In contrast, social memory after an IEI of 60 min for both ovariectomized females and juvenile males, was blocked after OTR-A administration in the lateral septum (Lukas et al., in preparation) (Figure 22). Therefore, the modulatory action of endogenous OT in the lateral septum seems to be of central importance in information processing for a broad variety of social stimuli. It should be mentioned that it was already demonstrated that OTR-A infused in the lateral septum did not block social recognition for juveniles after 30 min (Landgraf et al., 2003), but this could be due to a shorter IEI. However, if a basal level of OT is sufficient to assure social recognition in male rats or if OT is released endogenously in the medial amygdala and lateral septum during social memory consolidation, still needs to be determined.

The involvement of endogenous septal AVP in male social recognition and the memory-facilitating effect of synthetic AVP is known since first pharmacological studies from Dantzer (1988) that have been confirmed several times in rats (Landgraf et al., 1995b; Landgraf et al.,

2003) and mice (Bielsky et al., 2004). In Chapter 3, I demonstrated that the acquisition of social recognition memory is associated with a significant rise in extracellular AVP release within the lateral septum of juvenile and adult male rats. This is consistent with the finding that administration of AVP into the lateral septum prolongs social recognition memory in adult male rats for up to 2 h (Dantzer et al., 1988; Engelmann et al., 1994) or even 24 h (Landgraf et al., 2003).

6.3 Involvement of NPS in social preference and social recognition

In chapter 5, I was able to confirm findings from the literature that centrally infused NPS reduces anxiety-related behavior on the elevated plus-maze in rats and mice (Xu et al., 2004; Slattery et al., 2008). However, I was able to demonstrate that synthetic NPS did not increase social preference, nor did a specific NPS-A (Guerrini et al., 2010) trigger social anxiety in rats. Furthermore, icv infusion of synthetic NPS did not prolong social memory in male rats, although icv infusion of NPS during consolidation in an object discrimination paradigm prolonged memory from 120 to at least 240 min. This confirms the object memory-enhancing effect of NPS already known from mice (Okamura et al., 2011). Together with the finding that synthetic NPS did not influence social preference, this strongly indicates that NPS influences anxiety and memory in non-social settings, but is rather ineffective in a social context. OT, AVP, and various other neuropeptides, e.g. corticotrophin releasing factor, and neuropeptide Y, were modulate non-social as well as social behaviors, including spatial and social memory, non-social and social anxiety, and other social behaviors (e.g. sexual, aggressive, maternal) (Heinrichs, 2003; Argiolas and Melis, 2004; Painsipp et al., 2008; Veenema and Neumann, 2008; Bosch, 2011). The lack of NPS effects on social memory

and social anxiety is even more remarkable as icv NPS to triggers Fos expression, a marker for neuronal activity, in limbic brain structures involved in both emotional and social behaviors, such as the central and medial amygdala, lateral hypothalamus, arcuate nucleus and ventromedial hypothalamus (Kallupi et al., 2010). However, in order to further substantiate the context–dependent effects of NPS with preferential actions on non-social behaviors additional testing for social behavior needs to be employed, including intermale aggression, pair bonding, and sexual behavior.

3. OT and AVP release during social contact

Intracerebral release of OT and AVP in specific brain regions is increased in response to a variety of social stimuli in mammals, including mother-offspring interactions, maternal aggression, mating in males and females, and exposure to an adult con-specific during social defeat (reviewed in the introduction, see also Landgraf and Neumann, 2004; Veenema et al., 2008; Neumann, 2009). Thus, increased OT release could be identified within the hypothalamic PVN, SON, and olfactory bulb during parturition (Neumann et al., 1993; Lévy et al., 1995), in the central amygdala accompanying maternal aggression (Bosch et al., 2004; Bosch et al., 2005), and in the nucleus accumbens and PVN during socio-sexual interactions (Waldherr and Neumann, 2007; Ross et al., 2009). Increased AVP release during social stimulation was observed within the hippocampus during parturition (Landgraf et al., 1991), and within the septum in the context of inter-male aggression (Beiderbeck et al., 2007; Veenema et al., 2010b). The data I presented in chapter 3 substantially add to these findings demonstrating a rise of extracellular AVP in the septum of juvenile and adult male rats

during social memory acquisition of a neutral social stimulus (juvenile male rat). As aggressive as well as non-aggressive social interactions seem to trigger septal AVP release, this may propose that septal AVP release is of general importance for the evaluation of social stimulation. However, after a negative social stimulation, like social defeat, septal AVP release remained unchanged (Ebner et al., 2000). Notably, in the context of negative social stimulation, social defeat increases OT release within the SON and septum of male rats (Engelmann et al., 1999; Ebner et al., 2000), whereas AVP levels is elevated within the PVN (Wotjak et al., 1996). Apparently, social stress is able to directly influence neuropeptide release in limbic brain regions involved in social behavior, e.g. septum, amygdala, and hypothalamic nuclei (see above). The long-term effects of exposure to social stress on social behaviors and on the reactivity of intracerebral neuropeptide systems will be discussed in the following.

4. Effects of social stress on social recognition and social preference

Several genetic mouse models demonstrating impaired social abilities propose that social deficits are to a certain proportion due to genetic predisposition (Ferguson et al., 2000; McFarlane et al., 2008). However, environmental factors, especially different social experiences were shown to also play a role in the development of social deficits (Veenema, 2009). Therefore, I used rodent models of stressful social experiences like social defeat and MS to investigate their behavioral consequences on social preference and social recognition as well as their effect on the central OT and AVP system.

4.1 Effects of social defeat on social preference

In chapter 2, I demonstrated that acute social defeat by a male con-specific resulted in a loss of social preference in male rats. This finding is in line with several studies in rats and mice demonstrating avoidance of social contact to con-specifics after repeated negative social encounters (Avgustinovich et al., 2005; Berton et al., 2006). Only a few studies showed reduced social interaction after a single social defeat (Haller and Bakos, 2002). In contrast to these single defeat studies, I was only able to demonstrate a loss of social preference when the defeater rat was used as social stimulus, indicating social recognition in the defeated experimental rats. This phenomenon has also been observed in a study reporting social avoidance in golden hamsters (Lai et al., 2005). This implies that for eliciting social avoidance towards an unknown social stimulus in rats a stronger form of social defeat or repeated defeat by different defeater rats may be needed.

In my studies, social defeat resulted in even lower social investigation times than those directed towards the object stimulus, indicating not only loss of social preference, but even social anxiety. As social defeat elicits non-social anxiety in rats and mice (Heinrichs et al., 1992; Avgustinovich et al., 2005; Buwalda et al., 2005), it is possible that reduction in social preference after social defeat is due to increased general anxiety after the defeat. However, this is unlikely as I was able to demonstrate that the general anxiolytic agent PTZ did not reduce social preference, although it increased anxiety on the elevated plus-maze. Thus, the social preference paradigm appears to be specific for social stimulus-related anxiety.

Several studies measuring intracerebral release during stressful social encounters emphasized the important role of OT and AVP in this context (for review see above and

Engelmann et al., 2004). Non-social stressors, like forced swimming, trigger both OT and AVP release in the PVN, SON and septum, whereas in the periphery only OT is increased (Wotjak et al., 1998; Engelmann et al., 2000). In the context of social stress, OT release is increased in the septum and SON, but not PVN, of the defeated male rat during social defeat (Wotjak et al., 1996; Engelmann et al., 1999; Ebner et al., 2000). Within the PVN, increased OT release was only observed after positive social stimulation like mating (Wotjak et al., 1996; Waldherr and Neumann, 2007). In contrast, social defeat increases AVP levels in the PVN, but not in the septum or in the SON (Wotjak et al., 1996; Ebner et al., 2000). Interestingly, the secretion into the blood of both OT and AVP, is unchanged after social defeat (Wotjak et al., 1996; Engelmann et al., 2000). This indicates a specific release pattern for OT and AVP during an acute negative social stress experiences. Due to the pro-social effects of endogenous OT on social preference, an increase in the general availability of OT in the brain may be efficient to restore social preference in socially defeated rats.

Indeed, I restored social preference in socially defeated rats via icv infusion of synthetic OT. Despite its role in recognition of familiar con-specifics (Bielsky and Young, 2004; Lukas et al., in preparation), it is unlikely that these effects are due to OT-mediated alterations in social recognition, as the dose used in the current study was ineffective in social recognition (Benelli et al., 1995). Also, if anything, OT would have facilitated the recognition of the defeater rat, which would have promoted avoidance rather than restored social preference, as it was found in the present study. Attempts to localize the OT-mediated restoration of social preference behavior after social defeat in the medial and central amygdala were not successful. This supports my findings that blockade of endogenous OT in these amygdala subnuclei did not reduce natural social preference in male rats. Intriguingly, reduced social

interaction in prenatally stressed female rats can be rescued with intra-amygdala infusion of OT (Lee et al., 2007). The use of a different stress model may explain the differences in the local OT rescue of social approach behavior. I could further show that exogenous OT did not increase social preference under basal circumstances. Similarly, in human studies, intranasal OT application reduces hyperresponsivity of the amygdala to facial cues in socially anxious patients, while not affecting the response in healthy subjects (Labuschagne et al., 2010).

4.2 Effects of MS on social recognition

In chapter 3, I observed that postnatal stress in the form of MS did not affect social memory, i.e. social recognition abilities, of juvenile (5-weeks old) male rats. Social recognition in MS rats was present 30 min, but not 60 min after the initial social contact. Under control conditions, i.e. in un-separated adult male rats, the social memory for juvenile stimuli lasted for at least 60 min. Furthermore, social recognition, for juvenile stimuli, of female MS rats at an IEI of 60 min was unaffected (Figure 23).

Besides social recognition, MS alters more complex social behaviors, including an increase in aggression and a reduction of sexual behavior in adult male rats (Rhees et al., 2001; Greisen et al., 2005; Veenema et al., 2006). In the case of intermale aggression, alterations in social interactions occur more early in development, as juvenile male MS rats show an increase in nape attacks and biting, and a decrease in submissive behaviors during play-fighting (Veenema and Neumann, 2009). Together with the finding that MS impairs social recognition abilities, early life stress seems to alter important aspects of social communication. As MS impaired social recognition in adult male rats only, this may point

towards MS-induced changes during sexual maturation in neural circuits underlying social behavior, like alterations in the binding properties of OT and AVP to their respective receptors or in their intracerebral release. As males seem to be more affected concerning social recognition impairments, alterations in the brain AVP system may be more likely, especially due to its exclusive role in male social recognition (Bluthe and Dantzer, 1990) and its pronounced occurrence in the male rodent brain (De Vries et al., 1984b).

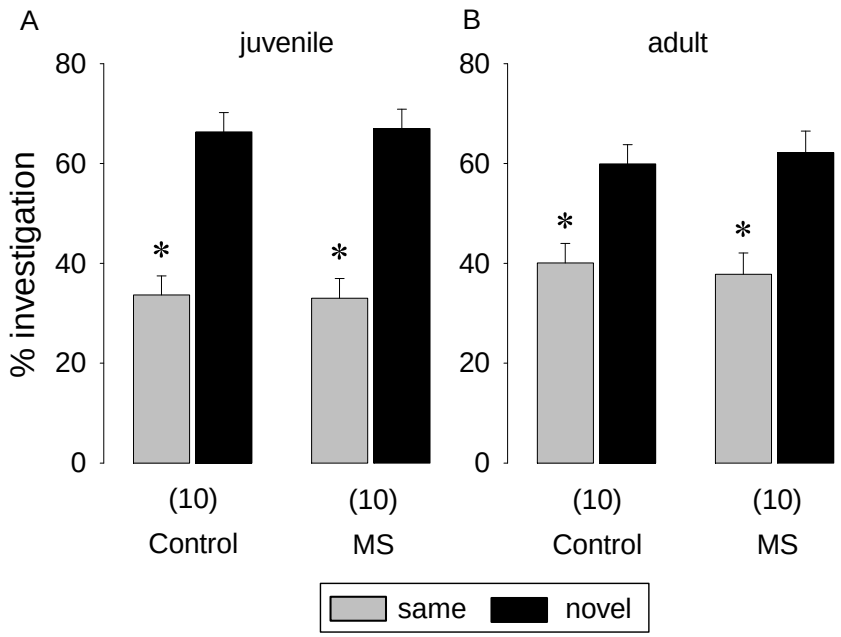

Figure 23: Effects of MS on social recognition in juvenile (A) and adult (B) female rats. Social discrimination abilities are reflected by the percentage of investigation time towards the *novel* and the *same* 3-week-old rat after an inter-exposure interval of 60 min in Control and MS rats. Numbers in parentheses indicate group size. Data are means + SEM, * $p < 0.05$ vs. novel rat, paired samples *t*-test (*same* × *novel*).

In line with this hypothesis, in chapter 4, I observed robust developmental changes in V1aR binding in several brain regions of juvenile and adult male rats. These developmental changes are in line with previous findings in rats (Tribollet et al., 1989; Tribollet et al., 1991) and are likely due to differences in receptor number rather than differences in their affinity

(Tribollet et al., 1991). Most developmental changes in V1aR binding were observed around the age of 8 weeks, which corresponds with adolescence in rats. During adolescence, pronounced remodeling of the brain occurs, which is required to achieve social and sexual maturity (Spear, 2000). The transition of V1aR binding to adult binding levels in adolescent rats might therefore contribute to sexual maturation and the expression of adult-typical behaviors.

Surprisingly, none of the MS-induced alterations in V1aR was stable across the three stages of development (i.e. juvenile, adolescent, adult). Although speculative, changes in V1aR gene expression and subsequent receptor binding may be induced by epigenetic modulation of respective gene transcriptions after MS. The epigenetic modulation of the glucocorticoid receptor altered stress responsiveness, but was potentially reversible later in life (Weaver et al., 2005). MS-induced changes in neuropeptide receptor binding that only occurred at juvenile and/or adolescent age (as was seen for V1aR binding in the lateral septum and dentate gyrus) could have interfered with the normal development of that particular brain region, and in turn, with the normal development of social or emotional behaviors. The MS effects on V1aRs are discussed below with an emphasis on those brain regions implicated in social behaviors.

MS accelerated the maturation of V1aR binding in the lateral septum in such a way that an adult-like V1aR binding pattern was already observed at juvenile age. V1aR gene expression in the septum is positively regulated by glucocorticoids (Watters et al., 1996b). In this respect, it is of interest to note that juvenile MS rats have higher baseline plasma

corticosterone levels compared to juvenile control rats (Veenema and Neumann, 2009). This suggests that the higher V1aR binding in juvenile MS rats could have been mediated by MS-induced changes in plasma corticosterone concentration. As already mentioned, the septal AVP system is strongly involved in male social recognition (Landgraf et al., 2003; Bielsky et al., 2005a) and complex social interactions dependent on the functioning of social recognition, like intermale aggression (Irvin et al., 1990; Koolhaas et al., 1991; Beiderbeck et al., 2007). As V1aR gene expression in the septum is positively regulated by glucocorticoids (Watters et al., 1996b), it is of interest to note that juvenile MS rats have higher baseline plasma corticosterone levels compared to juvenile control rats (Veenema and Neumann, 2009). This suggests that the higher V1aR binding in juvenile MS rats could have been mediated by MS-induced changes in plasma corticosterone concentration. The septal AVP system is strongly involved in male social recognition (Landgraf et al., 2003; Bielsky et al., 2005a) and complex social interactions dependent on functioning of social recognition, like intermale aggression (Irvin et al., 1990; Koolhaas et al., 1991; Beiderbeck et al., 2007).

Furthermore, MS significantly increased V1aR binding in the piriform cortex of adolescent and adult rats. The piriform cortex is part of a network involved in the processing of olfactory cues used for social communication (Richter et al., 2005; Ross and Eichenbaum, 2006; Petrulis, 2009). Activation of the piriform cortex was found to be crucial for the consolidation and for the recall of long-term social memory (Sanchez-Andrade and Kendrick, 2009).

Also, the amygdala is involved in the processing of olfactory and visual cues (Bressler and Baum, 1996; Ferguson et al., 2002; Adolphs and Spezio, 2006) and in fundamental emotions like anxiety and fear (LeDoux, 2000; Walker et al., 2003), which have a strong influence on

the expression of a variety of social behaviors. However, MS had no effect on V1aR binding in the amygdala, suggesting that the role of local V1aR is consistent across development.

Additionally, we showed that exposure to MS interferes with the normal development of OTR binding in specific forebrain regions, such as the agranular insular cortex, the lateral septum, and the ventromedial hypothalamus (Lukas et al., 2010) that might contribute to MS-induced alterations in aggressive (Veenema et al., 2006; Veenema and Neumann, 2009) and other social behaviors, like sexual behaviors or social cognition.

Overall, in chapter 4, I demonstrated that exposure to MS interferes with the developmental changes in V1aR binding in specific forebrain regions and contribute to the behavioral alterations found upon MS. However, whether these changes in receptor binding correspond with changes in AVP neurotransmission is not known. Therefore, it is necessary to examine to which extent the MS-induced alterations in local neuropeptide receptor binding are associated with changes in dynamic patterns of local neuropeptide release (Landgraf and Neumann, 2004; Neumann, 2008).

As discussed earlier, adult male MS rats were able to perform social recognition after an IEI of 30 min, but not after 60 min. In chapter 3, I observed that adult male MS rats failed to show a rise in septal AVP release during the acquisition of social memory that was formerly observed in non-stressed rats, suggesting a link between the blunted septal AVP release and MS-induced social recognition impairments. This finding further suggests that basal septal AVP release, which did not differ between MS and control rats, is sufficient for successful social discrimination at short intervals. This corresponds with the demonstration that a total lack of AVP in AVP-deficient male Brattleboro rats (Engelmann and Landgraf, 1994) or

blockade of septal V1aR in male Wistar rats (Dantzer et al., 1988) impaired social recognition at the short 30-min interval. As forced swimming induced a rise in septal AVP release in both control and MS rats the blunted response in septal AVP release after social stimulation strongly indicates stimulus-specificity of the impaired release. Moreover, this demonstrates that the blunted AVP release after social stimulation is not due to a reduced availability of AVP itself. Interestingly, the AVP release during social discrimination in juvenile male MS rats is unchanged compared to untreated controls. This indicates that the deficit in septal AVP release after social stimulation develops during aging and may be connected to the developmental changes observed in V1aR binding after MS.

In order to causally link the blunted septal AVP release with the MS-induced impairments in social recognition, I restored extracellular AVP release by retrodialysis of synthetic AVP into the lateral septum of adult MS rats. Thereby, I was able to reestablish social recognition in adult MS rats at the 60-min interval. This strongly suggests that the rise in septal AVP release observed during social memory acquisition is critical for successful recognition after a time period longer than 30 min. This is consistent with the finding that administration of AVP into the lateral septum prolongs social recognition memory in adult male rats for up to 2 h (Dantzer et al., 1988) or even 24 h (Landgraf et al., 2003).

5. Translational aspects of brain neuropeptides in social behaviors

As reviewed in the introduction, OT and AVP are involved in human social behaviors as well and elicit distinct effects on these behaviors when applied intranasally. I outlined in the

discussion that OT and AVP have the potential to restore environmentally-induced social deficits. Thus, it may be of interest to discuss the involvement of alterations in OT and AVP activity in human psychopathologies classified by deficits in social interactions, such as ASD and social anxiety disorder.

Association studies on several ethnic groups link polymorphisms in the OT receptor gene with ASD (Wu et al., 2005; Jacob et al., 2007; Lerer et al., 2007). Furthermore, another study was able to detect epigenetic modifications within the OTR promoter region of ASD patients, indicating altered levels of OT receptor expression (Gregory et al., 2009)

Implications for the involvement of the AVP system in autism are mainly based on several gene association studies that found a link between polymorphisms in the V1aR gene and ASD (Kim et al., 2002; Wassink et al., 2004; Yirmiya et al., 2006). Overall, association studies support, to some extent, the link between ASD and OTR as well as V1aR genes.

Another approach in this context may be to investigate alterations in the central and peripheral availability of OT and AVP that has been discussed to contribute to the development of social deficits in autism (Hammock and Young, 2006; Carter, 2007; Harony and Wagner, 2010). However, this kind of research is mainly based on OT so far. For example, plasma OT concentrations, which may reflect at least partly central OT system activity, are dysregulated in individuals with ASD or social anxiety disorder (Green et al., 2001; Hoge et al., 2008; Lane, 2009). Various studies indicate the potential use of OT applied intravenously or intranasally in the treatment of ASD. Thus, OT reduces repetitive behaviors in patients with Asperger's syndrome or autism (Hollander et al., 2002) and promotes prosocial behaviors in high-functioning autism (Andari et al., 2010). Moreover, OT improves affective speech comprehension in adults (Hollander et al., 2007) and emotion recognition in

youths with ASD (Guastella et al., 2010b). Therefore, OT was used as an adjunct to exposure therapy (Guastella et al., 2009) and attenuates amygdala reactivity to fear (Labuschagne et al., 2010) in social anxiety disorder. My finding in rats that administration of synthetic OT was able to prevent social avoidance, further supports the use of intranasal OT as potential treatment of social deficits in humans.

Beside these studies, using intranasal OT administration in patients and healthy subjects demonstrate that non-invasive intranasal administration is an adequate tool to investigate the effects of neuropeptides on clinical aspects of social behaviors, emotionality and neuronal activation patterns (for review see Heinrichs and Domes, 2008; Meyer-Lindenberg, 2008). The effects of intranasal NPS have not been tested in humans so far, although several human studies suggested an involvement of the brain NPS system in anxiety disorders (Laitinen et al., 2004; Domschke et al., 2010; Donner et al., 2010; Raczka et al., 2010). For example, a single nucleotide polymorphism in the human NPS receptor has been associated with an increased risk for panic disorder and over-interpretation of fear (Raczka et al., 2010), and the Ile^{107} receptor variant is associated with enhanced fear acquisition (Domschke et al., 2010). Intriguingly, this receptor variant has a 10-fold higher potency for NPS (Reinscheid et al., 2005). Although this seems to argue against a potential therapeutic effect of NPS in humans, studies performed in rat models for hyper-anxiety and depression-like behavior demonstrated that NPS exerts an acute anxiolytic effect even in such rodents (Slattery et al., 2008; Wegener et al., in press). Therefore, the nasal route of application may be promising to investigate the efficacy of nasal NPS in rodent models.

In chapter 5, I demonstrated for the first time that nasally applied NPS improved non-social memory and non-social anxiety in rats. Thus, nasal NPS mimicked the behavioral effects that were already shown after central administration of NPS in rats and mice (Xu et al., 2004; Okamura et al., 2011; Wegener et al., in press). In contrast to nasal application, sc administration of NPS at the same dose and time point was without any behavioral effect. Sc NPS needs to cross the blood brain barrier, which restricts neuropeptide transport in relevant amounts (Ermisch et al., 1985). The finding that behavioral effects of NPS were observed after nasal, but not sc, administration, is in line with the hypothesis that nasally applied NPS, at least in rats, reaches neuronal structures of the brain in significant amounts by bypassing the bloodstream and directly entering the CNS (Born et al., 2002).

Nasal NPS prolonged object memory at a ten-fold lower dose than needed for its anxiolytic effects. A possible explanation for that may be that different brain regions involved in memory and anxiety-related behavior vary in their accessibility by nasal NPS. It is also possible that different behavioral effects, i.e. memory-enhancing and anxiolytic effects require different doses of NPS. A follow up study investigating the behavioral efficacy of nasal NPS after blockade of central NPS receptors could be a possible approach to confirm that nasally applied NPS acts in the CNS.

Based on our finding that nasal NPS exerts both anxiolytic and memory enhancing effects, human studies are essentially needed to test the efficacy of intranasally applied NPS on anxiety, fear and stress responses, or memory tasks in healthy volunteers and relevant patient groups.

6. Concluding remarks

Psychopathologies like autism, schizophrenia, and social anxiety disorder that involve various forms of social deficits like social anxiety and reduced social cognition, gain more and more relevance. Unfortunately, the neurobiological underpinnings of these disorders are mainly unknown. Rodent and human studies suggest an involvement of highly conserved central neuropeptides like OT and AVP, in many mammalian social behaviors. However, as the investigation of neurobiological mechanisms in humans is limited, it is of main interest to further investigate the involvement of central neuropeptide systems in social deficits in rodent models.

In the present thesis, I could provide further evidence for the a prominent role of OT and AVP in basic social behaviors, i.e. social approach and social recognition in male rats, that are prerequisites for diverse complex social behaviors like aggression, sexual behavior, and pair bonding.

Naturally, male rats and mice demonstrate a preference for social stimuli over non-social stimuli. In this thesis, I demonstrated that social preference is dependent on endogenous OT. The pro-social effect of endogenous OT does not seem to be linked to a decrease in general anxiety and is, therefore, specific to social approach behavior. However, I failed to localize this pro-social effect of endogenous OT in one of the amygdala subnuclei, as it may be suggested by several human studies. Despite its endogenous effects during social preference, synthetic central OT was ineffective in further increasing natural social preference. OT seems to reverse social deficits only in rats with social impairments without influencing social preference in normal rats. Therefore, animal models with reduced

sociability may be an attractive opportunity to further investigate this issue. Furthermore, I could neither observe an involvement of endogenous AVP, nor endogenous and synthetic NPS in social preference behavior of male Wistar rats, although both regulate non-social anxiety, indicating that social anxiety might be regulated differently than non-social anxiety in rodents. However, at least in the case of AVP, this needs further investigation. Overall, I provided first evidence for the involvement of OT in social approach behavior and affiliation towards a same-sex social stimulus in rodents, which is thereby not linked to reproductive behavior.

Concerning the involvement of AVP in rodent social cognition, I demonstrated endogenous AVP release into the lateral septum of juvenile and adult male rats accompanying social memory acquisition during social discrimination, not only supporting the involvement of septal AVP in juvenile and adult male social recognition, but also providing first evidence that septal AVP gets released during a non-aggressive encounter. This further indicates the involvement of both AVP and social recognition in more complex social behaviors, e.g. intermale aggression, and in the evaluation of different social stimuli.

I also demonstrated that social stressors, like acute social defeat and MS, significantly impaired social preference and social recognition. Social defeat caused social avoidance in male rats when tested in the social preference paradigm. However, this effect was only visible when the former aggressor was used as the social stimulus, indicating an individual rather than a general involvement of social avoidance. Furthermore, I reversed this social defeat-induced impairment in social approach by central administration of synthetic OT.

Since localization of this OT-mediated rescue effect in the amygdala was not possible, further investigation is needed to localize the pro-social effects of OT in brain regions upstream of the amygdala, for instance.

Moreover, I demonstrated that MS impaired social recognition of adult male, but not of juvenile or female rats. These findings propose that social abilities, like individual social recognition, are shaped early in life and are, at least to some extent, influenced gender-specific by environmental factors like maternal care. Furthermore, MS interferes with the normal development of V1aR binding in specific forebrain regions, like the lateral septum and the piriform cortex. The alterations in V1aR binding likely contribute to the behavioral alterations found upon MS. Especially the MS-induced alterations in V1aR binding in the lateral septum might contribute to the MS-induced alterations in social recognition. As most of the effects did not result in permanent changes in receptor binding, these changes may rather have influenced central release patterns of AVP during development. By measuring AVP release during social recognition in adult male MS rats, I found a social stimulus specific blunted AVP release in response to the initial social stimulus during social recognition. As this impairment of septal AVP release was not observed in juvenile MS rats, it again indicates developmental effects of MS on the male AVP system. Finally, the impairment in social recognition at adult age were causally linked to the blunted AVP release during social memory acquisition, as administration of synthetic AVP enhanced social recognition abilities of adult MS rats to the level of unstressed rats. These findings strongly imply a prominent role of deficient central AVP signaling in rodent social cognition deficits that may be translated to human research as well.

Moreover, synthetic OT and AVP demonstrated therapeutic potential concerning social deficits induced by socially stressful experiences. Together, these findings may support the progress of clinical studies investigating human psychopathologies characterized by social interaction deficits like ASD.

Finally, I demonstrated that neither endogenous nor synthetic NPS is involved in social anxiety behavior and social memory processing. This indicates that the anxiolytic and memory enhancing effects of NPS are specific to a non-social context. Additionally, I showed for the first time that nasally applied NPS in male Wistar rats completely mimicked the non-social anxiolytic and memory enhancing effects of its central administration. As this finding confirms the effectiveness of non-invasive nasal application, this should strongly encourage potential clinical trials using nasal NPS administration as promising therapeutic agent in the context of panic and anxiety disorders.

In conclusion, this thesis significantly adds to the knowledge concerning the involvement of the neuropeptides OT, AVP, and NPS in social preference and social recognition in male rats and reveals several formerly unknown neurobiological underpinnings of stress-induced impairments in these behaviors.

Summary in German

Deutsche Zusammenfassung

Psychologische Erkrankungen, die soziale Angst und Defizite sozialer Kognition mit einschließen, nehmen in der heutigen Gesellschaft einen immer größer werdenden Stellenwert ein. Beispiele hierfür sind Schizophrenie, soziale Angsterkrankungen und Autismus, um nur einige zu nennen. Die neurobiologischen Ursachen dieser sozialen Defizite sind jedoch noch weitgehend unbekannt. Klinische Studien, sowohl an Patienten als auch an gesunden Freiwilligen, schreiben Störungen in zentralen Neuropeptid-Systemen eine wichtige Rolle dabei zu. Außerdem konnte bereits gezeigt werden, dass sich die intranasale Verabreichung der Neuropeptide Oxytocin und Vasopressin positiv auf verschiedene Aspekte sozialer Kognition, wie z.B. der Erkennung von Gesichtern, auswirkt. Ebenso wurde der Einfluss dieser Neuropeptide auf soziale Verhaltensweisen bei Nager beschrieben. Aufgrund der hohen evolutionären Konservierung der Neuropeptide innerhalb aller Wirbeltierklassen, speziell der Säugetiere, sind Belege zum Einfluss von Neuropeptiden auf das Verhalten niedriger Säuger besonders gut auf die spätere humane Anwendung übertragbar. Das Ziel dieser Arbeit war es, das Wissen über den Einfluss der Neuropeptide Oxytocin, Vasopressin und des erst kürzlich entdeckten Neuropeptids S auf das Sozialverhalten von Nagern zu ergänzen und mit Hilfe sozialer Stressmodelle soziale Defizite zu erzeugen, die mit Störungen zentraler Neuropeptid-Systeme in Verbindung gebracht werden können. Desweiteren wurde das Potential dieser Neuropeptide bei der Behandlung dieser sozialen Defizite getestet.

In Kapitel 2 und 3 dieser Arbeit habe ich den Einfluss von OT und AVP auf zwei grundlegende Verhaltensweisen sozialer Nager untersucht: Die Präferenz für soziale Stimuli gegenüber nicht-sozialer Stimuli und die Fähigkeit zur individuellen sozialen Wiedererkennung, der Einfachheit halber im Folgenden soziale Präferenz und soziales Gedächtnis genannt.

Bezüglich der sozialen Präferenz konnte ich zeigen, dass die Blockierung des endogenen Oxytocinsystems von männlichen Ratten zur Meidung des sozialen Stimulus und im Falle von Mäusen sogar zum totalen Verlust der sozialen Präferenz führt. Weder durch intraventrikuläre Infusion eines spezifischen Vasopressin 1a Rezeptor Antagonisten noch durch die Induktion nicht-sozialer Angst durch Pentylentetrazol war es möglich die soziale Präferenz der Ratten zu beeinflussen. Dies spricht für die Spezifität der durch das endogene Oxytocin vermittelten Effekte auf die soziale Präferenz männlicher Wistarratten. Desweiteren konnte ich zeigen, dass eine akute soziale Niederlage bei männlichen Ratten zur Reduktion der sozialen Präferenz führt. Da die Untersuchungszeit, die die Ratten nach sozialer Unterlegenheit mit dem sozialen Stimulus verbringen, sogar die Untersuchungszeit des nicht-sozialen Stimulus unterschreitet, kann man annehmen, dass soziale Angst erzeugt wurde. Mittels intraventrikulärer Infusion synthetischen Oxytocins, war es mir allerdings möglich, die soziale Präferenz in diesen Tieren wiederherzustellen. Interessanterweise führte die Infusion von synthetischem Oxytocin in nicht-gestressten Ratten nicht zu einer Erhöhung der sozialen Präferenz, was wahrscheinlich auf die bereits sehr hohe soziale Präferenz von Wistarratten zurückzuführen ist. Demnach ist es nicht auszuschließen, dass das Sozialverhalten von Versuchstieren mit geringerer sozialer Präferenz mittels OT beeinflussbar wäre. Lokalisationsstudien haben gezeigt, dass der prosoziale Effekt von Oxytocin nicht unmittelbar über die Amygdala vermittelt werden kann.

In Kapitel 3 und 4 wurden männliche Wistarratten während den ersten zwei Lebenswochen für jeweils 3 Stunden täglich von der Mutter getrennt. Dieses Stressmodel, auch maternale Separation (MS) genannt, wurde bereits mehrmals in Zusammenhang mit Veränderungen

emotionaler und sozialer Verhaltensweisen in juvenilen und adulten Ratten gebracht. Nach der MS-Prozedur wurden sowohl Veränderungen in der sozialen Gedächtnisleistung als auch im zentralen Vasopressinsystem bei juvenilen und adulten Tieren untersucht. Dabei konnte ich zunächst zeigen, dass adulte MS-Ratten Gedächtnisdefizite aufweisen, wenn sie 60 Min nach dem ersten Kontakt mit einem juvenilen Stimulus konfrontiert werden. Das soziale Gedächtnis juveniler MS-Ratten dagegen weißt keine Veränderungen gegenüber den jeweiligen Kontrolltieren auf. Außerdem war es mir möglich Veränderungen im Bindungsverhalten der Vasopressin-1a-Rezeptoren in für das Sozialverhalten relevanten Gehirnregionen, wie dem lateralen Septum und dem piriformen Kortex, festzustellen. Diese Veränderungen waren jedoch meist altersabhängig, d.h. in einem veränderten Entwicklungsmuster während der Entwicklung von der juvenilen und adoleszenten zur adulten Ratte zu beobachten. Desweiteren konnte ich eine reduzierte septale Vasopressinausschüttung während der Ausbildung des sozialen Gedächtnisses adulter männlicher MS-Ratten beobachten. Durch die septale Gabe von synthetischem Vasopressin während der sozialen Gedächtnisbildung, war es mir möglich das soziale Gedächtnis in adulten MS-Ratten wieder herzustellen. Dies bestätigte einen kausalen Zusammenhang zwischen den Defiziten im zentralen Vasopressinsystem postnatal gestresster Ratten und ihren Defiziten im sozialen Gedächtnis.

Schließlich war es mir möglich, erstmals nachzuweisen, dass weder exogenes noch endogenes Neuropeptid S die soziale Präferenz oder das soziale Gedächtnis männlicher Wistarratten beeinflusst. Dies war ein überraschendes Ergebnis, da zentral verabreichtes Neuropeptid S eine deutliche Verringerung nicht-sozialen Angstverhaltens und eine Verbesserung des nicht-sozialen Objektgedächtnisses zur Folge hatte. Desweitern konnte ich

zeigen, dass nasal verabreichtes Neuropeptid S, die angstlösenden und gedächtnisverbessernden Effekte, die bei zentraler Verabreichung auftreten, imitiert.

Zusammenfassend konnte ich in der vorliegenden Dissertation das Wissen über den Einfluss der Neuropeptide Oxytocin, Vasopressin und NPS bezüglich der sozialen Präferenz und des sozialen Gedächtnisses männlicher Wistarratten erweitern und es war mir möglich, einige der zugrundeliegenden neurobiologischen Mechanismen sozialer Defizite aufzuklären.

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Abbreviations

AVP	arginine- vasopressin
ANOVA	analysis of variance
CNS	central nervous system
EPM	elevated plus-maze
icv	intracerebroventricular
ip	intraperitonal
HPA	hypothalamic-pituitary-adrenal
NPS	neuropeptide S
NPS-A	neuropeptide S receptor antagonist
mRNA	messenger ribonucleic acid
OT	oxytocin
OTR	oxytocin receptor
OTR-A	oxytocin receptor antagonist
PTZ	pentylenetetrazol
PVN	paraventricular nucleus
sc	subcutaneous
SEM	standard error of the mean
SON	supraoptic nucleus
V1aR	vasopressin 1a receptor
V1aR-A	vasopressin 1a receptor antagonist

Acknowledgements

Acknowledgements - Danksagung

An erster Stelle möchte ich mich bei Prof. Dr. Inga Neumann bedanken, die es mir ermöglichte diese Arbeit an ihrem Lehrstuhl durchzuführen, aber vor allem war sie jederzeit dazu bereit sich Zeit zu nehmen laufende Projekte zu diskutieren um sowohl mit ihrem erfahrenem Rat als auch stets ehrlicher, konstruktivier Kritik zum Erfolg dieser Arbeit beizutragen.

Besonderer Dank gilt Prof. Dr. Alexa Veenema, die mir vor allem während der Anfangszeit meiner Promotion, und natürlich auch darüber hinaus, in allen Belangen geduldig und freundlich mit Rat und Tat zur Seite stand und damit wohl einen sehr prägenden Einfluß auf meine wissenschaftlich Denk- und Arbeitsweise hatte.

Desweiteren möchte ich mich bei Gabi Schindler, Rodrique Maloumby, Martina Fuchs und Andrea Havasi für ihre immer zuverlässige und kompetente Hilfe bei der Durchführung der OPs, der Versuche, der Histologie und der Rezeptorautoradiographie bedanken.

Besonderer Dank gilt außerdem meinen Kollegen Iulia Toth und Remco Bredewold die mit ihrem Einsatz bei zahlreichen Experimenten und wissenschaftlicher Diskussion eine unersetzliche Hilfe waren.

Ich möchte mich außerdem bei Birgit Müller, Björn von Rhein, Sahab Arinrat, Franziska Koller and Thomas Stolz für ihre großartige experimentelle Unterstützung im Rahmen der Durchführung ihrer Bachelorarbeiten bedanken. Auch ich hab sehr viel dabei gelernt.

Natürlich möchte ich mich auch bei den „senior scientist“ Dr. David Slattery, Dr. Daniela Beiderbeck, Dr. Stefan Reber, Dr. Erwin van den Burg und Dr. Oliver Bosch bedanken, die wann immer Hilfe von Nöten war ein offenes Ohr für mich hatten.

Außerdem bedanke ich mich bei allen Mitgliedern des Lehrstuhls Neumann für die immer gute Atmosphäre am Lehrstuhl. Besonders danke ich natürlich meinen treuen Zimmerkollegen Katharina Hillerer, Iulia Toth und Ben Jurek

Ich bedanke mich bei Prof. Dr. Rainer Landgraf für die Durchführung der zahlreichen Vasopressin Bestimmungen und bei Prof. Dr. Maurice Manning für die zur Verfügung Stellung der Oxytocin und Vasopressin Antagonisten.

Der allergrößte Dank gebührt natürlich meiner Familie bei der ich in allen Belangen und Lebenslagen stets Unterstützung und Verständnis fand.

CV and publications

Curriculum vitae

Dipl. biol. Michael Lukas

Schäffnerstraße 21, 93047 Regensburg

Personal details:

December the 27th, 1980 in Roth, Germany

unmarried, no children

1991 - 2000

Secondary school, Gymnasium Hilpoltstein, Bavaria, Germany

2000 - 2001

civilian service: nursing practice (Caritas Sozialstation, Hilpoltstein, Germany)

Scientific education:

2007 to date

PhD student in neurobiology in Prof. Neumann's group at the University of Regensburg

2006 - 2007

MSc (Diplom) student in biology in Prof. Neumann's group at the University of Regensburg (Diploma/Master thesis: "Early life stress-induced changes in the central vasopressin system: Effects on social memory in Wistar rats")

(grade: 1.0)

2004 - 2007

Graduate studies in biology at the University of Regensburg with focus on Zoology/Neurobiology (grade: 1.3), Cellbiology (grade: 1.0) and med. Microbiology (grade: 1.3)

2001 - 2004

Undergratuate studies in biology at the University of Regensburg, Germany

Publications:

Lukas M, Toth I, Reber SO, Slattery DA, Veenema AH, Neumann ID (2011) The neuropeptide oxytocin facilitates pro-social behavior and prevents social avoidance in rodents. Neuropsychopharmacology. [Epub ahead of print] doi: 10.1038/npp.2011.95

Lukas M, Bredewold R, Landgraf R, Neumann ID, Veenema AH (2011) Early life stress impairs social recognition due to a blunted response of vasopressin release within the septum of adult male rats. *Psychoneuroendocrinology* doi:10.1016/j.psyneuen.2010.11.007

Veenema AH, Beiderbeck DI, Lukas M, Neumann ID (2010) Distinct correlations of vasopressin release within the lateral septum and the bed nucleus of the stria terminalis with the display of intermale aggression. *Hormones and Behavior 58:273-281.* doi:10.1016/j.yhbeh.2010.03.006

Lukas M, Bredewold R, Neumann ID, Veenema AH (2010) Maternal separation interferes with developmental changes in brain vasopressin and oxytocin receptor binding in male rats. *Neuropharmacology 58(1):78-87.* doi:10.1016/j.neuropharm.2009.06.020

Lukas M and Neumann ID; Nasal application of neuropeptide S prolongs object, but not social memory, and reduces general anxiety. Neuropharmacology; In revision