Oxytocin: the neurochemical mediator of social life

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OXYTOCIN: THE NEUROCHEMICAL MEDIATOR OF SOCIAL LIFE

A pharmaco-behavioral and neurobiological study in male rats

Federica Calcagnoli

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AH	anterior hypothalamus
ALT	attack latency time
AN	accessory nucleus
AVP	vasopressin
AVPR	vasopressin receptor
BNST	bed nucleus of the stria terminalis
CeA	central amygdala
CSF	cerebrospinal fluid
DA	dopamine
DR	dorsal raphe
$D_{1\ (2\ or\ 3)}$-	dopamine receptor type 1 (2 or 3)
E_2	estradiol
5-HIAA	five-hydroxyindoleacetic acid
5-HT	five-hydroxy-tryptamine (serotonin)
$5\text{-}HT_{1A\ (1B)}$	five-hydroxy-tryptamine receptor type 1A (1B)
GABA	gamma (γ) aminobutyric acid
$GABA_A$	gamma (γ) aminobutyric acid receptor type A
GPCR	G protein-coupled receptor
HAB	high anxiety-related behavior
ICV	intracerebroventricular
IGR	intergenic region
IU	international unit
LAB	low anxiety-related behavior
LS	lateral septum
mRNA	messenger RNA (ribonucleic acid)
NAcc	nucleus accumbens
NP	neurophysin I
OXT	oxytocin
OXTR	oxytocin receptor
PP	partner preference
PVN	paraventricular nucleus
RI	resident-intruder
SON	supraoptic nucleus
SP	signal protein
$V_{1A\ (1B\ or\ 2)}$	vasopressin receptor type 1A (1B or 2)
VMH	ventromedial hypothalamus
VTA	ventral tegmental area
WTG	wild-type Groningen

"Men ought to know that from the brain, and from the brain only, arise our pleasures, joys, laughter, and jests, as well as our sorrows, pains, griefs and tears"

Hippocrates, "The sacred disease"

1

INTRODUCTION AND SYNTHESIS

Federica Calcagnoli

OXYTOCIN AS A NEUROPEPTIDE

Not long ago, oxytocin (OXT) was largely thought to be confined to its hormonal role in female reproduction. Its name, originally derived from the Greek words "quick birth", referred in fact, to its uterotonic activity (Dale, 1906) and facilitating action on milk-ejection (Ott and Scott, 1910). The groundbreaking discovery that changed the prevailing view of an exclusively peripheral neuroendocrine action of OXT was the induction of maternal behavior in virgin rats after its intracerebroventricular (icv) administration (Pedersen and Prange, 1979). Subsequently, the role of OXT in mate social recognition and pair bonding in prairie voles (Carter, 1998; Williams et al., 1994), and social recognition in mice (Ferguson et al., 2000) were reported. Hence, its role in social behavior became target of a large number of research projects.

Over the past decades, among many species, including humans, the central neuropeptidergic action of OXT has been demonstrated in a wide variety of social behaviors as summarized in Figure 1.

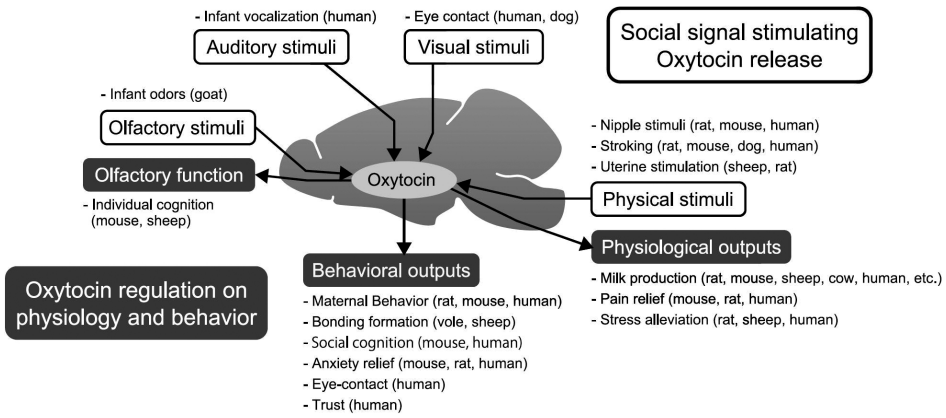

Figure 1. Summary of the social signals stimulating oxytocin release and of the behavioral and physiological oxytocin-induced effects (Nagasawa et al., 2012).

OXT is composed of nine amino acids (Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Leu–Gly-NH_2) with a disulfide bridge between the cysteines 1 and 6. This results in a peptide constituted of a rigid N-terminal cyclic 6-residue ring structure and a flexible COOH-terminal alfa-amidated three-residue tail (Figure 2) (Gimpl and Fahrenholz, 2001; Lee et al., 2009a; Tom and Assinder, 2010). OXT is the first peptide hormone to have its structure determined (Du Vigneaud et al., 1953) and to be chemically synthesized in a biologically active form (Du Vigneaud et al., 1954). The structure of the gene was elucidated in 1984 (Ivell and Richter, 1984), and the sequence of the OXT receptor (OXTR) gene was reported in 1992 (Kimura et al., 1992). The structure of OXT is very similar to another nonapeptide, entitled vasopressin

(AVP), which differs from OXT by only two amino acids in position 3 and 8 (Figure 2). OXT and AVP are both ancient neuropeptides which are evolutionary well conserved across phyla, and they have been found in species ranging from invertebrates to mammals (Caldwell and Young, 2006; Donaldson and Young, 2008). In all placental mammals examined to date, the amino acid sequence of OXT is identical, suggesting a strong selective pressure to withstand sequence variation (Lee et al., 2011). In the mouse, rat and human genomes, the OXT gene is located in the same chromosome as AVP, separated by an intergenic region (IGR) and in opposite transcriptional direction (Figure 2). Both genes are composed of three exons (shown as small solid arrows in Figure 2) separated by two introns.

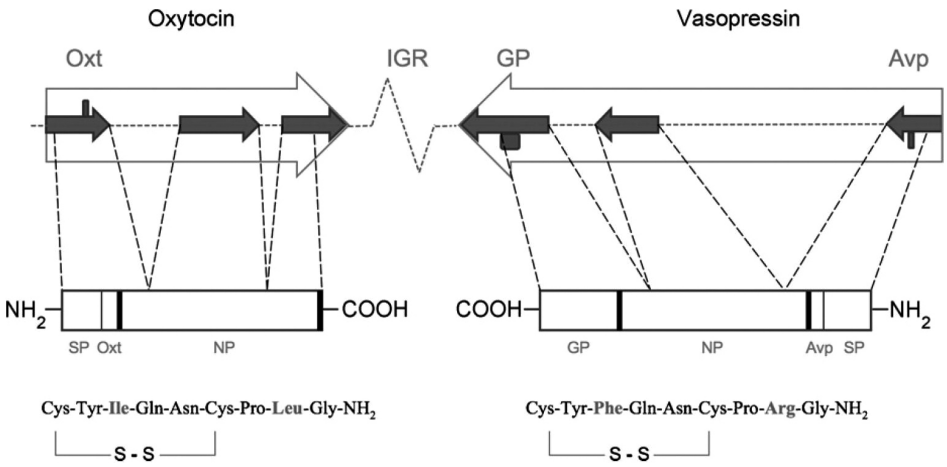

Figure 2. Schematic diagram of the oxytocin and vasopressin genes (large arrows), preprohormones (boxes), and neuropeptides (bottom) (Caldwell et al., 2008).

Oxytocin and its receptor in the brain

In mammals, OXT is primarily synthetized and expressed in the magnocellular neurons of the paraventricular (PVN), supraoptic (SON) and accessory (AN) nuclei of the hypothalamus (Gimpl and Fahrenholz, 2001; Landgraf and Neumann, 2004; Lee et al., 2009a; Sofroniew, 1983; Swanson and Sawchenko, 1983). Lower amounts of OXT are generated in parvocellular neurons of the PVN and, depending on species, the bed nucleus of the stria terminalis (BNST), medial preoptic area, as well as the lateral amygdala for release within the brain (Young and Gainer, 2003). OXT is synthesized as preprohormone and assembled in ribosomes at the level of the soma. The OXT precursor is then subsequently processed in neurosecretory vesicles and undergoes several post-translational processes such as phosphorylation, glycosylation or acetylation, which lead to the three final products: OXT, neurophysin I (NP) and a signal protein (SP) (Figure 2, left side) (Caldwell et al., 2008; Gimpl and Fahrenholz, 2001).

After this maturation process, the nonapeptide is transported via large neurosecretory axons to the posterior hypothalamus. Subsequently, it is moved to the posterior pituitary where it is stored in the Herring bodies, the terminal ends of the axons from the hypothalamus. In response to certain stimuli (Figure 1), OXT is released into the bloodstream from the axon terminals in the pituitary to exert its peripheral neuroendocrine effects (Gimpl and Fahrenholz, 2001; Insel, 2010).

In addition, from the hypothalamus OXT reaches various brain regions via volume diffusion and direct axonal transmission (Ludwig and Leng, 2006; MacDonald and MacDonald, 2010; McEwen, 2004) (Figure 3). For instance, parvocellular neurons of the PVN and AN project to the spinal cord and other parts of the brain such as the lateral septum (LS), the amygdala, the hippocampus, several nuclei of the hypothalamus, as well as diverse autonomic centers in the brainstem (Gimpl and Fahrenholz, 2001; Insel et al., 1999; Ishak et al., 2011; Landgraf and Neumann, 2004) (Figure 3). Moreover, although less extensively, magnocellular neurons have recently been found to project their axonal collaterals to structures (i.e., including the horizontal limb of the diagonal band of Broca, nucleus accumbens (NAcc), the central amygdala (CeA), LS, and ventral hippocampus) (Knobloch et al., 2012).

Therefore, besides acting as a peripheral hormone, OXT is a potent neuromodulator targeting the brain areas expressing its receptor, in particular within the so called "social behavior network". The "social behavior network" is a neuronal circuit implicated, across all vertebrates, in the control of multiple forms of social behavior (Goodson, 2005; Newman, 1999). These include aggression, appetitive and consummatory sexual behavior, various forms of social communication, social recognition, affiliation, bonding, parental behavior and response to social stressors (Ferguson et al., 2002; Gammie and Nelson, 2001; Kirkpatrick et al., 1994; Kollack-Walker and Newman, 1995). The social brain, originally described as a network of six nodes bidirectionally connected (Coolen and Wood, 1998; Dong and Swanson, 2004; Risold and Swanson, 1997), is largely widespread

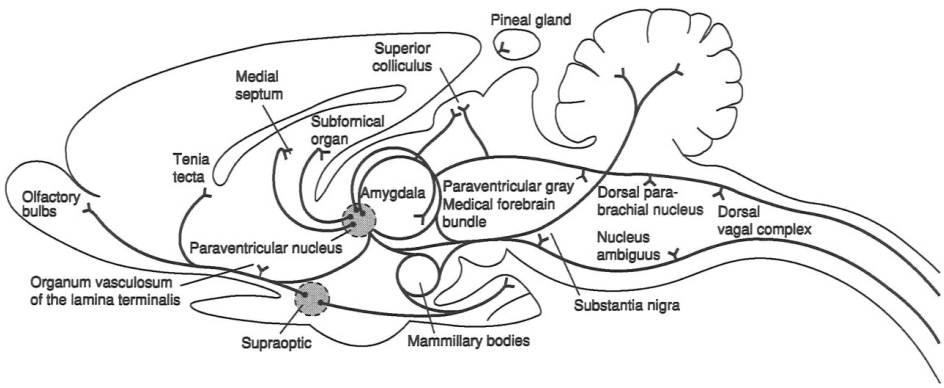

Figure 3. Major OXTergic pathways in the central nervous system of the rat and their sites of origin (McEwen, 2004).

within the central nervous system and includes regions where OXTergic connections and binding sites have been described.

OXT is currently known to have only one receptor, which belongs to the rhodopsin-type (class I) G protein-coupled receptor (GPCR) superfamily (Caldwell et al., 2008). These receptors are characterized by seven putative transmembrane domains, three extracellular and three intracellular loops (Gimpl and Fahrenholz, 2001; Young and Gainer, 2003). Agonists binding to GPCRs lead to receptor activation, phosphorylation, and translocation of beta-arrestin to the receptor complex. This last event, mediated by protein Gq11, disrupts the receptor/G protein interaction and turns off G-protein dependent signaling (Stoop, 2012). The OXTR can be coupled to different G proteins, leading to different intracellular pathways (Figure 4). It is possible that these various signaling pathways are differentially expressed in neuronal versus peripheral tissues.

The distribution of OXTR expression within the central nervous system is such that many brain regions are affected by this nonapeptide. In a number of species, including rat (De Kloet et al., 1985a; Freund-Mercier et al., 1987; Veinante and Freund-Mercier, 1997), mouse (Insel et al., 1991), vole (Insel and Shapiro, 1992), and human (Loup et al., 1991; Loup et

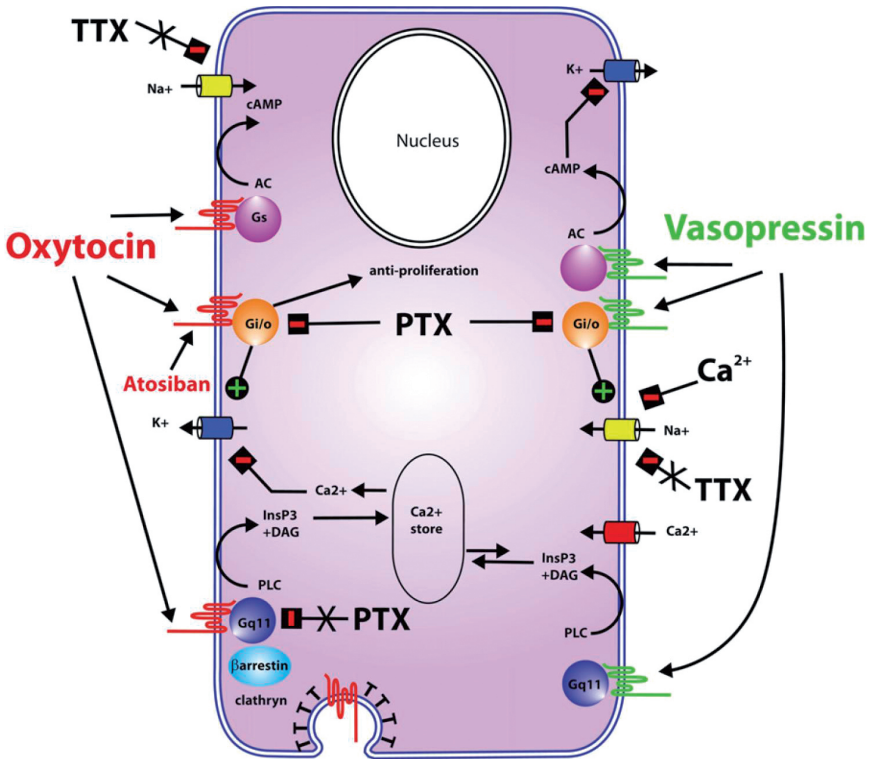

Figure 4. Different intracellular signaling pathways induced by the binding of oxytocin to the receptor, depending on the specific G proteins activated (Stoop, 2012).

al., 1989), this distribution has been studied using receptor autoradiography, an imaging technique that assesses the receptor location. In rodents, OXTR is especially prominent in the olfactory bulb and tubercle, neocortex, endopiriform cortex, hippocampal formation (especially subiculum), CeA and lateral amygdala, BNST, NAcc, ventromedial hypothalamus (VMH) and dorsal raphe (DR) (Insel et al., 1991; Veinante and Freund-Mercier, 1997; Yoshida et al., 2009). In humans, expression is prominent in the basal nucleus of Meynert, the nucleus of the vertical limb of the diagonal band of Broca, the ventral part of the LS, the preoptic/anterior hypothalamic area, the posterior hypothalamic area, the substantia nigra pars compacta, and the substantia gelatinosae of the caudal spinal trigeminal nucleus and of the dorsal horn of the upper spinal cord, as well as in the medio-dorsal region of the nucleus of the solitary tract (Loup et al., 1991; Loup et al., 1989; Zink and Meyer-Lindenberg, 2012).

Central functions of oxytocin

In mammals, the enhancement of brain OXT levels promotes affiliative and attachment behaviors (Insel, 2010; Lukas et al., 2011; Neumann, 2009), facilitates parental behavior (Atzil et al., 2012; Naber et al., 2010; Riem et al., 2011), social recognition and memory between conspecifics (Bielsky and Young, 2004; Ferguson et al., 2001; Ferguson et al., 2002; Gabor et al., 2012), but also emotional bonding between animals and caregivers (Coulon et al., 2013; Nagasawa et al., 2009). This pro-social "tend and befriend"-like action has conferred to OXT the title of "the peptide that binds" (MacDonald and MacDonald, 2010).

OXT, which has been baptized by the media as the "love hormone", is crucially involved in the neurobiology of intimacy facilitating sexual behavior (Argiolas and Melis, 2004; Gil et al., 2011; Melis et al., 2009), romantic attachment (Schneiderman et al., 2012), pair bonding and social preference for the partner over a novel companion (Liu and Wang, 2003; Williams et al., 1992; Williams et al., 1994; Young et al., 2011). In humans, it has been also shown to increase trust (Baumgartner et al., 2008; Kosfeld et al., 2005; Theodoridou et al., 2009; Zak et al., 2005) and generosity (Barraza et al., 2011; Barraza and Zak, 2009; Zak et al., 2007), to strengthen affective and cognitive empathy (Barraza and Zak, 2009; Domes et al., 2007b), as well as to reduce socio-anxiety and fear-related behavior (Ditzen et al., 2009; Domes et al., 2007a; Heinrichs et al., 2001; Neumann, 2007).

In line with these findings, disrupted social behavior profiles have been associated with lowered central endogenous OXTergic activity. In humans, at the age of 3 and 6 months, low level of cerebrospinal fluid (CSF) OXT corresponded with low soothability and social attention seeking (Clark et al., 2013). Low CSF and plasma OXT levels have also been correlated with high frequency of aggressive episodes in conduct disorder boys (Lee et al., 2009b). Moreover, higher OXT-reactive auto-antibodies were found in both conduct disorder subjects and prisoners although not fully clarified how this may influence the concentration of the neuropeptide in the brain (Fetissov et al., 2006). Two studies in human adults have shown that CSF OXT levels are diminished after childhood abuse and are negatively correlated with suicidal (auto-aggressive) behavior (Heim et al., 2009; Jokinen et al., 2012). Polymorphism-related alterations of OXTergic neurotransmission

have been associated with higher levels of anger and faster retaliation following betrayals in trust (Tabak et al., 2013), lower affective and cognitive empathy, higher physiological stress reactivity (Rodrigues et al., 2009), as well as lower beneficial effects of social support (Chen et al., 2011). Specific polymorphisms of the OXTR gene have also been associated with extreme, persistent and pervasive childhood-onset aggressive behaviors and with a higher intensity and frequency of expressed anger, aggression, and disruptive behaviors in men (Beitchman et al., 2012; Johansson et al., 2012a; Johansson et al., 2012b; Malik et al., 2012). In particular, genetic studies have shown that two common single nucleotide polymorphism variants in the OXTR gene are associated with individual variability in social behavior. The first variant, rs2254298, is associated with autism spectrum disorders (Jacob et al., 2007) and unipolar depression (Costa et al., 2009). The second variant, rs53676, is associated with decrease in psychological resources, such as optimism and self-esteem (Saphire-Bernstein et al., 2011), non-verbal intelligence (Lucht et al., 2009), behavioral and dispositional empathy (Rodrigues et al., 2009), positive affect (Lucht et al., 2009), and parental sensitivity (Bakermans-Kranenburg and van Ijzendoorn, 2008). DNA methylation of the OXTR genes decreases the transcriptional activity of the gene and high levels of methylation have been associated with autism spectrum disorders and psychopathy (Dadds et al., 2014; Jack et al., 2012).

In animals, strain- and species-comparisons have associated lower OXT immunoreactive cells in the hypothalamic nuclei or lower OXTR density in the areas of the mesolimbic system with solitary behavior, absence of partner preference, lower biparental and cooperative care, social cohesion and attachment (Insel and Shapiro, 1992; Kalamatianos et al., 2010; Olazabal and Young, 2006; Ross et al., 2009; Snowdon et al., 2010). Aberrant social behaviors displayed in young peer-reared rhesus monkeys have been associated with a lower CSF OXT level over the course of development as compared to maternally reared controls (Winslow et al., 2003). Diminished OXTR binding in various rat brain regions has been associated with impaired social functioning after poor social rearing conditions (Ahern and Young, 2009) or, and this has been shown in several species, after early life stress (Lukas et al., 2010). Depletion of OXTergic signaling via genetic alteration of the OXT gene or its receptor has resulted in persuasive social deficits, social amnesia, defects in lactation and maternal nurturing, and reduced infant ultrasonic vocalizations in response to social isolation, but normal parturition and sexual behavior (Ferguson et al., 2000; Lazzari et al., 2013; Lee et al., 2008; Nishimori et al., 1996; Sala et al., 2013; Takayanagi et al., 2005; Winslow et al., 2000; Winslow and Insel, 2002).

In summary, OXT is a neuromodulator involved in controlling and promoting a wide range of social behaviors, both in animals and in humans. Deficits in OXTergic signaling are associated with disrupted social behavior. However, the linkage OXT-behavior appears less clear when considering **aggressive behavior**, especially in animal studies. Evidence of both anti-aggressive and pro-aggressive properties of OXT treatment can be found in the literature, depending on species, strain, gender, hormonal, emotional, and social state of the experimental subjects, as well as upon the type of aggression analyzed.

Aim of this thesis was to elucidate the putative role of the central OXTergic system in the regulation and expression of aggressive behavior using male wild-type Groningen (WTG) rats challenged in a social context. This research combined the use of pharmacological and behavioral tools, with immunocytochemistry, receptor autoradiography and *in situ* hybridization techniques.

In **chapters 2** and **3**, the aim of the studies was to explore the acute and long-lasting behavioral effects on offensive aggression induced by acute or chronic icv infusion of different doses of synthetic OXT or a selective OXTR antagonist. Receptor specificity was assessed by pharmacologically blocking OXTR binding before the infusion of the agonist ligand. In line with the pro-social "tend and befriend"-like action described in human research, the results clearly revealed that both acute and chronic OXT infusion was able to reduce the expression of offensiveness in resident rats, and to facilitate the display of explorative interactions towards an unfamiliar intruder. Interestingly, chronic icv OXT infusion induced enduring behavioral effects that persisted 7 days after treatment cessation.

The findings of consistent central OXT-induced behavioral effects prompted to investigate the possible brain sites of action. Hence, **chapter 4** describes the behavioral effects of pharmacological manipulations selectively targeting the OXTergic system in the CeA and in the DR nucleus of male WTG rats. These brain sites were chosen because they are densely populated with OXT immunoreactive terminals and binding sites (Vaccari et al., 1998; Veinante and Freund-Mercier, 1997; Yoshida et al., 2009). Moreover, both regions are amply involved in the regulation of behavioral responses to agonistic encounters (Pan et al., 2010; Takahashi and Miczek, 2013). The results of this study point at the CeA as relevant node where enhancement of OXT level may result into a behavioral shift from offensive towards more socially explorative response in male rats.

Aim of **chapter 5** was to test the hypothesis whether the individual variation in basal aggression and in response to OXT treatment is related to individual differences in the endogenous OXTergic system. This hypothesis was based on the observation of greater OXT-induced anti-aggressive effects in animals characterized by higher baseline levels of aggression, whereas a trend in increasing offensiveness was reported after blocking the OXTRs in the least aggressive WTG rats and in the innately docile strain of Wistar rats (Figure 6A and B). Using receptor autoradiography and *in situ* hybridization techniques, individual variation in central OXTergic activity was indeed found to be linked to the expression of offensive behavior. Excessive levels and abnormal forms of aggression were associated with lower hypothalamic OXT availability (OXT mRNA), but with higher OXTR binding in the areas of CeA and BNST.

Finally, the translational value of the anti-aggressive and pro-social explorative effects found after icv OXT manipulation was elucidated by applying the nonapeptide intranasally, similarly to the potential clinical use and route of administration (**chapter 6**). Remarkably, both acute and repeated intranasal OXT applications selectively and potently reduced aggressive display, concomitantly with facilitating social exploration, and pair bonding. No unspecific change in the autonomic activity was observed after intranasal OXT

application. Moreover, by employing immunostaining of the neuronal activation marker Fos, activation of the PVN and SON OXTergic system was demonstrated after intranasal OXT application. Although the precise route and mechanisms of nose-to-brain transport and/or communication remain to be elucidated, the fact that exogenous OXT given nasally is able to self-stimulate its own endogenous hypothalamic system is a relevant finding to further investigate how and where changes of neuronal activity in the OXTergic system translate into changes in behavioral expression.

In summary, the studies presented in this thesis revealed that via either icv infusion (**chapters 2** and **3**), intranasal application (**chapter 6**), or local microinjection into the CeA (**chapter 4**), OXT significantly reduced intermale offensive aggression in adult WTG rats tested in a resident-intruder paradigm. These serenic effects have been observed after both acute (**chapters 2** and **6**) and chronic (**chapters 3** and **6**) pharmacological manipulation. Long-lasting effects have been found after cessation of continuous icv infusion (**chapter 3**), but not after repeated intranasal application (**chapter 6**). The reduction of aggression occurred simultaneously with the increase of social exploration, with both effects being blocked by the use of a selective OXTR antagonist prior to the synthetic OXT. In addition, extremely high levels and pathological forms of aggression have been associated with a potential low endogenous brain OXTergic activity, in support of clinical studies where violent behavior in humans has been associated with low brain OXT level.

VARIABLES MODULATING THE CENTRAL ROLE OF OXYTOCIN ON AGGRESSION

The results of the experiments summarized above show consistent OXT-induced anti-aggressive effects. However, over the years, contradictory behavioral evidence in literature gave rise to conflicting hypotheses on the regulating role of OXT in aggression. OXT and OXTRs are found throughout the social behavior neuronal network in a great variety of mammals, including humans. Considering the diversity existing between individuals in the way how they express evolutionarily and socially relevant behaviors, like aggression, one might expect that the characteristics and functioning of the central OXTergic system can vary depending on species, strains, sex, developmental influence, hormonal state, and social experience. Some of these inter- and intra-individual variables will be discussed below.

Types of aggression

Although all studies described in this thesis have focused on intermale offensiveness, aggression is anything but a unitary form of behavior. There are several forms of aggression such as maternal aggression, territorial aggression, predatory aggression, irritable aggression, etc., each with its own eliciting stimuli, neuroanatomical topography and neurochemical characteristics (Vitiello and Stoff, 1997). Hence, part of the confusion about the role of OXT in aggression originates from an unjustified generalization of results obtained from a specific

form of aggressive behavior towards general effects on aggression. This is nicely exemplified in lactating rats, in which OXT increases attacks towards intruders but simultaneously inhibits aggression directed towards the pups (Debiec, 2005; Pedersen, 2004).

The aggression displayed towards an intruder can also have different biological values, underlying neuropeptidergic mechanisms, and attack topography. The intermale aggression displayed by a territorial resident towards an intruder, despite the harmless behavior and smaller size of the intruder itself, is defined as an offensive type of aggression. The resident aims at ensuring supremacy on another male with regard to access to females and food. This is a proactive and rewarding form of aggression, with attacks and bites directed to the back and flanks of the intruder (Blanchard and Blanchard, 1977; Blanchard et al. 1977).

This type of aggression, defined also as "a challenge over adaptively important resources", differs from the defensive aggression that consists of "attacks in defense of the subject's own bodily integrity" and in response to an attack by another individual (Blanchard et al., 2003; Koolhass et al., 2013). Maternal aggression is an example of defensive type of aggression in which the mother aims defending the offspring and herself. It is therefore a reactive form of aggression evoked by the presence of a threat (Vitiello and Stoff, 1997). Defensive attacks are targeted at snout/head of the intruder (Blanchard and Blanchard, 1977; Blanchard et al. 1977).

Interestingly, OXT seems to modulate these two forms of aggression in opposite ways. In line with several other preclinical etho-pharmacological studies in male prairie voles and primates (Silakov et al., 1992; Winslow et al., 1993b), the data presented in this thesis consistently show that exogenous increase of brain OXT levels via icv infusion (**chapters 2** and **3**), intranasal application (**chapter 6**) or microinjection into the CeA (**chapter 4**) results in decreased offensive behavior in male resident rats. Although contrasting findings, for instance in dominant monkeys and in sexually experienced montane prairie voles (Winslow and Insel, 1991; Winslow et al., 1993b), this anti-aggressive function of brain OXT has been also supported by behavioral genetic studies showing increased offensiveness in mice lacking the ability of synthesizing OXT or its receptor (DeVries et al., 1997; Sala et al., 2011; Winslow et al., 2000).

On the other hand, several studies in hamsters and rats have shown that maternal aggression is enhanced by central infusion of the nonapeptide, and it is moreover correlated with elevated endogenous OXT release (Ferris et al., 1992; Neumann, 2002, 2003). Especially in dams bred for high anxiety, the level of OXT in the PVN and in the CeA correlates with the level of aggressive behavior displayed during the maternal defense test (Bosch et al., 2005), and high levels of OXT in the PVN (Blume et al., 2008; Waldherr and Neumann, 2007) and/or in the CeA are known to have anxiolytic properties and to reduce fear-induced freezing behavior (Knobloch et al., 2012; Viviani et al., 2011).

However, due to differences in manipulating methodologies and/or targeted brain regions, the relationship between brain OXT level and maternal aggression remains equivocal as other studies have found no interaction (Factor et al., 1992; Neumann et al., 2001) or even negative relationship between the two (Consiglio et al., 2005; Lubin et al., 2003).

In addition, evidence of OXT-induced increase of defensive aggression has been recently reported in clinical studies. In fact, heightened self-reported hostility has been found among mothers 5 days after parturition (Ledesma Jimeno et al., 1988; Mastrogiacomo et al., 1982), suggesting that maternal defense may indeed extend to humans. In line with the findings in lactating dams, increased aggression in breastfeeding women has been associated with lowered stress reactivity (Hahn-Holbrook et al., 2011) and heightened OXT levels during lactation (Light et al., 2000).

Another example of OXT-induced defensive aggression has been found in men (De Dreu et al., 2010). In a series of experiments (De Dreu et al., 2012; De Dreu, 2011, 2012; De Dreu et al., 2011), intranasal OXT has been reported to significantly increase non-cooperation only when likelihood of exploitation by the out-group was high, leading the authors to conclude that OXT stimulates humans to aggress against out-group threat in order to protect their in-group.

In conclusion, depending upon the biological value and the evolutionary roots of the various types of aggression, OXT may exert either anti- or pro-aggressive behavioral effects. Moreover, based on the context, valence of the stimulus, as well as the individual's traits, OXT can modulate the rewarding appetite to dominate or the perceived magnitude of the threat, by desensitizing or potentiating brain alerting function (Campbell and Hausmann, 2013).

Individual variation among and within species

There are significant differences among species and strains in the distribution of OXTRs (Insel et al., 1993; Kalamatianos et al., 2010) and some of these appear to explain the different patterns in social aggression and the differences in the behavioral responses to exogenous OXT treatment.

Comparison studies using voles of the genus *Microtus* have revealed that a high OXTR density in the prelimbic cortex, BNST, NAcc, midline nuclei of the thalamus, and the lateral amygdala characterizes prairie (*Microtus ochrogaster*) and pine (*Microtus pinetorum*) voles. These vole species typically form long-term monogamous relationships, show high levels of parental care and high territoriality, which usually correlates with high reproductive attachment and offensive displays towards unfamiliar adults (Insel and Shapiro, 1992). In contrast, low OXTR density has been reported in montane (*Microtus montanus*) and meadow (*Microtus pennsylvanicus)* voles that are typically polygamous, minimally parental, and live usually in isolated burrows, spending little time in contact with conspecifics (Insel and Shapiro, 1992). To note, relatively few behavioral effects have been reported after OXT treatment in the montane voles, except an increase in grooming and aggression following high icv OXT dose (500 ng). Prairie voles appear, instead, sensitive to lower doses of OXT, showing a similar increase in grooming following 5 ng and a decrease, instead of an increase, in aggression at icv doses from 5 to 500 ng (Insel et al., 1993).

Associations between the OXTergic system and differences in social structure have also been investigated within the South American tuco-tuco rodents of the genus *Ctenomys*

(Beery et al., 2008). While both male and female Patagonian tuco-tucos (*C. haigi*) are solitary, with each adult occupying its own burrow system (Lacey et al., 1998), the colonial tuco-tucos (*C. sociabilis*) lives in groups, with burrow systems occupied by up to six closely related adult females and a single unrelated adult male (Lacey et al., 1997; Lacey and Wieczorek, 2004). Almost all adult colonial tuco-tuco males (> 94%) share a burrow system with one or more females, whom they aggressively defend against other males (Lacey and Wieczorek, 2004). In *C. sociabilis* OXTR binding was found significantly greater in the piriform cortex, the thalamus and the CeA as compared to *C. haigi* (Beery et al., 2008). This interspecific difference may be particularly relevant, given that high OXTR binding in the central and lateral amygdala has been reported also in monogamous voles and in the most aggressive WTG rats (**chapter 5**), and that in all these species, adult males do not share burrows with other males and respond rather aggressively towards intruders to defend the territory and the female companions (Beery et al., 2008; Insel and Shapiro, 1992).

Differences in mean and distribution of offensive behavior have been consistently found between the wild type strains of animals and laboratory domesticated animals. Probably because of the absence of natural or artificial selection, domestication or breeding, the most common laboratory strains of animals lack the subgroup of highly aggressive animals, whereas the subgroup of low-to-medium aggressive individuals is similarly present among all laboratory rat strains (de Boer et al., 2003). A clear example of these inter-strain behavioral differences can be found when comparing the distribution curve of the individual aggression scores between male WTG rats and male Wistar rats (de Boer et al., 2003) (Figure 5).

In addition to this phenotypic variability in intermale aggression, a different response to pharmacological OXTergic manipulation has been found between the extremes in the population when centrally infusing the same dose of the same selective OXTR antagonist 10 min prior to the resident-intruder test (Figure 6). While no overall treatment effect was found in WTG rats (Figure 6A) (except for a trend in increasing aggression in the least aggressive rats (**chapter 2**)), a significant pro-aggressive effect was induced in Wistar male residents {overall treatment effect [$F_{3, 63} = 2.82$, $p < 0.05$, $\eta^2 = 0.12$]. OXTR antagonist $_{7.5\ \mu g}$ vs. vehicle ($p < 0.05$) and OXTR antagonist $_{15\ \mu g}$ vs. vehicle ($p < 0.05$)}. This effect occurred following an inverted U-shaped dose-effect relationship (Figure 6B), and it was not moderated by the baseline level of aggression. Moreover, it occurred concomitantly with a decrease in social exploration {overall treatment effect [$F_{3, 63} = 9.35$, $p < 0.001$, $\eta^2 = 0.31$]. OXTR antagonist $_{3.75\ \mu g}$ vs. vehicle ($p = 0.001$), OXTR antagonist $_{7.5\ \mu g}$ vs. vehicle ($p < 0.001$), and OXTR antagonist $_{15\ \mu g}$ vs. vehicle ($p = 0.001$)} (Calcagnoli et al., unpublished).

Considering the higher OXT mRNA expression found in less aggressive male WTG rats as compared to the most aggressive ones (**chapter 5**), it is tempting to speculate a higher OXTergic activity in the Wistar male rats as compared to the male WTG rats. This would be in line with several gene knockout studies in mice and with the hypo-OXTergic syndrome associated with neuropsychiatric disorders in humans. In particular, increased aggression is observed in mice that lack the ability to synthesize OXT or its receptor (Lee et al., 2008; Ragnauth et al., 2005; Sala et al., 2011; Takayanagi et al., 2005; Winslow et al., 2000). Similarly, aggression,

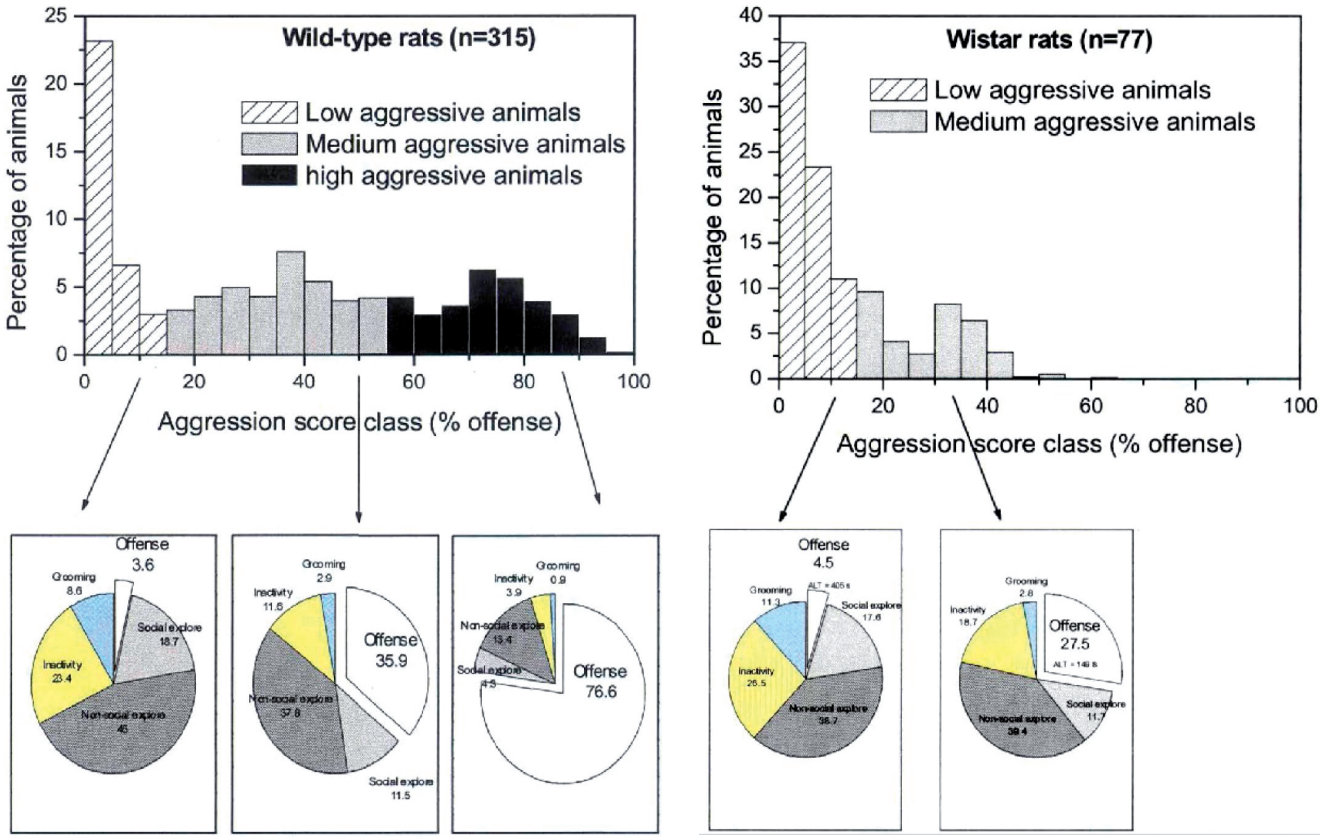

Figure 5. Offensive aggression score distribution of male wild-type Groningen rats (left upper panel) and male Wistar rats (right upper panel) and the respective ethograms of the low-, medium- and high-aggression groups (lower panels). Note the absence of high aggressive individuals in the Wistar strain as compared to the wild-type strain (de Boer et al., 2003).

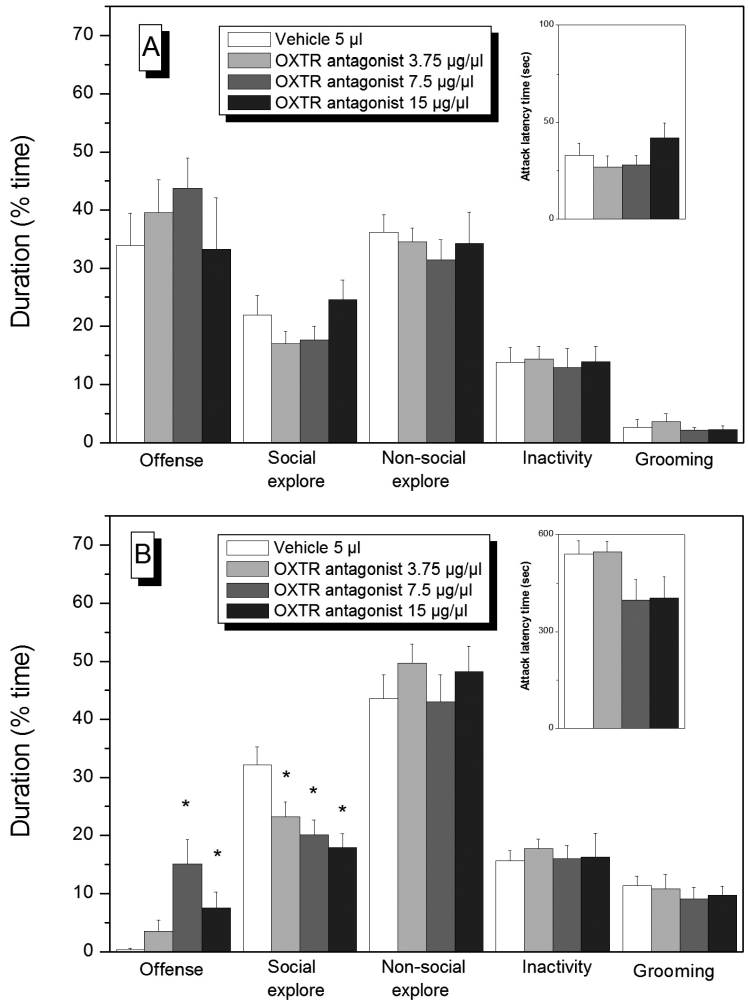

Figure 6. Behavioral changes induced by pharmacological manipulation of the central OXTergic system. Male resident wild-type Groningen (N = 12) (A) and Wistar rats (N = 22) (B) were exposed to an unfamiliar male intruder after acute icv administration of vehicle or a selective non-peptidergic oxytocin receptor (OXTR) antagonist, L368.899, at the doses of 3.75 µg, 7.5 µg, and 15 µg/5 µl. Insert graph depicts the treatment effects on attack latency time. Data are presented as group mean + SEM.

impulsivity and antisocial behaviors are associated with low central and peripheral OXT levels in antisocial personality disordered subjects (Fetissov et al., 2006; Jokinen et al., 2012; Lee et al., 2009b), or with OXTR deficiency in socially impaired individuals with autism (Gregory et al., 2009; Gurrieri and Neri, 2009; Jacob et al., 2007; Lerer et al., 2008; Wermter et al., 2010; Wu et al., 2012), or with loss of function OXTR gene variants (Malik et al., 2012).

In conclusion, the diversity in OXTergic substrates among individuals is likely to be responsible for the diversity of OXT-induced behavioral profiles. Studies that focus on

revealing the neuroanatomical differences between and within species appear a valuable tool to unravel candidate brain regions in which OXTergic activity may be important for the maintenance of specific patterns of sociality.

Sex, hormal state, emotional trait, and social experience

Animal studies indicate that sex-specific differences in response to OXT are common (Bales and Carter, 2003; Bales et al., 2007; Cho et al., 1999; Williams et al., 1994). Moreover, the histological structure of the OXTergic system is sexually dimorphic (de Vries et al., 2008), suggesting that sex steroids play a role in its morphogenesis and functioning.

In several brain regions, such as amygdala and hypothalamus, estrogens up-regulate OXT, OXTR production (Choleris et al., 2008; Patisaul et al., 2003; Windle et al., 2006), and OXTR binding (de Kloet et al., 1986; De Kloet et al., 1985b), whereas testosterone promotes both OXTR binding in the hypothalamus (Johnson et al., 1991), as well as production of AVP (Delville et al., 1996), which has many opponent actions to OXT (Neumann and Landgraf, 2012). Testosterone has been described to act following its conversion to estradiol (E_2) (Johson et al., 1991). In fact, administration of the aromatase inhibitor, androstatrienedione, in male rats mimicked the effects of castration in reducing the amount of OXTRs present in VMH and in the islands of Calleja (Tribollet et al., 1990). Interestingly, about 5% of testosterone undergoes 5α-reduction to form the more potent androgen, di-hydrotestosterone, which cannot be converted in E_2 and rather reduces the E_2-induced increase in OXTR mRNA and binding in rats (Bale and Dorsa, 1995). However, contrary to rats, castrated mice repeatedly exposed to pups have been found to show increased OXT immunoreactive neurons in the PVN and increased parenting behavior (Okabe et al., 2013).

The relation between gonadal hormones fluctuation, regulation of brain OXTR expression, and changes in aggressive behavior has been mainly studied in females during pregnancy and in males establishing dominance. During peripartum, the intensity of maternal aggression in rats changes dramatically likely due to hormonal fluctuations. It first peaks the day before parturition, drops immediately after parturition, and then increases to a maximum in the early lactation phase around day 4 to 7, and disappears at weaning (Caughey et al., 2011). Simultaneously, the brain OXTergic activity changes. At parturition, OXTR expression in rodents has been found to be elevated in the VMH and in the CeA as compared to during gestation (Bale et al., 1995). Binding is subsequently reduced during lactation in the VMH, but remains elevated in the CeA (Bale et al., 1995). Altogether, these findings suggest a potential role of OXTRs activation in the onset and regulation of peripartum maternal aggression in female rodents (Ferris et al., 1992; Pedersen et al., 1994; Rosenblatt et al., 1988).

On the other hand, in human males (Gossen et al., 2012), male squirrel monkeys (Winslow and Insel, 1991) and male rats (Postina et al., 1996) high social status is associated with increased agonistic behavior and elevated testosterone. Male rodents with high trait-level of aggression have been found to have high testosterone production and sensitivity (Compaan et al., 1992). Recently, in male resident WTG rats trained for aggression, excessive levels and abnormal forms of intermale offense were found to be

negatively correlated with the hypothalamic availability of OXT, but positively associated with the OXTR binding in regions, such as CeA and BNST (**chapter 5**). Similarly, in humans, testosterone seems to have opposite behavioral effects on the pro-social impact classically associated with OXT, decreasing trust, generosity, cooperation and empathy (Bos et al., 2010; van Honk and Schutter, 2007; Zak et al., 2009).

However, to complicate this picture, social experiences such as mating, and innate emotional traits such as anxiety may modulate the relation between OXTergic activity, gonadal hormones and aggressive display. In fact, in dominant squirrel monkeys, which have up to 50 times higher plasma testosterone level as compared to subordinates, OXT infused into the cerebral ventricles increases the offensive aggression, especially during mating season (Winslow and Insel, 1991). Similarly, OXT increases territorial aggression in sexually experienced male monogamous prairie voles, but not in sexually naïve males (Winslow et al., 1993b), nor in polygamous montane voles (Insel et al., 1995). In general, more self-orientated and self-rewarding choices in sexual and reproductive behaviors have been associated with OXT-induced increase of plasma testosterone in humans (Aron et al., 2005; Curtis and Wang, 2005), chimpanzees (Aragona et al., 2003) and other mammals (Aragona et al., 2003). Similarly, female aggression has been found to be dependent upon the estrous cycle of the resident in sexually experienced female resident rats (Ho et al., 2001), but not in sexually naïve female residents (de Jong et al., 2014).

Over the years, quite some effort has been spent to the attempt of linking aggression with anxiety. Aggression in males has mainly been coupled with low anxiety levels (Beiderbeck et al., 2007; Kantor et al., 2000; Neumann et al., 2010; Nyberg et al., 2003; Veenema et al., 2007). Similarly in females, higher levels of maternal aggression in mice are accompanied by lower anxiety (Maestripieri and D'Amato, 1991). However, in adolescent female rats selectively bred for high anxiety-related behavior, aggression is positively correlated with their innate level of anxiety (de Jong et al., 2014). This association disappears in adulthood (de Jong et al., 2014), but it is found back in lactating high anxious dams (Bosch, 2011).

These findings suggest a difference in the neurological underpinning of aggressive behavior in relation to innate anxiety and a difference in the biological meaning of aggression (non-maternal female aggression vs. maternal aggression). In fact, high anxious dams are known to over-express AVP due to a AVP promotor polymorphism as compared to low anxious dams (Murgatroyd et al., 2004). Moreover, adolescent female aggression is associated with reduced neuronal activity in the PVN, specifically in local OXTergic neurons, while in high anxious dams the level of aggressive behavior displayed during the maternal defense test correlates with the endogenous OXT release in the PVN and in the CeA (Bosch et al., 2005). Consequently, differences are also found in the behavioral response to pharmacological OXT manipulation. Central infusion of OXT reduces aggression in low anxious, but not in high anxious adolescent female rats (de Jong et al., 2014), while it increases maternal aggression in high anxious dams (Bosch et al., 2005).

In humans, anxiety disorders (especially social phobia) are often co-morbid with behavioral problems including aggression, especially during adolescence (Hodgins et al.,

2011). This link appears to be even stronger in girls compared to boys (Lehto-Salo et al., 2009). Interestingly, a recent study has shown that intranasal OXT diminishes the hostility typically expressed by highly state anxious women in a competitive aggression game (Campbell and Hausmann, 2013).

Altogether, these studies indicate that the functional role of endogenous brain OXT in aggression is most likely modulated by intrapersonal factors and individual traits, such as social status and anxiety, which may alter the individual's threshold to detect and respond to stimuli (Cisler and Koster, 2010). The knowledge of this individual-based variability might help optimizing the therapeutic use of exogenous OXT.

Methodology-biased effects, compensatory effects and interactive mechanisms

Another relevant aspect to consider is the great impact that the chosen methodological tool may have on the conclusions of one's research. For instance, as discussed in this thesis, a 7-day period of synthetic OXT administration was found to have long-lasting behavioral effects when using chronic icv infusion (**chapter 3**), but not when using intranasal application (**chapter 6**). Even more clear examples of methodology-induced discordant associations between OXT/OXTR gene and the expression of aggression/social deficits can be found scrutinizing the studies that employed knockout techniques.

Dhakar and colleagues have indeed shown that, compared to the controls, intermale aggression was elevated in mice in which the OXTR gene was depleted from the time of conception ($OXTR^{-/-}$), but not in mice with a specific predominant forebrain knockout ($OXTR^{FB/FB}$), in which the OXTR gene was not excised until approximately 21–28 days postnatally (Dhakar et al., 2012). Possible reasons for these differences may relate to the spatial or temporal differences in diminished OXTR expression between total and conditional knockout mice lines (e.g. different temporal onset and magnitude of binding loss in the forebrain region).

Similarly, after characterizing homozygous OXTR null mice ($OXTR^{-/-}$) as having pervasive social deficits, impaired cognitive flexibility, and increased aggression (Sala et al., 2011; Takayanagi et al., 2005), Sala and colleagues refined their previous conclusions reporting that mice heterozygous for the OXTR ($OXTR^{+/-}$) show impaired social behavior but not increased aggression or cognitive inflexibility (Sala et al., 2013).

Considering that OXT has organizational effects on the development of different neuromodulatory systems, such as serotonin (5-hydroxytryptamine; 5-HT) and dopamine (DA), and neuroanatomical substrates related to aggressive behavior (Baskerville and Douglas, 2010; Eaton et al., 2012; Yoshida et al., 2009), compensatory mechanisms may occur to counterbalance the reduced brain OXTergic function. Among others, OXT-AVP interaction is definitely to be mentioned as relevant and as one of the potential reasons of the contradictory findings about the role of OXT in modulating aggression (see Box 1, page 29).

Electrophysiological studies, for instance, have suggested that OXTergic neurotransmission could still occur in OXTR knockout mice via unselected binding of AVP on OXTRs or via

BOX 1. Vasopressin: closely similar in structure, yet so different in function

Structurally, AVP differs from its related nonapeptide OXT by only two amino acids in the 3rd and 8th position (Gimpl and Fahrenholz, 2001). Besides being produced in the PVN and SON, AVP synthesizing neurons have been found in the suprachiasmatic nucleus, BNST and medial amygdala (Caldwell et al., 2008). Within the mammalian central nervous system, the synaptic actions of AVP are mediated mainly by two of the three existing receptor subtypes: V_{1A} receptor subtype, found throughout the rodent brain (Muller and Wrangham, 2004), and the V_{1B} receptor subtype apparently present only in the anterior pituitary (Striepens et al., 2013). In addition to the specific binding to its own receptors, AVP has a high affinity to bind also OXTRs (De Kloet et al., 1985a; de Kloet et al., 1986). Considering the overlapping distribution between AVPRs and OXTRs within the social behavior network (Tribollet et al., 1988; Veinante and Freund-Mercier, 1997), cross-reactivity between the two systems is likely to occur. This consequently complicates the understanding of their respective physiological and behavioral profile.

OXT actions have been suggested to be directed towards "altruistic" maintenance of the social group and/or species (e.g. ovulation, parturition, lactation, sexual behavior and social bonding), while AVP actions directed towards protecting homeostasis and "selfish" attitude of the individuals (e.g. water retention, blood pressure and temperature regulation, increased arousal, and memory) (Stoop, 2012). This opposite yin/yang action seems to apply also to the way OXT and AVP differently regulate intermale offensive behavior.

There is indeed solid evidence about the fact that elevated brain AVP levels result in increased intermale aggression. Particularly, in male rodents, microinjection of synthetic AVP into the anterior hypothalamus (AH), BNST or medial amygdala has been reported to exert remarkably pro-aggressive effects. In line, the selective blockage of V_{1A} receptors has been shown to reduce offensive display (Bester-Meredith et al., 2005; Bester-Meredith et al., 1999; Caldwell and Albers, 2004; Ferris et al., 1997).

Similarly, in humans, Coccaro and colleagues reported a positive correlation of CSF AVP concentration with a life history of non-directed general aggression as well as aggression directed towards individuals (Coccaro et al., 1998). Moreover, in men but not in women, intranasal application of AVP decreases the perception of friendliness in the faces of unfamiliar men and stimulates agonistic facial motor patterns (Thompson et al., 2006).

As described for OXT, however, the ability of AVP to stimulate aggression appears to depend on individual's characteristics and social experience. AVP injected into the AH increases intermale aggression in hamsters that had been previously trained to fight other hamsters, and in hamsters that had been socially isolated for at least four weeks, but not in hamsters that had been housed in social groups (Huhman et al., 1998). This evidence is accompanied by data showing that V_{1A} receptor binding in several sub-regions of the AH is significantly higher in socially isolated males than in males living in social groups (Albers et al., 2006). Sexually naïve male voles are essentially non-aggressive, choosing to explore intruder males instead of attacking them. However, following mating-induced pair bonding, males display high levels of offensive and defensive aggression toward conspecifics and significantly more V_{1A} receptor binding in the AH as compared to sexually naïve males (Winslow et al., 1993a).

This overall picture of the link between AVP and intermale aggression, although rather simplistic, suggests that possibly occurring unspecific binding of OXT on AVPRs and vice versa could bias the assessment of OXT- and AVP-induced behavioral effects. Co-administrated infusion of agonist and selective receptor antagonist has been a pharmacological tool used in few studies of this thesis to verify the specific involvement of OXTRs in the OXT-induced anti-aggressive effects (**chapters 2** and **4**). Increasing the selectivity of synthetic ligands can also be a strategy to lower the risk of cross-reactivity and to ensure a more conclusive value to etho-pharmacological studies.

developmental compensation (Winslow and Insel, 2002). Neuronal responses to OXT were found more sensitive in male OXT knockout mice as compared to the wild-type mice, and most of the OXT-responsive neurons were also responsive to AVP (Carter et al., 1995). Considering that OXT has only 10-fold higher affinity for OXTR as compared to AVP receptors (AVPRs) (Audigier and Barberis, 1985; Postina et al., 1996), infusion of high dose of synthetic OXT may also induce unselective binding to AVPRs. Hence, cross-reactivity between these two systems might be the reason for the not linear dose effects on behavior after OXT or OXTR antagonist treatment. For example, the pro-aggressive effects of OXTR antagonist on Wistar rats (Figure 6B) followed an inverted U-shaped dose–response curve, thus suggesting unspecific binding at high dose.

However, so far it has often been difficult to define the contribution of individual OXT/ AVP receptors to specific behaviors unambiguously because of the use of rather unselective analogues or too high doses of selective analogues. As a proof of the *in vivo* unselective binding of synthetic OXT for OXTR and AVPR subtypes (Chini and Manning, 2007), infusion of OXT has been seen to rescue social deficits at the same doses in the OXTR $^{+/-}$ and OXTR $^{-/-}$ genotypes, suggesting that the rescue in OXTR $^{-/-}$ mice is mediated by binding of OXT to AVPRs type 1A (Sala et al., 2013).

In conclusion, considering the great scientific value that preclinical research has as a mean to understand and manipulate the OXTergic system, it is of crucial relevance to choose selective tools to pharmacologically and genetically dissect the specific roles played by closely related neuropeptidergic systems, such as OXTergic and AVPergic system.

PATHOLOGY IN THE ANIMAL MODEL OF HUMAN AGGRESSION

As repeatedly discussed in this thesis, the type of aggression studied and the aggressive phenotype expressed by the chosen experimental subjects are both important factors for interpreting experimental results.

To date, most of the preclinical studies have been conducted in highly domesticated rodent species, in which the intensity and diversity of aggressive behavioral traits have been drastically constrained due to selection and breeding processes. Consequently, studies of aggression in laboratory animals often use prolonged social isolation, presentation of aversive stimuli, electrical brain stimulation, brain lesions, pharmacological agents, and genetic manipulation or inbreeding to obtain measurable levels of aggression. However, these protocols might introduce confounding factors (i.e. social stress, avoidance behavior, fear and anxiety, etc.) to the neurotypical mechanisms of aggression.

As compared to other commonly used laboratory strains of rats, in baseline condition, adult male WTG residents are characterized by: (1) a higher level of intermale offensive behavior displayed, and (2) a wider individual variation in the quantity and quality of the displayed aggression when tested towards an unfamiliar male intruder (de Boer et al., 2003; Koolhaas et al., 2013). In baseline condition, same-age adult males differ among each other

in the duration (quantity) of the displayed offensive aggression. Within the population, the duration of aggression typically ranges from zero up to 80% of the total 10 min of social encounter, in contrast to a much more narrow variation in males of docile strains, such as Wistar rats, that show a maximum of around 25% offense when tested with the same behavioral paradigm. Interestingly, some WTG residents can escalate their aggression into excessively high duration and abnormal forms when challenged with repeated winning confrontations (de Boer et al., 2003). This is in line with the multiple reports of increased aggression induced by fighting experience in male rats and house mice, as well as in male and female California mice and Syrian hamsters (Fuxjager et al., 2011; Miczek et al., 2007; Schwartzer et al., 2013; Silva et al., 2010). This extremely high and abnormal aggressive phenotype is virtually absent in the Wistar strain (de Boer et al., 2003).

Based on the aforementioned characteristics, outbred WTG rats have been chosen as an animal model to assess the behavioral effects of exogenous OXTergic manipulation, and to investigate a potential link between phenotypic individual variation in aggressive behavior and endogenous OXTergic activity.

As explained in **chapter 5**, offensive aggression is defined as "excessive" when the frequency and/or duration of the aggressive acts are out of proportion to the causes and the representative threat of the target, while "abnormal" aggression refers to a qualitative connotation for offensive display such as attack of female or anesthetized conspecifics, or of vulnerable body parts (de Boer et al., 2009; Miczek et al., 2013). In a human perspective, studies on excessive levels and abnormal forms of aggression might better represent the human definition of violence, as considered an out-of-context social response and always out of proportion to any precipitating factors that might be present.

As mentioned earlier, the anti-aggressive properties of exogenous OXT has been consistently demonstrated with an interesting moderating effect of trait aggressiveness. In particular, greater OXT-induced reduction of aggression was observed in animals with a higher baseline level of aggression. In contrast, blockage of OXTergic neurotransmission induced significant pro-aggressive effects only in the less aggressive WTG animals (**chapter 2**) and in animals innately characterized by low aggressive-trait, such as Wistar rats (Figure 6B). From these findings, the hypothesis that the different sensitivity to the treatments could be due to individual variation in the brain OXTergic activity was tested and discussed in **chapter 5**. Repeated confrontations of the resident with an unfamiliar male intruder were used to allow the potential development of excessive levels and abnormal forms of aggression. Although we cannot exclude that the differences found in the OXTergic system might represent a consequence rather than the cause of the different behavioral responses, excessive and abnormal forms of aggression have been associated with a lower OXT availability in the PVN and SON, together with a higher binding capability in specific areas, like the CeA and the BNST. This up-regulated OXTR binding capability has been speculated to be a compensatory mechanism for the potentially lower OXT transcription (lower OXT mRNA level). According to this line of reasoning, a stronger efficacy of the synthetic agonist when exogenously applied may likely be expected in

the more aggressive subjects. Interestingly, although OXT-induced serenic effects have been reported along the continuum line of aggression levels, only excessive and abnormal aggression was associated with significantly altered functioning of the brain OXTergic system. This emphasizes the relevance of working with animal models that simulate the extreme manifestations of human aggression such as pathological violence.

To further substantiate the conclusions, further experiments should verify that the reduced OXT mRNA level found within the hypothalamic nuclei of the excessively aggressive wild–type Groningen rats is indeed accompanied by a blunted release of OXT either locally within the PVN and SON or within limbic target regions. Moreover, one relatively simple experiment that could help support the proposition that the different OXT and OXTR levels are innately existing and lead to the different behaviors might be the examination of experimentally-naïve wild–type Groningen rats. In this way it would be possible to assess whether OXT mRNA and OXTR binding levels in the aggression-experienced animals fall within or outside the range of experimentally-naïve rats.

Similarly, in clinical studies, a recent and rapidly expanding body of evidence indicates that genetic differences in aspects of the functional OXTergic system (the OXTR itself and the ectoenzyme CD38, which contributes to OXT secretion) contribute to measurable features of an individual's personality (Kumsta and Heinrichs, 2013). In fact, lower OXTergic tone or polymorphically altered neurotransmission have been associated with human violence, and with unemotional and callous aggressive bursts (Dadds et al., 2014; Johansson et al., 2012b; Jokinen et al., 2012; Lee et al., 2009b; Malik et al., 2012). Though no published studies in aggression research have examined the role of genetic variation in the OXTergic system in a person's clinical response to OXT, several recent studies in normal subjects indicate that this should be a relevant aspect to consider. Subjective responses to infant's or emotional faces have been found, for instance, to be moderated by certain genetic variations in the OXTR (rs53576G, rs53576, rs2254298, rs2228485) (Marsh et al., 2012; O'Connell et al., 2012).

Aside from its importance in terms of understanding individual variability in both neurotypical and clinically disordered populations, the use of information about individual's neuropeptidergic genotype may help identify the "OXT-sensitive" phenotype and may have advantageous implications in the selection and optimization of psychiatric treatment (Macdonald, 2012).

SITES AND POTENTIAL MECHANISMS OF ACTION

Although we have obtained firm evidence of the involvement of central OXT in the modulation of intermale aggressive behavior, the next important question is where in the brain exactly OXT exerts this action.

The CeA and the DR have been chosen among the areas densely populated by OXTRs and involved in social behavior control to investigate the functional role of the OXTergic system in regulating intermale aggression of WTG rats (**chapter 4**). Based on my own data, as well as on the current preclinical and clinical literature, I will discuss below some of the potential

mechanisms that may explain the behavioral effects observed when locally manipulating the OXTergic activity. Suggestions are made in relation to possible interactions between OXT and other local neurotransmitters. In addition, attention will be focused upon the mesolimbic area and the integrating role that the OXTergic system may have with the reward system.

Central amygdala: oxytocin and γ–aminobutyric acid (GABA)

In rats, the central nucleus of the amygdala is a sub-region where OXT binding (Elands et al., 1988), OXTR expression (Yoshimura et al., 1993) and functional OXTRs (Condes-Lara et al., 1994) have been especially localized. The CeA has the highest neuropeptide levels of all nuclear divisions in the amygdala and receives input from most sensory cortices or pathways, as well as other divisions of the amygdala (Swanson and Petrovich, 1998). Moreover, morphological and functional evidence has been reported for the presence of axonal endings through which OXT, produced in the hypothalamus, can reach the CeA and be locally released to exert direct effects both at the cellular and at the behavioral level (Knobloch et al., 2012).

A considerable and long-standing body of evidence indicates that OXT can locally exert important effects on anxiety and fear responses (Roozendaal et al., 1993; Roozendaal et al., 1992), especially in the context of maternal aggression. During a maternal defense test, increased OXT release was found into the CeA of especially highly anxious Wistar dams, and the amount of local OXT release correlates with the extent of aggressive displays. These effects were reversed by local OXTR antagonist infusion in low, but not in highly anxious female rats (Bosch et al., 2005; Bosch and Neumann, 2012). In support, repeated administration of OXT into the CeA enhances aggression towards a male intruder in lactating hamsters (Ferris et al., 1992). However, there are also studies which do not support the above-mentioned role of CeA OXT in maternal aggression, and the reasons might be species, strain or hormone-dependent (see above paragraph 2.3).

Chapter 4 of this thesis shows that local infusion of synthetic OXT into the CeA exerts robust anti-aggressive effects in male WTG rats, occurring concomitantly with enhanced social exploration. These behavioral changes were similar to the ones described after icv OXT treatment in the same rat strain, suggesting that enhancing CeA OXTergic activity is sufficient to regulate the behavioral response in a social context. Interestingly, OXTergic manipulation of a more medial sub-region of the amygdala tended to induce facilitation of the social exploration, but failed to modulate aggression.

The heterogeneous morphology that characterizes the structure of the CeA might explain the region selectivity of the effects. Using autoradiography of rat brain sections, Huber and colleagues observed that OXTRs are extensively present in the lateral and capsular areas, while AVPRs are found in the medial portion of the central nucleus. In the same study, the authors found two distinct populations of neurons in the CeA: one in the lateral and capsular areas that is excited by OXTR activation, and another in the medial area that is inhibited by OXTR activation but excited by stimulating AVPRs (Huber et al., 2005).

Interestingly, activation of OXTRs in the lateral and capsular areas has been shown to inhibit the neurons of the medial area evocating rapid increase of inhibitory γ–aminobutyric

acid (GABAergic) currents (Huber et al., 2005; Knobloch et al., 2012). This effect is blocked by application of OXTR antagonist or selective $GABA_A$ receptor antagonist (Huber et al., 2005). Therefore, OXT-induced inhibition of attacks may be due to a selective inhibition of spontaneous spiking frequencies from the CeA to other effector brain regions implicated in the species-specific expression of offensive behavior (Lima et al., 2008; Siegel and Victoroff, 2009). On the other hand, OXT infusion in the more medial part of the CeA might not effectively affect the intra-amygdala system of GABAergic connection, but might rather activate AVPRs and plausibly evocate opposite responses.

Similarly, in humans the neuronal activity of the amygdala is altered by intranasal OXT, leading to changes in behavior. In men, but not in women (Lischke et al., 2012), OXT has been amply shown to facilitate social approach behaviors reducing amygdala responsiveness to social stimuli in general (Domes et al., 2007a), and decreasing amygdala reactivity to social threat in particular (Coccaro et al., 2007; Kirsch et al., 2005; Viviani et al., 2011).

The specific role of GABA receptors in aggression seems to appear counterintuitive. In clinical practice, pharmacotherapies that indirectly activate GABAergic transmission are commonly used to treat aggressive patients (Comai et al., 2012). However, benzodiazepines, which are positive allosteric modulators of $GABA_A$ receptor, alter aggressive behavior in a biphasic manner with low doses increasing attacks and threats and high doses decreasing these behaviors (de Almeida et al., 2004; Miczek et al., 2002).

Behavioral effects of GABAergic potentiation into the CeA were tested in male WTG rats by bilaterally infusing in a random crossover design either vehicle, synthetic OXT or a selective $GABA_A$ receptor agonist, muscimol (Figure 7). An overall effect was found in the category of offensive behavior [$\chi^2 = 6.94$, df = 2, $p < 0.05$], social exploration [$\chi^2 = 6.22$, df = 2, $p < 0.05$], and inactivity [$\chi^2 = 13.55$, df = 2, $p < 0.001$]. The duration of aggression was reduced by both OXT ($p < 0.05$) and muscimol ($p = 0.01$), while the social exploration was enhanced only by OXT infusion ($p = 0.01$). Moreover, muscimol-treated animals showed an increased inactivity as compared to vehicle ($p < 0.01$) (Calcagnoli et al., unpublished).

Although, the data do not exclude the possibility that GABAergic potentiation may mediate the OXT-induced anti-aggressive effects, at the selected dose, the modulatory properties of the $GABA_A$ receptor agonist appear to be less behavioral specific as compared to the nonapeptide. Therefore, follow-up studies should involve dose-dependent testing and co-administered infusion of synthetic OXT and a selective $GABA_A$ receptor antagonist. However, more conclusive evidence of the functional interplay between OXT and GABA in the CeA requires a dynamic qualitatively and quantitatively monitoring of the local release of the amino acid after OXT infusion, during the challenging intermale encounter. Using this approach, Bosch and colleagues have indeed shown that local infusion of OXTR antagonist into the CeA increased the content of GABA in dams bred for high-anxiety related-behaviors (Bosch et al., 2007), whose high aggression level is typically correlated with elevated endogenous OXT release (Bosch et al., 2005).

To overcome region heterogeneity and the rather limited targeting selectivity of pharmacological manipulations, recently developed optogenetic techniques are promising

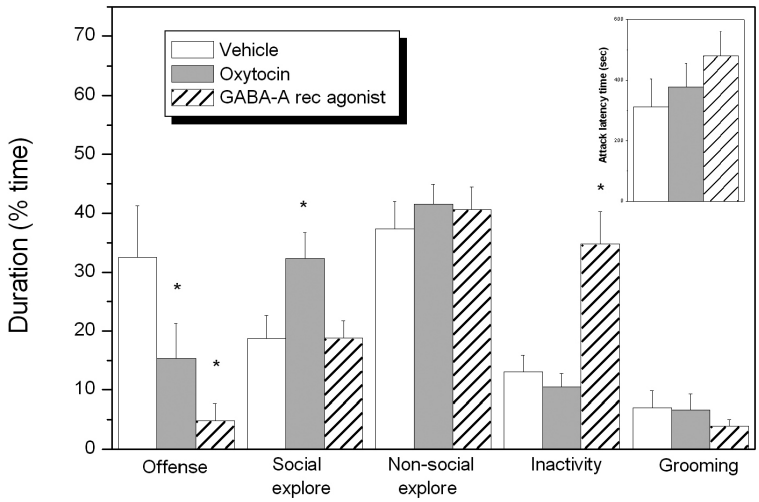

Figure 7. Behavioral changes induced by pharmacological manipulation of the OXTergic and GABAergic systems in the central amygdala. Male resident wild-type Groningen rats were exposed to an unfamiliar male intruder Wistar rat after acute bilateral administration of vehicle, synthetic oxytocin (30 ng/0.3 µl per side), or a selective $GABA_A$ receptor agonist, muscimol (0.5 µg/0.3 µl per side) into the central amygdala. Insert graph depicts the treatment effects on the attack latency time. Data are presented as mean + SEM (N = 9). *$p < 0.05$ indicates a significant difference in comparison with vehicle.

to explore the OXT-GABA interplay in vitro, and to assess in vivo the behavioral effects of evoked OXT release in the CeA with high neuronal selectivity (Knobloch et al., 2012).

Mesolimbic area: oxytocin and dopamine (DA)

Social interaction itself can act as a natural reward (Insel, 2003). Interestingly, across species, OXTRs are widely expressed in regions of the mesolimbic reward system where the DAergic system has its seeds (Insel and Shapiro, 1992). Moreover, particularly in the nuclei of the hypothalamus, main source of endogenous central OXT, DA receptors can be found on OXTergic neurons (Baskerville et al., 2009). These findings, together with a recent discovery of D_2-OXT receptor heteromers in the rat striatum (Romero-Fernandez et al., 2013), have prompted further investigation to explore possible interplay between these two neurotransmitters.

In male rats, OXT injection in the core region of this system, the ventral tegmental area (VTA) increases DA release in the NAcc, another core region of the mesolimbic DAergic system which receives projections from the VTA (Melis et al., 2007). In men, a recent study has provided evidence for an OXT-DA interaction during response to social stimuli, with the effects of endogenous central OXT secretion being strongly influenced by DA availability. OXT promotes interpersonal trust by inhibiting hostile behaviors and by enhancing the rewarding value of social encounters (Campbell, 2008). In particular, an amygdala-induced response to socially negative stimuli seems to be attenuated by OXT in

individuals with higher DA level in the prefrontal cortex, while it is enhanced in individuals with lower DA level in the prefrontal cortex (Sauer et al., 2013).

In animal models, interactions between the OXTergic and DAergic system have been investigated for a long time, mainly in the context of sexual behavior and pair bonding (Baskerville and Douglas, 2008; Liu and Wang, 2003). However, the mesolimbic reward system incorporates regions, such as NAcc and VTA, known to process stimulus salience and to regulate also aggressive behavior (Gil et al., 2013; O'Connell and Hofmann, 2011; Staffend and Meisel, 2012). Hence, it seems worthy to investigate the functional role of the OXT-DA interaction in the behavioral context of aggression, also considering that activation of the mesolimbic DAergic system itself has been shown to play a critical role in modulating aggressive behavior.

In Syrian hamsters, in which offensive aggression was enhanced by the use of anabolic androgenic steroid treatment, positive correlation was found between aggression level and changes in hypothalamic DA levels (Ricci et al., 2009), and D_2 receptors were the DA receptors subtype mediating the behavioral changes (Schwartzer and Melloni, 2010). Increases in tyrosine hydroxylase, the rate limiting enzyme in the synthesis of DA, and DA transporter mRNA levels have been found in the VTA of winner male mice compared with losers when they experienced repeated agonistic confrontations (Filipenko et al., 2001). A rise in DA levels was also reported in the NAcc of rats during and after a single aggressive episode (Ferrari et al., 2003). In a comparative study between two strains of mice, differences in the mesocorticolimbic DAergic system have been shown to contribute to differences in aggression. In particular, aggressive BALB/cJ mice exhibited lower DA utilization in the NAcc and prefrontal cortex, lower D_1 receptor expression in the rostral pole of the accumbens, and higher D_2 receptor expression throughout the accumbens as compared with the nonaggressive A/J mice (Couppis et al., 2008). Moreover, in a study comparing rats bred to be either high or low threat sensitive, DA receptor antagonist applied into the NAcc reduced aggression only in the low threat-sensitivity rats characterized by offensive/proactive coping style, but not in the high threat-sensitivity rats whose aggression seems to be more defensive/reactive (Beiderbeck et al., 2012).

In humans, lower CSF levels of homovanillic acid, a DA metabolite, have been detected in impulsively violent antisocial personality disordered subjects as compared with non-impulsively aggressive offenders (Linnoila et al., 1983). Moreover, DA D_3 receptor polymorphism has been associated with impulsiveness in violent offenders, but not in non-violent individuals (Retz et al., 2003).

In the attempt to understand the underlying mechanism of the OXT-induced behavioral changes reported in this thesis, interplay between OXT and DA should be considered, as both crucial in evaluating stimulus salience and controlling behavioral responses within the integrated social decision-making network (Bromberg-Martin et al., 2010; Insel, 2003; Sweidan et al., 1991).

In a social context such as a resident-intruder encounter, where both social exploration and establishment of dominance have motivational values, OXT and DA may together modulate the cognitive control, shift the motivational salience of the behaviors, and define the action selection.

Yet, OXT and DA might not be the only candidates required in the social reward network. A recent study in mice demonstrated that the social reinforcement signal exerted by OXT within the NAcc requires release of 5-HT through activation of presynaptic OXTRs localized on 5-HT-expressing cells on DR nucleus axon terminals within the NAcc (Dolen et al., 2013).

Dorsal raphe: oxytocin and serotonin (5-HT)

After the discovery of the substantial overlap between OXTRs and 5-HT-expressing cells in the DR of mice (Yoshida et al., 2009), this region has become of interest in my intent to localize the brain site of action of the observed OXT-induced behavioral effects.

The presence and the peculiar localization of OXTRs within the DR suggest a local functional role of endogenous OXT and invite to hypothesize an interaction between OXTergic and 5-HTergic system. Evidence for this interplay could be found when infusion of OXT into the medial part of the raphe nucleus was seen to increase the endogenous 5-HT release (Yoshida et al., 2009). In addition, a recent study has reported that OXTRs are active in stimulating the firing of DR 5-HT neurons (Spaethling et al., 2014). However, up till now, no study has directly manipulated the OXTergic system in this brain region, neither assessed the consequent behavioral effects or verified whether these are eventually mediated by 5-HT availability. Therefore, one of the studies included in this thesis (**chapter 4**) was aimed at pharmacologically manipulating the OXTergic system in the DR nucleus of WTG rats, especially targeting the sub-region populated by 5-HTergic neurons.

The DR is a structure of the brain stem that is considered to be one of the core regions involved in emotional states, in appetitive and aversive information processing, in reward-seeking behavior, in fear control and aggressive displays (Amat et al., 2005; Jacobs and Azmitia, 1992; Takahashi and Miczek, 2013; van der Vegt et al., 2003). It is also the main source of brain 5-HT neurotransmission that plays a crucial role in many impulsive types of aggressive behavior and violence in humans and other species (Caspi et al., 2009; Coccaro, 1989; Valzelli, 1981). For decades aggression research has investigated the relationship between 5-HT and aggression, and the general view is that 5-HTergic activity positively relates to normal forms of aggression, whereas there is evidence of an inverse relationship with impulsive-like violent aggression (Coccaro, 1989).

Studies previously performed on male WTG rats have shown that the basal CSF concentrations of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA), the major 5-HT metabolite, positively correlate with the trait aggression (i.e., an individual's propensity to respond aggressively) and that pharmacological treatments known to inhibit 5-HT transmission also inhibit state aggression (the actual display of aggressive behavior) (de Boer et al., 1999, 2000; Olivier et al., 1995). This suggests that acute stimulation of 5-HT neuronal activity may enhance aggression.

Hence, based on this hypothesis and on the observed 5-HT release induced by synthetic OXT infusion into the raphe of mice (Yoshida et al., 2009), local infusion of OXT into the DR of male WTG rats was expected to increase the 5-HT neuronal activity and to increase aggressive displays (**chapter 4**). Contrary to the expectation, there was only a

trend towards increasing the offensive element of keep-down. However, as discussed in **chapter 4**, several experimental limitations invite caution when interpreting the data. The study has been conducted on a relative small group size, applying only a single dose of synthetic OXT and OXTR antagonist; therefore, a potential dose-dependency effect might have been overlooked. Moreover, when trying to increase extracellular 5-HT, supposedly via OXT infusion, simultaneous compensatory inhibition of the neuronal activity might occur via 5-HT_{1A} autoreceptors.

5-HT_{1A} receptors are located on somata and dendrites in the DR, where they act as inhibitory autoreceptors (Miquel et al., 1992). 5-HT_{1A} receptors are also located postsynaptically in limbic areas acting as heteroreceptors on non-5-HTergic neurons (Barnes and Sharp, 1999). Local infusion of a highly selective 5-HT_{1A} receptor agonist that acts also as competitive antagonist at postsynaptic 5-HT_{1A} receptors has been reported to efficiently reduce inter-synaptic 5-HTergic transmission and mediate anti-aggressive action (**chapter 4**) (de Boer and Koolhaas, 2005; de Boer et al., 2000).

All those data converge on confirming the modulatory role of 5-HT into the DR in aggression, whereas the relationship between OXT and 5-HT remains still an unclear and challenging topic.

In conclusion, OXT may just be one of the key players orchestrating social behavior expression. Local pharmacological manipulation is only one possible strategy to reveal the site of action. Microdialysis technique may help to verify OXT-induced changes in local neurotransmission during aggressive behavior however, the time resolution of this technique may not be sufficient to measure the, most likely short lasting, changes in synaptic release, directly related to the display of a specific behavior. A possibility to measure OXT-induced activation of specific neurons would be to perform electrophysiological measurements, although this may be rather complex in freely moving animals, especially during direct agonistic confrontation. More innovative techniques should be employed in order to record real-time changes in neuronal firing with high spatial and time resolution. Among all neurotechniques, the recent emergence of optogenetics and pharmacogenetics technology is the current breakthrough in neuroscience and has the potential of shedding new lights on the causal relation between specific neuronal networks and behavior (Nieh et al., 2013).

ANTI-AGGRESSIVE EFFECTS: PRIMARY OR SECONDARY?

In all the pharmaco-behavioral studies described in this thesis, OXT-induced anti-aggressive effects have always occurred concomitantly with an increase of social explorative displays engaged by the resident male towards the intruder. The interdependency between these behavioral changes has opened the discussion about causality. From the data I have collected, no conclusive statement can be made whether OXT primarily reduces aggression with the consequence of enhancing social exploration, or vice versa. As discussed in **chapter 3**, OXT can either

(1) dampen the amygdala-mediated processing and responsiveness of social threat (Coccaro et al., 2007; Kirsch et al., 2005), directly reducing hostile response, and indirectly facilitating interactive engagement;
(2) or facilitate the mesolimbic-mediated processing of positive/neutral social cues, directing the social decision-making network towards explorative approaches, rather than towards the "winner effect" (Schwartzer et al., 2013);
(3) or inhibit the hypothalamus-pituitary-adrenal axis (re)activity (Neumann et al., 2000), while exerting unspecific anxiolytic effects, which lead to a less competitive and confronting behavior;
(4) or concomitantly orchestrate two or more of these hypothesized pathways.

In favor of a primarily pro-social explorative effect of OXT is the finding that in male mice the expression of social exploration appears to be particularly sensitive to even a partial reduction in *oxtr* gene expression, whereas the alteration of aggression requires complete inactivation of the *oxtr* gene (Sala et al., 2013; Sala et al., 2011). Similarly, in male WTG rats significant pro-explorative changes have been reported at a lower dose of icv OXT, as compared to the 4-fold higher dose required for anti-aggressive effects to appear (Figure 1 in **chapter 2**). Moreover, while the magnitude of the serenic effect is dependent upon the dose of OXT and the baseline aggression score, pro-social changes follows a U-inverted shape, with no significant effect at the highest dose, and independent of the baseline level of the behavior.

Another aspect that deserves consideration is the fact that the OXT-induced reduction of aggression always occurred without delaying the latency to the first attack. This behavioral profile indicates that OXT seems to act predominantly on the maintenance and/or termination aspects of aggressive behavior rather than the initiation phase. To date, most well-known serenic ligands typically modulate both the initiation, as well as the maintenance and/or termination phase of the offense, and concomitantly reduce the olfactory investigation (Blanchard et al., 2004; de Boer and Koolhaas, 2005; Takahashi et al., 2011). Hence, OXT does not seem to affect the initial processing of olfactory cues, but rather redefines the subsequent decision-making process.

A tool to advance the understanding of this putative relationship might be given by performing a detailed analysis of the constituent elements of the category of both social aggression and exploration. By using a high-speed video recordings and subsequent slow-motion analysis it could be indeed possible to reveal more precisely which behavioral element is significantly altered first. Moreover, representations of the behavioral profile of the resident animal over time-intervals shorter than the total 10 min might offer additional information about which phase of the social encounter is mostly affected by the nonapeptide. Alternatively, a path analysis might be useful as a method to describe the dependency among the set of behavioral categories we evaluated. The path analysis method decomposes correlations of behaviors within the behavioral profile into sequences and assesses the importance of relationship between those (Wright, 1921).

NOSE TO BRAIN OXYTOCINERGIC COMMUNICATION: A TOOL OR A TREATMENT?

Over the past decade, the possibility of intranasal drug administration to directly enhance brain levels of the administered ligand has received a great deal of clinical attention. Practical, non-invasive, rapid and simple, OXT nasal spray has been used in numerous clinical trials where empathic feelings, social cognition, anxiety, and eye gaze behavior have been investigated mainly in single-dose studies with healthy subjects (MacDonald et al., 2011; Striepens et al., 2011; Zak et al., 2005).

Up till now, only very few preclinical studies have successfully reported behavioral changes after intranasal OXT delivery (Bales et al., 2013; Chang et al., 2012; Parker et al., 2005). In particular, **chapter 6** of this thesis presents a series of studies conducted on male WTG rats where intranasal OXT treatment significantly induced anti-aggressive and pro-social changes, similarly to icv manipulation.

In general, the use of nasal spray as a tool for central delivery of drugs was promoted enormously after the revelation of a rise in the human CSF AVP level within 10 min after the intranasal AVP application (Born et al., 2002). Similarly, two studies, one in humans and one in rodents, have monitored the concentration of OXT in CSF and in plasma after intranasal OXT application. Without distinguishing between exogenous and endogenous source, a peak in OXT content in the extracellular fluid within the LS and the dorsal hippocampus has been found in rats and mice between 30 and 60 min after intranasal application of OXT (Neumann et al., 2013). In line, Striepens and colleagues have shown for the first time in humans increased CSF OXT concentration (+64%) 75 min after intranasal application of OXT at 24 IU dose (Striepens et al., 2013).

These studies, although minimal in number, seem to justify intranasal OXT application as methodology to manipulate the central OXTergic system, and consequently the behavior. However, the study of Striepens suggests that in the clinical studies where nasal OXT is used at the dose of 24 IU, the time interval between the intranasal application and any behavioral or neuroimaging experiments should be at least of 75 min, in contrast with the typical 45-50 min (Domes et al., 2010; Guastella et al., 2008; Macdonald and Feifel, 2013). Moreover, based on the different time-course and magnitude of the nonapeptides changes in the CSF of humans (Born et al., 2002; Striepens et al., 2013), it has been hypothesized that higher intranasal dose may result in a greater concentration gradient, especially detectable when sampling by lumbar spine puncture.

To possibly explain the entry of drugs into the central nervous system after intranasal application, several hypotheses have been formulated (Figure 8) (Dhuria et al., 2010; Thorne et al., 1995; Thorne et al., 2004).

(1) OXT may enter the central nervous system via adsorptive or receptor-mediated or non-specific fluid phase endocytosis into olfactory sensory neurons. These neurons are bipolar with one end located in the nasal olfactory epithelium on the roof of the nasal cavity, and the other end extending through the holes in the cribriform plate of the ethmoid bone and

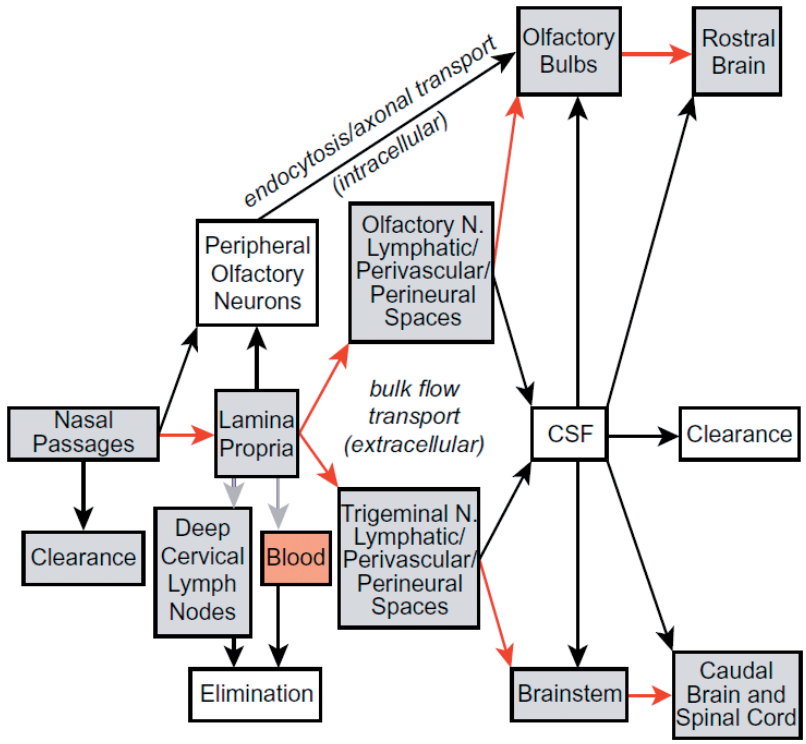

Figure 8. Proposed mechanisms for oxytocin transport into the central nervous system following intranasal application (Thorne et al., 2004).

ending in the olfactory bulb (Watelet and Van Cauwenberge, 1999). Although it has been suggested that there is no morphological barrier between the nasal submucosa interstitial space and the olfactory perineuronal space (Erlich et al., 1986; Jackson et al., 1979), this intraneuronal route has been reported to be slow (hours to reach the olfactory bulb and days to reach other brain region), not explaining the behavioral effects researchers have reported within minutes after the nasal puff (Chen et al., 1998; Frey et al., 1997).

(2) Alternatively, OXT might be transported through extraneuronal pathway, probably relying on bulk flow transport, through perineural channels. After reaching the lamina propria

   a. OXT may enter channels created by olfactory ensheathing cells surrounding the olfactory nerves, and directly access the brain parenchymal tissue and/or CSF. This pathway would allow OXT to reach the CSF within minutes (Chen et al., 1998; Frey et al., 1997).
   b. The trigeminal neuronal pathway may also be involved in rapidly delivering OXT to the brain or spinal cord, following the trigeminal nerve that innervates the respiratory and olfactory epithelium of the nasal cavity and enters the subarachnoid space through the cribriform plate near the olfactory bulb, and/or through the anterior lacerated foramen near the pons (Dhuria et al., 2010; Thorne et al., 1995).

Next to these hypothesis, researchers are also discussing the possibility that:

(3) increased central level of OXT after intranasal application could actually occur indirectly through "feed forward" stimulation of the endogenous OXTergic system. The signal might come via

   a. afferent feedback from the periphery, due to elevated circulating OXT (Churchland and Winkielman, 2012), or
   b. neuronal inputs from OXTR expressing cells in the inferior part of the brain (olfactory areas) (Ostrowski, 1998; Tribollet et al., 1992) activated by synthetic OXT.

In fact, in both humans (Born et al., 2002; Striepens et al., 2013) and rodents (Neumann et al., 2013), increased OXT concentration after nasal delivery has been consistently found also in the plasma with an earlier onset of the peak (10-15 min), greater magnitude (+225%) and faster back-to-baseline recovery (within 75 min) as compared to CSF changes (Striepens et al., 2013). This fast increase of OXT concentration in the blood may be due to its rapid absorption by the heavily vascularized nasal mucosa, draining then into the bloodstream through both fenestrated epithelium and facial veins (Macdonald and Feifel, 2013). The enzymatic degradation of OXT in the blood is then probably responsible for its fast decrease (Veening et al., 2010). In general, the lack of temporal correspondence between central and peripheral OXT concentrations invites caution in using the plasma OXT measurements as accurate reflection of central OXT levels (Kagerbauer et al., 2013), and does not exclude that the later rise in CSF OXT content may be due to indirect activation of the endogenous central OXTergic system. Interestingly, recent studies in male rats have reported increased hypothalamic OXTergic neuronal activity (Carson et al., 2010) and pro-social effects (Ramos et al., 2013) after intraperitoneal OXT injection.

The hypothesis of indirect activation of the endogenous OXTergic system after intranasal OXT application has been recently challenged in rodent studies, including the one descripted in **chapter 6** of this thesis. Contrary to Ludwig and colleagues who failed to show any change in behavior or neuronal activity after intranasal AVP application (Ludwig et al., 2013), increased neuronal activation in both OXTergic and other neurons was found in the PVN and SON of male WTG rats after intranasal application of OXT. This finding suggests that intranasal OXT may activate the endogenous hypothalamic OXTergic system and it leads to the hypothesis that intranasal OXT may also alter the neuronal activity of effector brain regions potentially responsible for the anti-aggressive and pro-affiliative effects observed in these animals after intranasal OXT application (**chapter 6**). However, activation of OXTergic neurons does not necessarily imply evidence of following endogenous OXT central release, neither does it exclude the entry of exogenous OXT.

In conclusion, more studies are needed to describe mechanistic nose-to-brain communication and to optimize the experimental conditions, considering how much influence these can have on the drug deposition within the nasal cavity and presumably also on the diffusion to the brain (Dhuria et al., 2010). Up till now, no studies have actually discriminated between the exogenous and endogenous source of the rise in CSF OXT level

after nasal application, neither have they temporally and spatially described the distribution of synthetic OXT into the brain. Moreover, in order to advance the current understanding of the pharmacokinetics, pharmacodynamics, methodologies and general safety related to intranasal OXT application, especially for clinical use, research should direct attention to the impact of dose-depend effects, multi-dose strategy, chronic treatment, long-term effects, as well as contextual cues and individual differences moderating the effects.

LIMITATIONS AND FUTURE PERSPECTIVES

The findings to date, including those presented in this thesis, support the general idea that OXT is involved in the regulation of social behaviors, including aggressive behavior and bonding, both in animals and in humans. However, to what extent is the clinical use of intranasal OXT to improve social relations and alleviate social deficits actually justified? Do we know enough about nose-to-brain route, dosing, therapeutic window, potential long-term effects and individual variability? Is it scientifically correct to advocate OXT for all the clinical trials that appear to be underway in several top "target "disorders, like schizophrenia, anxiety, and autism (www.clinicaltrials.gov)?

So far, the available knowledge about OXT behavioral properties in humans is mainly based on single application trials in healthy individuals, and mainly men (MacDonald et al., 2011). However, findings from healthy volunteers cannot be directly translated to the anticipated effects in patients with psychiatric illnesses, or treated with potentially interacting drugs (Macdonald and Feifel, 2013). Moreover, findings in one gender cannot reliably predict the outcome in the other (Lischke et al., 2012). Currently, only small sample studies have indicated that intranasal OXT administration for several days up to weeks may lead to improved functioning, and to reduced irritability and aggressive behavior in patients affected by schizophrenia and autism spectrum disorder (Kosaka et al., 2012; Striepens et al., 2011). However, studies on obsessive compulsive disorders have instead failed to show an effect of multiple doses of OXT (Epperson et al., 1996). In both genders, in healthy as well in disordered patients, multi-week, daily dose, and randomized placebo-controlled trials are therefore needed to "advance the field from the stage of optimistic speculation into the realm where definitive verdicts can be obtained" (Macdonald and Feifel, 2013).

Moreover, when trying to investigate the functional role of OXT in modulating behaviors like aggression, clinical studies may present several experimental limitations and shortcomings for which an integrated preclinical and clinical investigation may be advantageous. In general, studying human aggression and anti-social behaviors appears quite limited when performed under controlled laboratory conditions, with small sample sizes and testing tools that do not really allow a full behavioral expression of the aggressive outburst; i.e., self-reported questionnaires, or decision-making tasks simulating hostility and impulsivity. Hence, valid animal models for aggression are crucial to gain insights into the neurobiology, the mechanisms and the predictability of aggression, with the great advantage of working with large-scale studies and individual variability.

In general, more preclinical investigations are needed to gather evidence of the direct nose-to-brain transport, but are also necessary to advance the current knowledge about the pharmacokinetics, the pharmacodynamics, the dosing, the timing and frequency of intranasal OXT application. Moreover, since measuring of OXT level in the CSF of humans is limited by ethical considerations, it is fundamental that animal research gives attention to the physiological and functional relationship between central and peripheral OXT level, both as baseline and post-treatment measurements. It should be indeed noted that, under basal non-challenged conditions, concentrations and patterns of OXT release may differ between CSF and plasma (McEwen, 2004). For instance, OXT levels exhibit circadian rhythms in the CSF but not in the plasma (Amico et al., 1983; McEwen, 2004), and OXT has higher concentrations (Amico et al., 1983; Kagerbauer et al., 2013; Striepens et al., 2013) and longer half-life in CSF as compared to plasma (Veening et al., 2010). Lack of temporal correspondence has also been found between CSF and plasma after intranasal OXT application (Born et al., 2002; Striepens et al., 2013). Assuming these aforementioned differences being due to different metabolisms (metabolic enzymes might be less available in the CSF), the frequency of sampling might also influence conclusions regarding the rise of OXT after intranasal application (Churchland and Winkielman, 2012), especially in human trials where the number of samples is limited.

Another debate relates to the fact that beneficial effects of OXT seem to occur under rather specific circumstances, strongly depending on inter-individual differences and contextual factors (Bartz et al., 2011b; Olff et al., 2013). As previously discussed, OXTergic manipulation may show even dichotomous behavioral effects depending upon sex, hormones, gene functioning and context, while individual traits or experiences may moderate the magnitude of the effects. For example, there are indications of improved social skills only in groups of participants lacking of these skills such that they could indeed gain and benefit from the OXT-induced emotion regulation or attachment facilitation (Cardoso et al., 2012; Quirin et al., 2011). Some studies have challenged the well-known "pro-social" profile of OXT reporting increased anti-social, egoistic, and hostile behaviors after intranasal application (De Dreu et al., 2012; De Dreu et al., 2011). These contrasting findings have brought researchers to hypothesize that OXT might simply amplify the sensitivity to social salience with the overall effect being essentially dependent on the context or on pre-existing interpersonal experiences, be they positive or negative (Bartz et al., 2011a). Follow up research on the link between individual variability/context and efficacy of OXT treatment may lead to identify subjects who, based on their history, traits and genetic profile, could benefit more from the "pro-social" effects of the nonapeptide.

Finally, considering the neuronal and functional interplay that OXT appears to have with other neurotransmitters, future research might conceive OXT not only as a monotherapy but also suited to augment other established pharmacological, psychological and learning-based treatments (Macdonald and Feifel, 2013).

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2

ANTI-AGGRESSIVE ACTIVITY OF CENTRAL OXYTOCIN IN MALE RATS

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ABSTRACT

A substantial body of research suggests that the neuropeptide oxytocin (OXT) promotes social affiliative behaviors in a wide range of animals including humans. However, its anti-aggressive action has not been unequivocally demonstrated in male laboratory rodents. Our primary goal was to examine the putative serenic effect of OXT in a strain of rats (wild-type Groningen, WTG) that generally show a much broader variation and higher levels of intermale aggression than commonly used laboratory strains of rats. Resident animals were intracerebroventricularly (icv) administered with different doses of synthetic OXT and OXT receptor (OXTR) antagonist, alone and in combination, in order to manipulate brain OXT levels and to assess their behavioral response to an intruder. Our data clearly demonstrate that acute icv administered OXT produces dose-dependent and receptor-selective changes in social behavior, reducing aggression and potentiating social exploration. These anti-aggressive effects are stronger in the more offensive rats. On the other hand, administration of an OXTR antagonist tends to increase (non-significantly) aggression only in low-medium aggressive animals. These results suggest that transiently enhancing brain OXT levels has potent anti-aggressive effects whereas its reduction tends to enhance aggressiveness. In addition, a possible inverse relationship between trait-aggression and functional activity of the central OXTergic system is revealed. Overall, this study emphasizes the importance of brain OXT levels for regulating intermale offensive aggression. This study supports the suggestion that OXTR agonists could clinically be useful for curbing heightened aggression seen in a range of neuropsychiatric disorders like antisocial personality disorder, autism and addiction.

INTRODUCTION

During the past decade, a substantial body of human and animal research suggests that the neuropeptide oxytocin (OXT) plays a critical role in processing social aspects of behavior (Churchland and Winkielman, 2012; Lee et al., 2009a; MacDonald and MacDonald, 2010; Striepens et al., 2011; Veening et al., 2010). In humans, for example, intranasal administration of synthetic OXT has been shown to stimulate a host of pro-social behaviors, including empathy and "mind-reading" (Domes et al., 2007; Hurlemann et al., 2010; Wu et al., 2012), gaze to the eye region and face recognition (Guastella et al., 2008; Rimmele et al., 2009), trust, generosity (Barraza et al., 2012; Barraza and Zak, 2009; Zak et al., 2007), cooperation in economic games (Baumgartner et al., 2008; De Dreu, 2011; Kosfeld et al., 2005; Krueger et al., 2012; Theodoridou et al., 2009), in-group altruism (De Dreu, 2012; De Dreu et al., 2011), affiliation (Di Simplicio et al., 2009; Ditzen et al., 2009; Feldman, 2012) and parental caregiving (Gordon et al., 2010; Naber et al., 2010; Riem et al., 2011; Strathearn, 2011). Accordingly, a hypo-OXTergic function may be involved in the expression of abnormal aggressive and antisocial behaviors as indicated by the findings of low central and peripheral OXT levels in personality disordered subjects (Fetissov et al., 2006; Jokinen et al., 2012; Lee et al., 2009b), of OXT receptor (OXTR) deficiency in socially impaired individuals with autism (Gregory et al., 2009; Gurrieri and Neri, 2009; Jacob et al., 2007; Lerer et al., 2008; Wermter et al., 2010; Wu et al., 2005), and of the association between loss of function OXTR gene variants and childhood-onset aggression (Malik et al., 2012).

This putative affiliative and prosocial-like action of brain OXT is strongly supported by a large number of animal studies demonstrating that central OXT administration increases parental care and maternal defense of lactating mothers (Bosch, 2011; Bosch et al., 2005; Bosch and Neumann, 2012; Ross and Young, 2009), pair bonding (Cho et al., 1999; Liu and Wang, 2003; Williams et al., 1994a), sexual behavior (Gil et al., 2011; Snowdon et al., 2010) and social recognition (Choleris et al., 2009; Donaldson and Young, 2008). Accordingly, lowering central OXT functioning in animals by a variety of different manipulations, including gene knockout techniques, generally produces the opposite effects on these social behaviors, e.g. social withdrawal (Pobbe et al., 2012), impaired social cognition and social memory formation (Crawley et al., 2007; Higashida et al., 2012), and excessive aggression (Campbell, 2008; Dhakar et al., 2012).

Concerning the role of OXT in aggression regulation, however, the animal data at first sight seem to be contradictory with increases, decreases and no clear effects being reported. Previous animal research using a variety of lesion, pharmacological and genetic manipulation techniques to heighten or lower OXT function in the brain convincingly demonstrated a link between OXT and aggressive behavior, but the effects varied with gender, species, testing context and individual social status. For example, several studies in different species (hamsters, rats and mice) showed that maternal aggression in parous females correlates with elevated central OXT level, and is enhanced by central infusion of the peptide (Bales and Carter, 2003; Bosch et al., 2004; Ferris et al., 1992; Liu and Wang, 2003). This indicates

a defensive aggression-enhancing role of OXT. In contrast however, OXT infusion into the central amygdala and bed nucleus of the stria terminalis was also reported to have an inhibitory effect on the aggressive behavior of lactating female rats (Consiglio et al., 2005). Furthermore, OXT injected into the medial pre-optic area of the anterior hypothalamus of non-parous resident female Syrian hamsters decreases their aggression towards female intruders (Harmon et al., 2002). Also, Witt and colleagues (Witt et al., 1990) showed that estrogen-primed OXT-injected female prairie voles exhibited increased affiliative behaviors and a decreased aggressive response towards sexually experienced males.

Similar to the contradictory effects of OXT on aggression in females, several studies in males also suggest inconsistent effects of brain OXT on offensive aggressive behaviors. OXT infused into the cerebral ventricles of squirrel monkeys increases the offensive aggression of dominant monkeys towards a subordinate male and female (Winslow and Insel, 1991). Similarly, in polygamous montane voles, central administration of OXT increases the level of aggression that sexually-experienced males display towards a male intruder (Winslow et al., 1993b). In contrast, OXT micro-infusion into the central ventricles of monogamous male prairie voles was reported to result in a decrease of offensive behavior (Winslow et al., 1993b). Similar anti-aggressive effects of OXT were reported in rhesus monkeys and baboons by Silakov and colleagues (Silakov et al., 1992). Other relevant insights into the relationship between brain OXT and aggression come from the recent gene-knockout studies in mice. Increased aggression is consistently observed in mice that lack the ability to synthesize OXT (De Vries et al., 1997; Winslow et al., 2000) or its receptor (Lee et al., 2008; Ragnauth et al., 2005; Sala et al., 2011; Takayanagi et al., 2005). These behavioral genetic studies add support for the suggestion of an anti-aggressive function of brain OXT activity rather than an aggression-stimulating role. Surprisingly however, no behavioral pharmacological studies have been reported yet concerning the effects of directly and transiently enhancing or decreasing brain OXT levels on intermale offensive aggression in male rats. One reason for this omission may be the general low levels of aggression displayed in most commercially available rodent strains and hence the insensitivity to reliably induce or detect the anti-aggressive treatment effects.

Therefore, the primary goal of this study was to examine the putative serenic effects of OXT by employing a feral strain (wild-type Groningen, WTG) of rats that generally show a much broader variation and higher levels of intermale offensive aggression than commonly used laboratory strains of rats (de Boer et al., 2003). To achieve this goal, we first scrutinized the anti-aggressive action of intracerebroventricularly (icv) administered synthetic OXT, and tested its dose-dependency and behavioral specificity. Next, we intended to confirm the involvement of the OXTR in the mediation of the exogenous OXT-induced behavioral effects with a peptidergic OXTR antagonist pretreatment. Finally, we tested the role of endogenous central OXTergic system in modulating aggressive behavior by administering different doses of the highly selective non-peptidergic OXTR antagonist L368.899.

MATERIALS AND METHODS

Animals and housing condition

Young adult male WTG rats (*Rattus Norvegicus*), 4.5 months of age, were used as experimental subjects. This originally wild-trapped outbred strain of rats is well recognized for its high levels and broad range of aggressive behavior, as compared with laboratory strains of rats (de Boer et al., 2003). The animals, weighing between 400 ± 50 g at the time of the primary testing, were individually housed in observation cages (80 × 55 × 50 cm), each with an oviduct-ligated but gonadally-intact female to avoid social isolation, to allow normal sexual activity, and to facilitate territorial behavior. Animals had free access to food (Hope Farms, RMH-B) and tap water with a fixed 12 h light/12 h dark photoperiod (lights off at 13:00 h) in a temperature- (21 ± 2°C) and humidity-controlled room (50 ± 5%). All experimental and behavioral procedures were approved by the Animal Ethics Committee on Care and Use of Laboratory Animals (DEC 5824) of Groningen University and were conducted in agreement with Dutch laws (Wet op de Dierproeven 1996) and European regulations (Guideline 86/609/EEC).

Behavioral screening: resident-intruder test

After one week of habituation to the observation cage, the baseline level of offensive aggression was measured using the resident-intruder paradigm, carried out on four consecutive days. The female partner of the experimental rat was removed from the observation cage approximately 30 min prior to the start of the test. Naïve male Wistar rats (Harlan Laboratories, Horst, NL), socially housed in groups of 5-6 in transparent macrolon type IV cages (60 × 60 × 20 cm), were used as intruder animals (average weight 350 ± 50 g and 4 months old). For three consecutive days an attack latency test was performed by introducing an intruder, smaller in size, into the home cage of the experimental resident animal. The test was terminated shortly after occurrence of the first full attack. When the resident failed to attack within the first 10 min of testing, the attack latency time (ALT) was scored as 600 sec and the test was terminated. On the fourth day, the interaction between the resident and the intruder was videotaped for the 10 min following either the first attack or the introduction of the intruder, in case of no attack.

Videos were analyzed and the duration of all behaviors was manually scored on a custom-made data acquisition system (E-line). The observer was blind to the experimental treatments. Specific behavioral elements were grouped into the following broad behavioral categories in order to promote a clear representation of the data: (1) *offensive behavior* (lateral threat, clinch, keep down, chase, upright posture), (2) *social explorative behavior* (moving towards, investigation and ano-genital sniffing of the intruder, crawl over, social grooming), (3) *non-social exploration* (ambulation, rearing, sniffing, scanning, digging), (4) *inactivity* (sitting, lying, freezing), and (5) *self-grooming* (washing, scratching) (Koolhaas et al., 1980; Olivier et al., 1995). The duration of the different behavioral elements was expressed as a percentage in time of the total duration of the test. Behavioral tests were

performed in conformity with the resident-intruder offensive protocol (Koolhaas et al., 1980), and within the first 3-4 hours of the dark (active) phase, in order to avoid effects of circadian fluctuation and light exposure (Devarajan et al., 2005; Devarajan and Rusak, 2004). After this screening, animals were assigned to the different experimental groups, matched on the basis of their baseline level of aggression.

Surgical procedures

All stereotaxic surgical procedures were performed under isoflurane/oxygen anesthesia using semi-sterile conditions. Subcutaneous injection of 0.25 ml Penicillin and 0.1 ml Finadyne was given to respectively minimize the risk of infection and the discomfort directly linked to the operation. For the first 24 hr post-operation, rats were singly housed in their home cage, and then again housed together with the same female companion of the pre-surgery period. Animals were weighed daily to monitor their recovery, and handled daily to habituate them to the icv infusion procedure and to avoid non-specific stress responses during the experimental procedure. Animals were allowed to recover for at least 10 days before the start of behavioral testing.

Indwelling icv guide cannula

Central acute manipulation of the OXTergic system was achieved via icv microinjections through an implanted guide cannula as previously described (Blume et al., 2008; Neumann et al., 2000). Briefly, a guide cannula (22-gauge stainless steel cannulas, C313; Plastics One, Roanoke, VA) was placed according to the stereotaxic atlas of Paxinos and Watson (6^{th} edition, 2007) 2 mm dorsal to the lateral ventricle (AP: –1.0 mm from bregma, ML: +1.7 mm, DV: +3.1 mm below the surface of the dura mater, with the tooth bar set at -3.3 mm). The guide cannula was anchored to the skull with two stainless-steel screws using dental acrylic cement. The guide cannula was kept viable with a dummy cannula, which was removed daily and sterilized during the handling procedure.

Pharmacological manipulations and behavioral test

In all three experiments, the animals received all the different treatments. The sequence of the treatments was randomly assigned to each rat. Vehicle (sterile saline, pH 7.4), synthetic peptidergic OXT ($C_{43}H_{66}N_{12}O_{12}S_2$; MW 1007.19; Tocris, Germany) and two different types of OXTR antagonists, OXTR $antagonist_{non\text{-}peptidergic}$ (Onaka et al., 2003) and OXTR $antagonist_{peptidergic}$ (Blume et al., 2008), were used for pharmacological manipulations.

The OXTR $antagonist_{non\text{-}peptidergic}$ L368.899 ($C_{26}H_{42}N_4O_5S_2$; MW 591.23; Tocris, Germany), the first potent orally active non-peptidergic OXTR antagonist (IC_{50} = 8.9 nM) reaching clinical trials, was chosen because of its high selectivity (> 40 fold) for OXTRs over vasopressin receptors (AVPRs) type 1A and 2 ($V_{1A\text{-}2}$) (Williams et al., 1994b). In the co-administration study instead, we decided to use the OXTR $antagonist_{peptidergic}$ {desGly-NH_2,d(CH_2)$_5$[Tyr(Me)^2,Thr^4]OVT} (MW 992.2; kindly donated by Dr. Manning, University of

Toledo) because of its selectivity (18 times more potent as an OXTR antagonist in the rat than as a V_{1A} receptor antagonist) (Manning and Sawyer, 1989), and its similar molecular size as the synthetic OXT.

In all experiments a total volume of 5 µl was slowly infused into conscious rats using a 27-gauge injection cannula, which extended 2 mm beyond the guide cannula and remained in place for 30 s to allow proper diffusion of the injected solutions (Blume et al., 2008). The experimenter was blind to the type of treatment. Treatments occurred 10 min prior to behavioral testing (Neumann et al., 2000). A 5 days wash-out period was applied between different experimental treatment sessions. This time interval is considered to be long enough for the complete clearance of the compounds, based on our previous pilot study in which the restoration of pre-treatment aggression level occurred within the above mentioned period, independently on the treatments order. Moreover, literature amply reported the short life-span of OXT in the brain (Jones and Robinson, 1982; Mens et al., 1983; Robinson and Jones, 1982). The 10 min resident-intruder tests were performed following the same procedures described above for the primary testing. During each encounter, the resident was exposed to a different intruder.

Experiment 1

In the first experiment (Exp. 1) we tested the possible anti-aggressive effect of the synthetic OXT and assessed the putative dose-response relationship. We applied 3 different doses of OXT: 0.25 – 1 – 4 µg/5 µl following a randomized cross over design. In this experiment, 12 animals were studied.

Experiment 2

Experiment 2 (Exp. 2) was planned to verify whether the OXT-induced effects seen in Exp. 1 were selectively mediated by activation of OXTRs. Therefore, we performed a co-administration study in which OXT treatment was preceded by a pre-treatment of the OXTR antagonist 10 min before. The total injection volume of 5 µl was split into 2 micro-infusions of 2.5 µl each. In the control condition, the animals received two saline infusions (2.5 µl each). In the OXT condition, the OXT treatment (1 µg/2.5 µl) was preceded by a saline infusion (2.5 µl) or by OXTR $antagonist_{peptidergic}$ (0.75 µg/2.5 µl). The dose of the OXTR antagonist was calculated as equimolar to OXT itself. In this experiment, 17 animals were studied.

Experiment 3

A third experiment (Exp. 3) was carried out to test whether the endogenous brain OXTergic activation of OXTRs is a key mediator of resident's behavior towards an intruder. To address this specific question we performed a dose-response study using 3 doses of the highly selective OXTR $antagonist_{non\text{-}peptidergic}$ L368.899: 3.75 – 7.5 – 15 µg/5 µl, following a randomized cross-over design. In this experiment, 12 animals were studied.

Exclusion criteria

At the end of the study, the animals were rapidly euthanized with CO_2 and blue dye was injected via the infusion system into the guide cannula. Correct cannula placement was scored only when the dye was observed exclusively throughout the entire ventricular system of the brain. For this reason, one animal was discharged in experiment 1.

Other criteria for excluding animals were considered when the behavioral performance of the resident was altered by aversive post-injection reactions, or possible wounding during the encounter. For this last reason, another animal was discharged in experiment 1. This led to the following group sizes: experiment 1 N = 10, experiment 2 N = 17, experiment 3 N = 12.

Data analysis

Data are presented as group means + SEM of the ALT and the time spent in each behavioral category (indicated as percentage of the total 10 min test). Treatment effects on these behavioral parameters were statistically tested by repeated measures analyses of variance (ANOVA), using SPSS for Windows (version 18: SPSS Inc, Chicago, IL, USA). The ANOVA design consisted of one within-subjects variable with four levels (compounds or doses).

To account for possible violations of the sphericity assumption for factors with more than two levels, Huynh-Feldt adjusted *p*-values and the epsilon correction factor are reported, together with the unadjusted degrees of freedom and *F*-values. For all analyses the partial eta-squared effect sizes are reported with $\eta^2 < 0.06$ reflecting a small effect; $\eta^2 \geq 0.06$ a medium effect; and $\eta^2 \geq 0.14$ a large effect. If overall significance was obtained, *post-hoc* pairwise comparisons were carried out in order to reveal specific differences in treatment conditions. If these were found, the influence of individual variation in the baseline level of offensive aggression was investigated by entering this baseline level as a covariate into the design.

If a significant effect of the covariate on the overall treatment effect was found, Pearson's correlations were computed to find out whether effects were greater with lower or higher baseline level of aggression. To this end, effect measures were computed as the difference between the behavior observed during the treatment with vehicle and the one observed under different doses of OXT or OXTR antagonist. Furthermore, using a median split approach, the OXT and OXTR antagonist dose-effect curves on aggression were plotted for the low-medium aggressive (i.e., group average of the percentage of time spent in offensive behavior was 35 ± 4% in exp.1 and 18 ± 3% in exp. 3) and for the medium-high aggressive (i.e., group average of the percentage of time spent in offensive behavior was 65 ± 3% in exp.1 and 48 ± 4% in exp. 3) groups of subjects.

To verify the behavioral specificity of OXT treatment, ethograms of OXT-treated animals were compared to those of untreated animals, matched for the level of aggression. These comparisons were carried out by means of *t*-tests for independent samples.

All comparisons with a *p*-value ≤ 0.05 were considered to be statistically significant. *P*-values between 0.06 and 0.1 were noted as a trend towards significance.

RESULTS

Experiment 1: Oxytocin dose-response curve

All three doses of the OXT reduced offensive behavior of the experimental male resident towards the unfamiliar intruder during the social encounter ($F_{3,27}$ = 9.487, p = 0.001, η^2 = 0.513, ε = 0.840). This anti-aggressive effect occurred in a dose-dependent manner (*post-hoc* pairwise comparisons: vehicle vs. OXT 0.25 µg p = 0.079, vehicle vs. OXT 1 µg p = 0.013, vehicle vs. OXT 4 µg p = 0.003), with both the intermediate and the highest dose being significantly more effective than the lowest dose (OXT 0.25 µg vs. OXT 1 µg p = 0.028, OXT 0.25 µg vs. OXT 4 µg p = 0.006). Concomitantly, social explorative behavior was significantly enhanced ($F_{3,27}$ = 7.676, p = 0.001, η^2 = 0.460, ε = 0.967) by OXT 0.25 µg and OXT 1 µg compared to vehicle and OXT 4 µg (vehicle vs. OXT 0.25 µg p = 0.003, vehicle vs. OXT 1 µg p = 0.031, OXT 0.25 µg vs. OXT 4 µg p = 0.002, OXT 1 µg vs. OXT 4 µg p = 0.003) (Figure 1).

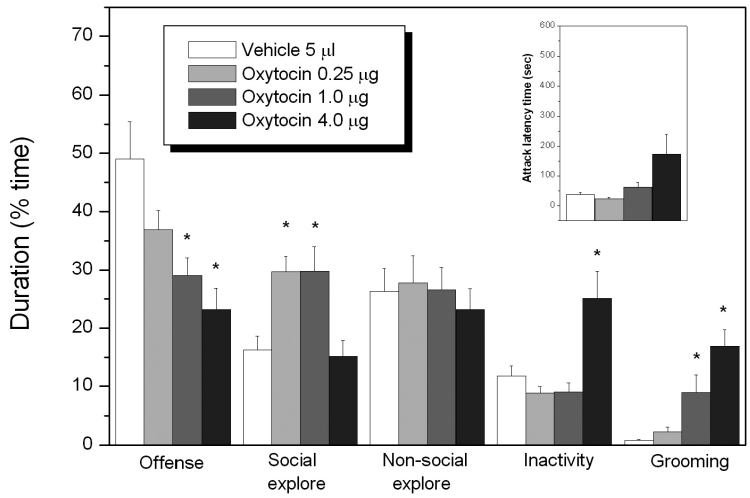

Figure 1. Behavioral changes induced by acute pharmacological manipulation of the central oxytocinergic system. Male resident wild-type Groningen rats were exposed to an unfamiliar male intruder Wistar rat after acute icv administration of vehicle (saline 5 µl; empty bars) or oxytocin at the doses of 0.25 µg (light gray bars) – 1.0 µg (gray bars) – 4.0 µg (dark gray bars)/5 µl. Insert graph depicts the treatment effects on the attack latency time. Data are presented as mean + SEM (N = 10). *p < 0.05 indicates a significant difference in comparison with vehicle.

Dose-dependent changes were also found in the modulation of self-grooming ($F_{3,27}$ = 12.743, p = 0.001, η^2 = 0.586, ε = 0.540). All doses resulted in an increased duration of this behavior compared to vehicle (vehicle vs. OXT 0.25 µg p = 0.053, vehicle vs. OXT 1 µg p = 0.021, vehicle vs. OXT 4 µg p < 0.001). Self-grooming after OXT 1 µg (p = 0.026) and OXT 4 µg (p = 0.001) was significantly more intense than after OXT 0.25 µg. Finally,

immobility was found to be enhanced by OXT 4 µg compared to all the other treatments ($F_{3,27}$ = 10.187, p = 0.001, η^2 = 0.531, ε = 0.699) (vehicle vs. OXT 4 µg p = 0.011, OXT 0.25 µg vs. OXT 4 µg p = 0.006, OXT 1 µg vs. OXT 4 µg p = 0.004). The overall treatment effect on ALT only showed a trend towards significance ($F_{3,27}$ = 3.880, p = 0.071, η^2 = 0.301, ε = 0.394). OXT 4 µg tended to prolong this latency compared to vehicle (p = 0.070) (Figure 1, insert). No treatment effects were found on the category of non-social behaviors.

Due to the wide within-group individual variation in intermale aggression, we tested the influence of baseline level of aggression on the significant overall treatment effects. Analysis of covariance revealed that the anti-aggressive effect induced by OXT 0.25 µg and OXT 1 µg indeed depended upon the baseline level of aggression, as the interaction of treatment with this baseline level was significant ($F_{3,24}$ = 8.379, p = 0.001, η^2 = 0.512, ε = 1.00) and adjustment reduced the previously found effects ($F_{3,24}$ = 2.756, p = 0.064, η^2 = 0.256, ε = 1.00) (OXT 0.25µg p = 0.023, OXT 1 µg p = 0.054, OXT 4 µg p = 0.173). Larger treatment effects were observed in the more aggressive animals as revealed by positive correlations between the baseline level of aggression and the effect measures (OXT 0.25 µg r = 0.827, p = 0.003; OXT 1 µg r = 0.835, p = 0.003) (Figure 4A). Baseline aggression level did not significantly interact with the treatment effect of OXT on social explorative behavior, self-grooming, immobility or ALT.

Experiment 2: Co-administration study

In line with the outcome of Exp.1, we replicated the anti-aggressive ($F_{3,48}$ = 4.400, p = 0.008, η^2 = 0.216, ε = 0.914) effect of OXT 1 µg which appeared significantly effective compared to vehicle (p = 0.002), OXTR antagonist (p = 0.010), and OXTR antagonist + OXT (p = 0.037). This dose also markedly intensified social explorative behavior ($F_{3,48}$ = 5.560, p = 0.002, η^2 = 0.258, ε = 1.00) compared to vehicle (p = 0.002), OXTR antagonist (p = 0.005), and OXTR antagonist + OXT (p = 0.025). When injected alone the OXTR antagonist treatment failed to show behavioral changes compared to vehicle. However, when applied prior to OXT treatment it completely blocked the synthetic OXT-induced effects. No overall treatment effect was found on self-grooming, or on locomotor activity, or on non-social behaviors, or ALT (Figure 2).

Experiment 3: Oxytocin receptor antagonist dose-response curve

Statistical analysis did not reveal any overall significant treatment effects in any of the five behavioral categories. However, a trend effect was found for the ALT ($F_{3,33}$ = 2.337, p = 0.099, η^2 = 0.175, ε = 0.899), where OXTR antagonist 15 µg delayed the first attack compared to OXTR antagonist 3.75 µg (p = 0.036), and OXTR antagonist 7.5 µg (p = 0.023), but not compared to vehicle. Yet, when the influence of the baseline level of aggression on the overall treatment effect was tested (as a covariate in the ANOVA), a significant effect in the category of offensive behavior was revealed ($F_{3,30}$ = 3.340, p = 0.032, η^2 = 0.250, ε = 1.00) (vehicle vs. OXTR antagonist 7.5 µg p = 0.060) (Figure 3). The negative correlation between the baseline level of aggression and the effect measures, although not significant,

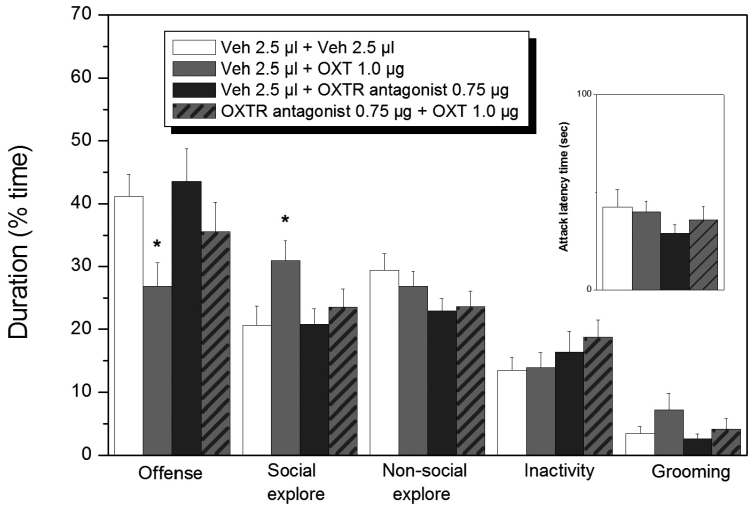

Figure 2. Behavioral changes induced by acute pharmacological manipulation of the central oxytocinergic system. Male resident wild-type Groningen rats were exposed to an unfamiliar male intruder Wistar rat after acute icv administration of vehicle (Veh; empty bars), oxytocin (OXT) (Veh 2.5 µl + OXT 1.0 µg/2.5 µl; gray bars), peptidergic oxytocin receptor (OXTR) antagonist (Veh 2.5 µl + OXTR antagonist 0.75 µg/2.5 µl; dark gray bars) or co-administration of both (OXTR antagonist 0.75 µg/2.5 µl + OXT 1.0 µg/2.5 µl; striped bars). Insert graph depicts the treatment effects on the attack latency time. Data are presented as mean + SEM (N = 17). *$p < 0.05$ indicates a significant difference in comparison with vehicle.

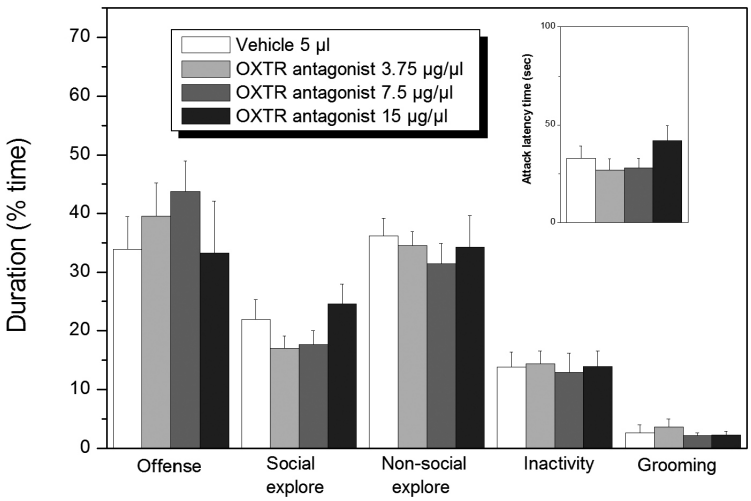

Figure 3. Behavioral changes induced by acute pharmacological manipulation of the central oxytocinergic system. Male resident wild-type Groningen rats were exposed to an unfamiliar male intruder Wistar rat after acute icv administration of vehicle (saline 5 µl; empty bars) or a non-peptidergic oxytocin receptor (OXTR) antagonist L368.899 at the doses of 3.75 µg (light gray bars) - 7.5 µg (gray bars) – 15 µg (dark gray bars)/5 µl. Insert graph depicts the treatment effects on the attack latency time. Data are presented as mean + SEM (N = 12).

suggests a greater increase of offensive aggression in animals with a lower level of baseline aggression (OXTR antagonist 7.5 µg r = -0.440, p = 0.153) (Figure 4B).

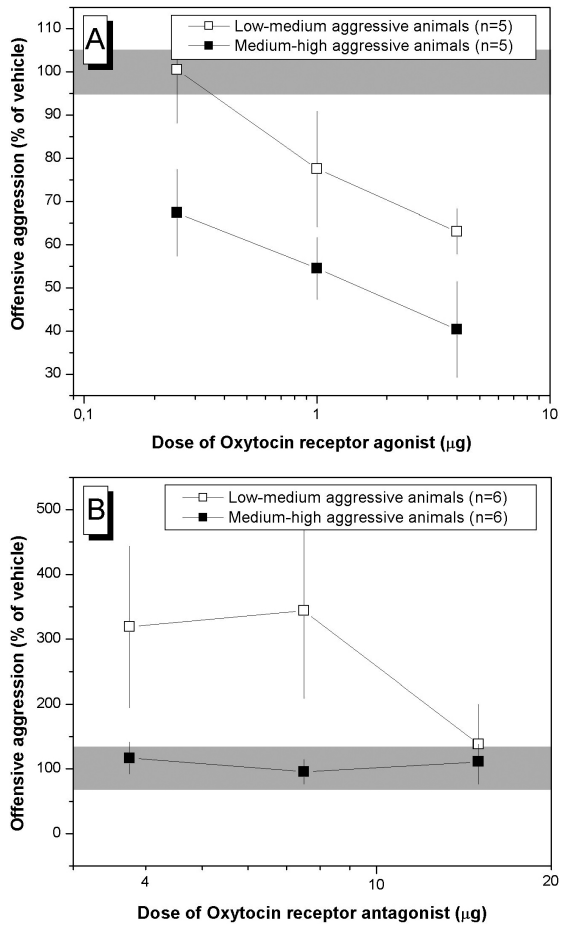

Figure 4. Potency of oxytocin (A) and the oxytocin receptor antagonist$_{non\text{-}peptidergic}$ (B) to change offensive behavior in low-medium (open squares) and medium-high (filled squares) aggressive groups of subjects. Gray area indicates SEM range of the vehicle-treated animals. Data are presented as mean ± SEM.

Behavioral specificity of OXT treatment

To validly control for the behavioral specificity of the synthetic OXT-induced effects, we compared the behavioral profile of OXT-treated with untreated WTG rats that are matched for their level of aggression. The analysis revealed that animals treated with OXT 0.25 µg spent more time in displaying social explorative behaviors (t_{18} = 5.991, p < 0.001), less time undertaking non-social behaviors (t_{18} = -2.138, p = 0.046), and slightly less immobility

(t_{18} = -1.750, p = 0.097) compared to the matched untreated animals. No differences were observed on self-grooming expression. The ethogram was changed in a similar way by OXT 1 µg with animals showing more social explorative interactions (t_{18} = 2.661, p = 0.016) and less non-social behaviors (t_{18} = -2.493, p = 0.023). Self-grooming and immobility appeared to be unchanged by OXT 1 µg. This specificity was compromised with the 4 µg dose of OXT. In this treatment condition, rats spent less time on social explorative (t_{18} = -2.421, p = 0.026) and non-social behaviors (t_{18} = -3.527, p = 0.002), but they increased self-grooming (t_{18} = 3.861, p = 0.001). Immobility was not altered by OXT 4 µg compared to no-treatment condition (Figure 5).

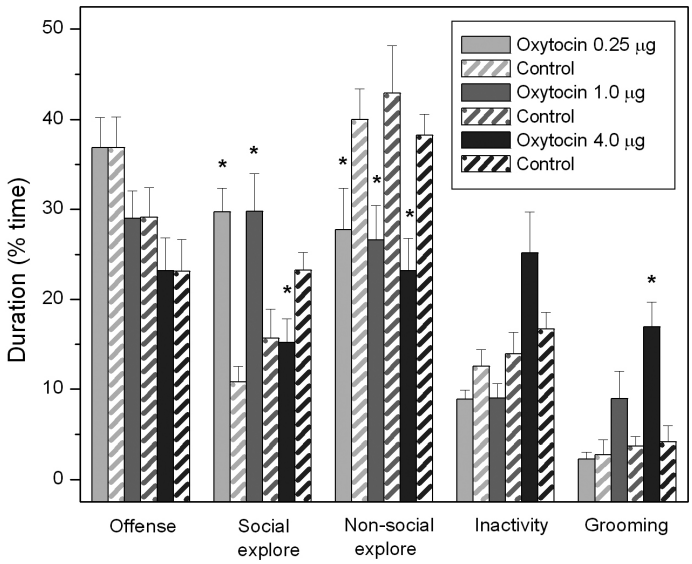

Figure 5. Comparison between the behavioral profile of pharmacologically treated and untreated male resident wild-type Groningen rats, matched for their basal level of aggression. Treated animals were tested after acute icv administration of oxytocin at the doses of 0.25 µg (light gray bars) – 1.0 µg (gray bars) – 4.0 µg (dark gray bars)/5 µl. Data are presented as mean + SEM. *p < 0.05 indicates significant difference in comparison with the matched control group.

DISCUSSSION

In this behavioral pharmacological manipulation study, we aimed at elucidating the putative serenic role of OXT in intermale aggressive conflicts. Our data clearly demonstrate that: 1) acute icv administration of synthetic OXT potently reduces offensive aggression and reinforces non-aggressive social interactions, as signified by the marked decrease in overall duration of fighting episodes undertaken by the resident rat; 2) this suppression of aggression occurs in a clear dose-dependent and behavioral-specific manner (i.e. without impairment of locomotor activity or other behaviors); 3) these exogenously OXT-induced

effects are blocked by pretreatment with a selective OXTR antagonist, confirming the specific involvement of OXTRs in mediating this anti-aggressive effect; 4) the efficacy of OXT and the OXTR antagonist treatments depends upon the individual's trait-like offensive aggressiveness, pointing to a possible inverse relationship between trait-like aggression and functional activity of the central OXTergic system.

As outlined in the introduction, previous animal studies investigating the nonapeptide OXT in the neural regulation of offensive aggression in males have been rather limited and/or have yielded highly variable effects (Lee et al., 2008; Sala et al., 2011; Winslow and Insel, 1991; Winslow et al., 1993b). The fact that the commonly used laboratory rat strains are rather docile and express considerably less offensive aggression in a resident-intruder setting than wild-derived rats (de Boer et al., 2003) may likely obscure to find a clear anti-aggressive effect of OXT. Clearly, our WTG rats generally displays a much larger variation in the level of intermale offensive behavior in a resident-intruder aggression test as compared with the most commonly used laboratory strains of rats (de Boer et al., 2003). Therefore, the highly aggressive ones are potentially more sensitive to putative serenic drug effects. Indeed, our data show robust and consistent anti-aggressive effects of icv OXT administration, particularly in the individuals demonstrating a high baseline level of offensive behavior. On the other hand, selective blockade of OXTRs tends to amplify aggression only in those animals that express low trait-like aggressive behavior. These findings indicate that the efficacy of central OXT manipulations seems to depend upon the individual's trait-like offensiveness, and suggest an inverse relationship between trait-aggression and endogenous brain OXTergic signaling in this rat strain. However, the different efficacies of the OXT manipulations in high versus low-aggressive animals may be (partly) amplified due to a rate-dependency effect, and therefore need to be interpreted cautiously. Obviously, direct assessment of brain OXT mRNA and peptide levels as well as its cognate receptors in high versus low-aggressive animals may elucidate this suggestion.

From our current findings in adult male feral rats, and from the previously reported inverse correlation between cerebrospinal fluid OXT level and life history of aggression in male social disordered patients (Fetissov et al., 2006; Jokinen et al., 2012; Lee et al., 2009b), we may hypothesize that highly offensive, and/or perhaps violent, aggressive individuals are characterized by a low endogenous release of brain OXT as compared to low aggressive individuals. Assuming unchanged OXTR properties, this would imply the occupation of a relatively smaller number of receptor binding sites in highly aggressive individuals. Consequently, more receptors remain available for OXT ligands to bind, and therefore, a larger behavioral response can be expected when OXT is exogenously supplied. Along the same line of reasoning, the greater increase in aggression found in less aggressive rats after OXTR blockade might reflect a higher basal OXTergic tone.

Alternatively, the data of our dose-response studies may also suggest differences in OXTR expression/binding properties between low and high aggressive WTG rats, with lower OXTR availability/binding hypothesized in high aggressive animals. Indeed, several reports of heightened aggression in OXT and OXTR knockout mice (De Vries et al., 1997;

Lee et al., 2008; Ragnauth et al., 2005; Sala et al., 2011; Takayanagi et al., 2005; Winslow et al., 2000) seem in line with this suggestion. However, future and more direct neuro-molecular investigations (e.g., OXT mRNA and protein levels, OXT release patterns and OXTR binding) are needed to verify the proposed individual differences in the endogenous OXTergic signaling components and their putative correlations with offensive behavior.

In contrast with the involvement of brain OXT level in adulthood reported in our findings, a previous study has shown that OXT plays an important role only during embryonic and early postnatal development in the organization of the neural circuitry that underlies aggressive behavior in adulthood (Bales and Carter, 2003). These data have been reinforced by a recent work using a conditional forebrain-specific knockout of the OXTR (OXTR $^{FB/FB}$ mice) reported heightened aggressive behavior only in mice with lifelong but not post-weaning knockout of the OXTR (Dhakar et al., 2012).

To test whether the OXT-induced behavioral effects were specifically OXTR-mediated, we performed a classic pharmacological co-administration study where a selective OXTR antagonist was injected prior to the agonist. The blockade of OXTRs with the specific OXTR antagonist virtually abrogates the behavioral effects of OXT treatment, thus proving the necessary and exclusive involvement of OXTRs in mediating the behavioral changes after OXT administration. However, higher doses of OXT or OXTR antagonists may be able to signal through AVPRs (Chini et al., 2008; Manning et al., 2012) Receptor cross-reactivity between the OXTergic and AVPergic systems may explain the trend towards a significant increase of ALT in the OXTR antagonist 15 µg, as AVP has consistently been implicated in the neural regulation of aggressive and affiliative behaviors across species (Altemus et al., 1992; Bester-Meredith et al., 1999; Bosch et al., 2010; Coccaro et al., 1998; Compaan et al., 1993; Everts et al., 1997). Previous studies in hamsters, mice and rats have indeed shown that selective V_{1A} and V_{1B} receptors antagonists increase sociability and/or decrease aggression in males (Blanchard et al., 2004; Ferris and Potegal, 1988; Koolhaas et al., 2010), but not in females (Gutzler et al., 2010). According to the literature, the mechanisms underlying the elevated social investigation after central blockade of OXT and/or AVP signaling might refer to a disrupted processing and/or integration of olfactory social cues leading to an impaired recognition and more intense investigation (Tobin et al., 2010; Winslow et al., 1993a). Polymodal effects of OXT have been reported for the modulation of social behaviors and social memory, where low doses of OXT facilitate and high doses inhibit social recognition of male rats towards a juvenile social conspecific (Benelli et al., 1995; Popik and van Ree, 1991; Popik and Vetulani, 1991; Popik et al., 1992).

The effects of OXT treatment were also examined with respect to behavioral specificity by comparing the complete ethogram of OXT-treated WTG rats with that of untreated WTG rats, the groups being matched for their level of offensive behavior. With this analysis, we reject the hypothesis that the anti-aggressive profile induced by OXT 4 µg might be a consequence of OXT-induced increased immobility, as the inactivity level of the two groups was similar. Moreover, we highlight the specific profile of OXT 0.25 and 1 µg that appeared to preferentially increase social explorative behavior. Several studies

across species and genders have reported the positive effects of OXT on social behavior and/or on the processing of social cues (Campbell, 2008; Churchland and Winkielman, 2012; Ditzen et al., 2009; Lee et al., 2005; Neumann, 2008; Ross and Young, 2009; Witt et al., 1992). Concerning the brain site of action of these effects, the medial amygdala is suggested to be one of the primary nodes within the social brain network. Among others it directly receives social odor cues from the olfactory system and becomes strongly activated after exposure to a conspecific (Bielsky and Young, 2004; Ferguson et al., 2001). Studies in OXT knockout mice have shown that OXT in the medial amygdala is essential for the processing or initial retention of social information (Ferguson et al. 2001; Lee et al. 2008). Moreover, OXT release in the central amygdala potently reduces the activation and reactivation of the emotional brain to fearful condition and social conflict (Kirsch et al., 2005; Knobloch et al., 2012), leading to a more passive coping style (Ebner et al., 2005). A passive behavioral coping style is generally reflected by an individual's low aggression level and low burying behavior in a conflicting/stressful testing context (Koolhaas et al., 2010). In relation to this, Linfoot at al. (2009) have reported that animals showing little to no defensive burying responses displayed relatively higher levels of OXT mRNA within the supraoptic nucleus and sub-regions of the paraventricular nucleus of the hypothalamus. Thus, brain OXT function seems to be associated with a passive and low aggressive coping style, in agreement with our findings of higher OXTergic activity in hypothalamic and amygdala regions in low-aggressive WTG rats (Calcagnoli et al., 2014).

When checking the behavioral specificity of OXT, an enhanced self-grooming after OXT 4 µg infusion was also reported, and this finding is consistent with previous reports in the literature. Drago et al. (Drago et al., 1986) showed that icv infusion of OXT is followed by an enhancement of novelty-induced grooming behavior in both male and female rats in a dose-dependent manner. Self-grooming is a spontaneous behavior that occurs widely in many species and it is associated with several hygienic and sex-related functions (Yu et al., 2010), but does also occur as a displacement or self-soothing behavior in situations in which the animal experiences conflict, or as a reaction to recent arousal or stress situations (Van Den Berg et al., 1999). However, in our study the increase of self-grooming was significant only after infusing the highest dose of OXT, which is substantially higher than the dosages necessary to suppress offensive behavior and increase social explorative behavior. This suggests that, among others, the social behavior network is the preferential target of central OXTergic action.

In conclusion, our data demonstrate an important role of the nonapeptide OXT in the neural regulation of adult intermale offensive aggression. Potentiation of central OXTergic activity, particularly in high-aggressive individuals, shapes the social behavioral profile facilitating the expression of more social explorative interactions and limiting overt aggressive reactions. On the other hand, pharmacological blockade of endogenous OXTergic signaling amplifies aggressive and conflicting reactions only in low-aggressive animals only. These findings support the feasibility of OXTR agonists to be employed clinically for curbing heightened antisocial aggressive behavior as seen in a range of neuropsychiatric disorders

like antisocial personality disorder, autism and addiction. Moreover, the suggested inverse relationship between trait-like aggression and endogenous OXTergic signaling might shed new light on the patho-physiology of aggression/violence in humans.

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3

CHRONIC ENHANCEMENT OF BRAIN OXYTOCIN LEVELS CAUSES ENDURING ANTI-AGGRESSIVE AND PRO-SOCIAL EXPLORATIVE EFFECTS IN MALE RATS

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ABSTRACT

Oxytocin (OXT) has been implicated in the regulation of social behaviors, including intermale offensive aggression. Recently, we showed that acute enhancement of brain OXT levels markedly suppressed offensive aggression and increased social exploration in resident rats confronted with an intruder in their home territory. Moreover, a different responsivity to the exogenous OXTergic manipulation was observed among individuals based on their baseline aggression. In this study we aimed at evaluating the behavioral response to chronically enhancing or attenuating central OXT levels, and at scrutinizing whether the trait-aggression moderates the treatment-induced behavioral changes. To this end, resident male wild-type Groningen rats were continuously (via osmotic mini-pumps) intracerebroventricularly infused with synthetic OXT or a selective OXT receptor (OXTR) antagonist for 7 days. Changes in behavior were assessed performing a resident-intruder test before and at the end of the treatment period, as well as after 7 days of withdrawal. Chronic infusion of OXT was found to selectively suppress aggression and enhance social exploration. Chronic blockage of OXTRs instead increased introductory aggressive behavior (i.e. lateral threat), yet without affecting the total duration of the aggression. The magnitude of the anti-aggressive changes correlated positively with the level of baseline aggression. Interestingly, OXT-induced behavioral changes persisted 7 days after cessation of the treatment. In conclusion, these findings provide further evidence that enhanced functional activity of the central OXTergic system decreases social offensive aggression while it increases social explorative behavior. The data also indicate that chronically enhancing brain OXT levels may cause enduring anti-aggressive and pro-social explorative behavioral effects.

INTRODUCTION

Oxytocin (OXT) is a cyclic nonapeptide synthesized principally by a relatively small clusters of neurons in the hypothalamic paraventricular, supraoptic and accessory magnocellular nuclei (Stoop, 2012). In addition to its well-known peripheral hormonal functions (i.e., induction of labor and milk ejection), OXT acts as an important neuronal messenger within the brain regulating social and emotional behaviors in a wide variety of animal species including humans (Lee et al., 2009a). Hence, disturbed brain OXTergic signaling has been implicated in several psychiatric disorders where social dysfunction is a core symptom (e.g., autism spectrum disorder, social anxiety, borderline personality disorder, addiction and schizophrenia) (Meyer-Lindenberg et al., 2011). In particular, low cerebrospinal fluid (CSF) OXT (Lee et al., 2009b), loss of functional polymorphisms of the OXT receptor (OXTR) gene (Beitchman et al., 2012; Malik et al., 2012) and epigenetic silencing (methylation) of the OXTR promoter have been related to impulsive and aggressive temperament, interpersonal violence and callous-unemotional traits in young boys (Kumsta et al., 2013). Similarly, animal research has reinforced the proposed functional role of the central OXTergic system in regulating the behavioral response in conflicting social contexts. For example, genetic knockout studies have demonstrated that abrogating OXTergic signaling results in escalated patterns and dysfunctional forms of intermale offensive aggression (Sala et al., 2011; Winslow et al., 2000). Recent etho-pharmacological studies carried out in our lab on a feral strain (wild-type Groningen, WTG) of male rats have revealed robust dose-dependent anti-aggressive and pro-social exploratory effects of acute intracerebroventricular (icv) administered OXT, especially in animals with high baseline aggression level. Conversely, acute and selective blockade of OXTergic signaling by administering a selective OXTR antagonist tended to potentiate aggressive displays especially in low aggressive individuals (Calcagnoli et al., 2013).

Considering the increasing scientific interest for the brain OXTergic social behavior network, and the increasing exploration of intranasally applied synthetic OXT in humans, it becomes relevant to expand our current psychoneuroendocrine knowledge of this neuropeptide. Before adopting synthetic OXTR agonists as a potential treatment for curbing social deficits and abnormal aggressive behaviors in humans, the effects of chronic OXT-treatment and possible long-lasting repercussions on behavior and physiology have to be investigated. To date, the data available on chronic OXT administration are limited to some preclinical studies that are mainly focused on drug addiction processes (Sarnyai and Kovacs, 1994), stress responsivity (Parker et al., 2005), anxiety (Slattery and Neumann, 2010; Windle et al., 1997), or male-female social interaction (Witt et al., 1992). No studies are yet available concerning the effect of chronic OXT on intermale social-aggressive behaviors in particular.

Therefore, in order to extend our recent findings of the acute central OXTergic manipulation effects on offensive and social explorative behaviors (Calcagnoli et al., 2013), a chronic icv administration study has been designed. In particular, we aimed at testing the hypothesis that chronic enhancement or attenuation of the central OXTergic activity

would result in the suppression or increase of intermale offensive behavior, respectively. We expected to replicate the selective changes in the social behavioral profile that were observed after acute OXT infusion, i.e., decreased offensive aggression concomitant with increased social exploration, without effects on non-social behaviors. Also, possibly enduring or rebound effects were monitored. Therefore, the behavioral effects of chronic OXTergic manipulation were tested using a standard resident-intruder test, performed before (day -1), immediately after a 7-day period of treatment (day 7), and again after 7 days of withdrawal from chronic treatment (day 14). Moreover, we expected to replicate the observation that the individual's initial aggressive phenotype might moderate the individual responsivity to the OXTergic manipulation. In particular, we hypothesized the most aggressive animals to be more sensitive to the exogenous synthetic OXT, as trained and aggression-experienced highly aggressive wild-type Groningen residents have been recently described to have potentially lower central OXT availability but higher OXTR binding capacities (Calcagnoli et al., 2014). On the other hand, we investigate the possibility that chronic OXTR antagonist infusion may induce pro-aggressive changes, as observed in one of our experiments with acute manipulation.

MATERIALS AND METHODS

Animals and housing condition

Adult male WTG rats (*Rattus Norvegicus*) were used as experimental subjects. This strain of rats descended from pairs of wild-trapped individuals that were outbred under conventionalized conditions for over 35 generations now in our laboratory. As compared to commonly used laboratory strains of rats, WTG rats display a much larger variation in the level of intermale offensive aggression (de Boer et al., 2003) and they are therefore a suitable model for clinical aggression research.

After weaning (postnatal day 23), the animals were socially housed with five non-sibling conspecifics in macrolon cages (55 × 34 × 20 cm). Around the age of 120 days, the animals were housed in large observation cages (80 × 55 × 50 cm), each with an oviduct-ligated but gonadally-intact female to avoid social isolation and to allow normal sexual activity, required to stimulate territorial behavior (Albert et al., 1988). Animals had free access to food (Hope Farms, RMH-B) and tap water with a fixed 12h light/12h dark photoperiod (lights off at 13:00 h) in a temperature- (21 ± 2ºC) and humidity-controlled room (50 ± 5%). All experimental and behavioral procedures were approved by the Animal Ethics Committee on Care and Use of Laboratory Animals (DEC 5824) of the Groningen University and were conducted in agreement with Dutch laws (WoD, 1996) and European regulations (Guideline 86/609/EC).

Behavioral characterization: resident-intruder test

After an acclimation period of 7 days in the observation cages, residents (average body weight 400 ± 50 g, 4.5 months old) were tested for their baseline level of offensive aggression using the standard resident-intruder (RI) test paradigm (Koolhaas et al., 2013; Koolhaas et al.,

1980). The companion female was always removed approximately 30 min prior to the start of the test and placed back afterwards. Naïve male intruder Wistar rats (Harlan Laboratories, Horst, NL) have been used as intruders (average body weight 300 ± 50 g, 4 months old). The lower range weight of the intruder guaranteed the assessment of dominance from the resident, with only one resident failing the attack during the baseline RI test.

The baseline behavioral screening consisted of an attack latency time (ALT) test repeated over three consecutive days, by introducing an unfamiliar intruder into the cage of the experimental animal. The intermale interaction was terminated as soon as the first clinch was recorded. When the resident failed to attack within the first 10 min of testing, the ALT was scored as 600 sec and the test was terminated. On the fourth day, while videotaped, the interaction was allowed to last for the 10 min following the first attack or, in case of no attack, to last for the 10 min following the placement of the intruder. A custom-made data acquisition system (E-line) was used to evaluate the video and to determine the duration of the following behavioral categories: (1) *offensive behavior* (lateral threat, clinch, keep down, chase, upright posture), (2) *social explorative behavior* (moving towards, investigation and ano-genital sniffing of the intruder, crawl over, social grooming), (3) *non-social behavior* (ambulation, rearing, sniffing, scanning, digging), (4) *inactivity* (sitting, lying, freezing), and (5) *self-grooming* (washing, scratching) (Koolhaas et al., 2013; Koolhaas et al., 1980).

All behavioral tests were performed within the first 3-4 hours of the dark (active) phase in a dimmed lights condition, to avoid effects of circadian hormonal fluctuation and light exposure (Devarajan et al., 2005). The baseline behavioral profile was assessed one day prior to the mini-pump implantation (day -1). The resident animals were then assigned to one of three experimental groups receiving either vehicle solution (N = 11), synthetic OXT (N = 12) or a selective OXTR antagonist (N = 12). Groups were matched on the baseline offensive behavior level.

Surgical procedures and chronic infusion

All surgical procedures were performed anesthetizing the animals with a mixture of isoflurane and oxygen, using sterile conditions. To minimize pain and risk of infection, directly linked to the surgery, rats were injected subcutaneously with analgesic (Finadyne, 0.1 ml) and antibiotic (Penicillin, 0.25 ml) compounds. For the first 24 hours post-operation, rats were singly housed in their home cage and then again housed together with the same female companion as during the pre-surgery period.

A guide cannula (22-gauge stainless steel cannulas, C313; Plastics One, Roanoke, VA, USA) was stereotactically placed according to the brain map of Paxinos and Watson (6th edition, 2007) 2 mm dorsal to lateral ventricle (AP: –1.0 mm from bregma, ML: +1.7 mm, DV: +3.1 mm below the surface of the dura mater, with the tooth bar set at –3.3 mm) and anchored to the skull with two stainless steel screws using dental acrylic cement. To chronically administer the drugs into the brain ventricles, an osmotic mini-pump (infusion rate 0.5 µl/h for 7 days; Alzet, Model 1007D, DURECT Corporation, CA, USA) was subcutaneously implanted. The pumps were filled with either vehicle (sterile, pyrogen-free saline, 0.9% Versylene®, Fresenius,

Kabi, France), or synthetic OXT ($C_{43}H_{66}N_{12}O_{12}S_2$; MW 1007.19; Tocris, Germany) (20 ng/µl) or a selective peptidergic OXTR antagonist (15 ng/µl) {desGly-$NH_{2(9)}$,$d(CH_2)_5$[$Tyr(Me)_2$,Thr_4]OVT} (MW 992.2; kindly donated by Dr. Manning, University of Toledo) (Manning and Sawyer, 1989). The doses were chosen based on effective treatment effects shown in a previous study where the same compounds were chronically infused in adult male rats for the same number of days and using the same model of osmotic mini-pumps (Slattery and Neumann, 2010). Mini-pumps were connected to the icv cannula via a 7.5 cm long polyethylene catheter tube (inside diameter of 0.69 mm; Alzet, brain infusion kit 2, DURECT Corporation, CA, USA). To optimize the operation of the mini-pump, both mini-pump and catheter were incubated in sterile saline at room temperature overnight. The 7-day treatment period started immediately after the surgical implantation of the mini-pump (day 0). Information about the degradation rate of the peptide over the 7 days was collected from a pilot study, revealing about 44% destruction of OXT over 10-day period *in vivo* (Witt et al., 1992).

Behavioral assessments

At days 7 and 14, the behavioral profile was again assessed using the 10 min RI test and then compared to the baseline ethogram recorded at day -1. In the time period between the RI tests, resident animals were kept housed with their companion female in their home cage.

Exclusion criteria

At the end of the experiment, the animals were rapidly euthanized with CO_2. To check for correct cannula placement and for proper attachment of the cannula to the mini-pump, blue dye was slowly injected into the guide cannula and through the catheter connecting the cannula to the mini-pump. Correct cannula placement was scored only when the dye was observed exclusively throughout the entire ventricular system of the brain. Animals that exhibited any indication of dye leakage at the connection sites of the catheter were excluded from the analysis. This led to the following group sizes: vehicle N = 11, OXT N = 10 and OXTR antagonist N = 12.

Data analysis

Treatment effects on the various behavioral variables were statistically tested by General Linear Model (GLM) repeated measures analyses of covariance (ANCOVA), while entering the corresponding baseline values as a covariate for the sake of the design's efficiency (power) and validity (Liu et al., 2009; Senn, 2006). We used SPSS for Windows; version 20: SPSS Inc, Chicago, IL, USA. The ANCOVA design consisted of one within-subjects (WS) variable with three measurement levels (time points: day -1, 7, and 14), and one between-subjects (BS) variable with three treatment levels (vehicle, OXT, and OXTR antagonist). If an overall significant interaction between the treatments (BS variable) and time points (WS variable) was found, post hoc pair-wise treatment group comparisons were carried out on the contrasts of the WS variable (day -1 vs. day 7; day -1 vs. day 14, and day 7 vs. day 14). The ability to compare all three group-dependent time contrasts made us chose

to enter the raw scores of the three measurements instead of change scores (Senn, 2006). To account for possible violations of the sphericity assumption for factors with more than two levels, Huynh-Feldt adjusted *p*-values and the epsilon correction factor are reported together with the unadjusted degrees of freedom and F-values.

For all comparisons, next to the *p*-values, Cohen's d or eta squared (η^2) are presented as measures of effect size, with $d < 0.5$ and $\eta^2 < 0.06$ reflecting a small effect; $d \geq 0.5$ and $\eta^2 \geq 0.06$ a medium effect; and $d \geq 0.8$ and $\eta^2 \geq 0.14$ a large effect.

Pearson's correlations were computed to find out whether the treatment effects were greater in animals with lower or higher baseline level of offensive behavior. To this end two types of change scores were computed, i.e. the difference scores between the pre-treatment measure (at day -1) and the two post-treatment measures obtained at day 7 and day 14, respectively.

We finally tested whether OXT effects on the time spent in the various behavioral categories might be interdependent. To this end correlations were computed between the above-described change scores referring to aggression and social explorative behavior respectively.

Data are graphically presented as group means of the time spent in each behavioral category (indicated as percentage of the total 10 min test) ± SEM.

RESULTS

Significant overall time*treatment interactions were found for only offensive behavior [$F_{4,58} = 8.81$, $p < 0.001$, $\eta^2 = 0.17$] and social exploration [$F_{4,58} = 6.75$, $p < 0.001$, $\eta^2 = 0.28$, $\varepsilon = 0.83$]. In particular, the measurements at both day 7 and day 14 of the two behavioral categories significantly differed from their respective baseline measurements [offensive behavior: contrast day -1 vs. day 7 ($F_{2,29} = 11.81$, $p < 0.001$, $\eta^2 = 0.18$) and day -1 vs. day 14 ($F_{2,29} = 15.46$, $p < 0.001$, $\eta^2 = 0.19$); social explorative behavior: contrast day -1 vs. day 7 ($F_{2,29} = 11.91$, $p < 0.001$, $\eta^2 = 0.38$) and day -1 vs. day 14 ($F_{2,29} = 5.48$, $p < 0.01$, $\eta^2 = 0.27$)]. As Figures 1 and 2 show, the overall effects were due to the fact that chronic OXT infusion (1) significantly attenuated the offensive display as compared to vehicle [$F_{2,36} = 7.10$, $p < 0.01$, $\eta^2 = 0.16$] and OXTR antagonist [$F_{2,38} = 25.40$, $p < 0.001$, $\eta^2 = 0.24$] (Figure 1) and (2) simultaneously enhanced the social exploration of the resident as compared to vehicle [$F_{2,36} = 6.28$, $p < 0.01$, $\eta^2 = 0.23$] and OXTR antagonist [$F_{2,38} = 13.16$, $p < 0.001$, $\eta^2 = 0.34$, $\varepsilon = 0.75$] (Figure 2).

Among the elements within the category of offensive behavior, a significant overall time*treatment effect was found in the lateral threat [$F_{4,58} = 5.12$, $p < 0.01$, $\eta^2 = 0.17$, $\varepsilon = 0.79$], the duration of which was lowered by OXT infusion [$F_{2,29} = 5.67$, $p < 0.01$, $\eta^2 = 0.17$] but increased by OXTR antagonist [$F_{2,29} = 15.87$, $p < 0.001$, $\eta^2 = 0.25$] as compared to vehicle (Table 1).

Interestingly, the OXT-induced anti-aggressive effects seen at day 7 [OXT vs. vehicle, contrast day -1 vs. day 7; $F_{1,18} = 10.68$, $p < 0.01$, $\eta^2 = 0.19$. OXT vs. OXTR antagonist, contrast day -1 vs. day 7; $F_{1,19} = 34.29$, $p < 0.001$, $\eta^2 = 0.23$] persisted over time for 7 days post-treatment [OXT vs. vehicle, contrast day -1 vs. day 14; $F_{1,18} = 14.23$, $p < 0.001$, $\eta^2 = 0.20$. OXT

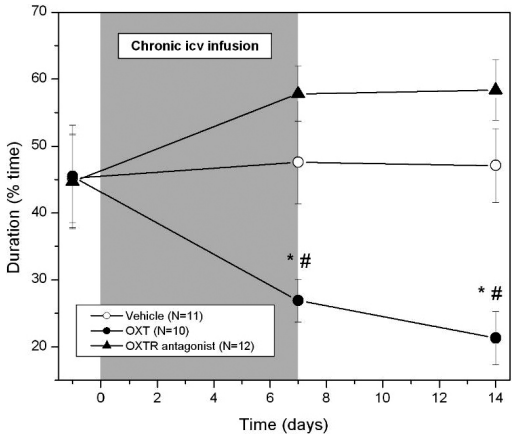

Figure 1. Changes in offensive aggression induced by chronic manipulation of the central oxytocinergic system. Male resident wild-type Groningen rats were exposed to an unfamiliar male intruder Wistar rat after chronic icv infusion of vehicle, synthetic oxytocin (OXT) or selective oxytocin receptor (OXTR) antagonist. The gray area indicates the 7-day treatment period. Procentual duration of offensive behavior is depicted at three time points: baseline measurement (day -1), at the end of the chronic treatment (day 7), and 7 days after the cessation of the treatment (day 14). Data are presented as mean ± SEM. * denotes significance (day 7: $p = 0.01$, $d = 1.30$ and day 14: $p = 0.001$, $d = 1.63$) between vehicle and OXT-treated groups. # indicates significance (day 7: $p < 0.001$, $d = 2.52$ and day 14: $p < 0.001$, $d = 2.65$) between OXT and OXTR antagonist treatments.

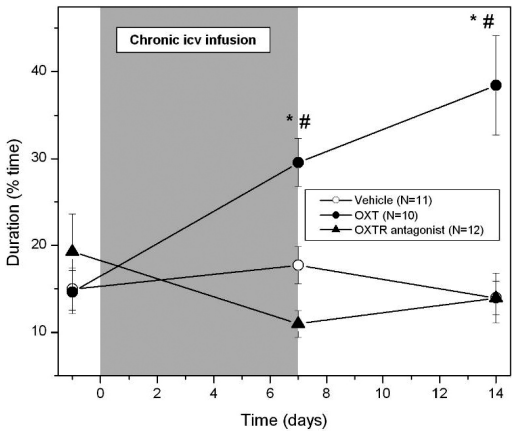

Figure 2. Changes in social explorative behavior induced by chronic manipulation of the central oxytocinergic system. Male resident wild-type Groningen rats were exposed to an unfamiliar male intruder Wistar rat after chronic icv infusion of vehicle, synthetic oxytocin (OXT) or selective oxytocin receptor (OXTR) antagonist. The gray area indicates the 7-day treatment period. Procentual duration of social explorative behavior is depicted at three time points: baseline measurement (day -1), at the end of the chronic treatment (day 7), and 7 days after the cessation of the treatment (day 14). Data are presented as mean ± SEM. * denotes significance (day 7: $p < 0.01$, $d = 1.48$ and day 14: $p = 0.001$, $d = 1.68$) between vehicle and OXT-treated groups. # indicates significance (day 7: $p < 0.001$, $d = 2.64$ and day 14: $p < 0.001$, $d = 2.05$) between OXT and OXTR antagonist treatments.

Table 1. Summary of the group means of the time spent in each element constituting the behavioral category of offensive behavior (indicated as percentage of the total 10 min test) ± the respective SEM. * denotes significance ($p < 0.05$) between vehicle and oxytocin (OXT) or oxytocin receptor (OXTR) antagonist treated groups.

		Day -1	Day 7	Day 14
		Average ± SEM	Average ± SEM	Average ± SEM
Lateral Threat	Vehicle	24.62 ± 4.50	26.75 ± 4.81	26.16 ± 4.11
	OXT	24.73 ± 4.59	15.53 ± 3.05*	11.53 ± 2.33*
	OXTR antagonist	22.01 ± 5.21	30.28 ± 4.16*	36.36 ± 3.36*
Clinch	Vehicle	3.11 ± 0.35	3.21 ± 0.63	2.52 ± 0.61
	OXT	3.44 ± 0.69	2.21 ± 0.28	2.08 ± 0.45
	OXTR antagonist	4.63 ± 1.01	3.88 ± 0.44	3.36 ± 0.60
Keep down	Vehicle	14.15 ± 2.74	15.87 ± 4.36	15.49 ± 5.69
	OXT	14.50 ± 4.28	7.65 ± 1.94	6.81 ± 2.05
	OXTR antagonist	15.58 ± 2.64	20.96 ± 4.87	14.28 ± 3.93
Chase	Vehicle	1.10 ± 0.30	0.85 ± 0.31	1.53 ± 0.44
	OXT	1.79 ± 0.93	0.53 ± 0.23	0.61 ± 0.18
	OXTR antagonist	1.38 ± 0.64	1.08 ± 0.21	1.84 ± 0.52
Upright posture	Vehicle	2.22 ± 0.62	0.89 ± 0.34	1.40 ± 0.31
	OXT	1.04 ± 0.21	0.95 ± 0.30	0.27 ± 0.15
	OXTR antagonist	1.09 ± 0.25	1.68 ± 0.30	2.54 ± 1.25

vs. OXTR antagonist, contrast day -1 vs. day 14; $F_{1,19} = 36.69$, $p < 0.001$, $\eta^2 = 0.27$] (Figure 1). Similarly, the pro-social explorative changes seen at day 7 [OXT vs. vehicle, contrast day -1 vs. day 7; $F_{1,18} = 8.35$, $p = 0.01$, $\eta^2 = 0.28$. OXT vs. OXTR antagonist, contrast day -1 vs. day 7; $F_{1,19} = 28.44$, $p < 0.001$, $\eta^2 = 0.45$] appeared to be long-lasting as well [OXT vs. vehicle, contrast day -1 vs. day 14; $F_{1,18} = 8.22$, $p = 0.01$, $\eta^2 = 0.29$. OXT vs. OXTR antagonist, contrast day -1 vs. day 14; $F_{1,19} = 9.67$, $p < 0.01$, $\eta^2 = 0.31$] (Figure 2). Importantly, for both offensive and social explorative behavior, no differences were found between day 7 and day 14. No significant effects were observed in any of the other behavioral categories of the ethogram, and, equally important, none of the treatments significantly affected the latency of the first attack (Table 2).

In addition, the OXT-induced changes in aggression were found to depend upon the baseline level of offensive behavior, while there was no such baseline dependency for the changes in social explorative behavior. In particular, Figure 3 shows that most of the OXT-treated rats lowered their offensive aggression and that a greater decrease was observed in animals with the highest baseline aggression scores (day 7 $r = -.93$, $p < 0.001$; day 14 $r = -.86$, $p = 0.001$).

Finally, the effects of OXT on aggression and on social explorative behavior appeared to be highly correlated; change scores at day 7 showed a correlation of $r = -.89$ ($p < 0.001$). Yet for day 14 it was no longer significant ($r = -.31$, $p = 0.38$).

Table 2. Summary of the group means of the time (indicated as percentage of the total 10 min test) spent in the behavioral categories evaluated during the intermale encounter (with the exclusion of the categories "offensive behavior" and "social explorative behavior"), and the group means of the latency time to the first attack (ALT; indicated in seconds) ± the respective SEM.

		Day -1	Day 7	Day 14
		Average ± SEM	Average ± SEM	Average ± SEM
Non-social exploration	Vehicle	35.11 ± 4.49	27.76 ± 3.18	28.70 ± 3.41
	OXT	38.45 ± 5.71	37.66 ± 3.66	39.36 ± 3.55
	OXTR antagonist	35.35 ± 4.75	25.93 ± 3.12	25.89 ± 3.20
Inactivity	Vehicle	8.77 ± 2.34	7.93 ± 1.54	11.48 ± 1.99
	OXT	5.80 ± 1.23	6.83 ± 1.20	13.71 ± 3.07
	OXTR antagonist	7.23 ± 1.71	6.05 ± 1.22	6.08 ± 0.89
Self-grooming	Vehicle	4.33 ± 1.30	6.95 ± 2.19	3.25 ± 0.75
	OXT	3.42 ± 1.97	6.18 ± 1.65	2.66 ± 0.89
	OXTR antagonist	2.70 ± 1.52	5.32 ± 1.15	3.33 ± 0.80
ALT	Vehicle	64.27 ± 16.70	54.82 ± 8.54	74.45 ± 13.56
	OXT	53.60 ± 9.46	76.20 ± 12.81	84.60 ± 12.27
	OXTR antagonist	70.58 ± 20.48	48.42 ± 6.85	43.25 ± 7.32

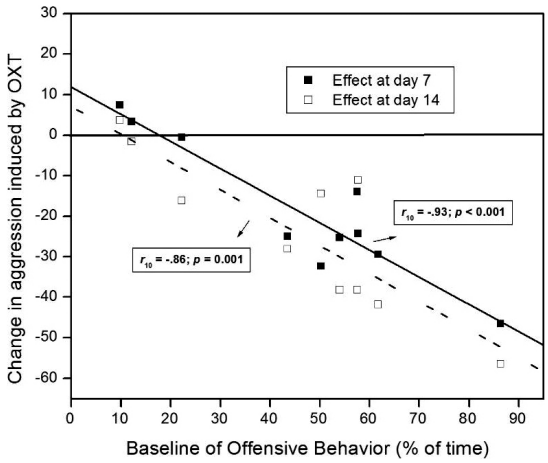

Figure 3. Correlation between the baseline level of offensive behavior and the relative change induced by oxytocin (OXT) at day 7 (filled squares, straight line) and day 14 (open squares, dashed line). Individual delta scores of aggression (y axis; difference between the behavioral score measured at day -1 and day 7, or between day -1 and day 14) are plotted with the individual duration of baseline offensive aggression (x axis, % of time in the 10 min test).

DISCUSSION

This study provides evidence that chronic central infusion of OXT suppresses intermale offensive behavior, particularly in animals with higher baseline level of aggression, while at the same time it enhances social explorative behavior. On the other hand, chronic infusion of the OXTR antagonist could be shown to specifically enhance introductory aggressive behavior (i.e. lateral threat), without affecting the display of agonistic contact or the total duration of the aggression. Surprisingly, the anti-aggressive and pro-explorative effects even persisted 7 days after the cessation of the chronic treatment, indicating protracted behavioral effects after a period of chronic OXT enhancement in the brain. Another interesting observation to note is that synthetic OXT specifically shortened the duration of the aggressive displays without significantly delaying the latency of the first attack. This suggests that OXT does not affect the initiation of an aggressive attack but rather the maintenance and/or termination aspects of offensive aggressive behavior. Moreover, the exogenously administered OXT selectively targeted social behavior components, without altering any of the other non-social behavioral categories evaluated during the resident-intruder test.

To our knowledge, this is the first chronic icv study reporting OXT-induced persistent behaviorally selective anti-aggressive and pro-explorative changes in male rats tested in a social conflicting context.

As extensively reported in the literature, exogenously administering OXT may alter the processing of and responding to social stimuli. In our current study, we defined social behavior as all types of interactive approaches and displays directed by the resident towards the intruder, aiming to either offend or explore. Our results revealed that chronic synthetic OXT infusion qualitatively re-shaped the social behavior profile, with a shift from offensive to social explorative behaviors. These findings are in line with our recent study in male WTG rats where acute pharmacological enhancement of brain OXT levels induced anti-aggressive and pro-social explorative effects that could be blocked by a selective OXTR antagonist (Calcagnoli et al., 2013). Moreover, deficits in social recognition, decreased social investigation, and increased offensive reactions have been reported in male mice when knocking out oxt or *oxtr* gene (Ferguson et al., 2001; Ferguson et al., 2000; Lee et al., 2008; Sala et al., 2013; Sala et al., 2011; Winslow et al., 2000; Winslow and Insel, 2002). However, there is evidence for a strong *oxtr* gene-dose dependency in terms of social exploration. A 50% reduction in the expression of the *oxtr* gene led to the same profound deficits in social behavior as that observed in *oxtr* $^{-/-}$ animals housed and tested under the same experimental conditions (Sala et al., 2011). It is worth noting that this was not observed for other behaviors such as aggression, for which the number of expressed OXTRs in *oxtr* $^{+/-}$ mice is compatible with normal functioning or is compensated for by other factors (Sala et al., 2013). These findings indicate that in males inactivation of the *oxtr* gene may affect specific behaviors in a dose-dependent manner: social exploration is particularly sensitive to even a partial reduction in *oxtr* gene expression, whereas the emergence of aggression may require complete inactivation of the *oxtr* gene. In our study,

the OXT-induced decreased duration of social aggression and increased duration of social exploration clearly appeared to be interdependent. The fact that the pro-social explorative properties of synthetic OXT have been reported more consistently in literature than anti-aggressive effects might favor a primarily pro-explorative action. However, causal relationship cannot be established from our correlational data, hence further studies should be conducted in order to prove whether OXT primarily reduces aggression with the consequence of enhancing social exploration, or vice versa.

To note, although dependent upon species, strain, hormonal state, social experience, as well as the brain region manipulated, many examples in the literature have indeed reported that an acute increase in CSF OXT potentiates social explorative activities (Insel, 1992; Witt et al., 1992), while activation of vasopressin receptors (AVPRs), especially in the anterior hypothalamus and lateral septum, promotes intermale aggressive behavior in hamsters and rats, respectively (Albers, 2012; Beiderbeck et al., 2007; Caldwell and Albers, 2004; Ferris et al., 1997). Haller and colleagues have also shown that CSF OXT levels directly correlate with the duration of social investigation, while changes in the measures of aggression significantly correlated only with CSF AVP level (Haller et al., 1996). Hence due to the strong molecular similarities between OXT and AVP and their potential cross-reactivity, the behavioral profile of the central AVPergic system should also be considered when investigating the primary functional role of OXT in modulating social behaviors. Moreover, dose-response curves, longer pharmacological manipulations and co-administration studies with OXT and OXTR or AVPR antagonist should be performed to verify behavior and receptor specificity, to disprove potential cross-reactivity and to assess the minimal dose needed to modulate a specific behavior, also for clinical translation.

Although our studies on male WTG rats have revealed a significant OXT-induced serenic profile, it is interesting to note that the latency to the first attack was not changed, neither in the present study, nor in our former acute administration study. In other words, synthetic OXT is ineffective in delaying the initiating phase of aggressive behavior, but selectively and potently inhibits the continuation of offensive displays. This selective action on primarily the consummatory phase of aggressive behavior suggests differences between the neuronal mechanism of this nonapeptide and other well-known serenic compounds such as the serotonin (5-HT) receptors type 1A and 1B agonists. This well-known class of serotonergics generally elicits anti-aggressive actions through both delaying the initiation and accelerating the termination of aggressive attack bouts, often in combination with shortened duration of total social engagements (de Boer and Koolhaas, 2005; Takahashi et al., 2011). Similarly, antagonism on AVPRs type 1B during intermale encounters resulted in a sharp reduction of the duration of aggressive behavior and olfactory investigation, as well as a significant increase of the latency to attack (Blanchard et al., 2004; Koolhaas et al., 2010).

Thus, the anti-aggressive effect of OXT seems to suggest a distinct mechanism of action, in which the reinforcement of positive/explorative social interactions may be responsible for the consequent attenuation of the hostile/offensive behaviors. Two explanations can be considered. (1) After the first aggressive action displayed by the resident, OXT may alter

the further processing of social information by, in the first place, reducing the saliency of negative/threatening cues of the intruder, with the consequence that social explorative behavior will increase. This hypothesis finds some support by several human and non-human studies reporting that OXT facilitated social approach behavior as a consequence of reduced amygdala responsiveness to social stimuli in general (Domes et al., 2007; Lukas et al., 2013), and decreased amygdala reactivity to social threat in particular (Coccaro et al., 2007; Kirsch et al., 2005; Viviani et al., 2011). (2) OXT might on the other hand affect the dopaminergic reward system with midbrain-striatal structures, such as the nucleus accumbens, being activated when social contact comes into play (Aragona et al., 2006). The pathways related to pro-social motivation and reward processing contain high levels of OXTRs (Insel and Shapiro, 1992), while furthermore OXT has been shown to facilitate dopamine release (Pfister and Muir, 1989). The dopaminergic system, when activated by OXT, might potentiate the positive valence of social interaction, directing the social decision–making network towards explorative approaches, rather than being implicated in the "winner effect" development (Schwartzer et al., 2013). Previous research has indeed shown that OXT facilitates affiliation and social attachment by inhibiting aggression and enhancing the value of social encounters in part by coactivation of dopaminergic circuits that are involved in motivation and reward (Campbell, 2008). As elevated inter-synaptic dopamine levels have been previously associated with increased OXT in the amygdala of dams (Johns et al., 2005), central infusion of the nonapeptide might reinforce the serenic effects and increase explorative contact in our rats via activation of dopaminergic reward circuits. Hence further research should locally investigate the interactive role of OXT with other neurotransmitters.

An intriguing and unexpected finding of the present study is that both the direction, the magnitude and the specificity of the behavioral effects still persisted 7 days after the cessation of the chronic icv infusion. Considering the very short half-life (about 28 min) of OXT in CSF (Veening et al., 2010) and the relatively short duration of the chronic manipulation (7 days), long-lasting effects as if due to persisting heightened levels of OXT would not have been expected. However, after chronic central infusion of OXT (100 ng/h for 10 days via an osmotic mini-pump), Insel and colleagues have reported a decrease in OXTR binding of as much as 95% at the time of pump removal, compared to artificial CSF-infused controls (Insel et al., 1992). As the reduction was observed in every brain region and as it remained for at least 24 h, it is likely that brain OXTRs are profoundly down-regulated as a consequence of sustained stimulation. However, it seems unlikely that such a compensatory neuromolecular change can explain our 7-day persistent anti-aggressive and pro-social effects. In fact, an OXTR down regulation/desensitization, possibly leading to reduced endogenous OXTergic signaling, would rather predict immediate 'withdrawal-like' pro-aggressive and anti-social effects. These rebound effects, however, may actually occur in the immediate withdrawal phase and requires testing the animals immediately after cessation of the treatment.

On the other hand, continuous infusion of synthetic OXT might have altered the transcription level of the nonapeptide in the hypothalamic production sites, i.e. the

paraventricular and supraoptic nuclei. It might also have elevated the background activity of slow-firing OXTergic neurons involved in the facilitatory control of the nonapeptide release (Freund-Mercier and Richard, 1984). Obviously, the current findings of enduring behavioral effects after a period of sustained enhancement of brain OXTergic signaling prompt future studies of potential treatment-induced alterations in the endogenous OXTergic system, at the level of OXTR expression or binding and mRNA peptide level or release patterns, keeping in mind that simultaneous compensatory alterations are likely to occur also in the AVPergic system. Moreover, although the different efficacies of the OXTergic manipulations between high and low aggressive animals may be amplified due to a rate-dependency and/or regression to the mean effect, it might be relevant to investigate further the link between individual treatment-induced alterations and the differences in trait-level of aggression and baseline properties of the OXTergic system.

Taken together, we report that a 7-day chronic infusion period with OXT selectively suppressed intermale offensive aggressive and enhanced social explorative behaviors in resident rats confronted with an unfamiliar intruder in their territory. On the other hand, chronic infusion of the OXTR antagonist increased introductory aggressive behavior. Moreover, the previously suggested inverse relationship between the trait-level of aggression and the anti-aggressive effects of exogenously administered OXT seems to be supported by the results of this chronic manipulation experiment. Finally, the persisting behavioral changes observed after OXT-treatment cessation suggest neuronal plasticity and prompt further studies to measure treatment-induced long-term alterations in the endogenous OXTergic system.

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4

OXYTOCIN MICROINJECTED INTO THE CENTRAL AMYGDALOID NUCLEI EXERTS ANTI-AGGRESSIVE EFFECTS IN MALE RATS

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ABSTRACT

We recently demonstrated that acute and chronic intracerebroventricular enhancement of brain OXT levels induces potent anti-aggressive and pro-social explorative effects during social challenges. However, the exact anatomical location in the brain where OXT exerts its action is still elusive. In the present study we targeted two critical brain areas, i.e., the central amygdala (CeA) and the dorsal raphe (DR), both containing high levels of OXT receptors (OXTRs) and constituting important nodes of the neural circuitry related to aggression. Behavioral effects of local micro-infusion of OXT and OXTR antagonist (alone and combined) were evaluated in resident male rats during confrontations with an unfamiliar male intruder. Our results show that OXT microinjected into the CeA markedly reduced resident's offensive behavior and facilitated social exploration, without affecting other non-aggressive behaviors. The receptor specificity of the behavioral effects was verified when a micro-infusion of a selective OXTR antagonist nullified the changes. Pharmacological blockade of CeA OXTRs per se was without clear behavioral effects suggesting that endogenous OXT within the CeA does not play a major inhibitory role on offensiveness. Anatomical specificity was indicated by the absence of behavioral effects when OXT was microinjected into more medial sub-regions of the amygdala. Likewise, within the DR neither OXT nor OXTR exerted significant effects on offensive aggression, while microinjection of the 5-HT_{1A} autoreceptor agonist in this region significantly suppressed aggression.

In conclusion, our results point at the CeA as an important brain site of action for the anti-aggressive and pro-social explorative effects induced by exogenous enhancement of brain OXT levels.

INTRODUCTION

Over the past decades, a number of studies have shown that the neuropeptide oxytocin (OXT) plays an essential role in regulating a variety of psychosocial functions in rodents and other mammals (Heinrichs et al., 2009; Neumann, 2008). It is crucially involved in social bonding and in-group co-operation, strengthening recognition memory for familiar conspecifics and increasing defensive behavior (Choleris et al., 2009; Hammock and Young, 2006; Winslow et al., 1993). It facilitates social approach by reducing anxiety and neuroendocrine stress/fear responses (Ebner et al., 2005; Heinrichs and Domes, 2008; Insel and Young, 2001; Knobloch et al., 2012). Moreover, it potentiates positive social interactions, increasing trust and reducing betrayal aversion in humans (Baumgartner et al., 2008; Kosfeld et al., 2005). In animals, OXT increases social exploration and decreases offensive behavior in animals (Calcagnoli et al., 2013).

Regarding social behaviors, our group recently demonstrated that both acute intracerebroventricular (icv) (Calcagnoli et al., 2013) and intranasal (Calcagnoli et al., 2014 in revision) administration of OXT reduces intermale offensive aggression and increases social exploration displayed by a resident rat towards an unfamiliar intruder. In addition, increased offensive aggression was reported in low aggressive residents after icv administration of a selective OXT receptor (OXTR) antagonist (Calcagnoli et al., 2013).

However, due to the widespread expression of OXTRs within the brain (Barberis and Tribollet, 1996), and especially within structures that encompass the social behavior neural network (Goodson, 2005), it is still unclear where exactly OXT mediates these socio-behavioral effects. In the currently described experiments, we tested the hypothesis that two core components of the social behavior network, i.e. the central amygdala (CeA) and the dorsal raphe (DR) nucleus, are important sites where central OXT regulates the behavioral response to threatening social challenges, such as the confrontation of a resident rat with an intruder. Several studies in humans and animals seem to support this hypothesis. From neuroimaging studies in men, intranasal OXT application has been found to dampen the amygdaloid neuronal activation and to reduce the functional connectivity between amygdala and brainstem regions involved in automatic fear reactivity, thereby attenuating the emotional and physiological response to fearful and threatening social stimuli (Coccaro et al., 2007; Gamer et al., 2010; Kirsch et al., 2005). However, contrary to these findings, in healthy women OXT appeared to increase amygdala reactivity to scenes depicting social and non-social threat (Domes et al., 2010; Lischke et al., 2012), suggesting a possible sexual dimorphism in the neuronal effects of OXT. In borderline personality disordered patients, where increased anger, aggression and impaired affective regulation have been associated with amygdala hyperactivity during confrontation with frightening situations (Donegan et al., 2003; Herpertz et al., 2001), OXT administration has been shown to normalize these abnormal behavioral and neural patterns (Bertsch et al., 2013).

In rodents, neuronal activation of the amygdala has been associated with agonistic encounters (Haller et al., 2006; Pan et al., 2010), while reduction of fear and social stress-

related behaviors has been found by optogenetically stimulating endogenous OXT release, particularly in the CeA (Knobloch et al., 2012). It is in these areas that the majority of OXT immunoreactive (OXT-ir) cells (Sofroniew et al., 1983) and OXT binding sites (Freund-Mercier et al., 1987; Veinante and Freund-Mercier, 1997) have been detected.

In lactating female rats acute bilateral infusion of OXT in the CeA decreases the frequency of defensive biting and frontal attack towards a male intruder (Consiglio et al., 2005), which is in line with the observation that local infusion of OXTR antagonist 4 h prior to the resident-intruder (RI) test significantly increases the fighting behavior of lactating rats (Lubin et al., 2003). However, this is in contrast with observations in female hamsters (Ferris et al., 1992) and with the positive correlation found between OXT release in the CeA and the level of maternal defense in female Wistar rats selected for high anxiety-related behaviors (Bosch et al., 2005).

Much less is known about the OXTergic activity in the DR. This nucleus is considered to be one of the core structures of the brainstem controlling aggression and violence (Takahashi and Miczek, 2013), via its extensive, serotonin (5-HT) containing, efferent projections to various forebrain nodes within the social behavior network (Amat et al., 2005; Jacobs and Azmitia, 1992; Nakamura et al., 2008; Takahashi et al., 2010; van der Vegt et al., 2003). Hence, dysregulation of the DR 5-HTergic system has been implicated in the pathophysiology of affective disorders including anxiety, depression and suicidal (auto)aggressive behavior (Arango et al., 2002; Stockmeier, 1997). Recently, a high density of OXTRs expressing cells was reported in the DR. These receptors have been co-localized with 5-HT neurons and, at the level of the median raphe nucleus, OXT infusion was reported to increase 5-HT release (Yoshida et al., 2009). Moreover, another study has shown that the activation of OXTRs could stimulate the firing of DR 5-HT neurons (Spaethling et al., 2014). However, despite the evidence of functional OXTergic binding sites in the DR, no study has locally investigated the socio-behavioral effects induced by manipulation of OXTergic activity.

The aim of the present study, therefore, has been to pharmacologically manipulate OXT levels in the CeA and in the DR of male resident rats, and to evaluate the behavioral effects during a direct intermale confrontation.

MATERIALS AND METHODS

Animals and housing condition

As in our previous etho-pharmacological studies (Calcagnoli et al., 2013; Calcagnoli et al., 2014 in revision; Calcagnoli et al., 2014), male wild-type Groningen (WTG) rats (*Rattus Norvegicus*) were used as experimental subjects (4.5 months old and average weight 400 ± 50 g). This strain of rats descended from pairs of wild-trapped specimens that were outbred under conventionalized conditions for over 35 generations in our laboratory (de Boer et al., 2003). Adult male specimens of this rat strain are very suitable for intermale aggression research as substantially higher levels of offensive behavior are displayed compared to virtually all other commercially available laboratory strains (de Boer et al., 2003). After 120 days of age, each

resident was housed in large observation cages (80 × 55 × 50 cm) together with an oviduct-ligated but gonadally-intact female to prevent social isolation and allow normal sexual activity, required to stimulate territorial behavior. Throughout the experimental period, the animals were maintained under standard housing conditions (12 : 12 h light/ dark photoperiod, lights off at 13:00 h; ambient temperature 21 ± 2ºC; humidity 50 ± 5%) with *ad lib*. access of food (Hope Farms, RMH-B) and water. All experimental and behavioral procedures (Figure 1) were approved by the Animal Ethics Committee on Care and Use of Laboratory Animals (DEC 5824) of Groningen University and were conducted in agreement with Dutch laws (Wet op de Dierproeven 1996) and European regulations (Guideline 86/609/EEC).

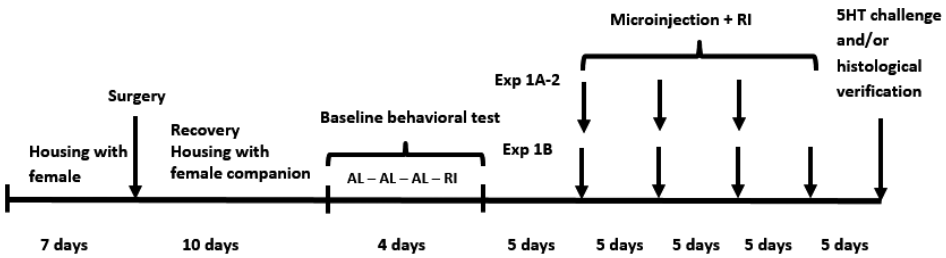

Figure 1. Schematic chronologic representation of the experimental and behavioral procedures. Briefly, each experimental animal was housed in their own cage with a female for 7 days. Cannula implantation was surgically performed and the rat had 10 days to recover, housed with the same female companion. During 4 days after the recovery period, we performed the baseline behavioral test to assess the pre-treatment individual ethogram (3 days of attack latency (AL) test and one day of resident-intruder (RI) test). Based on the displayed level of offensive behavior and social exploration, groups were created (3 (exp. 1A and 2) or 4 (exp. 1B) groups depending on the number of pharmacological treatments planned in the specific experiment). In each experiment, all animals underwent all the treatments with 5 days washout in between sessions. 10 min after each microinjection, the RI test was performed to assess the treatment-induced ethogram. At the end of the experimental period, a serotonin (5-HT) challenge and/or histological verification of the cannula placement was conducted.

Surgical procedures

All stereotaxic surgical procedures were performed under isoflurane/oxygen anesthesia, using sterile conditions. To minimize discomfort directly linked to the surgery, analgesic (0.1 ml Finadyne) and antibiotic (0.25 ml Penicillin) compounds were subcutaneously injected. Stainless steel guide cannulas (CeA: custom-made 15 mm, 23-gauge cannula; DR: 22-gauge, C313, Plastic One, Roanoke, VA) were placed stereotactically, according to the brain map of Paxinos and Watson (6th edition, 2007) with the cannula tip 2 mm above the target area (coordinates for the bilateral cannulation into the CeA: 2.2 mm posterior to bregma, ± 4.3 mm lateral to midline and 6.3 ventral to the skull, with a tooth bar at -3.3 mm. Coordinates for the cannulation into the DR: 7.6 mm posterior to bregma, 2.0 mm lateral to midline, under a 12º lateral angle and 6.8 ventral to the dura mater, with a tooth bar at -3.3 mm). The cannulas were affixed to the skull with anchoring screws and dental cement. Stylets were inserted into the cannulas to maintain patency. For the first 24 h post-operation, rats were singly housed in their

home cage, and then again housed together with the same tubal-ligated female companion of the pre-surgery period. During the 10-day recovery period, the weight and the well-being of the animals were daily checked. Moreover, animals were habituated to the infusion procedure in order to avoid non-specific stress responses during the experimental sessions.

Behavioral characterization

After full recovery, a baseline ethogram was assessed for each animal using a RI test, according to the standard protocol described by Koolhaas and colleagues (Koolhaas et al., 2013; Koolhaas et al., 1980; Olivier et al., 1995). Unfamiliar naïve male Wistar rats (Harlan, The Netherlands, 4 months old, average body weight 300 ± 50 g) socially housed in groups of five in transparent macrolon type IV cages (60 × 60 × 20 cm) were used as intruder animals. The lower weight of the intruder facilitated the establishment of resident's dominance. The female partner of the experimental rat was removed from the observation cage approximately 30 min prior to the start of the test.

As previously described (Calcagnoli et al., 2013), the baseline behavioral testing was performed during four consecutive days. During the first three days, an attack latency test was performed by introducing an intruder into the home cage of the experimental resident animal. The test was terminated shortly after the occurrence of the first full attack. When the resident failed to attack within the first 10 min of testing, the attack latency time was scored as 600 s and the test was terminated. On the fourth day, the interaction between the resident and the intruder was videotaped for the 10 min following either the first attack or the introduction of the intruder, in case of no attack (complete RI test). Videos were analyzed and all behaviors expressed within the 10-min observation were evaluated. Specific behavioral elements were grouped into the following broad behavioral categories in order to promote a clear representation of the data: (1) o*ffensive behavior* (lateral threat, clinch, keep down, chase, upright posture); (2) *social explorative behavior* (moving towards, investigation and ano-genital sniffing of the intruder, crawl over, social grooming); (3) *non-social exploration* (ambulation, rearing, sniffing, scanning, digging); (4) *inactivity* (sitting, lying, freezing) and (5) *self-grooming* (washing, scratching). Each behavioral category was graphically presented as a percentage of the total duration of the confrontation (10 min). Altogether, the behavioral elements recorded covered 100% of the observation time. This facilitates an unbiased interpretation of the results (Koolhaas et al., 2013).

The complete RI test (as performed on the fourth day of the baseline testing) was repeated during the experimental phase, 10 min after any pharmacological manipulation. All behavioral tests were performed within the first 3-4 hours of the dark (active) phase in a dimmed light conditions, to avoid effects of circadian hormonal fluctuation and light exposure (Devarajan et al., 2005).

Local pharmacological manipulation

Experimental groups were matched according to the level of offense and social explore scored during the baseline behavioral test. Similar to our previous acute pharmacological

manipulation (Calcagnoli et al., 2013), the animals of each experiment received different treatments and were randomly assigned to the possible different sequences. The researcher was blind to the experimental treatments. 5-day washout period was applied between the different treatment sessions. This time interval is considered to be long enough for the complete clearance of the compounds, based on our previous pilot study in which the restoration of pretreatment aggression level occurred within the above-mentioned period. Moreover, literature amply reported the short life span of OXT in the brain (Jones and Robinson, 1982; Mens et al., 1983; Robinson and Jones, 1982).

Drugs were slowly infused in conscious rats using a gauge cannula protruding 2.0 mm beyond the cannula tip and connected to 10-µL Hamilton microsyringes by polyethylene (PE-20) tubing. The volume was infused at the rate of 0.37 µL/min by an automated syringe pump (KD Instruments). The injection needle was retained within the implanted cannula for 20 s following the infusion to maximize diffusion and to prevent backflow of drug along the cannula track.

Pharmacological manipulation of the Central Amygdaloid nuclei

Experiment 1A

20 male WTG rats were used to study the behavioral effects of acute bilateral infusion into the CeA. Infused were: vehicle (0.3 µl per side of sterilized saline 0.9%, Versylene® Fresenius Kabi, France), synthetic OXT ($C_{43}H_{66}N_{12}O_{12}S_2$; MW 1007.19; Tocris, Germany) (30 ng/0.3 µl per side), and a highly selective OXTR antagonist$_{non\text{-}peptidergic}$ (L368.899; $C_{26}H_{42}N_4O_5S_2$; MW 591.23; Tocris, Germany) (250 ng/0.3 µl per side). The selected dose of OXT is within the range recently reported as effective in social behavioral manipulations (10-1000 ng) (Lee et al., 2005; Lukas et al., 2011). The dose of OXTR antagonist was consequently calculated as equimolar.

Experiment 1B

In 18 animals we bilaterally infused into the CeA vehicle, synthetic OXT, highly selective OXTR antagonist$_{non\text{-}peptidergic}$, and a co-administrated infusion in which OXTR antagonist (250 ng/0.15 µl per side) was infused 10 min before OXT (30 ng/0.15 µl per side). In the case of co-administrated treatment, the infused volume of each microinjection has be halved (0.15 µl) in order to maintain the total volume infused into the central amygdalae to 0.3 µl each side. This experiment was performed in order to replicate the outcome of experiment 1A and verify the receptor-specificity of the OXT-induced effects.

Pharmacological manipulation of the Dorsal Raphe nucleus

Experiment 2

9 male WTG rats were used to study the behavioral effects of acute infusion into the DR. Here again we used vehicle (0.5 µl), synthetic OXT (0.1 µg/0.5 µl), and the selective OXTR antagonist$_{non\text{-}peptidergic}$ (0.75 µg/0.5 µl). This dose has directly been calculated from

the lowest effective (both as anti-aggressive and pro-social) dose in the icv dose-response study (1 µg/5 µl) (Calcagnoli et al., 2013) adjusting for the infused volume (0.5 µl).

Verification of cannula placement and immunostaining

At the end of the experimental period, all animals were deeply anesthetized using CO_2 and perfused through the ascending aorta first with heparin (500 U/ml; 300 ml) in 0.9% saline solution, followed by 300 ml of 4% paraformaldehyde in 0.1M phosphate buffer solution (PBS). Brains were collected, post-fixed overnight in a solution of 4% paraformaldehyde in 0.1M PBS (pH = 7.4) at 4°C. After dehydratation in 30% sucrose solution (usually 48 h), brains were frozen and stored at -80°C until slicing in 25 µm coronal sections using a cryostat microtome. Cannula placement was verified using cresyl violet staining. Only animals with the needle tip located bilaterally into the CeA (experiments 1A and 1B) or into the DR (experiment 2), and without damage to the target tissue were included in the analysis.

In particular, since experiment 2 was based on the assumption that potential OXT-induced behavioral effects could be mediated by activation of 5-HTergic cells expressing OXTRs in the infused area of the DR, we indeed verified the presence of 5-HTergic neurons in the proximity of the cannula tip. For this purpose both a behavioral (A) and an immunohistochemical (B) approach was employed.

(A) Five days after the last treatment session (experiment 2), the behavioral profile of the residents was assessed in absence of treatment. As no difference was found compared to the pre-treatment baseline ethogram, a highly selective 5-HT_{1A} receptor ligand, S-15535 (4-(benzodioxan-5-yl)1-(indan-2-yl) piperazine; Servier, France) (25 µg/0.5 µl) was administered through the same cannula as used for the OXT manipulation. This was done 10 min before assessing the behavioral effects by the RI test. This compound behaves in vivo as a competitive antagonist at postsynaptic 5-HT_{1A} receptors and as an agonist at 5-HT_{1A} autoreceptors (Millan et al., 1993). In line with previous pharmaco-behavioral studies performed on male WTG resident rats (de Boer and Koolhaas, 2005), a strong anti-aggressive response to this 5-HT_{1A} receptor ligand would most likely confirm the presence of 5-HT neurons in the proximity of the cannula tip.

(B) From the animals of experiment 2, free-floating sections including the DR were collected and subsequently immunostained for 5-HT in order to identify 5-HT neurons in the proximity of the cannula. After pre-incubation overnight in 5% normal horse serum at 4°C, sections were incubated with the primary antibody (1:50.000, mouse anti-5-HT, Leger, France) for 3 hours at 37°C, 3 hours at room temperature and 3 days at 4°C, followed by incubation with biotinylated horse anti-mouse immunoglobulin (1:500, Vector Laboratories, CA,USA) for 2 hours at room temperature. Subsequently, sections were stained in 3,3'-diaminobenzidine (DAB) solution (15 mg DAB and 150 mg nickel ammonium sulphate in 100 ml Millipore water with 0.2% H_2O_2) for 5 min. In between incubation steps and after coloring, sections were rinsed in 0.1 M tris-buffered saline. Finally sections were mounted on slides (Figure 2).

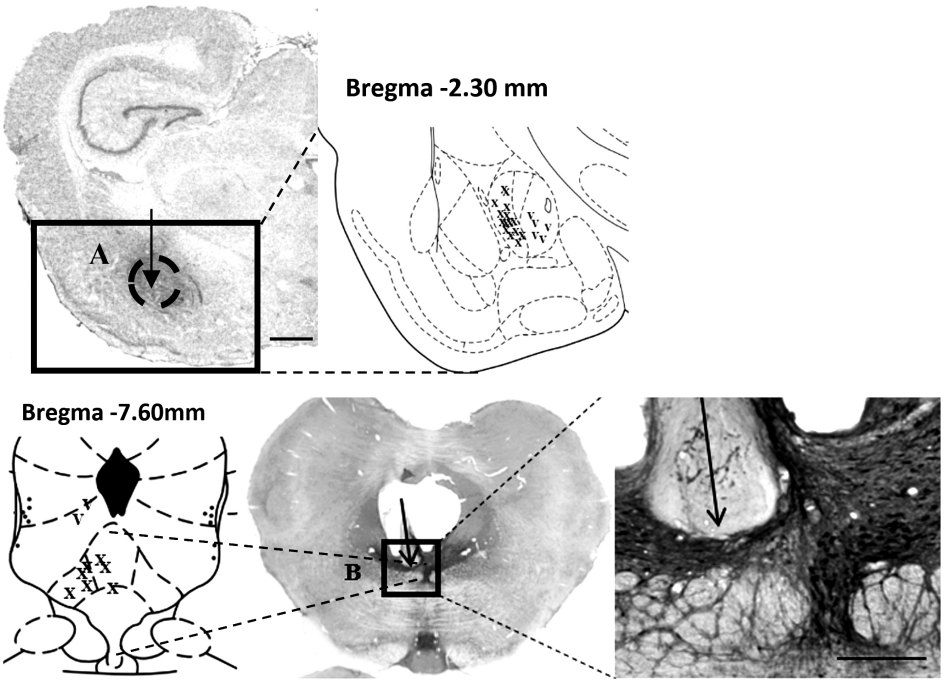

Figure 2. Schematic representation of the sites of cannula implantation within the CeA (A; upper panel), and in the dorsal raphe (B; lower panel) of rats used in the microinjection experiments as verified by histological examination. The "x" indicates individual injector tip placement of the subjects that were used in the statistical analysis, while the "v" indicates those of the rats that were excluded from the data. The magnification of picture B shows serotonin immunostained cells (dark dots) in proximity of the cannula tip (black arrow) in the dorsal raphe nucleus. Scale bar = 1 mm.

In 5 animals of experiment 1A the tips of the cannula were located in a more medial sub-region of the amygdalae, and therefore the related behavioral data were analyzed and presented separately (experiment 1C). From experiment 2 we excluded 2 animals because of cannula misplacement.

In conclusion, the final group size of each experiment was the following: N = 15 for experiment 1A, N = 18 for experiment 1B, and N = 7 for experiment 2.

Data analysis

Data are presented as group means + SEM of the time spent in each behavioral category (indicated as percentage of the total 10 min RI test) and of the attack latency time (expressed in seconds). Treatment-induced behavioral effects were statistically tested by using SPSS for Windows (version 20: SPSS Inc., Chicago, IL, USA).

Data from experiment 1A, 1B and 2 were analyzed using factorial repeated measures analyses of variance (ANOVA). The ANOVA design consisted of one within-subject variable with three (exp. 1A and 2: vehicle, OXT, and OXTR antagonist) or four (exp. 1B: vehicle,

OXT, OXTR antagonist, and co-administration) treatment levels. To check for a potential effect of the treatment sequence, we first conducted ANOVA's with moreover one between-subjects factor, i.e. the order of the treatments. Due to the limited N size of the resulting experimental groups (in case of the first experiment 1A, these would be six), we chose the between-subject factors to consist of only three levels: (1) order of treatment starting with vehicle; (2) order of treatment starting with OXT; and (3) order of treatment starting with the OXTR antagonist. The animals of experiment 1B that received the co-administration as first treatment were coded based on the second treatment. Assumption of homogeneity of variance (Level's test) and equality of variance (Box's test) were checked. To account for possible violations of the sphericity assumption for factors with more than two levels, Huynh–Feldt adjusted *p* values and the epsilon correction factor are reported, together with the unadjusted degrees of freedom and F values. For all analyses, the partial eta-squared effect sizes are reported with $\eta^2 < 0.06$ reflecting a small effect; $\eta^2 \geq 0.06$ a medium effect; and $\eta^2 \geq 0.14$ a large effect.

Nonparametric Friedman's test for comparing related samples was used to separately analyze the ethogram of the excluded cases from experiment 1A (N = 5, misplaced cannula).

In case of significant overall effects, the analysis was followed by *post hoc* pairwise t-test comparisons in order to reveal specific differences in treatment conditions. In case more than one behavioral category was altered by OXT infusion, we tested for potential interdependency between the induced changes by the use of Pearson correlation.

Moreover, in experiment 2, Wilcoxon signed-rank test was used to verify the behavioral responsiveness to the 5-HT_{1A} receptor agonist S-15535 as compared to vehicle.

All comparisons with a *p* value ≤ 0.05 were considered to be statistically significant.

RESULTS

Pharmacological manipulation of the Central Amygdaloid nuclei

Experiment 1A

First, multivariate tests revealed that there is no interaction between treatment and order of treatment (Wilk's Lambda $p > 0.05$). The same holds for the individual categories [Offensive; $F_{2,12} = 0.11$, $p = 0.89$, $\eta^2 = 0.02$. Social exploration; $F_{2,12} = 0.78$, $p = 0.48$, $\eta^2 = 0.11$. Non-social behavior; $F_{2,12} = 0.17$, $p = 0.85$, $\eta^2 = 0.03$. Immobility; $F_{2,12} = 0.66$, $p = 0.53$, $\eta^2 = 0.10$. Self-grooming; $F_{2,12} = 0.33$, $p = 0.70$, $\eta^2 = 0.05$. Attack latency time; $F_{2,12} = 0.33$, $p = 0.72$, $\eta^2 = 0.05$].

Having excluded the influence of sequence on treatment effects, we present the results from the within-subject analyses of the treatment effects. An overall treatment effect was found in the categories of offensive behavior [$F_{2,28} = 11.93$, $p < 0.001$, $\eta^2 = 0.46$], social exploration [$F_{2,28} = 9.21$, $p = 0.001$, $\eta^2 = 0.40$], and non-social behavior [$F_{2,28} = 5.00$, $p < 0.05$, $\eta^2 = 0.26$, $\varepsilon = 0.83$] (Figure 3). Among the elements constituting the category of offensive aggression, an overall treatment effect was found for the expression of lateral threat [$F_{2,28} = 3.97$, $p < 0.05$, $\eta^2 = 0.22$].

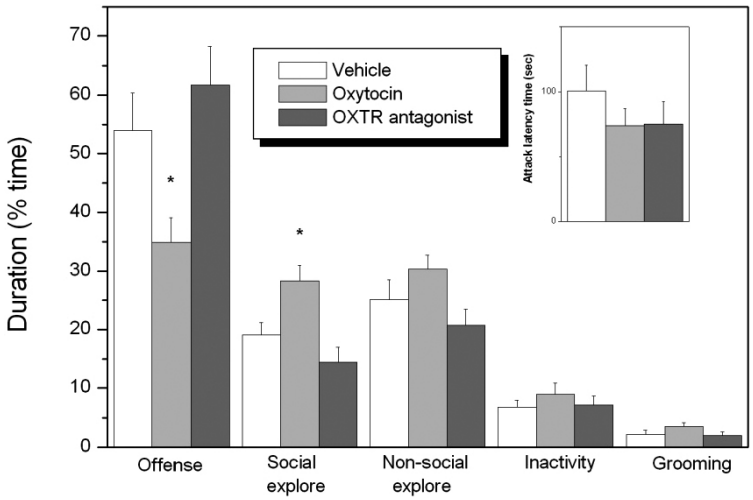

Figure 3. Behavioral changes induced by pharmacological manipulation of the central amygdala oxytocinergic system. Male resident wild-type Groningen rats were exposed to an unfamiliar male intruder Wistar rat 10 min after acute bilateral administration into the central amygdaloid nuclei of vehicle (saline; 0.3 µl per side), oxytocin (OXT; 30 ng/0.3 µl per side) or oxytocin receptor antagonist (OXTR antagonist; 250 ng/0.3 µl per side). Insert graph depicts the treatment effects on the attack latency time. Data are presented as mean + SEM (N = 15). *p < 0.05 indicates a significant difference as compared to the vehicle.

When examining the effects of the single treatments, pairwise comparisons showed that OXT infusion decreased the total duration of the offense display as compared to both vehicle ($p < 0.01$), and OXTR antagonist ($p < 0.001$). In particular, OXT selectively reduced the lateral threat as compared to vehicle ($p < 0.05$) and OXTR antagonist ($p < 0.01$). Moreover, the nonapeptide intensified the expression of social exploration towards the intruder as compared to both vehicle ($p < 0.05$) and OXTR antagonist ($p < 0.001$). In addition, animals receiving OXT infusion showed more non-social behavior as compared to OXTR antagonist ($p = 0.001$).

To note, the decrease of aggression induced by OXT as compared to the vehicle condition is appeared to be correlated with the OXT-induced increase of social exploration ($r_{15} = -.50$, $p = 0.05$).

Experiment 1B

Multivariate tests indicated that there is no interaction between treatment and the treatment order (Wilk's Lambda $p > 0.05$). For the individual categories significance was lacking as well [Offensive; $F_{2,15} = 2.31$, $p = 0.13$, $\eta^2 = 0.23$. Social exploration; $F_{2,15} = 0.02$, $p = 0.98$, $\eta^2 = 0.003$. Non-social exploration; $F_{2,15} = 3.23$, $p = 0.08$, $\eta^2 = 0.30$. Immobility; $F_{2,15} = 3.19$, $p = 0.07$, $\eta^2 = 0.29$. Self-grooming; $F_{2,15} = 3.62$, $p = 0.70$, $\eta^2 = 0.05$. Attack latency time; $F_{2,15} = 0.49$, $p = 0.62$, $\eta^2 = 0.06$].

Having excluded the influence of sequence on treatment effects, we present the results from the within-subject analyses of the treatment effects. Overall treatment effects were found

in the following behavioral categories: offensive behavior [$F_{3,51} = 7.03$, $p < 0.001$, $\eta^2 = 0.29$], social explorative behavior [$F_{3,51} = 11.41$, $p < 0.001$, $\eta^2 = 0.40$], and attack latency time [$F_{3,51} = 4.00$, $p < 0.05$, $\eta^2 = 0.19$, $\varepsilon = 0.66$] (Figure 4). Among the elements in the offensive category, an overall treatment effect was found in the lateral threat [$F_{3,51} = 2.92$, $p < 0.05$, $\eta^2 = 0.15$].

When examining the effects of the single treatments, pairwise comparisons showed that OXT infusion decreased the total duration of the offense display as compared to vehicle ($p < 0.05$), OXTR antagonist ($p < 0.001$), and co-administration group ($p < 0.001$). In particular, OXT selectively reduced the lateral threat as compared to vehicle ($p < 0.05$), OXTR antagonist ($p < 0.05$), and co-administration group ($p < 0.05$). Moreover, the nonapeptide intensified the expression of social exploration towards the intruder as compared to vehicle ($p < 0.05$), OXTR antagonist ($p < 0.001$), and co-administration group ($p < 0.001$). The attack latency time was significantly shortened by OXTR antagonist as compared to both vehicle ($p < 0.05$), and OXT ($p < 0.05$).

Here again, the decrease of aggression induced by OXT as compared to the vehicle condition turned out to be correlated with the OXT-induced increase of social exploration ($r_{18} = -.56$, $p = 0.01$).

Interestingly, in animals (N = 5) in which the cannula placement has been found to be bilaterally incorrect (in a more medial sub-region of the amygdalae, see "v" in Figure 2A) we observed only a trend towards significance in the overall treatment effects in the social

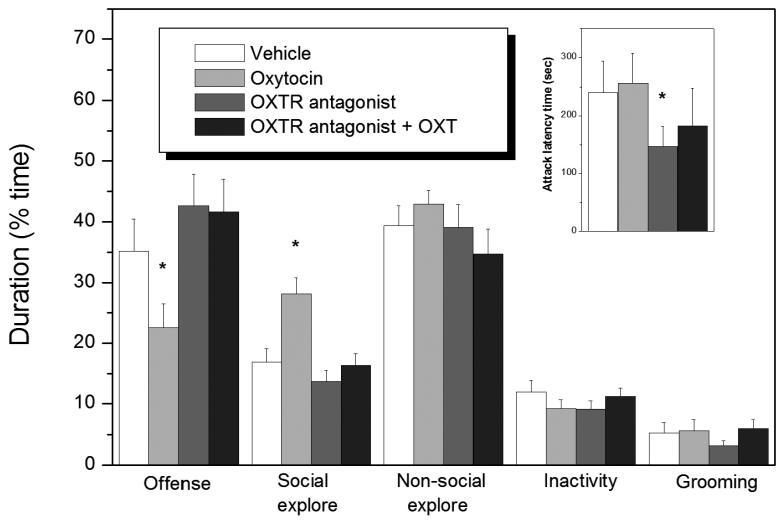

Figure 4. Behavioral changes induced by pharmacological manipulation of the central amygdala oxytocinergic system. Male resident wild-type Groningen rats were exposed to an unfamiliar male intruder Wistar rat 10 min after acute bilateral infusion into the central amygdaloid nuclei of vehicle (saline; 0.3 µl per side), oxytocin (OXT; 30 ng/0.3 µl per side), oxytocin receptor antagonist (OXTR antagonist; 250 ng/0.3 µl per side), or combination of OXTR antagonist and OXT (250 ng/0.15 µl + 30 ng/0.15 µl). Insert graph depicts the treatment effects on the attack latency time. Data are presented as mean + SEM (N = 18). *p < 0.05 indicates a significant difference as compared to the vehicle.

explorative category [$\chi 2 = 5.20$, df = 2, $p = 0.07$]. In particular, the social exploration in the OXT-treated animals was found to be higher as compared to the vehicle group ($p < 0.05$).

Pharmacological manipulation of the Dorsal Raphe nucleus

Experiment 2

Multivariate tests indicated that there is no interaction between treatment and the treatment order (Wilk's Lambda $p > 0.05$). The same holds for the individual behavioral categories and elements. The results from the within-subject analyses of the treatment effects revealed that pharmacological manipulation of the DR OXTergic system did not induce changes in any of the analyzed behavioral categories (Figure 5). However, when evaluating the behavioral elements constituting the category of offense, we reported only a trend towards significance in the overall treatment effect of keep down [$F_{2,12} = 4.03$, $p = 0.06$, $\eta^2 = 0.40$]. In particular, *post hoc* tests revealed that OXT tended to increase its duration as compared to vehicle ($p = 0.05$).

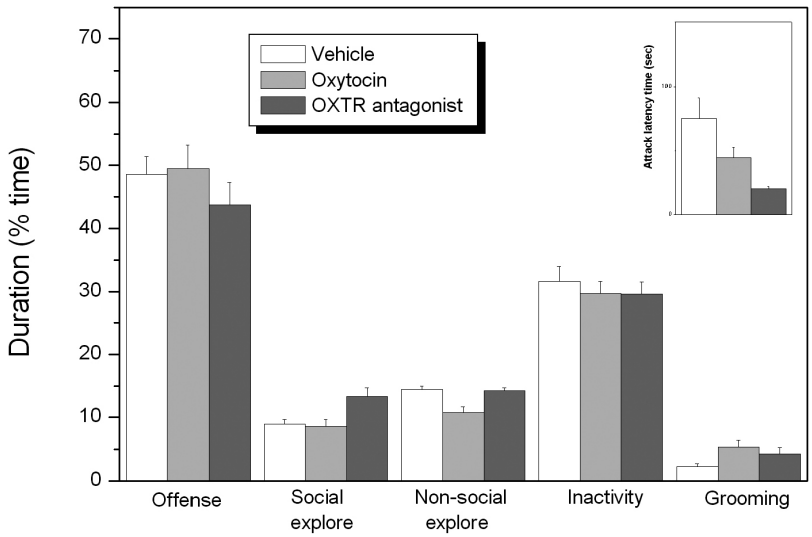

Figure 5. Behavioral changes induced by pharmacological manipulation of the dorsal raphe oxytocinergic system. Male resident wild-type Groningen rats were exposed to an unfamiliar male intruder Wistar rat after acute infusion into the dorsal raphe nucleus of vehicle (saline; 0.5 µl), oxytocin (OXT; 0.1 µg/0.5 µl) or oxytocin receptor antagonist (OXTR antagonist; 0.75 µg/0.5 µl). Insert graph depicts the treatment effects on attack latency time. Data are presented as mean + SEM (N = 7).

In contrast, acute infusion of 5-HT_{1A} receptor agonist decreased offensive behavior ($z = -2.37$, $p < 0.05$), enhanced self-grooming ($z = -2.12$, $p < 0.05$) (Figure 6). In particular, the duration of all the offensive elements, except for up-right posture, was reduced after microinjecting the 5-HTergic ligand (lateral threat $z = -2.20$, $p < 0.05$; keep down $z = -2.19$, $p < 0.05$; chase $z = -2.37$, $p < 0.05$).

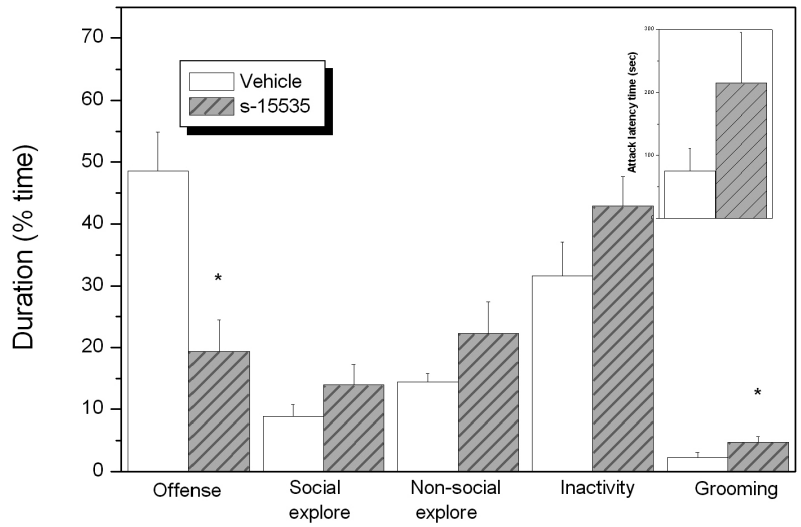

Figure 6. Behavioral changes induced by pharmacological manipulation of the dorsal raphe serotonin(5-HT)ergic system. Male resident wild-type Groningen rats were exposed to an unfamiliar male intruder Wistar rat after acute infusion into the dorsal raphe nucleus of either vehicle (saline; 0.5 µl), or S-15535, a selective 5-HT_{1A} presynaptic auto-receptor agonist (25 µg/0.5 µl). Insert graph depicts the treatment effects on attack latency time. Data are presented as mean + SEM (N = 7). *$p < 0.05$ indicates a significant difference in comparison with vehicle.

DISCUSSION

This study presents the CeA as an important brain site where pharmacological enhancement of OXT selectively regulates socio-behavioral expressions. In particular, we showed that microinjection of synthetic OXT within this brain region strongly suppresses the duration of intermale offensive behavior without delaying the latency to the first attack, and that it increases social explorative behavior. Both socio-behavioral effects are entirely blocked when the binding of the exogenously applied nonapeptide to OXTRs is impeded by pretreatment with a selective OXTR antagonist. On the other hand, infusion of the OXTR antagonist alone fails to show pro-aggressive changes, except for shortening the latency to the first attack in one of the experiments. No other behavioral category is affected by OXTergic pharmacological manipulation of the CeA.

Furthermore, no clear behavioral effects are revealed when manipulating the OXTergic system within the DR, while activating 5-HT_{1A} autoreceptors in this area is highly effective in suppressing aggression.

In line with our previous acute icv infusion (Calcagnoli et al., 2013; Calcagnoli et al., 2014) and intranasal application studies (Calcagnoli et al., 2014 in revision), "pro-social" modulatory properties have been found when synthetic OXT was infused into the CeA of male residents, although the unaltered behavioral profile of OXTR antagonist-treated animals did not suggest a direct regulating involvement of the local endogenous OXTergic system.

As described earlier, OXT-induced changes are specific for social behaviors, i.e. social offense and social explore. The ability of simultaneously modulating both behaviors in an opposite direction (decreasing aggression and increasing exploration) might be associated with the ability of OXT to both suppress and enhance local neuronal activation in response to negative or positive social stimuli (Gamer et al., 2010). Interestingly, in the CeA of rats, Huber and colleagues found two distinct groups of neurons differently responsive to a highly selective OXTR agonist: one group in the lateral and capsular areas that was excited by OXTR activation, and another in the medial area that was inhibited by activation of OXTRs but excited by vasopressin receptors AVPRs type 1A (Huber et al., 2005). Furthermore, due to the findings of γ-aminobutyric acid (GABA)–positive staining in the CeA and of GABAergic projections from its lateral/central part to the medial one, the inhibitory effects of OXTR activation has been thought to be mediated by GABA transmission. Several studies have verified this interplay showing that OXT application potentiates GABAergic inhibitory post-synaptic currents from the amygdala to more distal effector sites (Huber et al., 2005; Terenzi and Ingram, 2005; Viviani et al., 2010). This inhibition has been shown to prevent the expression of fear-induced autonomic and behavioral responses, normally under control of connected regions, such as periacqueductal gray and prefrontal cortex (LeDoux et al., 1988; Resstel et al., 2006). Therefore, although the specific role of GABA in aggression appears counterintuitive and dose-dependent (de Almeida et al., 2004; Earley and Leonard, 1977; Miczek et al., 2002), we cannot exclude that the reduction of aggression induced by OXT may be mediated by OXT-enhanced inhibitory neurotransmission.

Of increasing interest in the understanding of social behaviors is also the evidence of co-orchestrating action in the mesolimbic system between OXT and dopamine (DA) (Baskerville and Douglas, 2008; Liu and Wang, 2003). Depending upon the DA availability in the amygdala, central OXT has been seen to shape the amygdala-driven responses to social stimuli (Sauer et al., 2013). In addition, OXT directly activates the social reward circuit via increasing DA level in the nucleus accumbens (Melis et al., 2009), and via activating OXTRs-containing inputs from the DR, which provides 5-HT innervation to the nucleus accumbens (Dolen et al., 2013).

Evidence of such coordinated activity in rodents and in humans invites to speculate about a common neuronal circuit that may be differentially altered by OXTergic manipulation depending on the context, leading to differential behavioral outputs. It would therefore be challenging for future research to qualitatively and quantitatively monitor the local release of neurotransmitters after OXT infusion during different behavioral tests (Bosch et al., 2007). Such an approach, accompanied by a detailed analysis of the sequential behavioral elements, would give a better neuro-behavioral characterization of each phase of the aggressive display.

The morphological heterogeneity that characterizes the amygdaloid region in general, and the CeA in particular (Huber et al., 2005), may explain the anatomical specificity of the OXT-induced effects that we observed in rats cannulated in the central sub-region and rats cannulated in a more medial sub-region of this nuclei. Considering the high density of AVPRs reported in the medial portion of the CeA of rats (Huber et al., 2005)

and the binding affinity of synthetic OXT on AVPRs (Barberis et al., 1992), we cannot exclude that unselective binding of the nonapeptide to AVPRs may be the reason of the absence of OXT-induced effects in the rats cannulated in the more medial sub-region of the amygdalae. Immunohistochemical visualization of local OXTRs and AVPRs in the infusion areas should always be employed to confirm the selectivity of the manipulation. In our studies, the receptor selectivity of OXT-induced behavioral changes in the CeA has been verified by the fact that both anti-aggressive and pro-social changes were entirely blocked when the binding of exogenous OXT to the OXTRs was impeded by pretreatment with a selective OXTR antagonist.

In contrast to the findings in the CeA, the behavioral involvement of OXTRs in the DR failed to be proven, although the small group size requires caution when drawing conclusive statements. The rationale for targeting the DR was based on the discovery of substantial overlap between OXTRs and 5-HT-expressing cells in the DR of mice, on the evidence of increased endogenous 5-HT release after infusion of OXT into the median raphe nucleus (Yoshida et al., 2009), on the reported stimulated firing of DR 5-HTergic neurons via OXTRs activation (Spaethling et al., 2014), and on the modulating properties of local 5-HT on aggressive displays (Bannai et al., 2007; van der Vegt et al., 2003).

While trait-aggression has been generally associated with low tonic brain 5-HT brain activity (Chiavegatto and Nelson, 2003; de Almeida et al., 2005; Seo et al., 2008), rapid transient increases in 5-HT flow to the forebrain have been detected when an individual prepares or initiates imminent aggressive acts (i.e., state-aggression) (de Almeida et al., 2005; de Boer et al., 2009). In particular, a high intermale aggression level displayed during a RI test has been found to be correlated with high 5-HTergic activation in the DR of male WTG rats examined immediately after the aggressive encounter (van der Vegt et al., 2003).

However, when OXT was locally infused into the DR of our male resident rats, with the hypothesis of enhancing endogenous 5-HT release (Yoshida et al., 2009), we did not observe any pro-aggressive effects. Apart from the possible limitation by a small sample size, the lack of behavioral effect might be due to the heterogeneous OXTRs distribution within the DR (Gould and Zingg, 2003), possibly indicating that not all the cannulas were positioned in OXT receptive areas, even if they were all in proximity of 5-HTergic neurons responsive to the infusion of the selective 5-HT_{1A} autoreceptors agonist. Finally, the effect of infused OXT on the 5-HTergic system may be different for different sub-regions of the raphe nucleus or may be species-specific, making it difficult to compare our data from rats to the previous study in mice (Yoshida et al., 2009).

In conclusion, our data present the CeA as a brain region where exogenous enhancement of OXT levels leads to a behavioral shift from offensive/hostile response towards affiliative social exploration. In contrast, no direct link has been found between the OXTergic system of the DR and intermale aggression.

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5

LOCAL OXYTOCIN EXPRESSION AND OXYTOCIN RECEPTOR BINDING IN THE MALE RAT BRAIN IS ASSOCIATED WITH AGGRESSIVENESS

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ABSTRACT

We recently demonstrated in male wild-type Groningen (WTG) rats that enhancing brain oxytocin (OXT) levels acutely produces marked pro-social explorative and anti-aggressive effects. Moreover, these pharmacologically-induced changes are moderated by the individual's aggressive phenotype, suggesting an inverse relationship between aggressiveness and tonic endogenous OXT signaling properties. Aim of the present study was to verify the hypothesis that variations in OXT expression and/or OXT receptor (OXTR) binding in selected brain regions are associated with different levels or forms of aggression. To this end, male resident WTG rats that repeatedly contested and dominated intruder conspecifics were categorized as being low aggressive, highly aggressive or excessively aggressive. Their brains were subsequently collected and quantified for OXT mRNA expression and OXTR binding levels. Our results showed that OXT mRNA expression in the hypothalamic paraventricular nucleus (PVN), but not in the supraoptic nucleus (SON), negatively correlates with the level of offensiveness. In particular, the excessively aggressive group showed a significantly lower OXT mRNA expression in the PVN as compared to both low and highly aggressive groups. Further, the excessively aggressive animals showed the highest OXTR binding in the central amygdala (CeA) and bed nucleus of the stria terminalis (BNST). These findings demonstrate that male rats with excessively high levels and abnormal forms of aggressive behavior have diminished OXT transcription and enhanced OXTR binding capacities in specific nodes of the social behavioral brain circuitry.

INTRODUCTION

The neuropeptide oxytocin (OXT) is known to influence a variety of socio-emotional behaviors including parental care and affiliation, bonding and conflict behavior, in-group cooperation and out-group competition, social learning and recognition (De Dreu, 2012; Lee et al., 2009a; Lukas and Neumann, 2013; Neumann, 2009; Veening et al., 2010). OXT is believed to exert this important role by modulating the neuronal activity within several brain regions implicated in the regulation of these social behaviors like, for example, the amygdala, septum, hypothalamus, hippocampus and brain stem (Blume et al., 2008; Knobloch et al., 2012; Stoop, 2012).

From the clinical literature, there are data suggesting that different plasma levels of OXT are related to individual differences in social skills and interactions. Although human plasma OXT only poorly reflects central OXTergic neurotransmission (Neumann and Landgraf, 2012), higher plasma OXT levels have been associated with trust (Zak et al., 2005), positive parenting style (Gordon et al., 2010) and high social engagement (Forsman et al., 2012). Two studies in human adults have shown that cerebrospinal fluid (CSF) OXT levels are diminished after childhood abuse and are negatively correlated with suicidal (auto-aggressive) behavior (Heim et al., 2009; Jokinen et al., 2012). Finally, infants with higher CSF OXT levels appear to actively seek parental social interaction for soothing, and also have a greater interest in social interaction at 6 months of age (Clark et al., 2013). Moreover, Lee and colleagues have reported a negative correlation between both CSF and plasma OXT levels and the life history of aggression in male subjects with conduct disorder. Finally, specific polymorphisms of the OXTR gene have been found to be associated with a high frequency of disruptive behaviors and temper outbursts in young boys (Fetissov et al., 2006; Johansson et al., 2012; Lee et al., 2009b; Tauber et al., 2011).

These clinical results are in agreement with the preponderance of preclinical studies indicating that diminished OXT receptor (OXTR) binding in various rat brain regions is associated with impaired social functioning after poor social rearing conditions (Ahern and Young, 2009) or after early life stress (Lukas et al., 2010) in several species. Moreover, activating the brain OXTergic system by pharmacological manipulation was shown to promote affiliative and attachment behaviors (Insel, 2010; Lukas et al., 2011; Neumann, 2009). In contrast, however, a number of studies have also shown decreased social interaction and increased aggression with high levels of OXT or OXTR (Beery and Zucker, 2010; Olff et al., 2013; Winslow et al., 1993). Therefore, species and individual differences, as well as brain region specificity have to be critically considered when linking the OXTergic activity and social behaviors. Among other animals, the monogamous prairie voles (*Microtus ochrogaster*) that display higher levels of parental care, affiliation and pair bonding as compared to the solitary montane voles (*Microtus montanus*) also exhibit a higher density of OXTRs in the ventral tegmental area, nucleus accumbens and caudate putamen (Insel and Shapiro, 1992; Olazabal and Young, 2006a); but lower density in the lateral septum (LS) (Olazabal and Young, 2006b). In contrast with the virtually asocial

cape mole-rats (*Georychus capensis*), naked mole-rats (*Heterocephalus glaber*) represent the pinnacle of sociality for their remarkable high level of social cohesion, tolerance and cooperation in burrowing, foraging, and defending the colony (O'Riain et al., 2000). These animals also show greater OXTR binding in the nucleus accumbens, central (CeA) and medial amygdala, and bed nucleus of the stria terminalis (BNST) (Kalamatianos et al., 2010). Two studies have further shown that CSF OXT level is higher in more social bonnet macaques as compared to pigtail macaques, and that it is positively associated with affiliation in rhesus macaques (Rosenblum et al., 2002; Winslow et al., 2003). In addition, young peer-reared rhesus monkeys were found to display aberrant social behaviors; as a group, these monkeys have lower CSF OXT levels over the course of development when compared to maternally reared controls (Winslow et al., 2003). Linfoot and colleagues have reported higher levels of OXT mRNA in the magnocellular neurons of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of low burying male rats (Linfoot et al., 2009). Defensive burying behavior is a typical proactive coping strategy in rodents that vigorously displace bedding material towards a variety of noxious stimuli that pose a near and immediate threat, such as a wall-mounted electrified shock-prod (De Boer and Koolhaas, 2003). Several studies have demonstrated a close link between levels of shock-prod burying and levels of offensive aggression in a resident-intruder test indicating that low levels of burying behavior are characteristic for rodents with a low level of aggression (Koolhaas et al., 2007; Koolhaas et al., 2010).

These data strongly support the general idea that inter-individual differences in social skills and aggression may be closely related to individual differences in brain OXTergic neurotransmission. Indeed, in our previous studies we have found that the behavioral response of male rats to exogenous OXTergic manipulation was moderated by their individual baseline aggression score. Greater OXT-induced anti-aggressive and pro-social effects were observed in animals with higher baseline levels of aggression, whereas pro-aggressive and anti-social effects occurred after treatment with a selective OXTR antagonist in the least aggressive animals only (Calcagnoli et al., 2013).

Based on these findings, we hypothesized an inverse relationship between aggressiveness and brain OXTergic activity. The current experiments were principally aimed to verify this hypothesis. The wide variability in intermale offensive behavior in WTG rats reaching up to excessive and abnormal levels of aggression allowed us to associate quantitative and qualitative variations in aggression (de Boer et al., 2009) with the activity of brain OXTergic system. We define offensive aggression as "excessive", when the frequency and/or duration of the aggressive acts are out of proportion to the causes and the representative threat of the target; while "abnormal" aggression refers to a qualitative connotation for offensive display such as attack of female or anesthetized conspecifics, or of vulnerable body parts (de Boer et al., 2009). Therefore, our experimental design included groups of male WTG rats that developed excessive and abnormal forms of aggressive behavior upon repeated winning aggressive contests, in addition to normally low and high aggressive animals. OXT mRNA expression and OXTR binding were chosen

as neurobiological parameters reflecting the activity of the brain OXTergic system. OXT mRNA levels were assessed in both the PVN and SON as the two main hypothalamic sites of OXT synthesis and release of central OXT (Knobloch et al., 2012; Ludwig and Leng, 2006; Neumann, 2007). In addition, OXTR binding was quantified in the LS, CeA and BNST as part of the social behavior network. Within these regions high local density of OXTRs (Gimpl and Fahrenholz, 2001; Tribollet et al., 1989) and local effects of OXT on socio-emotional behaviors (Bale et al., 2001; Lukas et al., 2010; Meyer-Lindenberg et al., 2011; Neumann and Landgraf, 2012; Yamasue et al., 2012), including the discrimination of biologically relevant social cues (Lukas et al., 2013; Ophir et al., 2009) and the acquisition of appropriate social skills (Branchi et al., 2013), were described.

In summary, in this study we aimed at revealing the potential link between the individual variation in intermale offensive aggressive behavior including its escalation into excessive and abnormal forms of aggression and some functional properties of the endogenous OXTergic system including OXT mRNA expression and local OXTR binding.

MATERIALS AND METHODS

Animals and housing condition

Young adult male wild-type Groningen (WTG) rats (*Rattus Norvegicus*) (N = 21) were used as experimental subjects. This strain of rats descended from pairs of wild-trapped individuals that were outbred under conventionalized conditions for over 35 generations now in our laboratory. Throughout the experimental period, animals were held under standard conditions (12 h light/12 h dark photoperiod, lights off at 13:00 h; ambient temperature 21 ± 2ºC; humidity 50 ± 5%) with *ad lib*. access to food (Hope Farms, RMH-B) and water. After the age of 120 days, rats (body weight 350-400 g), previously housed with 5 non-sibling conspecifics in macrolon cages (55 × 34 × 20 cm), were then housed in observation cages (80 × 55 × 50 cm), together with an oviduct-ligated but gonadally-intact female to avoid social isolation, in order to allow normal sexual activity and to stimulate territorial behavior. As compared to commonly used laboratory strains of rats, this strain of rats expresses a more varied ethogram when socially challenged and a higher level of offensive behavior in conflicting/hostile context (de Boer et al., 2003).

All experimental and behavioral procedures were approved by the Animal Ethics Committee on Care and Use of Laboratory Animals (DEC 5824) of Groningen University and were conducted in agreement with Dutch laws (Wet op de Dierproeven 1996) and European regulations (Guideline 86/609/EEC).

Behavioral characterization and selection criteria of the experimental groups

After one week of habituation in the observation cage, each resident was repeatedly exposed to an unfamiliar male intruder Wistar rat (Harlan Laboratories, Horst, NL; body weight 300-350 g) for 10 times, evenly distributed over a period of 1 month, in order

to allow the development of potentially escalated and abnormal forms of aggression (Koolhaas et al., 2013). Each resident encountered a different intruder every time. The lower weight and general docility of the Wistar intruders guaranteed the expression of dominance from the experimental resident subjects.

Each test consisted of measuring the latency time (in sec) to the first full attack (attack latency time, ALT); the test was terminated immediately afterwards. Only during the 5th and 10th encounter, a complete 10-min resident-intruder (RI) test was performed and videotaped. The videos were evaluated using a custom-made analysis program (E-line). The duration of each behavioral element of interest was expressed as percentage of time of the total duration of the RI test. Behavioral elements were grouped into the following broad behavioral categories in order to promote a clear representation of the data: (1) *offensive behavior* (lateral threat, clinch, keep down, chase, upright posture), (2) *social explorative behavior* (moving towards, investigation and ano-genital sniffing of the intruder, crawl over, social grooming), (3) *non-social exploration* (ambulation, rearing, sniffing, scanning, digging), (4) *inactivity* (sitting, lying, freezing), and (5) *self-grooming* (washing, scratching) (Koolhaas et al., 2013; Koolhaas et al., 1980; Olivier et al., 1995). The female partner of the experimental rat was removed from the observation cage approximately 30 min prior to the start of each ALT or RI test and placed back afterwards. All behavioral tests were performed within the first 3-4 hours of the dark (active) phase, in order to avoid effects of hormonal circadian fluctuation and of light exposure on the endogenous OXT release (Devarajan and Rusak, 2004).

This training phase was then followed by three additional ALT tests to more specifically assess potential abnormal forms of aggression, each performed on separate days: 1) the resident was exposed to an unfamiliar naïve male Wistar intruder in a *novel cage*, 2) the resident was exposed to a freshly *anesthetized* male intruder in its own home cage, and 3) the resident was exposed to an unfamiliar sterilized *female* in its own home cage.

At the end of the testing period, three distinct groups of animals were created (Figures 1A and 1B) based on the percentage of time spent displaying offensive behavior during the 10th RI test. In addition, the accomplishment of the attack or the latency time to attack the intruder during the three additional tests for abnormal aggression (novel cage – anesthetized intruder – female intruder) gave a qualitative characterization of the aggressiveness of the animals. Accordingly, the "low aggressive" group consisted of the 7 animals with the lowest level of offensive behavior, and with the maximum (600 sec) attack latency time, which actually means absence of attack, recorded during all three additional tests for abnormal aggression. The "excessively aggressive" group consisted of the 7 animals with the highest level of offensive behavior scored during the 10th RI test, and with the shortest attack latency (group means $\leq$ 300 sec) recorded during all three tests for abnormal aggression. The remaining 7 animals showed "highly aggressive" behavior, with the duration of the offensive display significantly differing from both the low and excessively aggressive groups. These animals also scored a latency time of 600 sec in the ALT test towards a female intruder, and a latency time > 300 sec in the ALT test performed towards a male intruder in the novel cage and towards an anesthetized intruder.

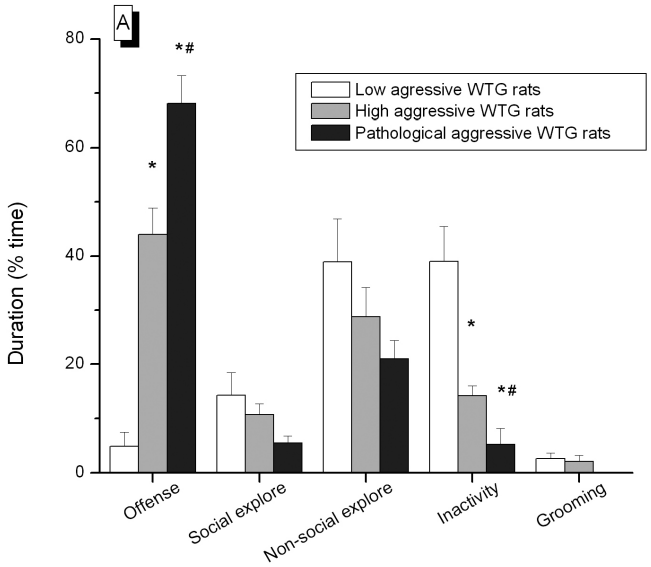

Figure 1A. Behavioral profile of male resident wild-type Groningen rats. Low, high or excessive level of aggression is displayed when the resident was exposed to an unfamiliar male intruder Wistar rat during the 10th resident-intruder test of the behavioral training. Data are presented as group mean + SEM (N = 7 each group). * and # indicate a significant difference ($p < 0.05$) in comparison with low aggressive animals and high aggressive animals, respectively.

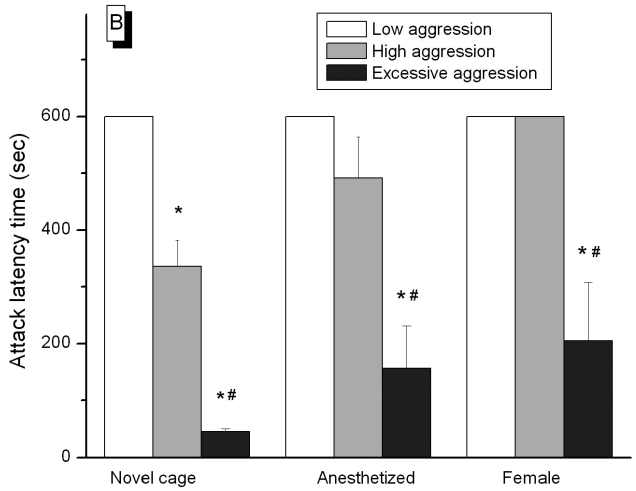

Figure 1B. Attack latency (sec) of male resident wild-type Groningen rats. Low, highly or excessively aggressive animals show different latency to the first offensive attack during the following behavioral challenges: 1) confrontation with a male intruder in a *novel cage*, 2) presentation of an *anesthetized* male, and 3) an unfamiliar *female* intruder. Data are presented as group mean + SEM (N = 7 each group). * and # indicate a significant difference ($p < 0.05$) in comparison with low aggressive animals and high aggressive animals, respectively.

Brain removal for in situ hybridization and receptor autoradiography

Three weeks after the last test for aggression, under basal and undisturbed conditions (housed in their own home cage together with the female companion), the resident rats were quickly decapitated under short isoflurane anesthesia, their brains removed and immediately frozen in dry-ice chilled n-methylbutane, and stored at -80ºC until cutting into 16-µm coronal cryostat sections. The slices containing the PVN, the SON, the LS, the BNST, and the CeA were mounted onto polysine glass microscope slides and stored at -20ºC.

In situ hybridization for OXT mRNA expression

The *in situ* hybridization followed a modified protocol based on the procedure originally developed by Young and colleagues (Young et al., 1986). Slide-mounted sections containing PVN and SON were thawed to 4ºC for 30 min and then left for 30 min at room temperature. They were then immersed in 4% paraformaldehyde solution for 8 min, rinsed in 0.1 M phosphate buffer (pH 7.2) (2 × 4 min), briefly dipped in distilled H_2O, followed by one rinse in 1.5% triethanolamine (TEA, pH 8.0) and incubation in 0.3% TEA/acetic anhydride (8 min). The slides were then rinsed in 0.03 M sodium citrate in 0.3 M sodium chloride buffer (2 × saline-sodium citrate (SSC), pH 7.0) (3 min), defatted in a graded ethanol series (50% - 85% - 100%, 3 min each step), dipped into chloroform (5 min) and again quickly washed in a graded ethanol series (100% - 95%, 3 min each step). Sections were air dried for 20 min and then incubated at 50°C with prehybridization solution for 2 h and hybridized with a highly specific 48 single-base, ^{35}S-labeled oligonucleotide probe (5'-CTC GGA GAA GGG AGA CTC AGG GTC GCA GGC GGG GTC GGT GCG GCA GCC-3'). The probe was applied to each section at a concentration of 10^6 counts per minute (cpm) per slide in 200 µl of hybridization solution for 18 h at 55°C in a humidified chamber. Post-hybridization washes consisted of three washes in 1 × SSC (15 min each) in a shaking bath at 50-55°C. The last wash step was repeated at room temperature. The slides were then dehydrated in a graded series of ethanol (50% - 85% - 100%, 3 min each step) and then quickly air dried. Finally slides were exposed to Kodak BioMaxMR film for 14 hours (Kodak, Rochester, NY, USA) and then developed (Kodak D19). Slides from all groups were processed simultaneously.

Receptor autoradiography for OXTR binding

Slides were processed for OXTR autoradiography as previously described by Bosch and colleagues (Bosch and Neumann, 2008, 2010). Slides were thawed to 4ºC for 30 min and then left for 30 min at room temperature. Slides were then fixed in 0.1% paraformaldehyde for 2 min followed by two rinses (10 min each) in 50 mM Tris buffer. Slides were then incubated for 60 min in 50 mM Tris buffer with 10 mM magnesium chloride ($MgCl_2$), 0.1% bovine serum albumin and 50 pM of the radioactive tracer (2000 cpm./10 µl). ^{125}I-ornithine vasotocin analog [^{125}I-d(CH_2)$_5$(Tyr(Me)^2, Thr^4, Orn^8)(^{125}I)Tyr^9-NH_2]; Perkin Elmer, Überlingen, Germany] was used for OXTR. Slides were then rinsed with Tris $MgCl_2$ buffer (4 × 5 min) followed by a 30-min wash (with a spinning magnetic stir bar) in the same buffer. After

briefly dipping the slides in distilled H_2O, they were air dried. Finally slides were exposed to Kodak BioMaxMR film for 6 days (Kodak, Rochester, NY, USA) and the autoradiographs were then developed (Kodak D19). Slides from all groups were processed simultaneously.

Quantification of OXT mRNA expression and OXTR binding

OXT mRNA expression and OXTR binding were quantified using NIH Image program (ImageJ V1.31; National Institutes of Health; http://rsb.info.nih.gov/ij/) as gray density. For each specific brain region of interest, bilateral measurements from four sections per rat were taken and the mean density measurement was calculated by subtracting the background activity, so that the resulting value reflected the specific signal only. Slides were coded so that the researcher was blind to the experimental group. Anatomical identification of the brain region of interested was based on the stereotaxic rat brain atlas of Paxinos and Watson (6th edition, 2007).

Data analysis

Behavioral data are presented as group means of the time spent in each behavioral category (indicated as percentage of the total 10 min test) or of the latency time (expressed in sec) + SEM. Results from the *in situ* hybridization and receptor autoradiography studies are presented as group means of gray density + SEM. Statistical analyses were carried out using SPSS for Windows (version 20: SPSS Inc, Chicago, IL, USA). Group differences were statistically tested by one-way analysis of variance (ANOVA) or, if data were not normally distributed, by the use of the non-parametric Kruskal-Wallis test. If overall significance was obtained, *post hoc* tests were carried out using either Tukey's HSD test or Mann-Whitney U test. Correlations were computed between the level of aggression (as measured during the 10th RI test) and the level of OXT mRNA expression or OXTR binding by the use of either Pearson's *r* or Spearman's ρ. All statistical tests with a *p*-value ≤ 0.05 were considered to be statistically significant. *P*-values between 0.05 and 0.1 were noted as a trend towards significance.

RESULTS

Oxytocin mRNA expression

Nonparametric Kruskal-Wallis test of the OXT mRNA expression in the PVN revealed an overall difference among the three experimental groups [χ^2= 10.61, df = 2, $p < 0.01$]. Testing the contrasts by means of the Mann-Whitney U test, the expression was found to be lower in the group with excessive offensive behavior as compared to the rats characterized by low ($p < 0.01$) and high ($p < 0.05$) level of aggression (Figure 2). Moreover, a negative correlation was found between the OXT mRNA expression in the PVN and the level of aggression assessed during the 10th RI test of the training procedure (ρ = -0.60, $p < 0.01$), which was performed more than three weeks before brains were removed (Figure 3). No differences between groups or behavioral correlations were found for the OXT mRNA expression in the SON.

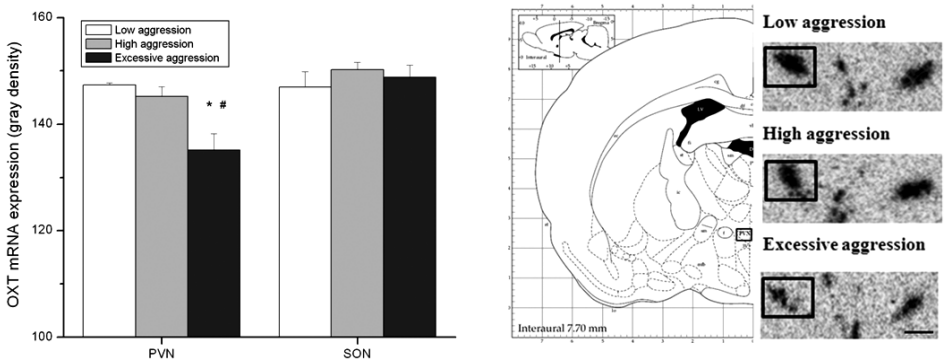

Figure 2. Oxytocin (OXT) mRNA expression in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei of male resident wild-type Groningen rats. Brains of animals behaviorally characterized as low, highly or excessively aggressive (N = 7 each group) were collected 3 weeks after exposure to the last test for aggression, and processed for OXT mRNA expression using *in situ* hybridization. Data are presented as the mean + SEM gray density (arbitrary units). * and # indicate a significant difference ($p < 0.05$) in comparison with low aggressive animals and highly aggressive animals, respectively. On the right, schematic drawing of the PVN (adapted from the brain atlas of Paxinos and Watson) and representative pictures of OXT mRNA expression in the PVN of low, highly or excessively aggressive male wild-type Groningen rats; scale bar = 0.5 mm.

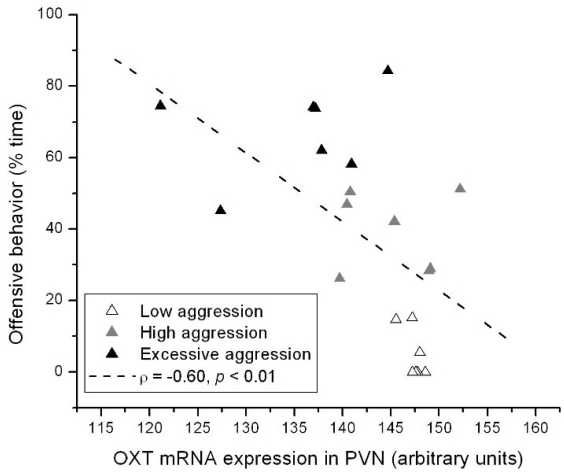

Figure 3. Correlation between oxytocin (OXT) mRNA expression in the paraventricular nucleus (PVN) and the offensive behavior. A significant negative correlation was found between the OXT mRNA expression in PVN and the duration of offensive behavior (% of time) displayed by low, highly or excessively aggressive male wild-type Groningen residents during the 10th resident-intruder test of the training period.

Oxytocin receptor binding

An overall difference in the level of OXTR binding among the groups was found in the CeA [normally distributed: $F_{2,18} = 4.08$, $p < 0.05$] and the BNST [not normally distributed:

χ^2= 9.38, df = 2, $p < 0.01$]. *Post hoc* tests revealed that the OXTR binding in these brain regions was higher in the excessively aggressive compared to low aggressive rats (CeA $p < 0.05$, BNST $p < 0.01$). Moreover, the OXTR binding in the BNST of the excessively aggressive group was also found to be higher compared to the highly aggressive rats ($p < 0.05$; Figures 4 and 5). OXTR binding in both regions correlated positively with the total

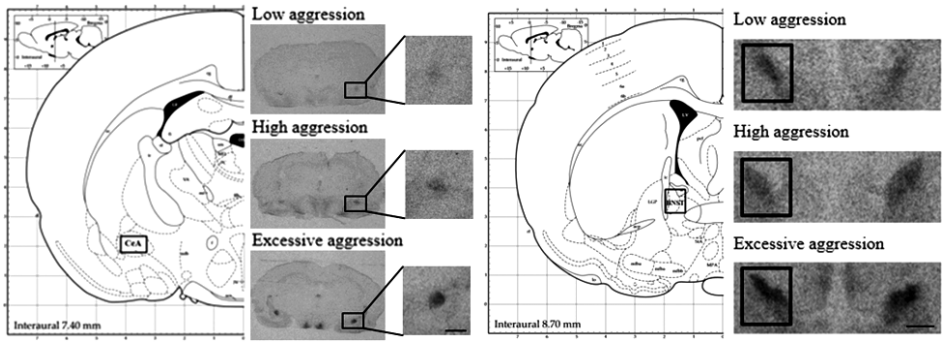

Figure 4. Oxytocin receptor (OXTR) binding within the lateral septum (LS), central amygdala (CeA) and bed nucleus of stria terminalis (BNST) of male resident wild-type Groningen rats. Brains of animals behaviorally characterized as low, highly or excessively aggressive (N = 7 each group) were collected 3 weeks after exposure to the last test for aggression, and processed for OXTR binding using receptor autoradiography. Data are presented as the mean + SEM gray density (arbitrary units). * and # indicate a significant difference ($p < 0.05$) in comparison with low aggressive animals and highly aggressive animals, respectively.

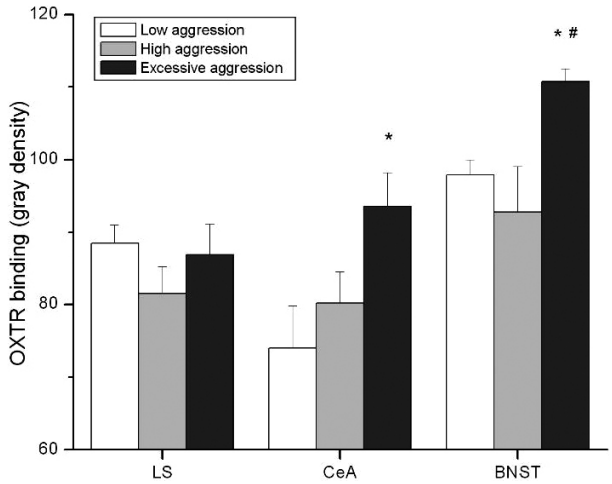

Figure 5. Representative pictures of oxytocin receptor (OXTR) binding in the central amygdala (CeA) and bed nucleus of the stria terminalis (BNST) of low, highly or excessively aggressive male wild-type Groningen rats. Schematic drawing of the CeA (right side) and BNST (left side) (adapted from the brain atlas of Paxinos and Watson) and enlargement of the related coronal sections of both brain regions; scale bar = 1 mm.

duration of offensive behavior shown during the 10th RI test (CeA: $r = 0.48$, $p < 0.05$; BNST: $\rho = 0.54$, $p < 0.05$; Figure 6A and B). No differences between groups or behavioral correlations were found for the OXTR binding in the LS.

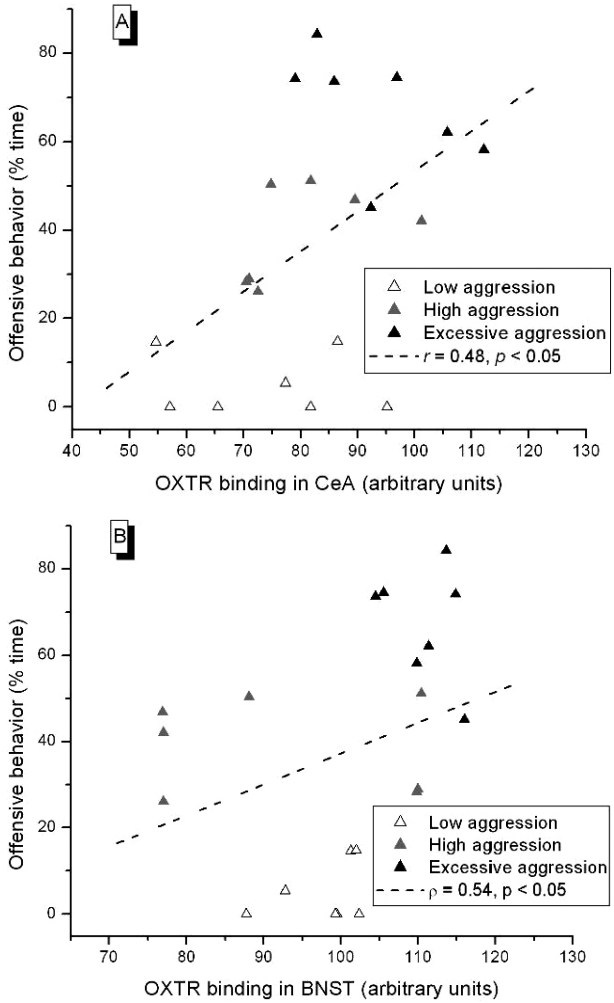

Figure 6A – B. Correlation between oxytocin receptor (OXTR) binding in the central amygdala (CeA; 6A) or in the bed nucleus of the stria terminalis (BNST; 6B) and the level of offensive aggression (% of time). A positive correlation was found between the OXTR binding in both CeA and BNST and the offensive behavior displayed by low, highly or excessively aggressive male wild-type Groningen residents during the 10th resident-intruder test of the training period.

DISCUSSION

Our findings provide evidence for a link between variations in aggression and the activity of the OXTergic system in specific brain regions. The data show that OXT mRNA expression, potentially indicating brain OXT peptide availability, in the PVN, but not in the SON, was inversely correlated with the aggression displayed by trained resident male rats. Interestingly, animals that developed excessive and abnormal forms of aggression upon repeated victorious encounters showed significantly lower OXT mRNA expression in the PVN as compared to both low and highly aggressive rats that did not develop such abnormal behavior. In contrast, OXTR binding in the CeA and BNST of the excessively aggressive group was found to be significantly higher as compared to both low and highly aggressive groups of animals.

The PVN is one of the main brain regions containing neurons that synthesize and release OXT either locally from the dendrites or from their axonal projections to other brain areas (Knobloch et al., 2012; Ludwig and Leng, 2006; Neumann, 2007). Therefore, the observed link between low OXT mRNA expression and excessively high levels of aggression suggests a lower central OXT availability in individuals that are more prone to escalate their aggression into abnormal forms during repeated social conflicts. The low level of basal *oxt* gene transcription observed in the excessively aggressive males may likely explain the more pronounced anti-aggressive efficacy of acute OXT treatment in highly aggressive WTG rats that we have previously reported (Calcagnoli et al., 2013).

The region-specific differences in hypothalamic OXT mRNA expression suggest a differential contribution of PVN and SON to sociality. While there is limited evidence for a contribution of the SON to social behavior regulation, OXT release within the PVN and its central target regions, such as the amygdala, has been shown to be involved in anxiety, stress coping and social behaviors (Blume et al., 2008; Lukas et al., 2013; Waldherr and Neumann, 2007).

However, from this study we cannot determine whether the observed OXT mRNA expression and/or OXTR binding differences are causally related to the expressed differences in aggressiveness. Since the experimental resident rats were repeatedly exposed to intruding conspecific challenge tests to reveal their distinctly expressed aggressiveness, this experimental procedure may also have induced long-lasting differences in *oxt* and/or *oxtr* gene expression. Although not incorporated in this study, it would have been relevant to verify whether the values of OXT and OXTR levels observed in our trained and aggression-experienced animals fall within the range present already in aggression-naïve rats in order to ascertain whether the differences found are already innately present and may lead to aberrant aggressiveness or are mainly due to the result of the experimental training procedure. Moreover, it needs to be demonstrated whether the reduced OXT mRNA level within the PVN in excessively aggressive individuals is indeed accompanied by a blunted release of OXT either locally within the PVN or within limbic target regions, e.g. the CeA or BNST. Putative differences in local neuropeptide availability may likely explain our finding of the aggressiveness-dependent OXTR binding within these brain regions that receive OXTergic axonal projections. In excessively aggressive animals, OXTR expression in the CeA

and BNST are likely to be up-regulated as a compensatory neuronal adaptation to overcome the putative low OXT availability resulting from the low transcriptional activity within the PVN. However, increased OXTR density might be on the other hand causing down-regulation of the OXT synthesis and/or release in the hypothalamic area. Moreover, higher OXTR binding combined with the potentially low OXT availability points to the limbic system as region of interest for local OXTergic manipulation. The higher OXTR binding capacity in highly aggressive animals might increase their sensitivity to the anti-aggressive effects of exogenously administered OXT. However, the behavioral significance of OXT within the CeA and BNST needs further clarification. Considering that local OXT has been associated with anxiety- and fear-related behaviors and stress responsiveness (Bale et al., 2001; Ebner et al., 2005), it is important to understand whether local changes in the OXTergic system directly affect fear and anxiety and indirectly the initiation of other behaviors, like aggression.

Besides the correlational evidence, we found that the OXTergic properties associated with excessive and abnormal aggression does not only differ from that of rats characterized as low aggressive, but also from that of the highly aggressive rats. Interestingly, no significant differences were found between low and highly aggressive animals in either OXT mRNA expression or OXTR binding. Thus, these findings suggest that major changes in these structural properties of the OXTergic system are mostly associated with alterations in the quality of the displayed aggression and hence are found only in excessive and abnormal aggressive phenotypes. However, a larger group size, more detailed pharmacological studies and/or molecular genetic manipulation studies are still needed to confirm and to interpret our findings.

Only recently we have started to focus on these excessive and abnormal forms of aggression that develop in a minority of male WTG rats after repeatedly permitting them to physically dominate other conspecifics (i.e., repeated winning experiences). Although the detailed neurobiological mechanisms underlying this abnormal aggression in WTG rats are still largely unknown, the (auto)regulatory components of brain serotonin (5-HT) neurotransmission have been shown to play a role (de Boer et al., 2009). While normal aggressive behavior aimed at securing territory, dominance and social coherence are positively related with 5-HT neuronal (re)activity, an inverse relationship develops between tonic, trait-like 5-HT activity and pathological forms of aggression. For example, a positive correlation was found between the level of adaptive intermale aggression and CSF concentrations of 5-HT and/or its metabolite (5-hydroxyindoleacetic acid, 5-HIAA) (van der Vegt et al., 2003). Moreover, although levels of 5-HT and 5-HIAA in the frontal cortex, implicated in cognitive and executive behavioral processes like impulse control, did not differ between low and highly aggressive animals, we found a negative correlation between aggression and frontal cortical 5-HT levels when samples from the abnormal and excessively aggressive trained resident animals were included (de Boer et al., 2009). In addition, functional changes in the premier auto-regulatory sites that control firing and 5-HT release of the serotonergic neurons, i.e., presynaptic 5-HT_{1A} autoreceptors, may underlie this transition of normal adaptive aggressive behavior into abnormal excessive

forms, as a profound hypersensitivity in the somatodendritic 5-HT_{1A} autoreceptors was observed in these excessively aggressive animals (Caramaschi et al., 2007; de Boer et al., 2009). A recently published study showed that a coordinated action of both 5-HT and OXT is required within the nucleus accumbens of mice to mediate the rewarding properties of social interaction (Dolen et al., 2013). Therefore, it will be of interest to investigate whether individual differences in processing the rewarding aspects of a winning experience may be explained by individual variation in the interplay between these neurotransmitters.

Concerning the status of other neuropeptidergic systems in male WTG rats, previous studies have shown a negative correlation between LS vasopressin immunoreactive fiber density and intermale aggression (Everts et al., 1997). In baseline condition, vasopressin content in the septal area was also found lower in highly aggressive animals as compared to low aggressive rats while OXT content did not appear to be different in this brain region (de Boer et al., 2003), but the local neuropeptide content might not predict release patterns per se (Neumann and Landgraf, 2012). Previous studies on male Wistar demonstrated a positive correlation between vasopressin release within the LS and intermale aggression (Veenema et al., 2010). However, in male Wistar rats selectively bred for low (LAB) anxiety-related behavior, high and abnormal aggression is accompanied by lower septal vasopressin release as compared to rats bred for high (HAB) anxiety-related behavior (Wigger et al., 2004). However, such locally released vasopressin does not seem to be directly involved in aggression regulation, but rather modulates anxiety-related behaviors (Beiderbeck et al., 2007). Rather, in LAB resident rats an increased neuronal activity of the nucleus accumbens was found during the RI test exposure accompanied by increased local dopamine release. As blockade of dopamine receptors has been shown to diminish intermale aggression in these rats, an activated reward system is likely to also contribute to high and abnormal aggression (Beiderbeck et al., 2012).

Overall, the results suggest that, within the LS, differences in local neuropeptide release, rather than in receptor binding, may determine the level of aggression. Thus, further studies are needed to monitor local release patterns of OXT to interpret our finding of similar septal OXTR binding in low, highly and excessively aggressive WTG rats.

For translational purposes, it is especially the abnormal aggressive group of WTG rats that seems to serve as a model for several neuropsychiatric disorders associated with pathological aggression and violence. Our current data concerning the neurobiology of abnormal aggressive male rats seem to be in line with findings from human studies where excessive and pathological forms of aggression, impulsivity, irritability and disrupted self-control have been associated with hypo-OXTergic function (Fetissov et al., 2006; Jokinen et al., 2012; Lee et al., 2009b; Malik et al., 2012). Yet, in order to more directly and conclusively delineate the central basal OXTergic tone in our animal model we need to assess the actual level of OXT released within the relevant limbic brain regions involved in intermale aggression using intracerebral microdialysis technique.

In conclusion, our findings strongly support the hypothesis that variations in signaling properties of the brain OXTergic system are linked to individual differences in aggression,

and likely play a role in the differential responsivity between high and low aggressive animals to exogenous OXT treatments.

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ACUTE AND REPEATED INTRANASAL OXYTOCIN ADMINISTRATION EXERTS ANTI-AGGRESSIVE AND PRO-AFFILIATIVE EFFECTS IN MALE RATS

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ABSTRACT

Socio-emotional deficits and impulsive/aggressive outbursts are prevalent symptoms of many neuropsychiatric disorders, and intranasal administration of oxytocin (OXT) is emerging as a putative novel therapeutic approach to curb these problems. Recently, we demonstrated potent anti-aggressive and pro-social effects of intracerebroventricular (icv) OXT administration in male rats. The present study tested whether similar behavioral effects are induced when OXT is delivered intranasally. Heart-rate and blood-pressure responses were telemetrically monitored to investigate whether peripheral physiological effects were provoked after intranasal OXT administration. Moreover, we tested whether intranasal OXT activates OXTergic neurons in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei. Intranasal OXT administration in resident animals reduced offensive aggression and increased social exploration towards an unfamiliar male intruder. Using a partner-preference test, intranasal OXT also strengthened the bonding between the male resident and its female partner. No changes in cardiovascular (re)activity were found, indicating an absence of direct peripheral physiological effects after intranasal OXT treatment. However, intranasal OXT increased neuronal activity in both OXTergic and other neurons of PVN and SON. In conclusion, this study demonstrated intranasal OXT to be an effective application method for suppressing intermale aggression and enhancing social affiliation. Although the precise route and mechanisms of nose-to-brain transport/communication remain to be elucidated, our data demonstrated activation of the endogenous brain OXTergic system after intranasal OXT application that may underlie the observed behavioral effects.

INTRODUCTION

Intranasal administration of oxytocin (OXT) has been shown to facilitate "pro-social" feelings and behaviors in healthy subjects (Domes et al., 2007; Kosfeld et al., 2005; Naber et al., 2010; Zak et al., 2007). Based on these findings, synthetic OXT analogues are emerging as novel therapeutic treatment approaches for mental disorders characterized by social dysfunction, such as autism (Guastella et al., 2010), social anxiety (Hall et al., 2012), and schizophrenia (Pedersen et al., 2011). Since minimal side-effects were reported across 38 randomized controlled trials (MacDonald et al., 2011), intranasal OXT administration found extensive use in clinical investigations for its easy and non-invasive delivery method and its putative rapid and direct access route to the brain.

The privileged access of the intranasal method is presumed to result from direct connections between the environment and the central nervous system afforded by the nasal mucosa (Guastella et al., 2013). To date, however, no clear evidence is available to support a direct transport pathway of OXT from the nasal cavity to the brain. Moreover, the mechanism and efficacy of penetration from the nose to either the cerebrospinal fluid (CSF) or the extracellular fluid is dependent on the distribution of the compound along the nasal epithelium.

A large expansion of trials testing nasal spray synthetic OXT effects on human social behaviors followed the initial study by Born and colleagues (Born et al., 2002), demonstrating a very small rise in human CSF vasopressin (AVP) level, i.e. of a nonapeptide structurally closely related to OXT, within 10 min after its intranasal application. Only very recently, Striepens and colleagues provided clear evidence that a behaviorally-effective dose of intranasal OXT (24 IU) elevated CSF (+60%) and blood (+250%) OXT concentrations in humans but that the kinetics in these compartments were considerably different (Striepens et al., 2013). Increased OXT concentration in human plasma (Gossen et al., 2012) and saliva (Weisman et al., 2012) has been reported more frequently and consistently after intranasal application, raising interpretative debates. Considering the great array of physiological activities affected by this peptide, a rise in plasma OXT level after intranasal application may provoke peripheral physiological changes thereby indirectly altering the behavioral performance with a similar, if not greater, impact than the effect induced by small rise in OXT CSF (Churchland and Winkielman, 2012). In primates, humans, and rats, for instance, peripheral administration of OXT is often associated with a decrease in blood pressure (Petersson et al., 1996), heart rate and body temperature (Hicks et al., 2014). Similarly, intracerebroventricular (icv) injected OXT decreased blood pressure, while inhibition of brain OXT synthesis by an anti-sense oligonucleotide increased blood pressure in rats (Maier et al., 1998). Moreover, deletion of the OXT gene in mice appeared to be associated with high blood pressure and heart rate (Bernatova et al., 2004).

Given that OXT is already prescribed off-label by health practitioners in the United States (Bales et al., 2013), animal studies should be pursued in a coordinated way with human studies, addressing research questions concerning the spatial and temporal dynamics of the intranasal route, the dose-dependent effects on behavioral changes, as

well as on the kinetics of both plasma and CSF OXT levels, while moreover verifying central availability of synthetic OXT after intranasal application.

Recent work by Neumann and colleagues showed, in rats and mice, increased OXT levels in the extracellular fluid of both brain regions that are targeted by OXTergic projections (amygdala) or regions that are free of them (dorsal hippocampus), providing evidence that intranasally-applied OXT is able to enhance CSF OXT (Neumann et al., 2013). Of relevance is also the recent study of Modi and colleagues reporting that aerosolized OXT resulted in significant increases in both lumbar CSF and plasma OXT levels over baseline for the full 120 min after administration (Modi et al., 2014).

Althought, based on the lack of a barrier between the extracellular fluid and the CSF, changes in CSF OXT concentration are likely to be indicative of changes in OXT concentrations in brain and thus its bioavailability for behavioral effects, no study has provided a definite description of the route and/or mechanisms by which intranasally delivered OXT enhances brain OXT levels.

To date in preclinical research, behavioral effects of synthetic OXT have mainly been examined after either an icv infusion or direct local delivery into a brain region. Only few animal studies have tried to employ the intranasal route for inducing behavioral changes. In macaques, inhaled OXT enhanced pro-social choices (reward to another monkey) when there was no potential cost to self, but provoked an increase in selfish decisions when there was potential for direct self-reward. Moreover, the OXT-treated group showed a significantly increased CSF OXT concentration compared to the vehicle group (Chang et al., 2012). Parker and colleagues described a significantly reduced stress-induced hypothalamic-pituitary-adrenal axis activation only after chronic, but not acute, intranasal OXT administration in adult female squirrel monkeys (Parker et al., 2005). Bales and colleagues showed an impairment in partner-preference formation in male voles when treated long-term with low doses of the neuropeptide, while the acute administration facilitated partner preference (Bales et al., 2013).

As we recently showed clear anti-aggressive and pro-social explorative effects after acute and chronic icv infusion of synthetic OXT in male wild-type Groningen (WTG) rats (Calcagnoli et al., 2013), our current focus is to replicate these behavioral effects by applying the neuropeptide intranasally. Hence, the effects of acute and repeated intranasal administrations of OXT on the behavioral response of male resident rats are assessed during a standard resident-intruder (RI) test. According to the literature (Cho et al., 1999; Williams et al., 1994), we also hypothesized that intranasally administered OXT would promote pair-bonding formation during a partner-preference (PP) test. Heart-rate and blood-pressure (re)activities were monitored after acute intranasal application of OXT in order to control for potential peripherally-provoked cardiovascular effects that may moderate the behavioral response to social challenges. Moreover, in order to investigate whether intranasal OXT application may cause some of its centrally-mediated behavioral effects by stimulating the endogenous OXTergic system (Kita et al., 2006), the expression of the neuronal activation marker Fos in OXTergic cells is assessed in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei.

MATERIALS AND METHODS

Animals and housing condition

Five cohorts of adult male WTG rats (*Rattus Norvegicus*) were used to perform the experiments as described below. All experimental and behavioral procedures were approved by the Animal Ethics Committee on Care and Use of Laboratory Animals (DEC 5824) of Groningen University and were conducted in agreement with Dutch laws (WoD 1996) and European regulations (Guideline 86/609/EEC).

Experiments

Each cohort of WTG rats was divided in 2 groups, and each group randomly assigned to either vehicle or OXT treatment condition. In experiments (1), (2), (3) and (5) the groups were matched according to the duration of offensive and social explorative behaviors displayed during the baseline RI test.

(1) thirteen animals received intranasal administration once a day, for 7 days of either vehicle (N = 6) or OXT (N = 7) and were tested using the RI test. This test was performed at baseline (day -4), and repeated at the start (day 1) and the end (day 7) of the treatment period, as well as 7 days after treatment cessation (day 14). In this way, we checked for acute (day 1 vs. day -4), repeated (day 7 vs. day 1 and vs day -4) and long-lasting effects (day 14 vs day -4);

(2) sixteen rats received intranasal administration once a day, for 7 days of either vehicle (N = 8) or OXT (N = 8) and their behavior was evaluated using the RI test. This test was performed at day -4, and then repeated at days 1, 7 and 14. Since the effects appeared to be washed out completely at day 14, the group-treatment combination was inverted in a cross-over design, i.e., animals that had received vehicle the first 7 days, were treated with OXT, and vice versa;

(3) sixteen rats received intranasal administration once a day, for 7 days of either vehicle (N = 8) or OXT (N = 8), and were tested using the PP test. The test was performed at days -4, 1, 7 and 14;

(4) thirteen animals were used for heart rate and blood pressure recordings before, during and after a single intranasal application of either vehicle (N = 6) or OXT (N = 7). After 5 days wash-out, the group-treatment combination was inverted in a cross-over design, i.e. the animals that received first vehicle, were then treated with OXT, and vice versa;

(5) fifteen animals were used for conducting double staining for Fos and OXT positive cells in the PVN and SON after a single intranasal administration of either vehicle (N = 7) or OXT (N = 8).

Behavioral characterization: resident-intruder and social preference tests

In experiment (1), (2), (3) and (5), after a 7-day habituation period, the baseline level of aggression was measured using the standard protocol of the RI test, as earlier described (Calcagnoli et al., 2013).

In experiment (3), one week after the baseline RI test, the baseline PP test was performed in the home cage of the resident. As social stimuli we used the companion female (partner) of the resident, that was removed from the home cage 1h prior the test, and a novel oviduct-ligated but gonadally-intact female WTG rat. Each female animal was encaged in a wire-meshed cage, allowing full visual interaction and olfactory communication. Both cages were simultaneously placed at the opposite walls of the observational cage. The test was performed and video recorded for 10 min. At the end of the test, both cages were removed. The companion female was placed back into the resident cage 1h after the test.

The evaluation of the videos from both the RI and PP test was conducted using a custom-made data acquisition system (E-Line), which allows the manual scoring of the duration of different behaviors. The researcher was blind to the treatment conditions.

For the evaluation of the RI test, we used the followed behavioral categories: (1) *offensive behavior* (lateral threat, clinch, keep down, chase, upright posture), (2) *social explorative behavior* (moving towards, investigation and ano-genital sniffing of the intruder, crawl over, social grooming), (3) *non-social behavior* (ambulation, rearing, sniffing, scanning, digging), (4) *inactivity* (sitting, lying, freezing), and (5) *self-grooming* (washing, scratching).

In the PP test, the behavioral assessment included (1) *investigation of the partner female*, (2) *investigation of the novel female*, (3) *exploration of the cage* (ambulation, rearing, scanning, digging), (4) *self-grooming* (washing, scratching), and (5) *inactivity* (sitting, lying, freezing). Investigation of the social stimulus was recorded only when the male resident was in the near proximity of the wired meshed cage, visibly engaged with the female animal (i.e., sniffing, nose-to-nose contact).

In both tests, the duration of each displayed behavior was expressed as the percentage of the total duration of the confrontation (10 min). The results of the PP test were graphically presented as ratio of the percentage of time spent investigating the partner above the novel female.

Telemetric measurements of blood pressure and heart rate

For the biotelemetric recordings of the cardiovascular signals, a blood pressure transmitter (PA-C40, Data Sciences International, St. Paul, MN, USA) was implanted surgically in the intraperitoneal cavity and the catheter of the transmitter was secured in the ventral aorta. Rats were anesthetized with a mixture of isoflurane and oxygen. After surgery, animals were allowed 10 days to fully recover. Receiver platforms were placed underneath each cage of the individually housed experimental animal and heart-rate and blood-pressure were recorded using Dataquest Labpro software. Data were sampled continuously from t = -15 min to t = 60 min. At t = 0 the animal was taken out of the cage for less than 2 min

in order to intranasally administer vehicle or OXT. The average of the samples between t = -15 and t = 0 was used as baseline. Data from heart rate and blood pressure recordings were expressed as Area Under the Curve (AUC) between t = 0 and t = 60.

Immunohystochemistry and quantification

A week after the baseline RI test, animals of experiment (5) received an acute intranasal administration of either vehicle or OXT. Ninety min later, animals were terminally anesthetized (overdose of CO_2) for brain fixation by cardiac perfusion first with heparin (5000 U/ml) in 0.9% saline solution followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer saline (PBS, pH = 7.4). Brains were removed and immersed overnight in a solution of 4% PFA in 0.1M PBS. Brains were then washed in PBS and placed in a solution of 30% sucrose in 0.1M PBS and left until the tissue had sunk. The brains were frozen and stored at -80 °C until cutting into 25 µm coronal cryostat sections. Brain slices containing PVN and SON were collected as free floating in 0.1M PBS.

Immunohistochemical visualization of Fos- and OXT-positive cells was carried out on free-floating sections using a protocol previously described by Kita and colleagues (Kita et al., 2006).

We quantified cells stained only for Fos, only for OXT, and cells double stained for Fos and OXT in the anterior parvocellular part of the PVN and in the SON, using a light microscope (MTV3, Olympus Inc., Zoeterwoude, The Netherlands) with integrated Leica camera (BH2, Leica Camera AG, Solms, Germany). The investigator was blind to the treatment at the time of the counting. The anatomical localization of labeled cells was aided by use of the illustrations in a stereotaxic atlas (Paxinos and Watson, 1998). The numbers of labeled cells in the PVN and SON were counted in 3 slices, bilaterally (Plate 23; Interaural 7.70 mm, Bregma -1.30 mm) (Figure 1). The number of counted cells was corrected for the surface area of interest. Thus, the data are expressed as density (cell/µm^2) + SEM.

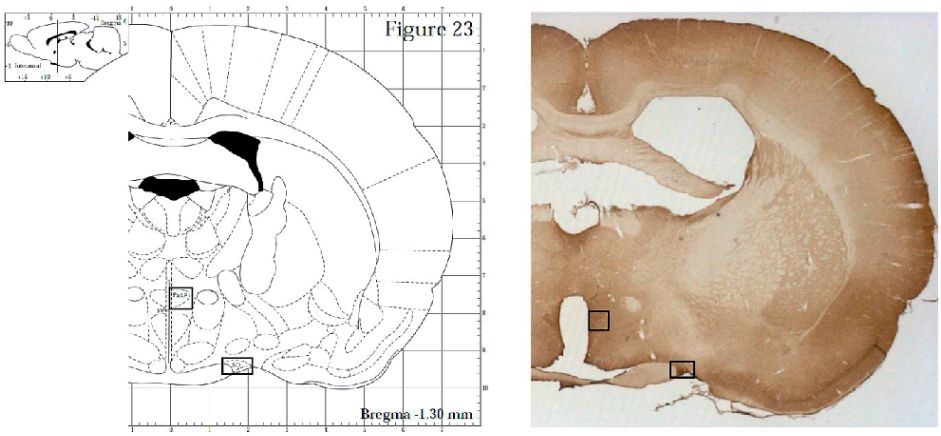

Figure 1. Overview of the investigated brain areas (PVN and SON) using the illustrations of the stereotaxic atlas by Paxinos & Watson (Paxinos & Watson, 1998). PaAP: Paraventricular hypothalamic nucleus, anterior parvocellular part; SO: Supraoptic nucleus.

Intranasal pharmacological treatment

We tested the effects of the following experimental treatments: vehicle solution (sterilized saline 0.9%, 20 µl) or synthetic OXT ($C_{43}H_{66}N_{12}O_{12}S_2$; MW 1007.19; Tocris, Germany; 1 µg/µl).

For the intranasal administration, we used the methodology described by Lukas and Neumann (Lukas and Neumann, 2012). To minimize non-specific stress responses, the experimental animals had one week of habituation to the holding position, as well as training to the procedure. The nasal application was carried out within the first 3-4 h of the dark phase, and 30 min prior to any behavioral test. The conscious rat was held by the experimenter in a supine position with a horizontal head position (Dhuria et al., 2010). The solution (2 × 10 µl) was bilaterally applied using a 100 µl pipette and equally distributed on the squamous epithelium of both the left and right rhinarium (Figure 2).

Direct contact of the tip of the pipette with the rhinarium, or direct application into one of the nostrils or in proximity of the philtrum was avoided to limit the drainage of the liquid into the esophagus and trachea. Each of the applications to the left and right rhinarium lasted about 1 min. After administration, the rats were returned to their home cage.

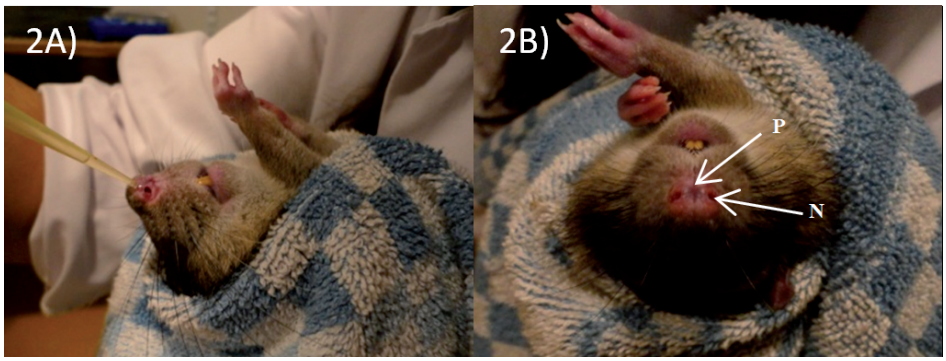

Figure 2. Holding of the conscious rat during nasal application while applying the solution intranasally (2A) and magnification of the nose region (2B). N: nostril; P: philtrum.

Exclusion criteria

In experiment 4, the blood-pressure response of one animal was excluded because of an unreliable signal. Therefore, in the analysis we included N = 13 animals for the heart-rate responses and N = 12 for the blood-pressure recordings.

In experiment 5, due to loss of tissue during cutting of the brains, the analysis of the quantification in the PVN was conducted with N = 6 in the vehicle-treated group and N = 8 in the OXT-treated group. The analysis of the quantification in the SON was conducted with N = 4 in the vehicle-treated group and N = 5 in the OXT-treated group.

Data analysis

Statistical analyses were carried out using SPSS for Windows; version 20. For all data, Shapiro-Wilk test was conducted to check for normality.

For experiments (1) and (3), treatment effects were tested by General Linear Model (GLM) repeated measures analysis of covariance (ANCOVA), while adjusting for baseline by entering the corresponding baseline values as a covariate for the sake of the design's efficiency (power) and validity (Liu et al., 2009; Senn, 2006). The design consisted of one within-subject variable (time) with the four measurement levels [baseline (day -4), acute (day 1), chronic (day 7), and wash-out (day 14)], and one between-subjects variable (treatment) with two levels (vehicle and OXT). If an overall significant time*treatment interaction was found, post-hoc comparisons were carried out on the contrasts of the within-subject variable (day -4 vs. day 1; day -4 vs. day 7, day -4 vs. day 14, and day 1 vs. day 7). The repeated measure analysis was then repeated for each experimental group separately, to reveal which treatment condition was determining the statistical difference.

For the analysis of experiment (2), we used a GLM repeated measures ANOVA, consisting of two within-subject variables: treatment (vehicle and OXT) and time [days -4, 1, 7, and 14], and one between-subjects variable (sequence) with two levels [sequence 1 (vehicle treatment first, followed by OXT) and sequence 2 (OXT treatment first, followed by vehicle)]. If an overall significant time*treatment interaction was found, post-hoc tests were carried out on the contrasts of the within-subject variable, as mentioned above.

To account for possible violations of the sphericity assumption for factors with more than two levels (such as the factor time), Huynh-Feldt adjusted *p*-values and the epsilon correction factor are reported together with the unadjusted degrees of freedom and F-values. To account for possible violations of the equality of variances, adjusted *p*-values are reported together with the unadjusted degrees of freedom and t-values.

Experiment (4) was analyzed by means of a repeated measure design with one within subject variable (treatment) with two levels (vehicle and OXT), and one between subjects variable (sequence) with two levels [sequence 1 (vehicle treatment first, followed by OXT) and sequence 2 (OXT treatment first, followed by vehicle)].

Due to the very small sample size, data of experiment (5) were analyzed by an Independent-samples non-parametric Wilcoxon rank-sum W-test.

For all comparisons, next to the p-values, partial eta squared (η^2) or the converted *z*-score (r) are presented as measures of effect size, with $\eta^2 < 0.06$ and $r < 0.3$ reflecting a small effect; $\eta^2 \geq 0.06$ and $r \geq 0.3$ a medium effect; and $\eta^2 \geq 0.14$ and $r \geq 0.5$ a large effect. $P = 0.05$ was adopted as the criterion for statistical significance.

RESULTS

EXPERIMENT 1: Acute, repeated and long lasting behavioral effects of intranasal OXT treatment

Aim of this experiment was to evaluate the effects induced by acute and repeated intranasal OXT application on the socio-behavioral profile of male residents when encountering an unfamiliar intruder, and to explore possible long-lasting effects.

Baseline-adjusted significant time*treatment effects were found only for the categories of offensive behavior [$F_{3,30}$ = 3.85, p < 0.05, η^2 = 0.28] and pro-social exploration [$F_{3,30}$ = 3.18, p < 0.05, η^2 = 0.24] (Figure 3 and Table 1, experiment 1).

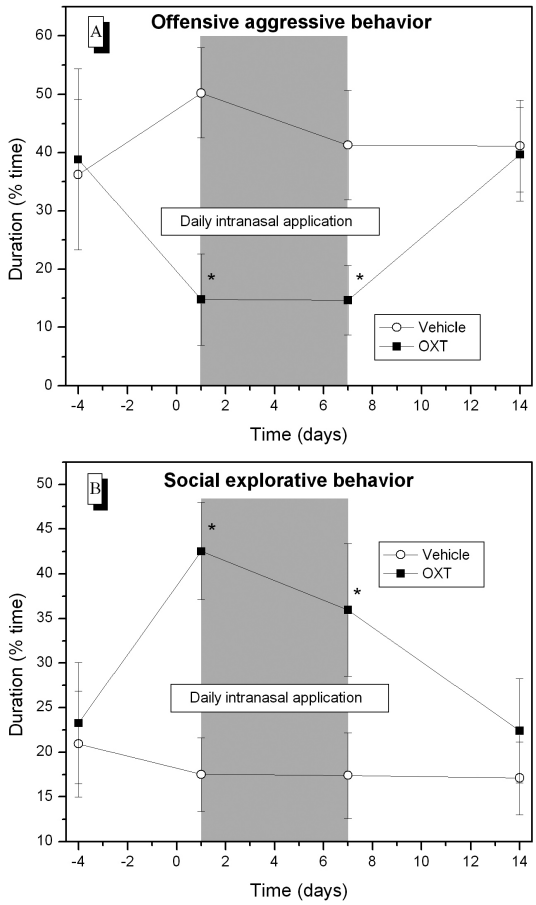

Figure 3. Changes in offensive aggression (A) and social exploration (B) before (day -4), during (days 1 and 7), and after daily intranasal treatment (day 14) of vehicle (20 µl) or oxytocin (OXT; 1 µg/µl, 2 x 10 µl). The gray area indicates the 7-day treatment period. Data are presented as mean ± SEM. Group differences at different time points are tested by means of t-test and * denotes significance (p < 0.05) between vehicle (N = 6) and OXT-treated (N = 7) groups.

Table 1 Summary of the durations (indicated as percentage of the total 10 min test) spent in the different behavioral categories evaluated during the intermale encounter (with the exclusion of the categories "offensive aggressive behavior" and "social explorative behavior"), and the group means of the latency time to the first attack (ALT; indicated in seconds) ± the respective SEM.

		Day - 4	Day 1	Day 7	Day 14
		Average ± SEM	Average ± SEM	Average ± SEM	Average ± SEM
Experiment 1; N =13					
Non-social exploration	Vehicle	40.50 ± 3.47	32.42 ± 5.29	44.87 ± 5.13	50.12 ± 6.61
	OXT	38.40 ± 6.70	34.61 ± 3.06	48.21 ± 5.06	44.19 ± 6.64
Inactivity	Vehicle	6.70 ± 1.32	5.82 ± 1.78	8.12 ± 1.25	7.18 ± 1.87
	OXT	6.53 ± 1.46	8.23 ± 1.44	6.97 ± 1.75	6.66 ± 1.32
Self-grooming	Vehicle	8.53 ± 2.66	5.38 ± 2.87	6.43 ± 3.03	5.68 ± 1.19
	OXT	3.36 ± 1.23	5.54 ± 1.60	6.39 ± 3.51	4.17 ± 1.34
ALT	Vehicle	492.50 ± 67.95	433.33 ± 49.01	428.33 ± 50.24	273.33 ± 46.61
	OXT	493.29 ± 49.97	458.00 ± 72.32	382.71 ± 64.19	211.43 ± 67.77
Experiment 2; N =16					
Non-social exploration	Vehicle	28.24 ± 2.72	31.88 ± 2.75	32.44 ± 2.01	31.59 ± 1.89
	OXT	29.50 ± 2.92	39.58 ± 2.53 *	42.01 ± 2.64 *	30.76 ± 2.27
Inactivity	Vehicle	21.84 ± 2.15	20.21 ± 1.81	22.08 ± 1.56	21.13 ± 1.88
	OXT	19.77 ± 3.33	18.06 ± 1.62	18.52 ± 2.09	22.32 ± 2.43
Self-grooming	Vehicle	8.73 ± 1.85	7.24 ± 1.50	10.16 ± 1.61	9.77 ± 2.13
	OXT	6.39 ± 1.00	6.51 ± 1.45	8.90 ± 1.53	11.34 ± 1.61
ALT	Vehicle	313.25 ± 53.11	306.38 ± 55.36	343.75 ± 56.70	307.81 ± 57.15
	OXT	321.06 ± 57.93	396.19 ± 63.72	396.69 ± 53.86	228.44 ± 42.79

In particular, in the offensive behavior time*treatment effects were found when comparing baseline (day -4) measure with day 1 [$F_{1,10} = 12.52$, $p < 0.01$, $\eta^2 = 0.56$], and day 7 [$F_{1,10} = 5.54$, $p < 0.05$, $\eta^2 = 0.37$]. No difference was found between day 1 and day 7, neither between day -4 and day 14 (Figure 3A). Further analysis revealed that the above mentioned effects on offensive behavior resulted from both its increase in the vehicle group {[$F_{3,12} = 3.83$, $p < 0.05$, $\eta^2 = 0.49$]; day -4 vs. day 1 [$F_{1,4} = 7.38$, $p = 0.05$, $\eta^2 = 0.65$]; day -4 vs. day 7 [$F_{1,4} = 12.55$, $p < 0.05$, $\eta^2 = 0.76$]} and its decrease in the OXT-treated animals {[$F_{3,15} = 8.52$, $p < 0.01$, $\eta^2 = 0.63$]; day -4 vs. day 1 [$F_{1,5} = 14.84$, $p < 0.05$, $\eta^2 = 0.75$]; day -4 vs. day 7 [$F_{1,5} = 33.53$, $p < 0.01$, $\eta^2 = 0.87$]}. To note, when inserting the baseline measure as covariate into the analysis, vehicle- but not OXT-induced time effects showed a p value near to significance with a high effect size [$F_{3,12} = 3.08$, $p = 0.065$, $\eta^2 = 0.43$], indicating the dependency of OXT effects on the baseline measure.

Similarly, for the social explorative behavior, time*treatment effects were found when comparing baseline (day -4) measure with day 1 [$F_{1,10} = 13.03$, $p < 0.01$, $\eta^2 = 0.56$]. When comparing baseline with day 7, the time by treatment interaction failed significance but showed a high effect size [$F_{1,10} = 4.17$, $p = 0.07$, $\eta^2 = 0.29$], inviting to further investigate this effect on a single treatment level (see below). No difference was found between day 1 and day 7, neither between day -4 and day 14 (Figure 3B). Independent of the baseline level of social exploration, OXT increased the duration of this behavior [$F_{3,15} = 4.13$, $p < 0.05$, $\eta^2 = 0.45$] after both single and repeated intranasal applications {day -4 vs. day 1 [$F_{1,5} = 14.07$, $p < 0.05$, $\eta^2 = 0.74$]; day -4 vs. day 7 [$F_{1,5} = 7.18$, $p < 0.05$, $\eta^2 = 0.59$]}. On the other hand, a baseline-dependent time effect was found in the vehicle condition [$F_{3,12} = 6.74$, $p < 0.001$, $\eta^2 = 0.63$] reflecting a significant decrease of social exploration compared to baseline (day -4)trend significant (day 1) or a significant (day 7 and 14) decrease of social exploration from baseline (day -4) {day 7 [$F_{1,4} = 33.12$, $p < 0.01$, $\eta^2 = 0.89$], and day 14 [$F_{1,4} = 12.08$, $p < 0.05$, $\eta^2 = 0.75$]}.

EXPERIMENT 2: Replication of the effects by a repeated measurement cross-over design

In this experiment, we aimed at confirming the findings of experiment (1), adopting a within-subject cross-over design. Inverting the initial group-treatment combination was possible since no long-lasting effects were found 7 days after the first treatment period [$F_{1,7} = 0.80$, $p > 0.05$, $\eta^2 = 0.10$].

No significance was found for the interaction treatment*time*sequence, excluding that treatment effects might have been due to the order of administration. Yet, significant overall treatment*time effects were found for both the category of offensive behavior [$F_{2,28} = 11.79$, $p < 0.001$, $\eta^2 = 0.46$] and pro-social exploration [$F_{2,28} = 13.00$, $p < 0.001$, $\eta^2 = 0.48$] (Figure 4). As shown in Figure 4A, only OXT treatment [$F_{2,30} = 4.57$, $p < 0.05$, $\eta^2 = 0.23$] remarkably reduced offensive behavior after both acute [$F_{1,14} = 14.12$, $p < 0.01$, $\eta^2 = 0.50$] and repeated [$F_{1,14} = 30.26$, $p < 0.001$, $\eta^2 = 0.68$] administration. Concomitantly, only OXT treatment [$F_{2,30} = 3.98$, $p < 0.05$, $\eta^2 = 0.21$] increased social exploration after both acute [$F_{1,14} = 25.04$, $p < 0.001$, $\eta^2 = 0.64$] and repeated [$F_{1,14} = 14.91$, $p < 0.01$, $\eta^2 = 0.52$] administration (Figure 4B). No statistical difference was found between measurements at day 1 and day 7 in any of the two categories, neither were long-lasting effects found 7 days after the cessation of the second treatment period [$F_{1,7} = 0.22$, $p > 0.05$, $\eta^2 = 0.06$]. No overall time*treatment was found in any of the other behavioral categories (Table 1, experiment 2).

EXPERIMENT 3: Acute, repeated and long lasting behavioral effects of intranasal OXT treatment on the partner-preference test

In this experiment we tested WTG rats in a PP test, in order to investigate the effects of acute and repeated intranasal OXT on pair-bonding behavior. We found a significant overall time*treatment interaction in the ratio score partner/novel [$F_{3,39} = 4.07$, $p < 0.05$, $\eta^2 = 0.24$] when comparing baseline (day -4) measure with day 1 [$F_{1,13} = 5.55$, $p < 0.05$,

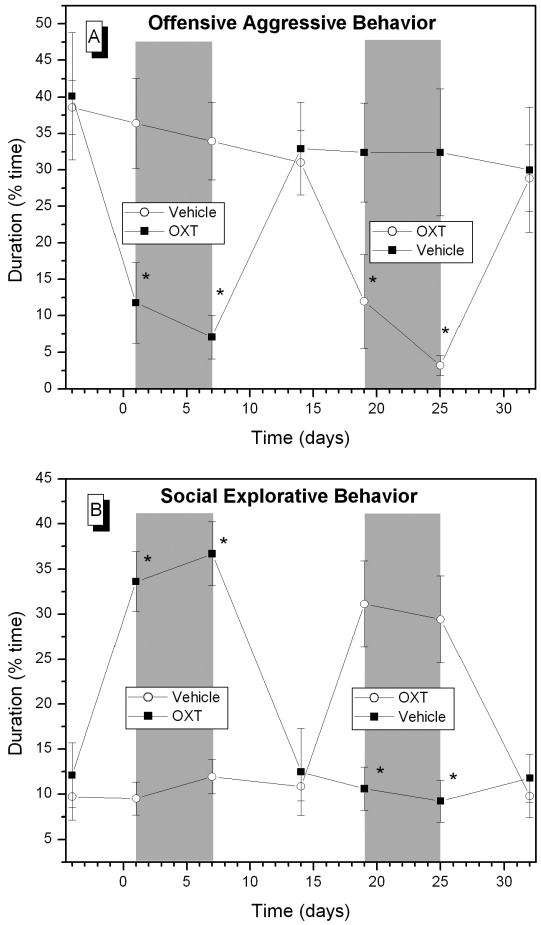

Figure 4. Changes in offensive aggression (A) and social exploration (B) before (days -4 and 14), during (days 1, 7, 19 and 25), and after (days 14 and 32) daily intranasal treatment of vehicle (20 µl) or oxytocin (OXT; 1 µg/µl, 2 x 10 µl). The gray areas indicate the 7-day treatment periods. Data are presented as mean ± SEM. Group differences at different time points are tested by means of t-test and * denotes significance ($p < 0.05$) between vehicle (N = 8) and OXT-treated (N = 8) groups.

$\eta^2 = 0.30$]. When comparing baseline with day 7 the interaction just failed significance, but yet showing a high effect size [$F_{1,13} = 4.28$, $p = 0.06$, $\eta^2 = 0.25$] (Figure 5). No difference was found between day 1 and day 7, neither between day -4 and day 14. Although the overall time effect failed significance when separately investigated in the OXT condition [$F_{3,18} = 2.90$, $p = 0.06$, $\eta^2 = 0.33$], the high effect size invited us to investigate single time contrasts. Here we found a greater preference to explore the partner rather than the novel female after both acute [$F_{1,6} = 10.13$, $p < 0.05$, $\eta^2 = 0.62$] and repeated [$F_{1,6} = 8.40$, $p < 0.05$, $\eta^2 = 0.58$] intranasal treatment. Even though the graph might suggest so, no effects were found for the vehicle group.

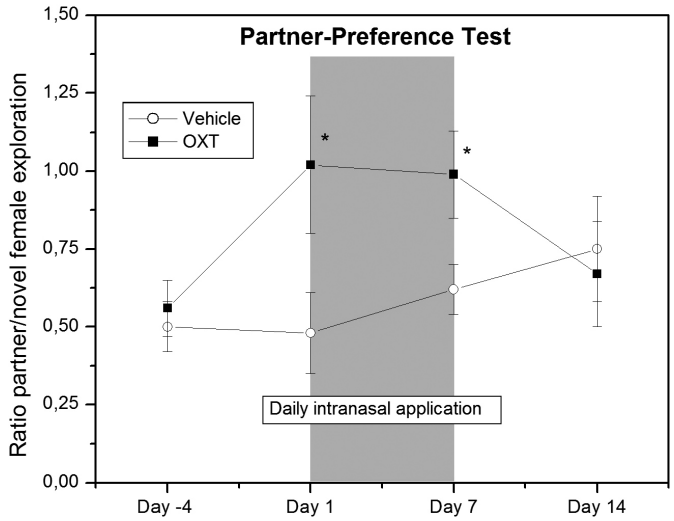

Figure 5. Changes in partner preference before (day -4), during (days 1 and 7), and after (day 14) daily treatment with either vehicle or oxytocin (OXT; 1 µg/µl, 2 x 10 µl). The gray area indicates the 7-day treatment period. Data are presented as mean ± SEM. Group differences at different time points are tested by means of t-test and * denotes significance ($p < 0.05$) between vehicle (N = 8) and OXT-treated (N = 8) groups.

On the other hand, time*treatment effects were also found in the category self-grooming [$F_{3,39} = 4.56$, $p < 0.05$, $\eta^2 = 0.26$]. Both vehicle [$F_{3,18} = 5.12$, $p < 0.05$, $\eta^2 = 0.46$] and OXT [$F_{3,18} = 9.55$, $p = 0.001$, $\eta^2 = 0.61$] treatment increased the duration of self-grooming at day 14 as compared to baseline {vehicle [$F_{1,6} = 11.68$, $p < 0.05$, $\eta^2 = 0.66$] and OXT [$F_{1,6} = 41.26$, $p = 0.001$, $\eta^2 = 0.87$]}. No increase in self-grooming was found during the treatment period, excluding the possibility of confounding effects. No difference was found in the general locomotor activity.

EXPERIMENT 4: Acute effects of intranasal OXT treatment on the cardiovascular baseline response

This experiment was designed to reveal potential intranasal OXT-induced peripheral effects that might bias centrally-induced behavioral effects. We found no difference in either heart rate or blood pressure recordings between intranasal vehicle and OXT treatment (Figure 6).

EXPERIMENT 5: Acute effects of OXT treatment on the neuronal activity of the endogenous OXTergic system

We performed double-staining for Fos and OXTergic cells in the PVN and SON after intranasal administration of either vehicle or synthetic OXT. Intranasal application of OXT significantly increased the total Fos positive cells in both PVN and SON as compared to intranasal vehicle solution (PVN; W = 29.00, $p < 0.05$, r = 0.55. SON; W = 10.00, $p < 0.01$,

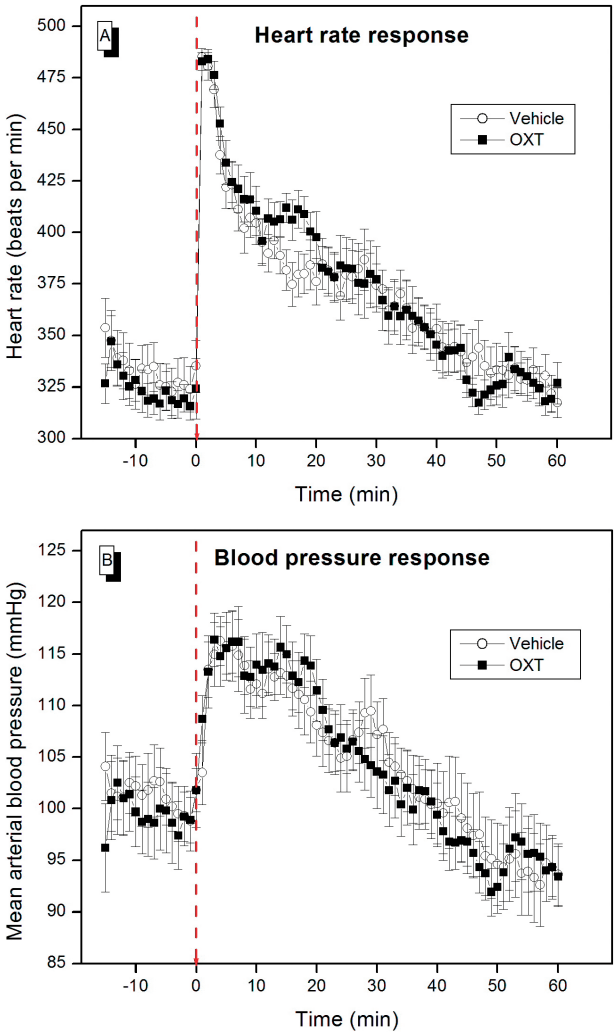

Figure 6. Heart rate (A) (N =13) and blood pressure (B) (N = 12) responses of male rats that intranasally received either vehicle or oxytocin (OXT; 1 µg/µl, 2 x 10 µl) at t = 0 (red dashed line).

r = 0.82). Although no difference in the total number of OXTergic cells was found between both treatments, OXT-treated animals showed more active OXT positive cells as compared to vehicle-treated rats (PVN; W = 29.00, $p < 0.05$, r = 0.55. SON; U = 10.00, $p < 0.01$, r = 0.82) (Figure 7). Within each region, the ranking of the data of the tested variables was the same; i.e. same W-value for total Fos positive cells and active OXT positive cells.

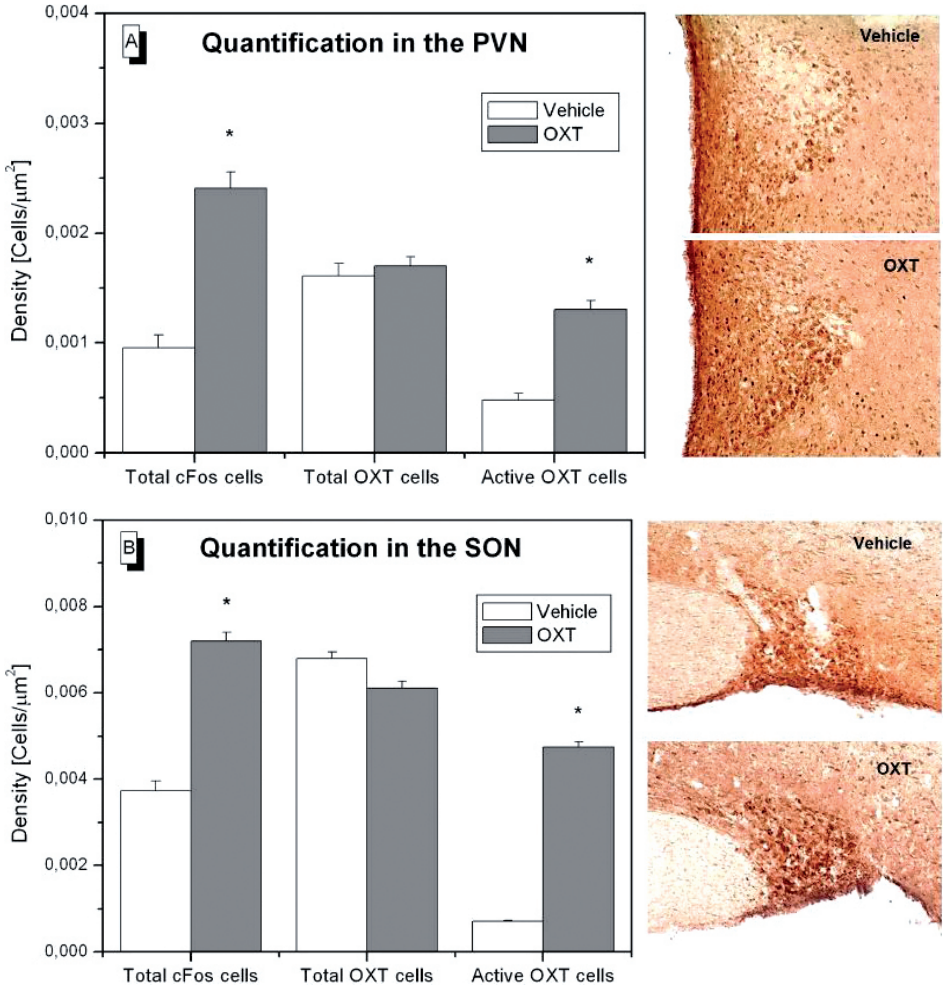

Figure 7. Quantification and corresponding photographic representation of total Fos-positive nuclei, total oxytocin (OXT)-positive cells and Fos-positive OXTergic cells in the paraventricular nucleus (PVN) (A) and supraoptic nucleus (SON) (B) of male rats after intranasal application of vehicle (20 µl) or oxytocin (OXT; 1 µg/µl, 2 x 10 µl). * denotes significance ($p < 0.05$) between vehicle (PVN; N = 6 and SON; N = 4) and OXT-treated (PVN; N = 8 and SON; N = 5) animals.

DISCUSSION

This study provides the first evidence of robust anti-aggressive and pro-social explorative effects after intranasal application of synthetic OXT in adult male resident rats. A single intranasal administration of the nonapeptide selectively shifted the social behavior profile from a hostile towards a more positive/explorative interaction. The efficacy and selectivity of the effects persisted after repeated OXT administrations. Moreover, both acute and repeated intranasal OXT treatments strengthened the attention for the female companion

in a PP test. No long-lasting effects were recorded 7 days after treatment cessation. In addition, increased neuronal activation of OXTergic cells in PVN and SON were found after acute intranasal OXT. As no alteration of heart rate and blood pressure was found after acute intranasal OXT application, we can exclude that effects on the cardiovascular system may have confounded the centrally OXT-induced acute behavioral changes.

The currently observed behavioral effects after intranasal OXT treatment resemble those found in our previous studies using acute and chronic icv administration (Calcagnoli et al., 2013) and are in line with other previous icv administration studies in animals (Williams et al., 1994; Young et al., 2011) and many clinical trials showing facilitated pro-social behavior, bonding formation and social engagement after intranasal OXT application (Bertsch et al., 2013; Liu et al., 2013).

Interestingly, we found that OXT decreased offensive behavior whereas an increase of offensive behavior was observed in the vehicle condition. Although this latter might be a batch-specific effect (it was not found in our second experiment), the phenomenon of increased aggressiveness with repeated exposure to a male intruder is well-known and likely to be due to repeated winning experience (de Boer et al., 2003). Hence the decrease resulting from intranasal OXT administration is even more remarkable. Moreover, as previously highlighted in our prior icv pharmacological manipulation (Calcagnoli et al., 2013), the efficacy of OXT in modulating offensive behavior appeared to be dependent on the baseline level of the behavior. Such dependency was not found for the OXT-induced effects in social explorative behavior. This prompts further research to investigate contextual and inter-individual factors that can moderate the effect of intranasal OXT, or even may confer a "tend and defend" response. In human trials, for instance, intranasal OXT was shown to decrease cooperation when participants interacted with strangers compared to familiar persons (Declerck et al., 2010). Similarly, OXT motivates non-cooperation in intergroup conflict to protect vulnerable in-group members (De Dreu et al., 2011), although a recent mata-analysis could not confirm that intranasal OXT significantly decreases out-group trust (Van and Bakermans-Kranenburg, 2012). Moreover, in women, state anxiety has been found to moderate the intranasal OXT-induced reduction of hostility expressed in a competitive aggression game (Campbell and Hausmann, 2013).

In addition to the contextual factors and inter-subject variability, OXT-induced behavioral responses vary depending upon the application method, the therapeutic window and dose range. In rhesus macaques, aerosolized OXT, but neither intranasal nor intraveneous OXT administration, resulted in significant increases in lumbar CSF OXT levels (Modi et al., 2014). The discordance in effect on CSF OXT concentrations between the two routes of nasal OXT administration, aerosolized and intranasal, suggests the two methods of application may have different dynamics as to the nasal passage of rhesus monkeys. In male WTG rats, enduring anti-aggressive and pro-social explorative effects were found after a 7-day period of chronic and continuous enhancement of brain OXT levels via osmotic mini-pumps (Calcagnoli et al., 2014), but not after a 7-day period of repeated intranasal delivery of OXT. Absence of persistent behavioral changes may indicate that

repeated OXT intranasal delivery is unable to provoke the putative lasting alterations in the endogenous OXTergic system (peptide expression, release patterns, receptor density, etc.) most likely occurring after continuous icv infusion of OXT.

Moreover, in male prairie voles, 3-weeks of low and medium doses of intranasal OXT during the developmental phase have been described to induce long-term impairment in partner-preference formation, despite the facilitation seen after acute intranasal treatment (Bales et al., 2013).

Although short-term administration may be safer or more effective than chronic administration, longitudinal studies should be carried out to assure safety, to exclude tolerance development, to determine the most effective therapeutic window and dose, and to verify the expected long-term effects. Moreover, studies exploring intranasal OXT effects should show corresponding central and plasma levels of OXT following intranasal dosing. In this way, we would know whether the rise in CSF OXT after intranasal application is appreciably greater than the one potentially triggered by the experimental challenges or contexts themselves. Collecting data of plasma OXT level after intranasal application is also of particular relevance when considering that pro-social effects (Ramos et al., 2013) and increased hypothalamic Fos expression (Carson et al., 2010) have been reported in male rats after intraperitoneal OXT injection.

The dose of OXT we applied intranasally is similar to what Neumann and colleagues have shown to induce increased brain OXT levels in adult rats (Neumann et al., 2013). Although those authors could not make a distinction between exogenous and endogenous OXT, the local rise in areas lacking OXTergic innervations (e.g., dorsal hippocampus and mediolateral part of caudate putamen) was interpreted as proof of transport of synthetic OXT from the nasal mucosa to the brain extracellular fluid. However, it cannot be excluded that the behavioral effect might also be due to endogenous OXT being released and transported from the hypothalamic area after binding of synthetic OXT on OXT-sensitive receptors located in the olfactory mucosal or brain regions (Yoshimura et al., 1993). Sensory input from the vomeronasal organ and main olfactory epithelium are received by and processed in the accessory olfactory bulb and main olfactory bulb, respectively (Sokolowski and Corbin, 2012). Already the olfactory bulb could be a site of action where OXT may alter the processing of social behavior-relevant olfactory stimuli (Dluzen et al., 1998), and therefore the behavioral response in social context and recognition task, such as intermale confrontation and partner-preference test. However, in our experiments, the unaltered latency to attack seems to reject the hypothesis of OXT-induced olfactory deficts.

As alternative mechianism, OXT may modulate the activity of brain areas that receive projections from the accessory and main olfactory bulb, such as the olfactory/piriform cortex and amygdala, especially the medial region (Swanson and Petrovich, 1998). The amygdala is generally believed to be a crucial processing station where the level of salience is attributed to a given stimulus (LeDoux, 1993); in particular, the lateral and basal nuclei of the amygdala are strongly involved in processing sensory information and in the detection of biologically relevant stimuli in the environment, including olfactory cues (Sah et al.,

2003). Microinjection of OXT into the central amygdaloid nuclei has been found to have potent anti-aggressive and pro-social exploratory effects in male WTG rats (Calcagnoli et al., 2014, in revision), similarly to what we have here described after intranasal OXT application. From human literature, the pro-social "tend and befriend"-like action ascribed to OXT appears to be associated with an OXT-dependent suppression of the amygdala activity in exposure to aversive and stressful conditions (Kirsch et al., 2005), and facilitated amygdala activity under positive/empathy-provoking conditions (Petrovic et al., 2008).

The amygdala is also known to have projections to the hypothalamus for further integration and coordination with the brain stem (Swanson and Petrovich, 1998). Therefore, the hypothesis that intranasal OXT applicaton might induce endogenous release of the peptide via activation of the OXTergic neurons in the PVN and SON cannot be ruled out. In the current study we have indeed found a higher neuronal activity in both hypothalamic nuclei after acute intranasal OXT treatment, and especially more active OXT positive cells. Such neuronal changes could be related to the behavioral changes observed acutely in the OXT-treated animals. Our results, however, are in contrast with data recently reported by the group of Ludwig and colleagues (Ludwig et al., 2013). In their study, Fos expression was evaluated in many areas of the olfactory system and in several brain regions, including PVN and SON, after either intranasal application or icv infusion of OXT or AVP. As expected, the authors reported increased neuronal activation in the amygdala, PVN, SON, and lateral septum after icv injection of the nonapeptides as compared to vehicle, but no activation effect was found after intranasal application. They also did not find effects of intranasal AVP on behaviors related to social discrimination, exploration or anxiety, well known to be centrally modulated by the nonapeptide. Contrary to what our data suggest, the authors conclude that very large doses of nonapeptides administered intranasally in rats do not enter the brain in amounts sufficient to exert clear neuronal effects. Therefore, it should not be ignored that differences in OXT dosage (we applied a higher dose as compared to Ludwig´s study) and method of nasal application may determine the effectiveness of the intranasal procedure.

Considering that a parallel but independent peripheral uptake is likely to occur after intranasal administration of OXT (Neumann et al., 2013; Striepens et al., 2013), more knowledge should also be gained about the contribution that peripheral OXT-evoked physiological changes may have to the centrally–mediated behavioral output. Studies have shown, for instance, that blockage of peripheral AVP receptors (AVPRs) not only prevented AVP-mediated physiological changes, but also abolished AVP-induced behavioral effects (Le Moal et al., 1981). In addition, inhaled AVP has been reported to increase sociality in rats, concomitantly with a reduction of temperature and heart rate (Ramos et al., 2014). Despite this evidence and the increased arterial pressure and bradycardia previously found after elevated peripheral OXT level (Ludwig et al., 2013), in our study we did not find any difference in cardiovascular (re)activity between vehicle and OXT-treated animals. Hence, the suggestion that our behavioral changes may have been (partly) caused by peripheral cardiovascular changes induced by plasma OXT can be refuted.

Another method to ascertain that the intranasal OXT-induced behavioral effects are principally centrally mediated is combining intranasal OXT with a selective OXT receptor antagonist given centrally. However, different diffusion rates of the intranasal and icv route might limit the validity and conclusive value of this type of experiment. Alternately, imaging studies have been used to prove the nose-to brain transport of relative big molecules in rats (Thorne et al., 2004). However, the influence that the radiolabelled ligand might have on the physical and chemical properties of the investigated molecule represents still the major concern for using this technique.

In conclusion, our findings provide evidence for the effectiveness of intranasal delivery of OXT in modulating social behavior in either an aggressive conflict with a conspecific or an affilitiative interaction with a familiar partner. The robustness and replicability of the behavioral effects encourage further investigation aimed at revealing the precise mechanistic underpinnings of the nose-to brain transport/communication route.

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NEDERLANDSE SAMENVATTING

Achtergrond

Agressie tussen soortgenoten is een karakteristieke vorm van gedrag dat in het gehele dierenrijk voorkomt, van insecten tot vogels, vissen en zoogdieren inclusief de mens. In al deze diersoorten heeft het een belangrijke adaptieve functie bij het vaststellen en handhaven van de sociale structuur en het verkrijgen en beschermen van zaken die voor de overleving en genetische 'fitheid' van het individu belangrijk zijn, zoals seksuele partners, nakomelingen, bronnen voor voedsel en water, enz. De fysiologische mechanismen die verantwoordelijk zijn voor agressief gedrag zijn ook evolutionair oud en goed geconserveerd. Dit betekent dat we veel over de (neuro)biologische basis van agressie bij de mens kunnen leren door het bestuderen van agressie bij (proef)dieren. Deze biologische kennis is noodzakelijk om te kunnen begrijpen hoe agressie kan ontsporen in meer pathologisch, gewelddadig gedrag. Dit is vervolgens weer van belang voor het ontwikkelen van doeltreffende therapeutische interventie- en preventie-strategieën. Hoewel slechts een betrekkelijk gering percentage van onze menselijke populatie buitensporige- en gewelddadige vormen van agressie vertoont, worden de omvangrijke negatieve medische en sociaaleconomische gevolgen van dit abnormale gedrag door de gehele maatschappij als zeer problematisch en ontwrichtend bestempeld. Daarnaast komen, in toenemende mate, extreme vormen van agressie, impulsiviteit en antisociale gedragingen veelvuldig voor bij diverse neuropsychiatrische en neurodegeneratieve stoornissen zoals autisme, dementie, verslaving, schizofrenie, etc.

Gedurende de laatste decennia is de aandacht van veel preklinisch en klinisch wetenschappelijk onderzoek uitgegaan naar oxytocine (OXT). Oorspronkelijk was OXT bekend als hormoon dat bij vrouwen betrokken is bij de bevalling (uteruscontractie) en borstvoeding (melkafgifte). Het heeft echter ook belangrijke en opmerkelijke neuromodulatoire eigenschappen bij de regeling van sociaal gedrag. Bij mens en dier versterkt OXT bijvoorbeeld ouderlijke zorg, samenwerking, vertrouwen, paarband en herkenning van familie en van soortgenoten. Op basis van deze pro-sociale "tend and befriend" werking is verondersteld dat OXT ook anti-agressieve eigenschappen zou hebben. Het onderzoek hiernaar is echter niet eenduidig en laat tegenstrijdige effecten zien mogelijk veroorzaakt door soort-, stam- en geslachtsverschillen, experimentele beperkingen, individuele en epigenetische factoren.

Onderzoeksvraag

Het promotieonderzoek dat in deze dissertatie is beschreven heeft zich gericht op de rol van OXT bij offensief agressief gedrag van de mannelijke rat. Offensieve agressie wordt geïnitieerd door een aanvaller en is gekenmerkt door een reeks van inleidende gedragingen zoals dreiggedrag voorafgaand aan de openlijke fysieke agressieve aanvallen zoals bijten. Agressie werd zowel kwalitatief als kwantitatief gemeten in een territoriale "resident-intruder" agressietest. In deze test wordt een territoriumeigenaar (resident) in zijn eigen

kooi geconfronteerd met een onbekende mannelijke soortgenoot (indringer). Dit leidt tot een rijk repertoire van offensieve gedragingen bij de resident, waarbij de intensiteit en de duur van dit gedrag sterk kan afhangen van het individu, diersoort of stam. Om extreem lage niveaus van agressie, zoals normaliter aanwezig bij de meeste laboratorium ratten, te voorkomen heb ik gebruik gemaakt van de zogenaamde wild-type Groningen rat. In deze rattenstam kan de duur en de intensiteit van offensieve agressie variëren van 0% tot 80% van de totale waarnemingstijd. Behalve een veel hoger populatiegemiddelde van agressie heeft deze stam ook een veel rijker repertoire van sociale gedragingen en een bredere individuele variatie binnen de populatie vergeleken met de sterk gedomesticeerde laboratorium rat. Deze fenotypische eigenschappen maken het mogelijk om het verband tussen de individuele variatie in agressie en variatie in het endogene OXT systeem te bestuderen. Dit is met name van belang voor het trekken van conclusies over de eventueel anti-agressieve eigenschappen van stoffen die aangrijpen op het OXT systeem.

De inhoud van dit proefschrift bestaat uit zes hoofdstukken. Een korte introductie (**hoofdstuk 1**) beschrijft de belangrijkste aspecten met betrekking tot het verband tussen OXT en agressie en bespreekt de belangrijkste resultaten van dit onderzoek in de context van de huidige literatuur. De resterende vijf hoofdstukken beschrijven experimenten gericht op de volgende onderzoeksvragen:

1. Beïnvloedt een acute toediening in het brein van synthetisch OXT offensieve agressie bij mannelijke ratten? Wat is de gedrags- en receptorspecificiteit en variatie tussen individuen?
2. Wat is het effect van een chronische behandeling met OXT in het brein op agressie en leidt dit tot blijvende veranderingen?
3. Welke hersengebieden zijn betrokken bij de modulatie van offensieve agressie door OXT?
4. Is de individuele variatie in agressie geassocieerd met individuele verschillen in het endogene OXT systeem in de hersenen?
5. Heeft een intranasale toediening van OXT dezelfde effecten op agressie als intracerebraal toegediende OXT?

De eerste reeks experimenten (**hoofdstuk 2**) hebben aangetoond dat een acute toediening van OXT rechtstreeks in de hersenventrikels leidt tot een dosis-afhankelijke daling van agressie. Verhoogde niveaus van OXT in de hersenen verlaagde de duur van offensieve gedragingen tijdens de agressieve interactie zonder dat het de aanzet tot de eerste agressie verminderde. Tevens verhoogde OXT het sociaal exploratieve gedrag van de resident naar de indringer. Deze duale werking werd verhinderd wanneer binding van de synthetische OXT werd geblokkeerd door het toedienen van een selectieve OXT receptor antagonist. De daling van agressie door OXT was het sterkst bij dieren die een hoog basaal niveau van agressie vertoonden. Farmacologische blokkade van de OXT receptoren leidde tot een toename in agressie bij dieren met een laag basaal agressieniveau. Deze eerste experimenten leiden tot de conclusie dat het endogene OXT systeem in het brein een remmende functie heeft op offensief agressief gedrag.

De eenduidige resultaten van de acute OXT toediening gaf aanleiding tot het testen van de effecten op agressief gedrag van een chronische verhoging (met een synthetisch OXT ligand) of een verlaging (met een OXT receptor antagonist) van de functionele activiteit van het centrale OXT systeem (**hoofdstuk 3**). Chronische veranderingen in het OXT systeem werd bewerkstelligd door gebruik te maken van onderhuids-geïmplanteerde osmotische mini-pompjes die werden verbonden met de eveneens geïmplanteerde canule in de laterale ventrikel van de hersenen. Deze pompjes leveren een continue en constante afgifte van de farmaca over een periode van zeven dagen. De proeven bevestigden de anti-agressieve en pro-sociale werking van OXT. Het was opmerkelijk dat deze gedragsveranderingen bleven bestaan tot minimaal zeven dagen na stopzetting van de behandeling. Dit laat zien dat het mogelijk is meer permanente veranderingen in agressief gedrag te bewerkstelligen via tijdelijke toediening van OXT. Hoewel de gebruikte centraal nerveuse toediening een methode is die niet direct toepasbaar is bij de mens, verdienen deze blijvende effecten nader onderzoek. Misschien is het op termijn mogelijk om via tijdelijke OXT manipulaties blijvende gedragsveranderingen teweeg te brengen in klinisch therapeutische situaties.

De eenduidige resultaten van zowel de acute als de chronische beïnvloeding van OXT gaven aanleiding tot de vraag waar precies in het brein OXT zijn werking op agressie uitoefent (**hoofdstuk 4**). De proeven richtten zich op een lokale toediening van OXT in de centrale amygdala van het limbische systeem en de dorsale raphe in de hersenstam. Beide hersengebieden zijn dicht bezet met OXT receptoren en in de literatuur zijn er sterke aanwijzingen dat deze hersengebieden een belangrijke rol spelen bij de gedragsmatige en emotionele reactie op sociale prikkels. Toediening van OXT in de dorsale raphe gaf verrassenderwijs geen duidelijke gedragsveranderingen te zien. Dit in tegenstelling tot de centrale amygdala waar OXT een robuuste anti-agressieve en pro-sociale werking had. Deze resultaten ondersteunen klinische studies die laten zien dat intranasale toediening van OXT leidt tot een reductie van de aversie voor negatieve en bedreigende sociale prikkels samenhangend met een daling in de reactiviteit van de amygdala.

In **hoofdstuk 5** wordt de hypothese getoetst dat individuele variatie in het endogene OXT systeem gerelateerd is aan de individuele variatie in basale agressie en de reactie op OXT toediening. Deze hypothese was niet alleen gebaseerd op onze eerdere gedragsfarmacologische resultaten, het was ook gebaseerd op de idee dat gewelddadig en antisociaal gedrag bij de mens geassocieerd is met lage niveaus van OXT in de hersenen. Om dit humane gegeven zo goed mogelijk te benaderen kregen de dieren herhaaldelijk eenzijdige winnaarservaring met gevechten. Onder deze condities ontwikkelen sommige (ongeveer 10-15 %) medium tot hoog-agressieve dieren namelijk een vorm van pathologische, geëscaleerde agressie. Deze vorm van gewelddadig agressief gedrag wordt niet alleen gekenmerkt door een hoge intensiteit van aanvallende agressie en verlies van inhibities over het uitvoerende gedrag, maar vooral door het onvermogen om alternatieve routes van conflict resolutie te bewandelen, het achterwege laten van geritualiseerde dreighoudingen die bijtaanvallen aankondigen en het negeren van signalen van de

tegenstander. Het mag dan ook geen verwondering wekken dat een dergelijke vorm van agressie leidt tot verwonding van de tegenstander. Deze gedragskenmerken suggereren een stoornis in hersenchemie. Het bleek inderdaad dat dieren die deze pathologische vorm van agressie vertoonden een laag niveau van OXT transcriptie hebben in de OXT producerende gebieden van de hypothalamus. Daarnaast hadden deze dieren een hogere OXT receptor binding in het limbische systeem. Deze bevindingen zijn relevant voor het begrijpen van het oorzakelijke verband tussen het functioneren van het OXT systeem in het brein en het ontstaan van pathologische vormen van sociaal gedrag.

De groeiende aandacht voor de gedragseffecten bij de mens van OXT, toegediend als nasale spray, en het gebrek aan inzicht in de achterliggende mechanismen vormde de basis voor de experimenten beschreven in **hoofdstuk 6**. Intranasale toediening van OXT bij de rat had een sterk anti-agressieve en prosociale werking tijdens de confrontaties met de onbekende mannelijke indringer. Daarnaast bevorderde het de paarband in een test waarin het proefdier de keuze kreeg uit de eigen vrouwelijke partner en een vreemd vrouwtje. Twee experimenten waren gericht op de vraag via welke mechanismen intranasale OXT het sociale gedrag zou kunnen beïnvloeden. In een experiment waarin met behulp van radiobiotelemetrie hartslag en bloeddruk kon worden gemeten konden niet-specifieke, perifere fysiologische effecten via veranderingen in autonome activiteit worden uitgesloten. In een tweede experiment werd de hypothese getoetst dat intranasaal OXT het endogene OXT systeem zou kunnen activeren. Intranasale toediening van OXT bleek inderdaad te leiden tot een verhoogde activiteit van OXT neuronen in de hypothalamus. Hoe dit in zijn werk gaat is echter nog onduidelijk. Onze studie is een van de weinige preklinische studies die gericht is op een mechanistische verklaring voor het transport van het OXT signaal van de neus naar het brein.

Is OXT een nieuwe anti-agressieve stof?

De gegevens verzameld in dit promotieonderzoek leiden tot de conclusie dat OXT werkt als een serenicum; onder invloed van OXT verschuift het gedrag van openlijke agressie naar sociaal exploratief en affiliatief gedrag. Tezamen met het succes van intranasale toediening van OXT op deze verandering in gedrag, geeft dit perspectief voor een mogelijke biomedische toepassing van synthetisch OXT bij neuropsychiatrische ziekten waarbij ongecontroleerde uitbarstingen van agressief gedrag een serieus probleem is voor zowel de patiënt als de verzorger. De blijvende toename van pro-sociaal gedrag na kortdurende subchronische toediening van OXT suggereert dat een effectieve therapeutische interventie met OXT of zelfs een preventieve benadering tot de mogelijkheden zou kunnen behoren. De studies wijzen ook op het feit dat het klinisch en preklinisch onderzoek meer aandacht moet besteden aan de individuele variatie in het OXT systeem en de daarmee samenhangende individuele verschillen in agressief gedrag. Het is daarnaast van groot belang dat het diermodel dat gebruikt wordt voor het testen van de effecten farmaca op agressie, de fenotypische eigenschappen heeft van humane pathologische agressie. De mogelijke hypo-activiteit van het OXT systeem bij ratten die

een ongeremde vorm van agressie vertonen dat duidelijk geen functie meer heeft in de normale sociale communicatie is daarvan een duidelijk voorbeeld.

Uit dit promotie onderzoek blijkt dat OXT een belangrijke signaalstof is in het neurale hersen netwerk voor sociaal gedrag; het is een belangrijke moleculaire kandidaat voor verder onderzoek naar de stoffelijke beinvloeding van offensieve agressie en de pathologie daarvan.

ENGLISH SUMMARY

Background

Aggression is one of the most widespread and characteristic behaviors in the animal kingdom that is critical for individual fitness and survival. Aggression is the behavioral weapon of choice in both animals and humans to defend themselves, secure food and mates, compete for resources, and maintain social hierarchies. However, despite its evolutionary conservation and importance for individual health and well-being, the neurobiology and psychopharmacology of aggressive behavior and of associated affective states, such as anger, hostility and rage, are sorely understudied topics in behavioral neuroscience and pharmacological research. This is even more surprising considering the fact that pathological antisocial behavior and violent outbursts represent one of the most significant problems for the public health and criminal justice systems, and takes a tremendous toll on our society. Unfortunately, despite this burden on our society, the current intervention strategies and treatment options are largely serendipitous, disappointingly inadequate and frequently accompanied by severe side effects. This is mainly due to our still very limited knowledge about the precise etiology and neurobiology of excessive aggressive behavior and the general lack of successful translation of putatively promising neuroscientific findings into clinically effective treatments.

During the last decades, the attention of many preclinical and clinical researchers has been devoted to the nonapeptide oxytocin (OXT). OXT was originally known as the neurohypophyseal hormone facilitating labor (uterus contraction) and lactation (milk ejection), however, important and remarkable neuromodulatory properties have been found in the regulation of social behavior. In humans and animals, for instance, OXT enhances parental care, cooperation, trust, pair bond and recognition among family members and peers. On the basis of these pro-social "tend and befriend" -like actions it has been suggested that OXT could have anti-aggressive properties. So far, however, the investigation remains inconclusive and the results are often contradictory, possibly due to species, strain and sex differences, experimental limitation, individual and epigenetic factors.

Research question

The PhD research described in this thesis has focused on the role of OXT in offensive aggression in male rats. Offensive aggression is initiated by the offender and is characterized by a series of preliminary behaviors such as threatening displays prior to overt aggression, such as biting. Aggression was both qualitatively and quantitatively measured using a territorial aggression test. In this test, a territorial male rat (resident) in its own cage is confronted with an unfamiliar male conspecific (intruder). This leads to a rich repertoire of offensive behaviors undertaken by the resident towards the intruder, in which the intensity and the duration of the offense may depend on the individual. To overcome the extremely low aggression levels typically expressed by the most commonly used laboratory animals, it has been used a strain of rodents originated from wild ancestors, so-called wild-type Groningen rat. In this strain of rats, the duration of the offensive aggression

displayed by adult male individuals can vary from 0 % to 80 % of the total observation time. Besides a larger population mean in baseline aggression, this strain has a much richer repertoire of social behaviors and a wider within-population individual variability as compared to the highly domesticated laboratory rats. These phenotypic properties allow studying the link between the individual variation in aggression and the variation in the endogenous OXTergic system. This is of particular importance for drawing conclusions about the possible serenic profile of substances that affect the OXTergic system.

The content of this thesis is divided in six chapters. A brief introduction (**chapter 1**) presents the core aspects related to the link between OXT and aggression, and discusses the main findings of this research in the context of the current literature. The following five chapters describe experiments focused on the research questions mentioned below:

1. Does acute administration of synthetic OXT in the brain affect offensive aggression in male rats? What is the dose-dependency, the behavior- and receptor-specificity, and the variation between individuals?
2. What is the effect of chronic OXT treatment on aggression and does it lead to long-lasting changes in aggressiveness?
3. Which brain regions are involved in the OXTergic modulation of offensive aggression?
4. Is the individual variation in aggression associated with individual differences in endogenous structural/functional properties of the brain OXTergic system?
5. Does intranasal administration of OXT have similar effects on aggression as intra-cerebral administered OXT, and if so via which mechanism?

The first set of experiments (**chapter 2**) has shown that acute administration of OXT into the cerebral ventricles leads to a dose-dependent decrease of intermale aggression, without delaying the onset of the first attack. In addition, OXT increases social exploratory behavior of the resident towards the intruder. These behavioral effects were prevented when receptor binding of the synthetic OXT was blocked by the co-administration of a selective OXT receptor antagonist. The OXT-induced decrease of aggression was greater in animals that exhibited high basal level of aggression. Pharmacological blockade of OXT receptors has led to an increase in aggression in animals with a low basal level of aggression. These first results lead to the conclusion that elevation of brain OXT levels has serenic properties on intermale offensive aggression.

The consistent results seen after acute OXTergic manipulation prompted to test the behavioral effects of a chronic elevation (with a synthetic OXT) or attenuation (with an OXT receptor antagonist) of the central OXTergic activity (**chapter 3**). Chronic manipulation of the central OXT levels was performed by implanting subcutaneously osmotic mini-pumps, which were connected to a cannula in the lateral ventricle of the brain. This methodology allowed a continuous and constant cerebroventricular drug infusion for seven days. The behavioral tests confirmed the robust anti-aggressive and pro-social efficacy of sustained enhancement of central OXT levels. A remarkable finding was that these behavioral

changes persisted for at least seven days after OXT treatment was stopped. This indicates a more lasting or permanent change in aggressive behavior induced by chronic OXT treatment. Although central infusion is a method not directly applicable to humans, the evidence of enduring effects and no rebound phenomena invites to further investigate potential long-lasting behavioral changes in a clinical therapeutic situation as well.

The unequivocal behavioral evidence for serenic effects of both acute and chronic OXT administration gave rise to the question of where exactly in the brain OXT exerts its effects on aggression (**chapter 4**). The experiments focused on local injections of OXT into the central nucleus of the amygdala within the limbic system, and the dorsal raphe in the brainstem. Both brain areas are densely populated with OXT receptors and the literature suggests that these brain regions play an important role in the regulation of behavioral and emotional responsivity to social stimuli. Administration of OXT in the dorsal raphe did not reveal clear behavioral effects. This is in contrast to the central amygdala where OXT micro-injection induced potent anti-aggressive and pro-social changes. These results are in support of clinical studies showing that intranasal administration of OXT reduces behavioral aversion to negative or threatening social stimuli through the dampening of amygdala reactivity.

In **chapter 5**, it was tested the hypothesis that the individual variation in basal aggression and the responsivity to OXT treatment are related to individual differences in the functional and/or structural properties of the endogenous OXTergic system. This hypothesis was based not only on our previous pharmacological results, but also on the idea that violent behavior in humans has been associated with low brain OXT levels and/or signaling activity. To better model human aggression and violence, the resident rats underwent repeated fighting experiences. Under these conditions, some animals develop excessive levels and abnormal/pathological forms of aggressive behavior. Interestingly, the animals that exhibited this pathological aggression were characterized with a low level of OXT mRNA transcription in hypothalamic regions. In addition, these animals had a higher OXT receptor binding in the limbic system (central amygdala and bed nucleus of the stria terminalis). These findings are relevant for understanding the causal link between the functioning of the OXTergic system in the brain and the development of pathological forms of social behavior.

The growing attention for the behavioral effects induced in humans by nasal OXT administration, and the lack of understanding of the underlying mechanisms were the basis for the experiments described in **chapter 6**. Intranasal OXT administration in male resident rats had a strong anti-aggressive and pro-social action during the confrontation with the unknown male intruder. In addition, it promoted pair bonding in a test in which the male resident was given to choose between its own female partner and a novel female rat. Two experiments were focused on investigating the possible mechanisms by which intranasal OXT delivery could affect social behavior. By employing radio-biotelemetry, heart rate and blood pressure responses were measured after intranasal OXT application. The lack of autonomic physiological changes excluded the possibility that enhanced peripheral circulation of OXT, possibly occurring after intranasal OXT delivery and absorption into the

nasal blood vessels, is provoking peripheral effects that in turn may affect behavior. In a second experiment, it was tested whether intranasal OXT could activate the endogenous OXTergic system. Although the underlying mechanism still needs to be elucidated, intranasal OXT administration was indeed shown to increase the activity of OXT neurons in hypothalamic regions. This study is one of the few pre-clinical studies that aimed at a mechanistic explanation for the putative direct OXT nose-to-brain transport.

Is OXT a novel serenic compound?

The data collected in this thesis lead to the overall conclusion that OXT acts as a serenic-like compound, concomitantly shifting the social behavior from aggressive towards socially explorative and affiliative. This, together with the behavioral effectiveness of intranasal OXT administration, is of translational value for a possible clinical application of synthetic OXT in neuropsychiatric diseases in which uncontrolled aggression and social deficits are serious problems. Moreover, the long-lasting pro-social behavioral effects seen after chronic OXTergic manipulation suggest that an effective enduring therapeutic intervention or even prevention with OXT may be possible. This research also points to the fact that clinical and preclinical research should focus more attention on individual variations in the OXTergic system and the related individual differences in aggressive behavior. Moreover, the fact that a hypo-OXTergic activity was found in pathological aggressive rats similar to what is observed in human patients that express excessive aggressive and antisocial behavior, demonstrates the relevance and validity of the current animal model. In conclusion, this thesis shows OXT as an important neuropeptidergic signaling molecule within the brain social behavior network, and as a valid molecular candidate for further research that aims at modulating the neuronal circuits of intermale aggression.

RIASSUNTO IN ITALIANO

Definizione del problema

L'aggressività è uno dei comportamenti più diffusi e caratteristici del regno animale ed è fondamentale per il benessere e la sopravvivenza dell'individuo. Per gli animali, incluso l'uomo, l'aggressività è uno strumento di difesa, una strategia per assicurarsi cibo e partners sessuali, per l'accaparramento delle risorse ambientali, e per mantenere le gerarchie sociali. Tuttavia, nonostante l'aggressività abbia un carattere adattativo che permette la conservazione della specie, la neurobiologia e la psicofarmacologia del comportamento aggressivo e degli stati affettivi associati, come rabbia, ostilità e ira, sono argomenti non sufficientemente studiati in neuroscienze comportamentali e ricerca farmacologica. Ciò è ancora più sorprendente se si considera che il comportamento antisociale e violento rappresenta uno dei problemi più rilevanti per la salute pubblica e il sistema giudiziario, con pesanti tributi per la nostra società. Purtroppo, nonostante questo onere piuttosto gravoso per la società, le attuali strategie di intervento e le opzioni di trattamento sono in gran parte fortuite, molto spesso inadeguate e accompagnate da gravi effetti collaterali. Ciò è dovuto principalmente alla nostra ancora limitata conoscenza della corretta eziologia e neurobiologia del comportamento aggressivo e alla mancanza di una validata applicazione delle incoraggianti scoperte precliniche in trattamenti clinici.

Negli ultimi decenni molti ricercatori preclinici e clinici hanno puntato l'attenzione sul neuropeptide ossitocina (OXT). Noto come l'ormone neuroipofisario dall'effetto uterotonico e facilitante l'allattamento, l'OXT possiede importanti proprietà neuromodulatorie per la regolazione del comportamento sociale. Nei mammiferi l'OXT intensifica l'espressione delle cure parentali, la cooperazione, il senso di fiducia, il legame di coppia e il riconoscimento e l'identificazione tra i familiari e conspecifici. Sulla base di questa azione pro-sociale, si è ipotizzato che l'OXT possa avere anche proprietà anti-aggressive. Finora, tuttavia, le ricerche si sono rivelate inefficaci e i risultati sono spesso contraddittori, probabilmente a causa della differenza tra specie, progenie e sesso oltre che per le limitazioni sperimentali e le differenze genetiche ed epigenetiche legate al singolo individuo.

Argomento dello studio

L'argomento della ricerca di questa tesi di dottorato si è focalizzato sullo studio del ruolo dell'OXT nel comportamento offensivo di ratti maschi. L'aggressione offensiva viene istigata volutamente dall'aggressore ed è caratterizzata da una serie di comportamenti preliminari di minaccia ed intimidazione che precedono il reale attacco-morso. L'aggressione è stata qualitativamente e quantitativamente misurata attraverso un test di aggressione territoriale. In questo test, il ratto maschio territoriale (residente) viene confrontato nella propria gabbia con un maschio conspecifico sconosciuto (intruso). Questo incontro scatena un ricco repertorio di comportamenti offensivi intrapresi dal residente verso l'intruso, la cui l'intensità e durata varia da individuo a individuo. Per ovviare alla scarsa aggressività tipicamente espressa dagli animali di laboratorio più comunemente utilizzati, in questa

ricerca sono stati utilizzati esemplari di una stirpe di roditori che originava da antenati selvatici, cosiddetti ratti selvatici di Groningen. L'intensità aggressiva dei maschi adulti di questa razza può variare dallo 0 % all' 80 % del tempo totale di osservazione. Oltre al fatto di avere un più alto livello di aggressione in condizioni basali, questo tipo di ratto mostra un più ricco bouquet di comportamenti sociali e una più ampia variabilità individuale all'interno della popolazione rispetto al comune ratto di laboratorio, altamente addomesticato. Queste peculiari proprietà fenotipiche permettono di studiare il legame tra la variazione individuale nella manifestazione dell'aggressività e la variazione nel sistema OXTergico endogeno. Questo tipo di studio è di particolare importanza per trarre conclusioni sul possibile profilo anti-aggressivo di sostanze che influenzano il sistema centrale dell'OXT.

Il contenuto di questa tesi è diviso in sei capitoli. Una breve introduzione (**capitolo 1**) presenta alcuni degli aspetti più importanti riguardanti il legame tra l'OXT e l'aggressività, e discute i principali risultati di questa ricerca contestualizzandoli con la letteratura corrente. I cinque capitoli che seguono descrivono gli esperimenti eseguiti per dare delle risposte alle seguenti domande:

1. La somministrazione acuta di OXT nel cervello influenza l'aggressività offensiva nei ratti maschi? È l'effetto dose-dipendente, specifico per il comportamento aggressivo, selettivo per il recettore, e diverso da individuo a individuo?
2. Qual è l'effetto del trattamento cronico dell'OXT sull'aggressività; ci sono cambiamenti del comportamento a lungo termine?
3. Quali sono le aree del cervello coinvolte nell'azione modulatoria dell'OXT sull'aggressione offensiva?
4. Qual è la relazione tra la variazione individuale nel comportamento aggressivo e le differenze individuali nelle proprietà strutturali/funzionali del sistema OXTergico centrale?
5. Può la somministrazione nasale dell'OXT avere effetti sull'aggressione simili a quelli della sua amministrazione intracerebrale; e se sì qual è il meccanismo d'azione?

La prima serie di esperimenti (**capitolo 2**) ha dimostrato che la somministrazione acuta di OXT nei ventricoli cerebrali porta ad una diminuzione dose-dipendente dell'aggressione espressa dal maschio residente, senza però ritardare lo sferrarsi del primo attacco. Inoltre, l'OXT aumenta il comportamento esplorativo sociale del ratto residente nei confronti dell'intruso. Questi effetti comportamentali sono assenti quando il legame OXT-recettore viene impedito dalla co-somministrazione di un antagonista selettivo per il recettore dell'OXT. La diminuzione dell'aggressione è risultata essere più intensa negli animali caratterizzati da un più alto livello basale di aggressività. Il blocco farmacologico dei recettori dell'OXT induce invece un aumento dell'aggressività negli animali con un basso livello basale di aggressione. Questi primi esperimenti portano alla conclusione che l'aumento dei livelli di OXT nel cervello ha proprietà anti-aggressive negli individui maschi.

I persistenti risultati osservati dopo la manipolazione acuta del sistema OXTergico sono stati da stimolo per testare gli effetti cronici di un aumento (tramite infusione di OXT) o

diminuzione (tramite infusione di un antagonista selettivo) dell'attività centrale dell'OXT (**capitolo 3**). La manipolazione cronica dei livelli centrali dell'OXT è stata eseguita mediante l'impianto sottocutaneo di una mini-pompa a principio osmotico collegata ad una cannula terminante nel ventricolo laterale del cervello. Questo metodo ha consentito un'infusione cerebro-ventricolare continua e costante di OXT per sette giorni. I test comportamentali hanno confermato l'efficacia anti-aggressiva e pro-sociale dell'aumento continuo dei livelli centrali di OXT. Di notevole importanza è stata la scoperta che tali cambiamenti comportamentali persistono per almeno sette giorni dopo l'interruzione del trattamento con l'OXT. Questo indica che il trattamento cronico con l'OXT induce un cambiamento più duraturo o permanente del comportamento aggressivo. Sebbene l'infusione cerebro-ventricolare non sia un metodo direttamente applicabile all'uomo, l'evidenza di effetti duraturi e l'assenza di recidive invitano ad approfondire, anche in campo clinico, questi potenziali cambiamenti comportamentali di lunga durata.

Gli inequivocabili effetti anti-aggressivi dell'OXT osservati sia dopo il trattamento acuto che quello cronico hanno suggerito di investigare dove esattamente nel cervello l'OXT eserciti questa sua azione modulatoria (**capitolo 4**). Gli esperimenti hanno riguardato infusioni locali di OXT nel nucleo centrale dell'amigdala nel sistema limbico, e nella zona dorsale del nucleo del rafe nel tronco encefalico. Entrambe queste aree del cervello sono densamente popolate di recettori dell'OXT e la letteratura le ha ampiamente descritte come regioni importanti per il controllo della risposta comportamentale ed emotiva agli stimoli sociali. La somministrazione di OXT nella zona dorsale del nucleo del rafe non ha evidenziato chiari effetti comportamentali. Al contrario, un'acuta infusione di OXT nel nucleo centrale dell'amigdala ha evidenziato potenti cambiamenti anti-aggressivi e pro-sociali. Questi risultati sono di sostegno a studi clinici che dimostrano che la somministrazione nasale di OXT riduce l'avversione comportamentale a stimoli sociali negativi attraverso la riduzione della reattività dell'amigdala.

Nel **capitolo 5**, si è testata l'ipotesi che la variazione individuale nell'espressione dell'aggressività e nella risposta al trattamento con l'OXT sia dovuta a differenze individuali nelle proprietà funzionali e/o strutturali del sistema OXTergico endogeno. Questa ipotesi è basata non solo sui risultati farmacologici sopra menzionati, ma anche sull'idea che il comportamento violento nell'uomo è stato associato a bassi livelli di OXT e/o a ridotta attività del sistema centrale OXTergico. Pertanto, per aumentare la validità del modello animale, i ratti maschi residenti sono stati ripetutamente sottoposti a confronti sociali con l'intruso. In queste condizioni, alcuni individui hanno manifestato livelli eccessivi e forme anomale/patologiche di aggressività. Interessante è stato notare che gli individui che avevano sviluppato forme aggressive patologiche avevano un basso livello di mRNA per l'OXT nelle regioni ipotalamiche. Inoltre, nel loro sistema limbico è stata trovata una maggiore densità di recettori per l'OXT (amigdala centrale e nucleo della stria terminale). Questi risultati sono di alto rilievo conoscitivo per la comprensione del nesso causale tra il funzionamento del sistema OXTergico nel cervello e lo sviluppo di forme patologiche del comportamento sociale.

La crescente attenzione per gli effetti comportamentali indotti sull'uomo dalla somministrazione nasale di OXT, e la mancanza di importanti tasselli per la comprensione dei suoi meccanismi sono stati la base per gli esperimenti descritti nel capitolo 6. La somministrazione intranasale di OXT nei ratti residenti maschi ha nuovamente rivelato una forte azione anti-aggressiva e pro-sociale durante il confronto con l'intruso maschio. Inoltre, l'OXT è stata vista promuovere il legame di coppia in un test di preferenza sociale in cui al maschio residente veniva data la possibilità di scegliere tra la propria compagna e una sconosciuta femmina di ratto. Due esperimenti si sono concentrati sullo studio dei possibili meccanismi attraverso i quali l'OXT intranasale possa influenzare il comportamento sociale. Con l'ausilio della tecnica di radio-biotelemetria, frequenza cardiaca e pressione sanguigna sono state misurate in risposta all'applicazione intranasale di OXT. La mancanza di cambiamenti fisiologici regolati dal sistema nervoso autonomo hanno permesso di escludere la possibilità che l'eventuale aumento di OXT nella circolazione sanguigna dopo l'applicazione intranasale possa provocare effetti periferici che a loro volta influenzino il comportamento. In un secondo esperimento, si è testata l'ipotesi che l'OXT per via nasale potesse attivare il sistema OXTergico endogeno. Sebbene il meccanismo sottostante debba ancora essere chiarito, la somministrazione intranasale di OXT ha rivelato un aumento dell'attività dei neuroni dell'OXT nelle regioni ipotalamiche del cervello. Questo studio è uno dei pochi studi pre-clinici, che mirano a formulare una spiegazione meccanicistica del putativo trasporto diretto dell'OXT del naso al cervello.

È l'OXT un composto con proprietà anti-aggressive?

I dati raccolti e presentati in questa tesi portano a concludere che l'OXT agisce come un composto anti-aggressivo, in concomitanza al fatto che l'OXT è in grado di intensificare il comportamento pro-sociale dell'animale maschio trattato. Questa osservazione, insieme all'efficacia del trattamento per via nasale, è di alto valore scientifico per una possibile applicazione clinica dell'OXT nelle malattie neuropsichiatriche in cui l'aggressività e i deficit sociali costituiscono un grave problema per l'individuo e per chi lo assiste. Inoltre, gli effetti pro-sociali visti a lungo termine dopo la manipolazione cronica, suggeriscono che con l'OXT sia possibile un efficace intervento terapeutico duraturo o addirittura preventivo. Questa ricerca sottolinea anche il fatto che la ricerca clinica e preclinica dovrebbe concentrare maggiormente l'attenzione sulle variazioni individuali del sistema OXTergico e sulle relative differenze individuali nell'espressione del comportamento aggressivo. Inoltre, il fatto che una ridotta attività dell'OXT centrale sia stata trovata nei ratti con patologia aggressiva similmente a quanto noto in pazienti violenti ed antisociali, dimostra l'importanza e la validità del modello animale utilizzato negli studi. In conclusione, questo lavoro di ricerca presenta l'OXT come un importante neuropeptide all'interno dei circuiti del cervello che regolano il comportamento sociale, e come valido candidato per successive ricerche che mirino a modulare i circuiti neuronali dell'aggressione tra individui maschi.

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Finally!!!

Writing this page feels like having found a refuge while still climbing the mountain of the "Scientific Revelation"…I am not on the peak yet, but still, finally I found some beverages and a less steep path...hence, for the time of few breaths, I can sit and look beneath…

I see people, I read words, I remember episodes…I feel good, it makes me happy.

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CURRICULUM VITAE

Federica Calcagnoli was born on 15 February 1986 in Macerata, Italy. In 1999 she commenced her scientific interest attending five years at the scientific high school "C. Onesti" in Fermo. In 2004 she started the University of Chemistry and Pharmaceutical Technology in Camerino. In 2008 she won a scholarship through the Erasmus international exchange program. She therefore moved to the University of Groningen in The Netherlands, where she performed a research project at the department of Neuroendocrinology under the supervision of prof. Anton J.W. Scheurink and Simon S. Evers. Her project was focused on the influence of topiramate on olanzapine-related metabolic dysfunctions in male rats. After her return to Italy, she graduated *cum laude* at the University of Camerino, and in 2009 she started a joint PhD project between the department of Behavioral Physiology of the University of Groningen and the department of Psychiatry of the University Medical Center Groningen, under the supervision of prof. Jaap M. Koolhaas, dr. Sietse F. de Boer and dr. Monika Althaus. The results of her research are summarized in this dissertation.