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OXYTOCIN-BASED PHARMACOTHERAPY FOR AUTISM SPECTRUM DISORDER

BEHAVIORAL AND NEURAL EFFECTS OF A PROMISING INTERVENTION APPROACH

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List of abbreviations

ABC	Aberrant Behavior Checklist
ACC	anterior cingulate cortex
ADOS	Autism Diagnostic Observation Scale
AI	anterior insula
A or AMY	amygdala
ANOVA	analyses of variance
ANS	autonomic nervous system
AQ	Autism Spectrum Quotient
ASD	autism spectrum disorder
Bst	brainstem
CARS	Childhood Autism Rating Scale
CBCL	Child Behavior Checklist
CGI	Clinical Global Impression
DANVA	Diagnostic Analysis of Nonverbal Accuracy
dmPFC	dorsomedial prefrontal cortex
DB	double-blind
DBC	Developmental Behavior Checklist
DSM	Diagnostic and Statistical Manual of Mental Disorders
ECG	electrocardiography
EEG	electroencephalography
fMRI	functional magnetic resonance imaging
GAF	Global Assessment of Functioning
GSAD	generalized social anxiety disorder
HRV	heart rate variability
IRSA	Interaction Rating Scale Advanced
IU	international units
IPPA	Inventory of Parent and Peer Attachment
mPFC	medial prefrontal cortex
MRI	magnetic resonance imaging
NAcc	nucleus accumbens
NT	neurotypical
OFC	orbitofrontal cortex
OT	oxytocin
PD	Prisoner's Dilemma

PL	placebo
PLD	point-light display
POMS	Profile of Mood States
pSTS	posterior superior temporal sulcus
PVN	paraventricular nucleus
RBS-R	Repetitive Behavior Scale – Revised
RMET	Reading the Mind in the Eyes Test
RRB	repetitive and restricted behavior
SAAM	State Adult Attachment Measure
SAD	social anxiety disorder
SB	single-blind
SD	standard deviation
SEM	standard error of the mean
Sep	septum
SON	supraoptic nuclei
SRS	Social Responsiveness Scale
STAI	State-Trait Anxiety Inventory
STS	superior temporal sulcus
Tha	thalamus
TSST	Trier Social Stress Test
vlPFC	ventral lateral prefrontal cortex
vmPFC	ventromedial prefrontal cortex
VTA	ventral tegmental area
WHO-QOL	World Health Organization Quality of Life
YBOCS	Yale Brown Obsessive Compulsive Scale

General Introduction

Chapter I

1 Autism Spectrum Disorder

"Since 1938, there have come to our attention a number of children whose condition differs so markedly and uniquely from anything reported so far, that each case merits - and, I hope, will eventually receive - a detailed consideration of its fascinating peculiarities."

Leo Kanner (1943)

1.1 Background

More than 75 years ago, both Leo Kanner and Hans Asperger nearly simultaneously described the first cases of an Autism Spectrum Disorder (ASD) resembling behavioral disorder in the scientific literature. Kanner (1943)[1] reported cases of eight boys and three girls with "autistic disturbances of affective contact", characterized by "extreme autistic aloneness, abnormal speech, and monotonous, repetitive behaviors with an "anxiously obsessive desire for sameness". According to Kanner (1943), these children "had come into the world with an innate inability to form the usual, biologically provided affective contact with people". Similarly, Asperger (1944)[2] described the cases of four boys with "autistic psychopathy of childhood", characterized by poor social and emotional relationships, high sensitivity, yet lacking feelings for others, and stereotypic behaviors and pervasive special interests. The exact definition of Kanner's syndrome, Asperger's syndrome or autistic disorder, however, has often changed throughout the years. Nowadays, according to the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-5), ASD is defined as a group of complex neurodevelopmental disorders mainly characterized by difficulties in social interaction and communication and restricted interests and repetitive behaviors[3]. These social impairments are reflected in more or less severe problems (depending on the individual) with the following behaviors as compared to neurotypicals: gaze and eye contact; emotion, face and biological motion processing; social attention, orienting and joint attention; social motivation; social reward processing; non-verbal communication; imitation; affective empathy and sympathy; pretend play; theory of mind or mental perspective taking; self-referential cognition; alexithymia (difficulty understanding and describing own emotions); and metacognitive awareness (reviewed in Lai et al. (2014)[4]). Despite the presence of these common features, individuals with ASD form a heterogeneous group, both at the level of behavioral characteristics and on the level of difficulties and needs in everyday life. Concurrent medical (i.e. sleep

disorders, epilepsy, gastrointestinal problems), developmental (i.e. intellectual disability, ADHD[i], language disorders) or psychiatric (i.e. anxiety, depression, OCD[ii]) conditions occur in more than 70% of individuals with ASD (reviewed in Lai et al. (2014)[4]). Also, the prevalence of ASD has increased the past decades, resulting in a prevalence of approximately 1% worldwide[4], with the disorder affecting 3.1 times more boys than girls[5]. Considering the increasing prevalence and high clinical, social and financial burden of ASD on society, there is a strong need for effective treatments. To date, however, no pharmacological treatment exists targeting the core characteristics of ASD.

1.2 Interventions in ASD

Currently, there is no existing cure for ASD. However, those interventions that have proven to be effective are mostly behavioral and educational in nature (and mostly targeted at helping children), whereas effective medications and behavioral interventions for core ASD characteristics in adults are limited (reviewed in Lai et al. (2014)[4]). Behavioral interventions can be classified into five complementary categories: (i) Applied Behavior Analysis (ABA) based interventions (e.g. the Early Start Denver Model); (ii) structured teaching (e.g. TEACCH[iii]); (iii) targeted skill-based interventions; (iv) targeted behavioral interventions for anxiety and aggression; and (v) parent-mediated early intervention. These interventions are mostly individualized (home- or school-based), multidimensional, multidisciplinary and take into account an individual's strengths to maximize children and adult's functional independence and quality of life through development and learning, improvements in social skills and communication, reductions in disability and comorbidity, promotion of independence, and provision of support to families[4].

Aside from behavioral interventions, the only two pharmacological therapeutics that have been approved by the US Food and Drug Administration (FDA) to treat young individuals with ASD are the antipsychotic medications Risperidone and Aripiprazole, both affecting dopaminergic and serotoninergic systems[6,7]. These medications, however, do not target the core ASD characteristics, but are aimed at decreasing associated (although not less troublesome) symptoms such as aggressive behavior, irritability, self-injury and severe tantrums or meltdowns. In sum, no pharmacological treatment currently exists targeting the core ASD characteristics. However, during the past 15 years, the neuropeptide oxytocin (OT) has gained increasing interest as a potential therapeutic approach for core ASD characteristics.

[i] Attention Deficit Hyperactivity Disorder
[ii] Obsessive-Compulsive Disorder
[iii] TEACCH = Treatment and Education of Autistic and related Communication-handicapped Children

2 Oxytocin

Labelled “the love hormone” by popular media and advertised online as a wonder drug to improve one’s social skills and trustworthiness on the job and at home, much has been said and written about oxytocin (OT). OT is a neuropeptide produced in the magnocellular neurons of the paraventricular and supraoptic nuclei of the hypothalamus, from where it is projected to the posterior pituitary[8]. From the posterior pituitary OT is released into the bloodstream where it functions as a hormone to affect bodily functions (peripheral circulation) (Fig. 1.1). Paraventricular nuclei neurons additionally project to multiple limbic, midbrain and hindbrain structures, regions characterized by having a large quantity of OT receptors (i.e. hippocampus, amygdala and nucleus accumbens) (central circulation). Aside from its hormonal function, OT also acts as a neurotransmitter and neuromodulator within the brain[8].

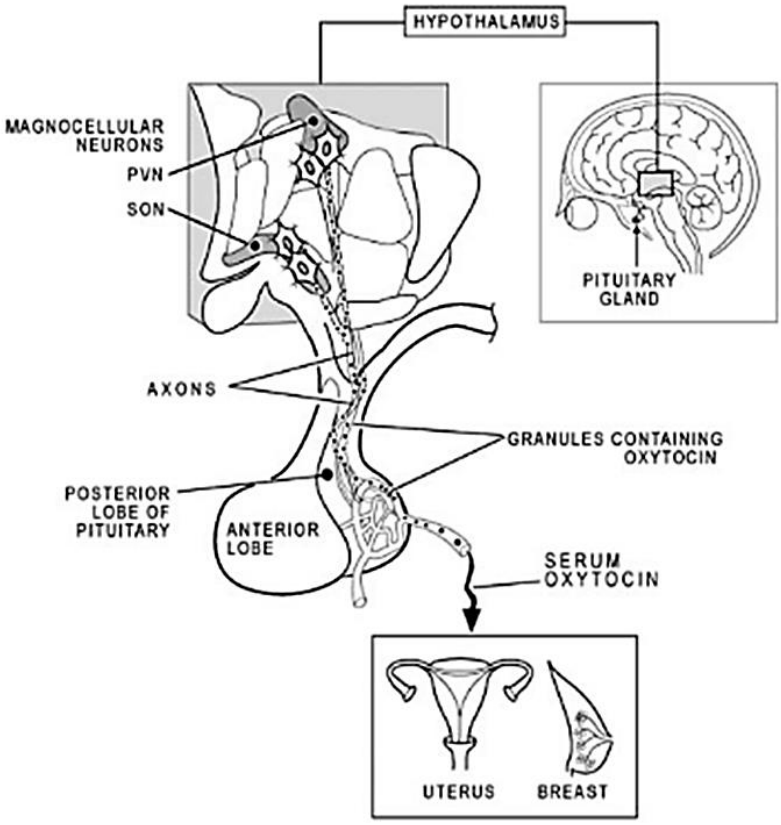

Figure 1.1. Neurophysiology of oxytocin. PVN = paraventricular nuclei, SON = supraoptic nuclei. Figure adapted from Macdonald & Macdonald (2010) [8].

2.1 How did oxytocin come into the ‘ASD-picture’?

Named after the Greek word for “quick birth”, OT is mostly known for its relation to childbirth, as OT promotes uterine contractions during labor and milk ejection during lactation[9] (peripheral circulation). However, centrally released OT (i.e. at the level of the brain) has been implicated in

the regulation of a variety of social behaviors in animals[9]. Initial studies in rats and sheep have shown that, aside from childbirth and nursing, OT also alters the mother's brain to induce the motivation to nurture its infant and to develop a selective mother-infant bonding[10–13]. Following studies in prairie voles, an exceptional model species to study complex social behaviors due to their monogamous pair bonding and biparental care of pups[14], uncovered a critical role for OT in the formation of pair bonding[15]. Subsequent research in animals (mainly rodents) also revealed that OT was not only involved in pair bonding itself, but also in the actions resulting in pair bonding, namely social approach and motivation (i.e. parental behavior, mother-infant interactions and adult affiliation), and social memory and recognition (i.e. duration of social investigation during exposure to other animals) (reviewed in Lim and Young (2006)[16]).

These findings from animal research have led researchers to hypothesize the existence of oxytocin abnormalities in humans with social impairments (e.g. ASD, schizophrenia, social anxiety). In support of this notion, multiple correlational studies have reported findings on the link between the level of peripheral OT and socially relevant behaviors. For example, higher levels of plasma OT have been associated with trust and trustworthiness[17], positive physical contact with a partner[18], reduced hormonal responses to a psychosocial stressor[19], whereas lower plasma OT levels have been discovered in patients with schizophrenia[20,21] and autism spectrum disorder (ASD)[22,23].

2.2 Oxytocin affects social behavior in neurotypicals

The first studies assessing the effects of single-dose, intranasal OT used paradigms related to social stress, social interaction, emotion and face processing, and social memory (i.e. behaviors often impaired in ASD) (reviewed in Meyer-Lindenberg et al. (2011)[24]). For example, Heinrichs et al. (2003)[25] explored the effects of both social support and a single dose of OT (24 International Units (IU)) during preparation for the Trier Social Stress Test (TSST)[iv] in 37 neurotypical men. They showed that cortisol levels were suppressed by social support and that anxiety levels in response to stress were lower after OT intake. Interestingly, the combination of both the social support and the administered OT resulted in the lowest cortisol and anxiety levels in response to stress. Another study from this lab showed that OT enhanced positive communication behaviors in both men and women during a couple conflict and reduced cortisol levels after the couple conflict[26].

[iv] The Trier Social Stress Test (TSST) is designed to assess the psychobiological stress response in a laboratory setting and consists of two parts: an anticipation period (10 min) and a test period (10 min) during which participants will have to deliver a speech and perform mental arithmetic in front of an audience[152].

In particular, the seminal paper by Kosfeld et al. (2005)[27] has caused a surge in studies investigating the effect of exogenously administered OT on social behaviors. Using a social trust game with monetary stakes, Kosfeld et al. showed that a single dose of OT significantly increased trust among humans, specifically by influencing a person's willingness to accept social risks arising through interpersonal interactions. Afterwards, a plenitude of studies have suggested that intranasal OT affects a variety of prosocial behaviors and social cognitive processes in humans, including trust, cooperative behaviors, face processing, emotion recognition, altruism and generosity (reviewed in Bartz et al. (2011)[28]). Similar to the findings by Kosfeld et al., Mikolajczak et al. (2010)[29,30] showed, in two separate studies, that participants who received OT showed higher levels of trust than the placebo group. In additional studies, OT was also shown to maintain trusting behavior after betrayal[31], increase the perceived attractiveness and trustworthiness of faces showing a range of expressions[32], increase generosity[33] and promote in-group (but not out-group) trust and cooperation[34–36]. Despite these findings, the OT-trust relationship is not robust. The seminal work by Kosfeld et al.[27] and later findings by Baumgartner et al.[31] have failed to replicate in multiple attempts and the combined effect size of all studies assessing the effects of OT in the trust game does not differ significantly from zero[37]. A comprehensive review by Bartz et al. also revealed that nearly half of the studies described in this domain reported no significant main effect of OT treatment and that more than half of the reported outcomes were moderated by situational or individual difference factors[28].

In addition to prosociality, another line of research has focused on the effects of intranasal OT on social cognition (e.g., emotion and face processing, emotion recognition, empathy). With respect to social memory, authors have reported that OT can selectively affect social memory. For example, one study revealed impaired implicit memory of socially relevant words but not neutral words after a single dose of intranasal OT[38]. Another study showed that securely attached men remembered their mother as more caring and close after a single dose of intranasal OT, while anxiously attached men reported the opposite effect[39]. Considering memory for faces, OT is shown to specifically enhance recognition memory for faces as opposed to non-social stimuli[40]. The timing of the OT administration, however, seems to be of relevance. Savaskan et al. (2008)[41] showed that post-learning, intranasal OT improved recognition memory specifically for faces with angry or neutral (but not happy) expressions, whereas Guastella et al. (2008)[42] found that pre-learning, intranasal OT enhanced recognition memory for happy faces (compared to angry and neutral). With respect to emotion recognition, Domes et al. (2007) investigated 30 participants' ability to infer the affective mental state of others during the

Reading the Mind in the Eyes Test (RMET)[v] after a single dose of intranasal OT (24 IU) or placebo[43]. They found that OT improved participants' test performance specifically for difficult stimuli and argued that OT-specific enhancement of eye gaze during face perception poses a potential mechanism underlying this effect. Guastella et al. (2008) later showed that compared to placebo, OT increased gaze towards the eye region of faces (increased number of fixations and total gaze time) during viewing of human male and female black and white neutral faces[44]. In another study, Domes et al. (2013) investigated whether a single dose of OT modulates the allocation of attentional resources to negative and positive social cues (i.e. angry and happy faces) and found that OT administration resulted in an attentional preference towards happy faces (when stimuli were presented with a short duration of 100ms)[45]. Another study from their lab also showed that OT affected visual attention towards the eye region of neutral and emotional facial expressions. Particularly, OT preserved increased eye gaze to happy faces, but decreased eye gaze to angry faces[46]. Further studies that investigated whether single-dose, intranasal OT selectively improved emotion recognition, however, revealed mixed results. While some researchers found no effect on emotion recognition (during a visual search task)[46,47] or specifically on recognition of sad, angry or fearful faces[48–50], others reported enhanced processing of positive (i.e. happy) facial expressions[51,52], increased recognition/perception of both positive and negative facial expressions[53,54] and decreased aversion to angry faces[55]. In another study, Bartz et al. (2010)[56] reported that a single dose of OT improves empathic accuracy (a perceivers' ability to accurately assess targets' emotions[57]), but only in the less socially proficient participants. Similarly, a single dose of intranasal OT was found to increase the subjective experience of secure attachment in insecurely attached men[58]. Both studies provided indications that OT might not be universally prosocial, but that individual differences can modulate the effect of the treatment. A meta-analysis of seven of these aforementioned studies revealed that intranasal OT administration enhanced overall emotion recognition of faces (albeit with a small effect size) and this specifically for happy and fearful facial expressions[59]. More recent meta-analyses also showed that, in neurotypicals, single-dose OT treatment significantly improved the recognition of basic emotions (i.e., happiness, sadness, fear, disgust, anger and surprise), especially fear, although effect sizes were small to negligible (effect sizes ranging between 0.13 and 0.28)[60]. In clinical samples (including ASD), however, single-dose OT did not significantly influence interpretation or expression of emotions[60].

Similar to the prosociality data, the majority of studies on OT treatment and social cognition showed effects determined by interactions with task or stimulus variables (both those reporting

[v] The Reading the Mind in the Eyes Test (RMET) is designed to assess the social cognitive abilities of adults with ASD. It involves describing (choosing) the emotional or mental state of a person based on only an image of their eyes (36 pictures) in a fixed-choice paradigm with four options[153].

significant main effects and null main effects)[28]. Together, these reviews and meta-analyses have suggested that the field should be cautious in terms of not over-interpreting the 'pro-social' effects of OT treatment. For example, Walum et al. (2016) highlighted substantial statistical and methodological limitations of several studies assessing the effects of intranasal OT on human social behavior[61]. Walum et al. reanalyzed the data from three meta-analyses[59,62,63] by calculating the average effect size for healthy subjects included in the meta-analyzed studies, weighted by sample size. They reported a mean effect size of d= .28 (small[64]) and an average sample size of 49 individuals, leading to a statistical power of 16% (i.e. a low likelihood of detecting a true effect) (when assuming an alpha level of 5%) for studies assessing the effects of intranasal OT treatment in neurotypical individuals[61]. Their analyses thus revealed that most intranasal OT studies are underpowered, which potentially induced an overestimation of the effects of the treatment[61]. As such, Walum et al. stated that the majority of the aforementioned positive findings might actually reflect false-positives[61].

Aside from the (potentially over-optimistic) abundance of positive or prosocial effects of OT, a number of studies have also reported negative or antisocial effects of single-dose OT administration. For example, one study showed that a single dose of OT increased feelings of envy and gloating (Schadenfreude) during a game of chance with money at stake[65]. Another study revealed that OT increased in-group favoritism, but also out-group derogation[34]. In addition, authors have also reported that OT increased mistrust[66,67] and insecure attachment[39]. However, a thorough review of these aforementioned studies revealed mixed results that are mostly linked to individual differences (e.g. attachment style) and contextual factors (e.g. presence or absence of social information)[28]. Subsequently, these authors have set up three potential, basic behavioral mechanisms of OT functioning related to a wide variety of social behavioral effects, namely anxiety reduction, affiliative motivation and perceptual selectivity or social salience[28] (discussed in more detail in the general discussion of this doctoral dissertation). To date, however, the exact mechanism behind OT's modulatory effects is still unknown.

2.3 Oxytocin affects brain functioning in neurotypicals

On a neural level, most studies have used fear and emotion processing paradigms to assess the effects of single-dose, intranasal OT administration on brain activity and connectivity, leading to a special focus on the role of the amygdala (reviewed in Bethlehem et al. (2013)[68] and Wigton et al. (2015)[69]). In a first study, Kirsch et al. (2005) showed that OT reduces amygdala reactivity in response to fear inducing stimuli[70]. Subsequent studies partially confirmed this OT-specific attenuating of amygdala reactivity to emotional faces regardless of valence (happy, angry and fearful expressions)[71], to conditioned emotional faces[72] and to fearful expressions (but increased

reactivity to happy expressions)[73]. Aside from the amygdala, Petrovic et al. (2008) also reported attenuated reactivity of the fusiform gyrus, anterior medial temporal and anterior cingulate cortices[72]. In addition to emotion processing, some studies specifically focused on OT's effects on anxiety. For example, in individuals with generalized social anxiety disorder (GSAD), who show increased amygdala reactivity in response to threatening stimuli compared to a neurotypical control group, Labuschagne et al. (2010)[74] showed that OT lowered this initially heightened amygdala reactivity in response to emotional facial expressions. The control group, however, did not show this attenuated amygdala reactivity, which is in conflict with the aforementioned studies. In a follow-up study, these authors also found that OT attenuated the initially heightened (compared to the control group) reactivity extending from the medial prefrontal cortex (mPFC) to the anterior cingulate cortex (ACC) in response to sad faces in individuals with GSAD to levels similar to those of the control group[75]. Other imaging studies also showed OT-specific attenuated amygdala, dorsal striatum and midbrain reactivity in relation to maintained trust after betrayal[31]; attenuated amygdala reactivity during painful stimulation in a trust game[76]; decreased amygdala reactivity and increased insula and inferior frontal gyrus reactivity in response to infant crying[77]; decreased amygdala reactivity in response to infant laughter[78]; increased amygdala and caudate nucleus reactivity in response to reciprocated cooperation during iterated Prisoner's Dilemma[vi] [79]; reduced (left) globus pallidus reactivity in response to passive viewing of pictures of fathers' own children and unfamiliar children (but not for familiar children)[80]; but also increased amygdala reactivity in response to social and non-social threatening scenes (in 14 women)[81].

Brain regions, however, do not function in isolation, but belong to networks of interconnected regions with functional specificities[82]. In an attempt to elucidate the interactions between the different brain regions affected by intranasal OT and eventually to uncover the working mechanisms of OT, some of the aforementioned imaging studies also explored the effects of intranasal OT on functional connectivity among neural regions. For example, Kirsch et al. (2005) found that OT compared to placebo reduced coupling of the amygdala to brainstem regions in response to fear inducing stimuli[70]. Rilling et al. (2012) later confirmed and extended these findings by showing decreased amygdala-brainstem functional connectivity, but also increased functional connectivity between the amygdala and ventral anterior insula, and the amygdala and the ventral lateral temporal cortex in response to reciprocated cooperation[79]. Subsequent studies reported enhanced functional connectivity between the amygdala and the orbitofrontal

[vi] The iterated Prisoner's Dilemma (PD) game is a model for relationships based on reciprocal altruism. In the game, two players choose to either cooperate or defect and receive a payoff that depends upon the interaction of their respective choices. Here, they used a sequential-choice PD game in which player 1 chooses and player 2 is then able to view player 1's choice before making his own choice. Each of the four outcomes is associated with a different payoff[79].

cortex (OFC), anterior cingulate (ACC), hippocampus, precuneus, supramarginal gyri and the middle temporal sulcus (during exposure to infant laughter)[78], but also decreased functional connectivity in response to socially salient stimuli in fronto-pallido-hippocampal networks in response to (un)familiar children[80].

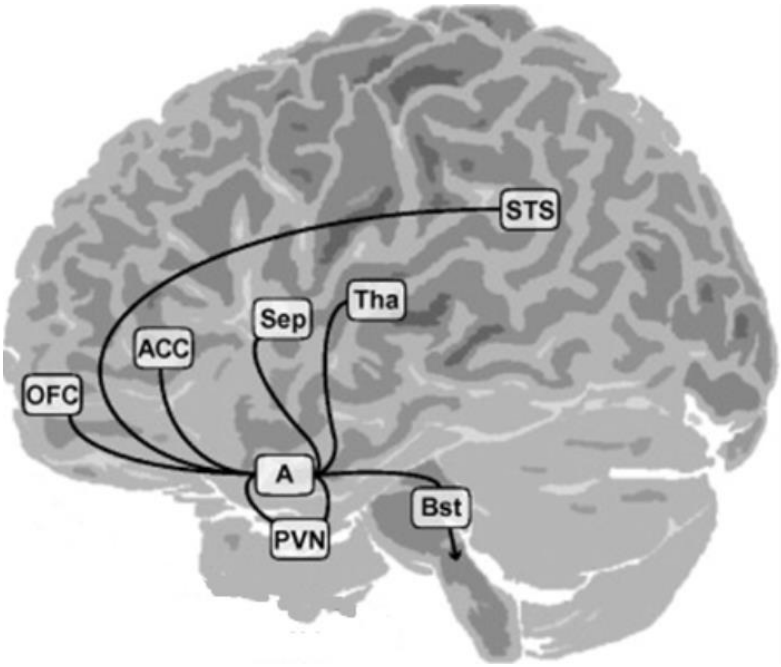

Figure 1.2. Neural model of the modulatory effects of OT as proposed by Bos et al. (2012). A= amygdala; Tha= thalamus; ACC= anterior cingulate cortex; Sep= septum; PVN= paraventricular nucleus; Bst= brainstem; OFC = orbitofrontal cortex; STS= superior temporal sulcus. Figure and figure legend adapted from Bos et al. in *Frontiers in Neuroendocrinology*[74].

In sum, research highlights the amygdala, temporal lobes and reward system as important regions for OT's modulatory effects. These brain regions, however, seem to respond differently to OT administration[68,69]. In terms of amygdala activity, OT has consistently shown to attenuate amygdala activity during implicit emotion processing in men (e.g., participants were not asked to indicate the emotion seen on the stimulus face, but rather the gender of the stimulus face), whereas OT-specific effects in the reward system and temporal lobes appear to depend on individual differences (e.g. gender) and contextual factors (e.g. task type). For example, in women, OT tended to increase activity in the temporal lobes during explicit emotion processing (e.g., participants were asked to indicate the emotion seen on the stimulus face), whereas, in men, OT tended to attenuate activity during implicit emotion processing[69]. In the reward system, OT has been shown to increase activity associated with social reward, but also to decrease learning social contingencies[69]. Together, connections between these regions might form the neural basis or network underlying the behavioral effects of OT. For example, Bos et al. (2012)[83]

proposed a network of brain regions involved in social-emotion processing that could be influenced by OT. Based on animal work and single-dose administration studies in humans, this network is suggested to include the amygdala, thalamus, PVN of the hypothalamus, septum and brainstem regions, prefrontal regions and their connections to the ACC and superior temporal gyrus[83] (Fig. 1.2).

Gaps in the literature identified

- Single-dose effects of intranasal OT treatment in neurotypicals are mixed.
- Multiple-dose effects of intranasal OT treatment in neurotypicals are unknown.
- The exact neurobehavioral mechanism by which OT affects social behavior is unknown, and therefore an active topic of investigation.

3 Does oxytocin alter social behavior and its associated brain regions in ASD?

3.1 Single-dose trials

Rather than the widely used intranasal OT administration (Table 1.1), the first study (reported across two publications) examining the effects of OT in ASD adopted an OT infusion (into the blood) approach to administer the treatment. In this study, Hollander et al. (2003, 2007) adopted a cross-over design to assess the effects of intravenous oxytocin on core autism characteristics (i.e. repetitive and restricted behaviors (RRBs) and affective speech comprehension) and revealed that a 4-hour intravenous OT infusion (compared to PL infusion) decreased the number and severity of RRBs and improved affective speech comprehension in 15 adult men with ASD[84,85]. Later, Guastella et al. (2010) reported the first clinical trial of intranasal OT in 16 adolescent boys with ASD aged between 12 and 19 years. The older participants (aged 16 to 19 years) received a dose of 24 IU, which was the 'standard' dose used in adult intranasal oxytocin administration studies, whereas the younger ones received 75% of that dose (18 IU). Guastella et al. assessed the effects of a single dose of OT (24 IU) on emotion recognition by using the Reading the Mind in the Eyes Test, a well-known task in research of social cognition in ASD, and revealed that a single, intranasal dose of OT can improve emotion recognition. Importantly, this study was the first to report beneficial effects of OT in younger participants,

highlighting the feasibility and efficacy of intranasal OT for adolescents and potentially younger children with ASD. Since then, subsequent studies have shown beneficial effects of OT on a range of experimental outcomes. Similar to the findings of Guastella et al. (2008)[44] in neurotypicals, Andari et al. (2010)[86] showed that, in adults with ASD (11 men and 2 women) compared to neurotypicals and those receiving PL, a single dose of OT (24 IU) improved the processing of socially-relevant information during a virtual, social 'ball-tossing' game. Specifically, OT was shown to increase gaze towards the eye region of the face and to increase feelings of trust and preference in the other players who were perceived as more cooperative. Later, using a cross-over design including 32 adult men with ASD and 34 neurotypical controls, Auyeung et al. (2015)[87] also showed that a single dose of OT (24 IU) increased gaze towards the eyes in both participants with ASD and neurotypical participants. In particular, in the ASD group, oxytocin has the most effect on fixation duration in individuals with impaired eye contact at baseline. Recently, Kanat et al. (2017)[88] used another visual attention paradigm to assess the effects of a single dose of intranasal OT (24 IU) on visual preference for social (faces) versus non-social (houses) stimuli in ASD. To do so, they adopted a placebo-controlled, crossover design in which 29 adult men with ASD and 30 control participants. Intranasal OT treatment was shown to restore the attentional preference for faces in the participants with ASD to the level observed in control participants under PL, whereas the participants with ASD under PL showed reduced attention to faces. Interestingly, secondary analyses also revealed that these OT-specific effects primarily occurred in participants with ASD who showed high levels of social anxiety (characterized by attentional avoidance of faces during PL intervention).

Aside from these behavioral effects, Lin et al. (2014)[89] also revealed OT-specific physiological effects in adult men with ASD. Specifically, Lin et al. showed that intranasal OT was able to increase the skin conductance response (used to measure autonomic arousal) to human versus non-human sounds in adult men with ASD compared to placebo and typically developing controls. Taken together, the findings of these single-dose intervention studies so far suggest that intranasal OT administration enhances social cognitive functions related to emotion recognition, social affiliation and specifically social attention; aspects of social behavior that are typically impaired in individuals with ASD.

On a neural level, only five studies have attempted to uncover the effects of single-dose OT administration on the neural circuitry of adults with ASD. Most of these studies have used functional magnetic resonance imaging (fMRI) to investigate regional brain activity during the processing of social stimuli. In a first study (reported across two publications) using a within-subject, cross-over design, Domes et al. (2013, 2014)[90,91] explored the neural effects of a single dose of intranasal OT (24 IU) during a face processing task and during an emotion processing (from faces) task in 13 adult men with ASD. Whereas participants with ASD showed reduced

right amygdala activity during processing of social stimuli (faces) as compared to non-social stimuli (houses) under PL, OT was shown to increase this previously shown underactivity (i.e. reduced activity) during the processing of social stimuli. In addition, OT was shown not only to improve emotion recognition performance , but also to increase reactivity of the amygdala, temporal pole and other cortical areas associated with face processing during emotion processing (from faces). In children with ASD, Gordon et al. (reported across two publications) revealed that a single dose of OT changed reactivity of brain regions in response to the emotion, biological motion and auditory processing (i.e. superior temporal sulcus (STS), amygdala, striatum, middle frontal gyrus, medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC))[92,93]. Specifically, this group's research showed that OT enhanced right posterior superior temporal sulcus (pSTS) activity during processing of point-light displays (PLDs) conveying biological motion[93]. In another study using a placebo-controlled, within-subjects, cross-over design, Aoki et al. (2014) assessed the effects of single-dose intranasal OT (24 IU) in 20 adult men with ASD on socio-emotional inference during a well-known first-order false belief task (Sally-Anne test[vii])[94]. Intranasal OT was shown to improve participants' socio-emotional inference and to enhance the originally diminished right anterior insula reactivity during inference of other's social emotions. In a follow-up study from the same lab, Watanabe et al. (2014)[95] also adopted a within-subject, cross-over design during which participants were administered a single dose of OT during making of social judgements based on non-verbal social information. OT was shown to improve task performance and to increase reactivity of the ventromedial prefrontal cortex (vmPFC) during the making of social judgements. In addition to the aforementioned fMRI clinical trials, only one study assessed the effects of a single dose of OT (24 IU) on evoked brain potentials (ERPs) to explore the OT-specific modulations of brain responses to social stimuli in 30 neurotypical men and 31 adults with ASD. There were no OT-specific changes in brain activity to the social stimuli. However, further analyses did reveal that OT could increase orientation towards affective social stimuli specifically in those participants (both with and without ASD) who reported higher levels of distress as measured by a personal distress scale.

Taken together, most studies have adopted an fMRI paradigm and within-subject (crossover) designs to assess the effects of single-dose OT on brain activity in individuals with ASD (Table 1.1). These studies revealed that a single dose of OT modulated activity in social-emotional processing regions such as the amygdala (during facial emotion recognition[91] or face perception[90,96]), medial prefrontal cortex (during a social decision-making task)[95], and anterior insula (during a false-belief task)[94], and the (p)STS (during emotion and biological motion processing)[92,93]. Although these studies have contributed important insights into the immediate

[vii] The Sally-Anne test a psychological test designed to assess a person's theory of mind (social cognitive ability to attribute false beliefs to others)[154].

behavioral and neural effects of single-dose OT administration, studies assessing the (long-term) effects of repeated OT administration are needed to explore the true therapeutic potential of OT for improving social impairments in ASD.

Table 1.1. Summary of single-dose clinical trials investigating the effects of oxytocin (versus placebo) in individuals with ASD.

Study	Design	Participants	Administration	Dosage	Duration	Phenomenon	Outcome measures	Main findings
Hollander et al. (2003, 2007)	DB, crossover (2-to-3-week-interval)	15 ASD adults (14M/1F) Age: 19-56	intravenous	1h: 10 ml 2h: 50 ml 3h: 100 ml 4h: 700 ml	4 h	RRBs Social cognition	RBS Comprehension of affective speech task	OT reduced RRBs OT improved affective speech comprehension
Guastella et al. (2010)	DB, crossover (1-week interval)	16 ASD adolescents (16M/0F) Age: 12-19	intranasal	18 IU (< 16 years) or 24 IU	1 dose	Social cognition	RMET (emotion recognition)	OT improved emotion recognition
Andari et al. (2010)	DB, crossover (1-week interval)	13 ASD adults (11M/2F) Age: 17-39	intranasal	24 IU	1 dose	Social functioning	Social ball-tossing game, face perception (eye gaze), plasma OT levels	OT enhanced the ability to process socially relevant cues and acquire their meaning in an interactive context; OT enhanced visual scanning of the eye region of faces; higher plasma OT levels
Domes et al. (2013, 2014)	DB, crossover (1-week interval)	14 ASD adults (14M/0F) Mean age: 24 14 NT adults	intranasal	24 IU	1 dose	Social cognition	Face discrimination, Emotion recognition fMRI	OT increased right amygdala activity to social compared to non-social stimuli in ASD; OT improved facial emotion recognition in ASD, linked to increased left amygdala reactivity
Gordon et al. (2013, 2016)	DB, crossover (3 to 78 days interval)	17 ASD children and adolescents (14M/3F) Age: 8-16	intranasal	12 IU (<12 years), 18 IU (12–15 years), or 24 IU (16 years)	1 dose	Social cognition	RMET Biological motion task Affective voices task fMRI	OT enhanced activity in brain regions implicated in reward and social cognition; OT did not improve emotion recognition; OT enhanced connectivity between brain regions implicated in reward and socio-emotional processing (preferentially for social compared to non-social stimuli)
Lin et al. (2014)	SB, crossover (1-week interval)	16 ASD adults (16M/0F) Age: 19-51 13 NT adults	intranasal	24 IU	1 dose	Autonomic response to social stimuli	Skin conductance	OT increased skin conductance to human sounds in both NT and ASD; in the ASD group, change in skin conductance was associated with autistic traits and measures of social functioning
Aoki et al. (2014)	DB, crossover	20 ASD adults (20M/0F)	intranasal	24 IU	1 dose	Social cognition	Modified Sally-Ann task	OT improved ability to correctly infer others' emotions; OT enhanced right

	(1-week interval)	Age: 22-41					fMRI	anterior insula activity during inferring others' emotions
Watanabe et al. (2014)	DB, crossover (1-week interval)	33 ASD adults (33M/0F) Age: ≥ 20	intranasal	24 IU	1 dose	Social cognition	Friend or foe task fMRI	OT increased nonverbal judgements, associated with mPFC activity; OT increased ACC activity; OT enhanced functional connectivity between ACC and mPFC
Auyeung et al. (2015)	DB, crossover (1-week interval)	32 ASD adults (32M/0F) Age: 18-56 34 NT adults	intranasal	24 IU	1 dose	Social functioning	Eye gaze during social interaction	OT increased eye gaze in both groups; in ASD group, OT was most effective on fixation duration in individuals with impaired levels of eye contact at baseline
Althaus et al. (2015)	DB, crossover (1-week interval)	32 ASD adults (32M/0F) Age: 18-34 30 NT adults	intranasal	24 IU	1 dose	Neurophysiological responses to affective pictures	State anxiety (STAI) EEG ECG	OT did not significantly affect state anxiety; evidence for individual differences
Quintana et al. (2017)	DB, crossover (minimum 24h interval; range: 1-72 days, on average: 13 days)	17 ASD adults (17M/0F) Age: 19-35	Intranasal (with Breath Powered device)	8 IU and 24 IU	1 dose	Social cognition	Emotion sensitivity, RMET, emotional dot probe task, emotional face-morphing task	8IU of OT increased the overt emotional salience of happiness in ambiguous faces compared to placebo; no significant improvements on secondary outcomes

Abbreviations: DB= double blind, SB= single blind, ASD: autism spectrum disorder, NT= neurotypical, RRBs= repetitive and restricted behaviors, M= male, F= female, IU= international units, h= hours, OT= oxytocin, PL= placebo, RMET= Reading the Mind in the Eyes Task, fMRI= functional Magnetic Resonance Imaging, ACC= anterior cingulate cortex, mPFC= medial prefrontal cortex, STAI= State-Trait Anxiety Inventory, EEG= electroencephalography, ECG= electrocardiography

Table 1.2. Summary of multiple-dose clinical trials investigating the effects of oxytocin (versus placebo) in individuals with ASD.

Study	Design	Participants	Administration	Dosage	Duration	Phenomenon	Outcome measures	Main findings
Kosaka et al. (2012)	Open-label, case study	1 girl with ASD 16 years old	intranasal	8 IU, 4IU twice daily	Twice-daily dose for 2 months	ASD characteristics	Clinical improvement	OT improved ASD characteristics (social functioning and global improvement)
Anagnostou et al. (2012)	DB, parallel	19 ASD adults (16M/3F) Mean age: 33.2	intranasal	48 IU, 24 IU twice-daily	Twice-daily dose for 6 weeks	Social cognition Social functioning RRBs	DANVA, RBS, SRS, RMET, YBOCS, WHOQOL	OT did not significantly improve primary outcome measures; OT improved emotion recognition and quality of life
Tachibana et al. (2013)	Open-label, single-arm	8 ASD children (8M/0F) Age: 10-14	intranasal	8 IU, 16 IU or 24 IU, each twice-daily	Twice-daily dose for 15-18 weeks (3 x 2 months) with 1-2 weeks of placebo between dose increases	ASD characteristics	Clinical improvement (assessed with ADOS, CBCL, ABC) Plasma OT Urinary OT	OT improved ADOS scores, but not CBCL or ABC (parent ratings); OT showed no side-effects; OT did not alter plasma OT levels; Increased urinary OT after administration
Dadds et al. (2014)	DB, parallel	38 ASD children (38M/0F) Age: 7-16	intranasal	12 IU (≤40 kg) or 24 IU	Once-daily for 4 consecutive days	Social functioning, social cognition, RRBs	Social interaction paradigm with questionnaires, Social Reciprocity Scale, Emotion recognition task	OT did not significantly improve primary nor secondary outcome measures
Anagnostou et al. (2014)	Open-label, modified maximum-tolerated-dose design	15 ASD children and adolescents (11M/4F) Age: 10-17	intranasal	0.2, 0.26, 0.33 and 0.4 (max) IU/kg/dose	Twice-daily for 12 weeks (+ 4-week retention)	Safety	Side-effects	No serious adverse events at any dose level nor clinical changes. Some participants reported clinical improvements, even until 3 months post-treatment (but not placebo-controlled)
Guastella et al. (2015)	DB, parallel	50 ASD children and adolescents (50M/0F) Age: 12-18	intranasal	18 IU (< 16 years) or 24 IU (36 IU or 48 IU in total/day)	24 IU twice-daily for 8 weeks (+ 3-month retention)	Social functioning, RRBs, social cognition	SRS Clinical improvement (CGI), DBC, RBS, RMET, Biological motion, DANVA	No beneficial effects of OT on primary or secondary outcomes; Parents' beliefs about the received treatment affected reported improvements

Watanabe et al. (2015)	DB, crossover (no interval)	20 ASD adults (20M/0F) Age: 24-43 (18 analyzed)	intranasal	48 IU	24 IU twice-daily for 6 weeks	ASD characteristics	ADOS, CARS, fMRI, SRS, RBS, AQ, STAI, Depression scale, WHOQOL, Clinical improvement (CGI, GAF)	OT improved social reciprocity (as assessed with the ADOS) and enhanced functional connectivity between ACC and dmPFC (during rest)
Yatawara et al. (2016)	DB, crossover (4-week interval)	31 ASD children (27M/4F) Age: 3-8	intranasal	24 IU	12 IU twice-daily for 5 weeks	ASD characteristics	SRS, RBS, ADOS, Clinical improvement (CGI)	OT improved social responsiveness (as assessed with the SRS)
Kosaka et al. (2016)	12 weeks DB, 12 weeks open-label, 8 weeks follow-up, parallel	60 ASD adults (47M/13F) Age ≥ 15 (53 analyzed)	intranasal	16 IU or 32 IU	1 daily dose for 12 weeks	Social functioning, RRBs	Clinical improvement, IRSA, ABC, plasma OT	OT did not significantly affect outcome measures; in men, OT showed clinical improvements in the high-dose but not the low-dose group (compared to placebo)
Munesue et al. (2016)	DB, crossover (no interval)	29 ASD adults with intellectual disability (29M/0F) Age: 15-40	intranasal	16 IU	8 IU twice-daily for 8 weeks	ASD characteristics, social functioning, clinical improvement	CARS, ABC, CGI, real-life assessments of social interaction, IRSA	OT showed no significant improvements on primary nor secondary outcomes
Parker et al. (2017)	DB, parallel	32 ASD children (27M/5F) Age: 6-12	intranasal	48 IU	24 IU twice-daily for 4 weeks	Social functioning, RRBs, safety/tolerability	SRS, RBS, side-effects, anxiety scale	OT improved social responsiveness (SRS); Lower pretreatment plasma OT levels are associated with the greater social improvement.
Yamasue et al. (2018)	DB, parallel	106 ASD adults (106M/0F) Age: 18-48 (103 analyzed)	intranasal	48 IU	24 IU twice-daily for 6 weeks	ASD characteristics, Social functioning	ADOS, eye gaze, anxiety scale, depression scale, clinical improvement (CGI)	OT did not significantly improve primary outcomes; OT improved RRBs (ADOS) and enhanced eye gaze

Abbreviations: DB= double blind, ASD: autism spectrum disorder, NT= neurotypical, RRBs= repetitive and restricted behaviors, M= men, F= women, h= hours, IU= international units, OT= oxytocin, PL= placebo, DANVA= Diagnostic Analysis of Nonverbal Accuracy, RBS= repetitive Behavior Scale, SRS= Social Responsiveness Scale, RMET= Reading the Mind in the Eyes Task, YBOCS= Yale Brown Obsessive Compulsive Scale, WHOQOL: World Health Organization Quality of Life questionnaire, ADOS= Autism Diagnostic Observation Scale, CBCL= Child Behavior Checklist, ABC= Aberrant Behavior Checklist, CGI= Clinical Global Impression, DBC= Developmental Behavior Checklist, CARS= Childhood Autism Rating Scale, AQ= Autism Spectrum Quotient, STAI= State-Trait Anxiety Inventory, GAF= Global Assessment of Functioning, fMRI= functional Magnetic Resonance Imaging, IRSA= Interaction Rating Scale Advanced, ACC= anterior cingulate cortex, mPFC= medial prefrontal cortex.

3.2 Multiple-dose trials

With respect to the repeated administration of OT (Table 1.2), Kosaka et al. (2012)[97] were the first to assess the behavioral effects of repeated intranasal OT administration (open-label) in a case study of a 16-year-old girl with ASD. OT was shown to improve the girl's social impairments as well as her secondary irritability and aggressive behavior. In a following single-armed, open-label study, Tachibana et al. (2013)[98] assessed the effects of 7-month, repeated OT administration intervention in eight adolescent boys with ASD aged between 10 and 14 years. Unlike any other study before, the dosage of OT was raised in a stepwise manner every two months from 8 IU/dose for the first 2-month period, to 15 IU/dose for the second 2-month period, to 24 IU for the third and last 2-month period, with each of the intervention periods preceded by one to two weeks of placebo intervention (as a washout period). Although participants and their caregivers reported improvements, these were not found to be significant. Importantly, however, participants reported good tolerability and no side effects, providing initial indications for the safety of repeated administration of intranasal OT for children and adolescents with ASD.

Anagnostou et al. (2012)[99] investigated – for the first time – the behavioral effects of a 6-week, daily (2 x 24 IU/day) OT administration in 19 adults with ASD and revealed that participants in the OT group compared to the PL group did not improve on measures of social cognition nor clinical ratings of improvement. Analyses of secondary outcomes (i.e. RMET, caregiver-reported quality of life and RRBs), however, did reveal improved emotion recognition, caregiver-reported quality of life and (lower-order) RRBs (stereotypy and self-injury). More importantly, this trial presented the first evidence of the safety and tolerability of repeated intranasal OT administration in adults with ASD. In a following study, Dadds et al. (2014)[100] reported no beneficial effects of intranasal OT (24 IU or 12 IU depending on weight of the child) after a 4-day intervention period in boys with ASD aged 7 to 16 years.

Note that the literature described until this point was the State of the Art at the start of the current doctoral project (April 2014). Since then, however, other researchers have conducted additional clinical trials that added to the existing knowledge, confirming the relevance of our project. For example, Guastella et al. (2015) for the first time not only assessed the immediate effects of an 8-week intranasal OT intervention in children with ASD, but also its potential retention effects until 3 months post-treatment[101]. To do so, Guastella et al. (2015) adopted a double-blind, placebo-controlled, parallel design to assess potential changes in social responsiveness and clinician-rated clinical improvement after an 8-week, twice-daily (18 IU or 24 IU, depending on weight) OT or PL intervention in 50 adolescent boys aged between 12 and 18 years. Their analyses revealed no beneficial effects of OT, but, interestingly, the study showed

that caregiver's beliefs regarding the received treatment influences caregiver's reports. In particular, caregivers who believed their child had received the OT treatment reported greater improvements than those who believed their child had received the PL treatment. Of importance, although results did not support the efficacy of repeated intranasal OT administration, they did show that a course of intranasal OT treatment in adolescents with ASD was well tolerated and did not cause negative side effects. Also using a placebo-controlled, cross-over design, a following study from the same lab assessed the behavioral effects of a 5-week, twice-daily (24 IU) intranasal OT or PL intervention in even younger children (27 boys, 4 girls) with ASD aged between 3 and 8 years[102]. Here, Yatawara et al. (2016) reported that compared to the PL group, the OT group showed significant improvements in (caregiver-rated) social responsiveness. In addition, the OT group again showed only minimal to moderate side effects and good tolerability[102]. In another randomized, placebo-controlled, cross-over study, Munesue et al. (2016) investigated the efficacy, feasibility and potential adverse events of an 8-week, intranasal OT intervention (16 IU/day) in 29 adolescent to adult men (aged between 15 and 40 years old) with ASD and comorbid intellectual disabilities[103]. Analyses revealed no significant differences between the OT and the PL group in primary and secondary outcomes. However, exploratory assessments revealed that both caregivers and researchers did notice more frequent social reciprocal behaviors in daily life and during play in the lab[103]. Similar to previous studies, Munesue et al. also concluded that intranasal OT intervention is tolerable and feasible in adolescent and adult men with ASD and intellectual disabilities (under careful supervision for adverse events such as seizures). Subsequently, Parker et al (2017) adopted a randomized, placebo-controlled, parallel design to assess the efficacy and tolerability of a 4-week, intranasal OT intervention in 32 children with ASD (aged between 6 and 12 years old)[104]. They showed that the OT treatment was well tolerated, and that OT improved social functioning, but not RRBs or anxiety[104]. Recently, Yamasue et al. (2018) conducted the largest clinical trial to date by using a placebo-controlled, parallel design to assess to behavioral effects of a 6-week, twice-daily (48 IU), intranasal OT intervention in 106 adult men with ASD (of which data of 103 participants was used)[105]. They found no beneficial effects of OT on primary outcomes (i.e. ADOS[viii] social reciprocity), but did find improvements in the secondary outcome measure, ADOS RRBs[105]. Note, however, that the ADOS is designed and validated as a diagnostic measure and not as an outcome measure and that it is considered inappropriate to use as a treatment endpoint[106,107].

Confirming anew the importance of our effort, a short time after the start of our project (April 2014), Watanabe et al. (2015)[108] reported results of an exploratory, randomized, placebo-controlled, cross-over study, in which they assessed the behavioral and neural effects of a 6-

[viii] The Autism Diagnostic Observation Schedule (ADOS) is an observation instrument used by a clinician to assess social communication, social interaction, RRBs and (imaginary) play in children, adolescents and adults with ASD[155].

week (48 IU/day), intranasal OT intervention in 20 adult men with ASD (data from 18 adult men with ASD analyzed). They revealed that, in adult men with ASD, a 6-week OT treatment improved core ASD characteristics related to social reciprocity, increased activation in the anterior cingulate cortex (ACC) and the dorsomedial prefrontal cortex (dmPFC) during social decision making and increased the functional connectivity between the ACC and dmFC during rest. They also showed, for the first time, that effect sizes of the multiple-dose treatment were not larger than those of the single-dose treatment[108]. However, more studies are needed to confirm this finding, considering the single-dose data and the multiple-dose data were collected in two different studies (but from the same participants). Also, the possibility that long-term OT treatment may induce long-lasting neuro-behavioral changes that outlast the period of actual administrations is currently unexplored.

Although these initial findings were again promising, a recent systematic review and meta-analysis by Ooi et al. revealed mixed findings concerning the behavioral effects of OT treatment[109]. In their review, Ooi et al. included 12 randomized controlled trials (RCTs)[85–87,91,92,95,99–101,110–112] assessing the effects of both single-dose and multiple-dose OT treatment on social cognition and RRBs in ASD. Although 7 out of 11 studies on social cognition described improvements and 1 out of 4 studies on RRBs reported improvements, meta-analyses suggested that the OT treatment had no significant effect on either of these domains[109]. Consistent with these findings, a more recent meta-analysis, including additional data of recent clinical trials, found that OT treatment had a small, yet non-significant, effect on the core characteristics of ASD[113]. Similar to studies including neurotypicals, these contradictory findings might be explained by methodological and statistical limitations of these studies (i.e., small sample sizes, underpowered studies). However, according to Quintana et al., it is uncertain whether these non-significant p-values in the current literature are indicative of the absence of a meaningful effect or whether the data were too insensitive to detect an effect[114]. Therefore, they aimed to improve the inference of non-significant results of the intranasal OT literature and to promote equivalence testing, a statistical tool used to assess evidence for the absence of meaningful effects[114]. To do so, Quintana et al. applied equivalence testing to recent meta-analytic outcomes[60] and suggested that 26.1% of non-significant meta-analytic findings from studies assessing the interpretation and expression of emotions after single-dose, intranasal OT treatment (both in neurotypical and clinical samples and both on a neural and behavioral level) were explained by data insensitivity rather than statistical equivalence between groups[114]. The authors, however, warrant caution to not attribute all non-significant results to data insensitivity, considering their results also demonstrated statistical equivalency of some effects[114]. Taken together, more well-designed studies are needed to further explore the potential beneficial effects of OT treatment to improve the core characteristics of ASD.

Gaps in the literature identified

- To date, no pharmacological treatment exists targeting the core characteristics of ASD.
- Behavioral and neural effects of single-dose, intranasal OT treatment in ASD are promising, yet behavioral effects of multiple-dose, intranasal OT treatment in ASD are mixed.
- Knowledge about the immediate neural effects of multiple-dose, intranasal OT treatment is limited.
- Knowledge about the potential retention effects of multiple-dose, intranasal OT treatment is lacking.
- The exact neural mechanism by which OT affects social behavior in ASD remains unclear.

4 Why is the superior temporal sulcus a potential, reliable target for oxytocin treatment?

The superior temporal sulcus (STS) is a brain region known as a key 'hub' in social information processing[115] by connecting distinct social brain networks underlying theory of mind (amygdala-orbitofrontal network)[116], action understanding (fronto-parietal action perception network or mirror neuron system)[117], and self-referential processing and mind-wondering (default mode network)[118]. On a behavioral level, STS (specifically right STS) has been linked to a variety of social processes, including processing of biological motion[119], speech perception[120], audio-visual integration[116], theory of mind[121] and perception of gaze[122] and face processing[123] (aspects of social behaviors known to be affected in individuals with ASD (reviewed in Lai et al. (2014)[4]). Neuroimaging studies have consistently shown diminished recruitment of STS regions in ASD during explicit processing of the biological motion content in point-light display (PLD) stimuli[124–130]. In particular, prior work by our lab using a social-emotional perception task involving the recognition of biological motion and emotional states from PLDs revealed pSTS underactivity and underconnectivity with the fronto-parietal action observation network in adult men with ASD[128,129]. In addition, recent meta-analyses of single-dose OT administration studies including healthy and patient populations consistently identified pSTS regions (middle and superior temporal gyrus) as robust areas of activation after OT administration[69,131,132]. Taken together, accumulating evidence points to the pSTS as a prime target for OT treatment in ASD.

5 Methodology

5.1 Point-light displays

Humans are skilled at recognizing and interpreting emotions in others and facial expressions are an important source of information to do so. However, facial expressions are not the only source for conveying emotional and socially relevant information. Particularly, when another's facial expressions are inconsistent or unavailable, his body language or bodily kinematics might provide equally important information to engage in social interaction. One widely adopted method to test bodily motion perception is the use of point-light displays (PLDs). PLDs are visual stimuli representing biological motion solely by a few moving dots corresponding to the body's main joints[133](Fig. 1.3). To assess the effects of OT on emotion processing from PLDs, participants will perform an emotion processing task (based on prior work from our lab[128,134,135]), involving the recognition of positive and negative emotional states (happiness, anger) from whole-body PLDs. The adopted PLD stimuli were adapted from motion captured data as previously described in detail[134]. In short, an eight-camera VICON system was used to obtain the motion data (capturing system measuring at 100 Hz, Oxford Metrics, Oxford, UK). With twelve reflective markers attached to the main joints of their body (shoulders, elbows, wrists, hips, knees and ankles), one male and one female actor were instructed to perform five actions (walking, jumping, kicking a ball using the right leg, drinking from a bottle and wiping the table), each carried out in four emotional states (neutral, happy, sad and angry). These recordings (after processing and converting) resulted in 3s movie clips, during which twelve moving white dots against a black background represented the motion of the joints of the human body during the aforementioned actions and from three different perspectives (front view (0◦), side view (90◦) and intermediate view (45◦)). As such, a total set of 120 PLD stimuli was created (2 actors x 5 actions x 4 emotions x 3 perspectives). Note, however, that in the current doctoral project only PLD stimuli representing 2 emotions (happiness or anger) and 3 actions (walking, jumping and kicking a ball with the right leg) were used, with an addition of the factor position (upright or inverted presentation of PLD stimuli), resulting in a test set of 72 PLD stimuli (2 actors x 3 actions x 2 emotional states x 3 perspectives x 2 positions).

In addition, a scrambled version of each PLD stimulus was created which consisted of the same individual dots, undergoing the same local trajectories as in the normal PLDs, but with the starting position randomly permutated. For the current doctoral project, this 'scrambling' resulted in 72 PLD stimuli displaying non-biological motions.

The current task paradigm was designed to specifically engage the pSTS and to assess whether OT could alter pSTS activity. Prior research has shown that children [136,137] and (adolescents and)

adults with ASD [128,135,138,139] showed more difficulties with accurately recognizing emotions from PLD stimuli as compared to TD children and adults. Furthermore, these biological motion depicting PLD stimuli have been known to involve the pSTS [140–146].

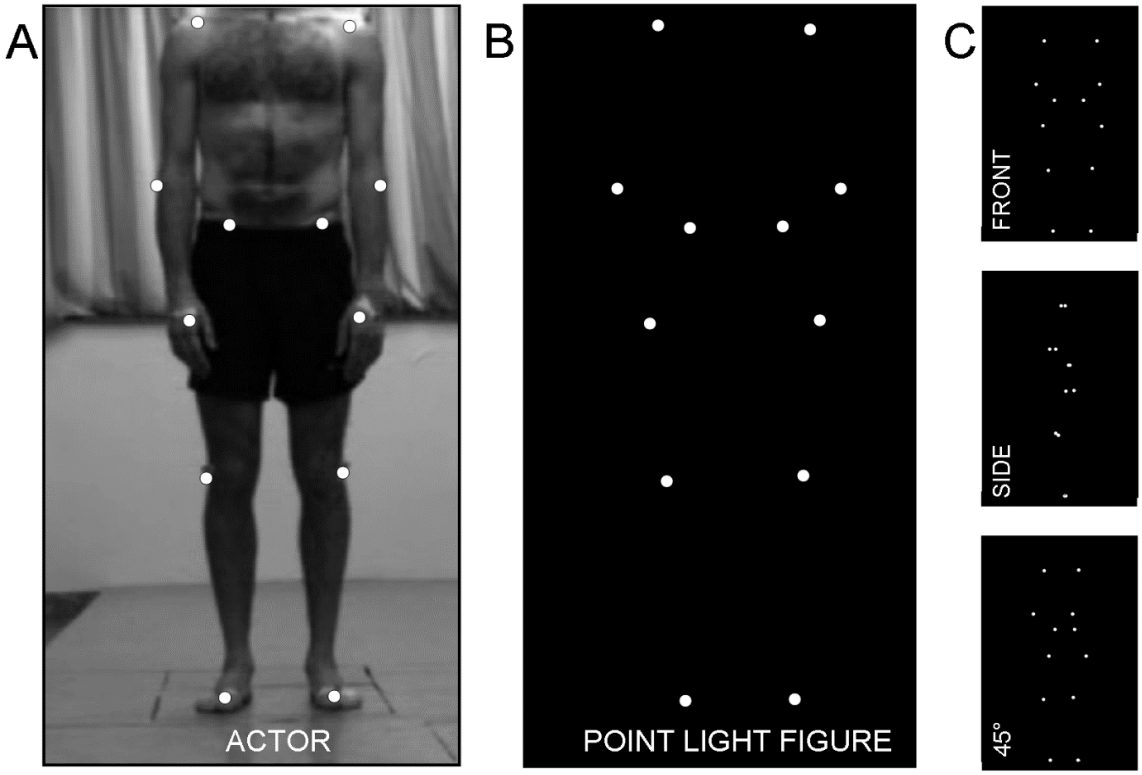

Figure 1.3. Point-light display stimuli. (A) An exemplary photograph of the male actor with the 12 markers attached to the body. (B) The corresponding point light figure. (C) Examples of point light figures, viewed from different perspectives i.e., the front, the side, and the 45° view. Figure and figure legend adapted from Alaerts et al. (2011) in *PLOS One*[105].

5.2 Functional Magnetic Resonance Imaging

Functional magnetic resonance imaging (fMRI) is a commonly used non-invasive imaging technique to study human brain functioning. The fMRI technique relies on the Blood Oxygenation Level Dependent (BOLD) contrast mechanism, which is sensitive to changes in cerebral blood flow, cerebral blood volume, and the cerebral metabolic rate of oxygen[147]. In order to function adequately, our brain requires glucose and oxygen. Considering the neurons in the brain cannot produce or store these energy sources themselves, blood functions as their transport mechanism.

When neurons belonging to certain brain regions become more active in response to certain stimuli, they require more glucose and oxygen to meet the demand of the action at hand. To do so, blood flow to these brain regions will increase. This mechanism of neurovascular coupling (i.e. changing local blood flow in response to neuronal activity) is called the hemodynamic response and typically consists of three parts. First, an initial 1-to-2-second dip in signal

intensity is triggered by an increase in neuronal activity, which in turn leads to increased oxygen consumption. Second, this increased demand of oxygen leads to an increase in local blood flow and supply of oxygenated blood, and consequently in signal intensity that peaks around 3 to 6 seconds. Third, a slight post-stimulus undershoot before returning to baseline after 12 to 30 seconds (Fig. 1.4)[147,148]. The signal intensity thus depends on the change in oxygenation when neurons in the brain become active. In the blood, the oxygen is carried by the protein hemoglobin. Oxygen-carrying hemoglobin (oxyhemoglobin) is diamagnetic (i.e. repelled by a magnetic field), whereas non-oxygenated hemoglobin (deoxyhemoglobin) is paramagnetic (i.e. attracted by a magnetic field). The changes in these magnetic properties cause differences in the BOLD signal, which in turn are measured by MRI. Note, thus, that the BOLD fMRI signal does not provide a direct measurement of neuronal activity, but provides an indicator of increased or decreased brain activity, as assessed indirectly through changes in hemodynamic responses to specific tasks or upon presentation of specific stimuli[147,148]. In the current doctoral project, we used fMRI to assess the effects of OT on pSTS and amygdala activity in response to emotion processing from PLDs conveying biological motion in adult men with ASD.

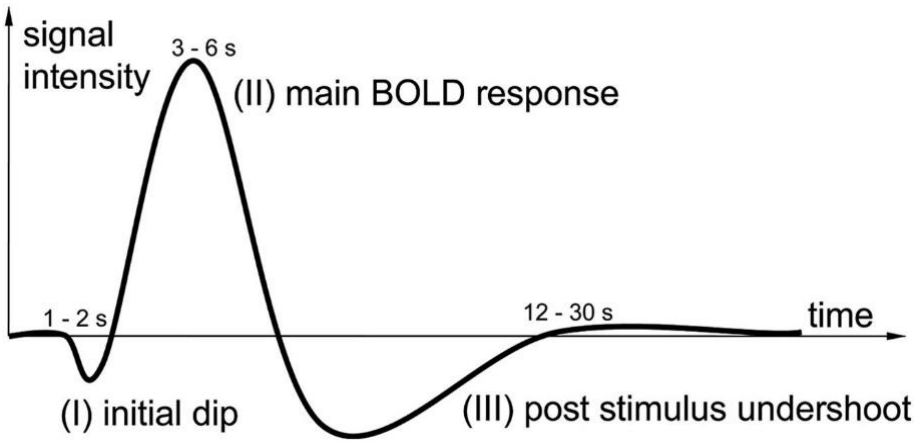

Figure 1.4. Illustration of the BOLD hemodynamic response function. Figure and figure legend adapted from Siero et al. (2013) in *PET Clinics*[108].

6 Objectives and anticipated impact of the project

6.1 Objectives and hypotheses

The general aim of this doctoral project is to gain more insight into the behavioral and neural effects of single-dose and multiple-dose administration of intranasal oxytocin both in the neurotypical population and in individuals with ASD. To address this general aim, the doctoral project was divided into three studies (described across four chapters, Fig. 1.5) with specific objectives addressed in each one of these studies.

Objective 1: Assess the immediate effect of single-dose OT treatment on emotion recognition in neurotypicals.

To date, research has mainly focused on exploring the effects of OT on emotion recognition from facial expressions. However, findings of these studies are mixed and no studies have directly investigated the effects of exogenously administered OT on the emotion processing from biological motion (or body language). We therefore aimed to investigate the effect of a single dose of OT on emotion recognition from biological motion. To do so, a double-blind, randomized, placebo-controlled trial was conducted during which participants performed a bodily emotion recognition task in which participants had to indicate the emotional state of a whole-body PLD figure before and after a single dose of intranasal OT.

We hypothesized that a single dose of intranasal OT (compared to placebo) would improve emotion recognition performance in these neurotypical participants.

This objective is addressed in **chapter 2**.

Objective 2: Assess the immediate effect of multiple-dose OT treatment on feelings of attachment, social responsiveness, quality of life and mood in neurotypicals.

A multitude of single-dose OT administration studies have explored the prosocial effects of OT. In particular, some studies showed that a single dose of OT can improve trust and feelings of (in)secure attachment[27,58,149,150]. However, evidence on the effects of multiple-dose OT treatment in neurotypicals is lacking. We therefore aimed to investigate the effects of two-week, daily intranasal OT administration on multiple socially relevant measures. To do so, a double-blind, randomized, placebo-controlled trial was conducted during which participants answered questionnaires assessing feelings of attachment, social responsiveness, quality of life and mood before and after a two-week treatment with intranasal OT.

We hypothesized that a 2-week, multiple-dose, intranasal OT treatment (compared to placebo) would improve self-report ratings of social responsiveness, experience of attachment, quality of life and mood in these neurotypical participants.
This objective is addressed in **chapter 3**.

Objective 3: Assess the immediate and long-term effects of multiple-dose OT treatment on core ASD characteristics (social responsiveness, restricted and repetitive behaviors (RRBs)), feelings of attachment and quality of life in individuals with ASD.
Initial clinical trials in ASD have provided evidence for beneficial effects of single-dose and multiple-dose OT treatment on ASD characteristics (e.g. RRBs) and social cognition (e.g. emotion recognition) in adults (reviewed in [151]), although multiple-dose effects in children with ASD are mixed[100–102,104]. To date, however, potential long-term effects of multiple-dose OT treatment that outlast the period of actual administration have not yet been addressed in adults with ASD. We therefore aimed to investigate the effect of a 4-week, intranasal OT treatment on multiple ASD-relevant measures as well as other socially relevant measures. To do so, a double-blind, randomized, placebo-controlled, parallel study was conducted during which participants answered questionnaires assessing social responsiveness (primary outcome) and RRBs, experience of attachment and quality of life (secondary outcomes) at multiple time points: (i) at baseline, (ii) immediately after four weeks of continual OT treatment, and at two follow-up sessions, (iii) one month (four weeks) and (iv) one year post-treatment.
We hypothesized that a 4-week, intranasal OT treatment would improve self-report and informant-based ratings of social responsiveness and RRBs, and ameliorate self-ratings of attachment characteristics and quality of life. A key question was to evaluate whether any beneficial effects of continual OT treatment would outlast the period of actual administration until one-month and or even one-year post-treatment.
This objective is addressed in **chapter 4.**

Objective 4: Assess the immediate and long-term effects of multiple-dose OT treatment on neural activity during emotion processing in individuals with ASD.
While initial neuroimaging studies have provided important insights into the immediate neural effects of single-dose OT treatment, our understanding of the potential of OT as a treatment for ASD has been hampered by a lack of insight into the effects of repeated OT administration on neural function. In addition, the possibility that long-term OT treatment may induce long-lasting neuro-behavioral changes that outlast the period of actual administrations is currently unexplored. We therefore aimed to investigate the effect of a 4-week, intranasal OT treatment on neural function in adult men with ASD. To address this aim, we conducted a double blind,

randomized, placebo-controlled, parallel study using functional magnetic resonance imaging (fMRI) after a single dose of OT (24 IU); after multiple doses of OT treatment (four weeks of daily administrations); and at follow-up sessions, one-month and one-year post-treatment; during the processing of emotional states from biological motion conveyed by whole-body point-light displays (PLDs).

We hypothesized that a single dose of OT would increase the recruitment of pSTS regions and alter recruitment of amygdala during the processing of emotional states from PLD biological motion stimuli. A key aim was to determine whether a four-week, multiple-dose treatment would induce similar or even augmented changes in pSTS and amygdala activity, and specifically, whether the changes in neural function would outlast the period of actual administration until one-month or even one-year post-treatment.

This objective is addressed in **chapter 5**.

6.2 Novelty and anticipated impact of the doctoral project

This doctoral project will expand knowledge on the immediate and – for the first time - long-term effects (until one year post-treatment) of OT administration on ASD characteristics, experience of attachment and the associated changes in brain activity (in particular in the pSTS and amygdala regions) in adult men with ASD. For the first time, the current project will not only include behavioral but also neural multiple-dose and retention measures until one year after the intervention. These insights are essential to evaluate the true therapeutic potential of OT for the social impairments experienced by individuals with ASD. Also, the neural insights will aid the field in discovering the working mechanism(s) of intranasal OT. More generally, the gained knowledge and insights may guide the design of new clinical trials not only for ASD but also for other neuropsychiatric disorders characterized by social impairments such as schizophrenia or generalized social anxiety disorder.

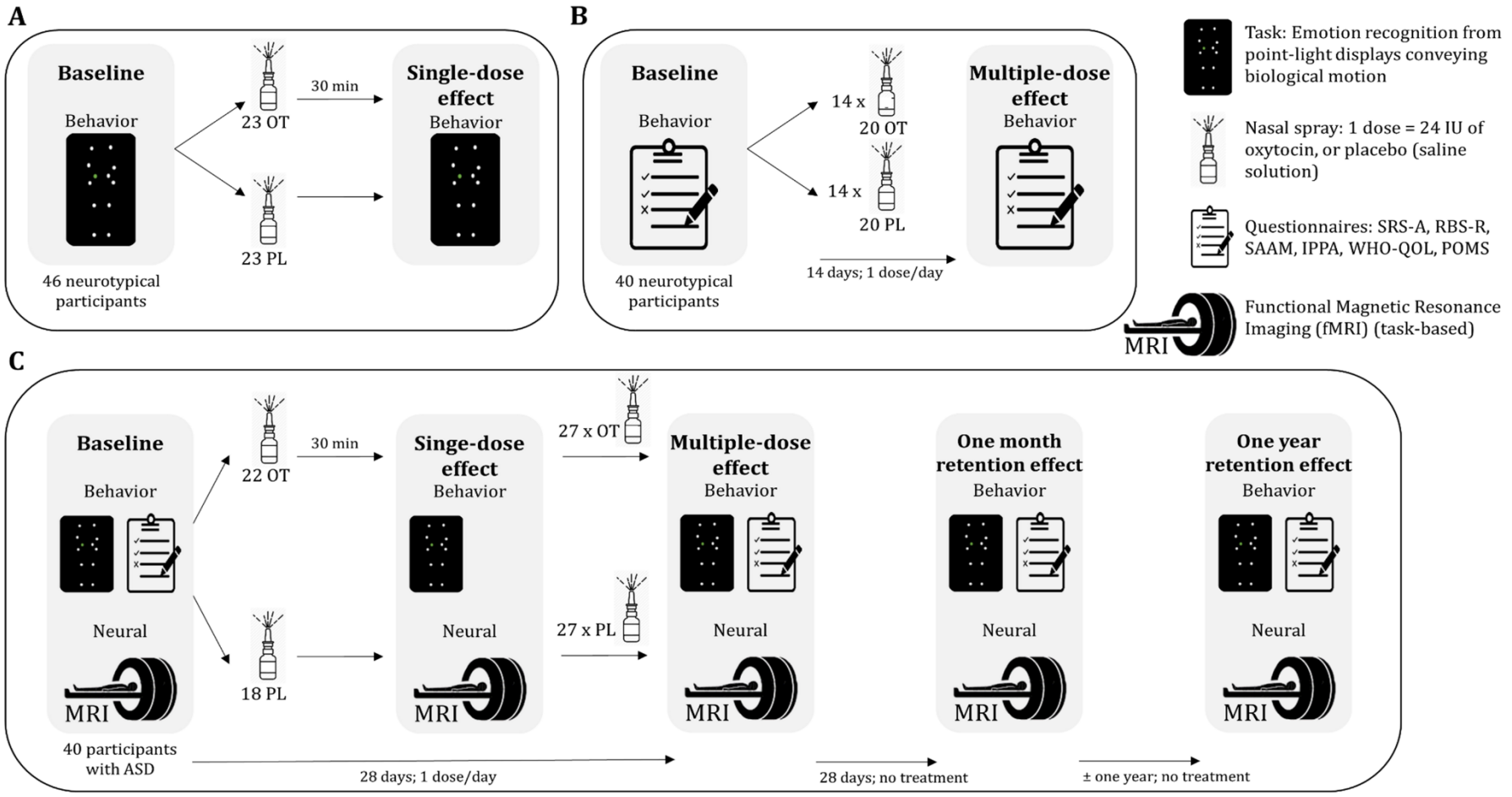

Figure 1.5. Overview of the doctoral project. Panel A represents the study described in chapter 2. Panel B represents the study reported in chapter 3. Panel C represents the study reported in chapters 4 and 5. ASD= Autism Spectrum Disorder, OT= Oxytocin, PL= Placebo, IU= International Units, SRS-A= Social Responsiveness Scale adult version, RBS-R= Repetitive Behavior Scale – Revised, SAAM= State Adult Attachment Measure, IPPA= Inventory of Parent and Peer Attachment, WHO QOL= World Health Organization Quality of Life questionnaire, POMS= Profile of Mood States questionnaire.

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Influence of oxytocin on emotion recognition from body language: A randomized, placebo-controlled trial

Chapter II

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Abstract

The neuropeptide 'oxytocin' (OT) is known to play a pivotal role in a variety of complex social behaviors by promoting a prosocial attitude and interpersonal bonding. One mechanism by which OT is hypothesized to promote prosocial behavior is by enhancing the processing of socially relevant information from the environment. With the present study, we explored to what extent OT can alter the 'reading' of emotional body language as presented by impoverished biological motion point light displays (PLDs). To do so, a double-blind between-subjects randomized placebo-controlled trial was conducted, assessing performance on a bodily emotion recognition task in healthy adult males before and after a single dose of intranasal OT (24 IU). Overall, a single dose of OT administration had a significant effect of medium size on emotion recognition from body language. OT-induced improvements in emotion recognition were not differentially modulated by the emotional valence of the presented stimuli (positive versus negative) and also, the overall tendency to label an observed emotional state as 'happy' (positive) or 'angry' (negative) was not modified by the administration of OT. Albeit moderate, the present findings of OT-induced improvements in bodily emotion recognition from whole-body PLD provide further support for a link between OT and the processing of socio-communicative cues originating from the body of others.

1 Introduction

The neuropeptide 'oxytocin' (OT) is a nonapeptide produced by the paraventricular and supraoptic nuclei of the hypothalamus and is known to play a pivotal role in a variety of complex social behaviors. Initial animal and human research mostly focused on exploring the function of OT in childbirth, lactation and mother-child bonding [1–4], but more recently, accumulating evidence also demonstrated a strong involvement of OT in promoting prosocial behavior [5], interpersonal bonding [6] and trust in adult relationships [7]. One mechanism by which OT is hypothesized to promote prosocial behavior is by enhancing the processing of socially relevant information from the environment [5]. Increasing evidence suggests that exogenous OT can mediate the processing of emotional and socially relevant information, such as emotional faces and facial expressions [8,9]. Particularly, a single dose of intranasally administered OT has been shown to enhance the detection of briefly presented facial expressions [9] as well as the ability to accurately identify positive emotional facial expressions [10]. Exogenously administered OT has also been shown to stimulate gaze towards the eye region [11,12] and increase eye contact [13].

However, facial expressions are not the only source for conveying emotional and socially relevant information. In everyday life, the processing of other sources of socially-relevant information - such as the communicator's body language or 'bodily kinematics' – may be equally important for stimulating interpersonal social cognitive processes [14]. To date however, only a handful of studies explored the effects of exogenously administered OT on the processing of biological motion originating from the body and bodily kinematics of others. One study by Kéri and Benedek (2009) used point light displays (PLDs) in which biological motion is presented by only a few moving dots that correspond to the movement of the body's main joints [15]. Although highly impoverished in terms of detail and background information, PLDs can readily evoke a vivid representation of a person. Interestingly, Kéri & Benedek (2009) showed for the first time that intranasal administration of OT can enhance the detection of biological motion PLD dots among a cloud of noise (mask) dots [15]. Perry et al. (2010) extended this work by showing that intranasal OT can significantly enhance the extent of EEG mu suppression over the sensory-motor regions in the brain during the observation of PLD biological motion [16]. Since mu suppression is considered a strong indicator of the extent by which observed actions are mapped or 'mirrored' onto the observer's own motor system, the results of Perry et al. (2010) provided initial neurophysiological evidence that OT can stimulate the processing of biological motion in the brain by altering 'mirror' motor resonance. Related to these findings, a more recent fMRI study provided indications that OT can alter the neural 'mirroring' of pain experienced by others [17]. Interestingly, Bos et al. (2015) showed that a single dose of OT significantly decreased neural activations in the insula and sensorimotor regions during the observation of painful stimuli experienced by others, which

likely relates to the pain-reducing properties of OT [17]. Aside the exploration of the effects of exogenously administered OT, Strauss et al. (2015) assessed levels of endogenous OT in blood plasma of patients with schizophrenia and showed that individual differences in plasma OT were associated with the 'reading of bodily expressions' [18]. Together, these studies provide strong indications of a link between OT and the processing of socio-communicative cues originating from the body of others.

To the best of our knowledge however, no studies to date directly investigated the effects of exogenously administered OT on the processing of emotional content embedded in biological motion kinematics or body language. To date, research mainly focused on exploring the effects of OT on emotion recognition from facial expressions. However, considering that bodily kinematics may provide subtle, but salient cues on the emotional state of others, it would be interesting to explore whether OT can affect this process.

In the present study, a double-blind between-subjects randomized placebo-controlled trial was conducted to specifically explore the effects of a single dose of OT on the 'reading' of emotional body language as presented in impoverished biological motion PLDs. To this end, a bodily emotion recognition task was adopted in which participants had to indicate the emotional state of a whole-body PLD figure before and after a single dose of intranasal OT.

2 Materials and methods

2.1 Study design

A randomized, double-blind, placebo-controlled, between-subjects design was used to test single-dose effects of intranasal oxytocin (OT) administration. Written informed consent was obtained from all participants prior to the study. Consent forms and study design were approved by the local Ethics Committee for Biomedical Research at the University of Leuven, KU Leuven (S56327) in accordance to The Code of Ethics of the World Medical Association (Declaration of Helsinki). The trial was registered with the European Clinical Trial Registry (Eudract 2014-000586-45) and the Belgian Federal Agency for Medicines and Health products.

2.2 Participants

A total of 46 healthy young adult men (23 OT, mean age = 21.50, S.D. = 2.02; 23 Placebo (PL), mean age = 21.78, S.D. = 2.11) were recruited to participate in the present study exploring single-dose effects of OT on the recognition of emotional states from point light displays (PLDs). Data from

three participants (2 OT, 1 PL) were excluded from the analysis due to technical problems during data collection. All participants were recruited through advertisement within the university, such that approximately 90% of the included sample were university students. Participants were randomly allocated to receive OT or placebo (PL) nasal sprays. All participants were right-handed (self-reported) and mean age did not differ between OT and PL groups in both experiments. Only male participants were recruited to avoid potential sex differences in OT response as well as the potential interaction with the female hormonal cycle. Exclusion criteria were (i) age below 18 or above 30 years old (ii) a diagnosed psychiatric or neurological disorder, (iii) intake of psychotropic medication, (iv) history of neurological disease, and (v) history or evidence of other diseases (cancer, hematologic illness, endocrine disease, cardiovascular disease, respiratory condition, renal disease, liver condition or gastrointestinal illness).

The sample size of the current study exploring the effect of OT on the recognition of emotional states from PLDs was similar to the included sample size of a related study exploring the effect of a single dose of OT on biological motion perception from PLDs [15]. In this study, a within-subject design with a sample of 20 participants was adopted and a large effect of OT (versus PL) was revealed on improving the recognition of biological motion (versus recognizing non-biological motion) [15] (more detailed information is provided in Supplementary Methods).

2.3 Drug protocol

Sprays were prepared by the KU Leuven University Hospital pharmacist. OT (Syntocinon®, Sigma-tau) and placebo (PL) (saline natriumchloride solution) were administered in amber 15 ml glass bottles with metered pump (ACA Pharma). Each puff per nostril contained 4 international units (IU) of OT. In healthy humans, the impact of OT on social cognition is commonly evaluated using a single dose of intranasal OT, typically given 30–45 min before the experimental task (see Guastella and Macleod (2012) for a review). The efficacy of this time interval for intranasal oxytocin administration has been confirmed by animal research [19,20] and human research [21]. Consequently, also in the present study, a single dose of 24 IU OT was delivered as 3 puffs per nostril, 30 min before the start of the experimental procedures.

All participants received clear instructions about the use of the nasal spray. At first use, air present in the nasal spray was removed by pumping the spray until a fine mist was observed. Participants were instructed to keep one nostril closed, to take a deep breath through the nose and to tilt their head slightly backwards during nasal administration in order to minimize gravitational loss of the spray. To assure proper use of the spray and to validate tolerability, each subject administered the first dose in front of the experimenter and commented on their experience (e.g. particular smell or taste). All participants were monitored onsite until approximately one hour after nasal

spray administration. All participants were screened for potential adverse events or side effects (see Supplementary Table 2.1 for side effects questionnaire).

Table 2.1 Change from baseline scores for each treatment group (oxytocin, placebo) and corresponding Cohen's d effect sizes (Performance change$_{OT}$ – Performance change$_{PL}$)/pooled SD).

Outcome measure	Oxytocin	Placebo	
	Change from baseline Mean ± SD	Change from baseline Mean ± SD	Cohen's d
Emotion Recognition Task			
ACC (%)	6.48 ± 10.22	-0.44 ± 9.51	0.70
RT (ms)	-14.41 ± 203.48	-79.34 ± 205.50	0.32
Discrimination Sensitivity (d')	0.59 ± 0.99	0.04 ± 0.60	0.68
Response Bias (criterion)	0.04 ± 0.40	-0.05 ± 0.44	0.21
Control Task			
ACC (%)	1.26 ± 4.65	-1.52 ± 7.45	0.44
RT (ms)	-30.97 ± 96.66	7.35 ± 95.04	0.40

Note. ACC = Accuracy rates in percentages; RT = reaction times for correct answers in milliseconds.

2.4 Task and Stimuli

A computer-based two-choice emotion recognition task was used as a behavioral measure to assess the effect of a single dose of OT administration. Participants also performed a two-choice control task matched on motor requirements and task demands, which enables to correct for potential unspecific changes in task performance from the baseline to the post-session (e.g., related to basic differences in task compliance or attention).

The emotion recognition task involved the recognition of 'emotional states' from bodily kinematics as depicted by point light displays (PLDs). The adopted PLD stimuli were based on motion capture data as previously described [22]. In short, all PLDs showed twelve moving white dots against a black background representing the motion of the joints of the ankles, knees, hips, wrists, elbows and shoulders of the human body (Figure 2.1). PLD stimuli represent an impoverished but highly controlled form of human motion, representing solely the bodily kinematics without any distracting details on form or background.

In the adopted PLD movies (duration 3 sec), male and female actors were displayed performing one of three actions (walking, jumping or kicking a ball using the right leg) in either a happy or angry emotional state. All movies were presented from three different viewpoints (front view (0°),

side view (90°) and intermediate view (45°) and either in upright or inverted (upside-down) position. As such, the total set of stimuli consisted of 72 movies (2 genders x 3 actions x 2 emotional states x 3 perspectives x 2 positions). At each trial of the emotion recognition task, participants were instructed to indicate as accurately and as quickly as possible whether the presented PLD figure was either happy or angry by pressing the respective response buttons (happy or angry) (Figure 2.1A). Participants also performed a control 'color' task, during which a 'scrambled' version of the PLDs were presented and participants were instructed to indicate color changes in the moving point lights (Figure 2.1B). Particularly, for each of the PLD movies used in the emotion recognition task, a scrambled version was created in which the same twelve dots undergo the same local trajectories as in the original PLDs, but with the starting position randomly permutated. In these stimuli, one dot changed color to red or green at a random time point and participants were instructed to indicate as accurately and quickly as possible the color of the dot by pressing the respective response buttons (green or red). Each participant executed the tasks in a quiet room on the same computer monitor. Instructions were provided verbally as well as on the computer monitor at the start of the test. No feedback was provided during the task. Accuracy and reaction times were recorded using E-Prime software (Psychological Software Tools).

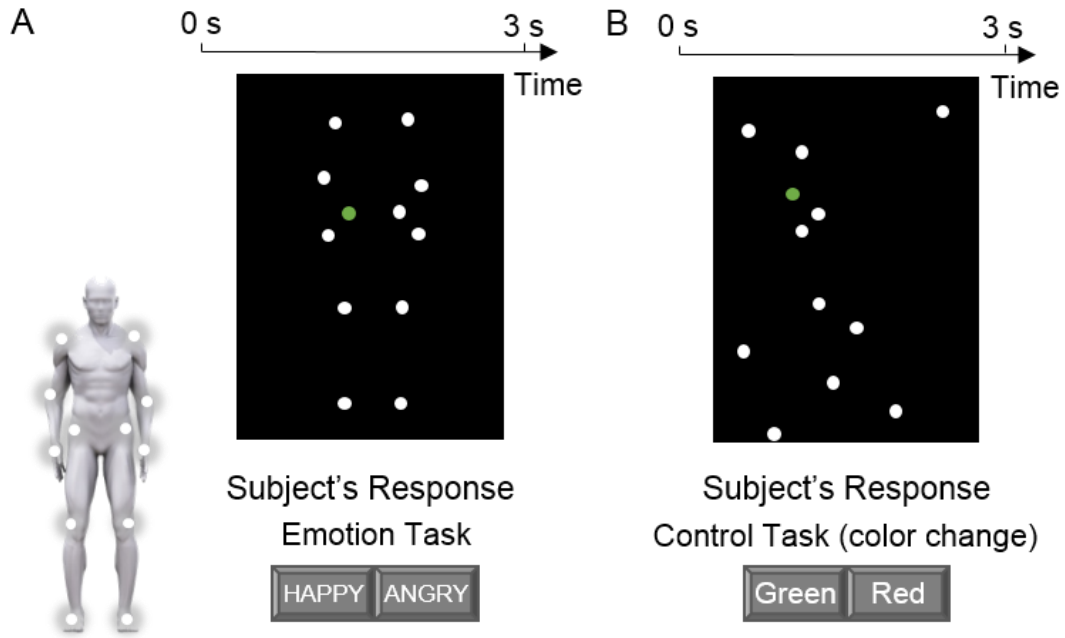


Figure 2.1. Participants determined the emotional state or color change of PLDs in which moving white dots reflected the main joints of the human body. (A) During the emotion task, participants were instructed to indicate the emotional state of the moving point light figure (happy or angry). (B) Scrambled versions of the PLD stimuli were presented in a two-choice control task matched on motor requirements and task demands. Here, one of the dots in the PLD briefly changed color to either red or green and participants had to indicate the color change.

2.5 Procedure

To assess the single-dose effect of OT on emotion recognition from PLDs, performance on the emotion task was assessed at baseline and after OT administration. A different set of PLD stimuli was presented before and after OT administration to avoid a repeated presentation (i.e., note that the use of *identical* sets of PLD stimuli before and after nasal spray administration was shown to induce general increases in performance from baseline to post-sessions, reflecting an overall memory effect based on the repeated presentation of stimuli (see Supplementary Figure 2.1)). In the current experiment, half of the PLD stimuli were randomly selected to assess emotion recognition performance at baseline and the other half of PLD stimuli was used to assess performance after OT administration. Note that across OT and PL groups, the same set of stimuli was adopted to assess 'baseline' or 'post' performance. At the start of the experimental session participants received verbal instructions on the purpose and procedure of the tasks and completed a short practice of ten trials to familiarize them with the stimuli and task instructions. Performance was then assessed immediately before (baseline measure) and 30 min after (post measure) a single dose of nasal spray administration (OT or PL).

2.6 Data analysis and statistics

For the emotion and control task, reaction times (RTs) and accuracy rates (percentage correct answers) were calculated to assess performance at the baseline and the post session.

For the emotion recognition task, we also derived the hit rate (h) and false alarm rate (f) to calculate the discrimination sensitivity [d'= z(h)− z(f)] and response bias [criterion=−1/2[z(h)+ z(f)]]. For each trial, participants were allowed to indicate their response within a time interval of 4000 milliseconds. Across all participants and trials, a few trials were lost due to 'no recorded response' [Emotion $task_{baseline}$: 3.07%; Emotion $task_{post}$: 1.65%; Control $task_{baseline}$: 0.16%; Control $task_{post}$: 0.47%]. No further trials were discarded based on outlier detection of the RT data (i.e., no RTs exceeded Q3 ± 3 × (Q3–Q1) with Q1 and Q3 denoting the first and third quartile over the whole set of trials for each subject) (Electronic Statistics Textbook, StatSoft, Inc. Tulsa).

For all performance measures (RTs, accuracy, discrimination sensitivity, response bias), Shapiro-Wilk's W tests were used to investigate the normality of data distribution. Only for the accuracy scores of the control task, data deviated from the normal distribution. As such, for these data, non-parametric Mann-Whitney U Tests for independent samples (OT, PL) were used to assess treatment-dependent changes in performance from baseline to post. For all other performance

measures, repeated-measures Analysis of Variance (ANOVA) were conducted with the between-subject factor 'group' (OT and PL) and the within-subject factor 'time' (baseline, post). Further, to assess the effect size of the OT treatment, Cohen's d [23] was calculated by subtracting the baseline-to-post change in performance of the PL group from the baseline-to-post performance change of the OT group ((Performance change$_{OT}$ – Performance change$_{PL}$)/ sqrt(SD$_{OT}$^2 + SD$_{PL}$^2)). All statistics were executed with Statistica 10 (StatSoft. Inc. Tulsa, USA). The significance level was set at $p < .05$ for all analyses.

3 Results

Performance measures are displayed separately for each treatment group (OT, PL) and session (baseline, post) in Figure 2.2 and 2.3 and in Supplementary Table 2.2. In Table 2.1, baseline-to-post performance changes are listed for each treatment group (OT, PL) and corresponding Cohen's d effect sizes are reported (Performance change$_{OT}$ – Performance change$_{PL}$)/pooled SD).

3.1 Accuracy

A repeated-measures ANOVA analysis with the between-subject factor 'group' (OT, PL) and the within-subject factor 'time' (baseline, post) was conducted to explore the effects of OT on performance accuracy of the emotion recognition task. A significant 'group x time' interaction was revealed ($F(1,41) = 5.30$; $p < .05$; $\eta^2 = .12$; power = .61), indicating that performance on the emotion recognition task significantly increased from the baseline to the post session in the OT group (Tukey's HSD test: $p < .05$), but not in the PL group (Tukey's HSD test $p = .99$) (Figure 2.2) (Cohen's d = .70, medium effect). Note that baseline performance was not significantly different between treatment groups (Tukey's HSD test p =.56). Also no main effects of 'group' ($F(1,41) = 0.10$; $p = .75$) or 'time' ($F(1,41) = 2.62$; $p = .11$) were revealed.

Non-parametric Mann-Whitney U Tests were performed to assess changes in performance accuracy of the control task from the baseline to the post session. As seen in Figure 2.2, pre-post changes in basic performance of the control task were tentatively more pronounced in the OT compared to the PL group, but the difference was not significant (Mann-Whitney U: $Z = 1.19$; $p = .23$) (Cohen's d = .44, small effect).

To directly explore whether treatment-dependent effects on the emotion task were potentially driven by unspecific changes in task performance from the baseline to the post session (e.g., related to session-dependent differences in task compliance or attention), a general linear regression analysis was conducted with performance on the control task as a covariate-of-no-

interest. This analysis revealed that the treatment-dependent effect on the emotion task persisted after correction for basic performance changes on the control task ($F(1,40) = 4.84$; $p < .05$; $\eta^2 = .11$; power = .58).

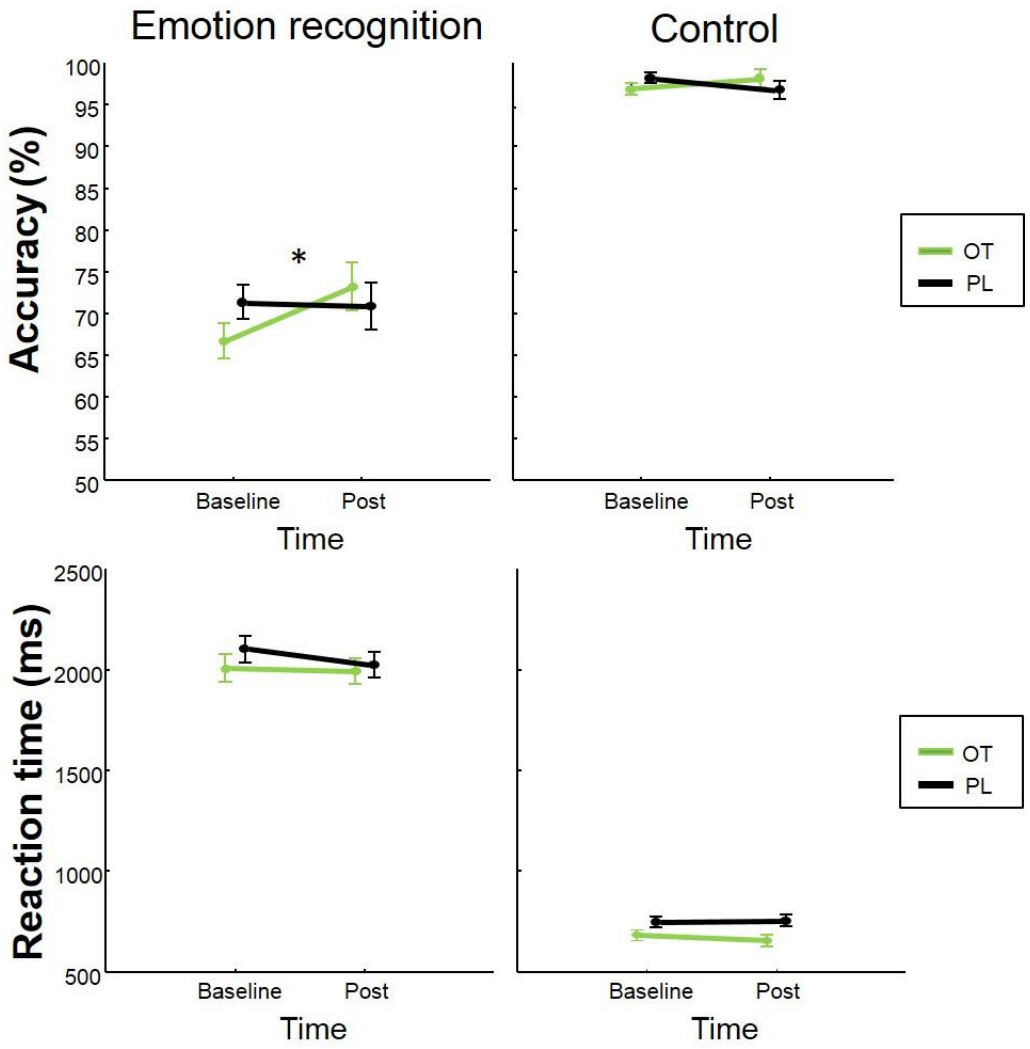


Figure 2.2. For each task (emotion recognition, control), accuracy (% correct scores) (upper graphs) and reaction times (lower graphs) are displayed separately for each treatment group (oxytocin (OT); placebo (PL)) and session (baseline, post). Vertical lines denote ± standard error.

3.2 Reaction times

Repeated-measures ANOVA analysis on the reaction time measures of the emotion task failed to reveal a significant main effect of 'time' ($F(1,41) = 2.26$; $p = .14$) or 'group' ($F(1,41) = 0.52$; $p = .47$) (Figure 2.2). Also no significant 'group x time' interaction effect was revealed ($F(1,41) = 1.08$; $p = .30$; $\eta^2 = .03$; power = .17) indicating that OT did not differentially influence reaction times (Cohen's d = .32, small effect).

Also for reaction times on the control task, no significant main effect of 'time' or interaction was revealed (both $F < 2.00$, $p > .19$) (Cohen's $d = .40$, small effect). However, on the control task, a main effect of group ($F(1,41) = 4.83$; $p < .05$) was revealed, indicating that reaction times were generally lower in the OT group compared to the PL group (Figure 2.2).

3.3 Discrimination sensitivity and response bias in emotion recognition

Repeated-measures ANOVA analyses with the between-subject factor 'group' (OT, PL) and the within-subject factor 'time' (baseline, post) were conducted to explore treatment-dependent changes in discrimination sensitivity (d') and response bias (criterion) for indicating the bodily emotional states in the emotion task (happy, angry).

For the discrimination sensitivity index, a significant 'group x time' interaction was revealed ($F(1,41) = 5.00$; $p < .05$; $\eta^2 = .11$; power = .59), indicating that discrimination sensitivity significantly increased from the baseline to the post session in the OT group (Tukey's HSD test: $p < .01$), but not in the PL group (Tukey's HSD test $p = .99$) (Figure 2.3) (Cohen's $d = .68$, medium effect).

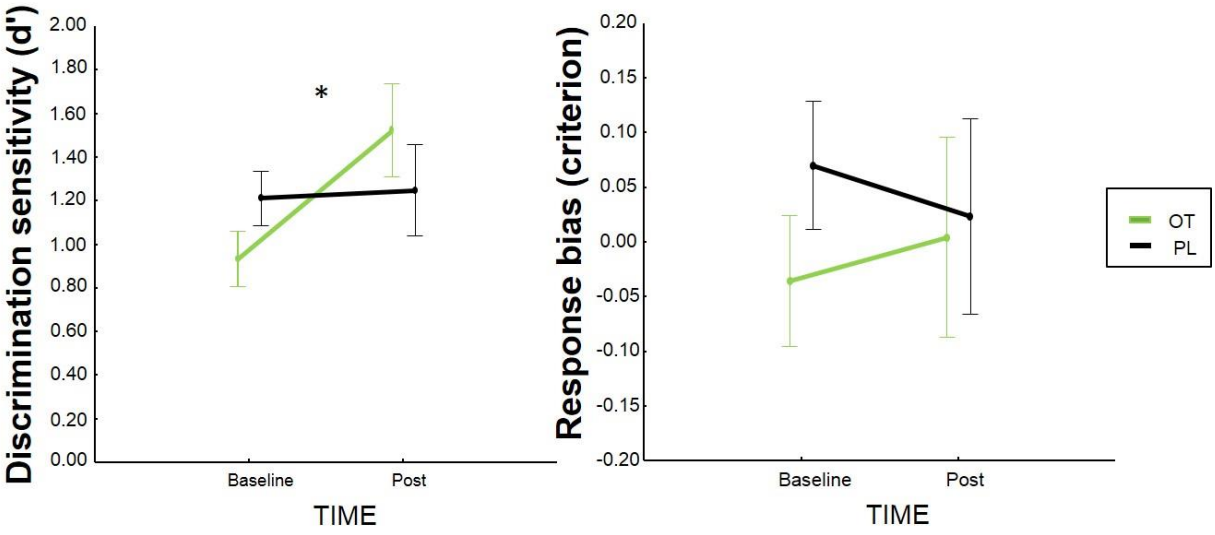


Figure 2.3. Discrimination sensitivity (d') and response bias (criterion) for indicating the bodily emotional states in the emotion task (happy, angry) are displayed separately for each treatment group (oxytocin (OT); placebo (PL)) and session (baseline, post). Vertical lines denote ± standard error.

In terms of response bias (criterion), no treatment-related effects were observed, indicating that OT treatment did not significantly alter the observers' tendency to label the presented emotional states as e.g. happy or angry ($F(1,41) = .45$; $p = .50$; $\eta^2 = .01$; power = .10). Also note that at both

sessions (baseline, post) and for each treatment group (OT, PL), criterion scores were not significantly smaller than zero (bias to respond 'happy') or higher than zero (bias to respond 'angry') (all, $p > .30$).

3.4 Secondary analyses

Secondary analyses were conducted to verify whether treatment-dependent effects on emotion recognition accuracy were modulated by the type of emotion. To do so, we repeated the 'group x time' ANOVA analysis with 'emotion type' (happy vs. angry) as an additional within-subject factor, forming a three-way 'group' by 'time' by 'emotion type' ANOVA model. This analysis failed to reach significance, however ($F(1,41) = 0.61$, $p = .44$), indicating that the enhancing effect of OT on emotion recognition was not differentially modulated by the type of the presented emotion.

Further secondary analyses were conducted to verify whether treatment-dependent effects were modulated by (i) the orientation of the presented PLD stimuli (upright, inverted) or (ii) the viewing perspective (front view (0°), side view (90°) and intermediate view (45°)).

For 'orientation', the three-way interaction 'group x time x orientation' was not significant, indicating that the enhancing effect of OT on emotion recognition was not differentially modulated by the orientation of the presented stimuli ($F(1,42) = .45$; $p = .47$). Note however that a significant main effect of 'orientation' was revealed, indicating that across sessions and treatment groups, performance accuracy was higher for recognizing emotional states from upright, compared to inverted PLD stimuli ($F(1,42) = 95.50$; $p < .001$).

For the three-way ANOVA analysis assessing the modulating effect of 'perspective', a significant three-way interaction was revealed ($F(2,84) = 4.64$; $p < .05$), indicating that overall, treatment-related effects were more pronounced for side view (90°) and intermediate view (45°) stimuli, compared to front view (0°) stimuli.

4 Discussion

The current study presents results of a double-blind between-subjects randomized placebo-controlled trial assessing the immediate (single-dose) effects of oxytocin (OT) on reading emotional body language. Overall, a single dose of OT administration had a significant effect of medium size on emotion recognition, indicating that a single dose of intranasal OT administration can enhance the reading of other's emotional body language from point light displays. Overall, these findings of OT-induced improvements in bodily emotion recognition from whole-body point light displays in young healthy adults extend previous results assessing the effects of OT on

biological motion processing (Kéri and Benedek, 2009; Perry et al., 2010) and the effects of OT on facial emotion recognition [8–10,25]. A study by Guastella et al. (2008) showed that OT can increase the number of fixations and total gaze time toward the eye region of other people [11]. Considering that the eyes represent a salient social cue of the face and a primary source for detection of interpersonal interest, and emotional states of others, the OT-induced enhancement of eye-region processing was suggested as the possible mechanism underlying the positive effects of OT on facial perception and interpersonal communication [8,12]. Together with the initial results of Kéri and Benedek (2009)[15], showing an effect of OT on basic biological motion processing, the findings of the present study provide indications that the mechanism by which OT induces prosocial behavior is not restricted to facial cues such as the eye region, but instead generalizes to other sources of socially-relevant information, such as cues originating from the bodily kinematics of others. To date however, the underlying neural mechanism by which OT can enhance the processing of biological motion information is largely unclear. One study by Perry et al. (2010) showed that intranasal OT can significantly enhance the extent of EEG mu suppression over the sensory-motor regions in the brain during the observation of PLD biological motion, which was hypothesized to reflect an enhanced 'mapping' or 'motor resonance' of observed bodily expressions in the 'mirror motor circuitry' of the brain [16]. Social neuroscience is increasingly focusing on the role of the observer's own motor system in understanding or 'reading' other's bodily kinematics [26–29]. Within the framework of the social-cognitive simulation theory [30,31] and the ideomotor theory [32], it was posited that the 'understanding of other's actions and behavior' may be essentially motor, rather than sensory in nature. Only recently, De Coster et al. (2014) explored the link between OT and motor simulation of observed actions and showed that intranasal administration of OT can increase automatic imitative behavior, which is supportive of the notion that OT can enhance the mirroring of other's actions [24].

While OT may act directly on the brain's mirror-motor circuitry to enhance the processing of facial or bodily cues, the possibility cannot be ruled out that the effects of OT on social information processing might be related to more general OT-induced modulations of attention orienting, thereby increasing the saliency of social cues in the observed environment [33]. Indeed, while changes in EEG mu-suppression were most evident over the sensorimotor cortex in the study by Perry et al. (2010), responses were not limited to this region [16], suggesting that also other perceptual and attentional processes are potentially influenced by OT. In support of this notion, several studies showed effects of OT on the orienting of attention in response to emotional gaze cues [34] and attentional shifts toward happy facial expressions [35]. Also more recently, Xu et al. (2015) showed that OT can improve the allocation of attentional resources towards neutral and positive facial expressions, but not for non-social stimuli or negative facial expressions [36]. On the other hand, an eye-tracking study of Lischke et al. (2012), failed to show an association between

the direction of overt visual attention and OT-induced improvements of facial emotion recognition, and this irrespective of the type of emotional expression [25]. In the present study, our bodily emotion recognition paradigm included two emotional states ('happiness' and 'anger'). While our results indicate that OT can increase the discrimination sensitivity for labeling these emotions, OT-induced improvements in emotion recognition were not differentially modulated by the emotional valence of the presented stimuli (positive versus negative). Also no OT-related changes in response bias were observed, indicating that the overall tendency to label an observed emotional state as 'happy' (positive) or 'angry' (negative) was not changed by the administration of OT. While these explorations on the modulating role of emotional valence are interesting, it should be noted that the effects of OT may not be restricted to the processing of emotional content per se. Particularly, in the study by Kéri and Benedek (2009), point light display stimuli were adopted without emotional content and results showed that the presence of biological motion (versus non-biological motion) was both necessary and sufficient to induce OT-related enhancements in perception [15]. In this view, it appears that OT may alter the processing of 'bodily' socially-relevant cues in general, but that the social saliency of these cues is not strictly determined by the presence or absence of explicit emotional content. With the present study, we showed that OT can enhance bodily emotion recognition and that the effects were not modulated by the valence of the emotional stimuli. For future experiments, it would however be interesting to directly compare the effects of OT on basic biological motion perception with the effects of OT on the perception of emotional content conveyed by the stimuli. Such designs would allow disentangling whether the additional presence of emotional content potentially increased the perceived social saliency of the presented cue, or whether the mere presence of a biological actor is equally salient to evaluate the presented stimulus as socially relevant.

Several functional neuroimaging studies from our [37] and other labs [38–40], as well as TMS/tDCS brain stimulation studies [41–44] and lesion studies [45] highlighted the importance of a cortical area in the superior temporal sulcus (STS) in biological motion processing. Also beyond biological motion detection, the STS has been shown to play an important role in several other social cognitive functions, including face perception, speech processing, directing of eye gaze and mentalization [46,47]. Overall, the STS is known to form an integral part of the brain's neural circuitry underlying social cognition, including the amygdala-orbitofrontal social brain, and also in relation to the human fronto-parietal mirror motor system, the STS has been hypothesized to form the main visual input area [48]. In the context of OT, results from a recent meta-analysis of human neuroimaging studies exploring the neural effects of single-dose OT administration specifically identified changes in brain activity in temporal areas including the STS as well as the insula during the processing of social stimuli [49]. Together, these findings highlight the STS as a possible neural target by which OT can exert increasing effects on bodily emotion recognition, either by directly

modulating the processing within this region, or indirectly by altering its connected neural circuitry. Future research is necessary however to further unravel the exact neural basis by which OT can mediate the processing of biological motion and bodily emotional expressions. Considering earlier reports on difficulties of patients with autism spectrum disorders (ASD) with the processing of biological motion [50,51] and emotion recognition more general (for review see [53]), these insights will be important to further evaluate the potential of OT as a novel treatment for ASD. In the present study, only healthy neurotypical males were included to assess the single-dose effects of OT administration on bodily emotion recognition and overall, the revealed effects were moderate. It would therefore be interesting for future studies to explore whether the present effects can be replicated or even enlarged in populations with particular deficits in the social domain, such as ASD.

Supplements

Supplementary Methods

The choice of sample size for the current study exploring the effect of OT on the recognition of emotional states from PLDs was mainly based on the included sample size and reported effect size of one previous study exploring the effect of a single dose of OT on biological motion perception from PLDs [15]. In this study, a within-subject design with a sample of 20 participants was adopted and large effects of OT (versus PL) were revealed on improving the recognition of biological motion (versus recognizing non-biological motion) [$F(1,38)= 30.53$, $p = .001$, $\eta 2= .45$, power= .99]. Based on this study, the minimum number of participants necessary to yield a similar power and effect size was estimated at 26 participants (13 OT, 13 PL) for a (two-tailed) between-subject design with the alpha-level of significance set at 0.05. Considering that this power calculation was based on a within-subject effect size, we chose to recruit a sample size of 46 participants (23 OT, 23 PL), which corresponds to the averaged sample sizes adopted in the study of Keri et al. (2009)[15] (20 OT, 20 PL, within-subject design) and a related study by De Coster et al., (2014)[24] investigating the effect of OT on motor simulation (24 OT, 24 PL, between-subject design).

Supplementary Tables

Supplementary Table 2.1. Side effects questionnaire

Please fill in the boxes (crosses) if the described side effect applies to you.							
Symptom	Y/N	Start of the reaction		Duration of the reaction (hours)	Severity of the reaction		
		Right after Administration	Within 2 hours		Mild	Moderately	Serious
Headache							
Drowsiness							
Dizziness							
Fainting							
Changes in heart rate or palpitations							
Shortness of breath							
Fever							
Sore throat							
Dry throat/dry mouth							
Hoarseness							
Coughing							
Coughing up mucus							
Congested nose							
Sneezing							
Nasal irritation							
Runny nose							
Burning sensation in nose and/or ears							
Sensitive to fragrances							
Watery eyes							
Nausea and/or vomiting							
Abdominal or stomach pain							
Decreased appetite							
Hungry							
Constipation							
Diarrhea							
Muscle pain/cramps							
Skin rash							
Increased fluid intake							
Water retention/bloating							
Insomnia/sleep difficult							
Nightmares							
Staring/daydreams							
Anaphylaxis							
Changes in perception of the tongue							
Back pain							
Bed wetting							

Weight gain							
Sweating							
Blurred vision							
Less talk to others							
Uninterested in others							
Persistent thoughts and/or feelings							
Development of repetitive behavior							
Increase in repetitive behavior							
Nail biting							
Annoyed, bored							
Sad							
Prone to crying, more emotional							
Anxious, worried, discomfort							
Happy, satisfied							
Euphoric, unusually happy, more energetic							
Calm, relaxed, comfortable							
More focused							
More confidence							
Did you experience other side effects that are not mentioned in the table above?							

Supplementary Table 2.2. Performance measures (accuracy, reaction times, discrimination sensitivity (d'), response bias (criterion)) are displayed separately for each treatment group (oxytocin, placebo) and session (baseline, post).

Outcome measure	Oxytocin		Placebo	
	Baseline	Post	Baseline	Post
	Mean ± SD		Mean ± SD	
Emotion Recognition Task				
ACC (%)	66.73 ± 10.33	73.21 ± 15.55	70.77 ± 8.82	70.90 ± 10.48
RT (ms)	1986.28 ± 313.59	1993.76 ± 322.55	2104.37 ± 307.19	2024.37 ± 273.20
Discrimination Sensitivity (d')	0.93 ± 0.62	1.52 ± 1.25	1.21 ± 0.55	1.25 ± 0.63
Response Bias (criterion)	-0.04 ± 0.18	0.00 ±0.42	0.07 ± 0.34	0.02 ± 0.42
Control Task				
ACC (%)	97.03 ± 3.22	98.15 ± 3.05	98.31 ± 2.71	96.72 ± 6.51
RT (ms)	678.77 ± 109.81	654.01 ± 116.58	738.67 ± 144.06	754.87 ± 151.40

Note. ACC = Accuracy rates in percentages; RT = reaction times for correct answers in milliseconds.

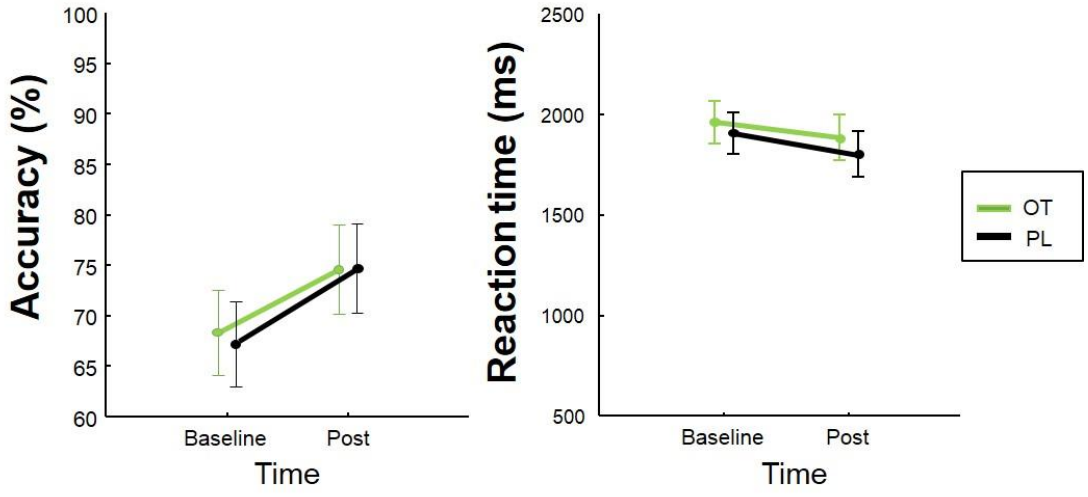
Supplementary Figures

Supplementary Figure 2.1.

The choice to explore OT-induced changes in performance using a *different* set of point light display (PLD) stimuli before and after OT administration in our main experiment was based on a prior experiment showing general performance increases from baseline to post sessions when *identical* sets of PLD stimuli are presented prior and after nasal spray administration. Supplementary Figure 2.1 briefly outlines the procedure and results of this prior experiment.

Forty subjects (20 OT, mean age = 21.10, S.D. = 2.88; 20 PL, mean age 21.60, S.D. = 2.26) participated in this prior experiment assessing the effects of OT nasal spray on emotion recognition with identical sets of PLD stimuli presented at baseline and post nasal spray administration (OT or PL).

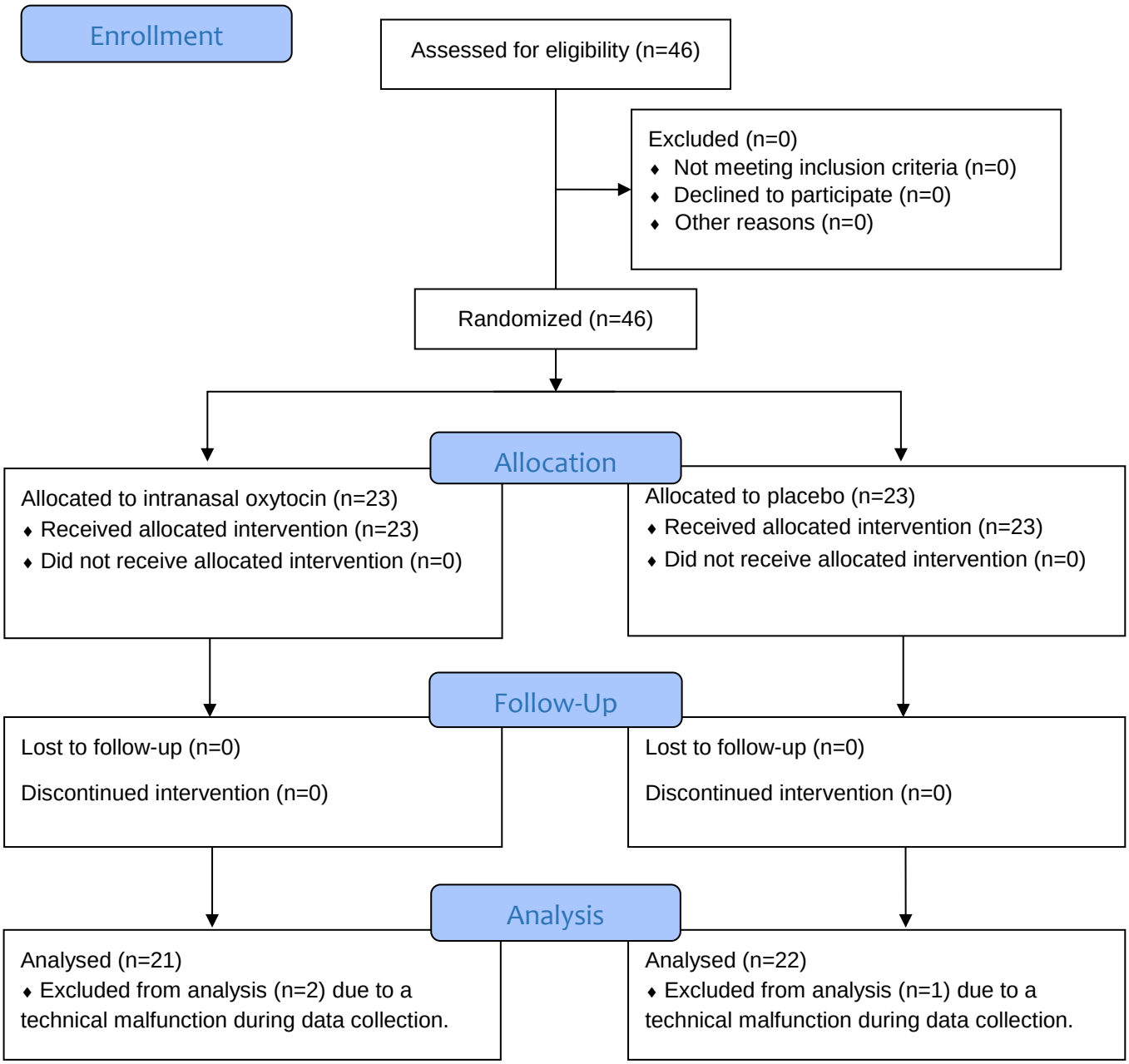
Repeated-measures ANOVA analysis with the between-subject factor 'group' (OT, PL) and the within-subject factor 'time' (baseline, post) revealed strong main effects of 'time' for both the accuracy measure ($F(1,38) = 27.92$; $p < .0001$) and the reaction time measure ($F(1,38) = 8.07$; $p < .01$); and no significant 'group x time' interaction effects were revealed (accuracy: $F(1,38) = 0.217$; $p = .42$) (reaction time: $F(1,38) = 0.22$; $p = .65$). This finding indicates that irrespective of the received treatment (OT or PL), performance generally increased from the baseline to the post session, most likely reflecting a memory effect of the presented stimuli when identical sets of PLD are used. Considering that the repeated presentation of the PLD stimuli likely elevated performance to ceiling levels in the post session, we chose to present different sets of PLD stimuli at baseline and post nasal spray administration in the main experiment.



Supplementary Figure 2.2. CONSORT Flow Diagram

CONSORT
TRANSPARENT REPORTING of TRIALS

CONSORT 2010 Flow Diagram



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Long-term oxytocin administration enhances the experience of attachment

Chapter III

Published as

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Abstract

The neuropeptide 'oxytocin' (OT) is known to play a pivotal role in a variety of complex social behaviors by promoting a prosocial attitude and interpersonal bonding. Previous studies showed that a single dose of exogenously administered OT can affect trust and feelings of attachment insecurity. With the present study, we explored the effects of two weeks of daily OT administration on measures of state and trait attachment using a double-blind between-subjects randomized placebo-controlled design. In 40 healthy young adult men state and trait attachment were assessed before and after two weeks of daily intranasal OT (24 IU) or placebo using the State Adult Attachment Scale and the Inventory of Parent and Peer Attachment. Mood, social responsiveness and quality of life were additionally assessed as secondary outcome measures. Reductions in attachment avoidance and increases in reports of attachment toward peers were reported after two weeks of OT treatment. Further, treatment-induced changes were most pronounced for participants with less secure attachment towards their peers, indicating that normal variance at baseline modulated treatment response. OT treatment was additionally associated with changes in mood, indicating decreases in feelings of tension and (tentatively) anger in the OT group, not in the placebo group. Further, at the end of the two-week trial, both treatment groups (OT, placebo) reported to experience an increase in social responsiveness and quality of life, but the effects were only specific to the OT-treatment in terms of reports on 'social motivation'. In summary, the observed improvements on state and trait dimensions of attachment after a multiple-dose treatment with OT provide further evidence in support of a pivotal role of OT in promoting the experience of attachment.

1 Introduction

The neuropeptide 'oxytocin' (OT) is known to play a pivotal role in a variety of complex social behaviors. OT is a nonapeptide produced by the hypothalamic paraventricular and supraoptic nuclei and is secreted into the bloodstream by the posterior pituitary gland. Based on initial animal and human research, the physiological role of OT in lactation and childbirth is well-established [1–4]. More recently, different lines of research have shown a strong involvement of OT in complex social behaviors including interpersonal bonding, maternal care, and the ability to establish trust and form social attachments (for reviews see [5–7], but also [5]).

From genetic research, important insights on the involvement of the oxytocinergic system in pair-bonding and attachment have emerged. For example, recent research showed that genetic variations in the OT-receptor gene (OXTR) of prairie voles are related to social attachment and partner preference [9], and that knock-down of the OT receptor inhibited social attachment and parental care [10]. Also in human infants, polymorphisms in the OXTR gene have been associated with variations in attachment security [11].

Associations have also been shown between endogenous levels of OT in plasma and maternal or paternal bonding behaviors, attachment-related thoughts and infant social engagement in naturalistic settings [12–14]. However, studies investigating a link between plasma OT levels and trust behavior in experimental settings (trust game paradigms), have yielded mixed results with some studies reporting tentative links [15,16], while others failed to reveal a significant correlation [17].

Other evidence for a role of OT in the establishment of trust and attachment came from studies investigating the effects of exogenously administered OT on behavior and neural functioning. For example, seminal work by Kosfeld et al. (2005) showed that intranasally administered OT can increase trust among human individuals [18]. Particularly, using a social trust game with monetary stakes, Kosfeld et al. (2005) showed that a single dose of OT significantly increased the readiness to bear social risks arising through interpersonal interactions [18]. Later, Buchheim et al. (2009) showed that OT can increase the experience of attachment security [19], while De Dreu (2012) proved that intranasal OT can facilitate the development of trust and cooperation in particular in adults with high attachment avoidance (by reducing betrayal aversion) [20]. Also self-reports on agency towards self or others were shown to be influenced by single doses of OT administration, indicating that in avoidantly attached individuals, OT positively influenced communal traits and agency towards others [21].

Interestingly, subsequent neuroimaging work provided indications that the effects of exogenously administered OT on trust and trust adaptation were associated with reductions in neural activity of brain regions that are implicated in fear processing (amygdala and midbrain regions) [22].

Together, these aforementioned studies provide promising indications that a single dose of exogenously administered OT can affect trust and attachment behavior in humans. To extend this line of work, the present study aimed to provide an initial investigation on the effects of multiple-dose treatments with OT on measures of state and trait attachment. To do so, we conducted a double-blind between-subjects randomized placebo-controlled trial assessing the effects of two weeks of daily OT administration using the State Adult Attachment Measure (SAAM) [23] and the Inventory of Parent and Peer Attachment (IPPA) [24] as primary outcome measures. The IPPA is constructed to measure perception of secure attachment towards peers, parents and significant others at a trait level. The SAAM on the other hand, is constructed to measure transient changes in attachment anxiety, attachment avoidance and attachment security at a state level.
To explore whether changes after multiple-dose OT intake were potentially related to changes in mood, we additionally assessed changes in mood states using the Profile of Mood States questionnaire (POMS) [25]. Additional secondary outcome measures were included to obtain an assessment of social responsiveness and general reports of quality of life. To this end, the adult self-report version of the Social Responsiveness Scale (SRS) [26] and the abbreviated version of the World Health Organization Quality of Life questionnaire (WHOQOL) (WHO, 1998) [27] were used, respectively.

2 Materials and methods

2.1 Study design

The study design involved a randomized, double-blind, placebo-controlled, between-subjects trial to assess multiple-dose effects of intranasal oxytocin (OT) administration. Written informed consent was obtained from all participants prior to the study. Consent forms and study design were approved by the local Ethics Committee for Biomedical Research at the University of Leuven, KU Leuven (S56327) in accordance to The Code of Ethics of the World Medical Association (Declaration of Helsinki). The trial was registered with the European Clinical Trial Registry (Eudract 2014-000586-45) and the Belgian Federal Agency for Medicines and Health products.

2.2 Participants

A total of 40 healthy young adult males were included in the study. Participants were randomly allocated to receive OT or placebo (PL) nasal sprays (20 OT, mean age = 20.70, S.D. = 2.72; 20 PL,

mean age = 21.55, S.D. = 2.39). All participants were right-handed (self-reported) and mean age did not differ between OT and PL groups.

All participants were recruited through advertisement within the university, such that 90% of the included sample were university students. Only male participants were recruited to avoid sex differences in OT response or potential interactions with the female hormonal cycle. Exclusion criteria were (i) age below 18 or above 30 years old (ii) a diagnosed psychiatric or neurological disorder, (iii) intake of psychotropic medication, (iv) history of neurological disease, and (v) history or evidence of other diseases (cancer, hematologic illness, endocrine disease, cardiovascular disease, respiratory condition, renal disease, liver condition or gastrointestinal illness).

2.3 Drug protocol

Sprays were prepared by the KU Leuven University Hospital pharmacist. OT (Syntocinon®, Sigma-tau) and placebo (PL) (saline natrium-chloride solution) were administered in amber 15 ml glass bottles with metered pump (ACA Pharma). Each puff per nostril contained 4 international units (IU) of OT. Each participant received a total of 14 doses of 24 IU, delivered daily over 14 consecutive days. All participants received clear instructions about the use of the nasal spray. At first use, air present in the nasal spray was removed by pumping the spray until a fine mist was observed. Participants were instructed to keep one nostril closed, to take a deep breath through the nose and to tilt their head slightly backwards during nasal administration in order to minimize gravitational loss of the spray. To assure proper use of the spray and to validate tolerability, each subject administered the first dose in front of the experimenter and commented on their experience (e.g. particular smell or taste). All participants were monitored onsite until approximately one hour after nasal spray administration. Participants were asked to take the nasal spray in the morning; and to keep a daily record of the time point of nasal spray administration and whether or not they were alone or in company of others the first two hours after administration (records were not returned by 2 OT participants and 2 PL participants). Percentage of days at which the spray was administered in the presence of others was not significantly different between treatment groups (OT: 75.6 % (SD 23.8); PL: 69.8 % (SD 28.6); $t(34)= .32$; $p = .75$).

All participants were screened for potential adverse events or side effects. The side effects questionnaire and a frequency table of reported side effects are provided as Supplementary Tables 3.1 and 3.2. As listed in more detail in Supplementary Table 3.2, only minimal side effects were reported and effects were independent of treatment (e.g., participants from both treatment groups reported to feel more focused (2 OT; 2 PL) or confident (1 OT; 1 PL)). Finally, at the end of

the trial, participants were asked if they thought they had received OT or PL. Only two participants correctly guessed to have received the OT treatment. All other participants thought they had received PL.

2.4 Procedure

Self-report questionnaires on attachment were used as primary outcome measures assessing the effects of two weeks of daily OT administration. Additionally, questionnaires on mood, social responsiveness and quality of life were used as secondary outcome measures.

2.5 Questionnaires

State Adult Attachment Measure (SAAM)

The SAAM is a questionnaire to assess temporary fluctuations in state attachment [23]. The questionnaire contains 21 statements where participants have to indicate their current state ('right now') on a seven-point Likert-scale. The questionnaire comprises three subscales assessing (i) attachment security (e.g., *"I feel like I have someone to rely on"*) (7 items; α = .82-.91); (ii) attachment anxiety (e.g., *"I feel a strong need to be unconditionally loved right now"*) (7 items; α = .81-.85); and (iii) attachment avoidance (e.g., *"If someone tried to get close to me, I would try to keep my distance"*) (7 items; α = .71-.87) [23].

Inventory of Parent and Peer Attachment (IPPA)

The IPPA is a questionnaire to assess trait attachment to (i) mother (25 items; α = .87); (ii) father (25 items; α = .89); (iii) peers (25 items; α = .92); and (iv) an important person of choice (25 items; no reported α) [24]. The IPPA questionnaire comprises a total of 100 items (25 for each section) with a four point Likert-scale to assess three dimensions of attachment: degree of mutual trust, quality of communication and extent of anger and alienation. The fourth part (attachment to an important person) was not mandatory and was only obtained from 11 OT and 16 PL participants (50% partner, 21.87% close friend, 28.13% unanswered). One participant of the OT group did not complete the section on 'father'.

Profile of Mood States (POMS)

A 32-item short version of the POMS questionnaire [25,28] was used as a measure of transient affective states in order to assess whether mood levels of participants changed over the course of the study. This instrument comprises emotional adjectives subdivided in five domains: 'tension'

(6 items; α = .84), 'depression' (8 items; α = .91), 'vigor' (5 items; α = .81), 'fatigue' (6 items; α = .90) and 'anger' (7 items; α = .87) which have to be rated on a five-point Likert scale. Participants were asked to rate their current mood state ('right now').

Social Responsiveness Scale (SRS) – Adult version

The Dutch adult self-report version of the SRS [29,30] was adopted in our study to assess social responsiveness at baseline and post OT-treatment. The SRS (64 items) assesses variations in social responsiveness in the typical population and autism spectrum disorders using a four-point Likert-scale. It encompasses four subscales, including social communication (22 items; α = .88), social awareness (19 items; α = .80), social motivation (11 items; α = .83) and rigidity/repetitiveness (12 items; α = .79). Higher scores indicate less social responsiveness.

World Health Organization Quality of Life (WHOQOL) - Bref

The abbreviated version of the WHOQOL is a 26-item questionnaire to assess general quality of life related to physical health, psychological health, social relationships, and environment (α = .84) (WHO, 1998) [27]. Ratings vary from 1 (very bad, very unsatisfied, totally not or never) to 5 (very good, very satisfied, totally or always).

2.6 Data analysis and statistics

For each questionnaire, total scores and/or subscale scores were calculated. Shapiro-Wilk's W tests were used to investigate the normality of data distribution. At the group level, extreme outliers were identified when scores were larger or smaller than Q3 ± 3(Q3-Q1), with Q1 and Q3 being the first and third quartile (Statistica 10; StatSoft. Inc. Tulsa, USA). No extreme outlier scores were identified in the distribution of the pre- and post-treatment scores for any of the questionnaires. However, a few extreme outliers were identified in the change-from-baseline scores (IPPA peer attachment: 1 PL outlier; IPPA father attachment: 1 PL outlier; SRS total: 2 OT outliers; POMS depression: 1 PL and 2 OT outliers; POMS angry: 2 OT outliers). For completeness, all primary statistical analyses are reported with and without exclusion of these outliers. Particularly, for all questionnaires, repeated-measures Analyses of Variance (ANOVA) were conducted with the between-subject factor 'group' (OT and PL) and the within-subject factor 'time' (Baseline, Post). Further, to assess the effect size of the OT treatment, Cohen's d [31] was calculated by subtracting the change-from-baseline scores of the PL group from the change-from-baseline scores of the OT group [(Score change$_{OT}$ – Score change$_{PL}$)/ sqrt(SD$_{OT}$^2 + SD$_{PL}$^2)]. All

statistics were executed with Statistica 10 (StatSoft. Inc. Tulsa, USA). The significance level was set at $p < .05$ for all analyses.

3 Results

Questionnaire scores are listed separately for each treatment group (OT, PL) and session (Baseline, Post) in Supplementary Table 3.3. Table 3.1 lists the change-from-baseline scores for each treatment group and the corresponding Cohen's d effect sizes.

3.1 Primary outcome measures: Trait and state attachment

Trait attachment: Inventory of Parent and Peer Attachment (IPPA)

Two-way ANOVA analysis revealed a significant 'group x time' interaction (with outlier: $F(1,38) = 6.49$, $p < .05$; $\eta^2 = .15$; power = .70; without outlier: $F(1,37) = 8.02$, $p < .01$, $\eta^2 = .18$; power = .79) for the subscale score 'Friends', indicating an increase in self-reported trait attachment towards peers in the OT group ($F(1,38) = 6.11$, $p < .05$), not in the PL group (with outlier: $F(1,38) = 1.28$, $p = .27$; without outlier: $F(1,37) = 0.00$, $p = 1.0$) (Cohen's d = .75, medium to large effect (with outlier); d = .64, medium effect (without outlier)) (Figure 3.1A (upper graph) and Table 3.1). Note that baseline scores in peer attachment were not significantly different between treatment groups ($F(1,38) = .01$, $p = .94$).

For the reports of trait attachment towards 'Mother' and 'Father', scores tended to increase after two weeks of OT treatment, but the change failed to reach significance (Mother: $F(1,38) = 1.97$; $p = .17$; $\eta^2 = .05$; power = .28) (Father: with outlier: $F(1,37) = 1.26$; $p = .03$; $\eta^2 = .05$, power = .20; without outlier: $F(1,36) = .34$; $p = .56$; $\eta^2 = .01$, power = .09). Reports of attachment towards an important person were only completed by a subset of participants (11 OT, 16 PL) and no significant treatment-related changes were revealed for this subscale ($F(1,25) = 0.09$, $p = .77$; $\eta^2 = .003$; power = .06). Across treatment groups, baseline reports on attachment towards peers, mother and father were significantly inter-correlated (all, $r > .62$; $p < .001$).

To explore whether inter-individual differences in baseline IPPA peer attachment scores were related to treatment-related effects on post-treatment peer attachment scores, a step-wise multiple regression analysis was conducted covarying for baseline scores and testing baseline-by-treatment interactions (Supplementary Table 3.4 and Figure 3.1A). Analyses showed that differences in post-treatment IPPA scores between the OT and PL treatment groups were only evident after correction for variance in baseline scores (treatment effect without correction for baseline scores: $\beta = .17$, $t(37) = 1.05$, $p = .30$) (treatment effect with correction for baseline scores:

β = .15, t(36) = 3.01, p < .01). Importantly, also a significant 'treatment group x baseline score' interaction effect was revealed (β = -.64, t(35) = -2.17, p < .05), indicating that the treatment effect differed according to initial (baseline) severity. Particularly, as visualized in Figure 3.1A (lower graph), the shape of the interaction pattern indicated that the difference in outcome between treatment groups increased with increasing baseline 'severity' (lower baseline peer attachment). In other words, the OT treatment appeared to enhance IPPA peer attachment particularly among those with low peer attachment at baseline.

State attachment: State Adult Attachment Measure (SAAM)

A significant 'group x time' interaction effect was revealed for the avoidance subscale (F(1,38) = 4.36, p < .05; η^2 = .10; power = .53), indicating that state attachment avoidance significantly decreased after the two-week treatment in the OT group (F(1,38) = 5.35, p < .05), but not in the PL group (F(1,38) = .41, p = .53) (Cohen's d = .63, medium effect) (Figure 3.1B (upper graph) and Table 3.1). Baseline scores in attachment avoidance were not significantly different between treatment groups (F(1,38) = .10, p = .77). No significant 'group x time' interaction effects were revealed for reports of 'attachment security' (F(1,38) = .50, p = .49; η^2 = .01; power = .11) and 'attachment anxiety' (F(1,38) = .001, p < .97; η^2 < .001; power = .05), indicating that the two-week OT treatment did not exert a significant effect on these subscales.

To explore whether inter-individual differences in baseline SAAM attachment avoidance were related to treatment-related effects on post-treatment attachment avoidance scores, a step-wise multiple regression analysis was conducted covarying for baseline scores and testing baseline-by-treatment interactions (Supplementary Table 3.4 and Figure 3.1B). Analyses showed a significant difference in post-treatment attachment avoidance scores between the OT and PL treatment groups irrespective of variance at baseline (treatment effect without correction for baseline scores: β = -.32, t(38) = -2.07, p < .05) (treatment effect with correction for baseline scores: β = -.28, t(37) = -3.21, p < .01). Although note that the treatment effect was more pronounced after removal of variance explained by the baseline scores. No significant 'treatment group x baseline score' interaction effect was revealed (β = -.08, t(36) = -.35, p = .73), indicating that the main effect of treatment (the vertical distance between the regression lines in Figure 3.1B) was constant irrespective of initial baseline score. Note however that for both groups (OT and PL), reductions in SAAM attachment avoidance were most pronounced for participants with high baseline attachment avoidance.

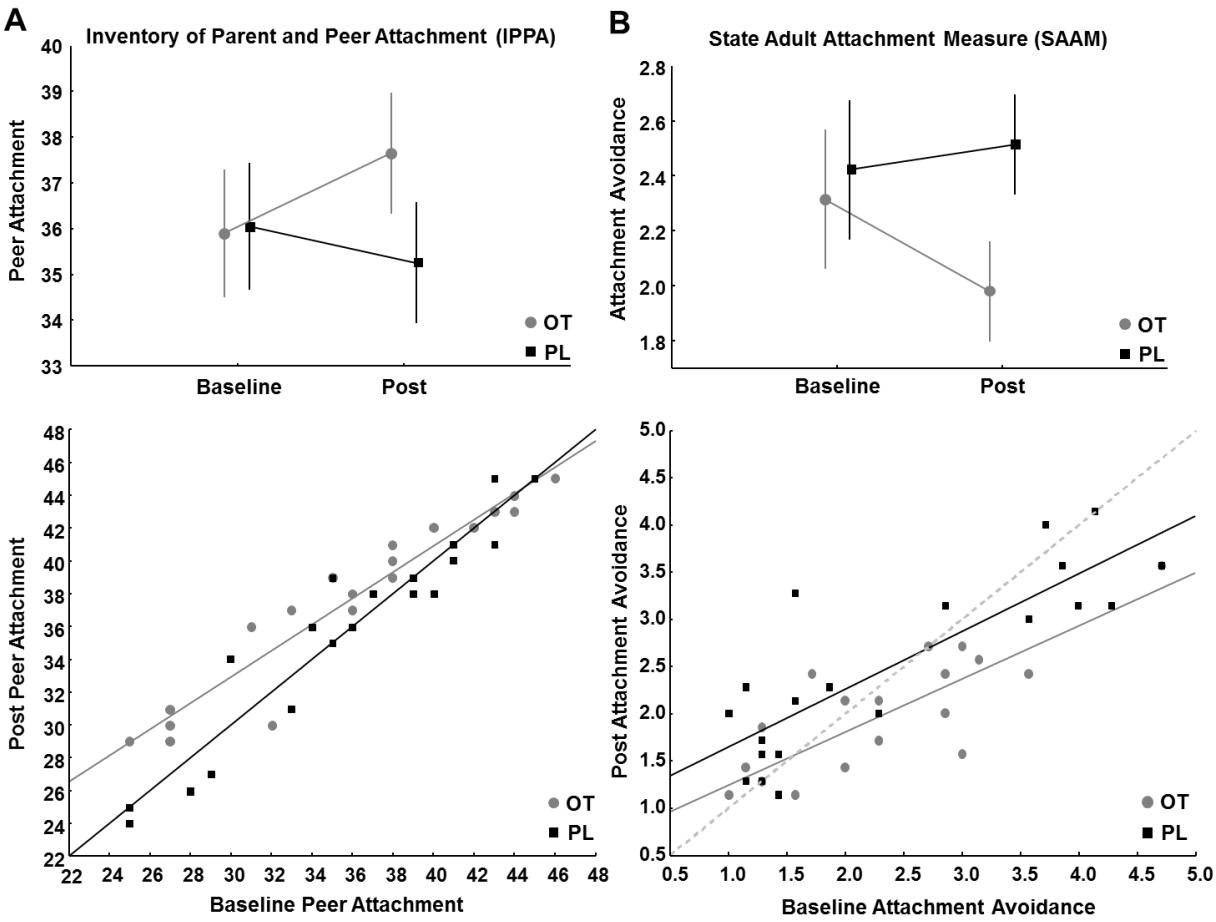


Figure 3.1. Effects of two-week treatment on the experience of attachment. The upper graph of Panel (A) visualizes reported changes in 'peer attachment' (IPPA) for each treatment group (oxytocin (OT) – placebo (PL)) at baseline and after (post) the two-week treatment. The upper graph of Panel (B) visualizes treatment-related changes in 'attachment avoidance' (SAAM) for each treatment group (OT - PL) at baseline and post treatment. The lower graphs visualize the individual treatment responses by plotting the relationship between the baseline (pre-treatment) scores (horizontal axis) and the post-treatment scores (vertical axis). In graphical terms, the main effect of treatment is visualized by the vertical distance between the regression lines of the OT group (grey line) and the PL group (black line). The dotted line represents a perfect linear relationship (x=y) indicating no change from pre-to-post treatment.
Note that for changes in IPPA 'peer attachment' (lower panel A), the regression line of the PL group largely overlapped with the perfect linear regression line, indicating no change from pre-to-post in the PL group. Importantly, for IPPA peer attachment, a significant 'treatment group x baseline score' interaction effect was revealed, indicating a difference in slopes of the regression lines for the OT treatment group and the PL group (i.e., regression lines are not parallel). Overall, the shape of the interaction pattern indicated that the difference in outcome between treatment groups increased with increasing baseline 'severity' (lower baseline peer attachment). In other words, the OT treatment appeared to enhance IPPA peer attachment particularly among those with low peer attachment at baseline.
For changes in SAAM 'attachment avoidance' (lower panel B), no significant difference was revealed between the slopes of the regression lines of the OT and PL treatment groups, indicating that the main effect of treatment (the vertical distance between the regression lines) was constant irrespective of initial baseline score. Note however that for both groups (OT and PL), reductions in SAAM attachment avoidance were most pronounced for participants with high baseline attachment avoidance.

Table 3.1. Change from baseline scores (with outliers) for each treatment group (oxytocin, placebo) and corresponding Cohen's d effect sizes (Performance change$_{OT}$ – Performance change$_{PL}$)/pooled SD).

Outcome Measure	**Oxytocin**	**Placebo**	
	Change from baseline	**Change from baseline**	
Self-report Questionnaires	Mean ± SD	Mean ± SD	**Cohen's d**
Trait Attachment - IPPA			
Friends	1.75 ± 1.97	-0.80 ± 4.02	0.75
Mother	0.65 ± 1.98	-0.20 ± 1.85	0.44
Father	0.90 ± 1.77	-0.35 ± 4.73	0.35
Important person	-0.09 ± 2.12	0.19 ± 2.54	0.10
State Attachment - SAAM			
Security	0.08 ± 0.40	0.18 ± 0.49	0.22
Anxiety	-0.17 ± 0.72	-0.18 ± 0.68	0.01
Avoidance	-0.34 ± 0.56	0.09 ± 0.72	0.63
Mood - POMS			
Tension	-1.50 ± 1.88	0.10 ± 2.90	0.63
Depression	-0.35 ± 1.23	0.60 ± 3.42	0.37
Anger	-1.10 ± 2.29	0.20 ± 1.58	0.63
Vigor	-0.55 ± 4.42	0.00 ± 4.58	0.12
Fatigue	-0.85 ± 3.25	-1.45 ± 4.83	0.15
Social responsiveness - SRS-Adult			
Social Awareness	-1.45 ± 2.74	-0.30 ± 3.91	0.34
Social Communication	-1.20 ± 3.05	-0.90 ± 2.90	0.10
Social Motivation	-2.05 ± 1.93	-0.65 ± 2.54	0.60
Rigidity and Repetitive behavior	-1.45 ± 2.16	-1.70 ± 2.92	0.10
Total	-6.15 ± 5.93	-3.55 ± 8.35	0.35
Quality of life - WHOQOL-Bref			
Total	1.25 ± 4.54	2.15 ± 6.23	0.17

Note. SAAM = State Adult Attachment Scale; IPPA = Inventory of Parent and Peer Attachment; POMS = Profile of Mood States; SRS-Adult = Social Responsiveness Scale, adult version; WHOQOL-bref = World Health Organization Quality of Life, abbreviated version.

Relationship between OT-related improvements in state (SAAM) and trait (IPPA) attachment

At baseline, significant inter-correlations were revealed between reports on peer/parent attachment (IPPA) and attachment security (SAAM) (all, $r > .50$; $p < .05$), whereas no significant relationships were revealed with baseline reports of attachment anxiety or avoidance (SAAM) ($p > .05$). Also no significant correlations were revealed between OT-related improvements in SAAM-based attachment avoidance and IPPA-based improvements in peer attachment (OT-group: $r = -.03$; $p = .92$). This finding indicates that high improvements on state attachment avoidance were

not necessarily predictive of high improvements in secure peer attachment (although as a group, participants receiving OT improved on both measures).

Treatment-related changes in mood: Profile of Mood States (POMS)

Compared to the PL group, participants receiving OT reported to feel significantly less tense ('group x time' interaction: $F(1,38) = 4.29$, $p < .05$; $\eta^2 = .10$; power = .52) and less angry ('group x time' interaction: $F(1,38) = 4.37$, $p < .05$; $\eta^2 = .10$; power = .53) after two weeks of nasal spray treatment (Figure 3.2), but note that the effect on angry mood states may have been driven by an outlier subject (i.e., no significant effect after removal of the outliers: $F(1,36) = 2.03$, $p = .16$; $\eta^2 = .05$, power = .28). No significant effects were found for the other mood states (depression: with outliers: $F(1,38) = 1.3$, $p = .25$; $\eta^2 = .03$; power = .21; without outliers: $F(1,35) = .00$, $p = 1.00$, $\eta^2 = .00$, power = .05) (vigor: $F(1,38) = .15$, $p = .70$; $\eta^2 = .003$; power = .06) (fatigue: $F(1,38) = .21$, $p = .65$; $\eta^2 = .006$; power = .07).

To explore whether inter-individual differences in OT-related improvements in attachment were related to inter-individual differences in mood changes, a general linear regression analysis was conducted with 'change in mood' as a covariate-of-no-interest. Correlation analysis revealed that treatment-related decreases in attachment avoidance (SAAM) were positively correlated with decreases in reports of anger (with outlier: $r = .52$; $p < .05$), but not tension ($r = .22$; $p = .33$), but note that the effect of angry mood states may again have been driven by an outlier subject (i.e., no significant effect after removal of the outliers: without outlier: $r = .23$, $p = .36$). Regression analysis further confirmed that the response to OT on attachment avoidance was - at least partly – accounted for by more basic changes in mood. Particularly, the OT-dependent improvements in attachment avoidance only tentatively persisted after correction for changes in 'tension' ($F(1,37) = 2.87$; $p = .09$) and no longer reached significance after correction for changes in 'anger' ($F(1,37) = 2.06$; $p = .15$). On the other hand, for reports on changes in peer attachment (IPPA), correlation analysis revealed no significant relationship with changes in mood ($p > .75$). Further regression analysis showed that the OT-related improvements in peer attachment remained significant after correction for changes in tension ($F(1,37) = 5.00$; $p < .05$), and tentatively after correction for changes in anger ($F(1,37) = 3.5$; $p = .07$).

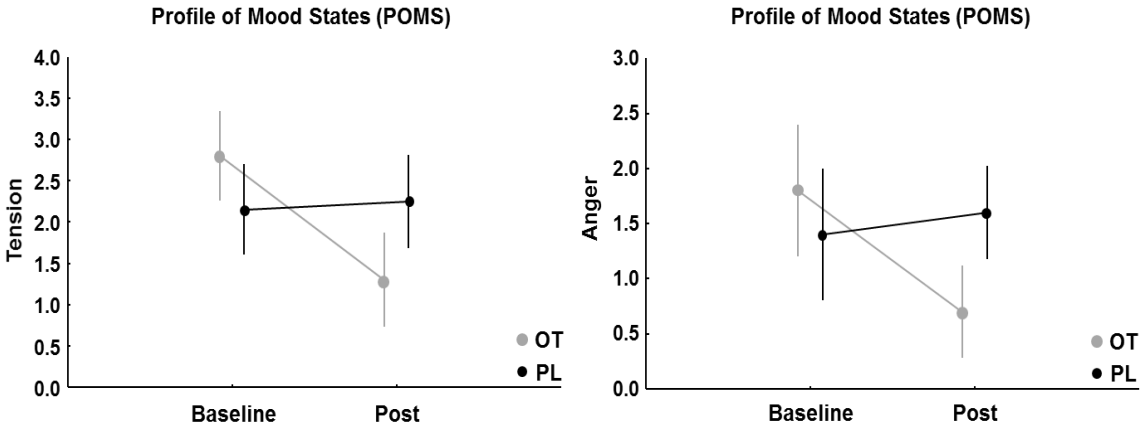


Figure 3.2. Effects of the two-week treatment on mood states. The left graph visualizes treatment-related changes in feelings of 'tension' (POMS) for each treatment group (oxytocin (OT) - placebo (PL)) at baseline and after (post) the two-week treatment. The right graph visualizes treatment-related changes in feelings of 'anger' (POMS).

3.2 Secondary outcome measures: Social responsiveness and overall quality of life

Social Responsiveness: Social Responsiveness Scale (SRS)

After two weeks of nasal spray administration, participants of both treatment groups reported lower total SRS-scores (increased social responsiveness) (main effect of time: with outliers: $F(1,38) = 17.94$, $p < .001$, without outliers: $F(1,36)$, $p < .001$). Although tentatively more pronounced in the OT group (with outliers: $F(1,38) = 14.42$; $p < .001$; without outliers: $F(1,36) = 23.44$, $p < .001$) compared to the PL group ($F(1,38) = 4.80$; $p = .034$), the difference in slopes was not significantly different, indicating that the effect was not specific to the OT-treatment (group x time: $F(1,38) = 1.30$, $p = .26$; $\eta^2 = .03$; power = .20) (Figure 3.3A and Table 3.1) (note however that the group x time interaction effect was marginally significant after removal of two outliers in the OT group: $F(1,36) = 3.51$, $p = .07$, $\eta^2 = .09$, power = .45). Total SRS scores at baseline were not significantly different between treatment groups ($F(1,38) = .40$, $p = .54$). Subsequent analysis of the subscale scores did reveal a treatment-specific effect for the subscale 'social motivation' (group x time: $F(1,38) = 3.85$, $p = .05$; $\eta^2 = .03$; power = .20), indicating an increase in self-reported social motivation in the OT group ($F(1,38) = 16.51$, $p < .001$), not in the PL group ($F(1,38) = 1.66$, $p = .21$) (Cohen's d = .60, medium effect) (Table 3.1). No significant treatment-related effects were found for the other subscales (social awareness: $F(1,38) = 1.16$, $p = .29$; $\eta^2 = .03$; power = .18) (social communication: $F(1,38) = 0.10$, $p = .75$; $\eta^2 = .003$; power = .06) (rigidity/repetitiveness: $F(1,38) = 0.09$, $p = .76$; $\eta^2 = .002$; power = .06). Also no significant inter-correlations were found between pre-to-post changes in attachment and changes in social responsiveness (all, $p > .12$).

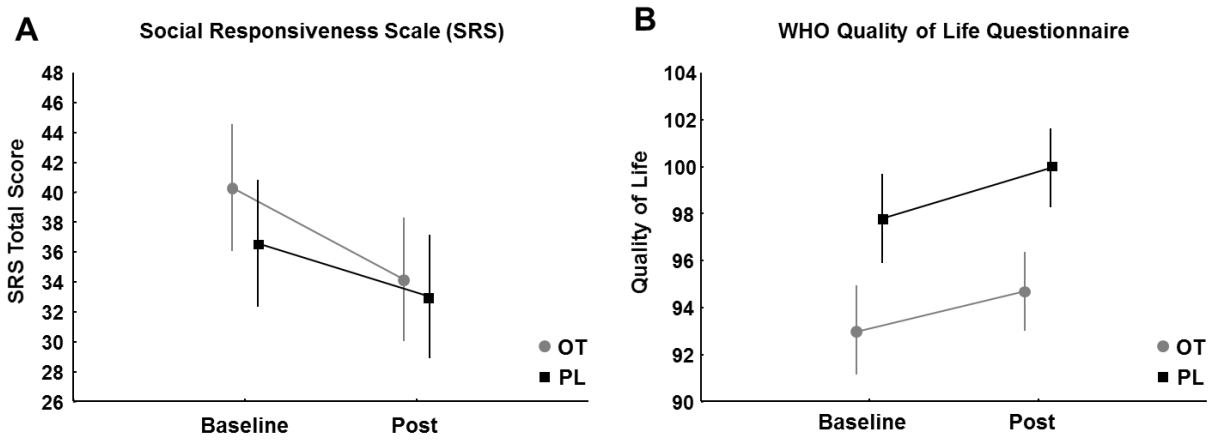


Figure 3.3. Effects of the two-week treatment on social responsiveness and quality of life. Panel (A) visualizes changes in 'social responsiveness' (SRS) for each treatment group (oxytocin (OT) - placebo (PL)) at baseline and after (post) the two-week treatment. Panel (B) visualizes changes in 'quality of life' (WHOQOL).

Quality of Life: World Health Organization Quality of Life Questionnaire (WHOQOL)

As shown in Figure 3B, a main effect of 'treatment' indicated that quality of life scores were overall higher in the PL group, compared to the OT group ($F(1,38) = 4.75$; $p < .05$) (both at baseline and post-treatment). In addition, a significant main effect of 'time' was revealed, indicating that after two weeks of nasal spray administration, both treatment groups reported an improvement in quality of life (main effect of time: $F(1,38) = 3.90$, $p = .05$). No significant 'group x time' interaction was revealed, indicating that the reported improvements were not specific to the OT-treatment ($F(1,38) = .27$, $p = .60$; $\eta^2 = .007$; power = .08). Only in the OT group however, not in the PL group, changes in quality of life were positively associated with changes in reported peer attachment (IPPA) (OT: $r = .50$, $p < .05$) (PL: $r = -.16$; $p = .50$).

4 Discussion

The current study presents results on a double-blind, between-subject, randomized placebo-controlled trial assessing the effects of two weeks of daily OT administration on measures of state and trait attachment. Reductions in attachment avoidance and increases in reports of attachment toward peers were reported after two weeks of OT treatment. OT treatment was also associated with changes in mood, indicating decreases in feelings of tension and anger in the OT group, not in the placebo group. Further, at the end of the two-week trial, both treatment groups (OT, PL) reported to experience an increase in social responsiveness and quality of life, but the effects were only specific to the OT-treatment in terms of reports on 'social motivation'.

The present study found a decrease in the experience of avoidant attachment after two weeks of OT administration as assessed using the State Adult Attachment Measure (SAAM) [23]. No significant changes in reports of secure attachment or anxious attachment were revealed. For a long time, an adult's attachment style was considered a relatively stable disposition, rooted in internal cognitive–affective working models (i.e., mental representations) of self and other, based on previous experiences in close relationships. More recently however, it has been suggested that attachment style can be transiently influenced or shaped by situational factors such as major life events or other contextual factors [32–37]. While notwithstanding the stability of attachment style, the SAAM questionnaire has been validated as a useful measure for capturing temporary fluctuations in the thoughts, feelings, and behaviors underlying attachment processes [23]. As a concept, *attachment anxiety* is characterized to reflect insecurity about one's own worth and abilities, extreme need for interpersonal closeness, love, and support, and worrying about being rejected or abandoned (e.g., *"I feel a strong need to be unconditionally loved right now"*). *Attachment avoidance* on the other hand, is characterized by the reluctance to trust others, an emphasis on autonomy and self-reliance, a relatively low tolerance for interpersonal intimacy and interdependence, and a tendency to down-regulate one's own emotions (e.g., *"If someone tried to get close to me, I would try to keep my distance"*). Both concepts are thought to reflect distinct dimensions of attachment style that are largely unrelated, a notion that is supported by the baseline reports of the current sample (i.e., no inter-correlation was revealed between reports of attachment anxiety or avoidance). Reports of attachment security on the other hand, were shown to be inversely related to attachment avoidance and attachment anxiety, which is in line with the conceptualization that attachment security reflects the relative absence of anxiety and avoidance as well as a sense of faith in the responsiveness of attachment figures, and comfort with intimacy and interdependence (e.g., *"I feel like I have someone to rely on"*).

Our results indicated that two weeks of OT treatment exerted a specific influence on decreasing a person's reluctance towards closeness or trust in others (decrease attachment avoidance), but that it has no specific effect on altering a person's feelings of insecurity about one's own abilities (attachment anxiety) or one's faith in the responsiveness of attachment figures (attachment security). Overall, the finding that OT may specifically influence one's reluctance to engage in closeness or intimacy with others may be interpreted within the framework of the recently proposed affiliative-motivation hypothesis [38] suggesting that OT may specifically act by increasing affiliative strivings and that individuals with a decreased tendency to affiliate (e.g. avoidantly attached individuals) may be most likely to benefit from OT treatment.

In addition to the assessments of changes in state attachment using the SAAM questionnaire, the Inventory of Parent and Peer Attachment (IPPA) questionnaire was administered prior and post OT administration to assess potential changes in trait-related conceptions of attachment towards

friends and parents. Results indicated that two weeks of daily OT administration induced a medium-large enhancement in self-reported attachment towards peers, whereas no significant changes were revealed for reports of parent-oriented attachment (although non-significant tendencies were revealed for reports of attachment towards 'mother'). Note however that the observation of more pronounced effects on peer attachment, compared to parent attachment may reflect a particularity of the included sample which consisted primarily of campus-based university students with limited or no parent-contact during the two-week trial.

We explored whether changes in reports of attachment were potentially related to basic changes in mood, by additionally assessing the Profile of Mood States questionnaire (POMS) at baseline and after the two-week treatment. While previous studies on the single-dose effects of OT often report no change in mood state [18,19,22,39], the current results indicate that after two weeks of treatment, feelings of tension and (tentatively) anger were significantly reduced in the OT group, not in the PL group. At least for reports on the IPPA of peer attachment, it appears that inter-individual differences in (baseline) attachment style may play a pivotal role in determining treatment response. The observation that normal variance at baseline can modulate the effects of OT is in line with results from previous studies exploring the effects of a single dose of OT. Particularly, De Dreu (2012) showed that OT administration significantly improved cooperation behavior, but only in individuals high on attachment avoidance [20]. Similarly, a more recent study showed that while OT produced a slight increase in communion for the average participant, avoidantly attached individuals were especially likely to perceive themselves as more communal ("kind," "warm," "gentle," etc.) after receiving OT [21]. Also in terms of social-cognitive competences, Bartz et al. (2010) showed that OT was able to improve empathic accuracy on an emotion recognition task, but only for less-socially proficient individuals [40]. While these studies and our study provide indications that OT may be particularly effective in specifically ameliorating domains that are affected in the treated individual, other lines of work provide indications that a more complex relationship might exist between variance in personality aspects and differentiated responses to OT treatment. For example, another study by Bartz et al. (2011) showed that individuals with low scores on attachment anxiety reported more positive childhood memories after OT administration (compared to placebo), while in individuals with high scores on attachment anxiety the inverse effect was found, indicating more negative recollections on the caring behavior of their mothers after OT administration [8]. Also positive effects of OT on one's willingness to donate money to charity [41] or prosocial behavior in a virtual ball-tossing game [42] were shown to be lowered or absent in individuals with high reports of parental love-withdrawal. Together, these observations further highlight the importance of adequately characterizing inter-individual differences in attachment style and/or other personality traits for evaluating expectancies on OT treatment outcome.

Finally, secondary outcome measures were included to assess the effects of OT treatment on social responsiveness and quality of life using the social responsiveness scale (SRS) and the World Health Organization Quality of Life questionnaire (WHOQOL), respectively. Interestingly, participants of both treatment groups (OT and PL) reported an improvement in quality of life after two weeks of nasal spray administration which may provide relevant insights in terms of the tolerability and safety of multiple-dose OT treatment by showing no adverse events on overall wellbeing. Further, both treatment groups also reported improvements in social responsiveness (SRS) although effects were tentatively more pronounced for the OT group, compared to the placebo group. Interestingly a specific significant effect of OT was revealed for the SRS subscale assessing 'social motivation', providing further support to the notion that OT may primarily exert its effects by increasing affiliative strivings and social motivation as postulated by the affiliative-motivation hypothesis [38].

To conclude, a two-week treatment with intranasally administered OT was shown to induce improvements in reports of attachment avoidance and secure peer attachment, particularly in individuals with avoidant or insecure attachment styles. The two-week treatment was well-tolerated by all participants with no reports of adverse events or impact on overall well-being. Instead, OT-specific effects were revealed for improving negative mood states and also unspecific increases in social responsiveness and quality of life were reported after participation in the two-week trial. Although overall, these results on multiple-dose OT treatment are promising, we note that the included sample was somewhat small and restricted to males. Future work is therefore needed to be conclusive on the generalizability of the reported effects.

Supplements

Supplementary Table 3.1. Side effects questionnaire

Please fill in the boxes (crosses) if the described side effect applies to you.							
Symptom	Y/N	Start of the reaction		Duration of the reaction (hours)	Severity of the reaction		
		Right after Administration	Within 2 hours		Mild	Moderately	Serious
Headache							
Drowsiness							
Dizziness							
Fainting							
Changes in heart rate or palpitations							
Shortness of breath							
Fever							
Sore throat							
Dry throat/dry mouth							
Hoarseness							
Coughing							
Coughing up mucus							
Congested nose							
Sneezing							
Nasal irritation							
Runny nose							
Burning sensation in nose and/or ears							
Sensitive to fragrances							
Watery eyes							
Nausea and/or vomiting							
Abdominal or stomach pain							
Decreased appetite							
Hungry							
Constipation							
Diarrhea							
Muscle pain/cramps							
Skin rash							
Increased fluid intake							
Water retention/bloating							
Insomnia/sleep difficult							
Nightmares							
Staring/daydreams							
Anaphylaxis							
Changes in perception of the tongue							
Back pain							

Bed wetting							
Weight gain							
Sweating							
Blurred vision							
Less talk to others							
Uninterested in others							
Persistent thoughts and/or feelings							
Development of repetitive behavior							
Increase in repetitive behavior							
Nail biting							
Annoyed, bored							
Sad							
Prone to crying, more emotional							
Anxious, worried, discomfort							
Happy, satisfied							
Euphoric, unusually happy, more energetic							
Calm, relaxed, comfortable							
More focused							
More confidence							
Did you experience other side effects that are not mentioned in the table above?							

Supplementary Table 3.2.

Supplementary Table 3.2 shows the frequency of each reported side effect during the two-week oxytocin trial. Frequencies are reported for each group (Placebo (PL) and Oxytocin (OT)) and for each measure of severity (Mild, Moderate and Severe).

Side effect	Severity					
	Mild		Moderate		Severe	
	PL	OT	PL	OT	PL	OT
More focused			2	2		
More confident			1	1	1	
More competitive		1				
Calm, relaxed	1					
Sweaty		1	1			
Itchy nose	1					

Supplementary Table 3.3. Questionnaire results.

Questionnaire scores (total and subscale scores) are displayed separately for each treatment group (oxytocin, placebo) and session (baseline, post) (with outliers).

Outcome Measure	Baseline	Post	Baseline	Post
	Oxytocin		Placebo	
Self-report Questionnaires	n = 20		n = 20	
Trait Attachment - IPPA				
Friends	35.90 ± 6.31	37.65 ± 5.25	36.05 ± 6.15	35.25 ± 6.50
Mother	36.95 ± 7.03	37.60 ± 6.55	37.60 ± 7.28	37.40 ± 7.42
Father	31.63 ± 6.80	32.58 ± 6.76	34.95 ± 7.36	34.60 ± 6.41
Important person	41.55 ± 3.70	41.45 ± 3.93	41.13 ± 6.69	41.31 ± 7.02
State Attachment - SAAM				
Security	5.66 ± 0.69	5.74 ± 0.68	5.79 ± 0.82	5.96 ± 0.70
Anxiety	3.96 ± 0.99	3.79 ± 1.06	3.83 ± 0.86	3.65 ± 1.11
Avoidance	2.31 ± 0.93	1.98 ± 0.65	2.42 ± 1.30	2.51 ± 0.95
Mood - POMS				
Tension	2.80 ± 2.14	1.30 ± 1.72	2.15 ± 2.70	2.25 ± 3.14
Depression	1.15 ± 2.03	0.80 ± 1.64	1.40 ± 2.70	2.00 ± 3.48
Anger	1.80 ± 3.09	0.70 ± 2.41	1.40 ± 2.30	1.60 ± 2.41
Vigor	11.90 ± 2.47	11.35 ± 3.34	11.40 ± 3.52	11.40 ± 3.57
Fatigue	3.60 ± 3.10	2.75 ± 2.53	4.60 ± 4.17	3.15 ± 3.00
Social responsiveness – SRS-Adult				
Social Awareness	10.40 ± 4.16	8.95 ± 4.73	10.05 ± 5.22	9.75 ± 4.55
Social Communication	13.65 ± 7.60	12.45 ± 7.38	11.45 ± 6.71	10.55 ± 7.52
Social Motivation	9.30 ± 3.59	7.25 ± 3.45	7.45 ± 5.76	6.80 ± 5.29
Rigidity and Repetitive behavior	6.95 ± 3.49	5.50 ± 3.90	7.60 ± 4.99	5.90 ± 4.72
Total	40.30 ± 16.81	34.15 ± 17.44	36.55 ± 20.93	33.00 ± 19.40
Quality of life - WHOQOL-Bref				
Total	93.05 ± 8.04	94.30 ± 6.60	97.80 ± 8.92	99.95 ± 8.33

Note. SAAM = State Adult Attachment Scale; IPPA = Inventory of Parent and Peer Attachment; POMS = Profile of Mood States; SRS-Adult = Social Responsiveness Scale, adult version; WHOQOL-bref = World Health Organization Quality of Life, abbreviated version.

Supplementary Table 3.4.

To explore whether inter-individual differences in baseline scores were related to treatment-related effects on post-treatment IPPA 'peer attachment' or SAAM 'attachment avoidance', a step-wise multiple regression analysis was conducted covarying for baseline scores and testing baseline-by-treatment interactions. Model A explores the effect of 'treatment' (OT, PL) (independent variable) on post-treatment scores (dependent variable) without covarying for variance in baseline scores. Model B explores the effect of 'treatment' on post-treatment scores with correction for variance in baseline scores. Model C additionally includes the 'treatment group x baseline score' interaction term to specifically explore whether the effect of treatment is modulated by variance in baseline scores.

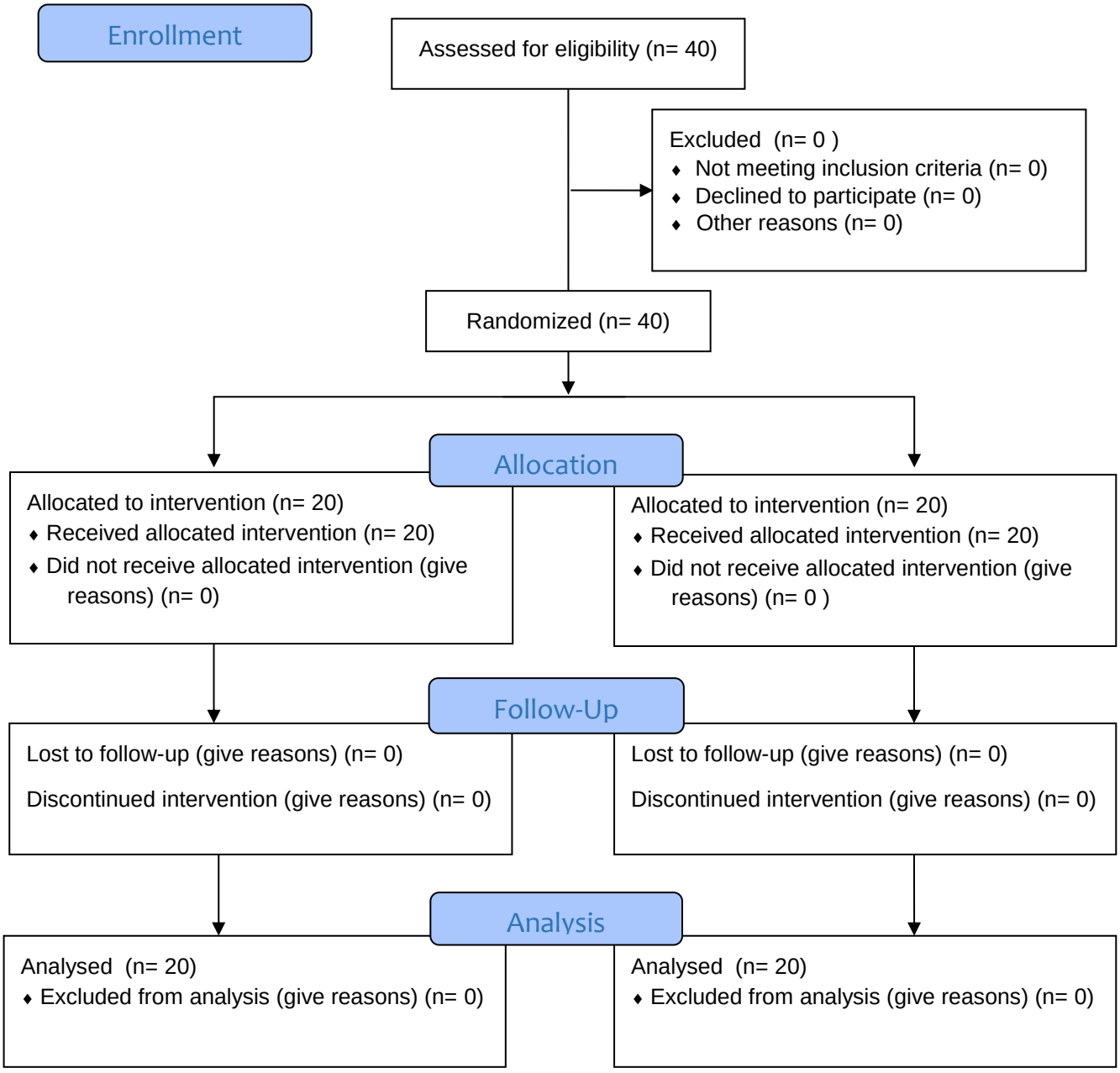
	Model A			**Model B**			**Model C**		
Covariates	**β**	**t**	**p**	**β**	**t**	**p**	**β**	**t**	**p**
Peer attachment (IPPA)									
Treatment group (OT, PL)	.17	1.05	.30	.15	3.01	< .01	.78	2.67	< .05
Baseline peer attachment				.94	18.35	< .001	.94	19.37	< .001
Treatment x Baseline peer attachment							-.64	-2.17	< .05
Attachment avoidance (SAAM)									
Treatment group (OT, PL)	-.32	-2.07	< .05	-.28	-3.21	< .01	-.21	-.98	0.34
Baseline attachment avoidance				.78	8.95	< .001	.77	8.26	< .001
Treatment x Baseline attachment avoidance							-.08	-.35	.73

Note: IPPA = Inventory of Parent and Peer Attachment, SAAM = State Adult Attachment Measure

Supplementary Figure 3.1. CONSORT Flow Diagram

CONSORT
TRANSPARENT REPORTING of TRIALS

CONSORT 2010 Flow Diagram



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Behavioral effects of multiple-dose oxytocin treatment in autism: A randomized, placebo-controlled trial with long-term follow-up

Chapter IV

Under review at *Molecular Autism*

Bernaerts S., Boets B., Bosmans G., Steyaert J., Alaerts K. Behavioral effects of multiple-dose oxytocin treatment in autism: A randomized, placebo-controlled trial with long-term follow-up.

Abstract

Intranasal administration of the 'prosocial' neuropeptide oxytocin (OT) is increasingly explored as a potential treatment for targeting the core characteristics of autism spectrum disorder (ASD). However, long-term, follow-up studies, evaluating the possibility of long-lasting retention effects are currently lacking. Using a double-blind, randomized, placebo-controlled, parallel design, we explored the possibility of long-lasting behavioral effects of four weeks of intranasal OT treatment (24 International Units once daily in the morning) in 40 adult men with ASD. To do so, self-report and informant-based questionnaires assessing core autism symptoms and characterizations of attachment were administered at baseline, immediately after four weeks of treatment (approximately 24 hours after the last nasal spray administration), and at two follow-up sessions, four weeks and one year post-treatment. No treatment-specific improvements were identified in the primary outcome assessing social responsiveness (Social Responsiveness Scale, self-report ($p = .37$) and informant-based ($p = .19$)). Medium- to large-sized effects were identified in two secondary outcomes, indicating treatment-induced improvements in self-reported repetitive behaviors (Repetitive Behavior Scale - Revised) ($p = .04$) and reduced feelings of avoidance towards others (State Adult Attachment Measure) ($p = .03$), which outlasted the period of actual administration until one month and even one year post-treatment. Screenings for changes in mood (Profile of Mood States) also revealed enhanced reports of vigor (more energetic, active, lively) both during and after the OT treatment (evident up to one year post-treatment) ($p = .03$).
The identification of long-term beneficial effects on repetitive behaviors, perceived attachment avoidance and vigor suggests that there is therapeutic potential of multiple-dose OT treatment in adult men with ASD. However, given that this is a pilot study, larger studies are warranted to evaluate the long-term effects of OT treatment further.
The trial was registered with the European Clinical Trial Registry (Eudract 2014-000586-45) on January 22, 2014 (https://www.clinicaltrialsregister.eu/ctr-search/trial/2014-000586-45/BE).

1 Introduction

Autism Spectrum Disorder (ASD) is characterized by lifelong impairments in social and communicative functioning, and the presence of stereotyped behaviors and interests [1]. In the past decade, intranasal administration of the neuropeptide oxytocin (OT) has increasingly been explored as a potential pharmacological treatment for targeting the core characteristics of ASD. Endogenous OT is synthesized in the hypothalamus where neurons of the paraventricular nuclei project to various areas of the central nervous system involved in complex (social) behaviors (e.g. amygdala). In typically developing individuals, OT has been linked to interpersonal bonding, parental care, and the ability to establish trust and form social attachments [2,3].

With respect to ASD, initial clinical trials have demonstrated that a single dose of OT can induce behavioral enhancements on tasks assessing repetitive behavior [4], affective speech comprehension (emotional intonations) [5], facial emotion recognition [6] and social decision making (cyberball computer game) [7]. Multiple-dose trials assessing the effects of OT treatment in adults with ASD have also shown beneficial effects. Anagnostou et al. [8] assessed the safety and efficacy of six weeks of intranasal OT treatment on core autism symptom domains (social cognition/function and repetitive behaviors) in 19 adults with ASD (16 men, 3 women), and showed improved emotion recognition, quality of life and tentative improvements in repetitive behaviors after OT treatment. In another study with 20 adult men with ASD, Watanabe et al. [9] studied the effects of six weeks of intranasal OT administration on core autism characteristics and showed significant improvements in social reciprocity and social functioning (social-judgement task). In a more recent large-scale trial, Yamasue et al. [10] also assessed the effects of six weeks of intranasal OT treatment on core autism symptom domains in 106 adult men with ASD and showed significant improvements in repetitive behaviors.

However, a more mixed pattern of results emerged from studies assessing the effects of multiple-dose OT treatment in children with ASD. For example, Dadds et al. [11] assessed behavioral effects of a four-day intranasal OT treatment in 38 boys with ASD (7 to 16 years old) during parent-child interaction training, but found no OT-specific improvements in repetitive behaviors or social responsiveness. Later, also Guastella et al. [12] failed to show beneficial effects after an eight-week OT treatment on core ASD characteristics in slightly older boys (12 to 18 years old). On the other hand, a more recent trial assessing the effects of five weeks of intranasal OT treatment on core ASD characteristics in 31 children with ASD (27 boys, 4 girls) was able to demonstrate improved social responsiveness as reported by the parents [13]. Similarly, Parker et al. [14] investigated whether a four-week intranasal OT treatment could improve core autism characteristics in 32 children (6-12 years old) with ASD and showed an increase in parent-reported social responsiveness. Taken together, findings of these initial multiple-dose trials provided indications for a more consistent

(positive) pattern of results for OT trials with adults with ASD [8–10], compared to trials with children with ASD [11–14].

To date, however, potential long-term effects of OT treatment that outlast the period of actual administration have not yet been addressed in adults with ASD. These assessments, however, would be of high relevance since repeated administrations over an extended period might induce long-lasting, experience-dependent adaptations within neural circuits. With respect to ASD, it has been postulated that early-life impairments in social attention/orienting may deprive patients of adequate social learning experiences that normally drive the typical development of social brain networks [15]. Considering that OT is hypothesized to increase the saliency of social cues [16] OT therapy might induce an enrichment of social experiences that stimulates long-term adaptations in social behaviors.

In the current study, we assessed - for the first time - the possibility of long-term retention effects of four weeks of intranasal OT administration on core autism symptom domains (including social responsiveness and restricted and repetitive behaviors), attachment characteristics and general aspects of quality of life in adult men with ASD. While prior multiple-dose trials mainly explored the effects of treatment on these core autism symptom domains, a recent study from our lab [17], showed that two weeks of OT treatment in typically developing young-adult men reduced self-reported feelings of attachment avoidance and increased self-reported feelings of secure attachment toward peers. Against this background and given the growing body of research demonstrating an important role of the oxytocinergic system in interpersonal trust and attachment [18–20], the current study also included an initial assessment of attachment measures to evaluate the long-term effects of OT treatment in adults with ASD. With respect to ASD, Rutgers et al. [21] suggested that most children with ASD (53%) are able to form a secure attachment to caregivers, but that they are significantly less likely to do so compared to typically developing children. In addition, a recent meta-analysis concluded that more severe autism characteristics are associated with less secure attachment in children with ASD [22].

The effects of treatment on core autism characteristics and attachment were assessed immediately after the four-week treatment period, and at follow-up sessions, four weeks and one year post-treatment, to assess the possibility of retention effects that outlast the period of actual administration. In line with prior multiple-dose studies [8,9,17], we hypothesized that multiple doses of OT would improve self-report and informant-based ratings of social responsiveness and repetitive behaviors, and ameliorate self-ratings of attachment characteristics and quality of life. A key question was to evaluate whether any beneficial effects of multiple-dose OT treatment would outlast the period of actual administration until one month (four weeks) and/or one year post-treatment.

2 Materials and Methods

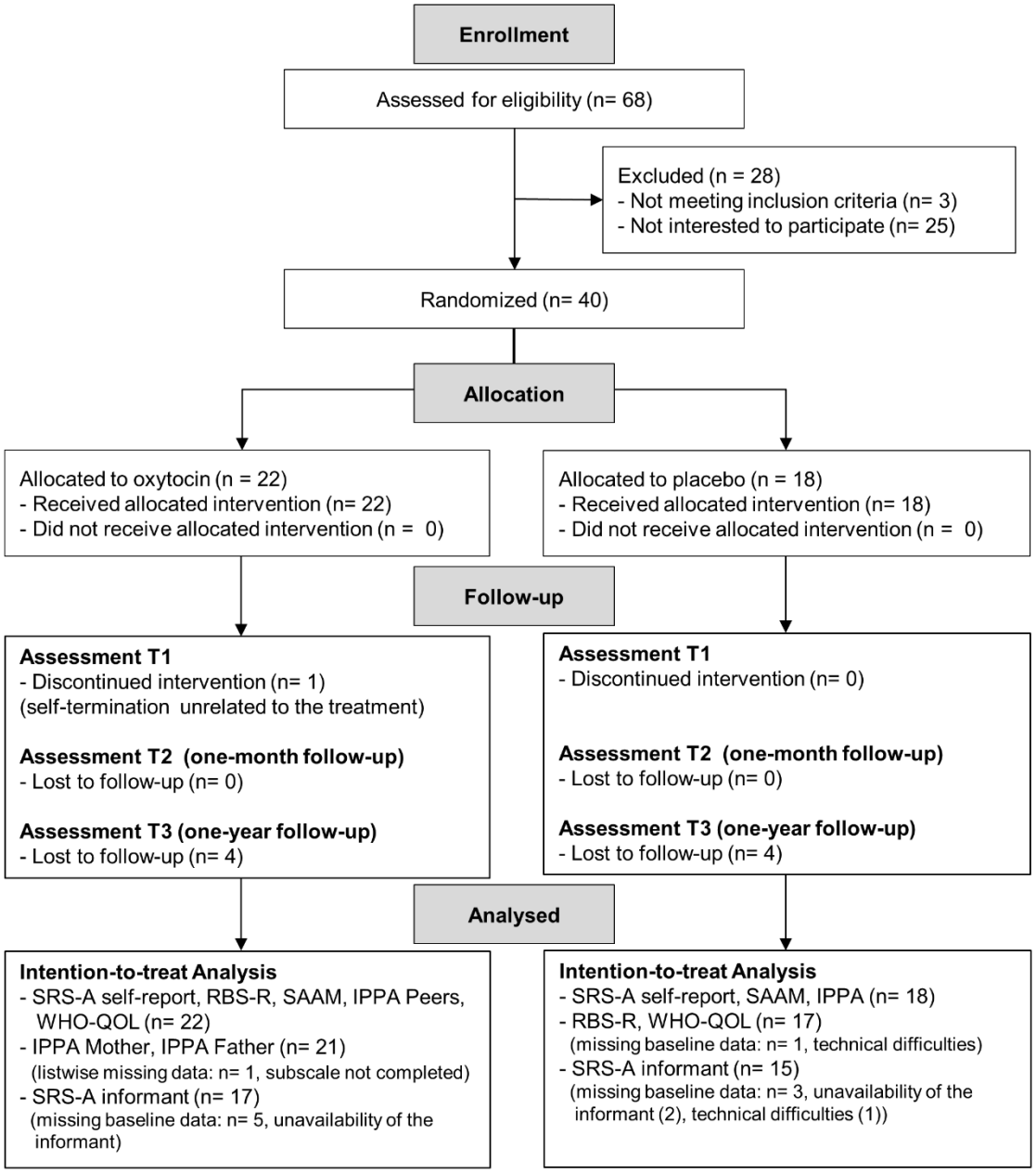


Figure 4.1. CONSORT flow diagram. Data were analyzed using an intention-to-treat format with last-observations-carried-forward to replace missing data. For participants with missing baseline data, analysis for that measure was excluded listwise. SRS-A: Social Responsiveness Scale adult version, RBS-R: Repetitive Behavior Scale Revised, SAAM: State Adult Attachment Measure, IPPA: Inventory of Parent and Peer Attachment, WHO-QOL: World Health Organization Quality of Life.

2.1 General study design

This two-arm, double-blind, randomized, placebo-controlled parallel study was performed at the Leuven University Hospital (Leuven, Belgium) to assess multiple-dose effects of intranasal

oxytocin (OT) administration on core autism characteristics and experience of attachment in male adults with ASD. A specific aim of the trial was to assess whether any treatment-induced effects would outlast the period of actual administration. To do so, changes-from-baseline (T0) in self-report and informant-based questionnaire scores were assessed immediately after four weeks of continual OT treatment (T1), and at two follow-up sessions, four weeks (T2) and one year post-treatment (T3) (See Figure 4.1, CONSORT Flow diagram for number of participants included in each assessment session). Written informed consent was obtained from all participants prior to the study. Consent forms and study design were approved by the local Ethics Committee for Biomedical Research at the University of Leuven, KU Leuven (S56327) in accordance to The Code of Ethics of the World Medical Association (Declaration of Helsinki). The trial was registered with the European Clinical Trial Registry (Eudract 2014-000586-45) and the Belgian Federal Agency for Medicines and Health products. Note that the data presented in the current report are part of a larger clinical trial in which neural measures (i.e. task-based and resting-state magnetic resonance imaging (MRI)) were assessed in addition to the questionnaire data presented below. These MRI modalities are however not part of the current report and will be reported elsewhere (manuscripts in preparation).

2.2 Participants

Forty high-functioning adult men with a formal diagnosis of ASD were recruited between April 2015 and December 2016 from the Autism Expertise Centre at the Leuven University Hospital. The diagnosis was established by a multidisciplinary team (child psychiatrist and/or expert neuropediatrician, psychologist, speech/language pathologist and/or physiotherapist) based on the strict criteria of the DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders[1]. Prior to the intervention, the Autism Diagnostic Observation Schedule (ADOS or ADOS-2) [23,24] and estimates of intelligence (6-subtest short version of the Wechsler Adult Intelligence Scale-IV Dutch version: Block design, Digit span, Similarities, Vocabulary, Symbol search and Visual puzzles [25] were acquired from all participants (Table 4.1). Inclusion criteria comprised a clinical diagnosis of autism spectrum disorder; gender (male); and age (18-35 years old). Exclusion criteria for participation comprised any neurological disorder (e.g., stroke, epilepsy, concussion); demonstrated genetic disorder; or any contraindication for MRI (note that the MRI data analyses are not part of the current report). Current psychoactive medication use and the presence of comorbid psychiatric disorders were screened (Supplementary Table 4.1). Forty participants were randomly allocated to either the OT (n=22) or the PL group (n=18). Data from two participants (1 OT, 1 placebo (PL)) were not included in the final analyses due to discontinued intervention (self-termination unrelated to the treatment) and missing data caused by technical

difficulties, respectively. For assessment sessions T1 and T2, final analyses were therefore performed on 21 OT and 17 PL participants (see Figure 4.1, CONSORT Flow diagram). Additionally, 4 OT and 4 PL participants were lost to follow-up one-year post-treatment (T3), such that final analyses of T3 were performed on 18 OT and 14 PL participants. The initial sample size (n=40) was set to be comparable to three prior studies showing significant effects of multiple-dose OT treatment on similar outcome measures [8,9,17].

Table 4.1. Participant characteristics

	Oxytocin	Placebo	T-value	p-value
Number of participants	22	18		
Age	25.00 ± 4.86	24.00 ± 5.55	0.62	0.54
IQ				
Total IQ	102.27 ± 12.45	104.61 ± 21.59	-0.43	0.67
VIQ	105.57 ± 9.27	108.72 ± 16.83	-0.74	0.47
PIQ	104.76 ± 18.35	102.39 ± 22.90	0.36	0.72
ADOS(2)				
Total	7.18 ± 4.22	8.06 ± 4.26	-0.65	0.52
Communication	2.05 ± 1.40	2.39 ± 1.54	-0.74	0.46
Social interaction	4.82 ± 3.50	5.67 ± 3.33	-0.78	0.44
Use of psychostimulant medication	6	2		
Comorbidity	8	2		

All data are shown as mean ± standard deviation. IQ = Intelligence Quotient, VIQ = Verbal IQ, PIQ = Performance IQ, ADOS = Autism Diagnostic Observation Schedule. Detailed information on medication use and comorbidities is provided in Supplementary Table 4.1. Note that for one participant of the OT group only total IQ information was available, but not VIQ or PIQ, so that the mean (± standard deviation) information of the VIQ and PIQ of the OT group are based on data from 21 participants.

2.3 Intervention

Participants were assigned to receive the OT or placebo (PL) treatment based on a computer-generated randomized order. Except for the manager of randomization, all research staff conducting the trial, participants and their parents and/or partners were blinded to treatment allocation. OT (Syntocinon®, Sigma-tau) and PL (saline natrium-chloride solution) were administered in amber 15 ml glass bottles with metered pump (ACA Pharma). Each puff per nostril contained 4 international units (IU) of OT. Participants self-administered a daily dose of 24 IU (3 puffs/nostril) over four consecutive weeks (28 doses in total). This dose is in accordance

with prior OT administration studies in neurotypical (young) adults [26,27] and adults and adolescents with ASD [6,28,29]. At this dosage, no side effects or contraindications of OT have been described [29]. All participants received clear instructions about the use of the nasal spray [17,30] and were monitored onsite until approximately two hours after first nasal spray administration. During the course of the treatment, participants were asked to administer the nasal spray in the morning, to keep a daily record of the time point of nasal spray administration, and whether or not they were alone or in company of others the first two hours after administration. Percentage of days at which the spray was administered in the presence of others was not significantly different between treatment groups (OT: 36.8 % (SD 29.9); PL: 36.0 % (SD 25.1); t(37)= .09; p= .93). Participants administered the nasal spray (OT or PL) daily during four consecutive weeks and at the end of each week participants were screened for potential adverse events, side effects or changes in mood. As listed in detail in Supplementary Table 4.2 and Supplementary Figure 4.1, only minimal, non-treatment specific side effects or changes in mood states were reported. Finally, at the end of the trial, participants were asked if they thought they had received OT or PL. The majority of participants thought they had received the PL treatment (78.95%). The proportion of participants that believed they had received the OT treatment was not significantly larger in the actual OT group (28.57%), compared to the PL group (11.76%) (p=.21). Nonetheless, secondary analyses were performed to explore whether treatment effects were modulated by the participants' own belief about the received treatment (Supplementary Results).

2.4 Outcome Measures

Self-report and informant-based questionnaires were assessed at baseline (T0), immediately after four consecutive weeks of nasal spray administration (approximately 24 hours after the last nasal spray administration) (T1), and at follow-up sessions, four weeks (T2) and one year post-treatment (T3).

The Social Responsiveness Scale (for adults) (SRS-A) total score was used as the primary outcome measure (self-report and informant-based versions). The other behavioral questionnaires were considered secondary: Repetitive Behavior Scale - Revised (RBS-R); State Adult Attachment Measure (SAAM); Inventory of Parent and Peer Attachment (IPPA); Quality of Life questionnaire of the World Health Organization (WHO-QOL) (all self-report). More detailed information on each of the adopted questionnaires is provided in Supplementary Methods.

In short, the SRS-A (64 items) [31] comprises four subscales examining social communication, social awareness, social motivation and rigidity/repetitiveness, using a four-point Likert-scale. SRS-A raw total scores were adopted.

The RBS-R (43 items) [32] examines a heterogeneous set of repetitive behaviors including stereotypic behavior, self-injurious behavior, compulsive behavior, ritualistic behavior, sameness behavior and restricted interests behavior, using a four-point Likert-scale. RBS-R raw total scores were adopted.

The SAAM [33] comprises three subscales examining attachment security (e.g., "I feel like I have someone to rely on") (7 items); attachment anxiety (e.g., "I feel a strong need to be unconditionally loved right now") (7 items); and attachment avoidance (e.g., "If someone tried to get close to me, I would try to keep my distance") (7 items) using a seven-point Likert-scale. SAAM raw subscale scores were adopted.

The IPPA [34] examines trait attachment to (i) mother (12 items); (ii) father (12 items); (iii) peers (12 items), using a four-point Likert-scale. The IPPA assesses attachment security along three dimensions: degree of mutual trust, quality of communication and extent of anger and alienation. IPPA raw subscale scores were adopted.

Finally, the abbreviated version of the WHO-QOL [35] assesses general quality of life related to physical health, psychological health, social relationships, and environment using a five-point Likert scale. WHO-QOL raw total scores were adopted.

2.5 Data Analysis

For each questionnaire, baseline differences between groups were assessed using two-sample t-tests. Pre-to-post difference scores were calculated for each assessment session (T1, T2, T3) (see Table 4.2) and difference scores were subjected to a linear mixed-effects model (one-tailed) with the random factor 'subject', and the fixed factors 'treatment' (OT, PL), 'session' (T1, T2, T3) and 'treatment x session' interaction, with an intention-to-treat format and last-observations-carried-forward to replace missing data. For participants with missing baseline data, analysis for that measure was excluded listwise (Figure 4.1 CONSORT Flow diagram).

For each questionnaire and session, Cohen's d effect sizes are reported in Table 4.2 (change from baseline_{OT}−change from baseline_{PL})/pooled standard deviation), where 0.2 is indicative of a small effect, 0.5 a medium effect and 0.8 a large effect (36). Additionally, single-sample t-tests are reported in Supplementary Table 4.3, assessing within-group changes from baseline, separately for the OT and PL group. All statistics were executed with Statistica 8 (StatSoft. Inc. Tulsa, USA). Given that this is a pilot study evaluating long-term retention effects of OT treatment, the significance level was set at $p < .05$ for all analyses, without correction for multiple comparisons.

Table 4.2. Mean pre-to-post change scores for each treatment group (oxytocin, placebo) and session (T1, T2, T3), and the corresponding Cohen's d effect sizes.

Outcome measure	Oxytocin		Placebo		Group difference effect size
	N	Mean ± SD	N	Mean ± SD	Cohen's d
Multiple-dose effect (T1)					
SRS-A self-report	22	-5.55 ± 11.40	18	-1.06 ± 10.01	-0.42
SRS-A informant-based	17	0.0 ± 15.86	15	-0.87 ± 12.83	0.10
RBS-R	22	-4.77 ± 6.47	17	-1.76 ± 4.75	**-0.63**
SAAM avoidance	22	-0.40 ± 0.71	18	0.06 ± 0.98	**-0.61**
SAAM security	22	0.27 ± 0.77	18	-0.05 ± 0.66	**0.63**
SAAM anxiety	22	-0.14 ± 0.75	18	0.28 ± 0.95	**-0.62**
IPPA Peers	22	1.45 ± 3.85	18	0.56 ± 4.05	0.30
IPPA Mother	21	-0.52 ± 2.71	18	0.44 ± 3.45	-0.39
IPPA Father	21	0.43 ± 3.30	18	-0.61 ± 3.81	0.29
WHO-QOL	22	1.77 ± 8.04	17	-1.35 ± 6.74	0.63
One-month Retention effect (T2)					
SRS-A self-report	22	-5.64 ± 12.57	18	-7.67 ± 12.09	0.22
SRS-A informant-based	17	-9.59 ± 10.98	15	-1.20 ± 10.73	**-0.83**
RBS-R	22	-4.91 ± 6.33	17	-2.35 ± 3.43	**-0.50**
SAAM avoidance	22	-0.38 ± 0.70	18	-0.06 ± 0.76	**-0.53**
SAAM security	22	0.04 ± 1.01	18	-0.40 ± 0.99	**0.62**
SAAM anxiety	22	0.08 ± 1.05	18	0.11 ± 0.87	-0.05
IPPA Peers	22	1.32 ± 3.71	18	0.06 ± 3.70	0.45
IPPA Mother	21	-0.38 ± 3.43	18	0.06 ± 4.35	-0.14
IPPA Father	21	0.52 ± 3.59	18	-0.33 ± 3.87	0.31
WHO-QOL	22	1.14 ± 5.48	17	0.35 ± 4.53	0.24
One-year Retention effect (T3)					
SRS-A self-report	22	-8.59 ± 20.95	18	-6.72 ± 21.01	-0.12
SRS-A informant-based	17	-7.41 ± 19.26	15	-4.13 ± 24.64	-0.18
RBS-R	22	-4.91 ± 9.46	17	-0.41 ± 4.27	**-0.98**
SAAM avoidance	22	-0.52 ± 1.18	18	0.0 ± 0.75	**-0.80**
SAAM security	22	0.20 ± 1.52	18	-0.14 ± 0.66	-0.05
SAAM anxiety	22	0.17 ± 0.94	18	0.11 ± 1.21	0.06
IPPA Peers	22	0.68 ± 6.26	18	1.28 ± 4.17	-0.20
IPPA Mother	21	0.33 ± 3.91	18	1.50 ± 5.44	-0.30
IPPA Father	21	0.57 ± 4.08	18	-0.50 ± 4.53	0.33
WHO-QOL	22	1.14 ± 8.37	17	0.29 ± 4.21	0.27

SRS-A = Social Responsiveness Scale adult version, RBS-R = Repetitive Behavior Scale – Revised, SAAM = State Adult Attachment Measure, IPPA = Inventory of Parent and Peer Attachment, WHO-QOL = World Health Organization Quality of Life questionnaire. For SRS-A, RBS-R and SAAM avoidance and anxiety, lower (or negative) scores indicate improvement. Cohen's d effect sizes (change from $baseline_{OT}$–change from $baseline_{PL}$)/pooled SD) are reported where 0.2 is indicative of a small effect, 0.5 a medium effect and 0.8 a large effect [36]. Data printed in bold show Cohen's d effect sizes equal to or larger than .50 (medium-sized effect) [36].

3 Results

3.1 Effects of OT treatment

No significant baseline differences between treatment groups were revealed for any of the questionnaires (see Supplementary Table 4.4) nor participant characteristics (see Table 4.1).

Social Responsiveness Scale (SRS-A)

Self-report SRS-A. No significant main effect of treatment ($F(1,76)= .12$, $p= .37$, $\eta^2= .00$), nor a treatment x session interaction effect was revealed ($F(2,76)= 1.17$, $p= .16$, $\eta^2= .03$), indicating that pre-to-post changes in self-reported social responsiveness were not significantly larger in the OT compared to the PL group (see Table 4.2 and Figure 4.2 for the effect sizes separately for each session). Also no main effect of session was revealed ($F(2,76)= 2.07$, $p= .07$, $\eta^2= .05$).

Informant-based SRS-A. Also for informant-based SRS-A scores, no significant main effect of treatment ($F(1, 60)= .78$, $p= .19$, $\eta^2= .03$), nor a treatment x session interaction effect ($F(2, 60)= .83$, $p= .22$, $\eta^2= .03$) was evident. Note however that a large-sized effect was identified at session T2 ($d= -.83$) indicating a larger pre-to-post improvement in informant-reported social responsiveness in the OT compared to the PL group at the one-month follow-up session (see **Table 2** and **Figure 2** for the effect sizes separately for each session). No main effect of session was revealed ($F(2,60)= 1.49$, $p= .12$, $\eta^2= .05$).

Repetitive Behavior Scale – Revised (RBS-R)

In terms of repetitive behaviors, a significant main effect of treatment was revealed ($F(1, 74)= 3.20$, $p= .04$, $\eta^2= .08$), indicating that across assessment sessions, pre-to-post improvements in repetitive behaviors were significantly larger in the OT compared to the PL group (see Table 4.2 and Figure 4.2 for the effect sizes separately for each session). No significant treatment x session interaction ($F(2, 74)= 1.04$, $p= .18$, $\eta^2= .03$), nor a main effect of session was evident ($F(2, 74)= .74$, $p= .24$, $\eta^2= .02$).

State adult attachment measure (SAAM)

Attachment Avoidance. In terms of attachment avoidance, a significant main effect of treatment was revealed ($F(1,76)= 3.70$, $p= .03$, $\eta^2= .09$), indicating that across assessment sessions, pre-to-post improvements in attachment avoidance were significantly larger in the OT compared to PL group (see Table 4.2 and Figure 4.2 for the effect sizes separately for each session). No treatment

x session interaction effect (F(2,76)= .27, p= .38, ŋ²= .01), nor a main effect of session was evident (F(2, 76)= .23, p= .40, ŋ²= .01).

Attachment Security. In terms of attachment security, no main effect of treatment F(1,76)= .88, p= .18, ŋ²= .02), nor a treatment x session interaction effect (F(2,76)= 1.08, p= .17, ŋ²= .03) was revealed. Note that medium-sized effects was identified at session T1 (d= .63) and T2 (d= .62) indicating a larger pre-to-post improvement in attachment security in the OT compared to the PL group immediately after the treatment (T1) and at the one-month follow-up session (T2) (see Table 4.2 and Figure 4.2 for the effect sizes separately for each session). No significant main effect of session was evident (F(2, 76)= 1.88, p= .08, ŋ²= .05).

Attachment Anxiety. In terms of attachment anxiety, no main effect of treatment (F(1,76)= .25, p= .31, ŋ²= .01), nor a treatment x session interaction effect (F(2,76)= 1.566, p= .10, ŋ²= .04) was revealed. Note that a medium-sized effect was evident at session T1 (d= .62) indicating a larger pre-to-post improvement in attachment anxiety in the OT compared to the PL group immediately after the treatment (T1) (see Table 4.2 and Figure 4.2 for the effect sizes separately for each session). No main effect of session was evident (F(2, 76)= .23, p= .40, ŋ²= .01).

Inventory of Parent and Peer Attachment (IPPA)

Pre-to-post changes in self-reported secure attachment towards peers and parents were not significantly larger in the OT compared to the PL group (no main effects of treatment: Peers: F(1,76)= .20, p= .33, ŋ²= .01; Mother: F(1,74)= .57, p= .23, ŋ²= .02; Father: F(1,74)= .78, p= .19, ŋ²= .02; nor treatment x session interaction effects: Peers: F(2,76)= 1.08, p= .17, ŋ²= .03; Mother: F(2,74)= .32, p= .36, ŋ²= .01; Father: F(2,74)= .03, p= .49, ŋ²= .00) (see Table 4.2 and Supplementary Figure 4.2 for the effect sizes separately for each session). There was no main effect of session for the peers subscale (F(2,76)= .10, p= .45, ŋ²= .00) nor father subscale (F(2,74)= .08, p= .46, ŋ²= .00). For the mother subscale there was a main effect of session (F(2,74)= 3.03, p= .03, ŋ²= .08), indicating that - irrespective of treatment group - participants reported higher secure attachment toward their mother at follow-up, one year post-treatment than immediately after the intervention or at follow-up, one month post-treatment.

World Health Organization Quality of Life (WHO-QOL) – Bref

Pre-to-post changes in self-reported quality of life were not significantly larger in the OT, compared to the PL group (no main effect of treatment: F(1,74)= .77, p= .19, ŋ²= .02, nor a treatment x session interaction effect: F(2,74)= .96, p= .19, ŋ²= .03) (see Table 4.2 and Supplementary Figure 4.2 for the effect sizes separately for each session). No main effect of session was evident (F(2,74)= .10, p= .45, ŋ²= .00).

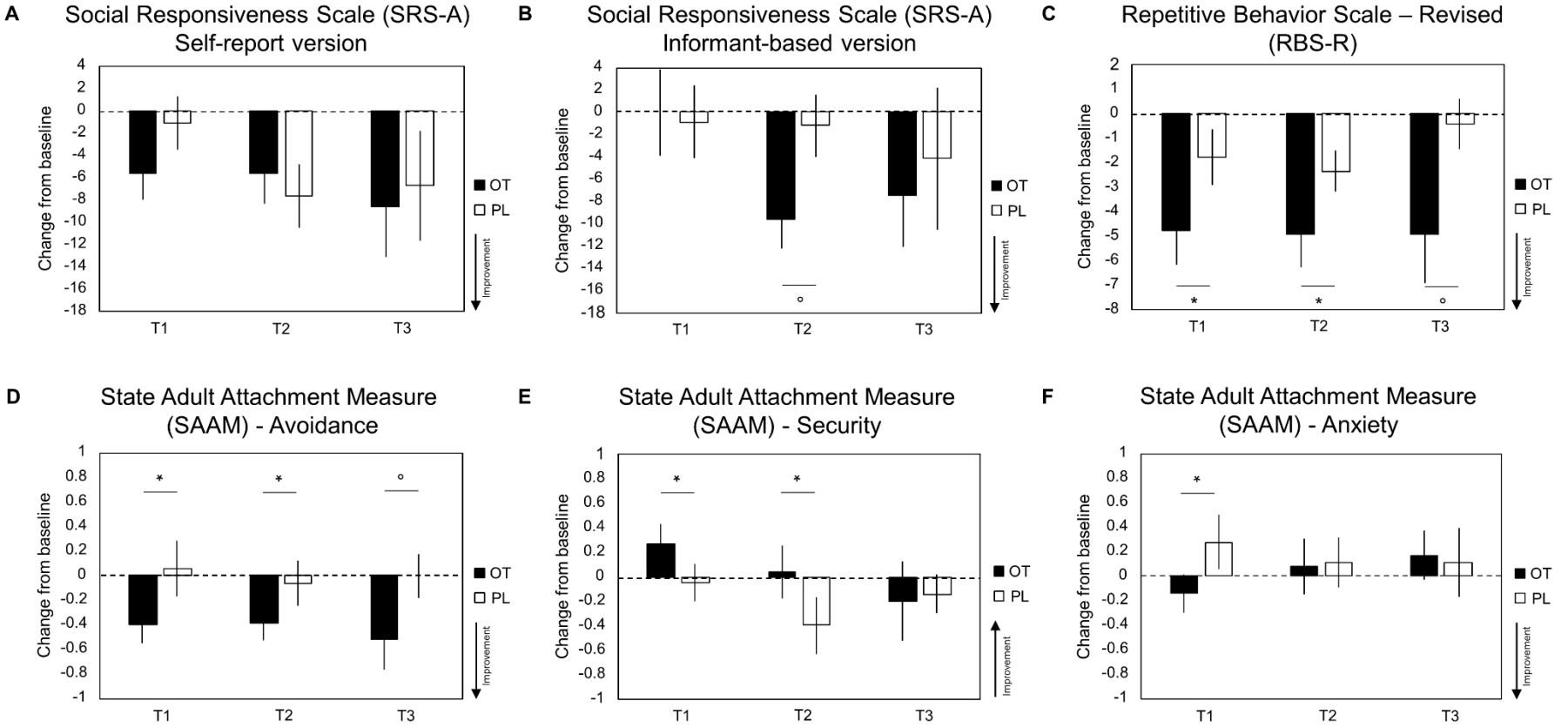


Figure 4.2. Behavioral effects of the four-week treatment. Mean changes from baseline on self-report and informant-based questionnaires are visualized for the oxytocin (OT) and placebo (PL) treatment groups at assessment session 'T1' (immediately after the four-week treatment), 'T2' (at follow-up, one month post-treatment) and 'T3' (at follow-up, one year post-treatment). Mean changes from baseline are visualized separately for (A) Social Responsiveness Scale (SRS-A) self-report version, (B) SRS-A informant-based version, (C) Repetitive Behavior Scale – Revised (RBS-R), (D) State Adult Attachment Measure (SAAM) Avoidance subscale, (E) State Adult Attachment Measure (SAAM) Security subscale, (F) State Adult Attachment Measure (SAAM) Anxiety subscale. Lower scores indicate improvement for the SRS, RBS-R, SAAM Avoidance and SAAM Anxiety questionnaires. For the SAAM Security questionnaire, higher scores indicate improvement. Vertical bars denote +/- standard errors. The asterisks (*) indicate Cohen's d > .50 (medium-sized effect). The circle (°) indicates a Cohen's d >.80 (large-sized effect)[35].

Table 4.3. Mean pre-to-post change scores of the Profile of Mood States (POMS) subscales for each treatment group (oxytocin, placebo) and session (T1, T2, T3), and the corresponding Cohen's d effect sizes.

Outcome measure	Oxytocin		Placebo		Group difference effect size
	N	Mean ± SD	N	Mean ± SD	Cohen's d
Single-dose (SD)					
Tension	22	-1.59 ± 2.11	18	-1.39 ± 2.15	-0.09
Anger	22	-0.09 ± 2.39	18	-0.28 ± 0.89	0.10
Depression	22	-0.23 ± 1.45	18	-0.67 ± 2.25	0.23
Vigor	22	0.27 ± 2.37	18	-0.50 ± 2.38	0.32
Fatigue	22	-1.00 ± 2.41	18	-0.72 ± 3.68	-0.09
Week 1 (W1)					
Tension	22	-1.41 ± 3.74	18	-1.50 ± 3.75	0.02
Anger	22	1.00 ± 2.54	18	0.39 ± 4.17	0.18
Depression	22	0.27 ± 3.81	18	0.28 ± 4.39	0.00
Vigor	22	0.36 ± 3.16	18	0.06 ± 4.32	0.08
Fatigue	22	-2.45 ± 3.86	18	-2.83 ± 3.17	0.11
Week 2 (W2)					
Tension	22	-1.36 ± 3.65	18	-1.39 ± 3.71	0.01
Anger	22	1.18 ± 5.84	18	-0.28 ± 3.04	0.31
Depression	22	0.23 ± 3.90	18	-.11 ± 4.60	0.08
Vigor	22	-0.32 ± 4.24	18	-0.89 ± 4.17	0.14
Fatigue	22	-2.23 ± 5.39	18	-3.00 ± 4.01	0.16
Week 3 (W3)					
Tension	22	-1.36 ± 2.98	18	-1.61 ± 3.87	0.07
Anger	22	1.14 ± 3.87	18	1.33 ± 4.56	-0.05
Depression	22	0.36 ± 4.81	18	1.83 ± 6.82	-0.25
Vigor	22	0.36 ± 4.39	18	-1.28 ± 2.70	0.45
Fatigue	22	-3.04 ± 5.21	18	-3.11 ± 4.54	0.01
Week 4 (W4)					
Tension	22	-1.36 ± 2.42	18	-1.17 ± 4.37	-0.06
Anger	22	0.68 ± 2.64	18	1.17 ± 3.60	-0.14
Depression	22	-0.14 ± 3.23	18	2.44 ± 6.84	-0.48
Vigor	22	0.95 ± 4.13	18	-2.06 ± 4.71	0.68
Fatigue	22	-3.32 ± 3.21	18	-1.72 ± 6.42	-0.31
Multiple-dose effect (T1)					
Tension	22	-2.00 ± 2.29	18	-2.39 ± 3.03	0.14
Anger	22	0.00 ± 4.05	18	-0.61 ± 2.73	0.18
Depression	22	-1.14 ± 4.50	18	-0.33 ± 2.81	-0.21
Vigor	22	-1.00 ± 2.53	18	-2.94 ± 3.64	0.62
Fatigue	22	-2.09 ± 3.99	18	-1.11 ± 5.12	-0.21
One-month Retention effect (T2)					
Tension	22	-2.64 ± 2.80	18	-2.11 ± 3.22	-0.17
Anger	22	0.36 ± 3.68	18	-0.39 ± 3.91	0.20
Depression	22	-0.82 ± 2.63	18	0.22 ± 3.41	-0.34
Vigor	22	0.14 ± 3.58	18	-1.44 ± 4.33	0.40
Fatigue	22	-2.69 ± 2.71	18	-2.33 ± 4.47	-0.09
One-year Retention effect (T3)					
Tension	22	-1.86 ± 2.29	18	-2.28 ± 3.46	0.14
Anger	22	0.59 ± 3.69	18	0.06 ± 3.84	0.14
Depression	22	0.50 ± 2.63	18	-0.28 ± 3.51	0.25
Vigor	22	1.14 ± 3.88	18	-0.61 ± 3.27	0.49
Fatigue	22	-0.23 ± 6.04	18	0.39 ± 4.73	-0.11

Cohen's d effect sizes (change from baseline$_{OT}$ –change from baseline$_{PL}$)/pooled SD) are reported where 0.2 is indicative of a small effect, 0.5 a medium effect and 0.8 a large effect [36]. Data printed in bold show Cohen's d effect sizes equal to or larger than .50 (medium-sized effect).

3.2 Screening of side effects and changes in mood

As listed in detail in Supplementary Table 4.2**,** only minimal, non-treatment specific side effects were reported. In terms of changes in mood, a main effect of treatment was revealed ($F(1,74)= 3.92$, $p= .03$, $\eta^2= .10$), indicating that participants of the OT group reported overall higher 'vigor', compared to the PL group (see Table 4.3 and Supplementary Figure 4.1**)**. No treatment-specific changes were identified in the other mood states (tension, anger, depression, fatigue). We additionally explored how these changes in 'vigor' were associated with the changes in repetitive behaviors (assessed with the RBS-R) and attachment avoidance (assessed with the SAAM). Higher self-reported 'vigor' (across treatment groups) was associated with lower scores on the RBS-R (less frequent and/or severe repetitive and restricted behaviors) at one year post-treatment (T3: $r= -.42$, $p= .008$), but not immediately after either treatment (T1: $r= -.02$, $p=.88$) or at one month post-treatment (T2: $r= .03$, $p= .87$). Higher self-reported 'vigor' (across treatment groups) was also significantly associated with lower perceived attachment avoidance at one year post-treatment (T3: $r= -.45$, $p= .004$), but not immediately after either treatment (T1: $r= .05$, $p= .75$) or at one month post-treatment (T2: $r= -.11$, $p=.51$).

3.3 Associations between ASD characteristics and attachment characteristics

Taken together, treatment-specific effects of a four-week OT treatment were most pronounced in terms of improvements in repetitive behaviors (RBS-R) and perceived attachment avoidance/security (SAAM). Here, we specifically explored whether and how the quantitative autism characteristics (SRS-A and RBS-R) (assessed at baseline) were associated with the adopted attachment characteristics (SAAM and IPPA). We additionally explored whether individuals with ASD displayed more impairments in attachment, when their baseline behavioral characterizations were compared to those previously obtained from a sample of neurotypical individuals (n=40, mean age = 21.1, S.D. = 2.6) (data adopted from [17]).

Higher self-reported SRS-A scores (at baseline) (more impairment in social responsiveness) were significantly associated with lower perceived secure attachment (IPPA) towards peers ($r= -.55$, $p< .001$), mother ($r= -.51$, $p= .001$) and father ($r= -.34$, $p= .034$) and with higher perceived attachment avoidance (SAAM) ($r= .38$, $p= .018$), but not with other reports of attachment characteristics (security: $r= -.26$, $p= .12$; anxiety: $r= .08$, $p= .63$). Further, higher scores on the RBS-R (more frequent and/or severe repetitive and restricted behaviors) were significantly associated with lower perceived secure attachment (IPPA) towards the mother ($r= -.56$, $p< .001$), but not the

father (r= -.31; p= .056) or peers (r= -.22, p= .19). Finally, higher scores on the RBS-R were also significantly associated with higher perceived attachment avoidance (r= .50, p= .002), but not with other reports of attachment characteristics (security: r= -.11, p= .50; anxiety: r= -.02, p= .89).
Notably, exploratory analyses also showed that as a group, the individuals with ASD reported significantly higher perceived attachment avoidance (t(76)=-2.51, p=.014), lower attachment security (t(76)=2.48, p=.015), and a trend towards lower perceived secure attachment towards peers (IPPA) (t(76)=1.74, p=.085), when compared to a neurotypical sample of adult men (data obtained from [17]) (Supplementary Table 4.5).

4 Discussion

With the present study we showed that four weeks of once-daily oxytocin (OT) treatment induced treatment-specific improvements in repetitive behaviors (RBS-R) and attachment characteristics in adult men with autism spectrum disorder (ASD). Screenings for changes in mood also identified an overall enhancement in feelings of 'vigor' in the OT, compared to the placebo group. Notably, the observed improvements outlasted the period of actual administration until one month and even one year post-treatment.
While the exact neuromodulatory mechanisms are unknown, OT has been implicated in enhancing the salience of socially-relevant cues, inducing reductions in (social) stress and anxiety, and modulating approach/avoidance motivational tendencies, presumably by impacting on limbic circuits (e.g. amygdala) and the central reward system (e.g. nucleus accumbens). As such, by enhancing social salience and reducing social stress/anxiety, the daily OT administrations over a course of four weeks, may have induced increased feelings of approachability (reduced avoidance) during social interactions. Furthermore, the observation that these beneficial effects of multiple-dose OT treatment on perceived attachment avoidance outlasted the period of actual administration until one month and one year post-treatment, provides support to the notion that repeated administrations over an extended period of time might induce long-lasting adaptations in social brain circuits, presumably in an experience-dependent manner. Indeed, through positive re-enforcement, the recursive experience of the social environment as more 'secure' or 'approachable' (during the period of actual OT administrations) can be anticipated to have contributed to the observed long-lasting adaptations in one's motivational tendencies (i.e. increased feelings of security/social approachability). Considering that attachment avoidance reflects a reluctance to trust others and an emphasis on autonomy, whereas attachment anxiety reflects insecurity about oneself (low trust in oneself) and fear of being rejected [33], our results suggest that the four-week OT treatment predominantly improved a person's reluctance towards

closeness or trust in others (improvements in attachment avoidance were evident at all assessment sessions), but that it could not induce long-term alterations in a person's feelings of insecurity about one's own abilities (improvements in attachment anxiety were only evident immediately post-treatment). The notion that OT may thus predominantly influence one's reluctance to engage in closeness or intimacy with others (rather than one's fear of being rejected) may be interpreted within the framework of the recently proposed affiliative-motivation hypothesis [37] suggesting that OT specifically acts by increasing affiliative strivings and that individuals with a decreased tendency to affiliate (e.g. avoidant attached individuals rather than anxiously attached individuals) may be most likely to benefit from OT treatment.

Overall, the observation of a beneficial effect of OT on feelings of approachability is in line with findings from a previous study from our lab [17], in which we demonstrated similar treatment-specific reductions in perceived avoidant attachment (as well as improvements in perceived secure attachment to peers) after a two-week course of OT treatment in neurotypical men. Our findings also extend previous studies showing beneficial effects of a single dose of OT on attachment security [20], development of trust and cooperation [18] and improved communal traits and altered agency [19].

In addition to the effects of OT on reducing perceived attachment avoidance, we observed long-lasting improvements in repetitive behaviors (assessed with the RBS-R). While the exact link between expressions of repetitive behaviors and difficulties in the social domain is unclear, it has been suggested that at least in a subset of individuals with ASD, the experience of the external (social) milieu as 'unapproachable' or even 'threatening' may result in an increased 'need for sameness' in order to sustain a level of control over the external surroundings [38,39]. The current study provides evidence that multiple-dose OT treatment may relieve an individual from this increased 'need for sameness' and the resulting need for engaging in repetitive and restricted behaviors. Overall, the observed effects on repetitive behaviors are in line with previous trials with adult men with ASD also showing beneficial effects of OT on repetitive behaviors after 4 hours of intravenous OT administration [4] and after 6 weeks of daily administrations [8,10]. Note however that one six-week trial with adult men with ASD did not show OT-specific improvements on informant-based reports of repetitive behaviors [9]. Also, in previous trials with children and adolescents with ASD, no improvements on repetitive behaviors were observed after 4 days or 4, 5, or 8 weeks of daily OT administration [11–14]. Notably, it appears that beneficial effects of OT treatment on repetitive behaviors were mostly demonstrated in studies that adopted assessments based on self-reports (current study, [4,8]), whereas no beneficial effects were evident in studies adopting informant-based reports of repetitive behavior (9–14, with the exception of 10). Together, these findings may therefore indicate that self-reports, as opposed to informant-based

reports, may be more sensitive for capturing subtle, self-experienced changes in repetitive behaviors.

With respect to self-reports of social responsiveness, we observed no treatment-specific effects, since comparable improvements were evident in both treatment groups (see Figure 4.2, and Supplementary Table 4.3). Correspondingly, in three previous long-term administration trials, improvements on the SRS-A were found across treatment groups, but with no specific benefit of OT over PL treatment [9,10,12]. Similarly, a more recent large-scale clinical trial also showed improvements in social reciprocity (ADOS subscale) both in participants receiving the OT treatment and in those receiving the placebo treatment [10]. As suggested by Yatawara et al. (2016), one explanation for these unspecific effects may be the presence of a placebo response, which has been shown to occur frequently in paediatric autism pharmacological and dietary placebo-controlled trials [40]. In the context of OT trials, increased public attention over the last decade may have influenced the expectations of patients or parents especially with respect to the anticipated effects of OT treatment on social functioning (hence the observed unspecific improvements on the SRS-A directly assessing the social domain).

With respect to the effect of multiple-dose OT treatment on informant-based reports of social responsiveness (SRS-A), no treatment-specific improvements were observed immediately after treatment, but a large-sized treatment-specific effect was revealed at the one-month follow-up session (see Table 4.2, Figure 4.2 and Supplementary Table 4.3), providing indications that adaptations in social responsiveness were only significantly observed by others one month after treatment cessation. In prior studies with young children with ASD, significant improvements in caregiver-rated social responsiveness were evident immediately after 4 or 5 weeks of OT treatment [13,14]. This difference in findings might be explained by the frequency of contact of children versus adult populations with their respective informants (i.e., more frequent, sustained child-informant contact). In this view, it can be argued that in the adult population, more subtle improvements in social functioning (such as those occurring during and immediately after the one-month treatment) were potentially less discernible to others and therefore remained undetected based on the informant-based reports.

With respect to the effect of OT on general aspects of quality of life, the current study identified no treatment-specific improvements. To date, evidence on the effects of OT on quality of life is relatively scarce since only two prior studies have addressed this topic. Contrary to our findings, Anagnostou et al. [8] reported an OT-specific improvement in quality of life (socio-emotional section) after a 6-week treatment in adult men with ASD. Watanabe et al. [9] on the other hand, only observed a trend towards improvement in quality of life immediately after a 6-week trial in adult men with ASD. Importantly, recent reviews stated that most individuals with ASD have poor quality of life (note that most studies included children with ASD) [41] or lower quality of life than

typically developing adults [42]. Note, however, that to date there is no comprehensive ASD-specific quality of life assessment tool validated and consequently, the tools used in the general population (i.e. WHO-QOL) might not be the most adequate to assess quality of life in ASD (and thus changes in quality of life after intervention) [42].

Finally, while not included as an explicit outcome measure, screenings for changes in mood states (performed during and after treatment) also revealed an overall enhancement in 'vigor' in the OT group (e.g., increased reports of feeling 'energetic', 'active', 'lively'). To our knowledge, this is the first study adopting the Profile of Mood States questionnaire (POMS) for screening mood states in an OT trial with ASD patients. In a previous study from our lab [17], the POMS questionnaire was also adopted to evaluate the effects of a two-week OT treatment in neurotypical men, and while here, no changes in vigor were detected, the POMS revealed improvements in feelings of tension and anger. The beneficial effect of OT administration on reports of vigor might be related to the (highly understudied, but in the ASD community heavily discussed) phenomenon of 'autistic burnout'. Individuals with ASD describe 'autistic burnout' as an extreme fatigue and inability to meet the demands of everyday life caused by a continuous attempt to mask and/or deal with their ASD symptoms (i.e. sensory disorders, repetitive behaviors [1]). The overall mitigation of repetitive behavior symptoms and increased feelings of social approachability by the OT treatment may therefore have been accompanied with overall increases in reports of feeling 'energetic', 'active', 'lively' (supported by the explorative correlation analyses in the current study). This interpretation, however, remains speculative and more research is needed to elucidate the effect of OT on positive mood.

In terms of associations between core autism characteristics and attachment characteristics, our study showed that impairments in social responsiveness and more frequent and/or severe repetitive behaviors were associated with a more avoidant attachment style and with less secure attachment towards significant others (especially the mother). Albeit exploratory, we also showed that, as a group, the individuals with ASD scored higher on avoidant attachment and lower on secure attachment, when compared to a sample of neurotypical individuals. Together, these findings provide indications that - at least to some extent - associations are evident between core autism characteristics and attachment characteristics, a notion that is generally supported by a recent meta-analysis showing an association between the severity of autism characteristics and less secure attachment in children with ASD [22], as well as by other studies showing more insecure attachment towards parents or romantic partners in unmarried [43] or married adults with ASD [44], respectively. However, since research on this topic to date is limited, it currently remains speculative whether the reported feelings of insecure attachment are a result of the social difficulties experienced by individuals with ASD, or conversely, whether difficulties in the social domain are – in part or within a subset of individuals with ASD - a result of a decreased tendency

or inability to form secure attachments. Nevertheless, elucidating the interaction between autism symptomology and attachment style may be of particular relevance in the context of OT treatment, since – according to the aforementioned affiliative-motivation hypothesis – especially individuals with a decreased tendency to affiliate (i.e., avoidant attached individuals) have been proposed to benefit the most from receiving OT treatment [37]. The current findings of significant ameliorations in attachment avoidance (and medium-sized effects indicating improved attachment security), but no treatment-specific effects on social responsiveness, are in line with this notion and together suggest that attachment characteristics may be more sensitive for evaluating treatment responses, as compared to evaluations based on core autism characteristics alone. Considering the mixed pattern of effects of OT treatment on core autism symptomatology (e.g. SRS-A, RBS-R, ADOS), it seems of great relevance for future multiple-dose clinical trials with individuals with ASD to additionally include more in-depth characterizations of attachment-related constructs both dimensionally and longitudinally (pre-post treatment). In view of the current observations, these explorations are anticipated to be informative for evaluating and predicting treatment responses, and potentially for delineating patient populations that will benefit the most from a course of OT treatment.

Limitations. Although the current study provides new insights regarding long-lasting effects of multiple-dose OT treatment in ASD and the relation between autism characteristics and attachment characteristics, several limitations need to be considered. First, although our sample size was comparable to that of prior similar clinical trials, studies with larger samples are urgently needed. Further, considering this is an initial pilot study exploring long-term effects of OT treatment (without correction for multiple comparisons), the findings of long-term improvements in repetitive behaviors and attachment avoidance should be interpreted with caution. Second, in the current study, participants administered the OT nasal spray once a day (in the morning) while the majority of prior multiple-dose OT studies administered two doses/day (one in the morning and one in the afternoon) (8–10, 12-14, but see 11). While elevated levels of OT have been demonstrated up to 7 hours after a single-dose administration [45], future studies are needed to identify at what point in time the effects of intranasal administration of OT fade out and when OT levels return back to baseline. Also potential interactions with diurnal patterns of endogenous OT levels need to be explored to identify the most optimal dosing and timing of intranasal OT administrations. Third, considering the adopted evaluations were predominantly based on self-report questionnaires, the possibility of subjective bias cannot be ruled out. Participants' own beliefs about the received treatment, however, were assessed and inclusion of this factor did not modulate the identified treatment effects. Finally, since only adult men with ASD were included, the current observations of beneficial effects of OT treatment cannot be extended to women or children with ASD.

5 Conclusions

To conclude, a four-week continual treatment with OT induced long-lasting, medium to large-sized improvements in repetitive behaviors and a reduction in perceived attachment avoidance, that outlasted the period of actual administration until one month and even one-year post-treatment. Overall, the observation that the OT treatment primarily targeted long-term adaptations in repetitive behaviors and perceived attachment characteristics indicates that these constructs are highly sensitive for capturing OT treatment effects in adult men with ASD. In line with the central role of the human oxytocinergic system in interpersonal bonding, trust and attachment, the current observations therefore particularly urge future multiple-dose clinical trials to continue to include characterizations of attachment-related constructs when evaluating the potential of OT treatment for ASD.

Supplements

Supplementary Methods: Detailed questionnaire descriptions.

Social Responsiveness Scale – Adult version (SRS-A)

The SRS-A is a questionnaire developed to measure social functioning in both clinical and nonclinical populations [31]. The Dutch-version of the SRS-A consists of 64 items that are rated on a four-point Likert scale ranging from 1 (untrue), 2 (sometimes true), 3 (often true) to 4 (almost always true) [46]. Raters were asked to refer to the previous month when completing the scale. The SRS-A encompasses four subscales, including social communication (22 items; α= 0.88), social awareness (19 items; α= 0.80), social motivation (11 items; α= 0.83) and rigidity/repetitiveness (12 items; α= 0.79). SRS-A raw total scores were adopted, for which higher scores indicate less social responsiveness.

Repetitive Behavior Scale – Revised (RBS-R)

The RBS-R is a questionnaire developed to assess the severity and frequency of restricted and repetitive behaviors (RRBs) observed in ASD [47]. The questionnaire consists of 43 items that are rated on a four-point Likert scale ranging from 0 (behavior does not occur), 1 (behavior occurs and is a minor problem), 2 (behavior occurs and is a moderate problem), to 3 (behavior occurs and is a severe problem). Raters were asked to refer to the previous month when completing the scale. The RBS-R encompasses five subscales, including stereotypic behavior (9 items; α= 0.85), self-injurious behavior (8 items; α= 0.84), compulsive behavior (6 items; α= 0.79), ritualistic/sameness behavior (12 items; α= 0.91) and restricted interests (3 items; α= 0.78) (5 items are not considered in scoring) [32]. RBS-R raw total scores were adopted, for which higher scores indicate a higher frequency and/or higher severity of restricted and repetitive behaviors.

State Adult Attachment Measure (SAAM)

The SAAM is a questionnaire designed to assess temporary fluctuations in state attachment [33]. For a long time, an adult's attachment style was considered a relatively stable disposition, rooted in internal cognitive–affective working models (i.e., mental representations) of self and other, based on previous experiences in close relationships [48]. More recently however, it has been suggested that attachment style can be transiently influenced or shaped by situational factors such as major life events or other contextual factors [49–55]. While not contesting the stability of attachment style, the SAAM questionnaire has been validated as a useful measure for capturing temporary fluctuations in the thoughts, feelings, and behaviors underlying attachment processes [33]. Although

the SAAM is more commonly used to assess attachment priming (e.g. (14–16) or as outcome measure to assess the effect of touch in neurotypicals (e.g. [59,60], prior work from our lab has indicated its relevance for human OT research [17]).

The questionnaire contains 21 statements to which participants have to indicate their current state ('right now') on a seven-point Likert scale ranging from 1 (totally disagree), 2 (disagree), 3 (rather disagree), 4 (agree/disagree), 5 (rather agree), 6 (agree), to 7 (totally agree). The SAAM comprises three subscales assessing (i) attachment security (7 items; α= 0.82–0.91); (ii) attachment anxiety (7 items; α= 0.81-0.85); and (iii) attachment avoidance (7 items; α= 0.71-0.87). As a concept, attachment anxiety is characterized to reflect insecurity about one's own worth and abilities, extreme need for interpersonal closeness, love, and support, and worrying about being rejected or abandoned (e.g., "I feel a strong need to be unconditionally loved right now"). Attachment avoidance on the other hand, is characterized by the reluctance to trust others, an emphasis on autonomy and self-reliance, a relatively low tolerance for interpersonal intimacy and interdependence, and a tendency to downregulate one's own emotions (e.g., "If someone tried to get close to me, I would try to keep my distance"). Reports of attachment security on the other hand, were shown to be inversely related to attachment avoidance and attachment anxiety, which is in line with the conceptualization that attachment security reflects the relative absence of anxiety and avoidance as well as a sense of faith in the responsiveness of attachment figures, and comfort with intimacy and interdependence (e.g., "I feel like I have someone to rely on").

SAAM raw subscale scores were adopted, for which higher scores indicate lower perceived secure attachment on the attachment avoidance and attachment anxiety subscales, and higher perceived secure attachment on the attachment security subscale.

Inventory of Parent and Peer Attachment (IPPA)

The IPPA is a questionnaire designed to assess trait attachment to (i) mother (12 items; α= 0.87); (ii) father (12 items; α= 0.89); (iii) peers (12 items; α= 0.92) [34]. More specifically, based on the attachment theory as formulated by Bowlby [48], the IPPA was developed to investigate how well parents and close friends serve as sources of psychological security, by assessing adolescents' perceptions of the positive and negative affective/cognitive dimension of relationships with their parents and peers (degree of mutual trust, quality of communication, and extent of anger and alienation). While commonly used as an instrument for assessing the relation between attachment characteristics and psychopathology (e.g. [61–63]), prior work from our lab has indicated its relevance for human OT research [17,64].

The questionnaire comprises 36 items (12 for each section) with a four-point Likert scale ranging from 1 (almost never), 2 (sometimes), 3 (often), to 4 (almost always). Each section consists of the same 12 items, but with a different person mentioned according to the section (e.g. "I wish I had

(a) different mother/father/peers")[34]. These items represent the three aforementioned dimensions: trust (e.g. "My mother accepts me as I am"), communication (e.g. "I tell my father about my problems and troubles"), and alienation (e.g. "I feel alone or apart when I am with my friends").

Note that one participant of the OT group did not complete the section on 'father' and another participant of the OT group did not complete the section on 'mother'. IPPA raw subscale scores were adopted, for which higher scores indicate increased feelings of secure attachment towards peers or parents.

World Health Organization Quality of Life (WHO-QOL) – Bref

The abbreviated version of the WHO-QOL is a 26-item questionnaire with a five-point Likert scale ranging from 1 (not at all, never, very dissatisfied or very poor) to 5 (an extreme amount/extremely, always, very satisfied or very good) (depending on the subscale) to assess general quality of life related to physical health (7 items; α= 0.82), psychological health (6 items; α= 0.75), social relationships (3 items; α= 0.66), and environment (8 items; α= 0.80) (2 items are examined separately) [35]. WHO-QOL raw total scores were adopted, for which higher scores indicate better quality of life.

Supplementary Results

Exploratory analyses assessing the effect of the participants' own belief about the received treatment on treatment response.

Note that the following secondary analyses are highly exploratory in nature and probably unreliable, considering the majority (79.5%) of participants believed that they had received the PL treatment.

		Actual treatment	
		OT	**PL**
Own belief	**OT**	6	2
	PL	15	16

Exploratory analyses were performed to assess whether treatment effects on self-report and informant-based social responsiveness (SRS-A), repetitive behaviors (RBS-R) and attachment characteristics (SAAM + IPPA) and quality of life (WHO-QOL) were modulated by the participants' own belief about the received treatment. To do so, change-from-baseline scores were subjected to a mixed-effects analysis with the factor 'subject' modeled as random effect and the factors 'treatment group' (OT, PL), 'session' (T1, T2, T3), 'own belief' (OT, PL), and the 'treatment x session' and 'treatment x own belief' interactions modelled as fixed effects.

For none of the outcome measures (except for the IPPA mother subscale) a significant main effect of 'own belief' was revealed (all $p > .05$), indicating that across treatment groups, the participants' own beliefs did not significantly affect the treatment response. For the IPPA mother subscale, a main effect of 'own belief' was found ($F(1,72)= 6.69$, $p= .007$), indicating that across treatment groups, those participants who believe to have received the OT treatment reported more secure attachment towards their mother compared to those who believed to have received the PL treatment.

For none of the outcome (except for the SAAM avoidance subscale) a significant 'treatment x own belief' interaction effect was revealed (all $p > .05$). For the SAAM avoidance subscale, a significant 'treatment x own belief' interaction effect was revealed ($F(1,74)= 4.54$, $p= .02$), indicating that in the PL group, participants who believed they had received the OT treatment, reported a greater reduction in perceived attachment avoidance, compared to those who believed to have received the PL treatment (post-hoc Tukey: $p= .02$). Unexpectedly, in the OT group, participants who believed to have received the PL treatment reported a greater reduction in perceived attachment

avoidance compared to those who believed to have received the OT treatment (post-hoc Tukey: p= .03). However, of those participants who believe to have received the PL treatment, those who actually received the OT treatment did indeed report a greater reduction in perceived attachment avoidance compared to those who had received the PL treatment (post-hoc Tukey: p= .0002). Nevertheless, this significant interaction did not change the main effect of treatment ($F(1,74)= 4.20$, p= .02), indicating that - across assessment sessions - improvements (from baseline) were significantly larger in the OT, compared to the PL group.

As stated before, considering the majority (79.5%) of participants believed that they had received the PL treatment, these analyses can be unreliable and their interpretation warrants caution.

Supplementary Tables

Supplementary Table 4.1. Detailed information on comorbidities and medication use for participants of the oxytocin and placebo treatment groups.

Comorbidities were screened through self-report (with the explicit mentioning of examples in the screening interview including e.g., ADHD, depression, dyscalculia, dyslexia). Current psychoactive medication use was defined as use within three months before study enrolment.

		Comorbidities	Medication use
Oxytocin			
	i	ADHD	Abilify, Tegretol
	ii	ADHD, Dyslexia	/
	iii	ADHD, Depression	/
	iv	ADHD, Bipolar disorder	Antipsychotics (not specified)
	v	Depression	Welbutrine XR, Leviron, Cymbalta
	v	Depression, ADD	Trazodone Mylan, Medikinet
	vii	Bipolar Disorder	Maniprex, Bellozal, Mometasone
	viii	Dyslexia	/
	ix	/	Risperdal, Venlafaxine
Placebo			
	i	ADHD	/
	ii	ADHD	/
	iii	/	Zolpidem, Remergon, Rilatine
	iv	/	Trazodone, Escitalopram

ADD = Attention Deficit Disorder, ADHD = Attention Deficit Hyperactivity Disorder.

Supplementary Table 4.2. Side effect screening

Side effect screening.

Participants administered the nasal spray (oxytocin (OT) or placebo (PL)) daily for four consecutive weeks. At the end of each week, participants were asked to report whether they presented any of the listed (or other) side effects and to indicate the severity of the side effect (mild, moderate, or severe). **Panel A** lists the proportion of OT or PL participants (%) that reported any mild, moderate or severe side effects (averaged across effects). **Panel B** lists, separately for each side effect, the proportion of OT or PL participants that reported the side effect (averaged across severity level (mild, moderate, severe)). No significant group differences were revealed in the total proportion of reported side effects (**Panel A**), or separately for each side effect (**Panel B**). Note that tentatively more 'mild' effects were reported by participants of the PL-group after week 4 of the treatment (p= .07) (indicated in italic in **Panel A**). Closer inspection showed that a tentatively larger proportion of PL participants reported to feel 'more calm, relaxed, comfortable' (p= .06) after week 2, 3 and 4 of the treatment and 'more confident' (p= .06) after week 4 (indicated in italic in **Panel B**). A marginal effect was also revealed for the side effect 'dry throat/dry mouth' (p= .06), indicating that a tentatively larger proportion of OT participants reported this side effect after week 1 (indicated in italic in **Panel B**). Overall, the most frequent reported side effect was 'runny nose', but the proportion of OT (17.9%) and PL (9.8%) participants reporting this effect was not significantly different (p= .47).

Panel A	**OT (%)**			**PL (%)**			**Group Difference (p-value)**		
	Mild	Moderate	Severe	Mild	Moderate	Severe	Mild	Moderate	Severe
Week 1	47.6	19.0	9.5	27.8	5.6	5.6	0.21	0.22	0.65
Week 2	38.1	9.5	0.0	27.8	11.1	5.6	0.50	0.87	0.28
Week 3	23.8	9.5	0.0	33.3	16.7	5.6	0.52	0.51	0.28
Week 4	42.9	9.5	0.0	22.2	11.1	5.6	0.18	0.87	0.28
Across weeks	38.1	11.9	2.4	27.8	11.1	5.6	0.50	0.94	0.61

Panel B	**Week 1**			**Week 2**			**Week 3**			**Week 4**		
	OT (%)	**PL (%)**	**p-value**	**OT (%)**	**PL (%)**	**p-value**	**OT (%)**	**PL (%)**	**p-value**	**OT (%)**	**PL (%)**	**p-value**
Headache	4.8	0.0	0.35	0.0	0.0	1.00	0.0	0.0	1.00	0.0	5.6	0.28
Drowsiness	4.8	0.0	0.35	4.8	5.6	0.91	4.8	0.0	0.35	4.8	0.0	0.35
Dizziness	4.8	0.0	0.35	4.8	0.0	0.35	0.0	0.0	1.00	0.0	0.0	1.00
Fainting	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Changes in heart rate or palpitations	0.0	0.0	1.00	0.0	0.0	1.00	4.8	0.0	0.35	0.0	0.0	1.00
Shortness of breath	0.0	5.6	0.28	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Fever	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Sore throat	9.5	0.0	0.19	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Dry throat/dry mouth	19.0	0.0	*0.06*	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Hoarseness	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Coughing	4.8	5.6	0.91	4.8	0.0	0.35	0.0	0.0	1.00	0.0	0.0	1.00
Coughing up mucus	4.8	5.6	0.91	9.5	5.6	0.65	4.8	5.6	0.91	4.8	5.6	0.91
Congested nose	9.5	16.7	0.51	4.8	5.6	0.91	9.5	0.0	0.19	4.8	5.6	0.91
Sneezing	9.5	0.0	0.19	0.0	5.6	0.28	0.0	5.6	0.28	4.8	0.0	0.35
Nasal irritation	4.8	11.1	0.47	4.8	11.1	0.47	4.8	16.7	0.23	9.5	16.7	0.51
Runny nose	23.8	11.1	0.31	14.3	5.6	0.38	23.8	5.6	0.13	9.5	16.7	0.51
Burning sensation in nose and/or ears	9.5	5.6	0.65	4.8	0.0	0.35	0.0	5.6	0.28	0.0	5.6	0.28
Sensitive to fragrances	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Watery eyes	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Nausea and/or vomiting	4.8	0.0	0.35	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Abdominal or stomach pain	4.8	0.0	0.35	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Decreased appetite	9.5	0.0	0.19	4.8	0.0	0.35	0.0	0.0	1.00	0.0	0.0	1.00
Hungry	0.0	5.6	0.28	0.0	0.0	1.00	0.0	0.0	1.00	0.0	5.6	0.28
Constipation	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Diarrhea	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Muscle pain/cramps	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00

Skin rash	0.0	0.0	1.00	0.0	0.0	1.00	0.0	5.6	0.28	0.0	0.0	1.00
Increased fluid intake	0.0	5.6	0.28	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Water retention/bloating	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Insomnia/sleep difficult	0.0	5.6	0.28	0.0	0.0	1.00	0.0	0.0	1.00	4.8	0.0	0.35
Nightmares	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Staring/daydreams	4.8	0.0	0.35	4.8	0.0	0.35	0.0	0.0	1.00	0.0	0.0	1.00
Anaphylaxis	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Changes in perception of the tongue	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Back pain	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Bed wetting	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Weight gain	0.0	0.0	1.00	4.8	0.0	0.35	0.0	0.0	1.00	0.0	5.6	0.28
Sweating	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Blurred vision	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Less talk to others	0.0	0.0	1.00	4.8	0.0	0.35	0.0	0.0	1.00	0.0	0.0	1.00
Uninterested in others	0.0	0.0	1.00	0.0	5.6	0.28	0.0	0.0	1.00	0.0	0.0	1.00
Persistent thoughts and/or feelings	4.8	0.0	0.35	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Development of repetitive behavior	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Increase in repetitive behavior	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Nail biting	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Annoyed. bored	4.8	0.0	0.35	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Sad	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Prone to crying. more emotional	0.0	0.0	1.00	0.0	0.0	1.00	4.8	0.0	0.35	4.8	0.0	0.35
Anxious. worried. discomfort	0.0	5.6	0.28	0.0	5.6	0.28	0.0	11.1	0.13	0.0	5.6	0.28
Happy. satisfied	4.8	11.1	0.47	4.8	16.7	0.23	4.8	11.1	0.47	4.8	11.1	0.47
Euphoric. unusually happy.	0.0	11.1	0.13	0.0	11.1	0.13	0.0	5.6	0.28	4.8	5.6	0.91
Calm. relaxed. comfortable	4.8	11.1	0.47	0.0	16.7	*0.06*	0.0	16.7	*0.06*	0.0	16.7	*0.06*
More focused	0.0	11.1	0.13	0.0	5.6	0.28	0.0	11.1	0.13	0.0	11.1	0.13
More confidence	0.0	11.1	0.13	0.0	11.1	0.13	0.0	11.1	0.13	0.0	16.7	*0.06*

Supplementary Table 4.3. Mean pre-to-post change scores for each treatment group (oxytocin, placebo) and assessment session (T1, T2, T3). T- and p-values correspond to single-sample t-tests assessing within-group pre-to-post changes, separately for the OT and PL group.

Outcome measure	Oxytocin				Placebo			
	N	Mean ± SD	T-value	p-value	N	Mean ± SD	T-value	p-value
Multiple-dose effect (T1)								
SRS-A self-report	22	-5.55 ± 11.40	-2.28	0.033	18	-1.06 ± 10.01	-0.45	0.66
SRS-A informant-based	17	0.0 ± 15.86	0.00	1.00	15	-0.87 ± 12.83	-0.26	0.80
RBS-R	22	-4.77 ± 6.47	-3.46	0.002	17	-1.76 ± 4.75	-1.53	0.15
SAAM avoidance	22	-0.40 ± 0.71	-2.63	0.016	18	0.06 ± 0.98	0.24	0.81
SAAM security	22	0.27 ± 0.77	1.62	0.12	18	-0.05 ± 0.66	-0.31	0.76
SAAM anxiety	22	-0.14 ± 0.75	-0.90	0.38	18	0.28 ± 0.95	1.24	0.23
IPPA Peers	22	1.45 ± 3.85	1.77	0.091	18	0.56 ± 4.05	0.58	0.57
IPPA Mother	21	-0.52 ± 2.71	-0.88	0.39	18	0.44 ± 3.45	0.55	0.59
IPPA Father	21	0.43 ± 3.30	0.60	0.56	18	-0.61 ± 3.81	-0.68	0.50
WHO-QOL	22	1.77 ± 8.04	1.03	0.31	17	-1.35 ± 6.74	-0.83	0.42
One-month Retention effect (T2)								
SRS-A self-report	22	-5.64 ± 12.57	-2.10	0.048	18	-7.67 ± 12.09	-2.69	0.015
SRS-A informant-based	17	-9.59 ± 10.98	-3.60	0.002	15	-1.20 ± 10.73	-0.43	0.67
RBS-R	22	-4.91 ± 6.33	-3.64	0.002	17	-2.35 ± 3.43	-2.83	0.012
SAAM avoidance	22	-0.38 ± 0.70	-2.58	0.018	18	-0.06 ± 0.76	-0.35	0.73
SAAM security	22	0.04 ± 1.01	0.18	0.86	18	-0.40 ± 0.99	-1.70	0.11
SAAM anxiety	22	0.08 ± 1.05	0.36	0.72	18	0.11 ± 0.87	0.54	0.60
IPPA Peers	22	1.32 ± 3.71	1.67	0.11	18	0.06 ± 3.70	0.06	0.95
IPPA Mother	21	-0.38 ± 3.43	-0.51	0.62	18	0.06 ± 4.35	0.05	0.96
IPPA Father	21	0.52 ± 3.59	0.67	0.51	18	-0.33 ± 3.87	-0.37	0.72
WHO-QOL	22	1.14 ± 5.48	0.97	0.34	17	0.35 ± 4.53	0.32	0.75
One-year Retention effect (T3)								
SRS-A self-report	22	-8.59 ± 20.95	-1.92	0.07	18	-6.72 ± 21.01	-1.36	0.19
SRS-A informant-based	17	-7.41 ± 19.26	-1.59	0.13	15	-4.13 ± 24.64	-0.65	0.53
RBS-R	22	-4.91 ± 9.46	-2.43	0.02	17	-0.41 ± 4.27	-0.40	0.70
SAAM avoidance	22	-0.52 ± 1.18	-2.07	0.05	18	0.0 ± 0.75	0.00	1.00
SAAM security	22	0.20 ± 1.52	-0.62	0.54	18	-0.14 ± 0.66	-0.91	0.37
SAAM anxiety	22	0.17 ± 0.94	0.83	0.42	18	0.11 ± 1.21	0.39	0.70
IPPA Peers	22	0.68 ± 6.26	0.51	0.61	18	1.28 ± 4.17	1.30	0.21
IPPA Mother	21	0.33 ± 3.91	0.39	0.70	18	1.50 ± 5.44	1.17	0.26
IPPA Father	21	0.57 ± 4.08	0.64	0.53	18	-0.50 ± 4.53	-0.47	0.65
WHO-QOL	22	1.14 ± 8.37	0.64	0.53	17	0.29 ± 4.21	0.29	0.78

SRS-A = Social Responsiveness Scale adult version. RBS-R = Repetitive Behavior Scale – Revised. SAAM = State Adult Attachment Measure. IPPA = Inventory of Parent and Peer Attachment. WHO-QOL = World Health Organization Quality of Life questionnaire. Data printed in bold show significant change from baseline (single-sample t-test. p<.05).

Supplementary Table 4.4. Assessment of baseline differences between groups.

Outcome measure	Oxytocin		Placebo			
	N	**Mean ± SD**	**N**	**Mean ± SD**	**T-value**	**p-value**
SRS-A self-report	22	72.82 ± 30.37	18	66.89 ± 23.95	0.67	0.50
SRS-A Informant-based	17	78.71 ± 30.43	15	71.47 ± 29.67	0.68	0.50
RBS-R	22	15.59 ± 16.16	17	8.82 ± 8.41	1.57	0.13
SAAM Avoidance	22	3.09 ± 1.26	18	2.90 ± 0.93	0.54	0.59
SAAM Security	22	5.27 ± 0.70	18	5.31 ± 0.82	-0.18	0.86
SAAM Anxiety	22	4.05 ± 1.11	18	3.83 ± 0.95	0.66	0.51
IPPA Peers	22	33.14 ± 6.68	18	33.89 ± 6.29	-0.36	0.72
IPPA Mother	21	36.33 ± 8.36	18	37.22 ± 5.40	-0.39	0.70
IPPA Father	21	31.57 ± 9.93	18	33.94 ± 9.03	-0.78	0.44
WHO-QOL	22	82.91 ± 14.04	17	85.24 ± 9.63	-0.58	0.56

SRS-A = Social Responsiveness Scale adult version, RBS-R = Repetitive Behavior Scale – Revised, SAAM = State Adult Attachment Measure, IPPA = Inventory of Parent and Peer Attachment, WHO-QOL = World Health Organization Quality of Life questionnaire.

Supplementary Table 4.5. Baseline attachment comparison between individuals with ASD and typically developing control subjects (data adopted from [17]) using SAAM and IPPA.

	ASD (N = 38)	CON (N = 40)	T-value	p-value
SAAM				
Avoidance	3.01 ± 1.14	2.37 ± 1.12	-2.51	0.01
Security	5.30 ± 0.76	5.73 ± 0.75	2.48	0.02
Anxiety	3.90 ± 1.02	3.89 ± 0.91	-0.03	0.98
IPPA				
Peers	33.47 ± 6.52	35.98 ± 6.15	1.74	0.09
Mother	36.78 ± 7.02	37.28 ± 7.07	0.31	0.76
Father	32.24 ± 9.51	33.33 ± 7.20	0.57	0.57

All data are shown as mean ± standard deviation. ASD = Autism Spectrum Disorder, CON = typically developing control subjects, SAAM = State Adult Attachment Measure, IPPA = Inventory of Parent and Peer Attachment.

Supplementary Figures

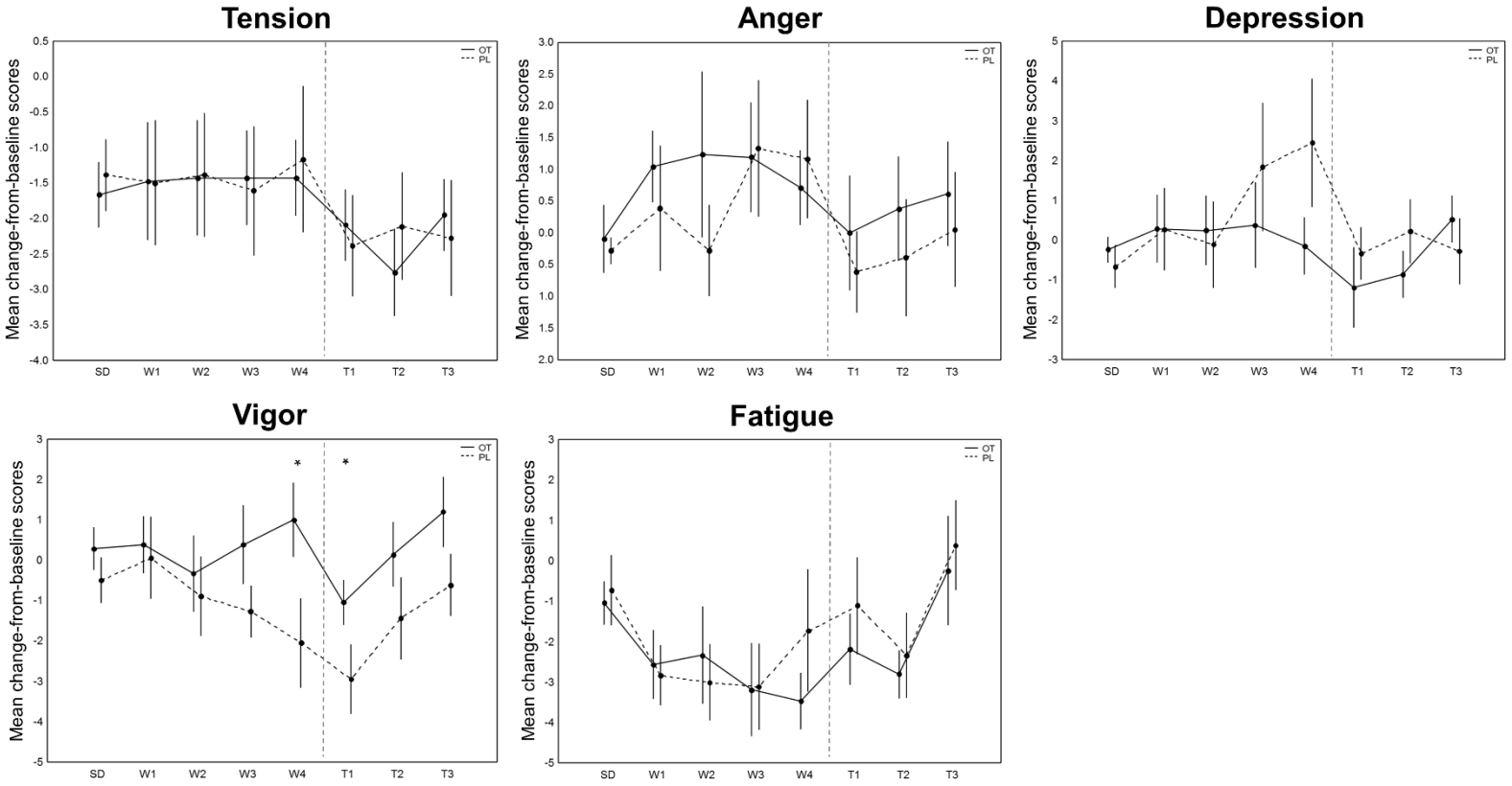
Supplementary Figure 4.1. Screening for changes in mood states.

A 32-item short version of the Profile of Mood States (POMS) questionnaire (26.27) was used as a measure of transient affective states in order to assess whether mood levels of participants changed over the course of the study. This instrument comprises emotional adjectives subdivided in five domains: tension (6 items; $\alpha = 0.84$). depression (8 items; $\alpha = 0.91$). vigor (5 items; $\alpha = 0.81$). fatigue (6 items; $\alpha = 0.90$) and anger (7 items; $\alpha = 0.87$) which have to be rated on a five-point Likert scale ranging from 0 (not at all), 1 (a little), 2 (moderately), 3 (quite a lot), to 4 (extremely). Only for the vigor scale, higher scores indicate improvement. Participants were asked to rate their current mood state ('right now'). For all participants, the POMS questionnaire was assessed at baseline, after administration of a single dose of nasal spray (SD); after four consecutive weeks of daily nasal spray administration (T1); and at a follow-up, four weeks (T2) and one year post-treatment (T3). Also, during the four consecutive weeks of daily nasal spray administration, participants were asked to complete the POMS questionnaire at the end of each week (W1, W2, W3 and W4). For each mood state, there were no baseline differences between treatment groups. Vertical bars denote +/- standard errors.

Similar to the main analyses, linear mixed-effects models were constructed (with the random factor 'subject', and the fixed factors 'treatment' (OT, PL), 'session' (T1, T2, T3) and 'treatment x session' interaction) to assess treatment-specific changes in mood states (one-tailed). For the 'vigor' scale, a significant main effect of treatment was revealed, indicating that participants of the OT group reported overall higher 'vigor' (e.g., feeling 'energetic', 'active', 'lively'), compared to the PL group ($F(1,76)= 4.09$, $p= .03$, $\eta^2= .10$). No significant effects of treatment (all $F< .33$, $p> .29$) or treatment x session interactions (all $F< 2.05$, $p> .07$) were revealed for the other scales, although note that for the 'fatigue' scale a main effect of session was evident ($F(2,76)=5.39$, $p=0.003$, $\eta^2= .12$) (i.e., indicating variable responses over sessions, irrespective of treatment group).

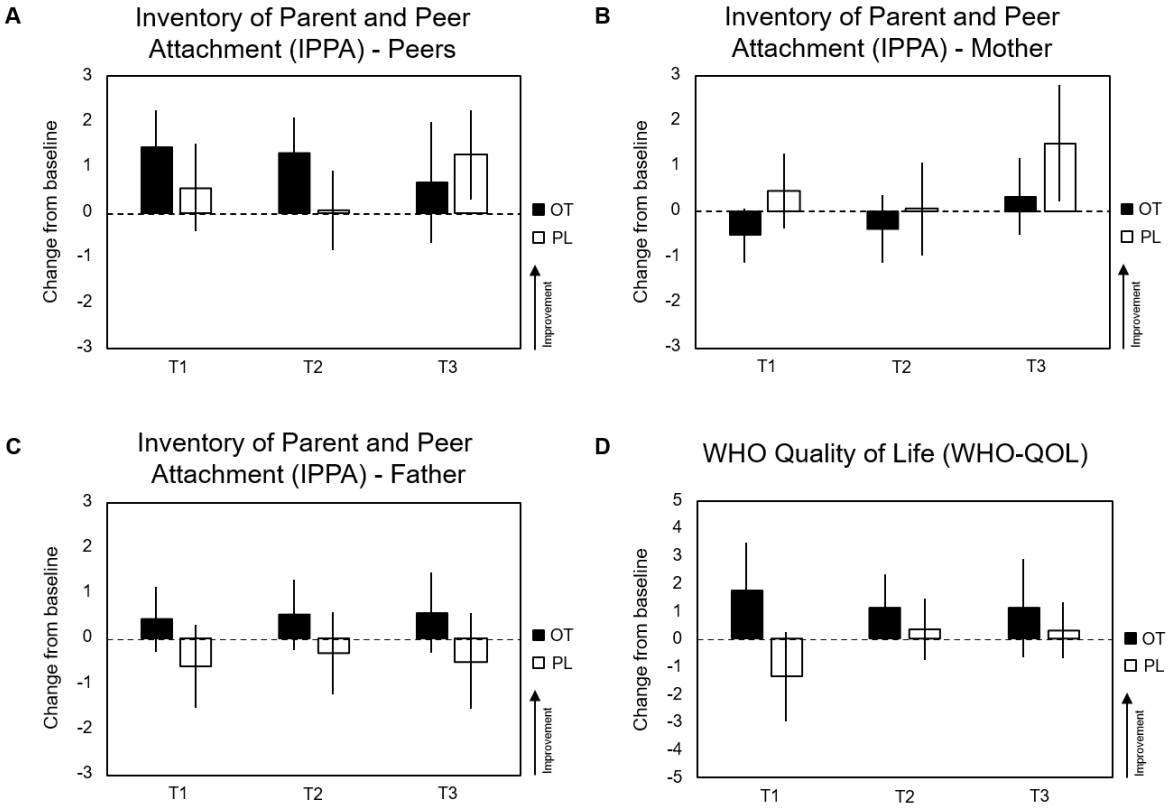
Linear mixed-effects models including the additional assessments of changes in mood states after the single-dose (SD) and during the course of treatment (weekly time points: W1, W2, W3, W4) (with the random factor 'subject', and the fixed factors 'treatment' (OT, PL), 'session' (SD, W1, W2, W3, W4, T1, T2, T3) and 'treatment x session' interaction) revealed a similar main effect of treatment for the 'vigor' scale (i.e. higher vigor in the OT compared to the PL group) ($F(1,266)= 3.18$, $p= .04$, $\eta^2= .08$). For the 'depression' scale, a significant treatment x session interaction was revealed

(F(7,266)= 1.80, p= .044, η^2= .05), indicating a mild-to-medium improvement in feelings of depression in the OT group during the last two weeks of treatment (W3: d= -.25; W4: d= -.48). No significant main effects of treatment (all F< .29, p> .29) or treatment x session interactions (all F< .79, p> .30) were revealed for the other scales. For all scales, a main effect of session was evident (i.e., indicating variable responses over sessions, irrespective of treatment group) (all F> 2.34, p< .02).



Supplementary Figure 4.2. Effects of four-week OT and PL treatment on attachment (IPPA) and quality of life (WHO-QOL).

Behavioral effects of four-week OT and PL treatment. Mean changes from baseline on self-report and informant-based questionnaires are visualized for the oxytocin (OT) and placebo (PL) treatment groups at assessment session 'T1' (immediately after the four-week treatment), 'T2' (at follow-up, one month post-treatment) and 'T3' (at follow-up, one year post-treatment). Mean changes from baseline are visualized separately for (A) Inventory of Parent and Peer Attachment (IPPA) Peers subscale, (B) Inventory of Parent and Peer Attachment (IPPA) Mother subscale, (C) Inventory of Parent and Peer Attachment (IPPA) Father subscale, and (D) World Health Organization Quality of Life questionnaire (WHO-QOL). Higher scores indicate improvement. Vertical bars denote +/- standard errors.



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Oxytocin treatment in adults with autism induces long-term changes in social brain activity until one year after treatment

Chapter V

Prepared for submission

Bernaerts S., Boets B., Steyaert J., Alaerts K. Oxytocin treatment in adults with autism induces long-term changes in social brain activity until one year after treatment.

Abstract

Intranasal administration of the 'prosocial' neuropeptide oxytocin (OT) is increasingly considered as a potential treatment for targeting the core symptoms of autism spectrum disorder (ASD), but the effects of chronic use on neural circuitry are fairly unexplored and long-term effects are unknown. In this double-blind, randomized, placebo-controlled study we assessed the possibility of long-term neural changes induced by chronic OT treatment (four weeks of daily (24 IU) administrations) in 38 adult men with ASD. Functional magnetic resonance imaging was performed during the processing of emotional states from point-light biological motion to assess treatment-induced changes in task-related brain activity of the posterior superior temporal sulcus (pSTS) and amygdala up to one month and one year post-treatment. The multiple-dose treatment with OT was shown to attenuate activity in bilateral amygdala and to induce a sustained recruitment of pSTS regions (especially right pSTS) during the emotion processing task up to one month and even one year post-treatment. Critically, the long-term attenuations in amygdala activity were associated with self-reported improvements in social functioning (Social Responsiveness Scale) and are anticipated to reflect OT's anxiolytic role in regulating negative affect and promoting social approach. Together, the observation that continual OT treatment was effective for inducing long-lasting neural adaptations in core regions of the social brain up to one year post-treatment holds important clinical implications for ASD and other neuropsychiatric disorders for which OT is considered as a potential treatment.

The trial is registered with the European Clinical Trial Register (https://www.clinicaltrialsregister.eu/) (Eudract 2014-000586-45).

1 Introduction

Intranasal administration of the 'prosocial' neuropeptide oxytocin (OT) is increasingly explored as a potential pharmacotherapy for targeting the core symptoms of autism spectrum disorder (ASD), a prevalent neurodevelopmental disorder characterized by impairments in social and communicative functioning and stereotyped and repetitive behavior [1]. OT is a neuropeptide produced by the paraventricular and supraoptic nuclei of the hypothalamus and has been implicated to act as an important neuromodulator for mediating a wide range of complex social behaviors, including interpersonal bonding, trust and affiliative and cooperative behavior (reviewed in [2–4]).

Initial single-dose administration studies in ASD consistently demonstrated behavioral improvements on various social tasks [5–8] and repetitive behaviors [9] (reviewed in [10]). Also several multiple-dose administration studies showed clinical improvements in the social domain after 4, 5 or 6 weeks of continual OT administration in adults [11,12] and young children with ASD [13,14], even though other multiple-dose studies failed to replicate positive clinical outcomes after 4-day or 8-week trials in children [15] and adolescents with ASD [16].

Recent mechanistic models have posited that OT's ability to influence behavioral responses to social stimuli is at least partially mediated by its capacity to increase the salience of social cues [3,17–20]. In line with this notion, initial neuroimaging studies (both in typically developing individuals (reviewed in [21]) and individuals with ASD [6,22–28]) provided evidence that single-dose OT administration can alter activations in cortical-amygdala and midbrain reward circuits implicated in social orienting by modulating social salience and guiding attention to biologically relevant stimuli, such as social information conveyed by eyes, faces or biological motion.

While these initial neuroimaging studies provided important insights into the immediate neural effects of single-dose OT administration, our understanding of the potential of OT as a treatment for ASD has been hampered by a lacking insight into the effects of repeated OT administrations on neural function. Likewise, the possibility that long-term OT treatment may induce long-lasting neurobehavioral changes that outlast the period of actual administrations is currently unexplored.

Using a randomized, double blind, placebo-controlled parallel design, the current study examined the possibility of long-lasting neural effects of four weeks of daily OT administrations in adult men with ASD. Neural functioning was assessed using functional magnetic resonance imaging (fMRI) after a single dose of OT (24 IU); after multiple doses of OT treatment (four weeks of daily administrations); and at follow-up sessions, one month and one year post-treatment. The fMRI paradigm involved processing of emotional states from biological motion conveyed by whole-

body point-light displays (PLDs). Accumulating evidence has demonstrated an early and marked disruption in the perception of point-light biological motion in patients with ASD (34–36,41,43,50, for review see 22, but also 37) and neuroimaging studies have consistently implicated the posterior superior temporal sulcus (pSTS) region as an area of dysfunction (33,38,49–53; but also 54). In line with the implicated role of OT in regulating the salience of social cues, previous OT administration studies have demonstrated improved biological motion perception [45–47] and increased pSTS activity [22] after a single dose of OT. Moreover, (single-dose) OT administration studies have also consistently revealed involvement of the amygdala [48], a key neural structure associated with the detection and processing of salient (social) stimuli (e.g. [49]). In line with these prior studies, we hypothesized that single-dose OT administration would increase neural activations within pSTS regions, as well as alter amygdala reactivity during the processing of emotional states from point-light biological motion stimuli. However, a key aim was to determine whether multiple-dose treatment could induce similar or even augmented changes in pSTS and amygdala activity, and specifically, whether the changes in neural function will outlast the period of actual administration until one month or even one year post-treatment.

2 Methods and Materials

2.1 General study design

This two-arm, double-blind, randomized, placebo-controlled parallel study was performed at the Leuven University Hospital (Leuven, Belgium) to assess single- and multiple-dose effects of intranasal oxytocin (OT) administration on brain activity during emotion processing from point-light biological motion in adult men with ASD. Changes-from-baseline (T0) in brain activity were assessed immediately after a single dose of OT treatment (T1), after four weeks of continual OT treatment (T2), and at two follow-up sessions, one month (four weeks) (T3) and one year post-treatment (T4) (Figure 5.1, CONSORT Flow diagram for number of participants included in each assessment session). Written informed consent was obtained from all participants prior to the study. Consent forms and study design were approved by the local Ethics Committee for Biomedical Research at the University of Leuven, KU Leuven (S56327) in accordance to The Code of Ethics of the World Medical Association (Declaration of Helsinki). The trial was registered with the European Clinical Trial Registry (Eudract 2014-000586-45) and the Belgian Federal Agency for Medicines and Health products.

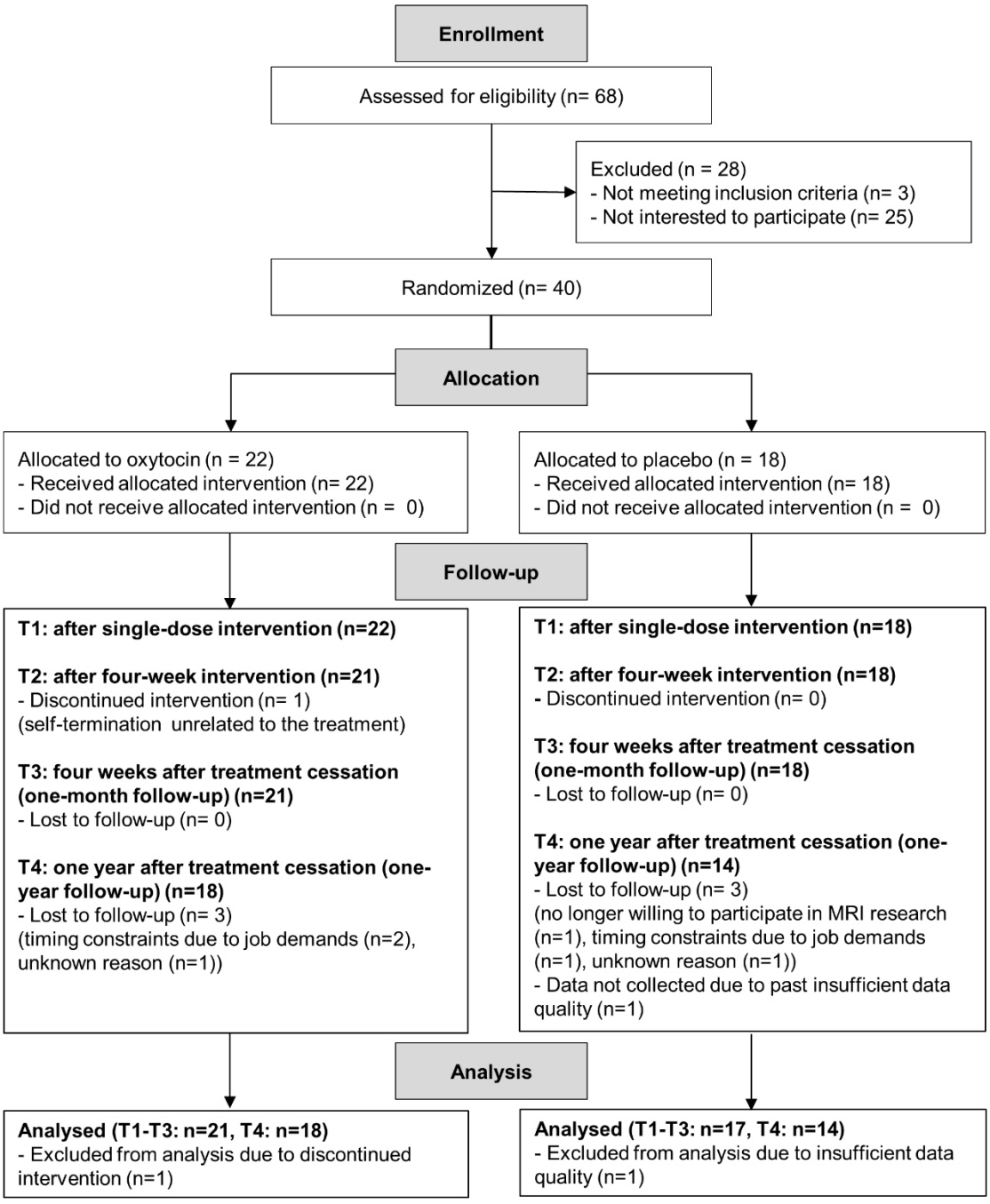


Figure 5.1. CONSORT Flow Diagram. MRI scanning was performed at baseline (T0); after a single-dose of OT or PL treatment (T1); after the four-week (OT/PL) treatment (T2); and at two follow-up sessions, four weeks (one month) (T3) and one year after cessation of the treatment (T4). One participant of the OT group discontinued the intervention after one week due to self-termination (unrelated to the treatment) and one participant of the placebo group completed the four-week treatment, but was excluded from analyses due to excessive in-scanner head motion (mean frame-wise displacement exceeding .50 mm.

2.2 Participants

Patients were mainly recruited from the Autism Expertise Centre at the Leuven University Hospital between April 2015 and December 2016. ASD diagnosis was made by a multidisciplinary

team based on the strict criteria of the DSM-IV-TR [50]. Prior to the treatment, the ADOS (Autism Diagnostic Observation Schedule) [51] and estimates of intelligence (6-subtest short-version of the Dutch Wechsler Adult Intelligence Scale-IV) [52] were acquired from all participants. Baseline symptom severity (ADOS), IQ-scores and age were not significantly different between participants randomized to receive the OT or placebo (PL) treatment (Table 5.1). Patients were also screened for medication use and comorbidities (Table 5.1, Supplementary Table 5.1). A detailed description of participant recruitment, eligibility criteria, patient characterizations and sample size is provided in Supplementary Methods.

Table 5.1. Participant characteristics

	Oxytocin	**Placebo**	**T-value**	**p-value**
Number of participants	21	17		
Age	24.76 ± 4.85	24.06 ± 5.54	0.42	0.68
Handedness	5 L/16 R	2 L/15 R		
IQ				
Total IQ	102 ± 13	107 ± 19	-1.08	0.29
VIQ	106 ± 9	111 ± 13	-1.60	0.12
PIQ	105 ± 18	104 ± 22	0.05	0.96
ADOS-2				
Total	7.2 ± 4.3	7.6 ± 3.9	-0.29	0.77
Communication	2.1 ± 1.4	2.2 ± 1.4	-0.20	0.84
Social interaction	5.1 ± 3.4	5.4 ± 3.1	-0.28	0.78
			Pearson Chi-square	**p**
Use of psychostimulant medication	5	2	.91	.34
Comorbidity	7	2	2.42	.12

Data are shown as mean ± standard deviation. L = Left-handed, R = Right-handed, IQ = Intelligence Quotient, VIQ = Verbal IQ, PIQ = Performance IQ, ADOS = Autism Diagnostic Observation Schedule. Detailed information on medication use and comorbidities is provided in Supplementary Table 5.1.

2.3 Intervention

Participants were assigned to receive the OT or PL treatment based on a computer-generated randomized order. Except for the manager of randomization, all research staff conducting the trial, participants and their parents and/or partners were blinded to treatment allocation. OT

(Syntocinon®, Sigma-tau) and PL (saline sodium-chloride solution) were administered in amber 15 ml glass bottles with metered pump (ACA Pharma). Each puff per nostril contained 4 international units (IU) of OT. Participants self-administered a daily dose of 24 IU (3 puffs/nostril) over four consecutive weeks (28 doses in total). Detailed information on administration instructions and side effect screening is provided in Supplementary Methods, Supplementary Table 5.2.

2.4 Functional MRI image acquisition

Anatomical and task-related fMRI images were acquired on a 3.0 Tesla Philips MR scanner (Best, The Netherlands) with an 8-channel phased-array head coil immediately after a single dose of OT treatment (task-based fMRI scanning was initiated approximately 45 min after administration) (T1), after four weeks of continual OT treatment (at least 24 hours after the final administration) (T2), and at two follow-up sessions, one month (four weeks) (T3) and one year post-treatment (T4). Detailed information on the MRI protocol and scanning parameters is provided in Supplementary Methods.

2.5 fMRI experimental paradigm

During task-based fMRI scanning, participants performed an emotion processing task (based on prior work from our lab [32,37,42,47,53]), involving the recognition of positive and negative emotional states (happiness, anger) from whole-body point-light displays (PLDs) (see [53] for a detailed description of the adopted PLDs)(Supplementary Figure 5.1A). During fMRI scanning, participants performed 6 blocks of the emotion task (12 trials/block, 4s each) interleaved with 6 blocks of a control task, involving detection of color changes in scrambled versions of the PLDs. Rest blocks (12s) were presented between task blocks (fixation on white cross). During each trial of the emotion task, participants were instructed to indicate as fast and accurate as possible whether the presented PLD figure conveyed happiness or anger by pressing the respective response buttons (happy or angry) (Supplementary Figure 5.1B). During the control task, participants were presented with a scrambled version of the PLDs in which one dot changed color to red or green at a random time point. Participants were again instructed to indicate as fast and accurate as possible the color of the dot by pressing the respective response buttons (green or red). No feedback was provided after the responses. Task instructions were provided verbally and on the monitor at the start of each block. Prior to scanning, participants practiced the tasks (12 trials/task). Reaction times and accuracy rates were recorded using E-Prime-software

(Psychological Software Tools). During each assessment session (T0, T1, T2, T3, T4), the same set of trials were presented in a randomized order.

2.6 fMRI data analysis

SPM8 (Wellcome Department of Imaging Neuroscience, University College London, UK) was used for image preprocessing and statistical analyses implemented in Matlab R2017b (Mathworks). Detailed information on preprocessing is provided in Supplementary Methods.

First-level analysis. For each subject, a general linear model was calculated with the time course of emotion and control blocks modeled as predictors and realignment parameters and task instructions as regressors of no interest. Separate epoch regressors were created for trials presenting happiness and anger. Contrast images were calculated for emotion (happiness) > control and emotion (anger) > control and were subjected to second-level random-effects models.

Group analyses. First-level contrast images were used to evaluate the effect of OT treatment on brain activity in predefined regions of interest (ROIs) (10-mm-radius spheres) centered over bilateral posterior superior temporal sulcus (pSTS) (MNI-coordinates [left: -55, -52, 12] [right: 55, -52, 10] based on [42]) a key social brain region implicated in point-light biological motion processing [43,54–59] and previously identified as an area of dysfunction in ASD [31,37–42,60]. Further, and in line with prior neuroimaging studies evaluating the effects of OT on brain activity [48], predefined ROIs centered over bilateral amygdala were also adopted to explore OT treatment effects (defined based on the FSL Harvard-Oxford subcortical atlas). Whole-brain one-sample t-test analysis identifying regions with brain activity during the emotion task (> control task) at the baseline session (T0) (across groups) ($p<.05$, familywise error (FWE) corrected for multiple comparisons) confirmed that both predefined ROIs (bilateral pSTS and amygdala) were reliably activated during the emotion task (Supplementary Figure 5.2 and Supplementary Table 5.3). To explore treatment-induced changes in average ROI activation levels (extracted using the MarsBar toolbox for SPM [61]), changes from baseline were calculated (separately for each assessment session (T1, T2, T3, T4)) and subjected to repeated-measures mixed-effects analyses. Single-dose administration effects were explored using a mixed-effects model with 'Subject' as random factor and the factors 'Treatment' (OT, PL), 'Emotion' (Happiness, Anger), 'Hemisphere' (Left, Right) and interactions with 'Treatment' as fixed factors. To assess (long-term) effects of the multiple-dose treatment, a mixed-effects model was adopted with 'Subject' as random factor and the factors 'Treatment' (OT, PL), 'Session' (T2, T3, T4), 'Emotion' (Happiness, Anger), 'Hemisphere' (Left, Right) and interactions with 'Treatment' as fixed factors. For each ROI, the activation levels (raw contrast estimates) are reported in Supplementary Table 5.4, separately for each treatment group and

assessment session. For completeness and hypothesis-generating purposes, treatment-induced group differences were also explored at the whole-brain level (Supplementary Table 5.5).

2.7 Behavioral data analysis

Performance accuracy (percentage of correct answers) and correct reaction times on the emotion task were assessed for each participant and assessment session, and a performance variable (accuracy divided by reaction time) was calculated. Note that across participants, a few trials of the emotion task were lost due to 'no recorded response' (T0: 3.58%, T1: 1.17%, T2: 2.12%, T3: 0.95%, T4: 1.87%). Similar to the neural analyses, mixed-effects analyses were performed to assess treatment-induced changes in behavioral performance. All statistics were performed with Statistica 8 (StatSoft. Inc. Tulsa, USA). The significance level was set at $p<.05$. Post-hoc analyses were Bonferroni-corrected for multiple comparisons.

3 Results

3.1 Behavioral effects of OT treatment

Mixed-effect analyses assessing treatment effects in behavioral performance on the PLD emotion recognition task revealed a main effect of session ($F(3,246)=4.99$, $p=.002$), indicating that both treatment groups showed improved performance with repeated presentation of the emotion task (Supplementary Table 5.6). However, no significant main effect of treatment ($F(1,246)=2.53$, $p=.12$), nor a treatment-by-session interaction ($F(3,246)=.45$, $p=.72$) was revealed. See Supplementary Table 5.7 for raw behavioral outcome measures.

3.2 Neural effects of single-dose OT treatment

Posterior superior temporal sulcus (pSTS). Mixed-effects analyses revealed a main effect of treatment ($F(1,110)=5.33$, $p=.03$, $d=.74$), indicating a significant increase in brain activity of the pSTS during emotion processing from point-light biological motion after a single dose of OT (Figure 5.2, Table 5.2). No significant effects of hemisphere ($F(1,110)=1.83$, $p=.18$) or emotion ($F(1,110)=1.10$, $p=.30$), nor interactions of these factors with treatment (treatment-by-hemisphere: $F(1,110)=.03$, $p=.87$; treatment-by-emotion: $F(1,110)=.81$, $p=.37$) were revealed, indicating that the main effect of treatment was evident irrespective of pSTS region (left, right) or processed emotional state (happiness, anger).

Amygdala. Amygdala activity during PLD emotion processing was not significantly altered after a single dose of OT (F(1,110)=.01, p=.92, d=.03). Also no significant main effects of hemisphere (F(1,110)=1.13, p=.29) or emotion (F(1,110)=1.19, p=.28) were revealed. As seen in Figure 5.3, attenuated amygdala activity was evident in both the OT and PL treatment groups. However, a significant treatment-by-emotion interaction was observed (F(1,110)=10.11, p=.002), indicating that in the OT group, amygdala attenuation was evident irrespective of emotional state (happiness versus anger: $p_{Bonferroni}$=1.00), while in the PL group, amygdala attenuation was more pronounced upon presentation of point-light biological motion conveying a negative (anger) compared to a positive (happiness) emotional state ($p_{Bonferroni}$=.02) (Supplementary Figure 5.5.a).

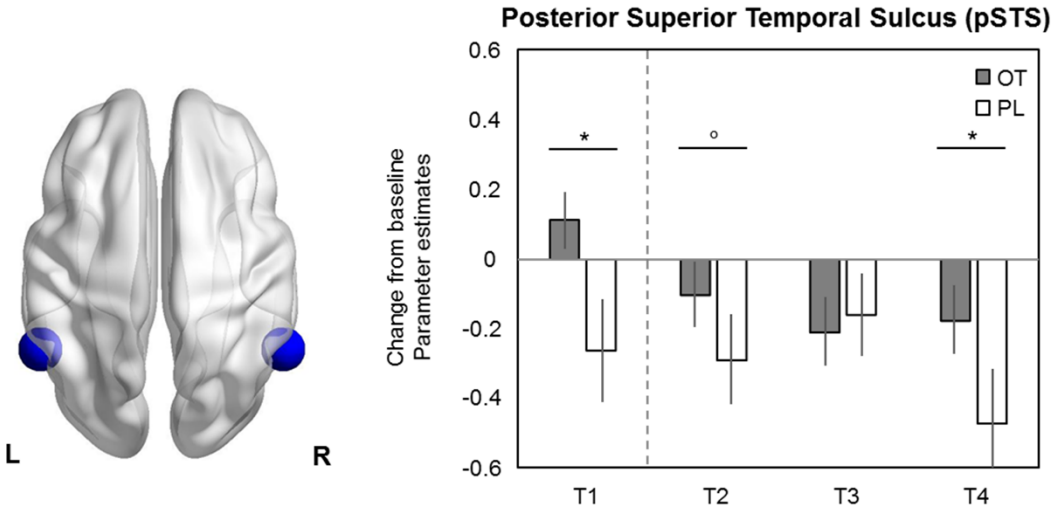


Figure 5.2. Oxytocin-induced changes (from baseline) in activity of bilateral posterior superior temporal sulcus (pSTS). Mean changes from baseline in pSTS activation during the processing of emotional states from point-light biological motion are visualized separately for the oxytocin (OT) and placebo (PL) treatment groups at assessment session T1 (after a single dose of treatment), T2 (immediately after the 4-week treatment), T3 (at follow-up, one month post-treatment) and T4 (at follow-up, one year post-treatment). Vertical bars denote ± standard errors. The asterisks (*) indicate Cohen's d > .50 (medium-sized effect). The circle (°) indicates a Cohen's d >.20 (small-sized effect) [62].

3.3 Neural effects of multiple-dose OT treatment

pSTS. A treatment-by-session interaction was revealed (F(2,386)=3.51, p=.03), indicating a significant effect of the multiple-dose treatment on pSTS activity at the one-year follow-up session (T4: $p_{Bonferroni}$=.03, d=.62), but not immediately after the multiple-dose treatment (T2: $p_{Bonferroni}$=.49, d=.39) nor at the follow-up session, one month post-treatment (T3: $p_{Bonferroni}$=1.00, d=-.10). PSTS activity during PLD emotion processing attenuated in both treatment arms, but the attenuation tended to be more pronounced in the PL compared to the OT group (Figure 5.2). Note that a treatment-by-hemisphere interaction was revealed at trend level (F(1,386)=3.10, p=.079),

indicating that the overall effect of treatment (across sessions) was significant for right pSTS (OT versus PL: $p_{Bonferroni}$=.014), but not for left pSTS ($p_{Bonferroni}$=1.00) (Table 5.2).

Amygdala. A main effect of treatment was revealed across assessment sessions (F(1,386)=4.41, p=.04), indicating that the multiple-dose OT treatment induced an overall attenuation in amygdala activity which lasted up to one month (T3: d=-.49) and even one year post-treatment (T4: d=-.77) (no significant treatment-by-session interaction was revealed: F(2,386)=.49, p=.61) (Figure 5.3, Table 5.2). Similar to the single-dose analysis, a tentative treatment-by-emotion interaction was revealed (F(1,386)=3.65, p=.056), indicating that in the OT group, amygdala attenuation was evident irrespective of emotional state (happiness versus anger: $p_{Bonferroni}$=1.00), whereas in the PL group, amygdala attenuation was more pronounced upon presentation of point-light biological motion conveying the negative (anger) compared to the positive (happiness) emotional state ($p_{Bonferroni}$=.02). (Supplementary Figure 5.5.b).

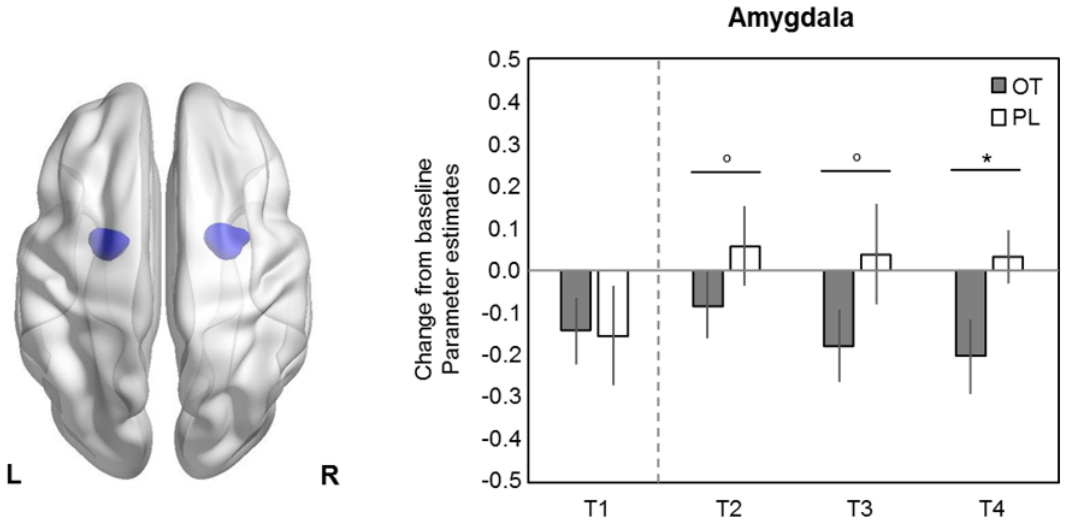


Figure 5.3. Oxytocin-induced changes (from baseline) in activity of bilateral amygdala. Mean changes from baseline in amygdala activation during the processing of emotional states from point-light biological motion are visualized separately for the oxytocin (OT) and placebo (PL) treatment groups at assessment session T1 (after a single dose of treatment), T2 (immediately after the 4-week treatment), T3 (at follow-up, one month post-treatment) and T4 (at follow-up, one year post-treatment). Vertical bars denote ± standard errors. The asterisks (*) indicate Cohen's d > .50 (medium-sized effect). The circle (°) indicates a Cohen's d >.20 (small-sized effect)[62].

Table 5.2. Mean change-from-baseline parameter estimates for each treatment group (oxytocin, placebo), session (T1, T2, T3, T4), and ROI.

Region of interest		Oxytocin		Placebo		Group difference effect size
		N	Mean ± SD	N	Mean ± SD	Cohen's d
T1	**Single-dose effect**					
	pSTS	21	.11 ± .38	17	-.26 ± .61	.74
	Left pSTS	21	.07 ± .41	17	-.32 ± .56	.78
	Right pSTS	21	.15 ± .47	17	-.21 ± .81	.55
	Amygdala	21	-.15 ± .36	17	-.16 ± .50	.03
	Left amygdala	21	-.11 ± .30	17	-.13 ± .43	.06
	Right amygdala	21	-.18 ± .47	17	-.19 ± .62	.01
T2	**Multiple-dose effect**					
	pSTS	21	-.10 ± .44	17	-.29 ± .52	.39
	Left pSTS	21	-.09 ± .60	17	-.31 ± .49	.41
	Right pSTS	21	-.11 ± .42	17	-.27 ± .72	.26
	Amygdala	21	-.09 ± .36	17	.06 ± .39	-.37
	Left amygdala	21	-.07 ± .34	17	.07 ± .46	-.36
	Right amygdala	21	-.10 ± .45	17	.04 ± .35	-.34
T3	**One-month retention effect**					
	pSTS	21	-.21 ± .46	17	-.16 ± .49	-.10
	Left pSTS	21	-.27 ± .63	17	-.08 ± .56	-.32
	Right pSTS	21	-.15 ± .41	17	-.24 ± .68	.16
	Amygdala	21	-.18 ± .40	17	.04 ± .49	-.49
	Left amygdala	21	-.13 ± .39	17	.03 ± .53	-.34
	Right amygdala	21	-.24 ± .47	17	.04 ± .51	-.56
T4	**One-year retention effect**					
	pSTS	18	-.18 ± .42	14	-.48 ± .53	.62
	Left pSTS	18	-.27 ± .57	14	-.38 ± .40	.23
	Right pSTS	18	-.09 ± .47	14	-.57 ± .80	.73
	Amygdala	18	-.21 ± .36	14	.03 ± .24	-.77
	Left amygdala	18	-.17 ± .41	14	-.01 ± .29	-.45
	Right amygdala	18	-.25 ± .43	14	.07 ± .24	-.91

Note: SD = Standard deviation, pSTS = posterior Superior Temporal Sulcus. Cohen's d effect sizes (change from $baseline_{OT}$ –change from $baseline_{PL}$)/pooled SD) are reported where 0.2 is indicative of a small effect, 0.5 a medium effect and 0.8 a large effect [62].

3.4 Association between neural and behavioral effects

While no behavioral improvements were observed after the OT treatment in terms of emotion recognition from point-light biological motion, we previously reported treatment-specific behavioral improvements until one month and even one year post-treatment in repetitive behaviors (assessed with the Repetitive Behavior Scale – Revised (RBS)[63]) and feelings of attachment avoidance (assessed with the State Adult Attachment Measure (SAAM)[64]) induced by the multiple-dose OT treatment (assessed within the same participant sample) (Bernaerts et al., under review). Also improvements in social functioning (assessed with the Social Responsiveness Scale (SRS)[65]) were identified, but these improvements were evident in both treatment arms.

Here, exploratory analyses were performed to assess whether these behavioral improvements were related to the observed neural effects in terms of treatment-induced changes in amygdala and (right) pSTS activity.

OT-induced decreases in amygdala activity (i.e. at sessions T2, T3 and T4) were shown to be associated with self-reported improvements in social functioning as assessed with the SRS (β=.32; t(53)=2.42; p=.02) (multiple-regression across sessions, corrected for session and subject) (Figure 5.4) (note that the relationship remained significant after removal of four influential data points (indicated in grey): β=.38; t(50)=2.93; p=.005). Inter-individual differences in treatment-induced improvements in repetitive behaviors and attachment avoidance were however not explicitly associated with changes in amygdala or right pSTS activity (all p>.05; Supplementary Table 5.8).

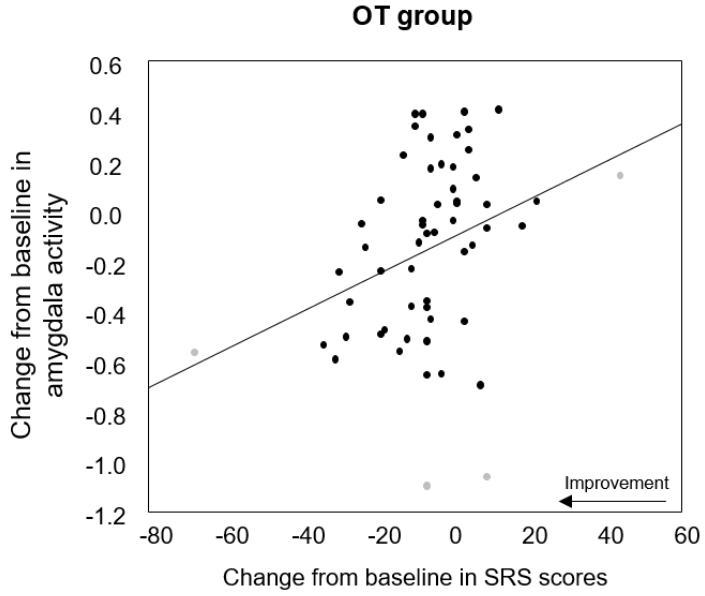


Figure 5.4. Association between neural changes in amygdala activity and behavioral improvements in social functioning. The relationship between changes (from baseline) in amygdala activity (y-axis) and behavioral improvements in social functioning (Social Responsiveness Scale (SRS)) (x-axis) is visualized. Participants of the OT group with the greatest improvement in social functioning (reduction in SRS scores indicates improvement) showed the strongest attenuation of amygdala activity (across assessment sessions T2, T3 and T4). Influential data points are indicated in grey.

4 Discussion

In the current study, we investigated the effects of single- and multiple-dose OT treatment on neural activation during the processing of emotional states from point-light biological motion in adult men with ASD. The multiple-dose treatment induced decreases in bilateral amygdala activity and sustained higher levels of task-related activity within (right) posterior superior temporal sulcus (pSTS). Importantly, these treatment-induced neural changes were shown to outlast the intervention until one month and even one year post-treatment, indicating that continual OT treatment can induce long-lasting neural changes in core social brain regions.

The observation of increased activation in pSTS regions after single-dose OT administration is consistent with a prior study showing enhanced pSTS recruitment during biological motion processing following OT administration in children with ASD [22]. Similarly, Gordon et al. (2013) showed that single-dose OT administration specifically increased activity in left pSTS during social judgements, but not during nonsocial judgments [23]. Recent meta-analyses of single-dose administration studies including healthy and patient populations consistently identified pSTS regions (middle and superior temporal gyrus) as robust areas of activation after OT administration [21,48,66]. In the current study, we extend findings from these single-dose administration studies by showing that (right) pSTS activation is also altered after multiple-dose treatment with OT. While pSTS activation was generally attenuated in both treatment groups upon repeated presentation of the PLD emotion stimuli, the OT treatment tended to reduce this attenuation/habituation, particularly at the follow-up session, one year post-treatment. Considering its implicated role in social perception, emotion processing and theory of mind, dysfunction of pSTS regions (particularly right pSTS) has been highlighted as an important neural substrate for the socio-emotional difficulties characteristic of ASD (reviewed in [67]). For example, compared to typical controls, individuals with ASD have been shown to display reduced pSTS activity upon biological motion processing tasks in previous studies from our lab [37,42] and other groups [31,38–41,44]. The current observation of OT-specific effects in pSTS highlights the potential of OT treatment for alleviating dysfunctions in this core social brain area. This notion is in line with the social salience hypothesis of OT, suggesting that OT may affect social functioning by enhancing the neural processing of socially-relevant stimuli [68]. While we revealed no significant behavioral effects of the OT treatment in terms of PLD emotion processing, previous OT administration studies have demonstrated improved biological motion perception after a single dose of OT [45–47]. In particular, using a similar task, prior work from our group showed that single-dose OT administration improved the recognition of emotional states from PLD when different sets of PLD stimuli were presented pre- and post-treatment [47]. In the current study, however, the same set of

PLD stimuli was presented at each assessment session (albeit in a randomized order), potentially causing treatment-related improvements in emotion recognition to be masked by general learning effects (i.e., both treatment groups showed significant improvements over time).

In terms of amygdala activity, our study revealed that multiple-dose OT treatment (not single-dose administration) significantly decreased bilateral amygdala activity (compared to PL) until one month and even one year post-treatment. From animal research, OT has been implicated in reducing amygdala reactivity through inhibitory GABAergic interneurons [69], a notion that is overall supported by previous human task-based fMRI studies showing attenuated amygdala responses after single-dose OT administration [70–92](in ASD [25,28]). However, a number of studies have also reported increased amygdala activation following OT administration [77,81,82,87,93–99] (in ASD patients: [6,22,24]), and several person-dependent (e.g., sex, psychopathology), and/or contextual factors (e.g., task type, stimulus valence, dose) have been put forward as potential sources of variability (reviewed in [48,66]). Mechanistically, OT's impact on human brain function has been proposed to involve a bottom-up anxiolytic effect to facilitate social approach behavior, and a top-down social salience effect to facilitate attention to, and perception of social signals [68,100,101]. Since the amygdala form an integral part of the threat-processing circuit, as well as the (social) salience network, opposite directions of OT's effect on amygdala activity have been interpreted to reflect this dual action [101]. While increased amygdala reactivity following OT administration may reflect a neural mechanism for facilitating attention to, and processing of salient (social) cues, attenuation of amygdala reactivity is anticipated to reflect OT's anxiolytic role in downregulating negative affect, social withdrawal and distress. Within the current study, we observed an overall pattern of attenuated amygdala reactivity after multiple-dose treatment with OT, and importantly, showed that the extent of amygdala attenuation was associated with the extent of (long-term) improvements in self-reports of social functioning as assessed with the Social Responsiveness Scale. In line with previous literature, we interpret the observed attenuating effects on amygdala activity to reflect OT's anxiolytic role for promoting social approach behavior, a notion that is supported by the observed association between amygdala attenuation and the extent of improvements in social functioning. At least within a subset of individuals with ASD, interactions with the social environment may be experienced as 'over-arousing' or even threatening due to excessive amygdala reactivity upon environmental socio-emotional cues. However, a recursive halting of this excessive amygdala reactivity from the repeated administrations of OT may have relieved individuals from this over-arousal, thereby allowing an increased propensity to socially engage. In turn, it can be anticipated that the resulting enhancement of 'positive' social experiences may have further contributed to an adaptive and recursive re-shaping of amygdala functioning in an experience-dependent manner (hence the observation of long-lasting neural and behavioral changes up to one year post-treatment). Since this is the first multiple-dose

administration study directly investigating changes in amygdala functioning following OT treatment, future studies may be warranted to explore whether the observed long-term attenuating effects up to one year post-treatment are specific to (male) adults with ASD, or whether they will generalize across gender, other (patient) populations and developmental stages. Indeed, meta-analyses of single-dose administration studies, have highlighted gender and psychopathology as important factors for moderating the directionality of single-dose OT's effects on brain function, indicating, for example, more consistent amygdala attenuation in men compared to women [21,101].

In the current study, effect sizes of the multiple-dose treatment were qualitatively different compared to the identified single-dose effect sizes. In terms of pSTS activity, the effect was most pronounced after single-dose administration, whereas in terms of amygdala attenuation, the effect was most pronounced after the multiple-dose treatment (Table 5.2). These observations provide indications that the neural effects after single-dose compared to multiple-dose OT treatment are different, and importantly, that the differentially induced single-dose versus multiple-dose effects may be region- or network-specific. In a prior multiple-dose administration study, Watanabe et al. (2015) investigated the neural effects of a six-week OT treatment on anterior cingulate and prefrontal cortex activity, but in this study, effect sizes of the multiple-dose treatment were found to be qualitatively similar to effect sizes of a previous single-dose administration study [25]. However, MRI scanning for assessing the 6-week treatment effect was performed only 15 or 40 min after the last nasal spray administration. Since this period corresponds to the optimal time frame for assessing acute, single-dose effects of OT administration, the possibility cannot be ruled out that the reported multiple-dose effect - at least in part - reflected an acute effect of exogenously administered OT. In contrast, in the current study, MRI scanning for assessing the multiple-dose effect (T2) was performed at least 24 hours after the last OT administration, rendering the observed neural effects at session T2 less susceptible to reflect an acute effect of OT administration. In addition, the neural changes at the one-month and one-year follow-up sessions are interpreted to solely reflect long-lasting adaptations in neural functioning due to OT's recursive action on these circuits.

Accordingly, our study highlights that in addition to dose, future clinical trials should be directed at identifying the optimal administration length and intervals, preferably adopting parallel, between-subject designs, as opposed to crossover designs with wash-out period. Also the possibility that OT's action may be further enlarged by simultaneously administering targeted behavioral therapies aimed at enhancing socio-communicative skills holds promise and should be addressed in future trials.

To conclude, our study revealed that multiple-dose OT treatment is effective for inducing long-lasting adaptations in core social brain regions (pSTS and amygdala) that outlast the four-week period of actual OT administration until one month and even one year post-treatment. These observations hold important clinical implications for ASD and other neuropsychiatric conditions for which OT is considered as a potential treatment.

Supplements

Supplementary Tables

Supplementary Table 5.1. Detailed information on comorbidities and medication use for participants of the oxytocin and placebo treatment groups.

Comorbidities were screened through self-report (with the explicit mentioning of examples in the screening interview including e.g., ADHD, depression, dyscalculia, dyslexia). Current psychoactive medication use was defined as use within three months before study enrolment.

		Comorbidities	Medication use
Oxytocin		**N=7**	**N=5**
	i	ADHD	Abilify, Tegretol
	ii	ADHD, Dyslexia	/
	iii	ADHD, Depression	/
	iv	Depression	Welbutrine XR, Leviron, Cymbalta
	v	Depression, ADD	Trazodone Mylan, Medikinet
	vi	Bipolar Disorder	Maniprex, Bellozal, Mometasone
	vii	Dyslexia	/
	viii	/	Risperdal, Venlafaxine
Placebo		**N=2**	**N=2**
	i	ADHD	/
	ii	ADHD	/
	iii	/	Zolpidem, Remergon, Rilatine
	iv	/	Trazodone, Escitalopram

ADD = Attention Deficit Disorder, ADHD = Attention Deficit Hyperactivity Disorder.

Supplementary Table 5.2. Side effect screening

Participants administered the nasal spray (oxytocin (OT) or placebo (PL)) daily for four consecutive weeks. At the end of each week, participants were asked to report whether they presented any of the listed (or other) side effects and to indicate the severity of the side effect (mild, moderate, or severe). **Panel A** lists the proportion of OT or PL participants (%) that reported any mild, moderate or severe side effects (averaged across effects). **Panel B** lists, separately for each side effect, the proportion of OT or PL participants that reported the side effect (averaged across severity level (mild, moderate, severe)). No significant group differences were revealed in the total proportion of reported side effects (**Panel A**), or separately for each side effect (**Panel B**). Note that tentatively more 'mild' effects were reported by participants of the PL-group after week 4 of the treatment (p= .07) (indicated in italic in **Panel A**). Closer inspection showed that a tentatively larger proportion of PL participants reported to feel 'more calm, relaxed, comfortable' (p= .052) after week 2, 3 and 4 of the treatment and 'more confident' (p= .052) after week 4 (indicated in italic in **Panel B**). In addition, a marginal effect was revealed for the side effect 'dry throat/dry mouth' (p= .07), indicating that a tentatively larger proportion of OT participants reported this side effect after week 1 (indicated in italic in **Panel B**). Overall, the most frequent reported side effect was 'running nose', but the proportion of OT (23.8%) and PL (11.8%) participants reporting this effect was not significantly different (p= .35).

Panel A	**OT (%)**			**PL (%)**			**Group Difference (p-value)**		
	Mild	Moderate	Severe	Mild	Moderate	Severe	Mild	Moderate	Severe
Week 1	47.6	19.0	9.5	29.4	5.9	5.9	0.26	0.24	0.68
Week 2	38.1	9.5	0.0	29.4	11.8	5.9	0.58	0.82	0.27
Week 3	19.0	9.5	0.0	41.2	17.6	5.9	0.14	0.47	0.27
Week 4	23.8	9.5	0.0	52.9	11.8	5.9	*0.07*	0.82	0.27
Across weeks	32.1	11.9	2.4	38.2	11.8	5.9	0.70	0.99	0.58

Panel B	**Week 1**			**Week 2**			**Week 3**			**Week 4**		
	OT (%)	**PL (%)**	**p-value**	**OT (%)**	**PL (%)**	**p-value**	**OT (%)**	**PL (%)**	**p-value**	**OT (%)**	**PL (%)**	**p-value**
Headache	4.8	0.0	0.37	0.0	0.0	1.00	0.0	0.0	1.00	0.0	5.9	0.27
Drowsiness	4.8	0.0	0.37	4.8	5.9	0.88	4.8	0.0	0.37	4.8	0.0	0.37
Dizziness	4.8	0.0	0.37	4.8	0.0	0.37	0.0	0.0	1.00	0.0	0.0	1.00
Fainting	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Changes in heart rate or palpitations	0.0	0.0	1.00	0.0	0.0	1.00	4.8	0.0	0.37	0.0	0.0	1.00
Shortness of breath	0.0	5.9	0.27	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Fever	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Sore throat	9.5	0.0	0.20	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Dry throat/dry mouth	19.0	0.0	*0.07*	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Hoarseness	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Coughing	4.8	5.9	0.88	4.8	0.0	0.37	0.0	0.0	1.00	0.0	0.0	1.00
Coughing up mucus	4.8	5.9	0.88	9.5	5.9	0.68	4.8	5.9	0.88	4.8	5.9	0.88
Congested nose	9.5	17.6	0.47	4.8	5.9	0.88	9.5	0.0	0.20	4.8	5.9	0.88
Sneezing	9.5	0.0	0.20	0.0	5.9	0.27	0.0	5.9	0.27	4.8	0.0	0.37
Nasal irritation	4.8	11.8	0.43	4.8	11.8	0.43	4.8	17.6	0.21	9.5	17.6	0.47
Runny nose	23.8	11.8	0.35	14.3	5.9	0.41	23.8	5.9	0.14	9.5	17.6	0.47
Burning sensation in nose and/or ears	9.5	5.9	0.68	4.8	0.0	0.37	0.0	5.9	0.27	0.0	5.9	0.27
Sensitive to fragrances	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Watery eyes	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Nausea and/or vomiting	4.8	0.0	0.37	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Abdominal or stomach pain	4.8	0.0	0.37	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Decreased appetite	9.5	0.0	0.20	4.8	0.0	0.37	0.0	0.0	1.00	0.0	0.0	1.00
Hungry	0.0	5.9	0.27	0.0	0.0	1.00	0.0	0.0	1.00	0.0	5.9	0.27
Constipation	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Diarrhea	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Muscle pain/cramps	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00

Skin rash	0.0	0.0	1.00	0.0	0.0	1.00	0.0	5.9	0.27	0.0	0.0	1.00
Increased fluid intake	0.0	5.9	0.27	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Water retention/bloating	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Insomnia/sleep difficult	0.0	5.9	0.27	0.0	0.0	1.00	0.0	0.0	1.00	4.8	0.0	0.37
Nightmares	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Staring/daydreams	4.8	0.0	0.37	4.8	0.0	0.37	0.0	0.0	1.00	0.0	0.0	1.00
Anaphylaxis	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Changes in perception of the tongue	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Back pain	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Bed wetting	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Weight gain	0.0	0.0	1.00	4.8	0.0	0.37	0.0	0.0	1.00	0.0	5.9	0.27
Sweating	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Blurred vision	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Less talk to others	0.0	0.0	1.00	4.8	0.0	0.37	0.0	0.0	1.00	0.0	0.0	1.00
Uninterested in others	0.0	0.0	1.00	0.0	5.9	0.27	0.0	0.0	1.00	0.0	0.0	1.00
Persistent thoughts and/or feelings	4.8	0.0	0.37	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Development of repetitive behavior	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Increase in repetitive behavior	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Nail biting	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Annoyed. bored	4.8	0.0	0.37	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Sad	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00	0.0	0.0	1.00
Prone to crying. more emotional	0.0	0.0	1.00	0.0	0.0	1.00	4.8	0.0	0.37	4.8	0.0	0.37
Anxious. worried. discomfort	0.0	5.9	0.27	0.0	5.9	0.27	0.0	11.8	0.12	0.0	5.9	0.27
Happy. satisfied	4.8	11.8	0.43	4.8	17.6	0.21	4.8	11.8	0.43	4.8	11.8	0.43
Euphoric. unusually happy.	0.0	11.8	0.12	0.0	11.8	0.12	0.0	5.9	0.27	4.8	5.9	0.88
Calm. relaxed. comfortable	4.8	11.8	0.43	0.0	17.6	*0.052*	0.0	17.6	*0.052*	0.0	17.6	*0.052*
More focused	0.0	11.8	0.12	0.0	5.9	0.27	0.0	11.8	0.12	0.0	11.8	0.12
More confidence	0.0	11.8	0.12	0.0	11.8	0.12	0.0	11.8	0.12	0.0	17.6	*0.052*

Supplementary Table 5.3.

Baseline activated brain regions across groups during emotion processing from point-light displays (vs control).

Hemisphere	Anatomical label	Peak MNI coordinates			Cluster size	T-value
		X	Y	Z		
R	Inferior temporal gyrus	46	-70	-8	7214	14.59
L	Fusiform gyrus	-42	-42	-20	4757	15.39
R	Inferior frontal - including anterior insula	52	34	14	4189	13.16
L	Inferior frontal - including anterior insula	-46	28	18	3440	12.04
L	Prefrontal - supplementary motor area	-2	24	50	850	10.99
L	Inferior parietal - intraparietal sulcus	-30	-54	42	522	7.35
R	Cerebellum	14	-76	-34	342	9.03
R	Amygdala	28	-4	-16	72	6.64
L	Amygdala	-26	-4	-20	45	7.11
L	Inferior parietal – supramarginal gyrus	-50	-36	24	37	6.30
R	Inferior temporal gyrus - fusiform face area	38	-4	-40	14	7.26
L	Medial temporal gyrus – including posterior superior temporal sulcus	-30	-4	-38	17	7.06

Whole brain one-sample analysis: p<.05, familywise error-corrected; Extent threshold: k=10 voxels. MNI= Montreal Neurological Institute. L= Left. R= Right.

Supplementary Table 5.4.

Average contrast estimates for each region of interest (posterior superior temporal sulcus (pSTS), amygdala), treatment group (oxytocin, placebo) and assessment session (T0, T1, T2, T3, T4).

Region of interest		Oxytocin		Placebo	
		N	Mean ± SD	N	Mean ± SD
T0	**Baseline***				
	Posterior Superior Temporal Sulcus	21	.89 ± .69	17	.65 ± .66
	Amygdala	21	.29 ± .31	17	.10 ± .39
T1	**Single-dose effect**				
	Posterior Superior Temporal Sulcus	21	1.00 ± .80	17	.39 ± .80
	Amygdala	21	.14 ± .34	17	-.06 ± .55
T2	**Multiple-dose effect**				
	Posterior Superior Temporal Sulcus	21	.79 ± .74	17	.36 ± .63
	Amygdala	21	.20 ± .31	17	.15 ± .44
T3	**One-month retention effect**				
	Posterior Superior Temporal Sulcus	21	.68 ± .77	17	.49 ± .70
	Amygdala	21	.11 ± .40	17	.13 ± .52
T4	**One-year retention effect**				
	Posterior Superior Temporal Sulcus	18	.75 ± .62	14	.30 ± .65
	Amygdala	18	.13 ± .34	14	.15 ± .56

Note: SD = Standard deviation. *No significant baseline differences were evident between treatment groups (pSTS: $t(36)=1.10$, $p=.28$; amygdala: $t(36)=1.69$, $p=.10$).

Supplementary Table 5.5.

For completeness and hypothesis-generating purposes, treatment-induced changes in brain activation were also explored at the whole-brain level. To do so, two-sample t-tests were implemented using a whole-brain $p < 0.001$ voxelwise threshold (uncorrected). The two-sample t-test analyses were masked to only include regions that were activated by the emotion task (> control task) at baseline across both groups (mask, thresholded at FWE$p < 0.05$).

Hemi	Anatomical label	Peak MNI coordinates			Cluster size	T-value
		X	Y	Z		
Single-dose effect						
(EmoT0 < EmoT1) x (OT > PL)						
R	Inferior frontal gyrus pars opercularis	32	10	30	31	4.87
R	Cerebellum	28	-44	-28	13	3.68
(EmoT0 < EmoT1) x (OT < PL): no activated regions						
Multiple-dose effect						
(EmoT0 < EmoT2) x (OT > PL): no activated regions						
(EmoT0 < EmoT2) x (OT < PL): no activated regions						
One month retention effect						
(EmoT0 < EmoT3) x (OT > PL): no activated regions						
(EmoT0 < EmoT3) x (OT < PL): no activated regions						
One year retention effect						
(EmoT0 < EmoT4) x (OT > PL)						
L	Superior parietal gyrus	-36	-48	58	18	3.85
R	Middle temporal gyrus	52	-46	-8	14	3.70
R	Postcentral gyrus	42	-34	58	10	3.61
(EmoT0 < EmoT4) x (OT < PL)						
R	Brainstem	4	-30	-46	17	4.03

Whole brain two-sample analyses: p<.001, uncorrected; Extent threshold: k=10 voxels. Hemi= Hemisphere, MNI= Montreal Neurological Institute. OT= Oxytocin, PL=Placebo, L= Left. R= Right.

Supplementary Table 5.6.

Mean change-from-baseline scores in behavioral performance (recognition of emotional states from point-light biological motion) listed separately for each treatment group (oxytocin, placebo), assessment session (T1, T2, T3, T4) and emotional state (happiness, anger).

Assessment session		Oxytocin		Placebo	
		N	Mean ± SD	N	Mean ± SD
T1	**Single-dose effect**				
	Happiness	21	3.4 ± 5.5	17	6.6 ± 7.3
	Anger	21	1.9 ± 7.9	17	5.5 ± 6.1
T2	**Multiple-dose effect**				
	Happiness	21	4.4 ± 8.1	17	7.6 ± 8.1
	Anger	21	3.5 ± 6.4	17	6.4 ± 5.0
T3	**One-month retention effect**				
	Happiness	21	7.5 ± 6.4	17	10.9 ± 9.9
	Anger	21	5.7 ± 9.9	17	6.2 ± 5.9
T4	**One-year retention effect**				
	Happiness	18	6.2 ± 5.4	14	11.2 ± 11.4
	Anger	18	8.6 ± 10.5	14	5.8 ± 6.1

Note: SD = Standard deviation. Behavioral performance was assessed by calculating a performance variable (accuracy rates (in percentages) divided by reaction times (in seconds)).

Supplementary Table 5.7.

Raw behavioral performance scores listed separately for each treatment group (oxytocin, placebo), assessment session (T0, T1, T2, T3, T4) and emotional state (happiness, anger).

Assessment session		Oxytocin		Placebo	
		N	Mean ± SD	N	Mean ± SD
T0	**Baseline***				
	Happiness	21	34 ± 10	17	31 ± 7
	Anger	21	.033 ± 9	17	31 ± 7
T1	**Single-dose effect**				
	Happiness	21	37 ± 11	17	38 ± 10
	Anger	21	35 ± 11	17	37 ± 10
T2	**Multiple-dose effect**				
	Happiness	21	38 ± 11	17	39 ± 10
	Anger	21	37 ± 11	17	38 ± 10
T3	**One-month retention effect**				
	Happiness	21	41 ± 12	17	42 ± 12
	Anger	21	39 ± 11	17	37 ± 9
T4	**One-year retention effect**				
	Happiness	18	39 ± 10	14	43 ± 12
	Anger	18	41 ± 13	14	36 ± 9

SD = Standard deviation. Behavioral performance was assessed by calculating a performance variable (accuracy rates (in percentages) divided by reaction times (in seconds)).*No significant baseline differences were evident between treatment groups (happiness: $t(36)=.97$, $p=.34$; anger: $t(36)=.86$, $p=.40$).

Supplementary Table 5.8.

Association between neural changes (right posterior temporal sulcus/amygdala activity) and behavioral improvements in social functioning, repetitive behaviors and feelings of avoidant attachment induced by the oxytocin treatment.

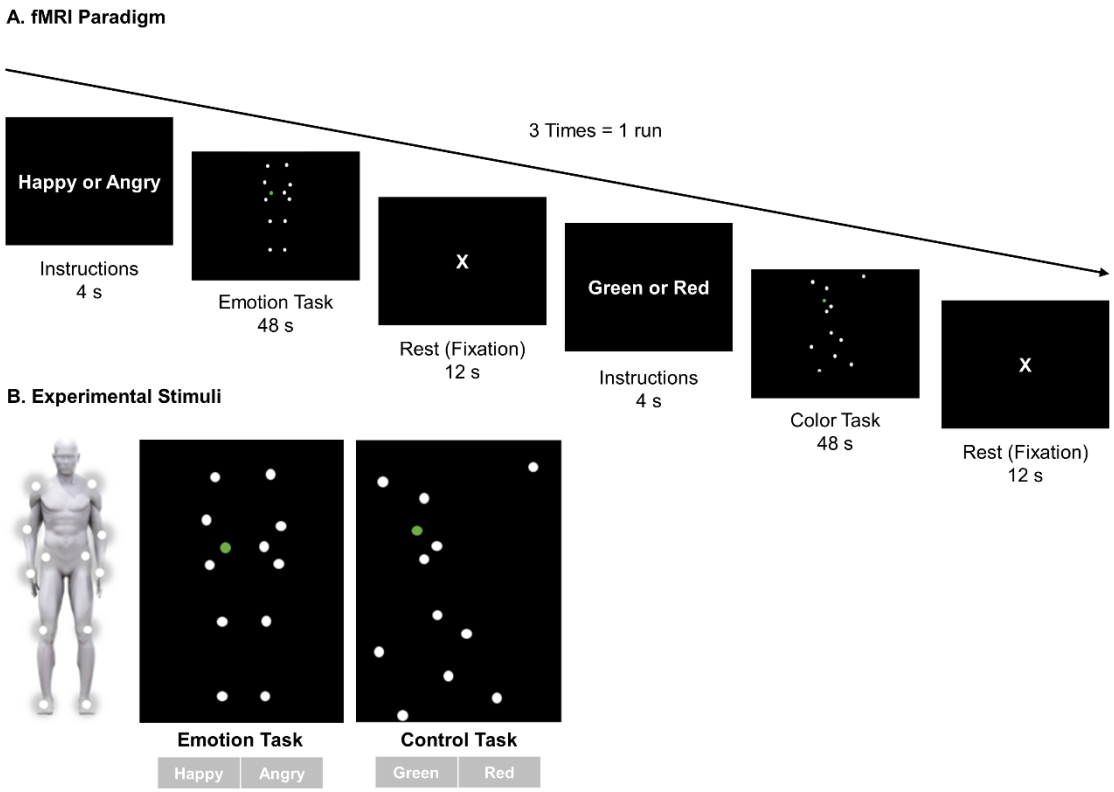
As outlined in detail in Bernaerts et al. (under review), social functioning was assessed using a self-report version of the Social Responsiveness Scale (SRS)[65]; repetitive behaviors were assessed using a self-report version of the Repetitive Behavior Scale - Revised (RBS-R)[63]; and feelings of avoidant attachment were assessed using the State Adult Attachment Measure (SAAM)[64].

ROI	Behavioral outcome	β	T-value	p-value
Right pSTS	SRS	.17	1.26	.21
	RBS-R	.17	1.27	.21
	SAAM avoidance	.20	1.49	.14
Amygdala	SRS	**.32**	**2.42**	**.019**
	RBS-R	.11	.79	.43
	SAAM avoidance	-.001	-.01	.99

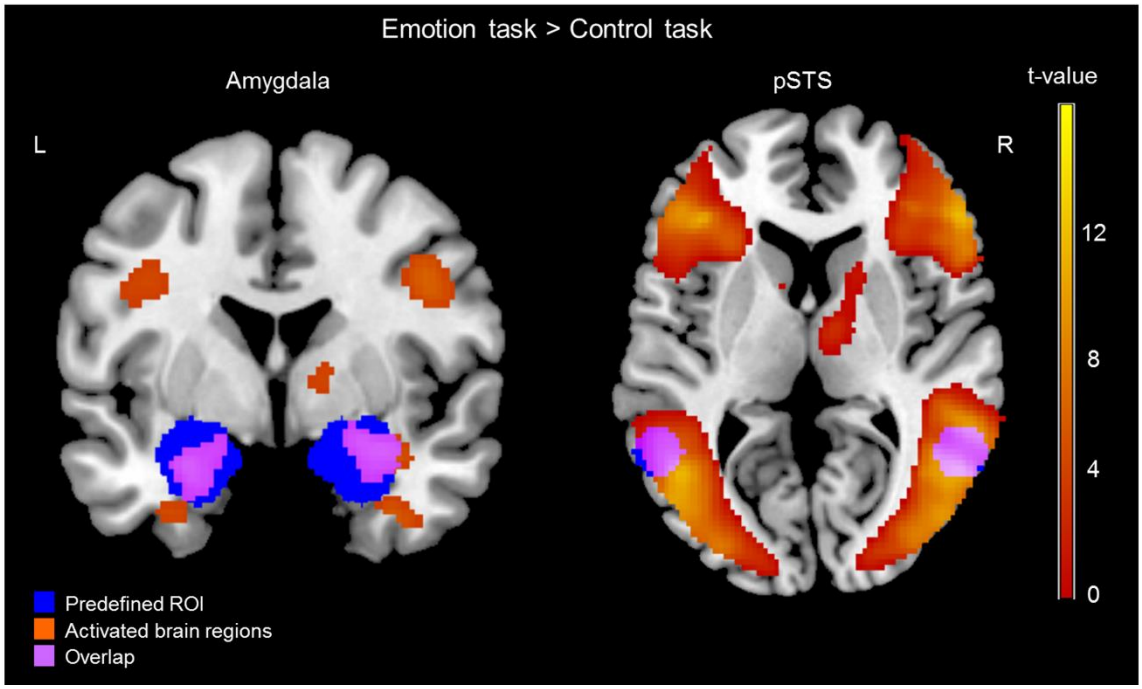
Note: ROI = region of interest, OT = oxytocin, PL = placebo, pSTS = posterior superior temporal sulcus, SRS = Social Responsiveness Scale, RBS-R = Repetitive Behaviors Scale-Revised, SAAM avoidance = State Adult Attachment Measure avoidance subscale.

Supplementary Figures

Supplementary Figure 5.1. Visualization of experimental design. (A) Participants completed two runs, each consisting of three blocks of the emotion recognition task, interleaved with three color task blocks (48s/block). All task blocks were separated by fixation blocks (12s rest period), during which participants fixated on a white cross. All trials lasted 4s, such that stimulus presentation was jittered with respect to image acquisition (TR = 3s). Instructions were provided verbally at the start of the test and on the monitor at the start of each test block (4s). (B) Point-light displays (PLDs) consisted of twelve moving white dots against a black background, representing the motion of the main joints of the human body (ankles, knees, hips, wrists, elbows and shoulders). Response options were displayed at the bottom of the screen, which corresponded to response buttons of the response box that the participants used while lying in the scanner.

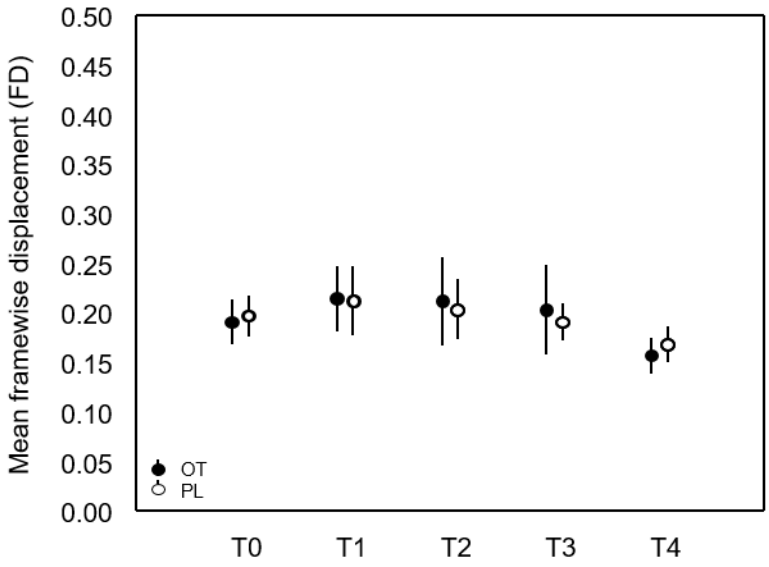


Supplementary Figure 5.2. Baseline task-based fMRI activity. Visualization of brain regions with reliable brain activity during the emotion task (> control task) at the baseline session (T0) (across groups) ($p < .05$, family-wise error corrected for multiple comparisons) (red-orange grading). As visualized, the adopted regions of interest (ROI) in bilateral posterior superior temporal sulcus (pSTS) (10-mm-radius spheres with MNI-coordinates [left: -55, -52, 12] [right: 55, -52, 10]) and amygdala (FSL Harvard-Oxford subcortical atlas) (visualized in blue) showed reliable brain activity during the emotion task (overlap visualized in purple).

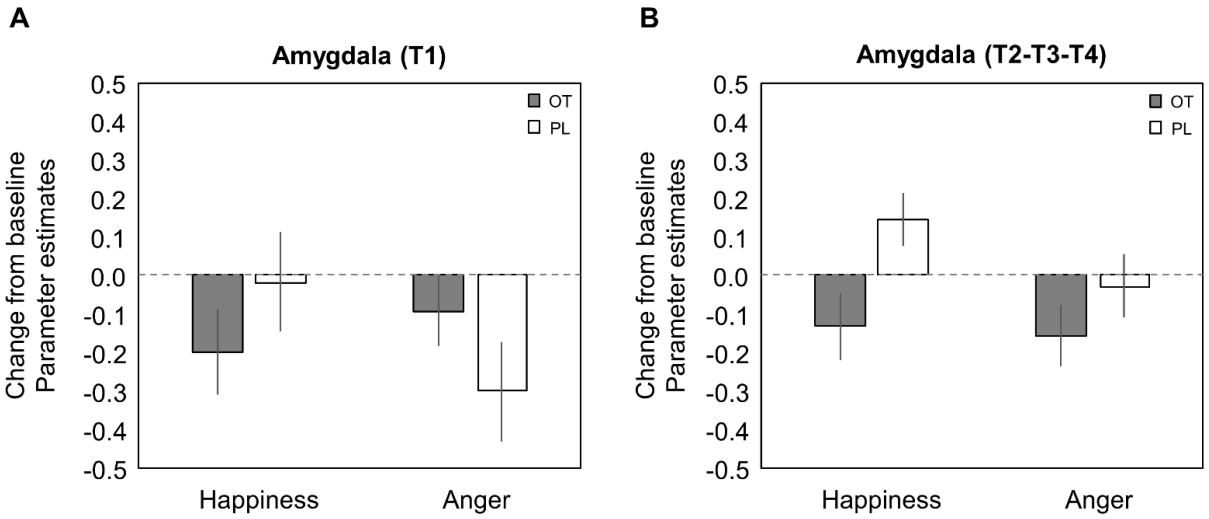


Supplementary Figure 5.3. Mean framewise displacement per group and assessment session.

Mean framewise displacement (FD) (in mm) was calculated for each participant to assess potential differences in in-scanner head movement between groups across sessions. A mixed-effects analyses with 'Subject' as random factor and the factors 'Treatment' (OT, PL), 'Session' (T0, T1, T2, T3, T4), and their interaction as fixed factor, revealed no main effects of treatment ($F(1,138)=.001$, $p=.97$) or session ($F(4,138)=2.06$, $p=.09$), nor a treatment-by-session interaction ($F(4,138)=.12$, $p=.97$), indicating no significant differences in mean FD between groups across test sessions.



Supplementary Figure 5.4. Additional results of the region-of-interest (ROI) analyses.
Panel A visualizes the mean change-from-baseline parameter estimates for each treatment group (OT, PL) and emotion (Happiness, Anger) after a single dose of treatment (T1). Panel B visualizes the mean change-from-baseline parameter estimates for each treatment group (OT, PL) and emotion (Happiness, Anger) across assessment sessions T2 (immediately after the multiple-dose treatment), T3 (at follow-up, one month post-treatment) and at T4 (at follow-up, one year post-treatment).



Supplementary methods

Participants

Forty high-functioning adult men with a formal diagnosis of ASD were recruited between April 2015 and December 2016 from the Autism Expertise Centre at the Leuven University Hospital. The diagnosis was established by a multidisciplinary team (child psychiatrist and/or expert neuropediatrician, psychologist, speech/language pathologist and/or physiotherapist) based on the strict criteria of the DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders [1]. Prior to the intervention, the ADOS (Autism Diagnostic Observation Schedule) [51] and estimates of intelligence (6-subtest short version of the Wechsler Adult Intelligence Scale-IV Dutch version: Block design, Digit span, Similarities, Vocabulary, Symbol search and Visual puzzles [52] were acquired from all participants (Table 5.1). Inclusion criteria comprised a clinical diagnosis of autism spectrum disorder; gender (male); and age (18-35 years old). Exclusion criteria for participation comprised any neurological disorder (e.g., stroke, epilepsy, concussion), demonstrated genetic disorder, or any contraindication for magnetic resonance imaging (MRI). Current psychoactive medication use and the presence of comorbid psychiatric disorders were screened (Supplementary Table 5.1). Forty participants were randomly allocated to either the OT (n=22) or the PL group (n=18). Data from two participants (1 OT, 1 placebo (PL)) were not included in the final analyses due to discontinued intervention (self-termination unrelated to the treatment) and unusable data caused by movement in the scanner, respectively. For assessment sessions T1-T3, final analyses were therefore performed on 21 OT and 17 PL participants (Supplementary Figure 5.1, CONSORT Flow chart). Additionally, 3 OT and 3 PL participants were lost to follow-up one-year post-treatment (T4), such that final analyses of T4 were performed on 18 OT and 14 PL participants.

Sample size

To date, several studies explored the effect of single-dose OT administration on changes in task-related fMRI brain activity in patients with ASD [22–25,102]. In only one previous randomized, placebo-controlled trial, Watanabe et al. [12] adopted a crossover design to assess the effect of multiple-dose OT treatment (six weeks of daily doses) on task-related fMRI activity during a social judgement task in 20 patients with ASD. Significant effects (large-size) were reported for 17 patients who completed the OT/PL crossover treatment. Considering this previous crossover study assessing the effects of OT on task-related fMRI activity in patients with ASD and the lack of prior studies using parallel designs, the current sample size was set at a comparable sample size.

Intervention

All participants received clear instructions about the use of the nasal spray[47,10347,103] and were monitored onsite until approximately two hours after first nasal spray administration. During the course of the treatment, participants were asked to administer the nasal spray in the morning, to keep a daily record of the time point of nasal spray administration, and whether or not they were alone or in company of others the first two hours after administration. Percentage of days at which the spray was administered in the presence of others was not significantly different between treatment groups (OT: 36.6% (SD 29.9); PL: 37.4% (SD 24.1); t(36)= -.09; p= .92). Participants administered the nasal spray (OT or PL) daily during four consecutive weeks and at the end of each week, participants were screened for potential adverse events and side effects. As listed in detail in Supplementary Table 5.2, only minimal, non-treatment specific side effects were reported. Finally, at the end of the trial, participants were asked if they thought they had received OT or PL. The majority of participants thought they had received the PL treatment (78.95%). The proportion of participants that believed they had received the OT treatment was not significantly larger in the actual OT group (28.57%), compared to the PL group (11.76%) (p=.21).

Functional MRI image acquisition

Scan sessions started with the acquisition of the anatomical scan, followed by a 7-min resting-state fMRI scan (data not part of the current manuscript) and the two task-related fMRI runs. Anatomical imaging consisted of a high resolution structural volume acquired using a coronal three-dimensional turbo field echo T1-weighted sequence with the following parameters: 182 contiguous coronal slices covering the whole brain and brainstem, slice thickness = 1.2 mm; repetition time (TR) = 9.4 ms; echo time (TE) = 3.0 ms; matrix size = 256 × 256; field-of-view (FOV) = 250 × 250 x 218.40 mm^3; acquisition time = 1 min 43s. For the two task-related fMRI scans a T2* weighted gradient echo - echo planar imaging (GE-EPI) sequence was used with the following parameters: TR = 3000 ms; TE = 30 ms; matrix size = 96 × 96; FOV = 210 x 210 x 140.20 mm^3; flip angle 90°; slice thickness = 2.5 mm, 0.2 mm gap; axial slices = 52; 127 functional volumes; total acquisition time = 6 min 39s.

Preprocessing

All functional images were corrected for differences in slice acquisition time by temporal interpolation to the middle slice (reference=26), realigned to the reference (mean) image and co-registered to each subject's T1 anatomical image. Images were then normalized to the standard EPI template of the Montreal Neurological Institute (MNI) space using the segmented anatomical image, resampled into 2 mm isotopic voxels and smoothed with an 8-mm full width at half maximum Gaussian kernel. A high-pass filter with a cutoff of 256s was used. Mean framewise

displacement (FD) was calculated for each participant to assess potential differences in in-scanner head movement between groups across sessions. There were no significant differences in mean FD between groups across assessment sessions (see Supplementary Figure 5.3).

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General Discussion

Chapter VI

The current doctoral project aimed to gain insight into the behavioral and neural effects of single-dose and multiple-dose administration of the 'prosocial' neuropeptide oxytocin (OT) both in the neurotypical population and in individuals with Autism Spectrum Disorder (ASD). Since ASD is mainly characterized by deficits in social interaction and communication, and OT has been shown to enhance social behaviors in animals and in humans with and without ASD, a surge of clinical trials emerged. These trials, however, mainly assessed the single-dose effects of OT, whereas knowledge of the multiple-dose effects and potential retention effects of oxytocin administration was lacking. These insights, however, are essential to uncover the true therapeutic potential of OT for the social impairments in ASD. To address this aim, the doctoral project was divided into three studies (reported across four chapters) with specific objectives addressed in each one of them. The general discussion will summarize the main findings of these studies, reflect on them and present suggestions for future research.

1 Summary of the main findings

Before starting the clinical trial designed to assess the behavioral and neural effects of single-dose and multiple-dose intranasal OT administration in adult men with ASD (that forms the core of the current doctoral project) (chapters 4 and 5), we first tested (parts of) the study procedure and outcome measures in a sample of typically developing adult men (chapters 2 and 3).

In chapter 2, we conducted a double-blind, randomized, placebo-controlled, between-subjects study with healthy adult men to assess the immediate effects of single-dose OT treatment on emotion recognition from point-light displays (PLDs) conveying biological motion. We hypothesized that a single dose of intranasal OT (compared to placebo) would improve emotion recognition performance in these typically developing participants. Overall, a single dose of OT administration had a significant, medium-sized effect on emotion recognition from PLDs. OT-induced improvements in emotion recognition were not differentially modulated by the emotional valence of the presented stimuli (positive versus negative) and the overall tendency to label an observed emotional state as 'happy' (positive) or 'angry' (negative) was not modified by the administration of OT. Albeit moderate, these findings of OT-induced improvements in bodily emotion recognition from whole-body PLDs provided further support for a link between OT and the processing of social information.

Findings of the study reported in chapter 2 were in line with a multitude of other single-dose administration studies revealing the prosocial effects of OT (reviewed in [1–3]). In addition, a complementary line of research has also highlighted the beneficial effects of intranasal OT administration on feelings of attachment. Moreover, evidence on the effects of multiple-dose OT treatment in typically developing individuals was limited. Accordingly, in chapter 3, we performed a double-blind, randomized, placebo-controlled, between-subject study, to assess the effects of two weeks of daily OT administration on measures of attachment feelings (State Adult Attachment Measure (SAAM) and Inventory of Parent and Peer Attachment (IPPA)), social responsiveness (Social Responsiveness Scale (SRS)), quality of life (World Health Organization Quality of Life questionnaire (WHOQOL)) and mood (Profile of Mood States (POMS)) in typically developing adult men. We hypothesized that a two-week, multiple-dose, intranasal OT treatment (compared to placebo) would improve self-report ratings of feelings of attachment, social responsiveness, quality of life and mood in these typically developing participants. Participants reported significant reductions in attachment avoidance and increases in reports of attachment toward peers after two weeks of OT treatment. In addition, treatment-induced changes were most pronounced for participants with less secure attachment towards their peers, indicating that normal variance at baseline modulated treatment response. OT treatment was additionally associated with changes in mood, indicating decreased feelings of tension and (tentatively) anger in the OT group, but not in the placebo group. Further, at the end of the two-week trial, both treatment groups reported to experience an increase in social responsiveness and quality of life, but the effects were not treatment-specific (i.e. both groups reported improvements, except for reports on 'social motivation'). In summary, the reported improvements in feelings of attachment after a multiple-dose treatment with OT provided further evidence in support of a pivotal role of OT in promoting the experience of attachment.

After the identification of effects of both single-dose and multiple-dose OT administration in typically developing adult men, we adopted the aforementioned outcome measures in a clinical trial with adult men with ASD (reported across two chapters). In chapter 4, we investigated the immediate and long-term effects of multiple-dose OT treatment on autism characteristics (social responsiveness as assessed with the SRS, restricted and repetitive behaviors (RRBs) as assessed with the Repetitive Behavior Scale – Revised (RBS-R)), feelings of attachment (SAAM, IPPA) and quality of life (WHOQOL) in adult men with ASD. In addition, we also used the Profile of Mood States (POMS) to screen for changes in mood. To do so, we adopted a double-blind, randomized, placebo-controlled, parallel design (clinical trial) during which participants filled in questionnaires at multiple time points: (i) at baseline, (ii) immediately after four weeks of continual OT treatment, and at two follow-up sessions, (iii) one month (four weeks) and (iv) one

year post-treatment. We hypothesized that a four-week, intranasal OT treatment would improve self-report and informant-based ratings of social responsiveness, and ameliorate self-ratings of RRBs, feelings of attachment and quality of life. In addition, a key question was to evaluate whether any beneficial effects of continual OT treatment would outlast the period of actual administration until one month or even one year post-treatment. In contrast to our hypotheses, no treatment-specific improvements in social responsiveness were identified. However, results revealed that the four-week, multiple-dose OT treatment induced medium- to large-sized improvements in RRBs and reduced feelings of avoidance towards others, which outlasted the period of actual administration until one month and even one year post-treatment. In addition, screenings for changes in mood (Profile of Mood States) also revealed enhanced reports of vigor (more energetic, active, and lively) both during and after the OT treatment (evident up to one year post-treatment). The identification of long-term beneficial effects on repetitive behaviors, perceived attachment avoidance and vigor suggests that there is therapeutic potential of multiple-dose OT treatment in adult men with ASD. However, considering this is a pilot study, larger studies are warranted to evaluate the long-term effects of OT treatment further.

Finally, in chapter 5, we investigated immediate and long-term effects of single-dose and multiple-dose OT treatment on neural activity during the processing of emotional states (i.e. emotion recognition) from point-light biological motion in adult men with ASD. Data were collected during the same double-blind, randomized, placebo-controlled, parallel-designed clinical trial as reported in chapter 4. To assess treatment-induced changes in task-related brain activity of the posterior superior temporal sulcus (pSTS) and amygdala up to one month and one year post-treatment, functional magnetic resonance imaging was performed during the processing of emotional states from point-light biological motion (i) at baseline, (ii) after a single dose of OT, (iii) immediately after four weeks of continual OT treatment (approximately 24 hours after the final nasal spray administration), and at two follow-up sessions, (iv) one month (four weeks) and (v) one year post-treatment. We hypothesized that a single dose of OT would increase activity in pSTS regions and alter amygdala activity during the processing of emotional states from point-light biological motion. A key aim was to determine whether a four-week, multiple-dose treatment would induce similar or even augmented changes in pSTS and amygdala activity, and specifically, whether the changes in neural function would outlast the period of actual administration until one month or even one year post-treatment. Results revealed that the multiple-dose treatment attenuated activity in bilateral amygdala and induced sustained higher levels of task-related activity within (right) pSTS. Importantly, these treatment-induced neural changes were shown to outlast the intervention until one month and even one year post-treatment, indicating that continual OT treatment induced long-lasting neural changes in core social brain regions.

Importantly, we also showed that the extent of amygdala attenuation was associated with the extent of (long-term) improvements in self-reports of social functioning. Critically, the long-term attenuations in amygdala activity were associated with self-reported improvements in social functioning (Social Responsiveness Scale) and are suggested to reflect OT's anxiolytic role in regulating negative affect and promoting social approach. Together, the observation that continual OT treatment was effective for inducing long-lasting neural adaptations in core regions of the social brain up to one year post-treatment holds important clinical implications for ASD and other neuropsychiatric disorders for which OT is considered as a potential treatment.

2 Critical reflections

2.1 Neurobehavioral models of oxytocin functioning

As described in the general introduction, OT is produced in paraventricular and supraoptic nuclei of the hypothalamus, from where OT containing neurons project to target regions within the central nervous system (i.e. the brain and spinal cord) coordinating brain and body states [4]. These regions include the lateral septum, nucleus accumbens, hippocampus and amygdala, but also brain stem regions including the ventral tegmental area and solitary tract nuclei down towards the spinal cord [4]. Of these regions, the amygdala has proven to be pivotal for social behavior [5], in particular, involving vigilance and the detection of (socially) relevant stimuli (i.e. salience) [6]. In a context of fear or stress, OT is known to inhibit output from the amygdala to brainstem regions, which in turn is suggested to dampen the autonomic expression of fear [7,8]. In addition, the amygdala is part of a larger socio-emotional network considering its connections with the thalamus, hypothalamus, septum, and prefrontal and brainstem regions, but also the anterior cingulate gyrus and the superior temporal gyrus involved in more complex emotion processing[9]. Interestingly, based on animal research and single-dose administration studies addressing these brain regions and associated behaviors, authors have proposed multiple potential mechanisms by which oxytocin is believed to modulate human social behavior (reviewed in [10–13]). For example, one influential theory states that OT modulates social behavior by reducing (social) anxiety and stress reactivity. The initial idea for this theory was based on studies showing that OT attenuated anxiety and fear in animals [14–17]. Accordingly, studies in humans revealed that OT reduced amygdala reactivity in response to fearful stimuli or conditioned fear in typically developing individuals [18–23] and individuals with generalized social anxiety disorder (GSAD) [24]. This theory, however, could not account for all the prosocial effects of OT. A second potential mechanism to explain OT's prosocial actions in humans was based on animal literature highlighting OT's role in

pair bonding and attachment [25]. This affiliative-motivation hypothesis proposed that OT might increase the desire to affiliate which in turn leads to more prosocial behavior [26]. This hypothesis was supported by findings in human research indicating that OT enhances trust, generosity, altruism and cooperative behavior (reviewed in [2,26]). However, Shamay-Tsoory et al. (2009) found evidence that OT not only increased prosocial behavior, but also negatively perceived behaviors such as envy and gloating (Schadenfreude) [27], leading to the proposal of a third potential mechanism, namely the social salience hypothesis of OT [11]. This hypothesis argues that OT regulates the salience of social cues through its interaction with the dopaminergic system and that OT's effects are influenced by contextual factors and individual differences, which can lead to both positive (i.e. prosocial) and negative (i.e. antisocial) effects. In particular, OT is believed to affect attention orienting towards salient contextual social cues [11]. Indeed, multiple reports have indicated that OT increased gaze towards the eye region [20,28], enhanced attention orienting in response to emotional gaze cues [29] and increased covert attention to happy faces[30]. Kemp and Guastella [12,31], however, interpreted the same research findings in the context of social approach and avoidance. With their social approach/withdrawal hypothesis they theorized that the variety of OT's effects might be explained by a dual mechanism that OT up-regulates social approach motivation, and down-regulates social avoidance motivation [12,31].

These theories, however, should not be considered mutually exclusive. In an attempt to combine these different accounts, Ma et al. (2016) proposed the Social Adaptation Model (SAM) of OT [13]. The SAM posits that OT promotes social adaptation by modulating emotional responses and adjusting behaviors during social interactions. In particular, Ma et al. proposed the following three neuropsychological mechanisms based on an in-depth review of neuroimaging studies assessing the effects of intranasal OT in the healthy and clinical population (Fig. 6.1).

First, by decreasing neural reactivity to negative emotions (e.g. anxiety and stress) in brain regions including the amygdala, anterior cingulate cortex and anterior insula, and by increasing neural activity during emotion regulation in multiple prefrontal regions, OT can reduce negative affect. Consequently, this improved regulation of negative affect should allow individuals to initiate and maintain social interaction, communication and relations. In line with this proposed neuropsychological mechanism, results of the current doctoral project indicated that the two-week OT treatment induced improvements in reports of negative feelings, namely tension (and tentatively anger) [32]. However, in our long-term clinical trial, participants with ASD did not report any improvements in mood states (Bernaerts et al., under review). In addition, we also found decreased amygdala activity after multiple-dose OT treatment in individuals with ASD and interpreted the observed attenuating effects on amygdala activity to reflect OT's anxiolytic role (i.e. reducing negative affect) for promoting social approach behavior (i.e. social adaptation),

which is supported by the observed association between amygdala attenuation and the extent of improvements in social functioning. Moreover, considering restricted and repetitive behaviors (RRBs) (at least 'need for sameness') are associated with stress and anxiety [33–35], the observed decrease in reports of RRBs after multiple-dose OT treatment might also provide (indirect) support to this mechanism. However, to elucidate this neuropsychological mechanism, future studies will need to assess the effects of OT on anxiety or stress (in ASD) and link these potential changes to neural changes and behavioral improvements.

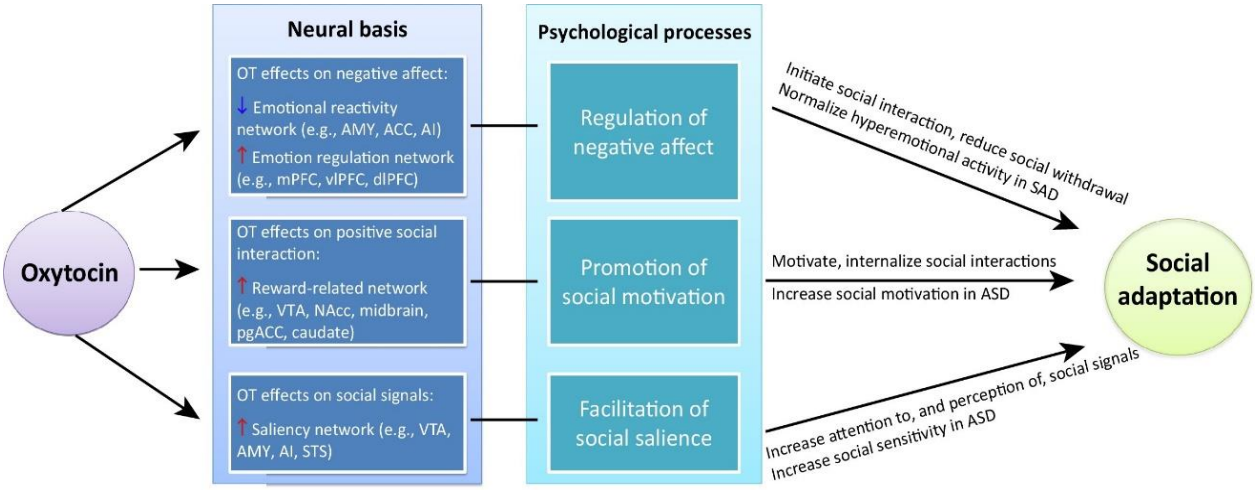

Figure 6.1. The Social Adaptation Model of oxytocin function as proposed by Ma et al. (2016)[13]. ↑ indicates increased neural activity by OT; ↓ indicates decreased neural activity by OT. ACC= anterior cingulate cortex, AI= anterior insula, AMY= amygdala, dlPFC= dorsal lateral prefrontal cortex, mPFC= medial prefrontal cortex, NAcc= nucleus accumbens, vlPFC= ventral lateral prefrontal cortex, STS= superior temporal sulcus, VTA= ventral tegmental area, ASD= autism spectrum disorder, SAD= social anxiety disorder. Figure and figure legend adapted from Ma et al. (2016) in *Trends in Cognitive Neurosciene*[13].

Second, by increasing activity in brain regions linked to the reward network including the ventral tegmental area, midbrain, caudate, nucleus accumbens and perigenual anterior cingulate cortex, OT can attribute reward value to social contexts. This, in turn, enhances social motivation, which also facilitates initiation and maintenance of social relations. In terms of our findings and in the context of the adopted task, we did not specifically study the aforementioned brain regions. However, results of the current doctoral project showed that multiple-dose OT treatment decreased attachment avoidance (i.e. a reluctance in closeness or trust towards others) in typically developing participants immediately after a two-week OT treatment, and until one year after the four-week OT treatment in participants with ASD. In this respect, we suggest that OT improved our participants' willingness to trust and approach others, providing (indirect) behavioral support to the proposed mechanism.

Third, by increasing activity in brain regions linked to the saliency network, such as the amygdala, anterior insula, superior temporal sulcus and ventral tegmental area, OT can facilitate social

salience and consequently improve attention to and perception of socially relevant signals. In line with this notion, results from our clinical trial, assessing the effects of multiple-dose OT treatment in adult men with ASD, revealed that a single dose of OT treatment indeed induced a relative increase in activation of the pSTS. Extending knowledge from single-dose studies, results also revealed that (right) pSTS activation was altered after multiple-dose treatment with OT. While pSTS activation was generally attenuated in both treatment groups upon PLD emotion processing (as opposed to increased after a single dose of OT), the OT treatment tended to reduce this attenuation, particularly at the follow-up session, one year post-treatment.

Together, these psychological processes are suggested to improve social adaptation towards a more 'normal' or 'healthy' reaction pattern by aiding individuals to initiate and maintain social communication, interaction and relations. Note, however, that considering only two studies to date have investigated and reported multiple-dose effects of OT on the level of the brain (Watanabe et al. (2015) [36] and one in the current doctoral project (Bernaerts et al., under review)), the SAM mainly represents a model for the effects of single-dose OT administration. Nonetheless, these proposed neuro-behavioral mechanisms based on single-dose studies provide the necessary first steps to test these specific hypotheses further on a neural level and show that the search for the neuro-behavioral mechanisms behind OTs modulating effects is still an active area of research.

Although the SAM provides a comprehensive framework for the seemingly discrepant effects of OT and the influence of personal and contextual factors on the effects of OT, another model has specifically highlighted the importance of one contextual factor. Bos et al. (2012) have suggested a neuroendocrine model of OT function related to the perceived safety of the environment [9]. They argued that OT's function is particularly important in safe environments where there is no direct need for vigorous action. Alternatively, in a stressful situation, OT serves the important function of reinstating homeostasis by enhancing parasympathetic nervous system activity, which promotes calming and caring behaviors. In this respect, OT is suggested to reduce stress and anxiety and encourage social motivation. In line with this dual function (i.e. increasing social salience in a low-stress environment and decreasing stress reactivity and anxiety in a stressful situation), Quintana et al. (2015) have recently proposed a two-level model of the modulatory effects of OT on social behavior and cognition based on three potential routes by which intranasal OT is believed to enter the central and peripheral nervous system: via the olfactory bulb, via the trigeminal nerve and absorption through blood capillaries [37] (Fig. 6.2). On the one hand, intranasal OT that reaches the amygdala, prefrontal cortex and brainstem modulates social information processing from the top down via connections with social brain circuits (e.g. STS regions). On the other hand, the intranasal OT that reaches the brainstem and systemic circulation modulates anxiolytic and approach-related behavior (considering the role of the autonomic nervous system in anxiety) from the bottom up. Taken together, they hypothesized that the brainstem would

provide the opportunity to change social behavior via decreased anxiety to facilitate approach behaviors, whereas the amygdala and prefrontal regions would steer the changes in social behavior by modulating social information (salience effect to facilitate attention to and perception of social signals) [37].

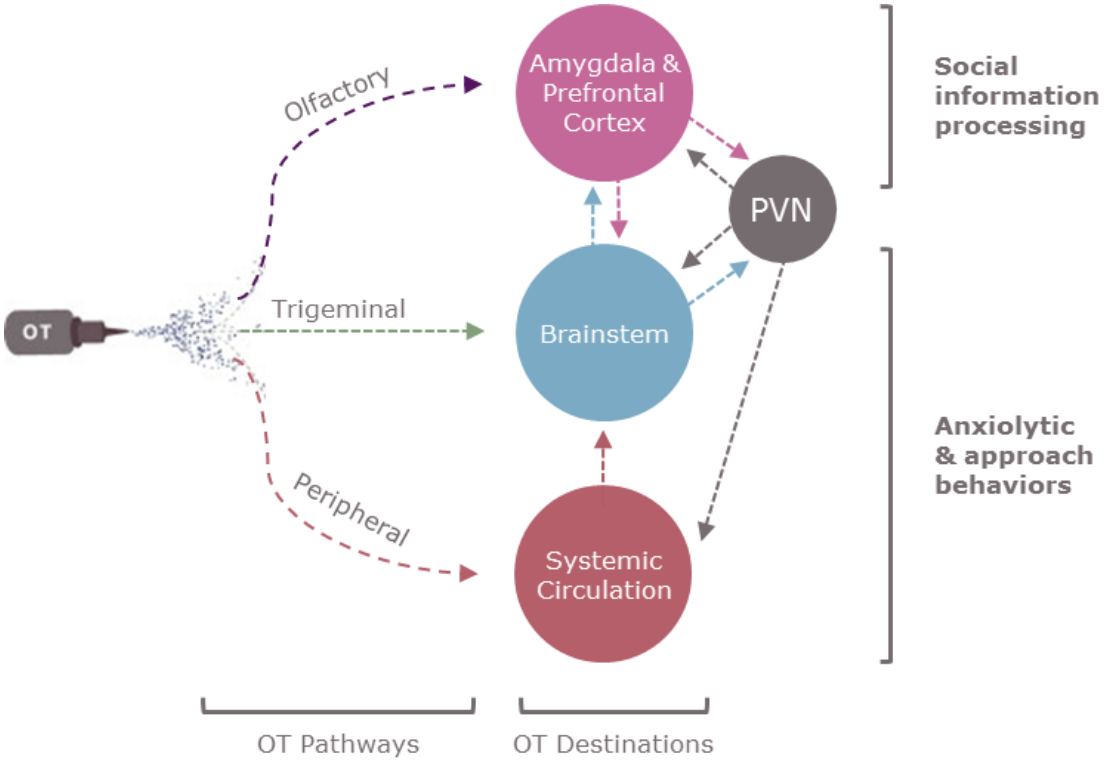

Figure 6.2. Schematic presentation of the two-level model of the modulatory effects of oxytocin on social behavior and cognition as proposed by Quintana et al. (2015)[37]. OT= oxytocin, PVN= Paraventricular nucleus. Figures and figure legends adapted from Quintana et al. (2015) in *Neuroscience and Biobehavioral Reviews* [37].

Further research is however needed to provide a more comprehensive and integrative understanding of the underlying mechanisms of OT treatment, encompassing an in-depth investigation of the central effects of OT on amygdala reactivity and its projections towards other social-emotion processing regions (e.g. brainstem regions) and how they are linked to the upregulation of social salience and/or downregulation of social stress and anxiety (via autonomic nervous system regulation). Nonetheless, an uncertain mechanistic understanding of OT function should not halt future research of intranasal OT administration as an intervention approach for the social and anxiety-related impairments in psychiatric conditions.

2.2 Single-dose vs multiple-dose oxytocin treatment in ASD

Considering, to date, only a handful of studies have investigated the effects of multiple-dose OT treatment and only two of those (including one as part of the current doctoral project) have assessed the neural effects of both single-dose and multiple-dose OT treatment, knowledge on the multiple-dose effects of OT is limited, in particular in comparison to single-dose effects.

Watanabe et al. (2015) [36] were the first to investigate the neural effects of a six-week OT treatment on anterior cingulate and prefrontal cortex activity, and to compare the effect sizes to those found in a prior single-dose administration study [38] from their lab. They reported that the effect sizes of the multiple-dose treatment were qualitatively similar to those of the single-dose treatment [36]. Notwithstanding the importance of their pioneering work, some aspects of their comparison, however, should be taken into consideration. First, the data were collected during two separate studies and thus in two different participant samples, potentially creating differences in results caused by individual and contextual differences (i.e. OT's effects can be modulated by personal and contextual factors [10]). Second, MRI scanning to assess the 6-week treatment effect was performed only 15 or 40 min after the last nasal spray administration. Since this period corresponds to the optimal time frame for assessing acute, single-dose effects of OT administration, the possibility cannot be ruled out that the reported multiple-dose effect - at least in part - reflected an acute effect of exogenously administered OT.

In contrast, in the current project, we assessed the neural effects of single-dose OT administration and multiple-dose OT administration during one clinical trial and thus in one participant sample. Additionally, MRI scanning for assessing the multiple-dose effect (i.e. after the four-week administration period) was performed at least 24 hours after the last OT administration, rendering the observed neural effects at this assessment session less susceptible to reflect an acute effect of OT administration. In contrast to previous findings, results of the current doctoral project revealed that effect sizes of the multiple-dose treatment were in fact qualitatively different from the identified single-dose effect sizes. While the single-dose OT administration strongly activated the pSTS as compared to the multiple-dose OT administration, the multiple-dose OT administration strongly attenuated amygdala activity as compared to the single-dose OT administration. Based on these observations, we hypothesize that the neural effects after single-dose compared to multiple-dose OT treatment are different, and importantly, that the differentially induced single-dose versus multiple-dose effects may be region- or network-specific.

In light of the neurobehavioral models described above, we hypothesize that the attenuation in amygdala activity at the one-month and one-year follow-up sessions reflects long-lasting adaptations in neural functioning due to OT's recursive action on the amygdala-centered social-

emotional network. We suggest that the four-week continual (exogenous) OT administration via the olfactory and trigeminal pathways might have stimulated a 'feedforward' production of endogenous OT from the paraventricular nucleus (PVN), delivering OT to the amygdala, prefrontal cortex and brainstem. Evidence for this feedforward production, albeit indirect, can be found in a study indicating elevated levels of salivary OT seven hours after administration [39]. We thus hypothesize that a single dose of (exogenous) OT administration exerts immediate responses in the social brain circuit, whereas multiple-dose (exogenous) OT administration might stimulate endogenous production of OT, potentially in an experience-dependent manner, which in turn could explain the long-lasting effects of the treatment found in the current doctoral project. Nonetheless, more research is needed to replicate our findings and support our hypothesis.

3 Limitations and methodological considerations

3.1 Sample size, dropout and power

In order to estimate the necessary sample size for the clinical trial described in chapters 4 and 5, a power analysis was performed based on the effect sizes reported in the meta-analysis by Wigton et al.[40] exploring the neural effects of single-dose OT administration in neurotypicals. Seven out of the 17 fMRI neuroimaging studies had adopted a between-subject design with an average sample size of 48 participants (24 in each group). The other 10 studies used a within-subject design with an average sample size of 16 participants. Across all studies, effect sizes of the neural measures ranged from 0.39 to 2.12 with an average effect size of 1.22 ± 0.44. To achieve a power of 0.8 for a between-subject design with the α-level of significance set at 0.001 (common for fMRI research), a sample size of 48 participants (24 in each group) was required. Note, however, that the study design used in chapter 4 and 5 is different from studies executed before, since we were - to the best of our knowledge at the start of the clinical trial - the first research group to assess the long-term effects of multiple-dose OT treatment in individuals with ASD both on a behavioral and neural level. In this respect, we lacked the necessary numbers for an adequate power analysis. As indicated, with the inclusion of 40 participants with ASD, the current clinical trial described in chapter 4 and 5 needs to be considered pilot research into the long-term effects of OT treatment and, therefore, effects need to be interpreted with caution.

In addition to the small sample size, 20% of participants (8/40) either dropped out of the study or had unusable data. There was, however, no difference in the proportion of dropout between treatment groups (p= .93). Specifically, four participants of the OT group (out of 22) and three of the PL group (out of 18) dropped out due to the following reasons: self-termination unrelated to

the treatment (1 OT), time constraints due to job demands (2 OT, 1 PL), no longer willing to participate in MRI research (1 PL) and unknown reasons (1 OT, 1 PL). Moreover, considering baseline characteristics (age, total IQ, ADOS total score) nor baseline pSTS or amygdala activity of the participants who dropped out differed from participants who completed the trial, we do not expect our findings to be strongly impacted.

To improve the reliability of intranasal OT research, the field would benefit from international collaborations and research consortia to increase sample sizes, but also careful consideration of treatment outcomes to increase statistical power of the tests used in these studies.

3.2 Compliance

Compliance is described as the degree to which a participant/patient correctly follows medical advice (i.e., correctly using medication or following a prescribed treatment regimen) and is necessary to acquire a valid assessment of treatment effects in research. One important aspect of our research was the correct administration of the nasal spray. Based on the guidelines of Guastella et al.[41], we used a number of methods to promote correct use of the nasal sprays and encourage compliance. First, participants were clearly instructed on the correct use of the nasal spray and were requested to administer the first dose in the lab in front of the examiner to allow for corrections, if needed (as described in detail in chapter 1). Second, in order to assess compliance in our participants with ASD, we provided them with a diary that was to be returned at the end of the trial. In that diary, participants were requested to provide the following information: timing of the daily dose (date and time) (i), whether the nasal spray was administered prior to or after breakfast (ii), whether or not they were in the presence of others during the two hours after the nasal spray administration (iii), and what activity they engaged in during the two hours after the nasal spray administration (iv). The diaries were also used to screen for potential adverse events, side effects or changes in mood with the Profile of Mood States (POMS) questionnaire. Aside from one (OT) participant dropping out, nearly all participants reported to have taken their daily dose of nasal spray. Out of 39 participants, 34 reported to have taken their daily dose (87%), 2 participants reported to have taken all but one of the daily doses (1 OT forgotten administration, 1 OT due to sickness), and 2 participants reported to have taken all but two of the daily doses (2 OT forgotten administration). Surprisingly, these less-compliant participants had all received the OT treatment. Closer inspection, however, revealed that they all believed to have received the PL treatment. Although participant compliance was good and guidelines promote the use of diaries to assess compliance[41], a downside of this method is that one has to rely on the honesty of the participants.

Note, however, that the diaries were only used during the four-week treatment period and not in the period between the later assessment sessions (until one month and one year post-treatment). Although unlikely, it was therefore not explicitly screened whether or not participants potentially purchased additional OT nasal sprays (e.g., available online via Amazon) for off-label self-administration. Conform guidelines, future research is advised to use additional methods to assess compliance, for example, measuring the remnant liquid of nasal spray bottles upon return or using bottles with dose counters.

3.3 Adopted fMRI task paradigm

Although the method of fMRI and the adopted fMRI task paradigm were deliberately decided upon for this doctoral project, some critical considerations can be made.

First, in OT research, MRI is a common method for assessing the treatment-induced effects on brain activation in individuals with ASD (indicated by differences in the blood-oxygen-level-dependent (BOLD) signal). However, with respect to ASD, evidence on the exact relationship between the BOLD signal and neuronal activity (i.e. neurovascular coupling or the hemodynamic response) is limited. In general, authors assume that observed differences in the BOLD signal reflect altered neuronal activity in studies using fMRI to compare participants with ASD and neurotypicals[42]. However, as proposed by Reynell and Harris (2013), in order to compare fMRI-based measures of brain activity between different groups reliably, neurovascular coupling must be the same in these different groups. Importantly, neurovascular coupling in ASD (as compared to neurotypicals) might be influenced by neurophysiological abnormalities present in ASD, such as abnormalities in neuronal activity, vasoactive mediators and energy use (reviewed in[42]). Regarding the findings described in chapter 5, the regions of interest (ROIs; pSTS and amygdala) were based both on prior work from our own lab as well as prior work in the field. However, to the best of our knowledge, none of these studies assessed whether neurovascular coupling differed between individuals with ASD and neurotypicals, thus assuming that neurovascular coupling was comparable between these groups. In this respect, the altered activity ascribed to these brain regions in ASD as compared to neurotypicals, might potentially reflect altered neurovascular coupling rather than actual altered neuronal activity between these groups. For example, Yan et al. (2018) recently showed that multiple brain regions related to ASD showed systematic differences in hemodynamic response (regarding resting-state functional connectivity)[43]. On the other hand, another study assessing fMRI BOLD time courses in children with ASD and neurotypical children during a simple visuomotor task, found no differences in the hemodynamic response between both groups[44]. As such, evidence on whether differences in the BOLD signal between individuals with ASD and neurotypical controls actually reflects differences

in neuronal activity remains unclear. Note, however, that the issues described in aforementioned studies [42–44] refer to the comparison of individuals with ASD and neurotypicals, while in our study, we compared two groups of participants with ASD. As such, we assume that any differences in neurovascular coupling or hemodynamic response are similar across all our participants with ASD. One way of dealing with this uncertainty in the future, is by including both fMRI and electroencephalography (EEG) measures to study designs. Considering fMRI measures the BOLD signal and EEG directly measures neuronal activity, the combination of both techniques might allow to disentangle the contributions of neurovascular coupling from those of neuronal activity to differences in BOLD signals between groups [42].

Second, in chapter 2, we revealed that a single dose of intranasal OT, as compared to PL, improved emotion recognition from point-light displays (PLDs) conveying biological motion in neurotypical participants (when different stimuli were used at both assessment sessions). However, we hypothesize that the effects of OT on emotion recognition might be related to more general OT-induced modulations perception of attention orienting, considering several studies have shown beneficial effects of OT on the orienting of attention in response to emotional cues[29,45,46]. In addition, OT has also been found to increase attention to the eye region[28] and to improve emotion detection in subliminally presented masked facial stimuli[47]. Therefore, the use of eye tracking in future research might solve the aforementioned uncertainty. On the other hand, in an eye-tracking study, Lischke et al. (2012) failed to show an association between the direction of overt visual attention and OT-induced improvements of facial emotion recognition[48]. In addition, the control task was easier to perform than the emotion recognition task, leading to ceiling performance. Future studies would benefit from including more challenging (non-emotional) control tasks, as this would allow a more direct disentanglement of potential effects of OT on basic attentional mechanisms from specific effects of OT on emotion processing. Note, however, that the control task was designed specifically to use in an fMRI scanner (chapter 5) to match the emotion recognition task on motor requirements and visual aspects.

Third, in chapter 5, we revealed no significant behavioral effects of the OT treatment in terms of PLD emotion processing. In contrast, using a similar task, prior work from our group showed that single-dose OT administration improved the recognition of emotional states from PLD when different sets of PLD stimuli were presented pre- and post-treatment [49]. In the current study, however, the same set of PLD stimuli was presented at each assessment session (albeit in a randomized order), potentially causing treatment-related improvements in emotion recognition to be masked by general practice or learning effects (i.e., both treatment groups showed significant improvements over time). Therefore, for future research, it is advised to use different stimuli during each assessment session to avoid learning effects.

3.4 Dosage and administration

In the studies reported in the current doctoral dissertation, we consistently adopted a dose of 24 IU, either as a single-dose administration or as a daily dose of 24 IU over a period of two or four weeks during the multiple-dose administration studies. Similar to other OT administration studies, the rationale behind the selection of this dose was, however, based more on precedent rather than empirical evidence [37]. Indeed, the majority of intranasal OT administration studies in both typically developing test samples and ASD samples have adopted the 24-IU-dose and have reported beneficial effects of intranasal OT administration. Few studies have used a dose differing from 24 IU with either a higher dose (i.e. 32 IU or 48 IU) or a lower dose (i.e. 8-18 IU (reviewed in [50]). The latter dose was more often used in OT administration studies that included children with ASD [51–57], although there was no clear evidence whether or not a higher dose would be unsafe for children.

Although the amount of administered intranasal OT was mostly similar across studies, the administration regime in the multiple-dose administration studies varied. For example, in one study, OT was administered during a period of four days [56], whereas other authors reported an administration period of three times two months[55]. Similar to our multiple-dose clinical trial in ASD, one study adopted an administration period of four weeks [58], whereas others used five weeks [59], six weeks [36,60,61], eight weeks [54,57,62] or twelve weeks [63,64]. Aside from the duration of the administration period, the amount of doses per day and moment of administration also varied across the multiple-dose administration studies. Similar to our multiple-dose clinical trial, two studies administered the OT treatment in the form of one dose [56,64], although it remains uncertain at what time the treatment was administered. In other studies, the OT treatment was administered twice daily, either in the morning and evening [54,59], or in the morning and afternoon [36,60,61,63] (or the exact regime was not mentioned [55,57,58,62]). However, a clear explanation for these choices is lacking.

Interestingly, recent efforts have been made to determine the ideal dose and timing to assess the efficacy of OT treatment. For example, to identify the most robust effects on amygdala reactivity, Spengler et al. (2017) [65] systematically varied both dose-test latencies (15-40 min, 45-70 min and 75-100 min) and doses of intranasal OT (12 IU, 24 IU and 48 IU) in a randomized, placebo-controlled, cross-over study including 116 typically developing adult men. They found that the OT-induced attenuation of amygdala responses to fear was most effective after a dose of 24 IU of intranasal OT and assessed within the 45-to-70-minute time window [65]. Kosaka et al. (2016) showed that a dose of intranasal OT larger than 21 IU was more effective than one lower than 21 IU in increasing reports of clinical improvement (as assessed with the Clinical Global Impression – Improvement Scale) in adult men with ASD [64]. Additionally, Shin et al. (2018) also showed

improved recognition of happy faces after a higher (40 IU) but not al 'lower' (32 IU) dose of intranasal OT [66]. In contrast, Quintana et al. (reported across two publications) compared the effects of four treatment conditions on amygdala activity in response to facial stimuli, including intravenous OT, two different doses of intranasal OT using a novel Breath Powered device (i.e. 8 IU and 24 IU), intravenous (IV) OT, and placebo. They revealed that the 8-IU-dose (compared to placebo) reduced amygdala activation in response to emotional faces and modulated the perception of anger in neutral facial stimuli [67,68]. Note, however, that the adopted Breath Powered device used in these latter studies, is different from a 'standard' nasal spray and is considered to be more efficient in transporting OT to the brain [67–69]. Interestingly, evidence from both human [70–72] and animal [73–75] research has revealed similar to stronger effects of lower doses of OT as compared to higher doses.

Notwithstanding these findings, one has to take into consideration that, to date, evidence on the actual amount of OT from these intranasal doses effectively reaching the brain is still uncertain. Taken together, more research is needed to uncover the most effective dosage, duration and device for OT treatment in ASD.

3.5 Generalizability

The studies reported in the current doctoral project only included typically developing adult men or adult men with ASD. Generalization of our findings to women or children with ASD is therefore limited. Also, considering our participants with ASD were predominantly high-functioning (average to above-average total IQ), our findings cannot be generalized to low-functioning individuals with ASD either.

Despite the commendable efforts to include women with ASD, only five (out of 23) OT administration studies have included women with ASD, albeit in small numbers. Hollander et al. [76,77], Andari et al. [78], Anagnostou et al. [61], Kosaka et al. [64], and Yatawara et al. [59] have included, respectively: one (out of 15), two (out of 13), three (out of 16), 13 (out of 60), four (out of 31) female participants with ASD. While these numbers reflect the 3/1 male-to-female ratio [79], they were considered too small to adequately and reliably assess potential gender differences. In addition, in typically developing individuals, single-dose OT administration studies have revealed differing effects in men and women. For example, while the OT-specific decrease in amygdala activity in response to negative affect (i.e. fearful stimuli, angry faces, pain,…) is mainly reported in men, authors have observed increased amygdala reactivity in response to fearful faces and threatening pictures in women [80,81] (but also [82]). Recently, these gender-dependent divergent effects of single-dose OT administration have been considered in light of their evolutionary adaptive value [13]. From an evolutionary perspective, men have experienced more intrasexual

competition and have favored risk-taking, whereas women have been focused on the care and protection of their offspring [83,84]. Accordingly, as stated by Ma et al. [13], lower fear of social threats (associated with decreased amygdala reactivity) can be beneficial for men in competition with other men, while a higher sensitivity and fear towards social threats (associated with increased amygdala reactivity) is beneficial in women to protect their offspring [84,85]. In order to fully understand the behavioral mechanisms of OT, more research in women is therefore necessary.
With respect to children with ASD, nine (out of 23) OT administration studies have included children and/or adolescents with ASD. These studies mainly assessed behavioral effects and showed mixed results [51–59,63]. Considering ASD is a neurodevelopmental disorder, it might however be particularly relevant to investigate the neural effects of multiple-dose OT treatment in children with ASD. Chevallier et al. (2012) argued that children with ASD, due to early-life impairments in social motivation (social orienting and/or social anxiety), might be deprived of the social learning experiences needed to build adequate social skills and develop the typical social brain circuits [86]. In this respect (and in light of our findings), administration of multiple-dose intranasal OT treatment during this developmental window in children with ASD might aid in improving social motivation and consequently increase experience-based adaptations in social brain circuits.

Considering we only included high-functioning, adult men with ASD, and considering the fewer findings in children and women with ASD showed differential effects of OT, more clinical trials are needed including larger sample sizes and more differing study samples (i.e. low-functioning ASD, women and children with ASD).

4 Future directions

4.1 Beyond Autism Spectrum Disorder

More research is needed to explore which individuals will benefit most from intranasal OT intervention. Although the current doctoral project, in particular, aimed to investigate the effects of OT treatment for improving the core characteristics of ASD, other studies have highlighted the potential of OT treatment for other conditions characterized (either primarily or secondarily) by social impairments, such as schizophrenia, social anxiety disorder, post-traumatic stress disorder, depression or obsessive-compulsive disorder.
Based on findings of the current doctoral project, one additional direction might be to explore individual differences in attachment and how these might modulate treatment responses. For example, as described in chapter 3, our results indicated that, in neurotypical individuals, two

weeks of OT treatment exerted a specific influence on decreasing a person's reluctance towards closeness or trust in others (decrease in attachment avoidance), but that it had no specific effect on altering a person's feelings of insecurity about one's own abilities (attachment anxiety) or one's faith in the responsiveness of attachment figures (attachment security). In addition, results also indicated that two weeks of daily OT administration induced an increase in self-reported attachment towards peers. In line with previous research[87–90], these treatment-induced changes were most pronounced for participants with less secure attachment towards their peers, indicating that normal variance at baseline modulated treatment response. The findings described in chapter 4 are in line with and extend these findings by showing that, in individuals with ASD, four weeks of OT treatment increased feelings of attachment avoidance immediately after the treatment until follow-up, one month and one year post-treatment.

These findings can be interpreted within the framework of affiliative-motivation hypothesis [91] suggesting that OT specifically acts by increasing affiliative strivings and that individuals with a decreased tendency to affiliate (e.g. avoidant attached individuals, individuals with ASD) may be most likely to benefit from OT treatment.

Together, these observations strengthen the notion that OT should not only be considered as a potential intervention approach for individuals with ASD, but also highlight the importance of adequately characterizing inter-individual differences in attachment characteristics for evaluating the effects of OT treatment both within and beyond individuals with ASD. In this respect, more research is needed to assess which individuals will benefit most from OT treatment and how individual differences in attachment characteristics might delineate different patient groups.

4.2 Epigenetics

Accumulating evidence supports the role of the Oxytocin Receptor Gene (OXTR) in the phenotypic expression of human social behaviors including pair bonding [92], empathy and stress reactivity [93], emotional support seeking [94], generous behavior [95], empathy and social communication [96], increased benefits from social support [97], face perception [98] and sociality [99]. Also with respect to ASD, genetic research has linked OXTR to the phenotypic expression of sociality [100–103]. These studies investigated polymorphisms in the DNA sequence of the OXTR gene, but more recently, interest is growing to additionally explore the potential contribution of epigenetic factors. Although much disputed in the past, it is now widely assumed that epigenetic modifications (chromosomal changes without alterations to the DNA sequence) can generate stable (neurobehavioral) phenotypes [104]. One of the mechanisms by which epigenetic modifications can be induced is DNA methylation, a process involving chemical modification of the DNA's cytosine-

guanine (CpG) dinucleotides, which in turn has been related to gene expression [105]. In particular, greater DNA methylation of a specific gene is associated with decreased expression of that gene. Therefore, epigenetic methylation of the OXTR will likely silence the OXTR and thus reduce the expression of the oxytocin receptors.

In the healthy population, imaging genetic studies assessing OXTR methylation have provided consistent evidence of a relation between OXTR methylation and individual social tendencies and activity in social brain regions (e.g. amygdala) [106,107]. With respect to ASD, only two studies explored methylation patterns of the OXTR and revealed mixed results with one study showing increased levels of methylation in ASD [108] while the other showed decreased levels of methylation [109].

Collectively, these prior studies have provided important initial evidence for the role of epigenetic variations of the OXTR in inducing inter-individual differences in social and emotional behavior. In this respect, and considering the large heterogeneity within the population of individuals with an ASD, it is pivotal for future research to investigate the potential role of epigenetic variations in ASD etiology as well as its relation to OT administration responses in both typically developing individuals and individuals with ASD.

4.3 Stress and autonomic nervous system physiology

Based on the above-mentioned neuroendocrine models [9,37], OT is suggested to increase the salience of social stimuli and consequently, via amygdala-prefrontal projections, to exert top-down modulation of social information processing. Compared to this 'prosocial mechanism', the 'anxiolytic mechanism' has received much less attention in the field, especially in ASD. Also in our multiple-dose OT administration study including participants with ASD, we did not include any stress-related measures or physiological measures intended to assess fear- or stress-related arousal (e.g. skin conductance, heart rate variability (HRV) or blood pressure). It is, however, suggested that, via brainstem structures (presumably also influenced by amygdala-prefrontal projections [9,37]), OT modulates stress reactivity and anxiety from the bottom up, by cardiovascular and autonomic nervous system (ANS) regulation. The ANS is part of the peripheral nervous system (i.e. the nervous system spread throughout the body except for the brain and spinal cord) and innervates internal organs including the heart, lungs, gastrointestinal organs, etc. In addition, the ANS itself comprises two branches: the sympathetic nervous system, involved in a general 'fight-or-flight-response', and the parasympathetic nervous system, involved in a general 'rest-and-digest-response' [110]. This latter system, and specifically autonomic cardiac regulation, is of particular interest for investigating the neuroendocrine mechanism behind OTs anxiolytic effects.

With respect to ASD, the regulation of ANS functioning might be particularly relevant considering, at least within a subset of individuals with ASD, the external (social) environment might be experienced as 'over-arousing' or even threatening, which in turn can lead to social withdrawal and/or the expression of RRBs (specifically insistence on sameness) [34,35]. Indeed, first studies have revealed that compared to healthy controls, individuals with ASD show reduced HRV [111–113]. HRV (the variation over time between consecutive heart beats) is believed to reflect the state of the ANS[114] and serves as a an index for autonomic cardiac regulation [115], whereby greater HRV is associated with both increased cardiac autonomic regulation and parasympathetic activity [37]. Interestingly, both in animals [116] and healthy controls [117,118], OT has already been shown to increase HRV. However, no clinical trial to date has evaluated the effects of multiple-dose OT treatment on ANS functioning or peripheral stress physiology (e.g. ANS regulation of cardiovascular systems). Considering the current identification of long-term neural effects in terms of attenuated amygdala activity in ASD after OT treatment, insights into the potential long-term changes in ANS stress physiology (i.e. assessed via HRV) and their relation to amygdala activity and/or amygdala-brainstem connectivity form promising directions to gain a more comprehensive understanding of the underlying (anxiolytic) mechanism of OT treatment in ASD.

5 Considerations for clinical practice

In light of the current debate in literature on the reliability of intranasal OT research for improving social behavior and considering this project is the first to study and uncover retention effects of intranasal OT treatment for improving ASD characteristics and altering social brain activity, any advice towards the use of OT in clinical practice is premature. However, certain aspects of intranasal OT treatment hold promise for future therapeutic approaches.

If intranasal OT treatment were to be used in clinical practice, one advantage is that the administration mode is highly accessible for home-based use. The use of OT in clinical practice is not new, considering it is widely used in the field of obstetrics (e.g., induction of labor[119]). In these situations, OT is administered intravenously in order to control the timing and amount of the dosing. However, intravenous administration of OT treatment is too invasive for both (experimental) research and clinical purposes, and oral administration is not suitable due to metabolic processes in the gastrointestinal tract and the liver[41]. Considering the nasal mucosa is the only location in the human body directly connecting the central nervous system and the environment, Guastella et al. (2012) recommended nasal spray administration, with nasal sprays causing minimal discomfort, being easy to use, and therefore typically resulting in higher

compliance[41]. A significant limitation of this approach, however, is that we currently do not know how nor how much OT or actually reaches the brain.

In addition to its easy use, another advantage is that, to date, authors have not reported any consistent severe side effects. In the current project, no significant differences were evident in frequency/proportion or severity of side effects between participants that had received the OT treatment compared to those that had received the PL treatment. Overall, participants reported rather mild to moderate side effects with the most frequently reported side effect being 'runny nose'. These findings are in line with prior research on the safety and tolerability of intranasal OT treatment. Although a more recent review is needed, MacDonald et al. (2010) have reviewed side effects and safety data on the use of intranasal OT treatment in 38 clinical trials, including both neurotypical participants and patient samples (i.e., ASD, SAD, PTSD, OCD), conducted between 1992 and 2010. At the time, 1529 participants had received intranasal OT treatment or placebo and only 279 reported mild side effects, mainly including: (1) increased calmness/euphoria or more energy; (2) light headedness, drowsiness and/or headache and (3) nasal irritation, dry mouth/throat[120]. Importantly, they found no differences in the type, frequency and severity of side effects reported by participants who had received the OT versus those who had received the PL[120]. More recently, Wang et al. (2019) assessed the safety of intranasal OT treatment for the core characteristics of ASD in 16 studies conducted between 2003 and 2018. In 10 of the 16 studies, no severe side effects were reported [121]. In 2 of the 16 trials[59,62], severe side effect were reported, including hyperactivity and aggression (participants receiving OT and 1 receiving PL), and seizures (1 participant both during OT and PL treatment in a cross-over trial and 1 participant during an off-treatment, follow-up period)[121]. Importantly, most reported side effects were mild to moderate and no differences between the severity or nature of the side effects in participants on the OT versus the PL treatment were evident[121].

As described above, we hypothesize that a single dose of intranasal OT treatment exerts immediate responses in the social brain circuit, whereas multiple-dose intranasal OT treatment might stimulate endogenous production of OT, potentially in an experience-dependent manner, which in turn could explain the long-lasting effects of the treatment found in the current doctoral project. In this respect, administering OT treatment during a period of behavioral therapy or social training might maximize therapeutic potential for individuals with ASD[122]. Supporting this notion, authors have already reported that OT combined with psychotherapy had beneficial effects for individuals with social anxiety disorder[123] and depression[124]. On the other hand, a recent study assessing the effects of intranasal OT treatment combined with cognitive-behavioral social skills training in participants with schizophrenia did not improve social cognition[125].

To conclude, although intranasal OT treatment is easy to administer and safe to use (in both healthy and clinical populations), evidence on the efficacy of the potential therapy is yet not strong

enough to encourage its use in clinical practice. For the future, however, we suggest that the integration of intranasal OT treatment with behavioral therapy or social training might form a successful combination.

6 Conclusions

This dissertation provides evidence that intranasal administration of OT, albeit in single-dose or multiple-dose form, is effective for inducing behavioral changes in typically developing adult men and behavioral and neural changes in adult men with ASD. In particular, for the first time, we show that multiple-dose intranasal OT administration is effective for inducing long-lasting improvements in repetitive and restricted behaviors, feelings of attachment and vigor. Additionally, our findings, indicating long-lasting attenuated amygdala activity and sustained higher pSTS activity after the multiple-dose OT treatment until one year post-treatment, provide first indications for the anxiolytic role and social salience boosting effect of OT in adult men with ASD. However, in order to generalize these findings, more research replicating these findings in larger and more diverse samples is necessary. Finally, we can conclude that the current doctoral project provides an important contribution to the field of OT research and puts researchers a step closer to finding an adequate intervention approach for the core characteristics of ASD.

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Summary

Autism Spectrum Disorder (ASD) is a complex neurodevelopmental disorder characterized by difficulties in social interaction and communication, and restricted interests and repetitive behaviors. Considering the increasing prevalence and high clinical, social and financial burden of ASD on society, there is a strong need for effective treatments. To date, therapeutic interventions mainly comprise behavioral social skills trainings, for which evidence is mixed. In addition, the only approved pharmacological therapies do not target the core characteristics of ASD. However, during the past 15 years, the neuropeptide oxytocin (OT) has gained increasing interest as a potential therapeutic approach for improving the core social impairments characteristic of ASD. OT is a neuropeptide with a dual function. As a hormone, OT is released into the bloodstream where it is known to affect bodily functions such as lactation and uterine contractions. Within the brain, OT acts as an important neuromodulator for modulating complex social behaviors such as interpersonal bonding, reciprocity and face processing, and the ability to establish trust and social attachment.
The current doctoral project aimed to gain insight into the behavioral and neural effects of single-dose and multiple-dose administration of OT both in the neurotypical population and in individuals with ASD. Before initiating the clinical trial designed to assess the behavioral and neural effects of single-dose and multiple-dose intranasal OT administration in adult men with ASD (that forms the core of the current doctoral project), we first tested (parts of) the study procedure and outcome measures in a sample of typically developing adult men.

In a first study, we assessed the immediate effects of single-dose OT treatment (compared to placebo (PL)) on emotion recognition from point-light biological motion and found that a single dose of intranasal OT had a significant, medium-sized effect on emotion recognition from point-light biological motion regardless of the emotional valence of the presented stimuli. Albeit moderate, these findings of OT-induced improvements in emotion recognition provided further support for a link between OT and the processing of social information. A complementary line of research has also highlighted the beneficial effects of intranasal OT administration on feelings of attachment, yet evidence on the effects of multiple-dose OT treatment in typically developing individuals was limited. Accordingly, in a subsequent study, we assessed the effects of two weeks of daily OT administration (compared to PL) on measures of attachment feelings, social responsiveness, quality of life and mood. Participants reported significant reductions in attachment avoidance and increases in reports of attachment toward peers after two weeks of OT treatment. In addition, treatment-induced changes were most pronounced for participants with less secure attachment towards their peers, indicating that normal variance at baseline modulated treatment response. OT treatment also improved participant's reports of mood with respect to tension and (tentatively) anger. Further, at the end of the two-week trial, both treatment groups (OT, PL) reported increased social responsiveness and quality of life, indicating that these effects were not treatment-specific. Again, the reported improvements in feelings of attachment after the

multiple-dose treatment with OT provided further evidence in support of a pivotal role of OT in promoting social behavior and, in particular, the experience of attachment.

After finding these beneficial effects of both single-dose and multiple-dose OT administration in typically developing adult men, we adopted the aforementioned outcome measures in a clinical trial with adult men with ASD. We investigated the immediate and long-term effects of multiple-dose OT treatment on a behavioral and neural level. To assess the behavioral effects of multiple-dose OT treatment, autism characteristics (social responsiveness, and restricted and repetitive behaviors (RRBs)), feelings of attachment and quality of life were assessed up to one month and one year post-treatment. To assess the neural effects of multiple-dose OT treatment, we performed functional magnetic resonance imaging (fMRI) in order to study the changes in task-related brain activity of the posterior superior temporal sulcus (pSTS) and amygdala up to one month and one year post-treatment. To do so, we adopted a double-blind, randomized, placebo-controlled, parallel design (clinical trial) during which we tested participants (i) at baseline, (ii) after a single dose of OT, (iii) immediately after four weeks of continual OT treatment, and at two follow-up sessions, (iv) one month (four weeks) and (v) one year post-treatment. A key question was to evaluate whether any beneficial effects of continual OT treatment would outlast the period of actual administration until one month or even one year post-treatment. On a behavioral level, results revealed that the four-week, multiple-dose OT treatment did not improve social responsiveness, but did induce medium- to large-sized improvements in RRBs, reduced feelings of avoidance towards others and increased feelings of vigor, which outlasted the period of actual administration until one month and even one year post-treatment. On a neural level, results revealed that the four-week, multiple-dose treatment attenuated activity in bilateral amygdala and induced sustained higher levels of task-related activity within (right) pSTS. Anew, these treatment-induced neural changes were shown to outlast the administration period until one month and even one year post-treatment, indicating that continual OT treatment induced long-lasting neural changes in core regions of the social brain circuit. Importantly, we also showed that the extent of amygdala attenuation was associated with the extent of (long-term) improvements in self-reports of social functioning (social responsiveness).

This dissertation provides evidence that intranasal administration of OT, albeit in single-dose or multiple-dose form, is effective for inducing behavioral and neural changes in typically developing adult men and adult men with ASD. In particular, for the first time, we show that these changes outlast the administration period until one year post-treatment. These findings provide first indications for the anxiolytic role and social salience boosting effect of OT in adult men with ASD. However, in order to generalize these findings, more research replicating these findings in larger and more diverse samples is necessary.

Samenvatting

Autismespectrumstoornis (ASS) is een complexe ontwikkelingsstoornis gekenmerkt door moeilijkheden met sociale interactie en communicatie, alsook repetititief gedrag en specifieke interesses. Gezien de toenemende prevalentie en hoge klinische, sociale en financiële druk van ASS op de samenleving, is er een grote nood aan effectieve behandelingen. Tot nog toe omvatten de beschikbare behandelingen hoofdzakelijk sociale vaardigheidstrainingen, waarvoor evidentie gemengd is. Bovendien zijn de enige goedgekeurde medicinale behandelingen niet primair gericht op het verbeteren van de hoofdkenmerken van ASS. De voorbije 15 jaar is de neuropeptide oxytocine (OT) echter steeds meer naar de voorgrond getreden als een potentiële interventie om de hoofdkenmerken van ASS te verbeteren. OT is een neuropeptide, geproduceerd in de magnocellulaire neuronen van de paraventriculaire en supraoptische kernen van de hypothalamus, waarna deze een dubbele functie uitoefent. Op hormonaal niveau wordt OT vrijgegeven in de bloedsomloop om lichamelijke functies als lactatie en baarmoedercontracties te beïnvloeden. Op niveau van de hersenen functioneert OT als een belangrijke neuromodulator om sociale gedragingen als interpersoonlijke binding, wederkerigheid en gezichtverwerking te beïnvloeden , alsook om interpersoonlijk vertrouwen en sociale gehechtheid tot stand te brengen. Met het huidige doctoraatsproject trachtten we meer inzicht te verkrijgen in de gedragsmatige en neurale effecten van een enkele dosis en meerdere dosissen OT, zowel in de typisch ontwikkelende populatie als in personen met ASS. Vooraleer we startten met de klinische studie om de gedragsmatige en neurale effecten van één dosis en meerdere dosissen intranasale OT te onderzoeken bij volwassen mannen met ASS (de kern van dit doctoraatsproject), hebben we eerst (delen van) de studieprocedure en uitkomstmaten getest in een groep typisch ontwikkelende volwassen mannen.

In een eerste studie onderzochten we de onmiddellijke effecten van één dosis intranasale OT op emotieherkenning via *point-light displays* die biologische beweging vertoonden (bewegende menselijke figuren voorgesteld a.d.h.v. stippen). Onze resultaten toonden dat één dosis OT de emotieherkenning van *point-light* biologische beweging significant kon verbeteren onafhankelijk van de emotionele valentie van de getoonde stimuli. Deze bevindingen boden verdere steun voor een verband tussen OT en de verwerking van sociale informatie. Naast de link met sociale informatieverwerking heeft een complementaire onderzoekslijn ook de gunstige effecten van intranasale OT voor gevoelens van gehechtheid onder de aandacht gebracht. Bovendien was er nog geen evidentie voor de effecten van meerdere dosissen intranasale OT bij typisch ontwikkelende personen. Daarom hebben we in een volgende studie onderzocht wat de effecten zijn van een tweeweekse behandeling met intranasale OT op gevoelens van gehechtheid, sociale responsiviteit, levenskwaliteit en het gemoed bij typisch ontwikkelende volwassen mannen. Na de inname van OT (gedurende twee weken) rapporteerden de deelnemers significante verlagingen in gevoelens van vermijdende gehechtheid en toenames in gevoelens van gehechtheid t.o.v. leeftijdsgenoten (vrienden). Bovendien bleken de door OT geïnduceerde veranderingen het grootst te zijn voor die deelnemers met een minder veilige gehechtheid t.o.v. hun leeftijdsgenoten bij aanvang van de studie. Daarnaast rapporteerden deelnemers na de OT

inname in vergelijking met PL inname (gedurende twee weken) ook verbetering in gemoed, meer bepaald minder spanning en (tentatief) minder boosheid. Beide groepen deelnemers rapporteerden echter een verbetering in sociale responsiviteit en levenskwaliteit (onafhankelijk van de behandeling die ze gekregen hebben). Weerom boden de gerapporteerde verbeteringen in gevoelens van gehechtheid na toediening van meerdere dosissen OT bewijs voor de belangrijke rol van OT in het promoten van sociaal gedrag, en specifiek, voor de beleving van gehechtheid.

Na de vaststelling van gunstige effecten van zowel één als meerdere dosissen intranasale OT bij typisch ontwikkelende volwassen mannen, hebben we deze uitkomstmaten gebruikt om de effecten van intranasale OT te onderzoeken bij volwassen mannen met ASS. We onderzochten de onmiddellijke en lange-termijn effecten van meerdere dosissen OT (één dosis per dag gedurende vier weken) op zowel gedragsmatig als neuraal vlak. Om de gedragsmatige effecten van deze OT behandeling te onderzoeken, gingen we de effecten ervan na op de hoofdkernmerken van ASS (sociale responsiviteit, en repetitief gedrag en beperkte interesses), alsook gehechtheid en levenskwaliteit. Om daarnaast ook de neurale effecten van de OT behandeling te onderzoeken, maakten we gebruik van functionele magnetische resonantie beeldvorming om de mogelijke veranderingen in hersenactiviteit in de posterieure superior temporale sulcus gebieden en de amygdala te bestuderen terwijl de deelnemers de hierboven beschreven emotieherkenningstaak uitvoerden. Deze potentiële gedragsmatige en neurale effecten onderzochten we vervolgens tot vier weken en één jaar na afronden van de eigenlijke behandeling. Om dit onderzoek uit te voeren, hebben we een dubbel-geblindeerde, gerandomiseerde, placebo-gecontroleerde, klinische trial opgesteld, tijdens dewelke we gegevens verzamelden op verschillende momenten, namelijk tijdens een baselinemeting (aan het begin van de studie) (i), na één dosis OT (ii), na de vierweekse behandeling (iii), vier weken na de stop van de behandeling (iv), en één jaar na de stop van de behandeling (v). Het voornaamste doel van deze klinische studie was om na te gaan of potentiële gunstige effecten onmiddellijk na de vierweekse behandeling nog aanwezig zouden zijn vier weken na de stop van de behandeling of zelfs één jaar later. Op gedragsniveau toonden de resultaten dat de vierweekse behandeling met OT de sociale responsiviteit van de participanten niet kon verbeteren, maar wel hun repetitief gedrag en specifieke interesses, hun gevoelens van vermijdende gehechtheid ten opzichte van anderen, alsook hun gemoed (met name gevoelens van kracht en energie). Op neuraal niveau toonden de resultaten dan weer dat de vierweekse behandeling met intranasale OT de hersenactiviteit in bilaterale amygdala verlaagde en hogere activiteit in de (rechter) pSTS ondersteunde. Zowel op gedragsmatig als neuraal niveau werden deze effecten gevonden tot vier weken en zelfs één jaar na de behandeling. Belangrijk hierbij is dat we ook aantoonden dat de mate van verlaagde amygdala activiteit gerelateerd is aan de mate van (lange-termijn) verbeteringen in zelf-gerapporteerde sociale responsiviteit.

Kortom, dit doctoraatsproject levert bewijs dat intranasale toediening van OT, zij het in de vorm van één dosis of als meerdere dosissen, zowel gedragsmatige als neurale veranderingen kan teweegbrengen bij zowel typisch ontwikkelende volwassen mannen als bij volwassen mannen met ASS. Meer specifiek tonen we – voor de eerste keer – dat deze veranderingen bij volwassenen mannen met ASS terug te vinden zijn tot wel vier weken en zelfs één jaar na de behandeling. Bovendien bieden de bevindingen initieel bewijs voor de angstdempende rol en het *social salience* verhogende effect van OT voor volwassen mannen met ASS. Om onze bevindingen te kunnen generalizeren is echter meer onderzoek met grotere en meer diverse testgroepen nodig.

Appendices

Tables

Supplementary Table 4.3. Mean pre-to-post change scores for each treatment group (oxytocin, placebo) and assessment session (T1, T2, T3). T- and p-values correspond to single-sample t-tests assessing within-group pre-to-post changes, separately for the OT and PL group.

Supplementary Table 4.4. Assessment of baseline differences between groups.

Supplementary Table 4.5. Baseline attachment comparison between individuals with ASD and typically developing control subjects using SAAM and IPPA.

Table 5.1. Participant characteristics.

Table 5.2. Mean change-from-baseline parameter estimates for each treatment group (oxytocin, placebo), session (T1, T2, T3, T4), and ROI.

Supplementary Table 5.1. Detailed information on comorbidities and medication use for participants of the oxytocin and placebo treatment groups.

Supplementary Table 5.2. Side effect screening.

Supplementary Table 5.3. Baseline activated brain regions across groups during emotion processing from point-light displays (vs control).

Supplementary Table 5.4. Average contrast estimates for each region of interest (posterior superior temporal sulcus (pSTS), amygdala), treatment group (oxytocin, placebo) and assessment session (T0, T1, T2, T3, T4).

Supplementary Table 5.5. For completeness and hypothesis-generating purposes, treatment-induced changes in brain activation were also explored at the whole-brain level.

Supplementary Table 5.6. Mean change-from-baseline scores in behavioral performance (recognition of emotional states from point-light biological motion) listed separately for each treatment group (oxytocin, placebo), assessment session (T1, T2, T3, T4) and emotional state (happiness, anger).

Supplementary Table 5.7. Raw behavioral performance scores listed separately for each treatment group (oxytocin, placebo), assessment session (T0, T1, T2, T3, T4) and emotional state (happiness, anger).

Supplementary Table 5.8. Association between neural changes (right posterior temporal sulcus/ amygdala activity) and behavioral improvements in social functioning, repetitive behaviors and feelings of avoidant attachment induced by the oxytocin treatment.

Figures

Appositions - Bijstellingen

To increase the relevance of autism research for both the autism community and the research field, the insights of individuals on the spectrum and their environment (via groups and/or societies) should be taken into account.
Om de relevantie van autismeonderzoek te vergroten voor zowel de autismegemeenschap als het onderzoeksveld, zouden de inzichten van mensen met autisme en hun omgeving (via groepen en/of verenigingen) meer gehoord moeten worden.

In light of the current 'green wave' concerning the environment and climate change in the western world and considering academics and researchers are the ones warning the world about the state of our climate and biodiversity, we (as academics and researchers) can set an example.

Rekening houdend met huidige 'groene golf' betreffende milieu en klimaatverandering in de westerse wereld en gezien het net academici en onderzoekers zijn die de wereld trachten te waarschuwen over de staat van ons klimaat en de biodiversiteit, kunnen wij (als academici en onderzoekers) het goede voorbeeld geven.

In light of the high prevalence of mental health issues in Belgium (stress, burn-out, depression) and increasing local and global concerns about the mental and physical well-being of PhD students and early career researchers, university policies need updating.

Rekening houdend met de hoge prevalentie van geestelijke gezondheidsproblemen in België (stress, burn-out, depressie) en de toenemende lokale en globale bezorgheid over het psychische en fysieke welzijn van doctoraatsstudenten en onderzoekers aan de start van hun carriere, is er nood aan een vernieuwd universitair beleid.

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Personal contribution

The studies in chapters 2 and 3 were designed by Prof Kaat Alaerts and data was collected by Emmely Berra (as part of a master thesis project) and the author of this doctoral manuscript (Sylvie Bernaerts). Data preparation, data analysis, data interpretation and writing of the manuscripts was conducted by Sylvie Bernaerts, under supervision of Prof Kaat Alaerts. Sylvie Bernaerts acquired, prepared and analyzed the data of the clinical trial with ASD participants (chapters 4 and 5), and drafted and revised the manuscripts for publication as well as the current doctoral manuscript. Prof Kaat Alaerts and Prof Jean Steyaert designed the initial clinical trial and Sylvie Bernaerts co-designed the one-year retention session of the clinical trial. Claudia Dillen aided with data collection at the start of the clinical trial. As co-authors of one or more chapters (scientific publications), Prof Kaat Alaerts, Prof Jean Steyaert, Prof Bart Boets and Prof Guy Bosmans provided intellectual contributions to one or more of the manuscripts and aided in revising these manuscripts for publication.

Conflict of interest

The author of this doctoral manuscript declares no conflict of interest for all of the contributing authors. The funding agencies had no role in the design of the studies, nor in the collection, analysis, interpretation or publication of the data, nor in the writing of this manuscript.

About the author

Sylvie Bernaerts was born in Mechelen (Belgium) on the 27th of December, 1991. In 2012, she obtained a Bachelor of Science in Psychology at KU Leuven (Cum Fructu), with a focus on clinical and health psychology. In 2014, she graduated Magna Cum Laude as a Master of Science in Psychology, with a specialization in clinical and health psychology for adults. Afterwards, she worked as a clinical psychologist in the multidisciplinary teams of the memory clinic and obesity clinic at AZ St. Dimpna in Geel (Belgium). However, with a strong interest in science and eagerness to learn, she switched her clinical work for scientific work and started her PhD in the Neuromodulation Lab of Prof Kaat Alaerts at the Department of Rehabilitation Sciences of the KU Leuven in 2015. During her PhD, she conducted a large clinical trial during which she investigated the immediate and long-term (until one year) neural and behavioral effects of a multiple-dose oxytocin intervention in adult men with ASD. In addition, during her doctoral training, Sylvie was also a member of multiple scientific organizations. She was a member of the Leuven Autism Research Consortium (LAuRes), which unites autism research groups in behavioral sciences, neuroimaging, cellular and molecular biology, genetics, and medical data processing at the KU Leuven, for which she also co-managed the website. Aside from LAuRes, Sylvie was also an active member of the Student and Trainee Committee of the International Society for Autism Research (INSAR), which supports INSAR's mission of promoting quality autism research to improve the lives of individuals affected by Autism Spectrum Disorder and to provide the next generation of autism researchers opportunities for collaboration, career development, and education. For the INSAR STC she was the newsletter editor in addition to general organizational functions. Finally, she was also a member of the following scientific organizations, Organization for Human Brain Mapping (OHBM), the Society of Biological Psychiatry (SOBP), and the International Society for Autism Research (INSAR).

When she is not working, Sylvie loves to cook and bake, travel the world, ski and spend time with friends and family.

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