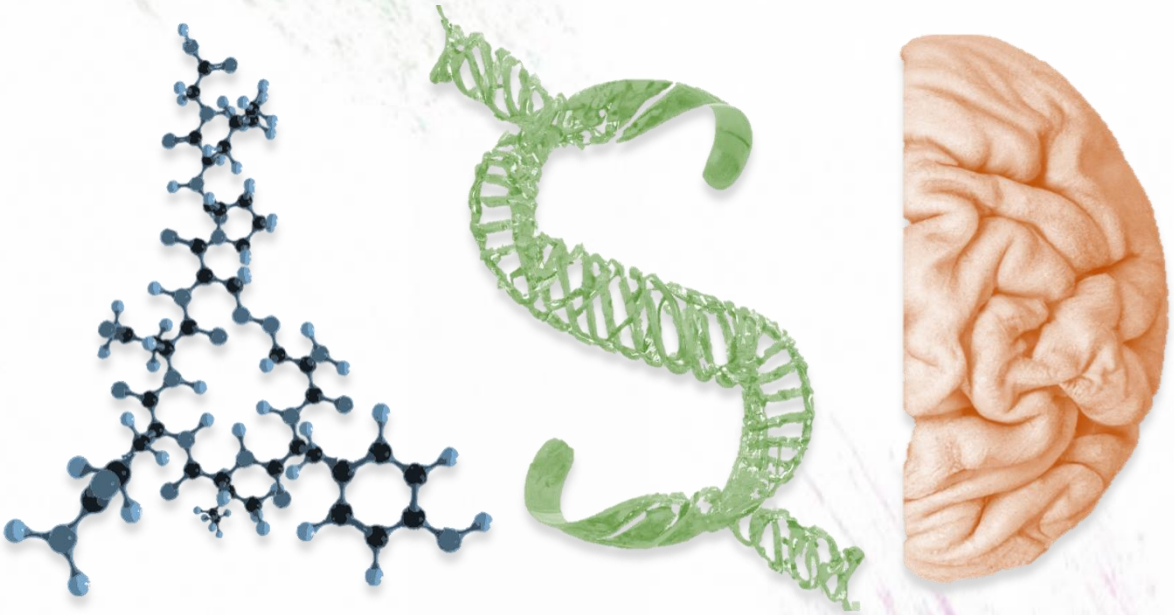
The role of oxytocin in autism



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De rol van oxytocine in autisme

Neurobiologische markers en interventies voor socio-communicatieve stoornissen bij autisme

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Preface

Oxytocin is a small protein produced in our brain and released in the body. A well-known function is to facilitate childbirth and milk-ejection in mothers, but oxytocin is also a major driver of *bonding* between people. For instance, our brains produce more oxytocin when we fall in love. For these reasons, in popular media, oxytocin is often called the "love hormone". Excitingly, we can easily boost our own oxytocin levels in various ways: hugging a loved one, petting our dog, putting on some good music, or enjoying a healthy workout… And some more good news, oxytocin levels appear to increase with age.

Researchers have pinpointed several ways how oxytocin mediates our social behaviour, for instance oxytocin has been linked to better recognition of emotions in a face, increased eye contact and being more trustful towards others. These characteristics made it very interesting to evaluate the associations between oxytocin and autism spectrum disorders (ASD), a neurodevelopmental condition where some of these socio-communicative abilities may be impaired. **The principal aim of this PhD thesis is to provide a comprehensive understanding of the role of oxytocin in ASD.** First, by summarizing the existing knowledge on the endogenous oxytocinergic system in ASD, and secondly by evaluating the behavioural and neural effects of a long-term oxytocin pharmacotherapy in children with ASD.

Chapter 1 | General Introduction

Autism spectrum disorder

Autism spectrum disorder or ASD is an early-onset neurodevelopmental condition, characterised by difficulties in two domains: social communication and interaction, and the presence of a pattern of restricted and repetitive behaviours, interests or activities (American Psychiatric Association, 2013). The diagnosis is made when these symptoms are present from early childhood and if they interfere with the daily functioning of the child. Difficulties in the social domain range from a lack of reciprocity (e.g., one-sided interaction) and limited sharing of interests and emotions to problems with understanding/applying more complex social signals (e.g., how and when to join an ongoing conversation). Additionally, non-verbal communication difficulties, for instance atypical eye contact and difficulties with facial expressions and body language, are common. Individuals with ASD often develop compensatory strategies to cope with these problems; this unfortunately requires a lot of mental effort and can be very stressful, particularly when confronted with complex social situations. Symptoms from the second domain (restrictive and repetitive behaviours, interests or activities) include for instance repetitive movements (e.g., hand flapping), repetitive language use (e.g., echolalia) and atypical sensory processing. The current PhD thesis mainly focusses on the social communication domain.

A recent study estimated the prevalence of ASD between 0.9% and 1.5%, although this should be interpreted carefully, since these numbers vary widely between regions and across time (Fombonne, 2020). Concerning the latter, the numbers have been rising over the last decades, possibly due to better-defined diagnostic criteria, improved awareness and increased screening frequency (Masi et al., 2017; Sharma et al., 2018). For every four boys diagnosed with ASD, there is only one girl diagnosed; females are commonly under-identified, possibly as a consequence of male-directed diagnosis-strategies and better camouflaging strategies (e.g., more proficient at imitating social behaviours or stronger non-verbal communication skills) (Rynkiewicz et al., 2016; Sanchack & Thomas, 2016). The precise neurobiology of ASD is not yet known. However, important risk factors have been identified over time. Genetic predisposition combined with environmental factors are believed to be the main drivers (Masi et al., 2017). Environmental factors include parental age, the use of psychotropic medication by the pregnant mother or viral or bacterial infection, exposure of the foetus to insecticides or when the child is born

prematurely or with a low birth weight (Sharma et al., 2018). From identical twin studies, we know that genetics play an important role in ASD. Specifically, if one identical twin is diagnosed with ASD, then the other has a chance between 36% and 95% of also receiving a diagnosis of ASD, this chance drops to 0% - 30% in non-identical twins (Sharma et al., 2018). A non-twin sibling of an individual with ASD has a chance of 2% - 8% of having the same disorder, but the risk increases to 12% - 20% if the child with ASD has more symptoms in several domains (Bolton et al., 1994). ASD symptoms are also often expressed in genetic syndromes, such as Down's syndrome or fragile X syndrome (Sharma et al., 2018). Genetic research has been able to identify numerous susceptibility genes (e.g. several GABA system genes on chromosome 15q11–13 and the serotonin transporter gene on chromosome 17q, DiCicco-Bloom et al., 2006), however, the contribution of a single gene (via duplications, deletions, or a single nucleotide polymorphism) to the development of ASD remains scarce (Masi et al., 2017).

An ASD diagnosis can be reliably established as young as four years old, yet commonly it is not made until children enter elementary school (McDonnell, Bradley, et al., 2019; Styles et al., 2020). This diagnosis is typically made by a multidisciplinary team (child psychiatrist and/or neuro-paediatrician, psychologist, speech pathologist and/or physiotherapist), based on standardized instruments such as patient/caretaker interviews and observational measures (DiCicco-Bloom et al., 2006; McDonnell, Boan, et al., 2019). Even though supported by these standardized instruments, a diagnosis still largely involves (subjective) clinical expertise. Furthermore, ASD symptoms are heterogeneous, making it difficult to establish a differential diagnosis (DiCicco-Bloom et al., 2006; Masi et al., 2017; McDonnell, Boan, et al., 2019). Currently, no objective quantitative (bio)marker exists for ASD (Goldani et al., 2014; Kapur et al., 2012; Loth et al., 2015; McPartland, 2016; Varcin & Nelson, 2016). A supplementary objective tool would be useful to support a correct diagnosis at an early age and to offer guidance for accurate treatment possibilities. An earlier diagnosis will allow earlier interventions, and both have been shown to improve a variety of developmental processes and long-term outcomes (Fernell et al., 2013). Unfortunately, the large phenotypic heterogeneity of ASD, the lack of a causal link between genetic mutations and the occurrence of idiopathic ASD are complicating the search for diagnostic biomarkers. Nevertheless, different types of biomarkers have been proposed, besides diagnostic biomarkers, such as various types of therapeutic biomarkers (e.g. response, predictive and prognostic biomarkers, see chapter

7 for more information) (FDA-NIH Biomarker Working Group, 2016). Therapeutic biomarkers can yield higher classificatory power because they focus on a simple behavioural construct (and its underlying neural or biological correlates) rather than on a set of behaviours that define a disorder. For instance, a response biomarker is a type of therapeutic biomarker that indicates response to treatment and acts as a proxy for clinical endpoints by measuring the physiological responses pre- and post-treatment (Aronson, 2005). Another type of therapeutic biomarker is a prognostic biomarker, which is used to follow the clinical evolution of a patient, independent from a specific treatment (Skov et al., 2021). Based on the information of a prognostic biomarker a clinician can decide whether it is useful to focus therapy on a certain skillset or to change strategy. For example, if an individual with ASD has difficulties with social communication, it may be more opportune to focus on alternative communication strategies. So besides diagnostic purposes, biomarkers in ASD may aid in treatment-outcome evaluation (McPartland, 2016, 2021). To achieve such biomarkers, it will be necessary to adopt methods that are economical, scalable, sensitive, and objective (McPartland, 2016). Against this background, **part of the current PhD thesis explored and validated the use of a novel, fast and robust, neurophysiological (EEG) technique, to quantify difficulties in socio-communicative sensitivity in ASD (i.e. a diagnostic biomarker) and to monitor response throughout an intervention (i.e. a response biomarker), see chapter 5.**

ASD may have a huge impact on the wellbeing of the individual with ASD and on his/her environment, and yields high societal and economic costs. Yet, treatment options for ASD are limited (Masi et al., 2017). Although non-pharmacological treatments (e.g., behavioural, speech, play or music therapy) are often highly effective, they are time-consuming and expensive (Guastella & Hickie, 2016; Lai et al., 2020; Masi et al., 2017; Sharma et al., 2018; Styles et al., 2020). The pharmacological treatment options approved for ASD (e.g., risperidone or aripiprazole) do not specifically target the social symptoms, but often control comorbidities such as anxiety, schizophrenia, attention-deficit hyperactivity disorder (ADHD), sleep disorders, obsessive compulsive disorder (OCD), epilepsy and tic disorders (Sharma et al., 2018; Styles et al., 2020). Unfortunately, these pharmacotherapies frequently result in various side effects, further affecting wellbeing (Masi et al., 2017). Currently, there is exciting support that the neuropeptide oxytocin has the potential to alleviate social impairments in ASD (Yamasue et al., 2018; Yamasue &

Domes, 2017). Yet, evidence is mainly based on acute single-dose effects, and to yield full therapeutic potential lasting long-term effects of multiple-dose treatments have to be established, which is currently unclear. This PhD thesis describes the results from a double-blind placebo-controlled multiple-dose intranasal oxytocin clinical trial performed in children with ASD, **see chapter 4, 5 and 6**.

The oxytocinergic system in autism

Oxytocin is a neuropeptide (i.e., a small protein active in the brain) that is synthesized in the neurons of the paraventricular and supraoptic nuclei of the hypothalamus (**Fig. 1**). The hypothalamus projects to the posterior pituitary, where oxytocin is released in the bloodstream and acts as a hormone to influence bodily functions (e.g., induction of childbirth, through uterine contractions, and lactation (Zingg & Laporte, 2003)). The hypothalamus also projects to various brain structures, including those that influence social information processing (e.g., the amygdala), where oxytocin acts as an important modulator of socio-cognitive functions and complex social behaviours (e.g., empathy, emotion recognition and affiliative behaviours) (Fineberg & Ross, 2017; Marlin & Froemke, 2017).

Prior studies have shown associations between differences in endogenous oxytocin concentrations and social difficulties, pointing to a potential role of oxytocin in the pathogenesis of the social deficits that characterize ASD (Quattrocki & Friston, 2014). An earlier meta-analysis integrating findings of six studies involving adult samples, reported no significant group differences in endogenous oxytocin levels comparing adults with ASD to neurotypical controls (Rutigliano et al., 2016). Yet, newer research suggested a developmental pattern in the relationship between endogenous oxytocin levels and socioemotional functioning, with a stronger correlation in younger age groups (Torres et al., 2018). Accordingly, the present PhD thesis includes an updated and extended **systematic review and meta-analysis on the differences in endogenous oxytocin concentrations in individuals with ASD compared to matched controls** across a wider age-range (including children, adolescents and adults), **see chapter 2**.

The role of the oxytocin system and its relation to ASD has also been explored in a broader sense, for instance from a genetic perspective (de Oliveira et al., 2018; Elagoz Yuksel et al., 2016; Gregory et al., 2009; Maud et al., 2018; Rijlaarsdam et al., 2017). Particularly the

oxytocin receptor gene (*OXTR*) has been the focus of research. This gene codes for the protein functioning as the receptor for oxytocin and is highly expressed in subcortical (e.g., hypothalamus, amygdala and hippocampus), temporal and olfactory brain regions (Quintana et al., 2019). Previous animal research provided evidence for the role of *OXTR* in social behaviour (Takayanagi et al., 2005; Winslow & Insel, 2002). For example, profound deficits in maternal behaviours and increased aggression were found in *OXTR* knockout mice (Ragnauth et al., 2005; Winslow & Insel, 2002). Besides genetic modifications (e.g., deletion/duplication of the DNA sequence) also epigenetic factors can influence the functioning of a gene. In this regard, DNA methylation is one of the most extensively studied epigenetic mechanisms and it is involved in the regulation of gene transcription. Specific regions of the DNA within a gene can become methylated (i.e., a methyl group can attach on the DNA) (Tchurikov, 2005). DNA methylation of a gene is generally associated with decreased gene transcription, and thus less expression of the respective protein, in our case the oxytocin receptor (Kusui et al., 2001). Several studies have suggested an association between *OXTR* DNA methylation and social behaviours (Kraaijenvanger et al., 2019; Maud et al., 2018). Yet, these studies included a broad range of psychopathologies (including ASD) and lack focus on the broad ASD symptomatology. Therefore, the current PhD thesis comprises **a systematic review on the association between *OXTR* DNA methylation and variations in social traits (i.e. social cognition/perception and social anxiety) in individuals with and without ASD**, **see chapter 3**.

Mechanistic models of oxytocin

Given that oxytocin might modulate (pro)social behaviour and given that difficulties in social behaviour are a hallmark of a broad range of psychiatric disorders (e.g. ASD, schizophrenia, anxiety disorder or post-traumatic stress disorder), oxytocin has increasingly been used as a pharmacological intervention to modulate social behaviour (Cochran et al., 2013; J. C. J. Liu et al., 2012). Since oxytocin is not able to cross the blood-brain barrier, oral or intravenous administration is fairly ineffective (Jones et al., 2017, but see Yamamoto et al., 2019). In contrast, nasal sprays are often employed in oxytocin clinical trials and seem to be most efficacious: intranasally administered oxytocin penetrates the brain and does not affect peripheral organs (**Fig. 1**) (Borie et al., 2021).

Moreover, this method is non-invasive, making it an accessible means of drug delivery, especially for children (with ASD) (Quintana et al., 2021). Intranasal oxytocin can reach relevant brain regions, and elicit its prosocial effects, via several routes (Jones et al., 2017). Primarily, intranasally administered exogenous oxytocin can enter the olfactory bulb or travel through the trigeminal nerve to the cerebrospinal fluid. Also, intranasal oxytocin could be taken up by the oral mucosa and the gastroenteric system. Interestingly, exogenous oxytocin has also been shown to indirectly induce the release of endogenous oxytocin through a feed-forward mechanism (Jones et al., 2017).

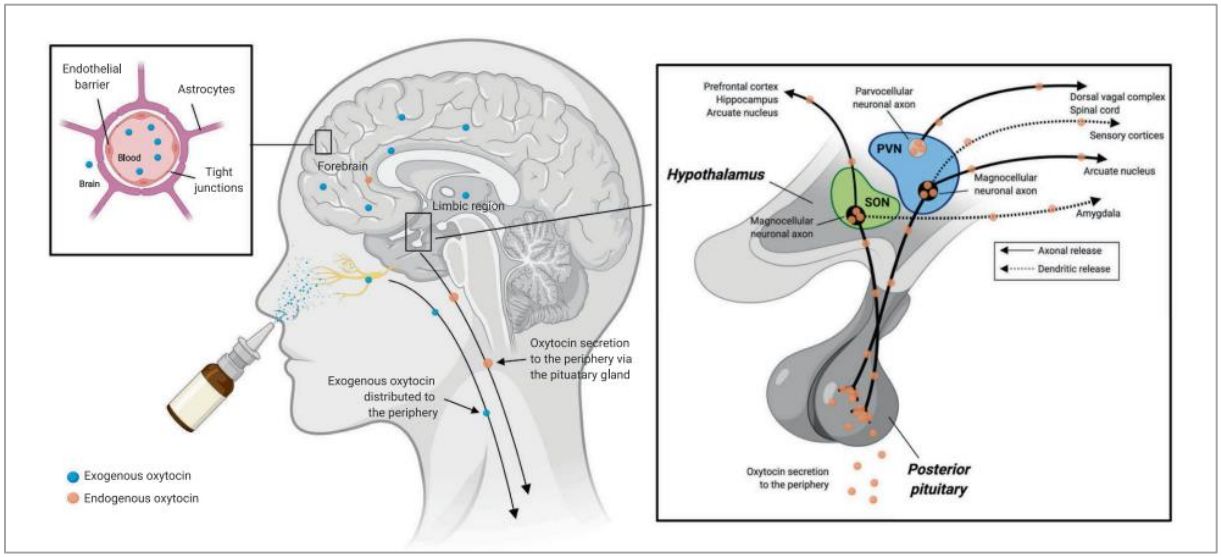

Figure 1. Endogenous oxytocin release and intranasal oxytocin administration. Endogenous oxytocin is mainly produced in paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. The posterior pituitary receives and stores oxytocin before releasing it into the bloodstream. Oxytocin is also transported to several brain regions via axonal and dendritic processes. Intranasally administered oxytocin is believed to reach the brain via the olfactory and trigeminal nerves, bypassing the blood-brain barrier. Figure reprinted from Quintana et al. (2021).

The fact that oxytocin can easily be administered intranasally via a nose spray (Quintana et al. (2021), contributed to the widespread exploration of its effects in various samples of psychiatric patients as well as in neurotypical controls. While oxytocin was initially simplistically announced as a "love or cuddle hormone", it should be noted that besides the mentioned prosocial and null effects of oxytocin, also *negative* effects on social behaviour have been reported. For instance, a single dose of oxytocin increased processing of negative social cues, such as the recognition of anger or fear (Leppanen, Wee, et al., 2017). Furthermore, in certain contexts, oxytocin can even promote negative social behaviour, such as envy, gloating or even defensive aggression towards out-group

members (de Dreu et al., 2010; Shamay-Tsoory et al., 2009). There are several mechanistic theories that attempt to elucidate these controversies and try to explain how oxytocin can mediate social behaviour.

One of the leading theories trying to account for the variety of reported social effects is the **social salience hypothesis of oxytocin** (Shamay-Tsoory & Abu-Akel, 2016). This theory combines (i) the observations that oxytocin is released during positive social interactions thus promoting prosocial behaviour (Heinrichs & Domes, 2008; Kemp & Guastella, 2011; Shamay-Tsoory & Abu-Akel, 2016) and (ii) the differential in-/out-group effects of oxytocin, that postulates oxytocin's role in human cooperation/conflict, specifically oxytocin increases in-group trusting behaviour versus out-group hate (Kemp & Guastella, 2011; Shamay-Tsoory & Abu-Akel, 2016). The social salience hypothesis suggests that oxytocin promotes the salience of social information by orienting attention towards social cues, which, depending on the context, can be both positive or negative (Shamay-Tsoory & Abu-Akel, 2016). For instance, within a trusting environment, oxytocin can boost cooperation, but in a threatening context it can decrease such prosocial behaviours (Declerck et al., 2010; Shamay-Tsoory et al., 2009).

In a similar vein, **the social-approach/withdrawal hypothesis of oxytocin** states that oxytocin impacts on social approach and avoidance motivation. Specifically, oxytocin enhances approach behaviour which can be positive (enthusiasm or trust, which encourages individuals to go towards a desired goal) or negative (anger, aggression or jealousy), whereas social withdrawal (fear or anxiety) is inhibited by oxytocin (Kemp & Guastella, 2011).

Next, **the general approach-avoidance theory of oxytocin** adds to the social salience and the social-approach/withdrawal hypotheses by stating that oxytocin does not only exert social effects but also *non-social effects*. A recent study indeed confirmed that oxytocin enhances behavioural and neural approach towards both social and non-social stimuli, but only towards those of high personal relevance (Alaerts, Taillieu, et al., 2021). Thus, oxytocin seems to affect social behaviour, irrespective of valence or sociality, but context and personal relevance are of key importance.

In another, yet related, line of thinking, the **anxiolytic account of oxytocin** proposes that oxytocin promotes social performance by attenuating (social) stress (Bartz et al., 2011; Heinrichs & Domes, 2008; Shamay-Tsoory & Abu-Akel, 2016). Animal studies have revealed oxytocin-induced reductions in reactivity in threat-processing brain regions

(e.g., amygdala), thereby effectively reducing stress-related activation of the hypothalamic-pituitary-adrenal (HPA) axis and the related somatic fear responses (Veening & Olivier, 2013). The amygdala plays a central role in the anxiolytic account of oxytocin, this brain region is part of the limbic system, and is involved in a wide range of cognitive processes, including fear conditioning but also social perception (Zalla & Sperduti, 2013). Initial (single-dose) fMRI studies in humans confirm these animal findings and indicate that exogenous oxytocin reduces amygdala activity and its connectivity to the brainstem, involved in the autonomic and behavioural fear response (Heinrichs & Domes, 2008; Kirsch, 2005).

Each of the abovementioned mechanistic models contributes to our understanding how oxytocin can influence social behaviour, but they are likely not mutually exclusive (Harari-Dahan & Bernstein, 2014). Indeed, the effects of oxytocin are complex and dependent on a variety of individual characteristics and contextual factors, and therefore several mechanisms might be in play (Bartz et al., 2011). **Chapter 5** of the current PhD thesis elaborates further on these oxytocin mechanisms (social salience versus anxiolytic) in light of the patterns of results obtained from the collected data.

Exogenous oxytocin administration as a therapy for ASD

Since oxytocin plays such a crucial role in facilitating social functioning, administration of exogenous oxytocin is increasingly being put forward as a potential pharmacological treatment for ASD (Jones et al., 2017; Yamasue & Domes, 2017).

Initial studies that employed a single-dose of intranasal oxytocin demonstrated improvements in social cognition and perception in individuals with ASD (Guastella & Hickie, 2016; Yamasue & Domes, 2017). For example, Andari and colleagues (2009) were the first to demonstrate that a *single-dose* of oxytocin in adults with Asperger syndrome improved interactions with the most socially cooperative partner during a simulated ball game, indicating increased social awareness. Additionally, participants reported enhanced feelings of trust and had increased gazing time towards the eye region of a face (Andari et al., 2010). Similarly, in adult men with ASD, a single-dose of 24 IU of oxytocin increased attention towards faces (Kanat et al., 2017) and improved facial recognition (Domes et al., 2014). However, to fully realize the therapeutic potential of oxytocin in ASD,

a comprehensive understanding of the (long-term) effects of *multiple-dose* oxytocin administration is necessary. Thus far, only a handful of studies involved multiple administrations of oxytocin over an extended period of time, and only five of them were performed in children (see **Table 1**). Regarding the effectiveness of treatment, a small number of studies showed positive effects of multiple-dose treatment in adults with ASD (for a review see: Huang et al., 2021). Yamasue et al., (2018), for instance, adopted a six-week, double-blind, randomized, placebo-controlled parallel design, where adult men with ASD received 48 IU of oxytocin or placebo daily. The authors report improvements in the oxytocin group on core ASD symptoms as measured using the Autism Diagnostic Observation Schedule (ADOS) (Yamasue et al., 2018). Likewise, using a four-week, double-blind, randomized, placebo-controlled, parallel design in adult males with ASD, Bernaerts and colleagues (2020) demonstrated oxytocin-induced improvements in repetitive behaviours and reduced feelings of avoidance towards others, interestingly, up to one month and even one year post-treatment (Bernaerts, Boets, Bosmans, et al., 2020). Since ASD is an early-onset developmental condition, it is crucial to extend these findings to child populations. Unfortunately, in children and adolescents with ASD results are more mixed, with some studies showing beneficial effects (Parker et al., 2017; Yatawara et al., 2016), but others were unable to replicate these positive effects (Dadds et al., 2014; Fastman et al., 2021; Guastella et al., 2015). For instance, in a recent, highly powered, placebo-controlled, multiple-dose clinical trial in children with ASD, improvements in the social domain were found after oxytocin treatment, but also after placebo, so no treatment specific effect could be demonstrated (Sikich et al., 2021). Of note, the study implemented a flexible dosing scheme for oxytocin administration, which could explain some of the variability. Also, placebo effects in oxytocin studies are not uncommon. It has even been argued that endogenous oxytocin release may be directly implicated in these placebo effects; due to the enhanced social connectedness (e.g., personal contact between patient and clinician during a clinical trial or between patient and a loved one) dopaminergic and opioidergic reward processes could be stimulated, which may constitute the neurobiological basis for the social effects of the treatment outcomes (Itskovich et al., 2022). Taken together, these puzzling results show that the neuromodulatory effects of oxytocin are quite heterogeneous, highlighting the need for additional (mechanistic) elaboration of theoretical oxytocin models.

Table 1. Overview of clinical trials investigating the effects of multiple-dose intranasal oxytocin administration in individuals with ASD

Authors	*Number*	*Gender*	*Age*	*Dose & Frequency*	*Duration*	*Design*	*Paradigm*
Anagnostou et al., 2012	19 ASD	M + F	Adults	24 IU (2x/day)	6 weeks	RCT parallel	Oxytocin improved social cognition
Dadds et al., 2014	38 ASD	M	Children & adolescents	12 -24 IU (1x/day)	4 days	RCT parallel	No oxytocin induced improvements
Yatawara et al., 2016	31 ASD	M + F	Children	24 IU (1x/day)	5 weeks	RCT crossover	Oxytocin improved social functioning
Guastella et al., 2015	50 ASD	M	Adolescents	18 -24 IU (2x/day)	8 weeks	RCT parallel	No oxytocin induced improvements
Watanabe et al., 2015	20 ASD	M	Adults	24 IU (2x/day)	6 weeks	RCT cross-over	Oxytocin improved social communication
Munesue et al., 2016	29 ASD met VB	M	Adolescents & adults	16 IU (8 IU 2x /day)	8 weeks	RCT cross-over	Oxytocin improved social functioning
Kosaka et al., 2016	60 ASD	M + F	Adolescents & adults	16 IU (1x/day)	12 weeks	RCT parallel	Oxytocin improved social functioning
Parker et al., 2017	32 ASD	M + F	Children	24 IU (2x/day)	4 weeks	RCT parallel	Oxytocin improved social functioning
Yamasue et al., 2018	106 ASD	M	Adults	24 IU (2x/day)	6 weeks	RCT parallel	Oxytocin improved social functioning and repetitive behaviours
Bernaerts et al., 2020	40 ASD	M	Adults	24 IU (1x/day)	4 weeks	RCT parallel	Oxytocin improved repetitive behaviours and attachment
Sikich et al., 2021	277 ASD	M + F	Children & adolescents	24 IU (2x/day) flexible	24 weeks	RCT parallel	No oxytocin induced improvements
Le et al., 2022	41 ASD	M + F	Children	24 IU (1x/every other day)	2x 6 weeks	RCT cross-over	Oxytocin improved social functioning and repetitive behaviours

The present PhD thesis comprises the data from a single-centre, randomized, double-blind, placebo-controlled clinical trial, with a parallel design (**chapter 4, 5 and 6**). Multiple-dose oxytocin treatment was applied for four weeks, involving twice-daily intranasal administration of 12 IU to 8-to-12-year-old boys and girls with ASD (N=40 for oxytocin / N=40 for placebo). The double-blind phase was followed by a four-week single-blind extension phase during which all ASD children received intranasal oxytocin. Following prior observations of long-lasting retention effects of oxytocin administration in adults with ASD (Bernaerts, Boets, Bosmans, et al., 2020), treatment effects were assessed immediately after the four-week treatment and at a follow-up session, four weeks after cessation of the daily administrations (both after the double-blind and single-blind phase), **see Fig. 2**. At baseline, we additionally included 40 matched neurotypical

control children, in order to investigate diagnostic-group differences in terms of baseline characteristics (neurotypical children did not receive any treatment). In the ASD group, we tested the **efficacy of intranasal oxytocin for improvement in social functioning, both at a behavioural level** (via various scales and questionnaires, see **chapter 4**) **and at a neural level** (via EEG and fMRI measures, see **chapters 5 and 6**, respectively), and we monitored the safety of the multiple-dose oxytocin treatment (**chapter 4**). Thus far, only two studies investigated neural effects of multiple-dose oxytocin in ASD, both in adults (Bernaerts, Boets, Steyaert, et al., 2020; Watanabe et al., 2015). Accordingly, this is the first trial investigating neural effects in children and the largest trial to date examining oxytocin treatment effects in a relatively narrow age range of pre-pubertal children with a consistent dosing scheme, thereby allowing to overcome some of the raised issues regarding sample and dosing heterogeneity. Additionally, the multifaceted design allows between-subject comparison, within-subject comparison and also the comparison between placebo-first/oxytocin-after versus oxytocin-first/oxytocin-after (i.e., participants that received multiple-dose oxytocin administration twice).

Note that the current PhD thesis includes behavioural results on the double-blind and single-blind phase, but only neural results of the double-blind phase. In addition to the data and measures included in this thesis, a whole series of additional data were also collected, among others: eye gaze during live interaction measured via eye-tracking, neural measures (EEG and fMRI) during (live) eye-contact paradigms and resting-state, stress-physiology measured during EEG and fMRI (i.e., heart-rate, skin-conductance and respiration) and saliva samples measuring peripheral endogenous oxytocin, cortisol and *OXTR* DNA methylation levels.

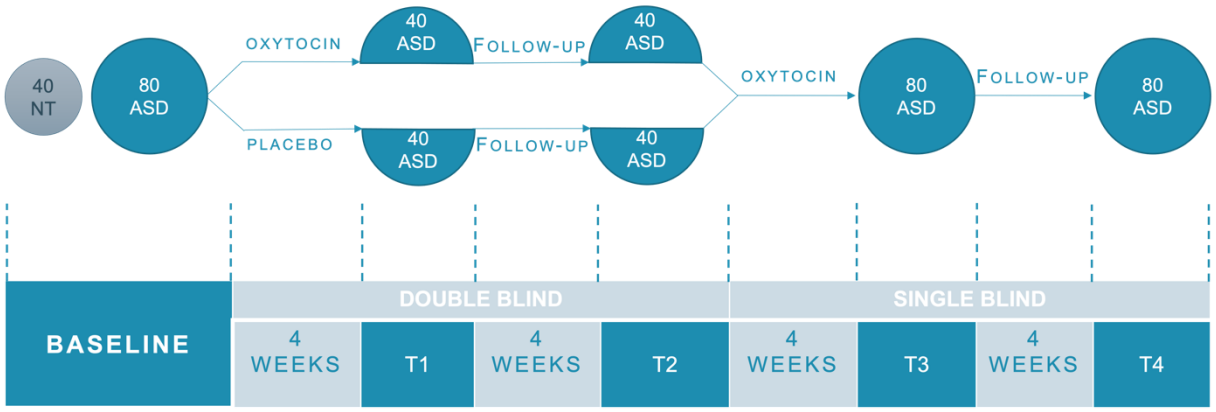

Figure 2. Clinical trial design and overview of participants in the trial. Forty neurotypical (NT) control children were enrolled and compared to 80 children with ASD at the baseline assessment. Individuals with ASD then underwent a double-blind phase during which they were allocated to receive either oxytocin or placebo (four weeks of twice daily intranasal administration), followed by an assessment immediately after the four-week treatment (time point T1, i.e. 24 hours after the last oxytocin administration) and at a follow-up session four weeks after cessation of the daily administrations (T2). Next, the ASD children flowed into a four-week single-blind extension phase, during which all participants received four weeks of intranasal oxytocin. Once more, treatment effects were assessed immediately after the four-week treatment (T3) and at a follow-up session one month later (T4). Results reported in the current thesis were obtained at varying timepoints: questionnaire data at T0-T4; frequency-tagging EEG at T0-T2; face processing fMRI at T0-T1.

Face processing in autism

As the present thesis crucially entails the administration of face processing paradigms in children with and without ASD, I will shortly introduce the topic here. The ability to quickly and accurately process faces is an important part of social interaction (Rossion, 2014). Our brains excel at extracting information from a face, including the person's identity, his or her feelings, and even intentions. The brain will use this information to activate memories, direct attention and stimulate social actions (Elfenbein & Ambady, 2002). Neuroimaging research has identified several important brain regions involved in different aspects of face processing. Haxby et al., differentiate between the "core" face processing regions, comprising the inferior occipital cortex (containing the "Occipital Face Area" or OFA), the fusiform cortex (containing the "Fusiform Face Area" or FFA) and the superior temporal sulcus (STS), and the "extended" face processing network, such as the anterior temporal cortex and the subcortical amygdala, **see Fig.** 3 (Haxby et al., 2000; Haxby & Gobbini, 2011). The core regions allow visual analysis of faces, specifically the perception of static features for identity recognition and dynamic features for recognition of facial gestures such as expression (Haxby et al., 2000). The extended network extracts information from faces (recognition of familiar faces, the meaning of expressions,…).

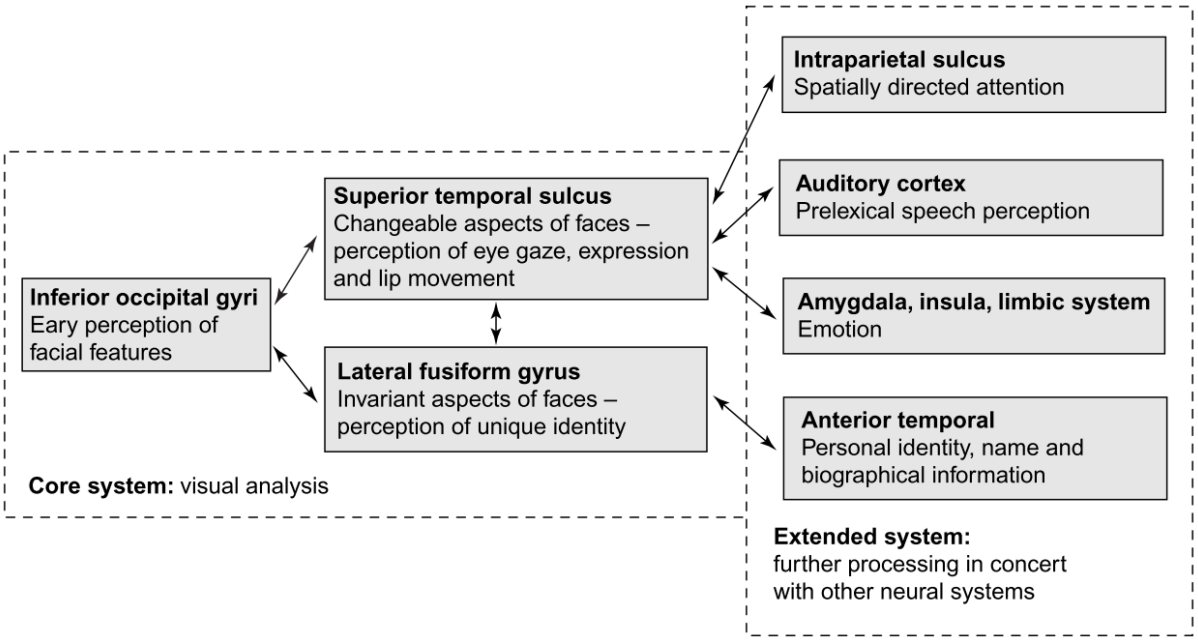

Figure 3. A neural model of the face processing network. The model contains the core face processing system, representing static (e.g., identity) and dynamic (e.g., biological motion) aspects of a face. These representations interact with the extended system to mediate processes such as emotion or attention direction. Functional interpretation of each region is tentative. Figure reprinted from Haxby et al. (2000).

One of the core clinical criteria of ASD involve deficits in nonverbal communication and body language (American Psychiatric Association, 2013). Against this background, face processing has frequently been studied in ASD, as it may contribute to the characteristic social communication and interaction deficits. Researchers have shown that individuals with ASD process facial information differently, it is argued that they rely on a feature-based processing style, instead of a holistic one, focusing on specific parts of the face rather than the overall configuration (Jemel et al., 2006). For instance increased looking-time towards trivial parts of the face (e.g., ears or chin), instead of the eyes or mouth. This could perhaps be due to a general lack of interest in social stimuli or because faces may be perceived as anxiety-inducing (Duchaine & Yovel, 2015; Jemel et al., 2006). Conversely, other researchers have demonstrated that holistic face processing is intact in individuals with ASD when processing expressive faces (Van der Donck et al., 2019; van der Geest et al., 2002). Seeing that the behavioural evidence is inconsistent (Leung et al., 2022; Sasson, 2006; Uljarevic & Hamilton, 2013), neurobiological research strategies (via EEG or fMRI) have been used to investigate the neural underpinnings of face processing impairments in ASD. Reviews mainly indicate reduced brain activity when looking at faces in individuals with ASD, specifically in the inferior occipital, fusiform, superior temporal and

inferior frontal regions, as well as in the amygdala (di Martino et al., 2009; Kleinhans et al., 2011; Nomi & Uddin, 2015; Philip et al., 2012). However, increased amygdala activation has also been found in individuals with ASD (Kleinhans et al., 2010), and this amygdala reactivity has been found to be associated with increased social anxiety in ASD (Kleinhans et al., 2010). These opposing findings may indicate that different underlying clinical characteristics relate to the social dysfunction in ASD (Dalton et al., 2005; Kleinhans et al., 2010). More specifically, for some individuals, reduced emotional salience (as indexed by reduced amygdala activation) may result in apathy towards social interactions, while for others, over-arousal towards faces (as indexed by increased amygdala activation) can result in aversive and avoidant social reactions (Kleinhans et al., 2010). Few neuroimaging studies have examined face processing in children with ASD, however existing fMRI literature points to reduced fusiform activity in ASD (Piggot et al., 2004; A. T. Wang et al., 2004). A recent study by Van der Donck and colleagues implemented a novel, fast, robust and implicit neurobiological technique to evaluate expressive face processing in boys with ASD: frequency-tagging EEG (Van der Donck et al., 2019, 2020). The core idea of frequency-tagging EEG is that the frequency of the electrophysiological response on the human scalp (measured with EEG) corresponds exactly with the periodicity of the visual stimulation (Norcia et al., 2015). The authors convincingly found reduced neural activity in the occipitotemporal regions in ASD compared to typically developing peers (Van der Donck et al., 2019, 2020). **Chapter 5** of this thesis describes frequency-tagging EEG results from a substantial group of boys *and* girls with ASD compared to matched controls, in order to validate the previous findings of Van der Donck, and to verify whether the frequency-tagging EEG approach allows to monitor subtle changes in neural sensitivity throughout an oxytocin clinical trial. In **chapter 6** we complement these EEG data with fMRI findings using a comparable fMRI face processing paradigm. One of the main goals of this PhD project was to corroborate the frequency-tagging EEG technique in its potential to pinpoint face processing sensitivity in ASD.

Effect of oxytocin on face processing

As mentioned before, oxytocin has been demonstrated to stimulate prosocial behaviour, reduce (social) anxiety and orient attention towards personally relevant stimuli (social or non-social). Accordingly, researchers have explored the effects of oxytocin on (expressive) face processing. Yet, in neurotypical individuals results are rather contradictory, with some reporting improvement of facial expression recognition upon intranasal oxytocin administration, but others only found emotion-specific improvements or no improvements at all (Bartz et al., 2011; Leppanen, Ng, et al., 2017; Shahrestani et al., 2013; Van der Donck et al., 2022). It is possible that the lack of oxytocin treatment effects might reflect a ceiling performance, allowing no further improvement in neurotypicals (Van der Donck et al., 2022). The effects on (expressive) face recognition and processing upon intranasal oxytocin administration has also been investigated in individuals with ASD. Initial single-dose studies reveal positive outcomes in terms of improvement on facial expression recognition (Althaus et al., 2015; Domes et al., 2014). However, there is limited research on the impact of multiple-dose oxytocin administration on face processing in children with ASD. Only recently Le et al., demonstrated enhanced social attention and increased looking time to the eye region upon (intermittent) multiple-dose oxytocin administration in children with ASD (Le et al., 2022). Yet, the exact (neural) mechanism of how oxytocin may affect face processing is largely understudied. In **chapter 5** we attempted to fill this gap by applying the frequency-tagging EEG technique to monitor expressive face processing sensitivity throughout the multiple-dose oxytocin clinical trial, in children with ASD. The effect of oxytocin on face processing was evaluated immediately after the four-week treatment, but also, for the first time, four weeks after cessation of the treatment, in order to screen for possible retention effects. In **chapter 6**, we take advantage of the high spatial resolution of the MRI scanner, to complement the EEG data in terms of identifying oxytocin effects on neural activity of face processing brain regions, by implementing a face processing fMRI task in the clinical trial.

Aim and Outline of the Thesis

The primary aim of this PhD thesis was to provide a comprehensive understanding of the role of oxytocin in ASD. First, we reviewed the literature on endogenous oxytocin concentrations in ASD (**chapter 2**) and the relation between *OXTR* DNA methylation (i.e., epigenetics) and ASD characteristics (**chapter 3**). Secondly, we conducted a randomized, double-blind, placebo-controlled, parallel, multiple-dose oxytocin clinical trial in children with ASD. Here, the efficacy of intranasal oxytocin therapy on behavioural improvement in social functioning was evaluated (**chapter 4**). Additionally, a novel neurobiological EEG-technique (frequency-tagging EEG) was validated to pinpoint socio-communicative sensitivity differences in children with versus without ASD (**chapter 5**). Subsequently, this EEG-technique was used to examine the effect of intranasal oxytocin on the neural socio-communicative sensitivity in children with ASD (**chapter 5**). Lastly, while frequency-tagging EEG provides a robust indicator of socio-communicative sensitivity, it does not inform us about the underlying neural mechanisms of aberrant face processing in ASD, therefore a comparable fMRI task was applied in the clinical trial to complement these EEG findings, before and after oxytocin treatment (**chapter 6**).

Overview of the chapters

Chapter 2 comprises a systematic review and meta-analysis investigating whether there are differences in endogenous oxytocin concentrations in children, adolescents and adults with ASD compared to matched neurotypical controls.

In **chapter 3**, we describe a systematic review that specifically aimed to explore the association between *OXTR* DNA methylation and variations in social characteristics in individuals with ASD, but also beyond categorical diagnosis, by assessing variations in other social dimensions, closely related to ASD (e.g., social cognition, perception and social anxiety).

In **chapter 4**, we present the results from a randomized, double-blind, placebo-controlled, parallel, multiple-dose oxytocin clinical trial (intranasal administration of 12 IU, twice daily for four weeks). We evaluated the efficacy on improvement in social functioning of multiple-dose oxytocin administrations in boys and girls with ASD (8-to-12-years old; 40 oxytocin versus 40 placebo). These effects were assessed immediately after treatment

and followed-up four weeks later. A consecutive single-blind phase followed the double-blind phase, where all ASD children received oxytocin nasal sprays for four weeks and were assessed again post-treatment and on follow-up. Lastly, we aimed to uncover possible moderating factors on treatment responses, such as gender, medication use, parental belief about the allocated treatment or concomitant (ongoing) psychosocial treatment.

In **chapter 5,** we discuss EEG data from the oxytocin clinical trial. Specifically, frequency-tagging EEG was applied to quantify neural facial expression processing sensitivity in a robust and implicit manner. First, we compared children with ASD versus matched neurotypical controls , in order to validate prior findings of reduced neural face processing sensitivity in ASD (Van der Donck et al., 2019, 2020). Secondly, we assessed the impact of a four-week course of multiple-dose oxytocin on the neural face processing sensitivity in children with ASD; immediately post-treatment and after a four-week follow-up.

In **chapter 6,** we extend the neurobiological findings of chapter 5, by applying a comparable emotional face processing fMRI task. First, the neural face processing signature in children with ASD was compared to matched neurotypical controls on baseline. Secondly, the effect of a multiple-dose oxytocin therapy on fMRI face processing responses was evaluated in ASD.

Chapter 7 summarizes and discusses the findings of chapters 2 through 6. Additionally, limitations of these studies are considered and opportunities for future research are presented.

Chapter 2 | Endogenous Oxytocin Levels in Autism — A Meta-Analysis

Moerkerke, M., Peeters, M., de Vries, L., Daniels, N., Steyaert, J., Alaerts, K., Boets, B. (2021). Endogenous Oxytocin Levels in Autism-A Meta-Analysis. Brain Sci, 11 (11). https://doi.org/10.3390/brainsci11111545

Abstract

Oxytocin (OT) circuitry plays a major role in the mediation of prosocial behaviour. Individuals with autism spectrum disorder (ASD) are characterized by impairments in social interaction and communication and have been suggested to display deficiencies in central OT mechanisms. The current preregistered meta-analysis evaluated potential group differences in endogenous OT levels between individuals with ASD and neurotypical (NT) controls. We included 18 studies comprising a total of 1422 participants. We found that endogenous OT levels are lower in children with ASD as compared to NT controls ($n = 1123$; $g = -0.60$; $p = 0.006$), but this effect seems to disappear in adolescent ($n = 152$; $g = -0.20$; $p = 0.53$) and adult populations ($n = 147$; $g = 0.27$; $p = 0.45$). Secondly, while no significant subgroup differences were found in regard to sex, the group difference in OT levels of individuals with versus without ASD seems to be only present in the studies with male participants ($n = 814$; $g = -0.44$; $p = 0.08$) and not female participants ($n = 192$; $g = 0.11$; $p = 0.47$). More research that employs more homogeneous methods is necessary to investigate potential developmental changes in endogenous OT levels, both in typical and atypical development, and to explore the possible use of OT level measurement as a diagnostic marker of ASD.

Introduction

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder characterized by deficits in reciprocal social communication and interaction, and by the presence of restricted, stereotyped and repetitive interests and behaviours (American Psychiatric Association, 2013). This early onset developmental disorder has a substantial genetic predisposition and affects mostly boys (Loomes et al., 2017). The multifactorial aetiology and underlying neurobiological mechanisms responsible for the heterogeneous clinical presentation of ASD are not yet fully understood, limiting diagnosis of ASD to rely solely on developmental history, behavioural and cognitive assessment. While current diagnostic approaches do not involve any biomarkers, recent evidence suggests that core symptoms of social deficits featuring ASD might be associated with oxytocin (OT) dysfunction in the central nervous system (Abdulamir et al., 2016; Alabdali et al., 2014; Bakker-Huvenaars et al., 2020; Feldman et al., 2014; Husarova et al., 2016; Modahl et al., 1998; Yang et al., 2015; Zhang et al., 2016).

OT is a hypothalamic nonapeptide with a wide variety of bodily functions. It is produced in the neurons of the paraventricular and supraoptic nuclei of the hypothalamus. These nuclei project to the posterior pituitary gland from where OT is released into the systemic circulation. In peripheral tissues, OT acts as a key mediator in labour induction and lactation (Zingg & Laporte, 2003). In the central nervous system, OT is an important modulator of socio-cognitive functions and complex social behaviours (e.g., empathy, emotion recognition, attachment development and affiliative behaviours) through action in different brain regions (e.g., amygdala) (Fineberg & Ross, 2017; Marlin & Froemke, 2017). Against this background, researchers have examined potential prosocial effects of intranasal OT administration, both in healthy individuals (Mierop et al., 2020) and in clinical populations (M. Bakermans-Kranenburg & van Ijzendoorn, 2013). In their systematic review of OT effects in healthy individuals, Mierop et al., (2020) describe large heterogeneity, limited and unsuccessful replication studies, and low statistical power, thus all pointing to a restricted possibility to disentangle true from false OT effects (Mierop et al., 2020). With respect to ASD, clinical trials generally report that intranasal OT has the potential to improve social impairment, or at least in particular individuals (Bernaerts, Boets, Bosmans, et al., 2020; Guastella & Hickie, 2016; Parker et al., 2017; Yatawara et al., 2016). Parker et al. (2017), for instance, found that the therapeutic effect

of intranasal OT administration was strongest in those ASD children showing the lowest endogenous OT levels pre-treatment (Parker et al., 2017). Likewise, Alaerts et al. (2021) showed improvements in social behaviour in adults with ASD after four weeks of intranasal OT administration, and these social improvements were accompanied by elevated endogenous OT levels until one month later (Alaerts, Steyaert, et al., 2021). These findings indicate the importance of endogenous OT levels as potential indicator of OT-therapy outcome.

Previous studies have shown correlations between individual differences in endogenous OT levels and social deficits, suggesting a potential role of endogenous OT in the pathogenesis of social impairments that characterize ASD (Quattrocki & Friston, 2014). However, an earlier meta-analysis of Rutigliano and colleagues (2016), integrating findings on six adult studies, reported no significant group differences in OT concentrations comparing adults with ASD versus neurotypical (NT) controls (Rutigliano et al., 2016). Though, previous evidence suggested a developmental trend in the association of endogenous OT levels and socioemotional functioning, with a more pronounced association in younger age groups (Torres et al., 2018). So, it is critical to consider a wider age range for the group comparisons. Accordingly, the current systematic review and meta-analysis aims at investigating whether there are differences in endogenous OT concentrations in individuals with ASD compared to matched NT controls across all ages.

Materials and Methods

A protocol describing the rationale and methods of this meta-analysis was registered on PROSPERO (registration number: CRD42021231207) on February 14th 2021. It is available at https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42021231207.

Studies were eligible for inclusion if a direct comparison of endogenous OT concentrations was reported between a group of human participants with a formal ASD diagnosis versus an NT control group. Reports of (group differences in) endogenous OT levels in blood serum, blood plasma, urine or saliva were required as outcome. Interventional studies were included if baseline pre-intervention OT levels were assessed

and compared between the ASD and NT groups. Participants of any age, sex and ethnicity were considered. No restrictions were imposed regarding methods of tissue sample extraction, sample measurement and statistical analysis of the OT levels. Concerning the target population, all participants received a diagnosis of ASD according to the Diagnostic and Statistical Manual of Mental Disorders, fourth (DSM-IV), fourth text reviewed (DSM-IV-TR) or fifth edition (DSM-5) prior to the study and had no physical or severe psychiatric comorbidities (e.g., schizophrenia; bipolar disorder). No exclusion criteria were defined based on disorder subtype (i.e., Asperger, autistic disorder or PDD-NOS) or symptom severity. In terms of NT control population, all included studies reported that the NT participants displayed no physical disease, neurological or mental disorder. We only included between-group designs, thereby excluding all studies involving single group designs, case studies, reviews, meta-analyses and animal studies. Only papers published in English were considered. No restrictions were imposed regarding publication year.

An electronic search strategy was constructed according to PRISMA guidelines, aimed at identifying all studies comparing endogenous OT concentrations between an ASD group and a NT control group. Electronic bibliographic databases Pubmed and Embase were searched up to December 1st 2020. The following string of key terms was used: (Oxytocin OR OT) AND (autism OR autism spectrum disorder OR ASD OR autistic). Search terms were applied to title, abstract and keywords. In the process of study selection, title and abstract of all studies that resulted from our systematic literature search were screened for eligibility. All duplicates and studies meeting one or more exclusion criteria were excluded. In addition, studies without available full text were excluded. Articles resulting from the screening process underwent full text analysis to evaluate if studies were to be included based on the previously determined eligibility criteria. In addition to the predefined electronic search strategy, reference lists of the selected articles were inspected for further empirical studies that may meet inclusion criteria.

Prior to statistical analyses, all included studies were reviewed and the following information was extracted: (i) participant characterization (sample sizes, age, sex distribution, diagnostic criteria), (ii) specimen type, method of sample collection and measurement, OT concentration, (iii) effect sizes of group comparisons and/or descriptive statistics allowing the calculation of standardized effect sizes. Studies qualified for inclusion in the formal meta-analysis if the means and standard deviations

for both groups were provided or could be retrieved. If the necessary study data was insufficient, the corresponding author of the study was contacted by e-mail to request provision of the missing data. A reminder request was sent within 14 days if the authors did not respond to the first e-mail. For two studies that did not report the necessary descriptive statistics in texts or tables but only graphically represented in a figure, an online plot digitizer was used to estimate the mean and standard deviation of the concentrations displayed in the figures (Ankit Rohatgi, 2017). The reliability of this digital tool was demonstrated in an earlier meta-analysis on the correlation of central and peripheral OT (Valstad et al., 2017), and was additionally validated and confirmed based on the current data (i.e., by comparing the visually estimated and the reported descriptive statistics). Quantitative analyses were conducted using RevMan 5.4.1 software (*RevMan | Cochrane Training*, n.d.). Overall effect sizes were calculated based on OT mean and standard deviations of ASD and NT group, weighted by sample or subsample size using a random effects model to adjust for standard errors. Effect sizes were expressed as the standardized mean difference measure Hedges' g. An effect size of 0.2–0.3 is often considered as a small effect, 0.5 as a medium effect and 0.8 or more as a large effect (Durlak, 2009).

We used the QUADAS-2 tool (Whiting et al., 2011), which is designed specifically to evaluate the quality of diagnostic accuracy studies, to assess the risk of bias in our included articles. The QUADAS-2 tool assesses study quality at four levels, i.e., patient selection, index test, reference standard and flow/timing. Patient selection was evaluated based on the type of participant sampling applied in the study (consecutive, random), amongst other parameters. The evaluation of the conduct and interpretation of the index test, OT dosing, involved for instance, whether this was done without knowledge of the reference standard. The reference standard, i.e., the diagnostic instrument used to classify the ASD group, was also assessed. The conduct of both index and reference test was assessed based on their timing and flow, for example the time between both and the order they were performed in. Finally, the participant flow was evaluated, to assess whether all studied participants were included in the analyses, because patients lost to follow-up can differ systematically from those who remain. Additionally, a judgment of applicability was made on each level, to assess whether the study matches the research question.

Results

Study selection and characteristics

The literature search yielded a total of 687 and 984 articles in Pubmed and Embase, respectively. The flowchart presented in **Figure 1** summarizes the number of studies that were screened, evaluated for eligibility, and included in the meta-analysis. From the pool of 23 studies eligible for quantitative analysis, four studies were excluded due to insufficient data. Considering that two of the included studies reported identical data on the same participant group, only the chronologically first reported was included in the analysis (Green et al., 2001; Modahl et al., 1998).

An overview of the included studies and participant characteristics is provided in Table 1. Data extracted and represented in Table 1 contains author and year of publication, total group size, number of ASD and NT participants per group, sex, age, tissue sample type, method of OT level measurement, OT concentration per group, and unit of measurement. A total of 18 studies comprising 1422 participants (699 ASD patients and 723 controls) were included in the final analyses.

To further explore what underlying mechanisms might be driving the general results, more detailed subgroup analyses were performed by age, by sex and by tissue sample type. Pertaining to age, 13 studies involved comparisons of groups of children, aged between 2 and 12 years (Abdulamir et al., 2016; Alabdali et al., 2014; Feldman et al., 2014; Fujisawa et al., 2014; J. D. Jacobson et al., 2014; Lakatosova et al., 2015; Mariscal et al., 2019; Modahl et al., 1998; Tanaka et al., 2020; Taurines et al., 2014; Yang et al., 2015; Zhang et al., 2016). Two studied an adolescent group, aged between 11 and 16 years (Bakker-Huvenaars et al., 2020; Miller et al., 2013). Three investigated groups of adults (Althaus et al., 2016; Fujioka et al., 2020; Procyshyn et al., 2020). Pertaining to sex, eight studies reported on exclusively male populations (Abdulamir et al., 2016; Alabdali et al., 2014; Althaus et al., 2016; Bakker-Huvenaars et al., 2020; Fujioka et al., 2020; Lakatosova et al., 2015; Modahl et al., 1998; Taurines et al., 2014) and one study adopted an exclusively female sample (Procyshyn et al., 2020). For the studies involving a mixed population, four studies reported separate outcomes of OT level measurement for male and female subgroups and were entered accordingly in the subgroup analyses (Fujisawa et al., 2014; J. D. Jacobson et al., 2014; Lakatosova et al., 2015; Miller et al., 2013). With regard to tissue sample type, twelve studies investigated blood plasma (Abdulamir et al.,

2016; Alabdali et al., 2014; Althaus et al., 2016; Husarova et al., 2016; J. D. Jacobson et al., 2014; Lakatosova et al., 2015; Mariscal et al., 2019; Miller et al., 2013; Modahl et al., 1998; Taurines et al., 2014; Yang et al., 2015; Zhang et al., 2016). Six studies took saliva samples for OT level measurement (Bakker-Huvenaars et al., 2020; Feldman et al., 2014; Fujioka et al., 2020; Fujisawa et al., 2014; Procyshyn et al., 2020; Tanaka et al., 2020).

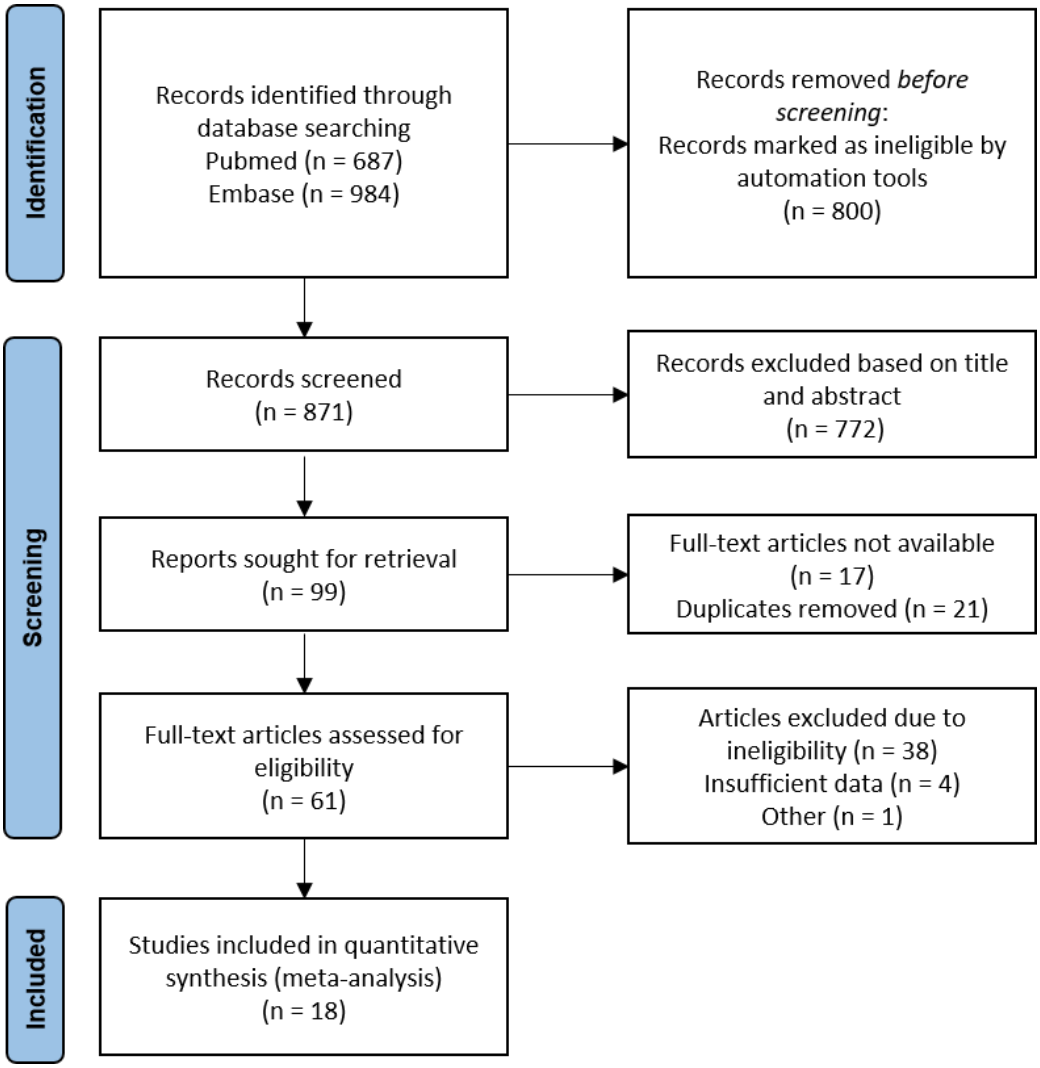

Figure 1. Schematic flowchart of the search method.

Table 1. Overview of included study data, participant characteristics, and outcome.

	Study	Subjects		Sample	Assay	[OT]		Unit	Results
		ASD	NT			ASD	NT		
	OT levels in children (13)								
1.	Abdulamir et al. (2016)	n = 60 (60M) Age (y): 7.28 ± 2.89 DSM-5	n = 26 (26M) Age (y): 6.92 ± 2.59	Plasma	ELISA	44.72 ± 36.1	102.1 ± 34.31	µIU/ml	Lower OT in ASD ($p < 0.001$)
2.	Alabdali et al. (2014)	n = 50 (50M) Age (y): 7.0 ± 2.34 DSM-IV; CARS, SRS	n = 30 (30M) Age (y): 7.2 ± 2.14	Plasma	ELISA	71.71 ± 18.09	139.22 ± 36.62	µIU/ml	Lower OT in ASD ($p = 0.001$)

	Study	Subjects		Sample	Assay	[OT]		Unit	Results
		ASD	NT			ASD	NT		
3.	Feldman et al. (2014)	n = 40 (34M/6F) Age (m): 63.38 ± 12.35 DSM-5; ADOS-2	n = 40 Age (m): 53.56 ± 13.83	Saliva	ELISA	4.25 ± 0.66	6.89 ± 1.03	pg/ml	Lower OT in ASD ($p < 0.05$)
4.	Fujisawa et al. (2014)	n = 15 (12M/3F) Age (m): 57.9 ± 13.6 DSM-5; DQ, PARS, SDQ	n = 58 (27M/31F) Age (m): 48.1 ± 22.7	Saliva	ELISA	39.33 ± 23.52 M: 40.3 ± 23.52 F: 35.7 ± 20.11	44.5 ± 24.89 M: 45.7 ± 29.78 F: 43.4 ± 20.15	pg/ml	No significant differences between groups ($p = 0.449$)
5.	Husarova et al. (2016)	n = 19 (19M) Age (m): 56.7 ± 25.4 ICD-10; CARS, ADI	n = 44 (44M) Age (m): 58.9 ± 23.0	Plasma	ELISA	124.10 ± 90.59	267.77 ± 212.37	pg/ml	Lower OT in ASD ($p = 0.0004$)
6.	Jacobson et al. (2014)	n = 37 (25M/12F) Age (y): 4.73 ± 0.61 DSM-IV-TR; ADI-R, ADOS	n = 41 (24M/17F) Age (y): 4.85 ± 0.61	Plasma	ELISA	M: 24.41 ± 7.45 F: 23.04 ± 6.97	M: 18,58 + 6.98 F: 22.59 + 8.82	pg/ml	Higher OT in male ASD only ($p = 0.022$)
7.	Lakatosova et al. (2015)	n = 104 (80M/24F) Age (y): 7 ± 5.5 DSM-IV	n = 128 (103M/25F) Age (y): 10.5 ± 7	Plasma	ELISA	M: 208.1 ±238.63 F: 282.9 ± 318.92	M: 281.7 ±200.85 F: 340.7 ± 340.70	pg/ml	Lower OT in male ASD only (M: $p = 0.0248$; F: $p = 0.5058$)
8.	Mariscal et al. (2019)	n = 34 (28M/6F) Age (y): 9.26 ± 0.37 DSM-IV-TR/DSM-V; ADI-R, ADOS	n = 30 (21M/9F) Age (y): 8.80 ± 0.40	Plasma	ELISA	8.62 ± 5.36	10.54 ± 5.37	pg/ml	No significant differences between groups ($p = 0.1564$)
9.	Modahl et al. (1998)	n = 29 (29M) Age (y): 8.1 + 1.7 DSM-IV	n = 30 (30M) Age (y): 8.8 + 1.8	Plasma	RIA	0.64 ± 0.58	1.16 ± 0.77	pg/ml	Lower OT in ASD ($p < 0.004$)
10.	Tanaka et al. (2020)	n = 12 (11M/1F) Age (m): 135 ± 16.7 DSM-IV-TR, DSM-V; CARS, ADOS, DISCO	n = 8 (4M/4F) Age (m): 107 ± 6.9	Saliva	ELISA	167.9 ± 62.01	161.5 ± 54.87	pg/ml	No significant difference between groups
11.	Taurines et al. (2014)	n = 19 (19M) Age (y): 10.7 ± 3.8 ICD-10; ADI-R, ADOS	n = 17 (17M) Age (y): 13.6 ± 2.1	Plasma	RIA	19.6 ± 7.1	14.4 ± 9.6	pg/ml	No significant difference between groups ($p = 0.132$)
12.	Yang et al. (2015)	n = 43 (35M/8F) Age (y): 7.51 ± 1.47 DSM-5; CARS	n = 40 (30M/10F) Age (y): 7.83 ± 1.63	Plasma	ELISA	116.47 ± 41.57	141.05 ± 51.61	pg/ml	Lower OT in ASD ($p = 0.022$)
13.	Zhang et al. (2016)	n = 84 (71M/13F) Age (y): 3.95 ± 1.26 DSM-IV-TR; CARS	n = 85 (71M/14F) Age (y): 4.80 ± 1.22	Plasma	ELISA	20.05 ± 13.88	25.76 ± 15.30	pg/ml	Lower OT in ASD ($p = 0.028$)
OT levels in adolescents (2)									
14.	Bakker-Huvenaars et al. (2020)	n = 49 (49M) Age (y): 15.0 ± 2.1 DSM-5; DISC-IV	n = 28 (28M) Age (y): 15.9 ± 1.8	Saliva	RIA	-0.22 ± 0.89	0.49 ± 0.97	z-score	Lower OT in ASD ($p = 0.002$)
15.	Miller et al. (2013)	n = 40 (21M/19F) Age (y): M: 12.24 ± 3.56; F: 11.79 ± 3.43 DSM-IV-TR; ADOS	n = 35 (19M/16F) Age (y): M: 11.74 ± 2.49; F: 12.94 ± 3.19	Plasma	ELISA	M: 357.12 ± 126.05 F: 525.23 ± 325.75	M: 361.52 ± 315.26 F: 434.33 ± 332.27	pg/ml	No significant differences between groups ($p = 0.270$)
OT levels in adults (3)									
16.	Althaus et al. (2016)	n = 31 (31M) Age (y): 22.67 ± 4.22 DSM-IV-TR; ADOS,	n = 30 (30M) Age (y): 22.67 ± 4.22	Plasma	RIA	1.34 ± 1.05	0.67 ± 0.77	pmol/l	Higher OT in ASD ($p = 0.006$)
17.	Fujioka et al. (2020)	n = 17 (17M) Age (y): 27.4 ± 7.2 DSM-IV; DISCO	n = 24 (24M) Age (y): 29.0 ± 9.8	Saliva	ELISA	36.2 ± 13.2	43.6 ± 17.0	pg/ml	No significant difference between groups ($p = 0.154$)
18.	Procyshyn et al. (2020)	n = 16 (16F) Age (y): 29.9 ± 8.4 DSM-IV	n = 29 (29F) Age (y): 27.2 ± 8.1	Saliva	ELISA	3.1 ± 0.5	2.8 ± 0.6	pg/ml	No significant difference between groups ($p = 0.064$)

OT = oxytocin (Mean ± SD), ASD = Autism Spectrum Disorder, NT = neurotypical, M = male, F = female, y = years, m = months, ELISA = enzyme-linked immunosorbent assay, RIA = Radioimmunoassay. DSM-IV or DSM-5 Diagnostic and Statistical Manual of Mental Disorders Version IV or 5. ICD-10 = International Classification of Diseases Version 10. When applicable: confirmation of the ASD diagnosis using ADOS = Autism Diagnostic Observation Schedule or ADI(-R) = Autism Diagnostic Interview (-Revised) or CARS = Childhood Autism Rating Scale or DISCO = Diagnostic Interview for Social and Communication Disorders or DISC-IV Diagnostic Interview Schedule for Children Version IV

Qualitative Risk of Bias analysis of included studies

The risk of bias evaluation for each individual study is shown in Table 2. None of the studies show a 'low' risk of bias on all four levels, most often the total risk of bias remains 'unclear', rather than 'high'. Applicability concerns are low. Whilst being aware of the moderate quality of the included studies, we nevertheless decided to include all studies in our analyses, because of the limited amount of available studies.

Table 2. The risk of bias evaluation for each individual study.

Study		RISK OF BIAS				APPLICABILITY CONCERNS		
		Patient Selection	Index test	Reference test	Flow and timing	Patient Selection	Index test	Reference test
1.	Abdulamir et al. (2016)	?	?	☹	☺	☺	☺	☺
2.	Alabdali et al. (2014)	☹	?	☺	☹	☺	☺	☺
3.	Feldman et al. (2014)	?	?	☺	☹	☺	☺	☺
4.	Fujisawa et al. 2014)	?	?	☹	?	☺	☺	☺
5.	Husarova et al. (2016)	?	?	☺	?	☺	☺	☺
6.	Jacobson et al. (2014)	☹	?	☺	?	☺	☺	☺
7.	Lakatosova et al. (2015)	?	?	☹	☹	☺	☺	☺
8.	Mariscal et al. (2019)	☹	☺	☺	?	☺	☺	☺
9.	Modahl et al. (1998)	?	?	☹	?	☺	☺	☺
10.	Tanaka et al. (2020)	☹	?	☺	☹	☺	☺	☺
11.	Taurines et al. 2014)	?	?	☺	?	?	☺	☺
12.	Yang et al. (2015)	☹	?	☺	☹	☺	☺	☺
13.	Zhang et al. (2016)	?	?	?	?	☺	☺	?
14.	Bakker-Huvenaars et al. (2016)	☹	?	☺	☺	☺	☺	☺
15.	Miller et al. (2013)	☹	?	☺	?	☺	☺	☺
16.	Althaus et al. (2016)	?	?	☺	?	☺	☺	☺
17.	Fujioka et al. (2020)	☹	?	☺	?	☺	☺	☺
18.	Procyshyn et al. (2020)	?	?	☹	?	☺	☺	?

☺ = Low Risk, ☹ = High Risk, ? = Unclear Risk.

Meta-Analysis of Peripheral OT Levels in ASD vs. NT Controls

Across all the 18 studies, meta-regression of differences in endogenous OT between ASD and NT populations resulted in a mean standardized difference of g = -0.42, which was significant (n = 1422, g = -0.42, Z = 2.36, p = 0.02, CI = [-0.78, -0.07]) and indicates that OT levels are generally lower in participants with ASD as compared to NT controls (**see Figure 2**). Yet, the effect was highly heterogeneous ($Tau^2 = 0.61$; $Chi^2 = 195.66$, df = 21 (P < 0.00001); $I^2 = 89\%$), thereby motivating a further subgroup analysis.

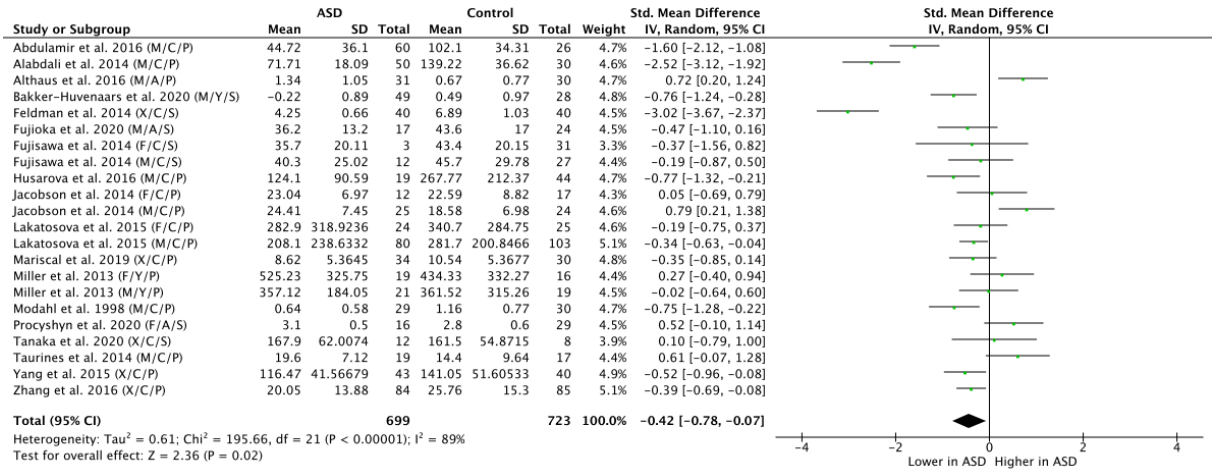

Figure 2. Meta-analysis of peripheral OT levels in ASD vs NT. M = male, F = female, X = mixed sexes; C = children, Y = youths/adolescents, A = adults; P = plasma, S = saliva.

Subgroup analysis by age

The subgroup analysis by age revealed that ASD children did display significantly lower OT levels compared to NT children (n = 1123; g = -0.60; Z = 2.75; p = 0.006; CI = [-1.03, -0.17]) (see **Figure 3**). However, there were no significant group differences in the OT levels for the adolescent populations (n = 152; g = -0.20; Z = 0.62; p = 0.53; CI = [-0.85, -0.44]), nor for the adult populations (n = 147; g = 0.27; Z = 0.75; p = 0.45; CI = [-0.43, -0.98]).

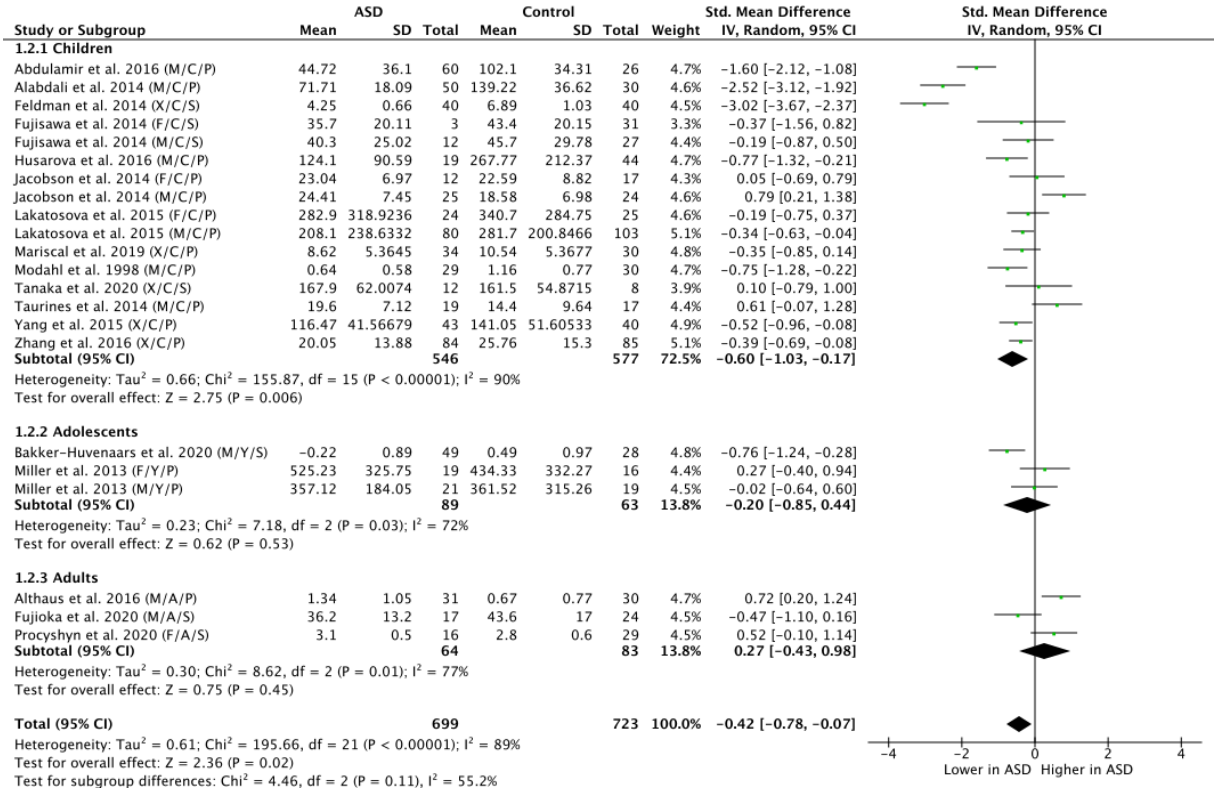

Figure 3. Subgroup analysis by age. M = male, F = female, X = mixed sexes; C = children, Y = youths/ adolescents, A = adults; P = plasma, S = saliva.

Subgroup analysis by sex

To explore the potential influence of sex on OT levels in ASD versus NT, a sub-group analysis by sex was carried out by grouping participants on the basis of sex and contrasting all exclusively male, female and mixed (i.e., papers where disentanglement was impossible) groups (**Figure 4**). This analysis revealed a trend-level effect of lower OT levels in the ASD group in the male population (n = 814; g = -0.44; Z = 1.75; p = 0.08; CI = [-0.94, 0.05]) but no significant effect in the female population (n = 192; g = 0.11; Z = 0.72; p = 0.47; CI = [-0.19, 0.42]). The mixed group, however, showed significantly lower OT levels in ASD versus NT (n = 416; g = -0.84; Z = 1.93; p = 0.05; CI = [-1.69, 0.01]).

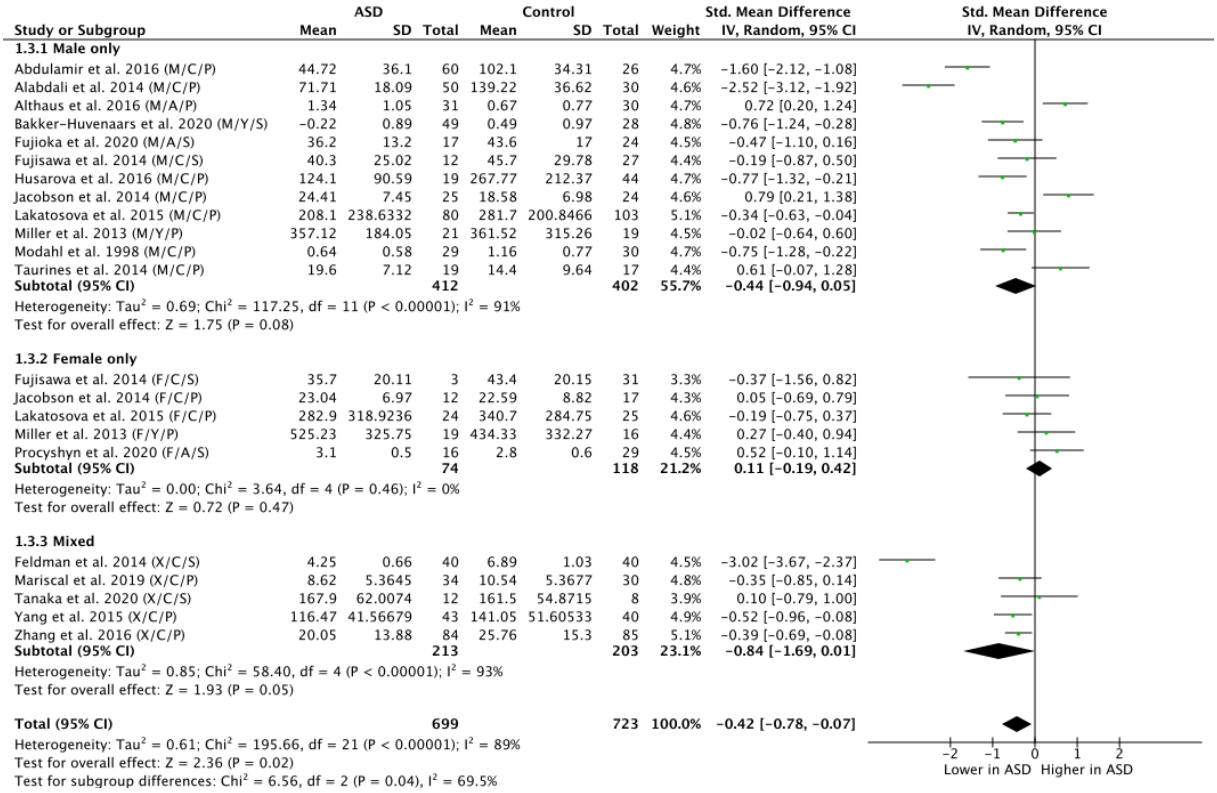

Figure 4. Subgroup analysis by sex. M = male, F = female, X = mixed sexes; C = children, Y = youths/ adolescents, A = adults; P = plasma, S = saliva.

Subgroup analysis by tissue sample

A subgroup analysis by tissue sample (blood plasma or saliva) showed a trend-level group difference in OT levels of ASD versus NT participants as assessed on the basis of blood plasma concentrations (n = 1086; g = -0.34; Z = 1.76; p = 0.08; CI = [-0.72, 0.04]) (**Figure 5**). While the effect size calculated on the basis of saliva as specimen for OT group comparison was high (g = -0.61), it did not reach significance, due to the larger variability and the smaller number of included participants (n = 336; g = -0.61; Z = 1.36; p = 0.17; CI = [-1.49, 0.27]). Statistically testing for subgroup differences did indeed confirm that the significant overall group effect was not modulated on the basis of tissue sample type subgroup differences ($Chi^2 = 0.30$, df = 1; p = 0.58; $I^2 = 0\%$).

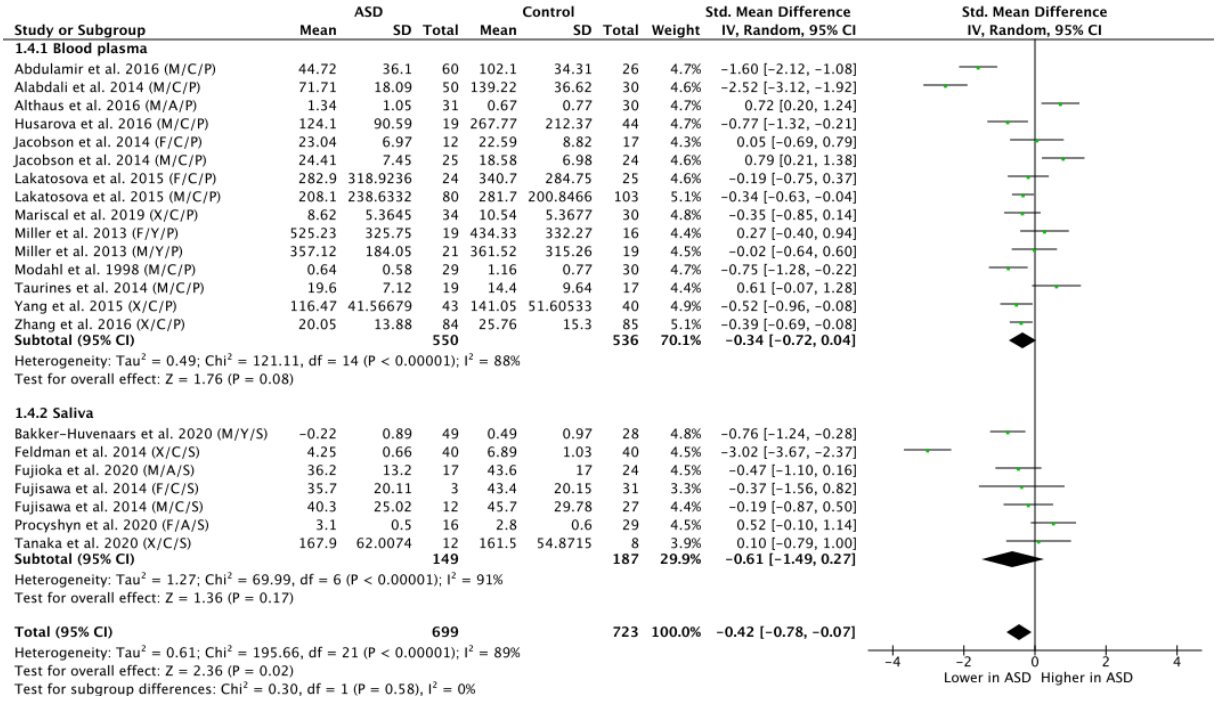

Figure 5. Subgroup analysis by tissue sample. M = male, F = female, X = mixed sexes; C = children, Y = youths/adolescents, A = adults; P = plasma, S = saliva.

Discussion

There is growing evidence that OT circuitry plays a major role in the mediation of prosocial behaviour, including empathy, emotion recognition, eye contact and attachment development (Macdonald & Macdonald, 2010). Individuals with ASD are characterized by impairments in social interaction and communication, and have been suggested to display deficiencies in central OT mechanisms. The current study evaluated potential group differences in endogenous OT levels between ASD and NT controls, and considered age, sex and tissue type as potential mediators of this group difference. We included 18 studies comprising a total of 1422 participants.

Statistical analysis of the total pool of participants showed that endogenous OT levels in ASD populations are significantly lower compared to NT controls. These results are in contradiction with an earlier systematic review and preliminary meta-analysis addressing peripheral OT in psychiatric disorders in general (Rutigliano et al., 2016), in which no significant difference was found in plasma or salivary OT between ASD and NTs. However, the studies included in this review only comprised adult participants. To elucidate this discrepancy, we performed a subgroup analysis by age, which showed that children with ASD do have significantly lower OT values compared to typically developing

controls, but this effect disappeared in the adolescent and adult groups. This developmental effect is even further specified when contrasting the child studies, based on the average age of the participants. In particular, in the group of <6-year-olds, 4 out of 5 studies found significantly lower OT levels in ASD (Feldman et al., 2014; Fujisawa et al., 2014; Husarova et al., 2016; J. D. Jacobson et al., 2014; Zhang et al., 2016). Likewise, in the group of 6-to-9 year olds, 4 out of 5 studies reported significantly lower OT levels in children with ASD (Abdulamir et al., 2016; Alabdali et al., 2014; Husarova et al., 2016; Lakatosova et al., 2015; Modahl et al., 1998). Yet, in the group of children >9 year olds, none of the three studies reported significantly lower OT in the ASD group (Mariscal et al., 2019; Tanaka et al., 2020; Taurines et al., 2014), indicating either a normalisation (increase) of OT levels in ASD or a reduction of OT levels in NT controls, after early childhood. Conversely, Lakatosova et al. divided their child sample into two groups according to age (younger versus older than 10 years) and found the decrease of plasma OT to be especially prominent in the older children (Lakatosova et al., 2015).

On average, significantly lower OT levels are not found in adolescents with ASD, but this may be due to the limited number of studies on this age group. Note that one study (Bakker-Huvenaars et al., 2020) did demonstrate lower OT levels in adolescents with ASD (Alvares et al., 2017), but the other (Miller et al. 2013) did not observe any group differences (Miller et al., 2013). However, pertaining to the adult populations, the general absence of observations of lower endogenous OT levels in adults with ASD seems robust and coincides with the results of the meta-analysis by Rutigliano et al. (Rutigliano et al., 2016). Notably, one adult study even found significantly higher OT concentrations in the ASD population (Althaus et al., 2016), substantiating a potential developmental effect.

Such an age-dependent aberrancy of the OT system has also been described in two earlier studies. First, Freeman at al., (2018) showed an early-life peak in OT receptor density in the ventral pallidum (part of the reward system in the brain) in NT children, but not in ASD children (Freeman et al., 2018). These authors suggest that the lack of this early life critical period, where this reward area becomes maximally sensitive to oxytocin binding, may impact social development and may result in social symptoms in ASD. Second, a recent review on oxytocin receptor (OXTR) gene DNA methylation suggested hypomethylation in children with ASD and hypermethylation in adults with ASD (Moerkerke et al., 2021). Tentatively, this opposite developmental pattern was interpreted as if the initial hypomethylation in children with ASD may underlie their

aversive and intrusive experience of social encounters, which they gradually counter by developing a hypermethylated (hence, dampened) OXTR system. Given the current observation of lower circulating OT levels in children with ASD and the lack of an early-life peak in OT receptor density, the suggestive OXTR hypomethylation in children with ASD could also be interpreted as an inefficient biological manner of coping with these OT system deficiencies.

Next, we performed a subgroup analysis per sex. This analysis yielded a marginally significantly lower OT level in males, but clearly no group difference in females. Note that the effect size in the male subgroup is considerable ($g = -0.44$, $p = 0.08$) and even exceeds the overall effect size across all studies ($g = -0.42$, $p = 0.02$), while the effect size in the females is negligible ($g = 0.11$, $p=0.47$). The male group is, however, larger ($n = 814$) than the female group ($n = 192$) which could also play a part here. Consistently, in a mixed group study, Lakatosova et al. reported relatively decreased plasma OT levels in boys with ASD but not in girls with ASD (Lakatosova et al., 2015). ASD is a male-dominant disorder and the diagnosis is three times more prevalent in boys compared to girls according to the meta-analysis of Loomes et al. (Loomes et al., 2017). The authors attribute this mainly to the fact that boys have a more distinct and recognizable phenotype of autistic traits whereas girls have a subtler presentation of autism characteristics and are more likely to camouflage their impairments, augmenting the risk of a late or overlooked diagnosis. As OT plays a key role in mediating social characteristics and behaviour, it is possible that this phenotypical disparity between ASD males and females is driven by sex specific differences in OT dysfunctions at the neurobiological level. We know from animal studies that OT levels are indeed sexually dimorphic, and are generally much higher in females than males, which could be due to its interactions with oestrogen and oestrogen receptors (Carter, 2007; Kramer et al., 2011; Patisaul et al., 2003; Witt et al., 1991). Against this background, it can be hypothesized that these generally higher OT levels in females may act in a protective manner, also in females with ASD profiles.

Obtaining central OT levels is challenging, therefore researchers acquire peripheral OT levels through blood plasma or saliva, thereby offering an accessible window on central OT circuitry. The subgroup analysis per tissue sample showed significantly lower OT levels in ASD as measured via plasma, but not via saliva. Yet, it should be noted that for both tissue types the effect sizes were considerable and comparable ($g= -0.69$ for plasma and -0.58 for saliva), as additionally confirmed by the lack of a sub-group difference.

Accordingly, the absence of a significant ASD versus NT group difference in the saliva samples largely depends on lacking statistical power due to the smaller number of participants. An active controversy regarding the measurement of OT in biological fluids concerns whether these peripheral measures are actually reliable and valid approximations for levels of OT in the central nervous system (Leng & Ludwig, 2016). In this regard, a recent meta-analysis revealed a positive correlation between peripheral OT levels and OT levels in the central nervous system, in particular after experimental stress induction. As no correlation was observed under baseline conditions, it remains questionable to what extent peripheral OT levels may approximate central OT levels (Valstad et al., 2017).

Against this background, one may also wonder via what mechanism reduced endogenous peripheral OT levels may impact on social functioning in children with ASD. At the peripheral level, OT has been postulated and demonstrated to exert an anxiolytic influence by regulating the cardiovascular and autonomous nervous system, and consequently reducing physiological stress reactivity and (social) anxiety (Kumsta & Heinrichs, 2013; Quintana et al., 2015). In as far as peripheral OT levels may also index central OT levels (Valstad et al., 2017), OT has also been suggested to act at the level of the amygdala and prefrontal cortex and enhance social functioning by enhancing the salience of socially relevant information, such as eye gaze and facial and vocal emotional information (Quintana et al., 2015; Shamay-Tsoory & Abu-Akel, 2016).

The current study has some limitations that need to be taken into consideration. Regarding the methodology of the included studies, there is a high variability in numerous aspects of the process that could attribute to dubious results. First, phenotypic characterization was variable across studies. This implies that possible differences in symptom severity of ASD subjects were not accounted for, which could correlate with the degree of impairment in the underlying OT biology. Second, different analysis approaches were used to measure OT concentration in samples (i.e., either ELISA or RIA techniques), which may also add to the heterogeneity of the findings (Engel et al., 2019). Third, the included studies show a high variability in terms of age, race, distribution of sex within ASD and control samples, and means of OT sampling measurement. While we incorporated the most prominent sources of variation in our analyses (i.e., age, sex and sample tissue), tissue extraction and analysis approach was not accounted for, which is known to yield a large variability (McCullough et al., 2013).

In conclusion, endogenous OT levels are lower in children with ASD as compared to NT controls, but this effect seems to disappear in adolescent and adult populations. Secondly, while no significant subgroup differences were found with regard to sex, the group difference in OT levels of individuals with versus without ASD seems to be only present in the studies with male participants. Finally, while no subgroup differences were found with regard to tissue sample, the ASD versus NT group difference in OT level may be slightly more pronounced for blood samples as compared to saliva samples. More research is required to investigate potential developmental changes in endogenous OT levels, both in typical and atypical populations. This research may also contribute to the design of more targeted therapies that can aid in mitigating differential OT development and its social consequences, e.g., through intranasal OT administration (Bernaerts, Boets, Bosmans, et al., 2020; Veening & Olivier, 2013) or via interpersonal sensorimotor synchronization therapies that may aid in heightening OT's endogenous production (Feldman, 2012; Papasteri et al., 2020). Further research employing more homogeneous methods is necessary to explore the possible use of OT level measurement as a diagnostic marker of ASD.

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Author contributions: Matthijs Moerkerke: Conceptualization, Methodology, Investigation, Data curation, Validation, Writing - Original Draft, Writing - Review & Editing, Visualisation, Project administration; Mathieu Peeters: Conceptualization, Methodology, Investigation, Data curation, Writing - Original Draft, Visualisation. Lyssa de Vries: Methodology, Investigation, Writing - Review & Editing, Visualisation; Nicky Daniels: Validation, Writing - Review & Editing; Jean Steyaert: Supervision, Validation, Writing - Review & Editing, Funding acquisition; Kaat Alaerts: Supervision, Validation, Writing- Reviewing and Editing, Funding acquisition; Bart Boets: Supervision, Conceptualization, Methodology, Validation, Writing- Reviewing and Editing, Funding acquisition.

Supplementary Material

A two-factor subgroup interaction of age and sex revealed (marginally) lower OT levels in boys (n =595; g = -0.60; Z = 1.75; p = 0.08; CI = [-1.27, 0.07]) and mixed boys/girls (n = 416; g = -0.84; Z = 1.93; p = 0.05; CI = [-1.69, 0.01]) groups of children with ASD but not in girls with ASD (n = 112; g = -0.13; Z = 0.63; p = 0.53; CI = [-0.55, 0.28]) (Figure S1).

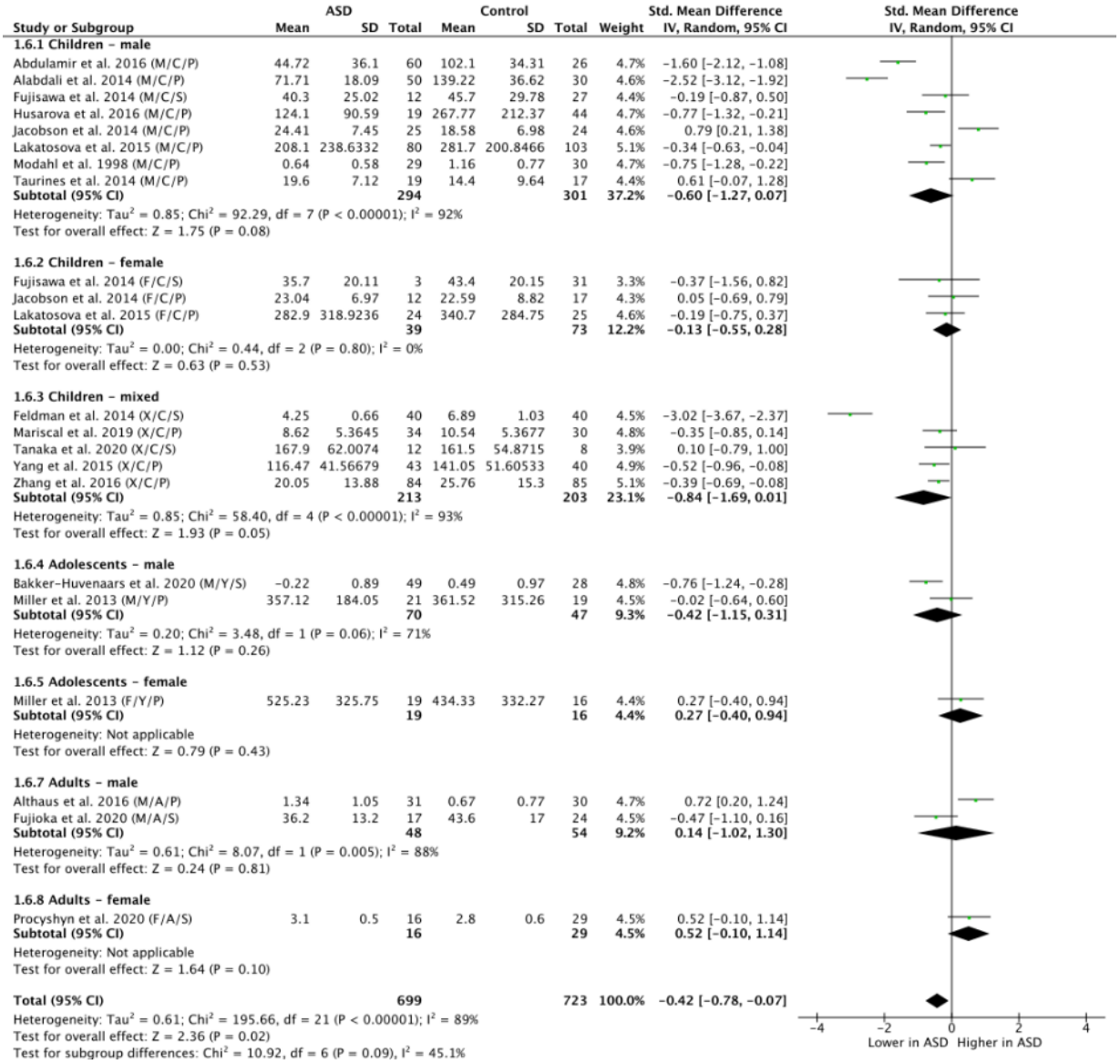

Figure S1. Subgroup analysis by age and sex. M = male, F = female, X = mixed sexes; C = children, Y = youths/ adolescents, A = adults; P = plasma, S = saliva.

Lastly, only 5 (out of the 18) papers have explicitly indicated that the OT sample was extracted. Evaluating these 5 in a separate subgroup revealed no group differences (n =421; g = 0.15; Z = 0.53; p = 0.60; CI = [-0.40, 0.69])

Chapter 3 | Oxytocin receptor gene (*OXTR*) DNA methylation is associated with autism and related social traits — a systematic review

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Moerkerke, M., Bonte, M-L. (shared first), Daniels, N., Chubar, V., Alaerts, K., Steyaert, J., Boets, B. (2021). Oxytocin receptor gene (*OXTR*) DNA methylation is associated with autism and related social traits-A systematic review. RESEARCH IN AUTISM SPECTRUM DISORDERS, 85.
https://doi.org/10.1016/j.rasd.2021.101785

Abstract

There is emerging evidence implicating oxytocin receptor gene (*OXTR*) DNA methylation (DNAm) in social behaviour. This review investigated its association with autism spectrum disorder (ASD) characteristics and related social dimensions, both in individuals with and without ASD. Twelve articles investigating OXTR DNAm in relation to ASD, social perception/cognition and social anxiety were included. We found that hypermethylation is associated with (i) higher quantitative autism traits in adults, reflecting a higher incidence of autism characteristics, (ii) increased brain activity while performing social tasks (indicating a higher need for resources) and (iii) decreased functional connectivity. (iv) Contradictory, hypomethylation was found to be present in children (especially boys) with ASD and was also associated with more social anxiety. While the included studies displayed a large variability, for example in terms of population characteristics, analysed OXTR DNAm regions, and adopted scales/questionnaires, an initial developmental pattern of results emerged, suggesting an association between hypermethylation of OXTR and autism traits in adults. Nonetheless, future studies are warranted to corroborate these initial conclusions.

Introduction

Oxytocin as a neuromodulator of social behaviour

The nonapeptide oxytocin (OT) is produced in neurons in the hypothalamic paraventricular and supraoptic nuclei and their accessory nuclei (Benarroch, 2013). The neurons project to the posterior pituitary where OT is released into the systemic circulation. OT is commonly known for its hormonal function inducing uterine contractions during childbirth and milk ejection during lactation (Zingg & Laporte, 2003). Additionally, OT acts as a neuromodulator in the central nervous system, where it plays a key role in a broad range of social and emotional behaviours (M. J. Bakermans-Kranenburg & van Ijzendoorn, 2014; de Oliveira et al., 2018; Kraaijenvanger et al., 2019). Accordingly, alterations in the OT system have been implicated in a range of neurodevelopmental conditions characterized by social impairments, such as autism spectrum disorder (ASD) (Elagoz Yuksel et al., 2016; Gregory et al., 2009; Kraaijenvanger et al., 2019; Maud et al., 2018; Rijlaarsdam et al., 2017). ASD is a neurodevelopmental condition, characterized by difficulties in social communication and interaction and the presence of restricted and repetitive behaviours and interests (American Psychiatric Association, 2013). It refers to a spectrum of developmental conditions with symptoms that are on a continuum ranging from mild to severe expression (Lauritsen, 2008).

The role of OT in social behaviour has been thoroughly studied. Exogenous intranasal OT administration in individuals with ASD is a relatively new psychopharmacological treatment possibility. Guastella et al. (2016) reviewed the existing literature about OT treatment for ASD (Guastella & Hickie, 2016) and concluded that OT has the potential to improve social impairment for some individuals, though, a personalized approach is needed to identify those individuals who might benefit of this particular treatment. Another meta-analysis of Wang and colleagues (2019), however, indicated only a small and non-significant OT effect on the core ASD symptoms (Y. Wang et al., 2019). Therapeutic exogenous OT efficacy could depend on, for instance, an individual's endogenous OT levels (Parker et al., 2017) or the genetic profile of the Oxytocin Receptor (OTR) (Ebner et al., 2019; Kosaka et al., 2016). Multiple studies offering intranasal administration of OT in neurotypicals (NT) indicated that OT can elevate the perception of trustworthiness and attractiveness and can also increase the encoding of positive social stimuli to make them more memorable (Guastella et al., 2008; Theodoridou et al., 2009).

In their review, Veening et al. (2013) found convincing fear reducing effects of intranasal OT, which impact on affiliative behaviour and social interactions (Veening & Olivier, 2013). Besides these positive effects, it should be noted that OT can enhance processing of negative social stimuli as well, such as the recognition of fear or anger (Leppanen, Wee, et al., 2017). Moreover, in certain contexts, OT can even enhance negative social behaviour such as envy, gloating or even defensive aggression towards out-group members (de Dreu et al., 2010; Shamay-Tsoory et al., 2009).

Variability in the OXTR and social behaviour

To date, a large body of research has explored the role of the OT system in relation to ASD from a genetic perspective (de Oliveira et al., 2018; Elagoz Yuksel et al., 2016; Gregory et al., 2009; Maud et al., 2018; Rijlaarsdam et al., 2017). Especially the OTR gene (OXTR), which codes for the protein functioning as the receptor for OT, has gained increasing research interest. The OTR is an encoded receptor that consists of a 388-amino acid polypeptide with 7 transmembrane domains (Kimura et al., 1992). The OTR belongs to the class I G protein-coupled receptors.

OXTR is a single copy, located on the third chromosome: 3p25-3p26.2 (hg38, 3:8750408-8769628) (Gimpl & Fahrenholz, 2001; Inoue et al., 1994) and is expressed in various tissues including many regions of the brain. Quintana et al. revealed that OXTR is highly expressed in subcortical (e.g., hypothalamus, amygdala and hippocampus), temporal and olfactory brain regions (Quintana et al., 2019). The gene is 17kb wide and exists of 3 introns and 4 exons (see Figure 2). Exons 1 and 2 are 5'-noncoding regions, while exons 3 and 4 encode the amino acids forming the OTR (Inoue et al., 1994). OXTR is liable to variations in the nucleotides. Such regions in the gene are called a single nucleotide polymorphism (SNP). Data from animal studies has shown that OXTR knockout mice demonstrate profound deficits in maternal behaviours (Takayanagi et al., 2005), impaired social memory - as measured via a social recognition test (Winslow & Insel, 2002) - as well as increased aggression (Ragnauth et al., 2005; Winslow et al., 2000). These findings support the notion that the *OXTR* is an important biological component involved in regulation of social behaviour.

Accumulating evidence supports the role of *OXTR* SNPs in human social behaviour such as pair bonding (Walum et al., 2012), empathy and stress reactivity (Rodrigues et al., 2009), emotional support seeking (Kim et al., 2010), generous behaviour (Israel et al.,

2009), empathy and social communication (Tost et al., 2010), increased benefits from social support (Chen & Johnson, 2012), face perception (Westberg et al., 2016) and sociality (Li et al., 2015). Also with respect to ASD, genetic research has linked *OXTR* to different social dimensions (Harrison et al., 2015; Kranz et al., 2016; LoParo & Waldman, 2015; Yamasue, 2013). Multiple SNPs have been identified, but one SNP has received particular attention in research on social behaviour and ASD: the rs53576 located in the third intron (Li et al., 2015). According to the Genotype-Tissue Expression Portal the rs53576 SNP is associated with OTR expression in several brain regions (Andari & Rilling, 2020). This SNP contains two alleles, a G-allele (Guanine substitution) and an A-allele (Adenine substitution). Multiple studies have suggested that the A-allele might entail risk for ASD and reduced sociability (Kogana et al., 2011; Lucht et al., 2009; Wu et al., 2005). Abovementioned studies however have mainly investigated the DNA sequence of *OXTR* without giving empirical attention to the role of epigenetic factors.

DNA methylation as an epigenetic mechanism

Nevertheless, more recent studies suggest that the aetiology of neurodevelopmental disorders as well as complex behavioural traits, can be partially attributed to molecular epigenetic mechanisms (Kubota & Mochizuki, 2016; Scaini et al., 2014). Conrad H. Waddington introduced the concept of an "epigenetic landscape", almost 70 years ago (Waddington, 1957). In his theory, Waddington suggests that interactions between genes and environment can influence the developmental pathway that a cell can take during differentiation and that this will shape the final phenotype (Waddington, 1957). Since then, the field of epigenetics has much evolved and in a broad context, it studies the complex biological mechanisms that affect and regulate transcriptional activity of a gene without altering the actual DNA sequence. In addition, it has been shown that epigenetic mechanisms can be dynamic throughout the lifespan and are sensitive to environmental influences (Fraga et al., 2005; Gouin et al., 2017).

DNA methylation (DNAm) is one of the most extensively studied epigenetic mechanisms and it is involved in the regulation of gene transcription. Specific cytosines in DNA can become methylated, implying that a methyl group is added to the carbon-5 position in the cytosine ring (Tchurikov, 2005). Cytosines of CpG dinucleotides (i.e., a cytosine followed by a guanine, connected by a phosphate) are preferably methylated. Some DNA regions are rich in CpG dinucleotide and form clusters known as CpG islands, that are on average

1000 base pairs long, most of which are unmethylated (Deaton & Bird, 2011). Such CpG islands most often coincide with transcription initiation sites. Yet some are situated distant from such a site, but most still show evidence for promotor function (Deaton & Bird, 2011; Illingworth et al., 2010). DNAm takes place after a new chain is synthesized with unmethylated dinucleotides in positions opposite of the methylated old DNA chain. The DNAm enzyme, DNMT1, is responsible for reproducing the DNAm pattern in the new DNA chain (Tchurikov, 2005). Though, de novo DNAm occurs as well (Deaton & Bird, 2011).

DNAm is generally associated with decreased gene transcription, and therefore less expression of the respective protein, OTR in this case (Kusui et al., 2001). The current review focusses on DNAm as it has been the most widely studied epigenetic mechanism by far, because of both its biological relevance for the regulation of gene transcription and the availability of techniques for DNAm analysis. However, it should be acknowledged that other epigenetic mechanisms exist as well, such as histone modification, RNA regulation, chromosomal silencing and transpositioning of mobile elements.

OXTR DNA methylation and social behaviour

Several studies have shown an association between *OXTR* DNAm, social behaviour and related psychopathologies. Recently, two review articles have been published, investigating DNAm of *OXTR* in relation to social functioning. Maud et al. stated on the basis of a narrative review that no concluding evidence was found for the role of *OXTR* DNAm in social and emotional behaviour (Maud et al., 2018). Kraaijenvanger et al. provided an overview regarding epigenetic variability of *OXTR* as a possible pathway towards various psychopathologies (Kraaijenvanger et al., 2019). While both reviews included articles about ASD and autism characteristics, they also covered many other psychiatric pathologies such as psychotic disorders, anorexia nervosa, obsessive compulsive disorder, post-traumatic stress disorder, (postpartum) depression and conduct disorder.

Because of the phenotypic heterogeneity of genes, which is apparent in ASD, the current review specifically aimed to investigate the association between *OXTR* DNAm and variations in social traits in participants with ASD, but also beyond categorical diagnosis, by assessing variations in other social dimensions, closely related to ASD, in neurotypicals. We based these social dimensions on the DSM-5 A-criteria for ASD

(American Psychiatric Association, 2013), in particular impairments in socio-emotional reciprocity and deficits in nonverbal communicative behaviour, such as facial expression processing, eye contact and body language. At an underlying neurocognitive level, these impairments may be related to alterations in social perception (i.e., the ability to interpret another's mental state based on behavioural signals (Apperly & Butterfill, 2009)) and social cognition (i.e., how people process and respond to social information (Hunt et al., 2012)), even though these alterations are not specific for ASD per se. Lastly, due to the high comorbidity between ASD and social anxiety (Matson & Goldin, 2013), we additionally investigate the relation between *OXTR* DNAm and social anxiety, in individuals with and without Social Anxiety Disorder (SAD).

Materials and Methods

A search of two databases, PubMed and Embase, was performed to conceive relevant literature (ultimate search date = February 2021), as displayed in **Figure 1**. The current systematic review was performed in accordance with the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" (PRISMA) guidelines. The following search string was used: '(Oxytocin OR Oxytocin receptor OR oxytocin receptor gene OR OXTR OR OT OR OXT) AND (methylation OR DNA methylation OR epigenetics) AND (autism OR autism spectrum disorder OR ASD OR autistic traits OR ASD traits OR social OR socio OR non-verbal OR nonverbal OR face OR faces OR eye OR eyes OR eye contact OR communication OR interaction OR perception OR cognition OR relation OR body language OR emotion)'. A total of 117 papers were found in Pubmed and 335 papers in Embase. These articles were screened based on title and abstract. Only articles describing *OXTR* DNAm and ASD or autism traits or social behaviour, perception and cognition in human study groups were included. All papers had to be peer-reviewed and written in English. Articles reporting animal studies or prenatal/postnatal oxytocin effects or covering restricted and repetitive patterns of behaviour and interests or articles not involving epigenetics were excluded in the primary search. A total of 34 articles was retained and was subjected to full paper analysis. Nine of these articles did not meet inclusion criteria, 5 were books and 8 reviews, so finally 12 studies were included in the present review.

The final selection of articles was categorised along three broad themes, i.e., (1) 'ASD', (2) 'social perception / social cognition', and (3) 'social anxiety'. For the first category, only 4

articles, comparing *OXTR* DNAm in ASD versus neurotypicals, were found. The second category includes 6 papers, evaluating *OXTR* DNA methylation and social perception / social cognition. For the last category we found 2 papers, evaluating *OXTR* DNA methylation and social anxiety.

The protocol for this review was registered on PROSPERO (CRD42020160372) and can be accessed at https://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42020160372.

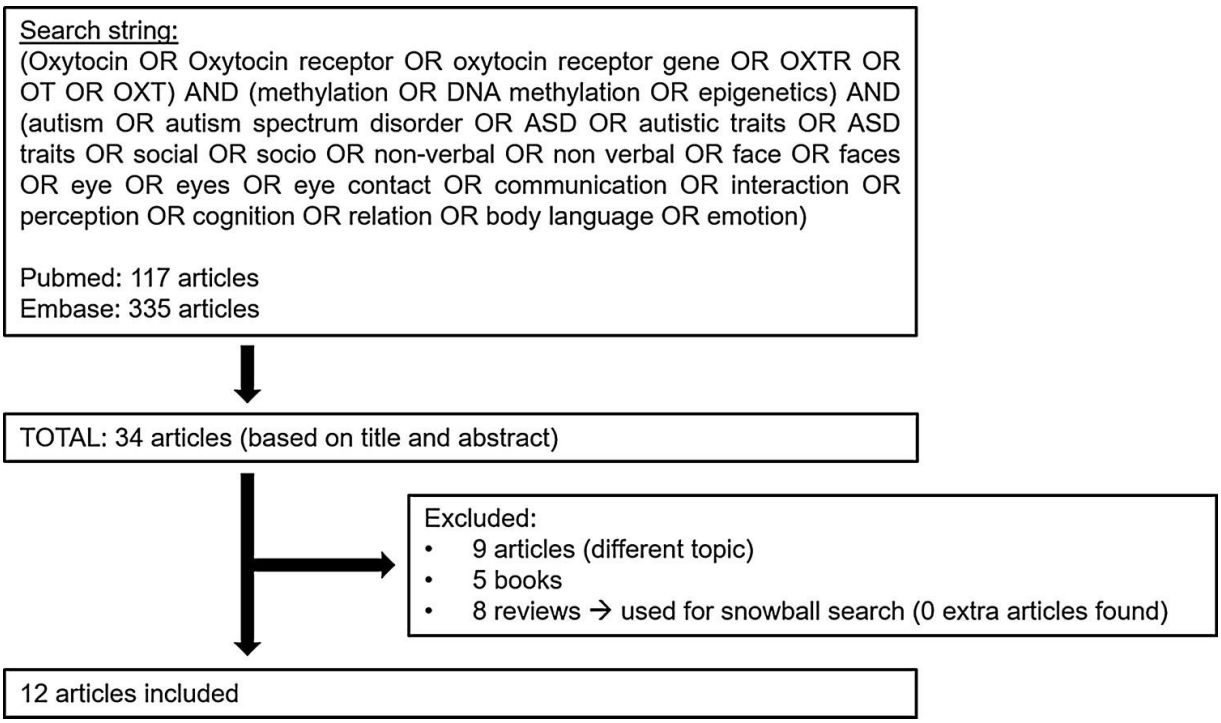

Figure 1. Schematic flowchart of the search method used in this review.

Results

A detailed overview of research findings can be found in Table 1.

OXTR DNA methylation and ASD

Only four studies investigated DNAm of *OXTR* in individuals with ASD in comparison to NT controls, but contradictory results were found (Andari et al., 2020; Elagoz Yuksel et al., 2016; Gregory et al., 2009; Siu et al., 2021).

In 2009, in an early pioneering study, Gregory et al. compared the genomic profiles of individuals with ASD (DSM-IV) to those of phenotypically normal controls (Gregory et al., 2009). They used genome-wide tilepath micro-arrays and array comparative genomic hybridization to identify copy number variants (CNV) in 119 idiopathic ASD patients (i.e.,

probands) and their first-degree family members (54 controls). The age of the participants was not mentioned (only for the smaller sub-study investigating post mortem brain tissue in 8 ASD versus 8 NT participants, it was mentioned that the subjects were aged 4 – 30 years). In the first part of this study, genomic profiles of the probands and their controls were obtained. One hundred and thirteen loci contained at least 1 genomic deletion or duplication. The most interesting finding was a heterozygous deletion of *OXTR* (3p25.3 deletion) in an ASD patient and his mother. The mother was not diagnosed with ASD but had an obsessive-compulsive disorder (OCD). As the proband also had an affected brother with ASD without the deletion, other mechanisms beside this particular genomic deletion had to be involved as well. Accordingly, this family was further investigated with bisulfite sequencing analysis of 2 CpG islands (island A involving exon 1, 2 and 3, and island B involving intron 3, see **Figure 2**) on *OXTR*. The dinucleotides in island B were heavily methylated in all family members, but in island A DNAm levels differed between the subjects. In particular, CpG at sites -860, -901, -924, -934 and -959 were more methylated in the diagnosed ASD patients only. In a second part of the study, peripheral blood of 20 ASD patients and 20 unrelated NT controls was compared. Individuals with ASD showed increased DNAm at -860, -934 and -959 compared to controls. Third, DNAm status in the cortical brain was investigated using post mortem frozen brain tissues of 8 ASD patients and 8 NT controls. Individuals with ASD showed hypermethylation of sites -860, -901, -924 and -934 of the temporal cortex tissue (brain region relevant for human behaviour) compared to healthy controls. Additionally, to correlate DNAm within the temporal cortex and the expression of *OXTR*, a PCR of analysis mRNA was performed, showing that increased DNAm of the promotor on *OXTR* correlates with decreased expression of the gene in a brain region relevant to the development of ASD (Gregory et al., 2009). Thus, altogether, this initial study provided threefold evidence for the involvement of *OXTR* hypermethylation in ASD.

In 2016, Elagoz et al. conducted a similar study investigating *OXTR* DNAm in children (aged 22 – 94 months) with ASD versus NT control children (Elagoz Yuksel et al., 2016). Peripheral blood samples of 27 children diagnosed with ASD (DSM-IV; 23 boys) and 39 control children were used for DNA analysis in 4 regions of *OXTR* (i) exon 1, (ii) intron 1, (iii) exon/intron 2 and (iv) part of exon 3. In contrast to Gregory et al., they found a significant decrease in DNAm status of exon 1 (44.4% in ASD vs 71.8% in NT) and

exon/intron 2 (29.6% in ASD vs 61.5% in NT) in ASD compared to NT controls. There was no group difference in DNAm at the other two regions (intron 1 and exon 3).
Andari et al. (2020), investigated *OXTR* DNAm, from saliva samples, comparing 40 adults with ASD (mean age: 27.02 years) versus 74 NT control adults (mean age: 28.22 years) (Andari et al., 2020). In line with Gregory et al., intron 1 showed hypermethylation in the ASD group, at CpG site -989. Additionally, methylation levels at exon 1 showed a positive correlation with scores on the Social Responsiveness Scale (SRS), a quantitative scale assessing autistic traits (J. N. Constantino & Todd, 2005), indicating that higher methylation levels relate to reduced social responsiveness. In a second part of the study, resting-state functional brain connectivity was evaluated using functional magnetic resonance imaging (fMRI). Here, hypermethylation at site -989 correlated with lower functional connectivity between the superior temporal sulcus and posterior cingulate cortex, two brain areas involved in theory-of-mind processing (which is often altered in individuals with ASD).
In 2021, Siu and colleagues obtained blood samples from 248 children with ASD and 151 NT controls (aged 2 – 18 years) for *OXTR* DNAm analysis at 9 CpG sites located in intron 1. In contrast with Gregory et al. and Andari et al., the authors observed hypomethylation at site -982 in the ASD group, but only in males only (N_{ASD} = 120 vs $N_{control}$ = 58; not in females nor across both sexes combined). However, a substantial proportion of their ASD sample (N = 35, of which 26 male) had outlying DNAm values (compared to the greater ASD cohort), and 76% of these outliers involved hypermethylation in intron 1. Accordingly, the authors reported that extreme *OXTR* hypermethylation might be specific for some individuals with ASD, despite the general tendency of hypomethylation in ASD males compared to NT controls.

So, thus far, the four studies investigating DNAm of *OXTR* in individuals diagnosed with ASD yielded contradictory results, with two studies pointing towards hypermethylation in ASD and two towards hypomethylation. Yet, several methodological differences between the studies should be noted, such as the investigated tissue types and the age of the participants. For instance, Gregory et al. and Andari et al. found hypermethylation in adult ASD populations, whereas Elagoz et al. and Siu et al. provided evidence for hypomethylation in ASD children (especially boys). Moreover, when closely inspecting the specifically investigated CpG sites, the inconsistencies between the studies seem to

partially resolve. For instance, Gregory et al. and Andari et al. showed hypermethylation in ASD in part of intron 1, containing the CpG island of *OXTR* promotor, (see **Figure 2**), whereas Elagoz et al. reported equal DNAm (thus no hypomethylation) across both groups in this same region. Likewise, Elagoz et al. showed hypomethylation in ASD in exon 1 and exon/intron 2, whereas Gregory and colleagues reported equal DNAm (thus no hypermethylation) across both groups in very similar regions. On the other hand, Siu et al. showed hypomethylation in intron 1 in ASD; however, they note that hypermethylation could be specific in some individuals with ASD because a subpopulation of their ASD sample had outlying hypermethylation values in intron 1 (compared to the greater ASD cohort).

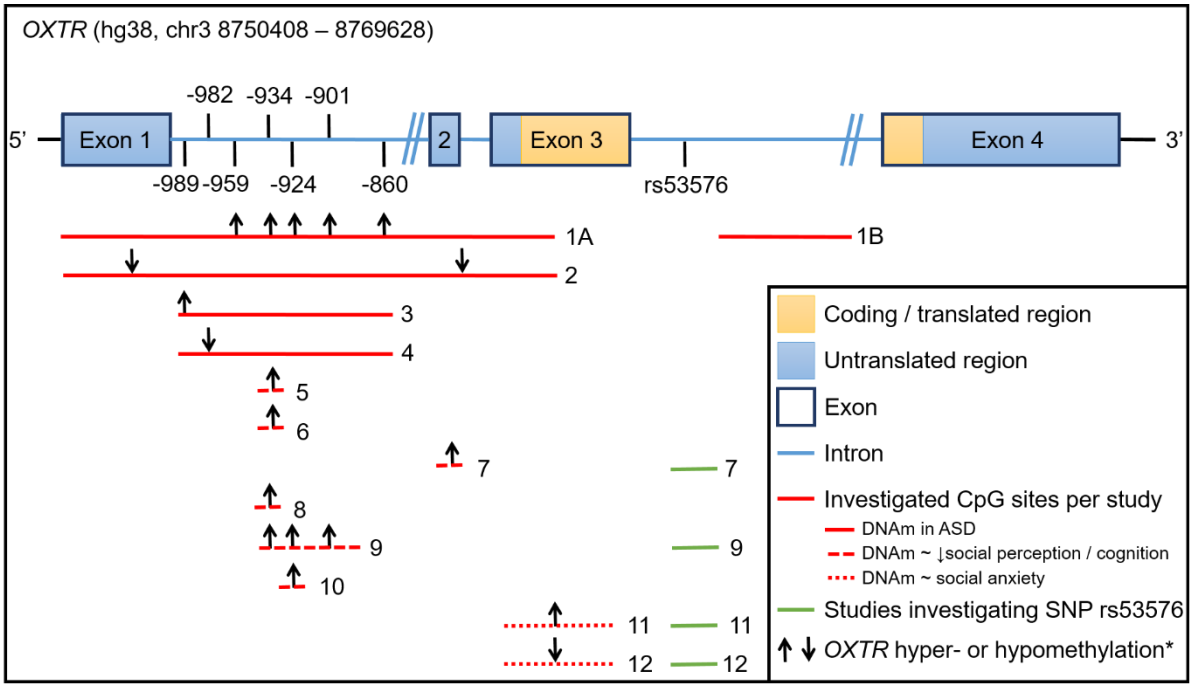

Figure 2. The structure of *OXTR* with a visualisation of the regions investigated in the reviewed articles. *OXTR* is a single copy, located on chromosome 3 (hg38, 3:8750408-8769628). The gene contains 3 introns and 4 exons. This figure shows the structure of *OXTR* with the approximate location of the CpG sites -860, -901, -924, -934, -959, -982, -989 (upstream from the translation start site) and the rs53576 SNP. The red lines indicate the investigated CpG sites/regions and the green lines show studies investigating the SNP rs53576 as well, for the included studies in this review: **1. (Gregory et al., 2009),** 1A: exons 1-3, introns 1 (sites -860; -901;-924; -934; -959) and 2, and 1B. intron 3; **2. (Elagoz Yuksel et al., 2016),** exon 1-3 and intron 1 and 2; **3. (Andari et al., 2020),** intron 1 (site -989); **4. (Siu et al., 2021),** intron 1 (site -982); **5. (Jack et al., 2012),** intron 1 (site -934); **6. (Puglia et al., 2015),** intron 1 (site -934); **7. (Rijlaarsdam et al., 2017),** exon 2 (average of 3 sites); **8. (Puglia et al., 2018),** intron 1 (site -934); **9. (Chen et al., 2019),** intron 1 (sites -934, -924 and -901); **10. (Krol et al., 2019),** intron 1 (site -924); **11. (Chagnon et al., 2015),** exon 3; **12. (Ziegler et al., 2015),** exon 3 (average over 12 sites). * The arrows schematically show *OXTR* hyper- (↑) or hypomethylation (↓) in ASD or in association with autism traits, social perception/cognition or social anxiety per study. (adapted from Kraaijenvanger et al., 2019).

OXTR DNA methylation and social perception / social cognition

In this second part, we broadened our scope beyond the ASD diagnosis. We found 6 papers in neurotypical controls investigating the relationship between individual differences in autism traits and/or social perception/cognition on the one hand and epigenetic variability of *OXTR* on the other.

In 2012, Jack et al. examined whether *OXTR* DNAm was predictive of neural responses to social stimuli (Jack et al., 2012). Forty-three healthy subjects (age 18 – 30 years) performed a social cognition task while undergoing fMRI. They were asked to observe shapes moving along the screen that either involved goal-directed behaviour involving social cognition (e.g., chasing each other) or random behaviour (i.e., bouncing around following straight paths). The authors investigated the brain regions that are selectively sensitive for perceived goal-directed behaviour and found that the degree of DNAm at site -934 was associated with neural activity in two brain regions particularly involved in social cognition, i.e., the temporal parietal junction and the dorsal anterior cingulate cortex (dACC). In particular, individuals with higher DNAm showed greater activity in these regions for the "goal-directed" versus "random behaviour" contrast. The authors interpret this increased activity as evidence that the processing of these social stimuli may have required more resources in individuals showing hypermethylation and that therefore more neural activity was mobilized.

Puglia et al. (2015) administered an emotional face-matching task during fMRI to investigate the influence of *OXTR* DNAm at site -934 on the neural processing of the emotions anger and fear in 98 healthy adults (age 18 – 30 years) (Puglia et al., 2015). An effect of *OXTR* DNAm on the response to angry and fearful faces in left amygdala was shown, indicating that individuals with higher levels of DNAm also showed increased left amygdala reactivity upon processing negative emotional states from faces. Notably, a similar pattern of results was evident in several other brain areas, implicated in face perception and emotion processing, including the fusiform gyrus, the insular cortex, the dorsal anterior cingulate cortex (dACC) and the posterior superior temporal gyrus. Further, hypermethylation of *OXTR* was also shown to be associated with reduced levels of functional connectivity between these brain areas, indicating reduced network integrity among face processing areas in individuals with low levels of *OXTR* expression.

Rijlaarsdam et al. (2017) examined the relationship between autistic traits and *OXTR* DNAm in 6-year old children from the general population (Rijlaarsdam et al., 2017). A specific emphasis was put on investigating DNAm of exon 2, specifically of the rs53576

(G/A) SNP since previous research highlighted the importance of rs53576 A-allele variation in determining variations in social behaviour (Reiner et al., 2015). Umbilical cord blood samples of 743 children collected at birth were used for DNA genotyping. Social behaviours were assessed using various questionnaires, including the SRS and the pervasive developmental problems subscale of the Child Behaviour Checklist (CBCL) assessing behavioural and emotional problems, as reported by parents and/or teachers (Achenbach & Ruffle, 2000). While no overall effect of rs53576 allele variability was found on *OXTR* DNAm, the authors did identify a significant relationship between *OXTR* DNAm and social communication problem scores (SRS) as well as pervasive developmental problems (CBCL) depending on the rs53576 genotype. In particular, only in children with rs53576 homozygous for the G-allele, higher levels of *OXTR* DNAm were associated with higher socio-communicative problem scores on the SRS and CBCL.

In 2018, Puglia et al. investigated associations between *OXTR* DNAm and neural response during a selective social cognition fMRI paradigm (Puglia et al., 2018). Fifty-four healthy adults (age 18 – 30 years) completed a one-back selective cognition task with composite figures of houses and faces, while undergoing fMRI. According to previous research, selectively attending to faces is associated with a higher activation of brain regions of the face perception network. *OXTR* DNAm was investigated at CpG site -934. Results indicated that subjects with higher DNAm levels also showed higher recruitment of brain areas regulating the attentional control network, including the bilateral dorsolateral prefrontal cortex (DLPFC) and the bilateral parietal lobule. Increased *OXTR* DNAm was also associated with reduced connectivity within the salience network, in particular between the DLPFC, the right insula and the bilateral superior temporal gyrus. Secondly, the authors tested whether broader autism related traits would moderate the relationship between *OXTR* DNAm and neural response during selective social attention (Puglia et al., 2018). To do so, participants completed the Autism Quotient Questionnaire (AQ), an ASD screening tool assessing the presence of ASD symptoms in adults and children with an average intelligence (Woodbury-Smith et al., 2005), as well as the Social Interaction Anxiety Scale (SIAS) a self-report scale measuring experienced anxiety levels when interacting with other people (Heimberg et al., 1992). A whole-brain regression analysis was performed, assessing the linear relationship between neural responses to faces and *OXTR* DNAm or autistic traits (AQ or SIAS) scores. A positive relationship was found between the *OXTR* DNAm and activation within the visual cortex and ventromedial

prefrontal cortex in individuals with high AQ scores, indicating that individuals with more autistic characteristics (higher AQ scores) and higher DNAm levels, showed an increased need for attentional-control resources. In contrast, a negative relationship was found for SIAS anxiety scores, indicating that individuals with higher DNAm levels and higher social anxiety (high SIAS scores) showed reduced visual cortex recruitment during facial processing. Chen et al. (2019) investigated if *OXTR* DNAm would modulate the impact of intranasal OT administration on brain activity in response to positive and negative social interactions (Chen et al., 2019). Three hundred and four subjects (age 18 – 22 years) were randomized in three groups, with one group receiving treatment with intranasal OT, another group receiving intranasal vasopressin, and the third group receiving placebo. Saliva samples were used for genotyping *OXTR* in general and the SNP rs53576 specifically. The epigenetic analysis was focused on CpG sites -901, -924 and -934. The participants played the Prisoner Dilemma game (i.e., a model for relationships based on reciprocal altruism) while undergoing fMRI. The contrast of interest was the effect of intranasal OT versus placebo on brain activity, in responses to positive (partner cooperation) and negative (partner defection) social interactions. Firstly, concerning brain activity towards positive social interactions, it was found that (i) OT administration decreased visual brain activity in women with higher DNAm at site -901, whereas it increased brain activity in women with lower DNAm at site -901, (ii) this pattern was not present in men, and (iii) for women nor men, were there significant associations nor interactions with DNAm at sites -924 or -934. So only in women, and particularly in those with low *OXTR* DNAm levels at site -901, intranasal OT could increase the salience of visual feedback of positive social interactions. Secondly, concerning brain activity towards negative social interactions, different associations were observed. (i) OT administration decreased responses in the right precuneus, the right occipital pole and the left postcentral gyrus in men with higher DNAm at sites -924 and -934, and it increased activity along these same brain regions in men with lower DNAm at sites -924 and -934, (ii) this pattern was not present in women, and (iii) neither for women nor men, were there significant associations nor interactions with DNAm at sites -901. So only in men with high *OXTR* DNAm levels at site -924 and -934, intranasal OT could decrease precuneus, occipital pole and the postcentral gyrus activity towards negative social interactions.

Additionally, the authors specifically investigated associations for individuals with the rs53576 GG genotype (N_{male}=41 and N_{female}=38). This particular sub-analysis revealed an opposite effect while experiencing positive interactions: OT increased precuneus activity in individuals with higher DNAm, and decreased brain activity in those with lower DNAm. Secondly, regarding negative social interactions, the rs53576 GG genotype showed that (i) in men with higher DNAm at -901, OT could increase the activation of the visual cortex, and (ii) vice versa. (iii) There were no significant interactions in men at -924/-934, nor in women and at -901, -924 or -934.

Finally, Krol and colleagues (2019) investigated associations between *OXTR* DNAm brain responses towards facial emotional expressions, both in infants and adults. Saliva samples were collected from 84 5-month-old infants and *OXTR* DNAm was measured over site -924. At 7 months, the children underwent functional near-infrared spectroscopy (fNIRS) and brain responses to happy, angry and fearful expressions were assessed, particularly over inferior frontal cortex (IFC). Lower neural responses to happy faces in right IFC in infants were associated with higher *OXTR* DNAm at site -924. An opposite relationship was found for neural responses to angry and fearful faces, indicating that infants with higher *OXTR* DNAm showed higher neural responses to negative emotional expressions. No effects were evident in left IFC, indicating a right-lateralization of the observed effects in the infant brain.

In a next phase, Krol et al (2019) also explored the impact of *OXTR* DNAm on facial emotion processing in the adult brain by using adult fMRI data from a previous study. Here, only angry and fearful faces were presented during the fMRI scanning. In the adult sample, in both left and right IFC, there was a significant positive association between DNAm at site -924, assayed from peripheral blood mononuclear cells, and brain responses toward angry faces, and a marginally significant positive association with brain responses towards fearful faces. Thus, both in infants and adults, higher DNAm levels were associated with higher neural IFC responses toward angry and fearful faces.

Table 1. Summary overview of articles investigating *OXTR* DNA methylation in relation to ASD, autism characteristics and social behaviour.

STUDY	SUBJECTS	PRINCIPAL MEASURES	TISSUE	*OXTR* REGION	RESULTS
***OXTR* DNA methylation and ASD**					
1. Gregory et al., 2009	A. 119 idiopathic autism probands (93 male) and 54 phenotypically normal controls. B. 20 ASD and 20 controls (blood analysis). C. 8 ASD and 8 controls (post mortem study). Age: 4 – 30 years. Caucasian (USA).	Autism spectrum disorder (DSM-IV)	Peripheral blood (after centrifuging) and whole blood (before centrifuging). Post mortem: frozen brain tissue.	A. *OXTR* exons 1, 2 and 3 (sites -860; -901; -924; -934; -959) B. *OXTR* intron 3	A. ASD family: ↑ DNAm in intron 3 and in exon 1, 2 and 3 at site -860, -901, -924, -934 and -959. B. ASD vs controls: ↑ DNAm of -860, -934 and -959. C. Brain cortex (ASD vs controls): ↑ DNAm at -860, -901, -924 and -934.
2. Elagoz et al., 2016	27 children with ASD (23 male) and 39 controls (33 male). Age: 22 – 94 months. Caucasian (Turkey).	Autism spectrum disorder (DSM-IV)	Peripheral blood	*OXTR* exon 1-3 and intron 1 and 2	ASD vs controls: ↓ DNAm in ASD compared to controls in exon 1: (44.4% ASD/ 71.8% controls) and exon/intron 2 (29.6% ASD/ 61.5% controls).
3. Andari et al., 2020	40 adult males with ASD. Age: mean 27.02 years. Caucasian (USA), African American, other. 74 controls (57 male). Age: mean 28.22 years. Caucasian (USA), African American, Asian, other.	Autism spectrum disorder (DSM-V) Resting-state fMRI	Saliva	*OXTR* intron 1 (27 CpG sites)	°ASD vs controls: ↑ DNAm in ASD compared to controls in intron 1 at site -989. °↑ DNAm decreased functional connectivity between superior temporal sulcus and posterior cingulate cortex (brain areas involved in theory-of-mind).
4. Siu et al., 2021	248 children with ASD (120 male for site -982) and 151 controls (58 male for site -982) Age: 2 – 18 years. Canadian (race not listed).	Autism spectrum disorder (confirmed by ASI-R and ADOS)	Whole blood	*OXTR* intron 1 (9 sites)	ASD vs controls: °Sexes combined and females: no significant differences in DNAm. °Males: ↓DNAm in ASD compared to controls in intron 1 at site -982.
***OXTR* DNA methylation and social perception / social cognition**					
5. Jack et al., 2012	43 healthy subjects (23 male). Age: 18 – 30 years. Caucasian, Asian, Black, mixed origin (USA).	Classic social cognition task under fMRI.	Peripheral blood	*OXTR* intron 1 (site -934)	↑ DNAm greater BOLD activity in temporal parietal junction and dorsal anterior cingulate cortex.
6. Puglia et al., 2015	98 healthy subjects (42 male). Age: 18 – 30 years. Caucasian (USA).	Emotional face-processing under fMRI.	Peripheral blood	*OXTR* intron 1 (site -934)	°↑ DNAm increased BOLD activity in brain areas for the perception of faces and the processing of emotions (left amygdala, insular cortex, dACC, fusiform gyrus, posterior superior temporal gyrus). °↑ DNAm decreased functional connectivity between neural systems for social perception.
7. Rijlaarsdam et al., 2017	743 general population children. Age: birth – 6 years. European (race not listed) (Netherlands).	Child autistic traits: Social Responsiveness Scale (SRS) and Child Behaviour Checklist (CBCL).	Cord blood	*OXTR* exon 2, average of 3 sites (cg02192228, cg04523291, cg15317815) Included *OXTR* SNP rs53576	° ↑ DNAm higher social communication problem scores (stronger association for rs53576 GG genotype). °↑ DNAm higher CBCL PDP scores (stronger association for rs53576 GG genotype). °↑ DNAm higher SRS and CBCL scores in rs53576 GG genotype.

STUDY	SUBJECTS	PRINCIPAL MEASURES	TISSUE	*OXTR* REGION	RESULTS
8. Puglia et al., 2018	54 healthy subjects (31 male). Age: 18 – 30 years. Caucasian (USA).	Selective social attention fMRI paradigm, Autism Quotient Questionnaire (AQ) the Social Interaction Anxiety Scale (SIAS).	Peripheral blood	*OXTR* intron 1 (site -934)	°↑ DNAm more recruitment of areas controlling attention (DLPFC and parietal lobule). °↑ DNAm lower connectivity between DLPFC and areas of salience network (right insula and superior temporal gyrus). °Positive correlation between DNAm and AQ scores interaction in visual cortex and ventromedial prefrontal cortex. °Negative correlation between DNAm and SIAS interaction in visual cortex.
9. Chen et al., 2019	304 healthy subjects (153 male). Age: 18 – 22 years. Caucasian, Asian, African American, Latino, other (USA).	Prisoner Dilemma game in fMRI.	Saliva	*OXTR* intron 1 (sites -934, -924 and -901) Included *OXTR* SNP rs53576	°Men: ↓ DNAm at -924 and -934 increased precuneus activity to negative social interactions (CD) after OT administration, and vice versa. ↑ DNAm at -924 and -934 more activation to CD with placebo. At rs53576 ↑ DNAm at -901 had increased activity to positive social interactions (CC) and CD after OT administration. °Women: ↑ DNAm at -901 decreased response to CC after OT administration. At rs53576 ↑ DNAm at -924/-934 decreased activation to CC after OT administration.
10. Krol et al., 2019	84 infants. Age: birth – 7 months. 206 adult controls (94 males). Age: mean 36.9. Caucasian (Germany).	fNIRS (response to happy, angry, fearful facial expressions).	Infant study: Saliva Adult study: peripheral blood mononuclear cells	*OXTR* intron 1 (site -924)	°Infants: negative association of DNAm and right IFC in response to happiness. Positive association of DNAm and right IFC in response to anger and fear. No effect in left IFC. °Adults: positive association of DNAm and angry faces in right and left IFC.
***OXTR* DNA methylation and social anxiety**					
11. Chagnon et al., 2015	19 female patients with anxiety disorder and/or depression and 24 female controls. Age: > 65 years. Caucasian (Canada).	Anxiety disorder and depression (DSM-IV)	Saliva	*OXTR* exon 3 Included *OXTR* SNP rs53576	°No overall difference between patients and controls. °↑ DNAm in anxiety/ depression patients, in rs53576 AA genotype
12. Ziegler et al., 2015	110 patients (34 male) with social anxiety disorder (SAD) and 110 controls (33 male). Age: mean 30.1 - 30.9 years. Caucasian (Germany).	Social anxiety disorder (DSM-IV), SIAS, SPS, cortisol response to TSST, fMRI during social-phobia-related word processing.	Whole blood	*OXTR* exon 3, average over 12 sites within region chr3: 8 809 281–8 809 534 Included *OXTR* SNP rs53576	°↓ DNAm in A allele carriers of *OXTR* rs53576. °Negative correlation DNAm and max cortisol response to TSST (in controls and SAD). °Negative correlation DNAm and scores on SPS and SIAS. °↓ average DNAm in SAD, compared to controls. °SAD: negative correlation DNAm and amygdala responsiveness to social phobia-related words.

OXTR DNA methylation and social anxiety

The literature search rendered two published articles investigating *OXTR* DNAm in relation to social anxiety.

Chagnon et al. (2015) investigated DNAm in 4 candidate genes, including *OXTR*, in 19 elderly women (age >65 years) with anxiety disorder and/or depression (conform DSM-IV criteria) and 24 controls (Chagnon et al., 2015). Saliva samples were used for DNA genotyping, involving DNAm analyses of CpG sites located in the coding region of exon 3 and examination of allele variability in *OXTR* SNP rs53576. Compared to healthy controls, patients with anxiety disorder and/or depression, showed higher levels of *OXTR* DNAm within exon 3. Note however that this effect was driven by a small subgroup with the rs53576 AA genotype ($N_{anx/depr}$=5, $N_{control}$=3).

In 2015, Ziegler et al. investigated both *OXTR* DNAm of exon 3 (averaged over 12 CpG sites) and the rs53576 genotype variability in relation to SAD (Ziegler et al., 2015). 110 patients with SAD (DSM-IV) and 110 matched controls (mean age 30.9 years) were assessed using the Social Interaction Anxiety Scale (SIAS), the Social Phobia Scale (SPS), the Trier Social Stress Test (TSST) and an fMRI task measuring the responsiveness of the amygdala to words related with social phobia. Interestingly, DNAm levels across CpG sites of *OXTR* were shown to be significantly lower in patients with SAD compared to controls, especially in rs53576 A allele carriers. Further, in patients with SAD, lower DNAm was associated with higher amygdala responsiveness to social phobia-related words. In healthy controls, a negative association between *OXTR* DNAm levels and maximal cortisol responses during the TSST was found, as well as a negative correlation with the scores on the SPS and the SIAS. This implies that lower methylation levels were associated with high responses of the stress hormone cortisol and higher anxiety levels in social situations.

Discussion

OXTR DNAm is a relatively new area of investigation in epigenetics, specifically its role in human social behaviour. Overall, as summarized in the current review, hypermethylation of *OXTR* does not unequivocally reflect an increased risk for developing symptoms related to ASD or lower levels of social proficiency, yet some trends are apparent.

To date, only four studies have directly investigated differences between *OXTR* DNAm levels in ASD versus neurotypical controls (Andari et al., 2020; Elagoz Yuksel et al., 2016;

Gregory et al., 2009; Siu et al., 2021), and they yielded seemingly contradictory results. While Gregory et al. (2009) and Andari et al. (2020) showed hypermethylation of multiple CpG sites in adults with ASD, Elagoz et al. (2016) and Siu et al. (2021) showed hypomethylation in children with ASD as compared to controls. This divergent pattern of results possibly relates to variability in the investigated region of CpG sites, with the results of Gregory et al. and Andari et al. pertaining mostly to intron 1, whereas Elagoz et al. mostly focused on exon 1 and exon/intron 2 (see **Figure 2**). On the other hand, Siu et al. did also report hypomethylation in ASD within intron 1. However, this finding should be interpreted with caution as they excluded numerous outliers, most of which showed hypermethylation in intron 1. Gregory et al. showed that hypermethylation in *OXTR* intron 1 corresponds directly with reduced expression of *OXTR* (Gregory et al., 2009), underlining intron 1 as a key regulatory region, perhaps more potent than other regions. Besides differences in the investigated gene region, age might also play a role in these conflicting findings. Gregory et al. and Andari et al. found hypermethylation in adult ASD populations, whereas Elagoz et al. and Siu et al. provided evidence for hypomethylation in ASD children. This may point towards a developmental effect, where increased methylation of *OXTR* sets off in ASD somewhere during childhood or adolescence. According to this hypothesis, OTR levels should be equal in children with ASD, as compared to NT children, and decrease with time. Indeed, Freeman and colleagues discovered that OTR decreases with age, in children with and without ASD, in the ventral pallidum (a brain region involved in reward and motivation) (Freeman et al., 2018). Interestingly, in this same brain region, they also revealed a temporary peak of OTR concentration, in 2-5 year old NT children which was absent in children with ASD (Freeman et al., 2018). Possibly, this OTR peak at this critical age, may boost early social development and reward in the NT children, a process which is missed in the ASD population.

The findings regarding the impact of *OXTR* DNAm on social perception and social cognition in neurotypical populations are more conclusive. In adults, hypermethylation of *OXTR* is associated with increased activity in multiple brain regions involved in social cognition and emotional face processing. Such regions include the temporo-parietal junction, dACC, amygdala, fusiform gyrus, posterior superior temporal gyrus, DLPFC and insular cortex, suggesting that more neural resources are needed to process these type of stimuli (Jack et al., 2012; Krol et al., 2019; Puglia et al., 2015, 2018). Aberrant activity in

these brain regions has previously been linked to psychopathological conditions, including ASD, Post Traumatic Stress Disorder and SAD (Boehme et al., 2014; Koch et al., 2014; Müller & Fishman, 2018). These same regions have previously also been shown to be sensitive to the treatment effects of OT. For instance, intranasal OT administration in individuals with ASD can modulate the neural activity in the "social brain" (Domes et al., 2013) and can attenuate the brain activity related to the processing of social fear (Petrovic et al., 2008).

According to Krol et al., the association of *OXTR* DNAm to emotion processing is emotion-specific in infants, with hypermethylation being related to a higher inferior frontal cortex responsivity for negative emotions like anger and fear, and lower DNAm being related to a higher sensitivity for happy emotions (Krol et al., 2019). In adults, a similar positive association was shown for angry and fearful faces (Krol et al., 2019). These findings are also in line with Chen et al., who observed increased brain response in the precuneus and visual cortex in reaction to social stimuli in individuals with hypermethylation , (Chen et al., 2019). The precuneus is a brain region involved in social information processing and theory-of-mind reasoning (which is often altered in individuals with ASD) (Chen et al., 2019; Cheng et al., 2015). Moreover, intranasal OT attenuated these responses towards social stimuli (Chen et al., 2019). That OT administration might improve emotion recognition and increase social attention was already known, however Chen et al. were the first to investigate the moderating effect of *OXTR* DNAm on its therapeutic effect (i.e., social stimulation) (Domes et al., 2014; Guastella et al., 2008, 2010). Taken together, this is evidence for the hypothesis that *OXTR* hypermethylation increases the need for neural resources to process these type of stimuli.

Conversely, when looking at the rs53576 GG genotype specifically, Chen et al. found opposite effects. In this subsample *OXTR* hypermethylation was associated with lower precuneus and visual cortex activation and intranasal OT elevated these responses (Chen et al., 2019). Interestingly, Rijlaarsdam and colleagues showed that individuals with this rs53576 GG genotype and *OXTR* hypermethylation had more socio-communicative problems based on the SRS and CBCL scores (Rijlaarsdam et al., 2017). This pinpoints the importance of the specificity of the investigated genotype.

Previous studies have shown the implication of proper functional brain connectivity in the processing of social information (Denny et al., 2014). Andari et al. was the first to report that hypermethylation of *OXTR* was correlated with decreased functional

connectivity between brain regions involved in theory-of-mind in ASD (Andari et al., 2020). For the general population, Puglia et al. found that hypermethylation of *OXTR* was associated with a decrease in functional connectivity between brain areas associated with emotion processing and face perception, such as fusiform gyrus, the insular cortex, dACC and the posterior superior temporal gyrus (Puglia et al., 2015). Additionally, hypomethylation has been associated with an increased brain connectivity in response to negative social stimuli (Puglia et al., 2015). In a follow-up study in 2018, Puglia et al. showed that *OXTR* hypermethylation was also associated with reduced connectivity within the salience network, in particular between the DLPFC, the right insula and the bilateral superior temporal gyrus. Consequently, these authors suggest that *OXTR* hypermethylation and decreased functional connectivity could lead to less desensitization and thus less habituation processes resulting in a higher probability of a scattered social perception (Denny et al., 2014).

Hypermethylation of *OXTR* positively correlated with scores on the SRS in ASD, indicating more social responsiveness problems. In the general population, *OXTR* hypermethylation is also associated with higher scores on the SRS and the CBCL, in particular more communication problems, and individuals with higher AQ scores showed increased association between *OXTR* DNAm and neural responses during selective social attention, indicating a higher need for resources (Puglia et al., 2018; Rijlaarsdam et al., 2017). Higher scores on these questionnaires are indicative of more autistic traits and more socio-communicative problems. This confirms the general hypothesis that hypermethylation of *OXTR* is linked to the broad ASD symptomatology.

Regarding the association of *OXTR* DNAm and social anxiety, Puglia et al. (2018) found that neurotypical individuals with high self-reported social anxiety levels as measured by SIAS showed a negative association between *OXTR* DNAm and neural responses during selective social attention. Thus, SIAS scores moderated the relationship between *OXTR* DNAm and neural response during selective social attention in an opposite manner to AQ scores.

Similarly, Ziegler et al. reported that hypomethylation in *OXTR* was associated with a higher cortisol stress response to the TSST and with higher SPS and SIAS scores in the control group (Ziegler et al., 2015). Accordingly, these results contradict the intuitive hypothesis that hypermethylation would be associated with higher anxiety scores. They also contradict previous research showing that higher endogenous OT levels (in

peripheral blood) were associated with lower cortisol levels during the TSST (Pierrehumbert et al., 2010). Possibly, a hypomethylated status of *OXTR* may induce an increased sensitivity towards social situations, thereby turning social stimuli extremely saliently, possibly resulting in elevated levels of social anxiety.

On the other hand, in a clinical population, Chagnon et al. found that elderly women with anxiety disorder and/or depression showed a hypermethylation of *OXTR* as compared to healthy controls (Chagnon et al., 2015). However, after adequate statistical testing, this group difference became insignificant. Thus, based on the reviewed studies, hypomethylation seems to be more often associated with increased social anxiety. Yet these findings should be interpreted carefully and the large heterogeneity between different studies should be taken into account.

The current review has some limitations. Firstly, this review included only 12 articles that were subdivided by topic, implying that each subdivision comprised a relatively small number of studies. The subdivisions of ASD and social anxiety consisted of only four and two studies, respectively, making it difficult to reach a conclusion, especially since the findings of each of these studies contradicted each other. With regard to social perception and social cognition, six articles were included, encompassing a rather broad range of questionnaires, scales and measures. Secondly, there was considerable methodological heterogeneity between studies; e.g., in terms of (i) the included age range of the participant (infants vs. children vs. adults vs. elderly) (ii) diagnostic classification (ASD vs. NT vs. SAD), (iii) tissue samples for assessing *OXTR* DNAm (blood vs. saliva vs. frozen brain tissue), (iv) assessed *OXTR* CpG site, and (v) range of analysis techniques (e.g., gene primer; see Supplementary Table 1). Although the current review revealed some patterns in this heterogeneity, for instance the role of intron 1 and a possible developmental effect, future research should corroborate these findings. Accordingly, since each of the included studies was too idiosyncratic in terms of methods, outcomes and population characteristics, no formal meta-analysis was performed on the statistical data.

While the reviewed literature allowed to draw some tentative conclusions with regard to the relationship between epigenetics of the oxytocinergic system and social functioning, further research into the association between *OXTR* DNAm and ASD characteristics is urgently needed to allow drawing more general conclusions.

Conclusion

This review investigated the association between *OXTR* DNAm and ASD characteristics and related social dimensions, both in individuals with and without ASD. Twelve articles investigating *OXTR* DNAm in relation to ASD, social perception/cognition and social anxiety were included. We found that hypermethylation is associated with (i) higher quantitative autism traits in adults, reflecting a higher incidence of autism characteristics, (ii) increased brain activity while performing social tasks (indicating a higher need for resources) and (iii) decreased functional connectivity. (iv) Contradictory, hypomethylation was found to be present in children with ASD and was also associated with more social anxiety. Generally, this opposite pattern may tentatively be interpreted as follows: *OXTR* hypermethylation inhibits the sensitivity for social cues, with ASD as its extreme, whereas *OXTR* hypomethylation enhances the sensitivity for social cues, with oversensitivity in SAD as its extreme. Speculatively, the initial hypomethylation in children with ASD may underlie their aversive and intrusive experience of social encounters, which they gradually counter by developing a hypermethylated OXTR system. While the included studies displayed a large variability in terms of population characteristics, *OXTR* CpG site, and adopted scales and questionnaires, an initial pattern of results emerged, suggesting an association between hypermethylation of *OXTR* and autism traits in adults. Nonetheless, future studies are warranted to corroborate these initial conclusions.

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Author contributions: Matthijs Moerkerke: Conceptualization, Methodology, Investigation, Validation, Writing - Review & Editing, Visualisation, Project administration; Marie-Laure Bonte: Conceptualization, Methodology, Investigation, Data curation, Writing - Original Draft, Visualisation. Nicky Daniels: Validation, Writing - Review & Editing; Jean Steyaert: Supervision, Validation, Writing - Review & Editing, Funding acquisition; Viktoria Chubar: Validation, Writing - Review & Editing; Kaat Alaerts: Supervision, Validation, Writing- Reviewing and Editing, Funding acquisition; Bart Boets: Supervision, Conceptualization, Methodology, Validation, Writing- Reviewing and Editing, Funding acquisition.

Chapter 4 | Effects of multiple-dose intranasal oxytocin treatment on social responsiveness in children with autism — A randomized, placebo-controlled trial

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Daniels, N., Moerkerke, M. (shared first), Steyaert, J., Bamps, A., Debbaut, E., Prinsen, J., Tang, T., Van der Donck, S., Boets, B., Alaerts, K. (2022). Effects of multiple-dose intranasal oxytocin treatment on social responsiveness in children with autism: A randomized, placebo-controlled trial. Preprint available at https://doi.org/10.1101/2022.04.20.22274106
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Abstract

Background. Intranasal administration of oxytocin is increasingly explored as a new treatment for reducing the core symptoms of autism spectrum disorder (ASD). The efficacy of multiple-dose oxytocin treatment in children with ASD is, however, not well established.

Methods. A double-blind, randomized, placebo-controlled trial was completed including 77 children with ASD (61 boys, 16 girls) aged 8-12 years. Primary outcome was the parent-rated Social Responsiveness Scale (SRS-2). Secondary outcomes included questionnaire-based assessment of repetitive behaviors, anxiety, and attachment. The double-blind phase was followed by a four-week single-blind extension phase during which all participants received oxytocin.

Results. In the double-blind phase, no treatment-specific effects were identified in the primary or secondary outcomes. However, exploratory moderator analysis indicated that children who received oxytocin in combination with concomitant psychosocial treatment displayed greater benefits than those who received oxytocin alone. Furthermore, parents who believed their child received oxytocin reported greater benefit than those who believed their child received placebo. Finally, participants who were first allocated to receive the placebo treatment and later crossed over to receive oxytocin during the single-blind extension phase displayed a significant improvement in social responsiveness, over and above the placebo-induced improvements noted in the first phase.

Conclusions. While no overall treatment-specific improvements were identified, our results provide important indications that clinical efficacy can be augmented when oxytocin administration is paired with targeted psychosocial interventions that similarly stimulate socio-communicative behaviors.

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by impairments in social communication and interaction, combined with restricted and repetitive behaviors and interests (American Psychiatric Association, 2013). Thus far, the means of treatment of ASD's core symptoms are primarily based on behavioral interventions (e.g., stimulation of social communication, lessening the impairment due to restricted and repetitive behaviors), since biomedical or pharmacological therapies targeting social impairment or repetitive behaviors are largely unproven.

In the past decade, intranasal administration of the neuropeptide oxytocin (OT) has been increasingly explored as a new treatment option for reducing ASD's social symptoms (Huang et al., 2021). OT is an endogenous neuropeptide that is mainly produced in paraventricular nuclei of the hypothalamus. In the brain, OT acts as an important neuromodulator for a broad range of affiliative and prosocial behaviors, including interpersonal bonding, social attunement and attachment (M. Bakermans-Kranenburg & van Ijzendoorn, 2013; Bartz et al., 2011; Jurek & Neumann, 2018), presumably mediated through its postulated top-down enhancing effect on 'social salience' and bottom-up effect on regulati(Guastella & Hickie, 2016; Shamay-Tsoory & Abu-Akel, 2016)Shamay-Tsoory & Abu-Akel, 2016).

Following a myriad of single-dose proof-of-principle studies (Alvares et al., 2017; Huang et al., 2021), an initial multiple-dose pilot study assessed the safety and efficacy of six weeks of chronic intranasal OT treatment on core autism symptoms in 19 adults with ASD (10 receiving OT, 9 receiving placebo), and showed improved emotion recognition and quality of life, and tentative improvements in repetitive behaviors after OT treatment (Anagnostou et al., 2012). Later, significant improvements on the Clinical Global Impression-Improvement scale after twelve weeks of OT treatment in adult men with ASD were shown, albeit only in the subgroup of participants receiving the high-dose treatment (32 IU/day; n = 13), and not in the low-dose (16 IU; n = 15) or placebo groups (n = 16) (Kosaka et al., 2016). In an exploratory cross-over study (Watanabe et al., 2015), the effects of six weeks of daily intranasal OT administration on core autism characteristics were studied in 20 adult men with ASD, and significant improvements in social reciprocity and social functioning (social-judgement task) were identified. A confirmatory trial with an identical protocol as in (Watanabe et al., 2015) in 106 adult men with ASD (53 OT / 53

placebo) similarly identified significant improvements in repetitive behaviors, but the effects on social reciprocity and social functioning could not be replicated (Yamasue et al., 2018). These observations were further extended in an exploratory sample of 40 young adult men with ASD (22 OT / 18 placebo), demonstrating long-term improvements in repetitive behaviors and feelings of attachment after a four-week course, with improvements outlasting the period of administration till one year post-treatment (Bernaerts, Boets, Bosmans, et al., 2020).

Given that ASD is an early-onset neurodevelopmental condition, it is important to extend these insights to pediatric populations, allowing evaluations of OT treatment efficacy within an early developmental window and whether it can be facilitatory for enriching social behaviors and experiences from an early age onwards. To date, a handful of trials explored the effects of multiple-dose OT administration in children with ASD. Two initial trials reported a consistent pattern of results, indicating improvements in the social domain (parent-reported social responsiveness) after five weeks of intranasal OT treatment in 3-to-6-year-old children with ASD (n = 31, cross-over; (Yatawara et al., 2016)) and after four weeks of treatment in 6-to-12-year-old children with ASD (14 OT / 18 placebo; (Parker et al., 2017)). No significant improvements on core autism symptoms were demonstrated, however, after an eight-week OT treatment in adolescent boys with ASD (26 OT / 24 placebo, 12-18 years; (Guastella et al., 2015)) or in a preliminary 12-week administration trial encompassing a broad age range of 5-to-17-year-old children (8 OT / 10 placebo) with Phelan-McDermid syndrome (characterized by ASD symptoms; (Fastman et al., 2021)). Also, in a recent confirmatory trial including 3-to-17-year-old children with ASD (139 OT / 138 placebo) and an age-adjusted dosing scheme ranging from 8-80 IU, no improvements on outcomes of social functioning were evident after 24 weeks of OT treatment (Sikich et al., 2021).

Several factors have been put forward to understand these inconsistent results, ranging from heterogeneity in trial design (e.g., parallel versus cross-over design, adopted outcomes, dosing scheme) to variation in participant characteristics. For instance, the well-powered confirmatory trial covered a broad age range (3-17 years) (Sikich et al., 2021), encompassing a critical period of pubertal development, which could have rendered heterogeneity due to differential physiologic effects of OT during different developmental stages (Geschwind, 2021).

Here, results are presented from a single-center, randomized, double-blind, placebo-controlled clinical trial (RCT with parallel design), testing efficacy on improvement in social functioning and safety of multiple-dose OT treatment (four weeks of twice daily intranasal administration of 12 IU) in a representative sample of 8-to-12-year-old children with ASD (40 OT / 40 placebo). Accordingly, this is the largest trial to date, examining OT treatment effects in a relatively strict age range of pre-pubertal, school-aged children, thereby allowing to overcome some of the raised issues regarding sample heterogeneity. Further, following prior observations of long-lasting retention effects of OT treatment in adults with ASD (Bernaerts, Boets, Bosmans, et al., 2020), the current trial also included a follow-up session four weeks after cessation of the daily OT administrations, testing the possibility of crucial retention effects in the current pediatric sample. Finally, possible variations in treatment responses depending on several moderating variables were assessed, examining for example, whether the gender of the participant, medication use, or parental belief about allocated treatment impacted treatment responses. Also the impact of concomitant engagement in ongoing psychosocial treatment was assessed, considering recent notions that the efficacy of OT treatment may be augmented when administered in socially stimulating contexts (Ford & Young, 2021; Le et al., 2022).

Materials and methods

General study design

The RCT assessing the effect of multiple-dose OT treatment in children with ASD was performed at the Leuven University Hospital (Belgium). The double-blind phase (phase I) was followed by a four-week single-blind extension phase (phase II) during which all participants received intranasal OT. In both phases, treatment effects were assessed immediately after the four-week treatment (post) and at a follow-up session, four weeks after cessation of the daily administrations (follow-up). See **Figure 1**, CONSORT Flow diagram for number of participants randomized and analyzed. Written informed consent from the parents and assent from the child were obtained prior to the study. Consent forms and study design were approved by the Ethics Committee for Biomedical Research at the University of Leuven, KU Leuven (S61358) in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The trial was registered at the

European Clinical Trial Registry (Eudract 2018-000769-35) and the Belgian Federal Agency for Medicines and Health products.

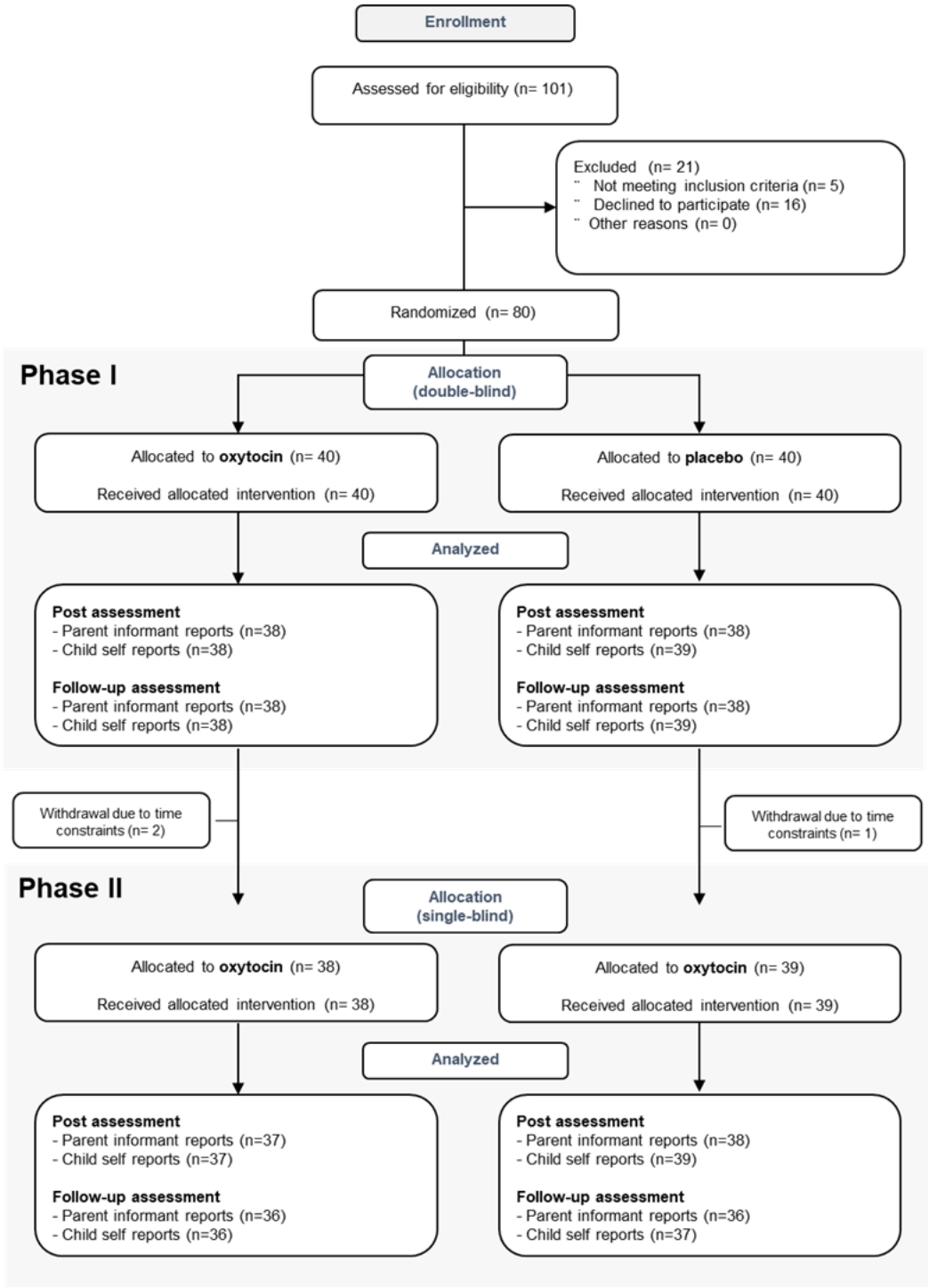

Figure 1. CONSORT flow diagram of participants in the trial. Participants first underwent a double-blind phase (phase I) during which they were allocated to receive either oxytocin or placebo (four weeks of twice daily intranasal administration), followed by a four-week single-blind extension phase (phase II) during which all participants received four weeks of intranasal oxytocin. In both phases, treatment effects were assessed immediately after the four-week treatment (post) and at a follow-up session four weeks after cessation of the daily administrations (follow-up). For each assessment session, completed assessments are indicated separately for parent informant- and child self-reports.

Participants

Children with a formal diagnosis of ASD were recruited through the Autism Expertise Centre at the Leuven University Hospital between July 2019 and January 2021. The diagnosis was established by a multidisciplinary neuropediatric team based on the strict criteria of the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders) (American Psychiatric Association, 2013). Prior to randomization, the Autism Diagnostic Observation Schedule (ADOS-2) (Lord et al., 2012) and estimates of intelligence (four subtests of the Wechsler Intelligence Scale for Children, Fifth Edition, Dutch version) (Wechsler, 2018) were acquired (**Table 1**). The verbal intelligence quotient (IQ) was derived from the subtests Block Design and Figure Puzzles. The performance IQ was derived from the subtests Similarities and Vocabulary.

Inclusion/exclusion criteria. Principal inclusion criteria comprised a clinical diagnosis of ASD, age (8-12 years old), intelligence quotient (IQ) above 70, native Dutch speaker, a stable background treatment for at least four weeks prior to the screening and no anticipated changes during the trial. Only premenstrual girls were included. Principal criteria for exclusion comprised any neurological (e.g., stroke, epilepsy, concussion) or significant physical disorder (liver, renal, cardiac pathology) or prior treatment with OT (see **Supplementary Table 1**).

***Sample size determination*.** A sample size of 40 participants in each treatment group was determined to be able to detect a medium effect size (d = 0.60) with α = 0.05 and 80% power, corresponding to effect sizes previously reported in a four-week oxytocin trial with school-aged children (Parker et al., 2017).

Medication use, comorbidities and concomitant treatment participation. The presence of comorbid psychiatric disorders (with the explicit mentioning of examples in the screening interview including e.g., attention deficit hyperactivity disorder, depression, dyscalculia, dyslexia), current psychoactive medication use (defined as use within four weeks before study enrolment), and concomitant participation in psychosocial therapies were screened through parent-report. Detailed information on comorbidities and medication use is provided in **Supplementary Table 2.** If concomitant participation in psychosocial therapies was present (minimum of one session/month), frequency was determined as number of sessions/month. Reported psychosocial therapies include: Theory of Mind training, emotion recognition training, social skills training, cognitive

behavioral therapy, psychotherapy, self-esteem training, mood regulation, music therapy, hippotherapy and an autism coach.

Table 1. Demographic characteristics of the trial participants, separately for the oxytocin and placebo treatment groups.

	Oxytocin		**Placebo**			
	n	**Mean ± SD**	***n***	**mean ± SD**	***t*-value**	***p*-value**
Age	38	10.5 ± 1.3	39	10.4 ± 1.2	0.3	0.78
Gender	30 M / 8 F		31 M / 8 F			
Handedness	35 R / 3 L		33 R / 6 L			
WISC-V*						
Verbal IQ	37	105.8 ± 14.4	38	109.4 ± 15.8	-1.0	0.31
Performance IQ	38	104.1 ± 15.4	38	101.7 ± 12.8	0.7	0.46
ADOS-2						
Total	33	9.5 ± 3.8	32	9.2 ± 4.2	0.3	0.74
Social Affect	31	7.1 ± 3.6	32	7.5 ± 3.7	-0.4	0.71
Restricted and Repetitive Behavior	31	2.1 ± 1.2	31	1.7 ± 1.3	1.2	0.23
Primary Outcome						
SRS-2	38	89.3 ± 21.7	39	87.9 ± 20.0	0.3	0.77
Secondary outcomes - parent report						
RBS-R	38	27.3 ± 15.2	39	26.6 ± 16.4	0.2	0.86
SCARED parent	38	39.7 ± 21.7	39	45.2 ± 18.3	-1.2	0.24
Secondary outcomes - self report						
SCARED child	38	38.3 ± 21.0	39	39.1 ± 20.2	-0.2	0.87
ASCQ Anxious	38	13.5 ± 5.2	39	12.9 ± 4.2	0.6	0.58
ASCQ Avoidant	38	13.8 ± 4.0	39	14.1 ± 3.8	-0.3	0.75
ASCQ Secure	38	20.0 ± 3.5	39	19.2 ± 2.8	1.0	0.31
Attachment Mother Anxiety	38	4.7 ± 2.9	39	4.8 ± 2.8	-0.1	0.90
Attachment Mother Avoidance	38	9.1 ± 4.7	39	8.0 ± 4.0	1.1	0.29
Attachment Mother Secure	38	16.8 ± 4.2	39	17.9 ± 3.1	-1.3	0.20
					Pearson chi square	***p*-value**
Comorbidity	15		15		0.01	0.927
Psychoactive Medication	22		23		0.01	0.923
Psychosocial Therapy (*n* sessions/month)	15	3.3 ± 1.0	14	3.3 ± 1.1	0.10	0.746

M: male, F: female, R: right, L: left, WISC-V: Wechsler Intelligence Scale for Children, ADOS: Autism Diagnostic Observation Schedule, SRS-2: Social Responsiveness Scale, RBS-R: Repetitive Behavior Scale-Revised, SCARED: Screen for Child Anxiety Related Disorders, ASCQ: Attachment Style Classification Questionnaire. Values printed in bold are significant differences with *p*-values smaller than 0.05.

Intervention

Study medication. Participants were randomized to receive OT (Syntocinon®, Sigma-tau) or placebo nasal sprays, administered in identical blinded amber 10 ml glass bottles with metered pump. The placebo spray consisted of all the ingredients used in the active solution except the OT compound. Nasal spray preparation, packaging, blinding and randomization (permuted-block randomization, RITA software (Pahlke et al., 2004)) was performed by the pharmacy of Heidelberg University Hospital (Germany). Participants were randomly assigned in a 1:1 ratio, with stratification according to gender. During the initial double-blind phase (phase I), all research staff conducting the trial, participants and their parents were blinded to treatment allocation. During the subsequent single-blind extension phase (phase II), experimenters were aware that all participants received intranasal OT, but participants and parents were still fully blinded regarding treatment allocation.

Dosing. Children (assisted by their parents) were asked to self-administer a daily dose of 2 x 12 IU nasal spray or placebo equivalent (3 puffs of 2 IU in each nostril), 12 IU in the morning and 12 IU in the afternoon (similar to the conservative dosing scheme adopted in young children with ASD (Yatawara et al., 2016)). The nasal spray was administered during 28 consecutive days during the initial double-blind phase (phase I), and for another 28 days during the single-blind extension phase (phase II). The duration of four weeks was similar to prior trials in children (Parker et al., 2017) and adults (Bernaerts, Boets, Bosmans, et al., 2020) with ASD. Participants received clear instructions about use of the nasal sprays through a demonstration together with the experimenter (Guastella et al., 2013).

Compliance monitoring. Compliance was assured using a daily medication diary that recorded date and time of administration and the total amount of administered fluid was also monitored.

Side effects. During the course of the treatment, participants were screened for potential adverse events (weekly parent report) or changes in affect and arousal (daily diary by child and parent). Overall, reports of side effects were minimal and not treatment-specific (see **Supplementary Tables 3** and **4**).

Parent reported treatment beliefs. At the end of each trial phase (I and II), parents reported beliefs about treatment allocation via a questionnaire.

Outcome Measures

The primary outcome measure was change from baseline in parent-rated social responsiveness on the Social Responsiveness Scale-Children, second edition (SRS-2) (J. Constantino & Gruber, 2012; Roeyers et al., 2015), which comprises four subscales examining social communication, social awareness, social motivation, social cognition and rigidity/repetitiveness, using a four-point Likert-scale (65 items). Higher scores indicate greater deficit.

Secondary outcome measures included changes from baseline in parent-rated repetitive behaviors (Repetitive Behavior Scale-Revised; RBS-R) (Bodfish et al., 2000), self and parent-rated presence of anxiety symptoms (Screen for Child Anxiety Related Emotional Disorders; SCARED-NL)) (Muris et al., 2007), and changes from baseline in constructs of self-rated attachment towards their mother (Attachment Questionnaire child-report) (Bosmans et al., 2014) and peers (Attachment Style Classification Questionnaire child-report) (Finzi et al., 2000) (**see Table 2** and **Supplementary Table 5**).

All outcomes were assessed five times: (i) at baseline, (ii) immediately after the four-week double-blind treatment (phase I - post); (iii) at a follow-up session, four weeks after cessation of the double-blind treatment (phase I - follow-up); (iv) immediately after the four-week single-blind treatment (phase II - post); and (v) at a follow-up session four weeks after cessation of the single-blind treatment (phase II - follow-up). Post sessions were scheduled approximately 24h after the last administration, follow-up sessions within 28 ± 7 days.

Data Analysis

Analyses were performed using a modified intention-to-treat approach that included all randomized participants who completed the baseline session and at least one post or follow-up session (**Figure 1**, **CONSORT flow-chart**). All statistics were executed with Statistica 14 (Tibco Software Inc.).

First, possible baseline differences on the questionnaires were assessed between randomized treatment groups, indicating no statistically significant differences (**Table 1**).

Next, between-group differences in treatment responses of *phase I (double-blind)* on the primary and secondary outcome measures were assessed, by subjecting change from baseline scores of the post and follow-up session to independent sample t-tests. Cohen's *d* effect sizes (change from $baseline_{OT}$ - change from $baseline_{PLACEBO}$)/pooled SD) are also reported, where 0.2 is indicative of a small effect, 0.5 a medium effect and 0.8 a large effect. Additionally, single-sample *t*-tests were adopted to assess within-group changes (compared to baseline) in the OT and placebo group separately (**Table 2**).

Subsequent exploratory analyses of the primary outcome were performed to investigate the potential influence of possible moderator variables on phase I treatment outcome. To do so, change from baseline scores of the primary outcome (SRS-2) were subjected to general linear models with the within-subject factor 'assessment session' (post, follow-up) and the between-subject factors 'treatment' (OT, placebo) and specific moderator variables. Separate models were constructed to assess the modulating effect of concomitant psychosocial treatments (present, not present); medication use (present, not present; as listed in **Table 1**); gender (boy, girl); and parent reported beliefs (OT, placebo).

To evaluate the effect of phase, within-subject changes from *phase I (double-blind)* to *phase II (single-blind extension)* were assessed, across groups and separately within the OT-first and placebo-first groups. To do so, change from baseline scores of the primary outcome (SRS-2) were subjected to general linear models with the within-subject factors 'assessment session' (post, follow-up) and 'phase' (phase I, phase II).

Finally, to assess whether the magnitude of the observed change from baseline scores at the last session of the trial were reliable for individual participants (more than can be expected by measurement error), the Reliable Change Index (RCI) (N. S. Jacobson et al., 1999) was calculated, based on the test-retest reliability of the adopted Dutch parent-reported SRS scale (Cronbach's alpha = .94) and corresponding standard error of measurement (SEM = baseline SD * SQRT(1 - Cronbach's alpha) = 5.19) using the formula: RCI = 1.96*SEM*SQRT(2) = 14.4. Change scores higher than the RCI-value (14.8) were considered reliable.

Results

Compliance monitoring and parent reported treatment beliefs.

Medication compliance percentage was similar between groups in phase I (OT: 96.75 ± 5.26%; placebo: 96.11 ± 5.29 %; $t(74) = .52$, $p = .603$) and in phase II (OT-first: 94.55 ± 11.69%; placebo-first: 92.98 ± 13.92 %; $t(74) = .53$, $p = .597$). The total amount of administered fluid was also similar in phase I OT (14.86 ± 2.37 ml; placebo: 13.79 ± 2.35 ml; $t(75) = 2.00$, $p = .050$) and phase II (OT-first: 13.72 ± 3.47 ml; placebo-first: 12.83 ± 3.52 ml; $t(74) = 1.10$, $p = .275$). The proportion of parents that believed their child had received the OT treatment in phase I was similar in both treatment arms: 39.5% in the OT group, 35.9% in the placebo group (p = .75). In the OT group 18.4% of parents indicated to 'have no explicit belief' about treatment allocation versus 10.3% in the placebo group. In phase II, during which all participants received the actual OT treatment, 51.9% of the parents believed their child received the OT treatment, 35.1% believed their child received the placebo treatment and 13.0% indicated to 'have no explicit belief'.

Double-blind phase (phase I)

No significant effect of treatment was revealed on parent-reported social responsiveness (SRS-2), either immediately post-treatment ($p = .839$), or at the four-week follow-up session ($p = .626$) (see **Table 2** and **Supplementary Table 6**). Both groups displayed similar significant pre-to-post-treatment improvements in social responsiveness (reduced SRS-2 scores) immediately after treatment (OT: $p = .017$; placebo: $p = .009$) and at the follow-up session (OT: $p = .001$; placebo: $p = .017$). A similar pattern of non-treatment-specific improvements was evident for the secondary outcomes (**Table 2**).

Table 2. *Effects of oxytocin treatment on primary and secondary outcome measures of the double-blind phase I.*

	Within-group										Between-group		
	Oxytocin group					Placebo group							
Outcome measure	***n***	**mean**	**± SD**	***t*-value**	***p*-value**	***n***	**mean**	**± SD**	***t*-value**	***p*-value**	**Cohen's *d***	***t*-value**	***p*-value**
Post assessment Primary Outcome													
SRS-2	38	-4.08	± 10.05	-2.50	**0.017**	38	-4.55	± 10.23	-2.74	**0.009**	0.05	0.20	0.839
Secondary Outcomes -													

parent report													
RBS-R	38	-6.53	± 11.26	-3.57	**<u>0.001</u>**	38	-6.76	± 9.92	-4.20	**<u>0.000</u>**	0.02	0.10	0.923
SCARED Parent	38	-0.47	± 11.61	-0.25	0.803	38	-4.95	± 11.93	-2.56	**<u>0.015</u>**	0.38	1.66	0.102
Secondary Outcomes - self report													
SCARED Child	38	-4.74	± 11.47	-2.55	**<u>0.015</u>**	39	-3.38	± 11.32	-1.87	0.070	-0.12	-0.52	0.604
ASCQ Secure	38	-0.89	± 2.42	-2.27	**0.029**	39	-0.92	± 2.93	-1.97	0.057	0.01	0.05	0.963
ASCQ Anxious	38	-0.92	± 3.24	-1.75	0.088	39	-1.00	± 2.50	-2.50	**<u>0.017</u>**	0.03	0.12	0.905
ASCQ Avoidant	38	-0.29	± 3.73	-0.48	0.636	39	-1.08	± 3.30	-2.04	**0.049**	0.22	0.98	0.330
Attachment Mother Anxiety	38	0.76	± 2.95	1.59	0.120	39	-0.33	± 2.57	-0.81	0.423	0.40	1.74	0.086
Attachment Mother Avoidance	38	-0.21	± 3.18	-0.41	0.686	39	-0.82	± 3.94	-1.30	0.201	0.17	0.75	0.458
Attachment Mother Secure	38	0.24	± 4.18	0.35	0.729	39	-0.18	± 2.55	-0.44	0.663	0.12	0.53	0.598
Follow-up assessment Primary Outcome													
SRS-2	38	-6.76	± 11.19	-3.73	**<u>0.001</u>**	39	-5.38	± 13.42	-2.51	**<u>0.017</u>**	-0.11	-0.49	0.626
Secondary Outcomes - parent report													
RBS-R	38	-4.55	± 10.76	-2.61	**<u>0.013</u>**	39	-4.41	± 8.28	-3.33	**<u>0.002</u>**	-0.01	-0.07	0.948
SCARED Parent	38	-2.92	± 11.53	-1.56	0.127	39	-5.38	± 9.52	-3.53	**<u>0.001</u>**	0.23	1.02	0.309
Secondary Outcomes - self report													
SCARED Child	38	-5.97	± 9.84	-3.74	**<u>0.001</u>**	39	-6.36	± 13.38	-2.97	**<u>0.005</u>**	0.03	0.14	0.886
ASCQ Anxious	38	-1.00	± 3.92	-1.57	0.125	39	-2.38	± 3.75	-3.98	**<u>0.000</u>**	0.36	1.58	0.117
ASCQ Avoidant	38	-0.84	± 4.04	-1.28	0.207	39	-1.46	± 3.09	-2.95	**<u>0.005</u>**	0.17	0.76	0.452
ASCQ Secure	38	-1.37	± 3.34	-2.53	**<u>0.016</u>**	39	-0.82	± 3.78	-1.35	0.184	-0.15	-0.67	0.503
Attachment Mother Anxiety	38	0.84	± 3.11	1.67	0.103	39	0.08	± 3.07	0.16	0.877	0.25	1.09	0.281
Attachment Mother Avoidance	38	-0.61	± 3.89	-0.96	0.343	39	-0.67	± 3.50	-1.19	0.241	0.02	0.07	0.942
Attachment Mother Secure	38	0.66	± 3.93	1.03	0.308	39	0.13	± 2.83	0.28	0.779	0.16	0.68	0.498

Treatment changes are listed as change from baseline scores, separately for the post assessment session (immediately after the four-week treatment) and the follow-up assessment (four weeks after cessation of the treatment). Values printed in bold are significant differences with *p*-values smaller than 0.05, values that are significant after false discovery rate (FDR) correction with $q < 0.05$ (Benjamini and Hochberg, 1995) are <u>underlined</u>. SRS-2: Social Responsiveness Scale, RBS-R: Repetitive Behavior Scale-Revised, SCARED: Screen for Child Anxiety Related Disorders, ASCQ: Attachment Style Classification Questionnaire. For all outcomes, except ASCQ secure and Attachment mother secure, negative change from baseline scores indicate pre-to-post improvement.

Interestingly, exploratory moderator analyses showed a significant interaction between treatment and the presence of **concomitant psychosocial treatment** ($F(1,72) = 6.87$; $p = .011$; **Figure 2**), indicating that, across assessment sessions (post, follow-up), participants who received the OT treatment combined with psychosocial treatment displayed greater benefits compared to children receiving only psychosocial treatment (combined with placebo; $t(26) = 2.40$; $p = .024$) or only the OT treatment (trend: $t(36) = 1.90$; $p = .066$). In children without psychosocial treatment, OT treatment responses were not significantly different from those in the placebo group ($t(46) = 1.40$; $p = .17$). None of the other main or interaction effects (e.g., with assessment session) were significant (all, $p > .05$).

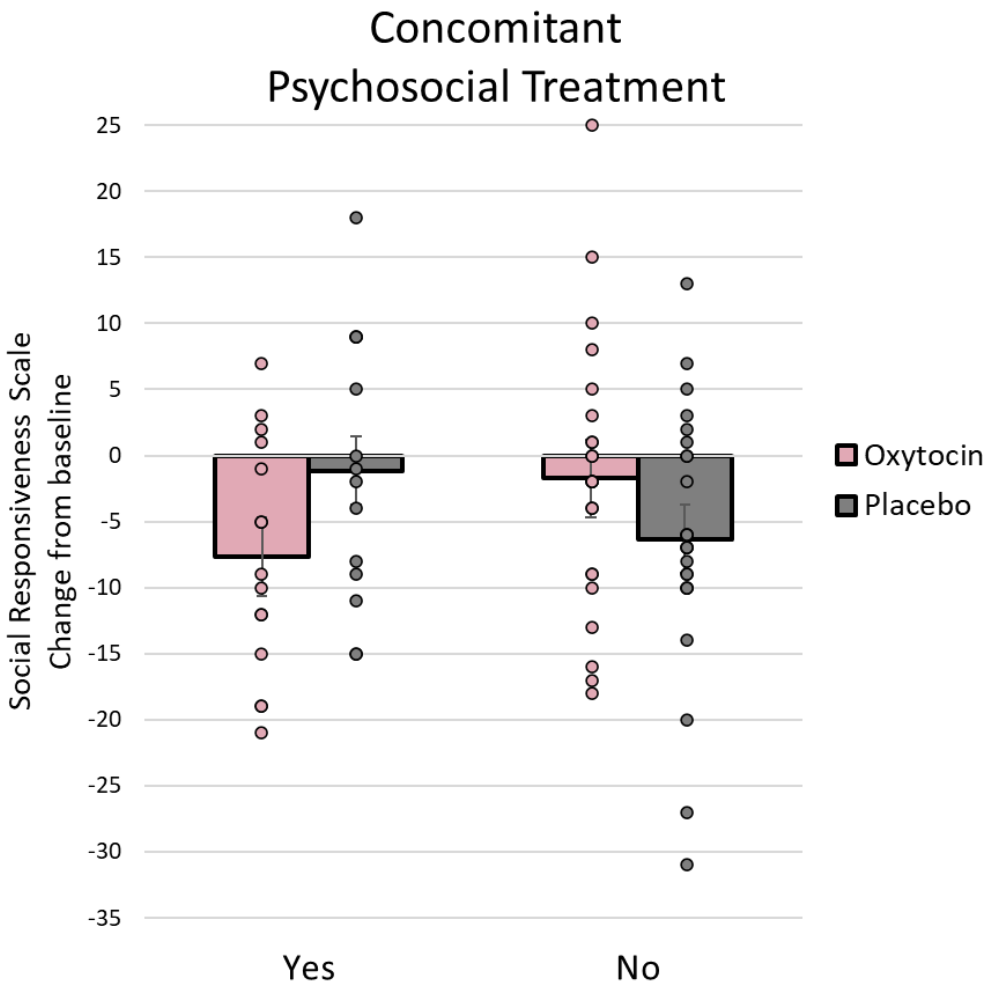

Figure 2. Change in treatment responses according to the presence of concomitant psychosocial treatment. Visualization of changes from baseline in parent-reported social responsiveness (SRS-2) of the double-blind phase (phase I), separately for children receiving only the oxytocin (n = 23) or placebo (n = 25) treatment and children receiving oxytocin (n = 15) or placebo (n = 13) treatment in combination with concomitant psychosocial treatment (pooled across the immediate post and four-week follow-up session). Lower scores indicate improvement. Vertical bars denote ± standard errors.

In terms of modulating effects of **parent reported beliefs**, a trend-level interaction with 'assessment session' was evident ($F(1,61) = 3.22$; $p = .077$), indicating that the parents' own belief about allocated treatment differentially modulated treatment responses at the post and follow-up assessment session (**Figure 3**). Direct exploration of this effect, separately for each treatment group, showed that for participants receiving the actual OT treatment, parents' own belief moderated treatment immediately post-treatment ($t(29) = -3.18$; $p = .002$), but no longer at the follow-up session ($t(29) = -1.02$; $p = .32$). Specifically, parents who believed their child had received OT, reported significantly greater improvements in social responsiveness immediately post-treatment, compared to those who believed their child had received placebo. In the placebo group, no significant modulations of treatment responses were evident, either immediately post-treatment ($t(32) = -.32$; $p = .75$) or at the follow-up session ($t(32) = .37$; $p = .72$). None of the other main or interaction effects were significant (all, $p > .05$). Further, for **concomitant medication use** or **gender**, no significant treatment modulating effects were identified (all, $p > .148$).

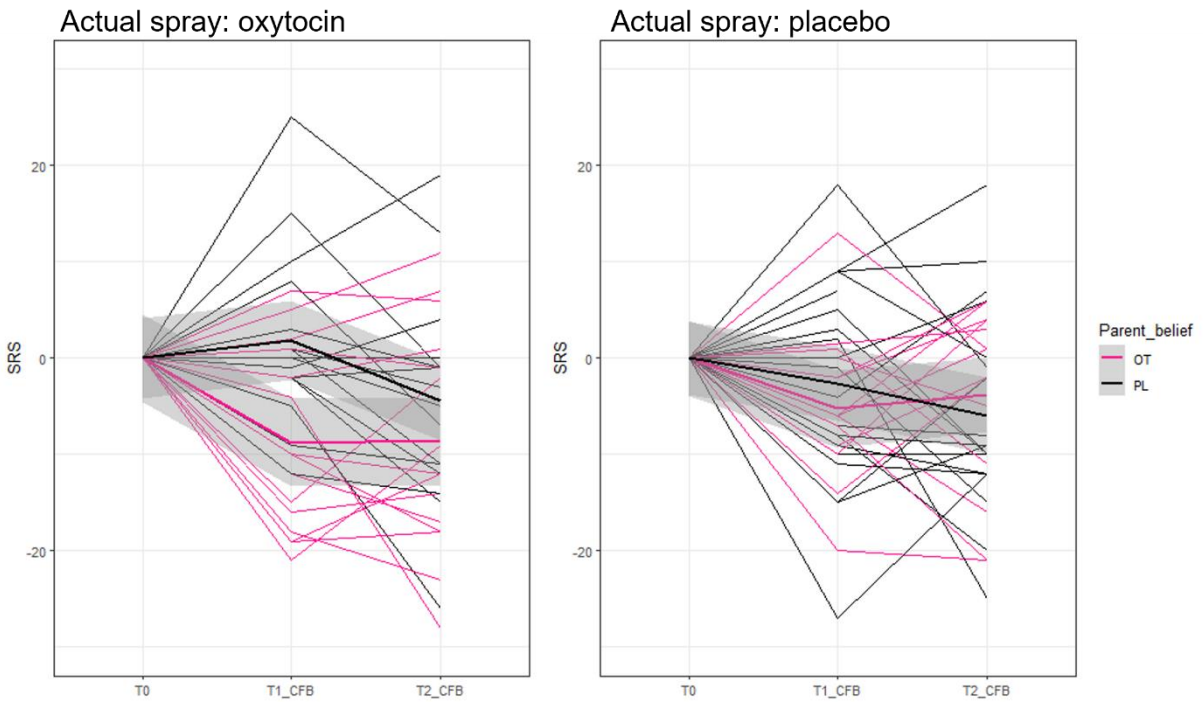

Figure 3. Change in treatment responses according to parent reported beliefs about the allocated treatment. Visualization of changes from baseline (CFB) in parent-reported social responsiveness (SRS) of the double-blind phase (phase I) at the post (T1) and four-week follow-up (T2) session, separately for each treatment group (actual spray: oxytocin or placebo) and according to parent reported beliefs about the allocated treatment (oxytocin or placebo): $oxytocin_{spray}/oxytocin_{belief}$: n = 15; $oxytocin_{spray}/placebo_{belief}$: n = 16; $placebo_{spray}/oxytocin_{belief}$: n = 13; $placebo_{spray}/placebo_{belief}$: n = 21. Lower scores indicate improvement.

Single-blind extension phase (phase II)

Examination of within-subject changes from phase I to phase II yielded a significant effect of phase ($F(1,70) = 12.94$; $p < .001$), but no phase × treatment interaction ($F(1,70) = 2.24$; $p = .14$), indicating a further improvement in social responsiveness across treatment groups from phase I to phase II (**Figure 4**; **Supplementary Table 6** for the raw scores). The main effects of treatment and assessment session were not significant ($p > .05$).

When examined separately for each treatment group, the effect of phase was particularly strong in the placebo-first group ($F(1,35) = 14.54$; $p < .001$), indicating that for children who crossed over from placebo (in phase I) to OT treatment (in phase II), improvements in social responsiveness were significantly more pronounced in phase II, during which the child received the actual OT treatment (**Figure 4**, right panel). Within the OT-first group, only non-significant within-subject changes from phase I to phase II were noted ($F(1,35) = 1.99$; $p = .17$), indicating that OT treatment effects of phase I were not significantly augmented by receiving the additional four-week OT treatment of phase II. Analysis of the OT-first group did reveal a significant effect of session, indicating that irrespective of phase, treatment-related improvements were more pronounced at the follow-up session, compared to the post session ($F(1,35) = 6.11$; $p = .018$), with maximal treatment responses at the last assessment session of the trial (four-week follow-up of phase II; **Figure 4**, left panel).

Accordingly, at the last session of the trial, both the OT-first (receiving a total of eight weeks of OT treatment) and the placebo-first group (receiving a total of four weeks of OT treatment) displayed significant pre-to-post improvements in social responsiveness (OT-first; pre-post change: -9.61 ± 12.18; $t(35) = -4.74$; $p < .001$; placebo-first; pre-post change: -9.81 ± 14.83; $t(35) = -3.97$; $p < .001$). In the OT-first group, 27 (out of 36: 75%) participants displayed a pre-to-post improvement, and this change was identified to be reliable for 12 participants (higher than the Reliable Change Index: > 14.8), and 7 out of these 12 received concomitant psychosocial therapy. Similarly, also in the placebo-first group, 27 (out of 36: 75%) participants displayed a pre-to-post improvement at the last session of the trial, which was reliable for 11 participants, interestingly none of these 11 received concomitant psychosocial therapy.

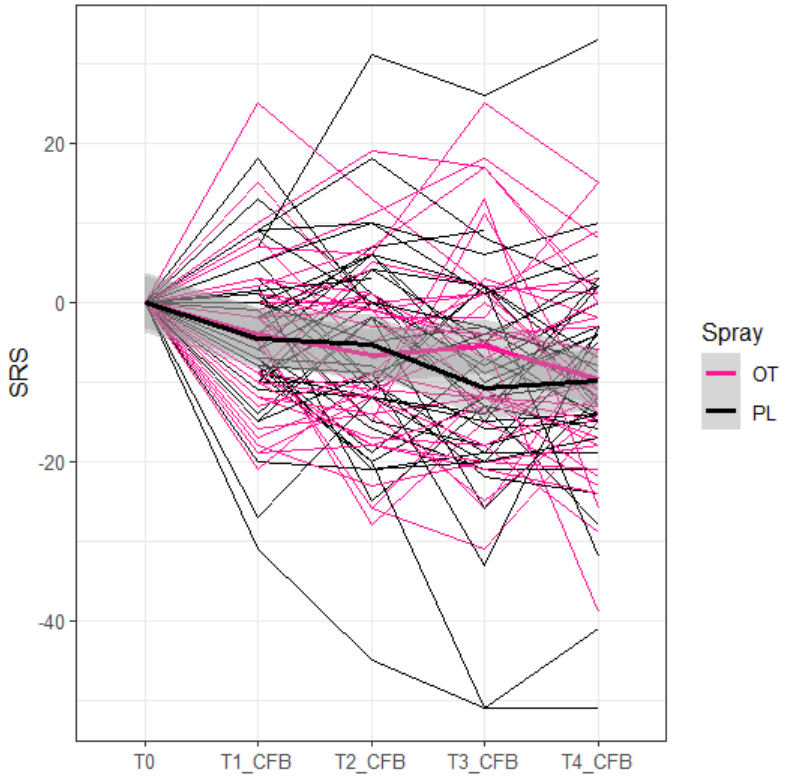

Figure 4. Treatment responses of the complete clinical trial. Visualization of changes from baseline (CFB) in caregiver-reported social responsiveness (SRS) of the double-blind phase (T1-T2, oxytocin, placebo) and the single-blind extension phase (T3-T4, oxytocin-first, placebo-first), separately for each original treatment group (oxytocin or oxytocin first (OT) and placebo or placebo-first (PL)) and assessment session (immediate post (T1 & T3) and four-week follow-up (T2 & T4). Lower scores indicate improvement.

Discussion

The current pediatric trial demonstrated no significant treatment-specific effects of a four-week OT treatment on social responsiveness (SRS-2), nor on the secondary outcomes. Both the OT and the placebo group displayed similar improvements, both immediately after the multiple-dose treatment and at the four-week follow-up session. Notably, exploratory analyses showed that children who received the OT treatment in combination with concomitant psychosocial treatment displayed a greater improvement in social responsiveness than those who received psychosocial treatment or OT alone. A modulating effect of parents' belief about allocated treatment was also identified, indicating that parents who believed their child had been assigned the active treatment reported greater benefit than those who believed their child received placebo, particularly in the experimental group receiving actual OT. Finally, participants who were allocated to receive the placebo treatment during the first double-blind phase of the trial and crossed-over to receive the active treatment during the second (single-blind) phase,

displayed a significant improvement in social responsiveness, over and above the placebo-induced improvement noted in the first phase.

Results of earlier multiple-dose OT trials in children with ASD have been equivocal: some with beneficial outcomes (Parker et al., 2017; Yatawara et al., 2016), others without significant effect (Fastman et al., 2021; Guastella et al., 2015; Sikich et al., 2021). While it is difficult to pinpoint the different factors contributing to variability in study results, several key differences in adopted dosing scheme, trial design, and participant demographics have been put forward as important moderators. Furthermore, the particular context in which the OT treatment is administered is also increasingly put forward as a vital factor for understanding variability in treatment responses within and across studies. Initial single-dose administration studies already noted that acute effects of OT can be modulated by contextual factors, indicating for instance that OT-induced facilitation of cooperation and trust is most pronounced towards in-group members (de Dreu et al., 2010; Mikolajczak et al., 2010). Also, stress-reducing effects of OT were significantly augmented when accompanied by a supportive context (i.e., social support from a friend) (Heinrichs et al., 2003).

Against this background, it has been theorized that OT may open a 'window of opportunity' to enhance prosocial behavior, but that its potential can only be fully realized when OT treatment is administered within a (socially) stimulating context, such as effective concomitant behavioral interventions that can support social skill development and improve prosocial behavior (Ford & Young, 2021; Geschwind, 2021). In line with this notion, exploratory assessments within our study revealed a significant synergetic modulation of treatment outcome related to the presence of **concomitant psychosocial treatment** during the course of the OT trial, indicating maximal treatment effects in children receiving the OT treatment in combination with ongoing psychosocial treatment. Administration of OT as an adjunct to other therapeutic approaches has been explored before. For example, in a study with schizophrenic patients, six-week social cognition training was combined with OT administration, yielding significant improvements in empathic accuracy (Davis et al., 2014). Also, in patients with social anxiety disorders, OT treatment administered as an adjunct to 4 sessions of public speaking-exposure therapy induced significant improvements in mental representations of the self (Guastella et al., 2009). While preliminary, a recent 6-week OT administration study in which parents were stimulated to systematically engage with their child in a positive social interaction

or play session in the first hour after spray administration, yielded unanimously positive treatment outcomes in 46 3-to-8-year-old children with ASD, both in terms of social improvements and repetitive behaviors (Le et al., 2022). Together, these and our study highlight the relevance of context and urge future clinical trials to further elucidate whether clinical efficacy can be augmented when OT administration is paired with targeted behavioral interventions that support similar states and (social) behaviors (Ford & Young, 2021).

Another notable observation was the identification that the **parent's belief about allocated treatment** constituted an important moderator of treatment response, indicating that parents who believed their child had been assigned the active treatment reported greater benefit than those who believed their child received placebo. Notably, the modulation was only significant in the group receiving the actual OT treatment, not in the placebo group, and only for the immediate post-treatment outcome assessment, not for the four-week follow-up assessment. These results therefore only partly concur with a prior OT trial in which moderator effects by parent-belief were evident, both in the actual OT group, as well as in the placebo group (Guastella et al., 2015). One the one hand, the modulation by parents' belief may reflect an expectancy bias, as noted in many prior pediatric studies (Guastella et al., 2015; King et al., 2009). However, since the modulating effect was specific to the OT group, the possibility cannot be ruled out that parents may have actually correctly identified real treatment responders, yielding maximal treatment responses in a particular subgroup of children that displayed actual beneficial effects. Further, in line with the notion that context may constitute an important moderator of treatment, one could also envisage that parents who believed their child received the active treatment, may have provided their children with more active socio-interactive family contexts during the four-week treatment period, i.e., prompting them to increasingly engage in social experiences and learning, thereby effectively boosting treatment responses.

Another important result relates to the observation that children who **crossed over** from placebo (in phase I) to the actual OT treatment (in phase II), showed a significant further improvement in social responsiveness over and above the substantial placebo-induced improvement noted in phase I. Trial designs in which a phase of blinded placebo intervention is administered before actual treatment allocation have been put forward as an effective method to control for placebo effects and to improve detection of 'real'

therapeutic responses (Yatawara et al., 2016). The current observation of a significant further improvement from a blinded placebo phase to the active treatment provides support to this notion.

Further, in our trial, children who received the actual OT treatment in the first phase and crossed over to a second phase of active treatment, showed only non-significant within-subject improvements from phase I to phase II, particularly at the four-week follow-up session of phase II - supporting prior observations of a retention of OT's beneficial effects, also after cessation of the daily nasal spray administrations (Alaerts et al., 2020; Bernaerts, Boets, Bosmans, et al., 2020). It is noted indeed, that at the last follow-up session of the trial, the majority of children of both the OT-first group and the placebo-first group displayed (reliable) beneficial effects in social responsiveness, indicating that both an eight-week (with a four-week break in the middle) or a continual four-week OT treatment were similarly able to induce a significant beneficial outcome on a core ASD symptom domain. This observation adds to the field's uncertainty regarding to-be-administered dosing schemas and durations. In multiple-dose OT trials with individuals with ASD, daily dosing ranged from 8-80 IU and durations from 4 continual days to 24 weeks, but strong empirical support for favoring one dosing scheme over another is currently lacking. Some earlier single-dose trials suggested dose-response curves to exhibit U-shaped forms (Lieberz et al., 2019; Spengler et al., 2017), a notion that is supported by a recent chronic four-week OT administration trial in ASD, identifying a daily total dose of 6 IU of TTA-121 (a new formulation of intranasal OT spray) to be the most efficacious one, compared to a lower (3 IU) or higher (10 IU) daily dose (Yamasue et al., 2022). Furthermore, in terms of dosing scheme, recent work showed that intermittent (every other day) administration may be therapeutically more efficient than continual administration to obtain anxiolytic effects (Kou et al., 2020). These observations were attributed to reflect a desensitization of the endogenous oxytocinergic system upon too high concentrations and/or too high frequencies of exogenous OT administration. The current observation that a single four-week course can yield the same beneficial effects as a twice four-week course therefore reinforces the notion that longer treatment durations do not necessarily facilitate higher treatment responses. Similarly, in a recent large-scale trial administering OT over a 24-week period, it was noted that the long duration might have attenuated initial early responses to OT (Sikich et al., 2021). In light

of these observations, future trials should be directed at identifying the optimal dosing, administration length, and intervals of intranasal OT administration.

While the study provides novel insights into the effects of OT treatment in school-aged children with ASD, the following limitations are noted. First, the current study included a relatively strict age range of pre-pubertal, school-aged children with ASD limiting generalizability to other age ranges. Also, considering the aforementioned uncertainty regarding dosing schemes, it is uncertain whether the identified effects will replicate using differential dosing schemes/durations. Further, the identified modulation by parental belief about allocated treatment on the SRS questionnaire further highlights the need for more objective (biological) outcomes for evaluating treatment efficacy. Finally, considering the identified moderating effect of concomitant psychosocial treatment, future studies are urged to monitor more closely and/or standardized any ongoing behavioral intervention to elucidate its potential for modulating treatment efficacy.

To conclude, while the current study showed no overall treatment-specific improvements, important moderator effects were identified, providing important indications that clinical efficacy can be augmented when OT administration is paired with targeted behavioral interventions.

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Supplementary Material

Supplementary Table 1. Full list of inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Diagnosed with ASD by a multidisciplinary team of experienced clinicians as defined by the DSM-IV-TR or DSM-IV-TR criteria (Diagnostic and Statistical Manual of Mental Disorders).	History of any neurological disorder (stroke, concussion, epilepsy etc).
Age-range of 8 to 12 years old.	Significant hearing or vision impairments.
Premenstrual girls (girls with onset of menstruation during the course of the trial are allowed to continue the treatment).	Active medical problems: unstable seizures, significant physical illness (e.g., serious liver, renal, or cardiac pathology).
Intelligence Quotient above 70 (either full-scaled IQ, verbal IQ or performance IQ).	Regular nasal obstruction or nosebleeds.
Dutch native speaker.	Subjects who have had previous chronic treatment with oxytocin.
Stable background treatment during four weeks prior to screening.	Participation in another Clinical Trial.
No planned changes in psychosocial interventions during the trial.	Known hypersensitivity to active substance or excipients in nasal sprays.
	(Significant) change in background treatments.
	For MR assessment: any contraindication to MRI research (pacemaker, implanted defibrillator, ear implant / a cochlear implant, insulin or implanted pump, a neurostimulator or VP shunt, any metallic object in the eyes (metallic fragments)*.

*As indicated in the registration at the European Clinical Trial Registry (Eudract 2018-000769-35), behavioral data collections were part of a larger project assessing (MRI) neurophysiology and biological outcomes.

Supplementary Table 2. Detailed information on medication use and comorbidities for participants of the oxytocin and placebo treatment groups. Current psychoactive medication use was defined as use within four weeks before study enrollment. Comorbidities were screened through parent-report (with the explicit mentioning of examples in the screening interview including e.g., ADHD, depression, dyscalculia, dyslexia). All participants had a stable background treatment for at least four weeks prior to the treatment allocation and changes in medication regime were screened and logged.

	Oxytocin (n = 38)		Placebo (n = 39)		Pearson Chi square	*p*-value
	no.	(%)	no.	(%)		
Psychoactive Medication						
Antianginal agents	1	(2.5%)	0	(0%)	1.04	0.31
Anticholinergic agent	3	(7.5%)	0	(0%)	3.20	0.07
Anti-depressants	3	(7.5%)	1	(2.5%)	1.11	0.29
Antipsychotics	6	(15%)	7	(17.5%)	0.06	0.80
Sleep Aids	6	(15%)	12	(30%)	2.41	0.12
Stimulants	11	(27.5%)	9	(22.5%)	0.34	0.56
Other Medication						
Allergy and asthma medications	3	(7.5%)	1	(2.5%)	1.11	0.29
Gastrointestinal medications	0	(0%)	2	(5%)	2.00	0.16
Nutritional Supplements	2	(5%)	4	(10%)	0.67	0.41
Statins	1	(2.5%)	0	(0%)	1.04	0.31

Comorbidity					
ADHD	11	(28.9%)	10 (25.6%)	0.11	0.74
DCD	3	(7.5%)	1 (2.5%)	1.11	0.29
Dyslexia	2	(5%)	5 (12.5%)	1.33	0.25
Dysorthography	0	(0%)	1 (2.5%)	0.99	0.32
OCD	1	(2.5%)	0 (0%)	1.04	0.31

ADHD, Attention Deficit Hyperactivity Disorder; DCD, Developmental Coordination Disorder; OCD, Obsessive-Compulsive Disorder.

Side effect screening

Participants (with the help of their parents) were asked to administer the nasal spray (oxytocin or placebo) daily for four consecutive weeks in phase I (double-blind phase) and another four weeks in phase II (single-blind extension phase). At the end of each week, parents were asked to report whether their child presented any of the listed (or other) side effects and to indicate the severity of the side effect (mild, moderate, or severe). Safety analyses included all participants that received the allocated intervention. **Supplementary table 3 - Panel A** lists the proportion of oxytocin or placebo participants (%) that reported any mild, moderate or severe side effects (averaged across effects). P-values correspond to hypothesis tests for difference in proportions between the two treatment groups. Data printed in bold show p-values equal to or smaller than 0.05.

Supplementary table 3 - Panel B lists, separately for each side effect, the proportion of oxytocin (OT) or placebo (PL) participants that reported the side effect (averaged across severity level: mild, moderate, severe).

Supplementary table 3 - Panel C lists any other incidental adverse event, spontaneously reported by the parents.

While on average, no group differences were evident in the total proportion of reported side effects, a group differences was observed in the proportion of participants who reported moderate side effects in the last week of administration, indicating that the participants of the oxytocin group reported a slightly higher number of moderate side effects during that week.

When examined separately for each side effect, a higher proportion of participants of the oxytocin group reported to experience a 'headache' or 'abdominal/stomach pain' during the third week of the phase I treatment. Also a higher proportion of participants of the placebo group were noted to experience a 'sore throat' and to feel 'more confident', compared to the oxytocin group, but only during the first week of the phase I treatment.

In the single-blind phase (phase II), no significant group differences were revealed, either in the total proportion of reported side effects (Panel A) or separately for each side effect (Panel B).

Supplementary Table 3. Side effect screening.

Panel A

Phase I (double-blind)

	Oxytocin (%)			Placebo (%)			Group difference (*p*-value)		
	Mild	**Moderate**	**Severe**	**Mild**	**Moderate**	**Severe**	**Mild**	**Moderate**	**Severe**
Week 1	30.00	27.50	2.50	32.50	15.00	0.00	0.81	0.17	0.31
Week 2	37.50	25.00	2.50	22.50	17.50	0.00	0.14	0.41	0.31
Week 3	22.50	20.00	5.00	22.50	10.00	5.00	1.00	0.21	1.00
Week 4	35.00	20.00	2.50	30.00	5.00	2.50	0.63	**0.04**	1.00
Across weeks	31.25	23.13	3.13	26.88	11.88	1.88	0.67	0.19	0.72

Phase II (single-blind)

	$\text{Oxytocin}_{\text{first}}$ (%)			$\text{Placebo}_{\text{first}}$ (%)			Group difference (*p*-value)		
	Mild	**Moderate**	**Severe**	**Mild**	**Moderate**	**Severe**	**Mild**	**Moderate**	**Severe**
Week 1	34.21	21.05	7.89	23.08	23.08	10.26	0.28	0.83	0.72
Week 2	28.95	23.68	13.16	17.95	17.95	2.56	0.25	0.54	0.08
Week 3	23.68	13.16	5.26	17.95	7.69	2.56	0.54	0.43	0.54
Week 4	34.21	26.32	2.63	35.90	28.21	0.00	0.88	0.85	0.31
Across weeks	30.26	21.05	7.24	23.72	19.23	3.85	0.52	0.84	0.51

Panel B

Phase I (double-blind) (OT *n* = 40; PL *n* = 40)

	Week 1			Week 2			Week 3			Week 4		
	OT (%)	**PL (%)**	***p***	**OT (%)**	**PL (%)**	***p***	**OT (%)**	**PL (%)**	***p***	**OT (%)**	**PL (%)**	***p***
Headache	10.00	12.50	0.72	7.50	7.50	1.00	12.50	0.00	**0.02**	5.00	2.50	0.56
Drowsiness	2.50	2.50	1.00	7.50	5.00	0.64	7.50	0.00	0.08	2.50	0.00	0.31
Dizziness	7.50	2.50	0.30	2.50	2.50	1.00	7.50	0.00	0.08	2.50	0.00	0.31
Fainting	0.00	0.00	1.00	0.00	0.00	1.00	2.50	0.00	0.31	0.00	0.00	1.00
Changes in heart rate or palpitations	0.00	0.00	1.00	2.50	0.00	0.31	2.50	0.00	0.31	0.00	0.00	1.00
Shortness of breath	0.00	0.00	1.00	0.00	0.00	1.00	0.00	2.50	0.31	0.00	0.00	1.00
Fever	0.00	2.50	0.31	0.00	0.00	1.00	0.00	0.00	1.00	2.50	0.00	0.31
Sore throat	0.00	12.50	**0.02**	5.00	7.50	0.64	5.00	0.00	0.15	0.00	0.00	1.00
Dry throat/dry mouth	0.00	5.00	0.15	0.00	0.00	1.00	2.50	0.00	0.31	5.00	0.00	0.15
Hoarseness	0.00	5.00	0.15	0.00	2.50	0.31	0.00	0.00	1.00	2.50	0.00	0.31
Coughing	5.00	5.00	1.00	2.50	2.50	1.00	2.50	0.00	0.31	5.00	0.00	0.15
Coughing up mucus	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Congested nose	10.00	7.50	0.69	5.00	10.00	0.40	10.00	5.00	0.40	5.00	5.00	1.00
Sneezing	2.50	2.50	1.00	0.00	2.50	0.31	7.50	2.50	0.30	7.50	2.50	0.30
Nasal irritation	5.00	5.00	1.00	5.00	0.00	0.15	7.50	2.50	0.30	2.50	0.00	0.31
Runny nose	2.50	2.50	1.00	2.50	2.50	1.00	2.50	0.00	0.31	7.50	0.00	0.08
Burning sensation in nose and/or ears	5.00	0.00	0.15	5.00	0.00	0.15	5.00	0.00	0.15	2.50	0.00	0.31
Sensitive to fragrances	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	2.50	0.00	0.31

Watery eyes	0.00	0.00	1.00	2.50	0.00	0.31	0.00	0.00	1.00	2.50	0.00	0.31
Nausea and/or vomiting	2.50	7.50	0.30	5.00	2.50	0.56	2.50	0.00	0.31	0.00	0.00	1.00
Abdominal or stomach pain	12.50	10.00	0.72	15.00	7.50	0.29	12.50	0.00	**0.02**	7.50	7.50	1.00
Decreased appetite	2.50	5.00	0.56	2.50	2.50	1.00	5.00	0.00	0.15	2.50	0.00	0.31
Hungry or increased appetite	0.00	2.50	0.31	0.00	2.50	0.31	2.50	0.00	0.31	0.00	0.00	1.00
Constipation	7.50	2.50	0.30	5.00	0.00	0.15	7.50	0.00	0.08	7.50	2.50	0.30
Diarrhea	0.00	2.50	0.31	2.50	2.50	1.00	2.50	0.00	0.31	0.00	0.00	1.00
Muscle pain/cramps	5.00	0.00	0.15	5.00	0.00	0.15	5.00	0.00	0.15	5.00	2.50	0.56
Skin rash	2.50	0.00	0.31	2.50	0.00	0.31	0.00	0.00	1.00	5.00	0.00	0.15
Increased fluid intake	0.00	0.00	1.00	0.00	0.00	1.00	2.50	0.00	0.31	0.00	0.00	1.00
Water retention/bloating	0.00	0.00	1.00	2.50	0.00	0.31	0.00	0.00	1.00	2.50	0.00	0.31
Insomnia/sleep difficulties	5.00	0.00	0.15	5.00	5.00	1.00	5.00	0.00	0.15	0.00	5.00	0.15
Nightmares	2.50	0.00	0.31	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Staring/daydreams	0.00	0.00	1.00	2.50	0.00	0.31	0.00	0.00	1.00	2.50	0.00	0.31
Anaphylaxis	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Changes in perception of the tongue	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Back pain	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Bed wetting	0.00	2.50	0.31	0.00	5.00	0.15	0.00	0.00	1.00	0.00	0.00	1.00
Weight gain	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Sweating	0.00	0.00	1.00	2.50	0.00	0.31	0.00	0.00	1.00	0.00	0.00	1.00
Blurred vision	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Less talk to others	0.00	0.00	1.00	0.00	2.50	0.31	2.50	0.00	0.31	2.50	2.50	1.00
Uninterested in others	0.00	0.00	1.00	0.00	2.50	0.31	0.00	0.00	1.00	2.50	0.00	0.31
Persistent thoughts and/or feelings	2.50	7.50	0.30	0.00	5.00	0.15	2.50	2.50	1.00	0.00	2.50	0.31
Development of repetitive behavior	0.00	0.00	1.00	2.50	0.00	0.31	0.00	2.50	0.31	0.00	0.00	1.00
Increase in repetitive behavior	2.50	0.00	0.31	2.50	0.00	0.31	0.00	2.50	0.31	0.00	0.00	1.00
Nail biting	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	2.50	0.00	0.31
Irritability or Anger	0.00	7.50	0.08	2.50	7.50	0.30	7.50	5.00	0.64	10.00	7.50	0.69
Sad	2.50	0.00	0.31	0.00	7.50	0.08	2.50	5.00	0.56	5.00	5.00	1.00
Prone to crying or more emotional	2.50	5.00	0.56	2.50	12.50	0.09	7.50	12.50	0.46	5.00	7.50	0.64
Anxious, worried or discomfort	0.00	0.00	1.00	5.00	0.00	0.15	5.00	2.50	0.56	2.50	2.50	1.00
Happy or satisfied	10.00	25.00	0.08	7.50	15.00	0.29	17.50	15.00	0.76	7.50	12.50	0.46
Euphoric or unusually happy	5.00	10.00	0.40	7.50	7.50	1.00	7.50	5.00	0.64	10.00	2.50	0.17
Calm, relaxed or comfortable	10.00	25.00	0.08	15.00	17.50	0.76	12.50	22.50	0.24	7.50	10.00	0.69
More focused	0.00	2.50	0.31	0.00	5.00	0.15	2.50	2.50	1.00	0.00	2.50	0.31
More confidence	0.00	12.50	**0.02**	7.50	7.50	1.00	7.50	5.00	0.64	5.00	7.50	0.64

Phase II (single-blind)
(OT_{first} n = 38; PL_{first} n = 39)

	Week 1			**Week 2**			**Week 3**			**Week 4**		
	OT_{first} (%)	**PL_{first} (%)**	***p***	**OT_{first} (%)**	**PL_{first} (%)**	***p***	**OT_{first} (%)**	**PL_{first} (%)**	***p***	**OT_{first} (%)**	**PL_{first} (%)**	***P***
Headache	5.26	10.26	0.41	2.63	7.69	0.32	10.53	5.13	0.38	5.26	2.56	0.54
Drowsiness	2.63	0.00	0.31	2.63	2.56	0.99	0.00	0.00	1.00	0.00	0.00	1.00
Dizziness	0.00	2.56	0.32	0.00	2.56	0.32	0.00	0.00	1.00	0.00	0.00	1.00
Fainting	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00

Changes in heart rate or palpitations	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	5.26	0.00	0.15
Shortness of breath	0.00	0.00	1.00	0.00	0.00	1.00	0.00	2.56	0.32	0.00	0.00	1.00
Fever	0.00	2.56	0.32	0.00	2.56	0.32	0.00	0.00	1.00	0.00	2.56	0.32
Sore throat	2.63	5.13	0.57	2.63	7.69	0.32	5.26	5.13	0.98	5.26	0.00	0.15
Dry throat/dry mouth	0.00	2.56	0.32	7.89	2.56	0.29	5.26	2.56	0.54	5.26	2.56	0.54
Hoarseness	0.00	0.00	1.00	2.63	0.00	0.31	2.63	2.56	0.99	2.63	0.00	0.31
Coughing	2.63	2.56	0.99	2.63	0.00	0.31	2.63	2.56	0.99	5.26	0.00	0.15
Coughing up mucus	0.00	0.00	1.00	2.63	0.00	0.31	2.63	0.00	0.31	0.00	0.00	1.00
Congested nose	2.63	5.13	0.57	5.26	5.13	0.98	5.26	7.69	0.67	7.89	0.00	0.07
Sneezing	0.00	0.00	1.00	0.00	2.56	0.32	0.00	2.56	0.32	2.63	0.00	0.31
Nasal irritation	2.63	5.13	0.57	0.00	0.00	1.00	5.26	2.56	0.54	7.89	0.00	0.07
Runny nose	2.63	0.00	0.31	0.00	2.56	0.32	2.63	5.13	0.57	0.00	5.13	0.16
Burning sensation in nose and/or ears	0.00	2.56	0.32	0.00	0.00	1.00	2.63	0.00	0.31	0.00	0.00	1.00
Sensitive to fragrances	0.00	0.00	1.00	2.63	2.56	0.99	2.63	0.00	0.31	2.63	0.00	0.31
Watery eyes	0.00	0.00	1.00	0.00	0.00	1.00	2.63	0.00	0.31	2.63	0.00	0.31
Nausea and/or vomiting	2.63	0.00	0.31	5.26	0.00	0.15	2.63	2.56	0.99	2.63	0.00	0.31
Abdominal or stomach pain	5.26	2.56	0.54	13.16	7.69	0.43	5.26	2.56	0.54	2.63	2.56	0.99
Decreased appetite	2.63	0.00	0.31	7.89	7.69	0.97	2.63	5.13	0.57	2.63	2.56	0.99
Hungry or increased appetite	0.00	0.00	1.00	2.63	2.56	0.99	0.00	0.00	1.00	0.00	0.00	1.00
Constipation	0.00	0.00	1.00	2.63	0.00	0.31	0.00	0.00	1.00	0.00	0.00	1.00
Diarrhea	5.26	0.00	0.15	5.26	0.00	0.15	0.00	0.00	1.00	0.00	0.00	1.00
Muscle pain/cramps	7.89	0.00	0.07	2.63	5.13	0.57	2.63	0.00	0.31	5.26	0.00	0.15
Skin rash	2.63	0.00	0.31	5.26	0.00	0.15	2.63	0.00	0.31	0.00	2.56	0.32
Increased fluid intake	0.00	2.56	0.32	2.63	0.00	0.31	5.26	2.56	0.54	2.63	0.00	0.31
Water retention/bloating	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Insomnia/sleep difficulties	0.00	0.00	1.00	0.00	2.56	0.32	2.63	2.56	0.99	5.26	2.56	0.54
Nightmares	2.63	0.00	0.31	2.63	0.00	0.31	0.00	0.00	1.00	0.00	0.00	1.00
Staring/daydreams	2.63	0.00	0.31	2.63	2.56	0.99	0.00	0.00	1.00	0.00	0.00	1.00
Anaphylaxis	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Changes in perception of the tongue	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Back pain	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Bed wetting	0.00	0.00	1.00	2.63	0.00	0.31	0.00	0.00	1.00	0.00	0.00	1.00
Weight gain	0.00	0.00	1.00	0.00	2.56	0.32	0.00	0.00	1.00	0.00	0.00	1.00
Sweating	0.00	0.00	1.00	2.63	0.00	0.31	2.63	2.56	0.99	2.63	2.56	0.99
Blurred vision	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Less talk to others	0.00	0.00	1.00	0.00	7.69	0.08	0.00	2.56	0.32	2.63	2.56	0.99
Uninterested in others	0.00	0.00	1.00	0.00	2.56	0.32	2.63	0.00	0.31	2.63	0.00	0.31
Persistent thoughts and/or feelings	0.00	2.56	0.32	2.63	5.13	0.57	2.63	0.00	0.31	5.26	0.00	0.15
Development of repetitive behavior	5.26	0.00	0.15	0.00	0.00	1.00	0.00	2.56	0.32	0.00	0.00	1.00
Increase in repetitive behavior	0.00	0.00	1.00	2.63	2.56	0.99	0.00	0.00	1.00	0.00	0.00	1.00
Nail biting	2.63	0.00	0.31	2.63	0.00	0.31	0.00	0.00	1.00	0.00	2.56	0.32
Irritability or Anger	2.63	2.56	0.99	7.89	10.26	0.72	5.26	5.13	0.98	5.26	0.00	0.15
Sad	2.63	0.00	0.31	7.89	5.13	0.62	2.63	0.00	0.31	0.00	0.00	1.00
Prone to crying or more emotional	2.63	2.56	0.99	13.16	2.56	0.08	7.89	0.00	0.07	2.63	5.13	0.57
Anxious, worried or discomfort	2.63	0.00	0.31	5.26	2.56	0.54	5.26	0.00	0.15	2.63	0.00	0.31

Happy or satisfied	21.05	12.82	0.33	15.79	12.82	0.71	7.89	7.69	0.97	5.26	7.69	0.67
Euphoric or unusually happy	5.26	2.56	0.54	5.26	0.00	0.15	2.63	2.56	0.99	2.63	0.00	0.31
Calm, relaxed or comfortable	18.42	15.38	0.72	13.16	12.82	0.96	10.53	10.26	0.97	7.89	12.82	0.48
More focused	5.26	0.00	0.15	5.26	5.13	0.98	0.00	5.13	0.16	0.00	0.00	1.00
More confidence	7.89	5.13	0.62	7.89	5.13	0.62	0.00	7.69	0.08	0.00	7.69	0.08

Panel C	**Phase I (double-blind)**								**Phase II (single-blind)**							
	Week 1		**Week 2**		**Week 3**		**Week 4**		**Week 1**		**Week 2**		**Week 3**		**Week 4**	
Number of other incidental events in spontaneous reports	**OT**	**PL**	**OT**	**PL**	**OT**	**PL**	**OT**	**PL**	**OT_{first}**	**PL_{first}**	**OT_{first}**	**PL_{first}**	**OT_{first}**	**PL_{first}**	**OT_{first}**	**PL_{first}**
Nose bleeding	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0
Fracture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Earache	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Inappropriate Affect	0	1	0	0	0	1	0	1	0	1	0	1	1	0	1	1
Mood swings	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Less focussed	1	0	0	0	0	0	1	0	3	0	2	0	0	0	0	0
Fecal incontinence	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Feeling cold	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Daily screenings of changes in affect and arousal.

Both participants and their parents completed a structured daily diary for four consecutive weeks in phase I (double-blind phase) and another four weeks in phase II (single-blind extension phase). Once daily, parents were asked to complete two 9-point Manikin rating scales, rating their child's perceived arousal (1= calm, 9= excited) and valence (1= feeling pleasant/happy, 9= feeling unpleasant/unhappy; (Bradley & Lang, 1994)). Children also completed a self-report of the arousal and valence scale, twice-daily, once at noon and once in the evening. See **supplementary Table 4** for full results.

To examine treatment-related differences in the daily screenings, weekly averages were calculated for each rating and subjected to a general linear model with the within-subject factor 'week' (week 1-4) and the between-subject factors 'treatment' (oxytocin, placebo).

None of the scales yielded a significant main effect of 'treatment' **across weeks** (all, $p > .05$), indicating no overall significant group differences in ratings of valence or arousal, either by the parents or by the child, both in phase I and phase II.

Closer analysis of treatment effects **separately for each week** revealed a significant group difference in child-ratings of valence (in the evening), indicating higher feelings of

'unpleasantness' in the oxytocin, compared to the placebo group during the second week of the phase I treatment. Also in the first week of phase II, a similar effect was noted, indicating that children who crossed over from the placebo treatment (in phase I) to the oxytocin treatment (in phase II) (placebo-first group) reported slightly higher feelings of 'unpleasantness', compared to children who were receiving their second course of oxytocin treatment (oxytocin-first group).

In the table below, weekly averages (and average responses across the four weeks) are reported separately for each treatment group (oxytocin, placebo) and phase (phase I and II). P-values correspond to independent-sample t-tests (or F-tests) assessing between-group differences in ratings of arousal and valence. Data printed in bold show p-values equal to or larger than 0.05.

Notably, for several of the scales, the general linear model also revealed a main effect of 'week', indicating that across treatment groups, both parents and children reported improvements in reports of valence (more pleasant) and arousal (more calme) from the first to the last week of the trial, particularly in phase II, when all children received the 'actual' oxytocin treatment (phase II, parent-reported valence: $F(3,216) = 4.14$; $p = .007$; child-reported valence (noon & evening): $F(3,213) > 3.09$; $p < .028$; child-reported arousal (noon & evening) $F(3,213) > 6.88$; $p < .001$). Also in phase I, an overall effect of 'week' was evident in terms of child-reported valence (only at noon) ($F(3,195) = 5.62$; $p = .001$).

In addition to the ratings of arousal and valence, parents were also asked to indicate how they experienced the interaction with their child while completing the daily diary together, using a 5-point scale (1 = unpleasant/difficult; 5 = pleasant/easy). Generally, child-parent interactions while completing the daily diaries were experienced to be overall 'pleasant/easy', with slightly more pleasant experiences of the child-parent interaction in the oxytocin group, compared to the placebo group in phase I of the trial (oxytocin (n = 38): 4.32 ± 0.47; placebo (n = 36): 4.06 ± 0.58; $t(72) = 2.26$, $p = .027$). Differences in the experiences of the child-parent interaction were no longer evident in phase II of the trial, when all children received the actual oxytocin treatment (oxytocin-first (n = 36): 4.14 ± 0.73; placebo-first (n = 37): 3.84 ± 0.84; $t(71) = 1.62$, $p = .11$). Generally, these overall positive ratings provide an indication that the completion of the daily diaries was well-tolerated, both by the children and their parents.

Supplementary Table 4. Daily screenings of changes in affect and arousal.

	Oxytocin				Placebo					$Oxytocin_{first}$				$Placebo_{first}$				
	N	Mean	±	SD	N	Mean	±	SD	*p*-value	N	Mean	±	SD	N	Mean	±	SD	*p*-value
Phase I (double-blind)	**Week 1**									**Week 2**								
Informant report																		
Valence	37	3.10	±	0.92	36	3.03	±	1.17	0.780	37	3.23	±	1.04	36	2.96	±	1.32	0.323
Arousal	37	3.70	±	1.13	36	3.64	±	1.24	0.831	37	3.93	±	1.45	36	3.55	±	1.36	0.246
Self report																		
Valence - noon	36	2.71	±	1.15	35	2.68	±	1.19	0.905	36	2.78	±	1.25	35	2.31	±	1.20	0.109
Arousal - noon	36	3.30	±	1.20	35	3.20	±	1.25	0.749	36	3.58	±	1.66	35	3.00	±	1.53	0.135
Valence - evening	36	2.69	±	1.15	35	2.70	±	1.13	0.957	36	3.01	±	1.28	35	2.32	±	1.15	**0.019**
Arousal - evening	36	3.47	±	1.29	35	3.16	±	1.07	0.279	36	3.70	±	1.49	35	3.04	±	1.38	0.057
	Week 3									**Week 4**								
Informant report																		
Valence	37	3.19	±	1.09	36	2.95	±	1.08	0.345	35	3.11	±	0.81	36	2.89	±	1.26	0.383
Arousal	37	3.86	±	1.38	36	3.32	±	1.31	0.092	35	3.79	±	1.35	36	3.23	±	1.31	0.081
Self report																		
Valence - noon	36	2.47	±	1.15	34	2.25	±	1.15	0.434	34	2.51	±	1.09	34	2.23	±	1.17	0.306
Arousal - noon	36	3.21	±	1.47	34	3.01	±	1.67	0.588	34	3.03	±	1.37	34	2.91	±	1.42	0.721
Valence - evening	36	2.73	±	1.11	34	2.39	±	1.27	0.230	34	2.72	±	1.18	34	2.51	±	1.25	0.467
Arousal - evening	36	3.26	±	1.33	34	3.17	±	1.73	0.801	34	3.35	±	1.23	34	3.16	±	1.65	0.607
Phase II (single-blind)	**Week 1**									**Week 2**								
Informant report																		
Valence	36	3.74	±	1.54	38	3.55	±	1.23	0.565	36	3.55	±	1.55	38	3.32	±	1.11	0.461
Arousal	36	3.05	±	1.03	38	3.30	±	1.36	0.366	36	2.79	±	0.96	38	2.99	±	1.27	0.446
Self report																		
Valence - noon	37	2.36	±	1.11	39	2.67	±	1.32	0.280	35	2.02	±	0.94	38	2.41	±	1.24	0.139
Arousal - noon	37	3.07	±	1.51	39	3.27	±	1.57	0.578	35	2.92	±	1.71	38	2.89	±	1.40	0.922
Valence - evening	37	2.25	±	1.07	39	2.92	±	1.32	**0.017**	35	2.32	±	1.04	38	2.53	±	1.21	0.441
Arousal - evening	37	3.10	±	1.67	39	3.49	±	1.48	0.283	35	3.20	±	1.69	38	2.94	±	1.37	0.477
	Week 3									**Week 4**								
Informant report																		
Valence	37	3.83	±	1.97	39	3.23	±	1.41	0.124	37	3.63	±	1.86	39	3.13	±	1.33	0.179
Arousal	37	3.03	±	1.24	39	2.80	±	1.19	0.422	37	2.89	±	1.28	39	2.79	±	1.15	0.724
Self report																		
Valence - noon	36	2.03	±	1.04	38	2.29	±	1.08	0.286	36	2.03	±	0.98	38	2.08	±	0.95	0.801
Arousal - noon	36	2.80	±	1.68	38	2.62	±	1.37	0.618	36	2.85	±	1.74	38	2.57	±	1.43	0.450
Valence - evening	36	2.20	±	0.99	38	2.37	±	1.15	0.504	36	2.18	±	1.05	38	2.34	±	1.10	0.533
Arousal - evening	36	2.96	±	1.77	38	2.52	±	1.24	0.226	36	3.07	±	1.79	38	2.79	±	1.45	0.462
Informant report	37	3.83	±	1.97	39	3.23	±	1.41	0.124	37	3.63	±	1.86	39	3.13	±	1.33	0.179
Across weeks																		
Informant report	**Phase I (double-blind)**									**Phase II (single-blind)**								
Valence	37	3.17	±	0.70	36	2.96	±	1.00	0.302	36	3.69	±	1.60	38	3.26	±	1.00	0.163
Arousal	37	3.83	±	1.15	36	3.43	±	1.06	0.131	36	2.94	±	0.95	38	2.93	±	1.05	0.969
Self report																		
Valence - noon	36	2.62	±	0.98	35	2.36	±	1.10	0.282	37	2.18	±	1.02	39	2.42	±	1.09	0.333
Arousal - noon	36	2.79	±	1.02	35	2.46	±	1.02	0.175	37	2.24	±	0.86	39	2.59	±	1.06	0.120
Valence - evening	36	3.29	±	1.23	35	3.01	±	1.29	0.355	37	2.92	±	1.54	39	2.88	±	1.38	0.917
Arousal - evening	36	3.45	±	1.14	35	3.11	±	1.28	0.247	37	3.06	±	1.62	39	2.99	±	1.24	0.818

Valence scale: Higher scores denote feeling more unpleasant/unhappy ((1= feeling pleasant/happy, 9= feeling unpleasant/unhappy). **Arousal scale:** Higher scores denote feeling more excited (1= calm, 9= excited).

Supplementary Table 5. Detailed description of questionnaires adopted as descriptive, primary or secondary outcomes.

Outcome measures	Construct	Type of outcome	Type of report	Number of items	Range of scores	Rating scale (points)	Meaning of higher scores	Reference
Autism Diagnostic Observation Schedule (ADOS-2)	Symptom severity	Descriptive	Observation	Per module	1-10	-	More severe symptoms of autism spectrum disorder	Lord et al., 2012
Wechsler Intelligence Scale for Children (WISC-V-NL)	Verbal Intelligence Quotient	Descriptive	Observation	Similarities Vocabulary	40 - 145	-	Higher verbal abilities	Wechsler, 2018
	Performance Intelligence Quotient			Block Design Visual Puzzles	40 - 145	-	Higher visual spatial abilities	
Social Responsiveness Scale-Children (SRS-2)	Symptom severity	Primary	Parent	65	0 - 192	0-3	Greater deficits in social responsiveness	Constantino & Gruber, 2012; Roeyers et al., 2015
Repetitive Behavior Scale-Revised (RBS-R)	Repetitive behavior	Secondary	Parent	43	0 - 129	0-3	More severe repetitive behavior	Bodfish et al., 2000; Lam & Aman, 2007
Screen for Child Anxiety Related Emotional Disorders (SCARED-NL)	Anxiety	Secondary	Parent and self	69	0 - 207	0-2	Higher risk for anxiety disorders	Muris et al., 2007
Attachment Style Classification Questionnaire (ASCQ) **Anxious** **Avoidant** **Secure**	Attachment	Secondary	Self	15	Per subscale 5 - 25	1-5	More anxious, avoidant or secure attachment toward their peers	Finzi et al., 2000

Attachment questionnaire **Anxious** **Avoidant** **Secure**	Attachment	Secondary	Self	9	Per subscale 3 - 21	1-7	More anxiety, avoidance or trust toward their mother	Bosmans et al., 2014

Supplementary Table 6. Mean questionnaire scores for each treatment group and assessment session. For each questionnaire, raw mean scores and standard deviations are listed separately for each treatment group (oxytocin, placebo) and each assessment session (baseline, post, follow-up) of each phase (phase I and II).

	Phase I (double-blind)															
	Baseline						**Post**						**Follow-up**			
	Oxytocin			**Placebo**			**Oxytocin**			**Placebo**			**Oxytocin**			**Placebo**		
Outcome Measure	**N**	**Mean**	**± SD**	**N**	**Mean**	**± SD**	**N**	**Mean**	**± SD**	**N**	**Mean**	**± SD**	**N**	**Mean**	**± SD**	**N**	**Mean**	**± SD**
Primary Outcome																		
SRS informant based	38	89.3	± 21.7	39	87.8	± 20.0	38	85.2	± 23.6	38	83.7	± 21.9	38	82.5	± 25.6	39	82.5	± 23.9
Secondary Outcomes - informant report																		
RBS	38	27.3	± 15.2	39	26.6	± 16.4	38	20.8	± 13.3	38	19.5	± 14.7	38	22.7	± 14.2	39	22.2	± 16.7
SCARED Parent	38	39.7	± 21.7	39	45.2	± 18.3	38	39.3	± 21.5	38	40.2	± 18.3	38	36.8	± 21.0	39	39.8	± 17.7
Secondary Outcomes - self report																		
SCARED Child	38	38.3	± 21.0	39	39.1	± 20.2	38	33.6	± 20.7	39	35.7	± 21.2	38	32.3	± 20.6	39	32.7	± 17.9
ASCQ Secure	38	20.0	± 3.5	39	19.2	± 2.8	38	19.1	± 3.2	39	18.3	± 3.4	38	18.6	± 3.4	39	18.4	± 3.8
ASCQ Anxious	38	13.5	± 5.2	39	12.9	± 4.2	38	12.5	± 5.0	39	11.9	± 3.8	38	12.5	± 5.1	39	10.5	± 3.4
ASCQ Avoidant	38	13.8	± 4.0	39	14.1	± 3.9	38	13.5	± 3.9	39	13.0	± 4.0	38	13.0	± 4.4	39	12.6	± 3.8
Attachment Mother Anxiety	38	4.7	± 2.9	39	4.8	± 2.8	38	5.5	± 2.7	39	4.5	± 2.0	38	5.6	± 2.9	39	4.9	± 2.3
Attachment Mother Avoidance	38	9.1	± 4.8	39	8.0	± 4.0	38	8.8	± 5.0	39	7.2	± 2.8	38	8.5	± 4.7	39	7.3	± 3.3
Attachment Mother Secure	38	16.7	± 4.2	39	17.9	± 3.1	38	17.0	± 3.6	39	17.7	± 3.4	38	17.4	± 3.6	39	18.0	± 2.9

	Phase II (single-blind)											
	Post						**Follow-up**					
	Oxytocin$_{first}$			**Placebo$_{first}$**			**Oxytocin$_{first}$**			**Placebo$_{first}$**		
Outcome Measure	**N**	**Mean**	**± SD**	**N**	**Mean**	**± SD**	**N**	**Mean**	**± SD**	**N**	**Mean**	**± SD**
Primary Outcome												
SRS informant based	37	83.8	± 25.1	38	77.4	± 22.8	36	79.2	± 24.3	36	78.1	± 23.8
Secondary Outcomes - informant report												
RBS	37	20.6	± 16.5	38	19.1	± 16.7	36	19.6	± 15.6	36	17.1	± 12.8
SCARED Parent	37	37.4	± 22.6	38	37.9	± 19.0	36	33.8	± 21.9	36	35.8	± 17.2
Secondary Outcomes - self report												

SCARED Child	37	30.4 ± 20.5	39	31.8 ± 21.1	36	29.6 ± 20.4	37	29.2 ± 17.3
ASCQ Secure	37	18.3 ± 3.5	39	18.4 ± 3.9	36	18.2 ± 2.8	37	18.2 ± 3.4
ASCQ Anxious	37	12.2 ± 5.4	39	10.4 ± 3.8	36	11.8 ± 4.8	37	10.9 ± 4.2
ASCQ Avoidant	37	12.5 ± 3.6	39	12.8 ± 4.2	36	12.6 ± 3.9	37	13.2 ± 4.0
Attachment Mother Anxiety	37	5.7 ± 3.6	39	5.1 ± 3.3	36	5.7 ± 4.0	37	5.0 ± 3.0
Attachment Mother Avoidance	37	8.3 ± 5.1	39	6.9 ± 3.3	36	7.9 ± 5.2	37	7.2 ± 3.4
Attachment Mother Secure	37	17.0 ± 3.4	39	17.0 ± 4.2	36	17.1 ± 4.4	37	17.5 ± 3.1

SRS Social Responsiveness Scale, RBS-R Repetitive Behavior Scale-Revised, SCARED Screen for Child Anxiety Related Disorders, ASCQ Attachment Style Classification Questionnaire.

Chapter 5 | Can repeated intranasal oxytocin administration affect reduced neural sensitivity towards expressive faces in autism? — A randomized controlled trial

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Moerkerke, M., Daniels, N. (shared first), Van der Donck, S., Tibermont, L., Tang, T., Debbaut, E., Bamps, A., Prinsen, J., Steyaert, J., Alaerts, K., Boets, B. (2022). Can repeated intranasal oxytocin administration affect reduced neural sensitivity towards expressive faces in autism? A randomized controlled trial. Submitted and under review in the *Journal of Child Psychology and Psychiatry*

Abstract

Background: Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by difficulties in social communication and interaction. Crucial for efficient social interaction is the ability to quickly and accurately extract information from a person's face. Frequency-tagging EEG is a novel tool to quantify face-processing sensitivity in a robust and implicit manner. In terms of intervention approaches, intranasal administration of oxytocin (OT) is increasingly considered as a potential pharmacological treatment for improving socio-communicative difficulties in ASD, through enhancing social salience and/or reducing (social) stress and anxiety.

Methods: In this randomized, double-blind, placebo-controlled treatment-mechanism clinical trial, we implemented frequency-tagging EEG to examine the impact of repeated OT administration (four weeks, 12 IU, twice daily) on neural sensitivity towards happy and fearful facial expressions in children with ASD (8-12 years old; OT: n=29; placebo: n=32). At baseline, neural assessments of children with ASD were compared to those of an age- and gender-matched cohort of neurotypical (NT) children (n=39).

Results: Children with ASD demonstrated reduced neural sensitivity towards expressive faces, as compared to NT children. Upon nasal spray administration, children with ASD displayed a significant increase in neural sensitivity at the post and follow-up sessions, but only in the placebo group, likely reflecting an implicit learning effect. Strikingly, in the OT group, neural sensitivity remained unaffected from the baseline to the post session, likely reflecting a dampening of an otherwise typically occurring implicit learning effect.

Conclusion: First, we validated the robustness of the frequency-tagging EEG approach to assess reduced neural sensitivity toward expressive faces in children with ASD. Secondly, while children receiving placebo displayed an increase in neural sensitivity, this effect was dampened upon receiving the four-week OT treatment. Thus, in contrast with the social salience account, repeated OT treatment may reduce neural sensitivity towards emotionally-evocative faces, likely reflecting OT's (social) stress reducing effects.

Introduction

Autism Spectrum Disorder or ASD refers to a range of neurodevelopmental conditions characterized by difficulties in social reciprocity and communication, combined with restricted, repetitive and stereotyped patterns of behaviour, interests or activities (American Psychiatric Association, 2013). These difficulties frequently result in distress for these individuals and their environment. For society in general, the increasing prevalence of ASD poses high clinical, social and economic challenges (Solmi et al., 2022). Addressing these challenges requires more effective assessment and treatment of the core social symptoms of ASD.

Frequency-tagging EEG as a novel method for quantifying socio-communicative sensitivity

Impairments in the identification of body language and the interpretation of emotional states of others play a major role in socio-communicative difficulties experienced by individuals with ASD (Aoki et al., 2015). The human brain excels in extracting social meaning from a face, including the mental state, feelings and intentions (Haxby & Gobbini, 2011). Deficits in facial expression processing have often been reported in ASD, but the behavioural evidence is mixed and inconsistent (Leung et al., 2022; Uljarevic & Hamilton, 2013). Recently, fast periodic visual stimulation frequency-tagging electroencephalography (EEG) was proposed as a highly reliable, objective and implicit technique to pinpoint individual-subject face processing sensitivity (Rossion et al., 2015). The main principle of frequency-tagging EEG is that the periodicity (frequency) of the electrophysiological response on the human scalp corresponds exactly with the periodicity of the visual stimulation (Norcia et al., 2015), thereby allowing to label (or "tag") streams of particular stimulus categories. Pioneering studies applying this technique within the context of ASD demonstrated that brains of boys with ASD, compared to typically developing boys, were equally sensitive for the detection of a face within a scene, but were significantly less sensitive to rapid changes in facial identity (Vettori et al., 2019) and facial expression (Van der Donck et al., 2019, 2020).

Modulating socio-communicative sensitivity via oxytocin pharmacotherapy

Recently, intranasal administration of oxytocin (OT) is increasingly put forward as a potential treatment for alleviating the social difficulties characteristic of ASD. OT is an endogenous neuropeptide synthesized in the hypothalamus. Via the posterior pituitary, it is released in the bloodstream, where it acts as a hormone to influence bodily functions. The hypothalamus also projects to various brain structures, including those involved in social information processing (e.g., the amygdala), where OT acts as a neuromodulator, mediating a broad range of affiliative and prosocial behaviours (M. Bakermans-Kranenburg & van Ijzendoorn, 2013; Bartz et al., 2011; Jurek & Neumann, 2018; Landgraf & Neumann, 2004). Initial studies applying a single-dose of intranasal OT provided evidence for improvements in emotion recognition and prosocial functioning, both in neurotypical and in ASD populations (Andari et al., 2010; Domes et al., 2014; Guastella & Hickie, 2016; Okamoto et al., 2016). Yet, to yield full therapeutic potential in ASD, insights into the effects and (neural) mechanisms of multiple-dose (i.e., repeated) OT administration are needed. In terms of treatment efficacy, a handful of studies demonstrated beneficial effects on social functioning of repeated treatment in adults with ASD (Huang et al., 2021). In children and adolescents with ASD, on the other hand, the pattern of results appeared to be inconclusive, with some studies demonstrating beneficial effects (Parker et al., 2017; Yatawara et al., 2016), while others failed to replicate the positive outcomes (Dadds et al., 2014; Fastman et al., 2021; Guastella et al., 2015), also in highly powered studies (Sikich et al., 2021). Together, these elusive outcomes highlight that OT's neuromodulatory effects may be highly variable, emphasizing the need for a further mechanistic elaboration of theoretical OT models.

Mechanistic models of OT pharmacotherapy: anxiolytic versus social salience effects

To date, two main - not mutually exclusive - hypotheses have been put forward regarding OT's neuromodulatory function in regulating social behaviour. First, the anxiolytic account emphasizes that OT primarily acts as a regulator of (autonomic) stress and (social) anxiety responses, thereby facilitating social approach behaviour during social interactions (Bethlehem et al., 2014; Ma et al., 2016; Quintana et al., 2015; Stoop, 2014). Secondly, the social salience hypothesis proposes that OT primarily improves attention to

and perception of social cues by prioritizing the allocation of neural resources to the processing of these social cues (Shamay-Tsoory & Abu-Akel, 2016).

In support of the anxiolytic account, animal studies have demonstrated OT-induced reduction of amygdala reactivity, thereby effectively reducing stress-related activation of the hypothalamic-pituitary-adrenal (HPA) axis and the related somatic fear responses (Veening & Olivier, 2013). Likewise, in humans, attenuating effects of OT on amygdala activity have been shown to decrease social stress and facilitate social interaction (for review see (Ma et al., 2016). In a seminal study in neurotypical adult males, Kirsch et al. showed that a single-dose of OT significantly reduced amygdala activation and its connectivity to the brainstem, involved in the autonomic and behavioural fear response (Kirsch, 2005). Similarly, a unique multiple-dose OT study in adults with ASD evidenced long-term attenuation of amygdala reactivity and amygdala-frontal connectivity while processing emotionally charged point-light displays expressing body language (Alaerts et al., 2020; Bernaerts, Boets, Steyaert, et al., 2020). Besides the amygdala, single-dose intranasal OT administration in neurotypical adult males has also been shown to reduce neural activity in other brain regions underlying emotion regulation, such as the anterior cingulate cortex (Kanat et al., 2015; Petrovic et al., 2008), anterior insula (Bos et al., 2015; Kanat et al., 2014) and orbitofrontal cortex (Singer et al., 2008; Zunhammer et al., 2015). Additionally, (Y. Liu et al., 2022) recently demonstrated a dampening effect of single-dose OT in neurotypical adult males on early face processing activity in the occipital cortex. Together, these studies demonstrate that OT administration can reduce neural reactivity to social cues, such as faces, presumably reflecting OT's neuromodulatory role in dampening emotional arousal.

Quite in contrast and pertaining to the social salience effect, previous research also reported an enhancing effect of OT on brain activity/connectivity within and between neural structures of the social brain and the salience network, including for example, the amygdala, insula, and superior temporal sulcus (Domes et al., 2013; Gordon et al., 2013, 2016; Watanabe et al., 2014). With regard to the amygdala, OT has not only been shown to induce attenuation. Also increased amygdala activity has been observed, e.g., in response to happy faces in neurotypical male adults (Gamer et al., 2010), and this increased reactivity has been shown to be directly associated with improved face recognition in adults with Asperger syndrome (Domes et al., 2014). In children with ASD, single-dose intranasal OT was also shown to increase activity in brain regions related to

social attention and perception (posterior superior temporal sulcus (pSTS), posterior cingulate and premotor cortex) (Gordon et al., 2013). Likewise, in a prior study with adults with ASD, a single-dose of OT was shown to induce a reliable increase in pSTS brain activity during the processing of emotionally charged point-light displays expressing body language, whereas no consistent change in pSTS activity was induced after repeated OT treatment, indicative of a differential effect of acute versus repeated OT treatment (Bernaerts, Boets, Steyaert, et al., 2020).

To understand these contrasting neural effects, it has been proposed that the direction of the induced effects may depend on interindividual variability in person-dependent characteristics. According to the 'social adaptation hypothesis of OT' people with excessive social fear and stress may primarily experience an attenuation of amygdala reactivity thereby dampening emotional arousal (Labuschagne et al., 2010; Ma et al., 2016), whereas individuals with low social sensitivity may experience a social salience enhancing effect by increasing amaygdala reactivity (Gordon et al., 2013, 2016). A similar notion has been put forward by the allostatic theory of OT, suggesting that not only person-dependent factors, but also context will shape OT's neuromodulatory effects, in order to adapt to and promote stability within shifting environments (Quintana & Guastella, 2020).

The present study

We conducted a double-blind, randomized, placebo-controlled mechanistic-EEG trial assessing the neural effects of a four-week course of repeated OT administrations on the implicit processing of expressive faces in children with ASD. In line with the social salience account, it can be hypothesized that OT induces an enhancement of the salience of the presented social stimuli, in this case we expect to find increased neural sensitivity for processing facial expressions. However, in line with the anxiolytic account of OT and similar to previous multiple-dose treatment studies (Alaerts et al., 2020; Bernaerts, Boets, Steyaert, et al., 2020), the possibility cannot be ruled out that repeated OT administrations may induce a general attenuation of emotional reactivity as indexed by an overall attenuation of neural sensitivity towards emotionally-evocative facial expressions.

Furthermore, to allow a comparison of 'baseline' EEG neural sensitivity of children with ASD (prior to treatment allocation) to a group of typically developing children, a single recording session of frequency-tagging EEG was performed in a sample of matched

neurotypical controls. With regard to diagnosis-related differences in neural sensitivity, we expected to replicate prior findings of children with ASD displaying reduced neural sensitivity for facial expressions as compared to their neurotypical peers (Van der Donck et al., 2019, 2020).

Materials & Methods

Clinical trial design

A single-center, two-arm, double-blind, randomized, placebo-controlled parallel study was conducted at the Leuven University Hospital (Belgium) to assess the effects of four weeks intranasal administration of OT (twice daily) on neural sensitivity for implicit facial expression discrimination (see **Fig. 1** for the CONSORT flow diagram visualizing the number of participants randomized and analysed). Children with ASD underwent three neural assessment sessions: at baseline (T0); immediately post-treatment (T1) (24 hours after the last nasal spray administration); and at a follow-up session, four weeks after cessation of the daily administrations (T2). Additionally, a single recording session of frequency-tagging EEG was performed in a sample of neurotypical (NT) controls (T0) to allow assessment of diagnosis-related differences in baseline neural sensitivity. Children of the control group did not receive any treatment. All study procedures and consent forms were approved by the local Ethics Committee for Biomedical Research at the University of Leuven, KU Leuven (S61358) in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The trial was registered at the European Clinical Trial Registry (EudraCT 2018-000769-35).

Participants

Children with a formal diagnosis of ASD were recruited through the Leuven Autism Expertise Centre between July 2019 and January 2021. NT control children were recruited through elementary schools. For children with ASD, the Autism Diagnostic Observation Schedule, 2nd edition (ADOS-2; (Lord et al., 2012) was administered. The parent-rated Social Responsiveness Scale-Children, 2nd edition (SRS-2; (J. Constantino & Gruber, 2012) and verbal and performance intelligence quotients (IQ; WISC-V-NL; (Wechsler, 2018) were acquired for all children (ASD and NT). As outlined in **Table 1**, groups were matched on age and gender, although verbal and performance IQ were significantly higher in the control group compared to the ASD group. Prior to treatment, children of the ASD group

were randomly allocated to receive OT or placebo (PL). There were no statistically significant differences between randomized treatment groups in terms of age, gender, IQ and ASD symptomatology (Table 1). See Suppl. Mat., for more participant information.

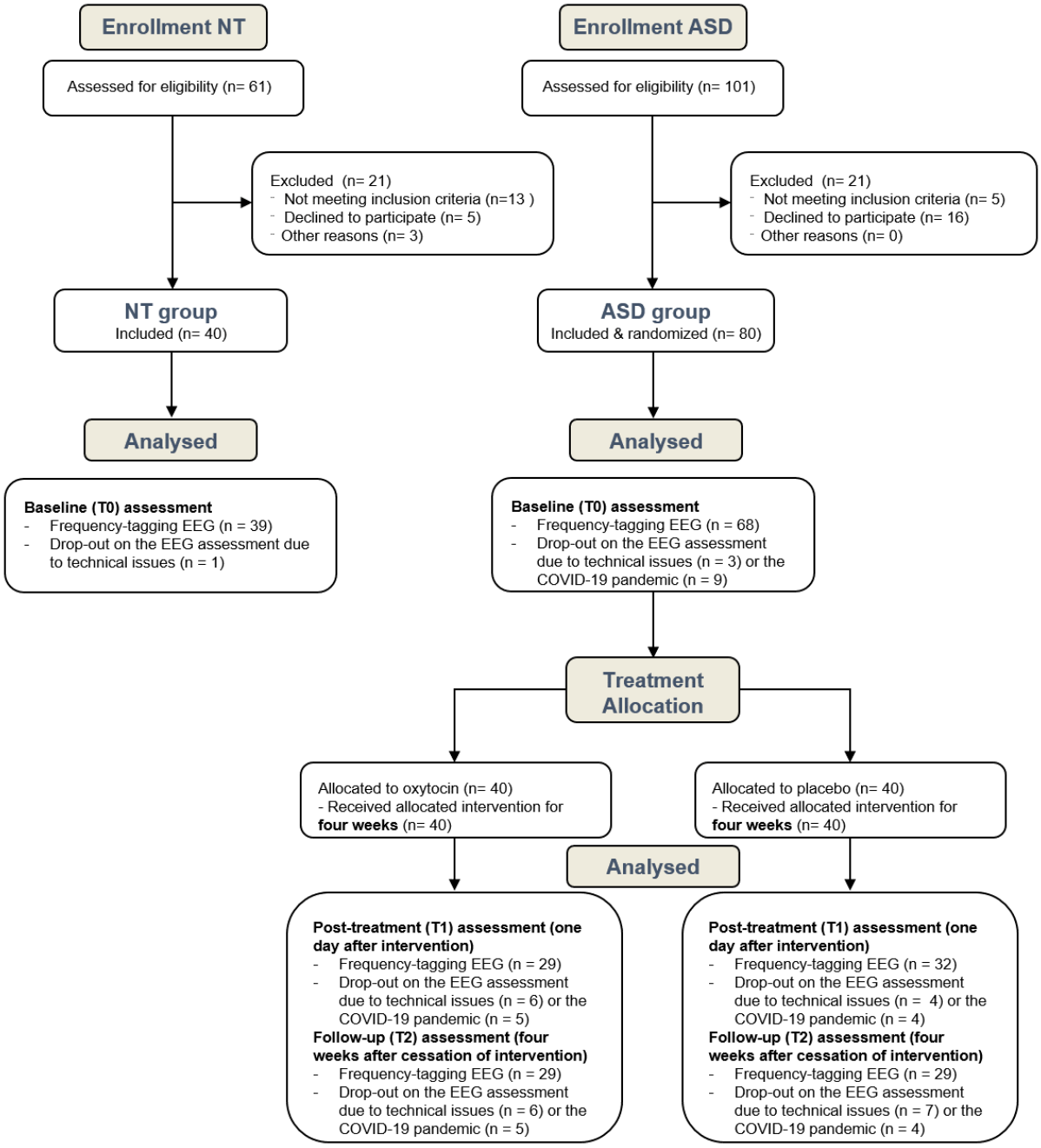

Figure 1. CONSORT flow diagram. Participants of the neurotypical (NT) or autism spectrum disorder (ASD) group were recruited and assessed at baseline (T0). Next, the ASD group was allocated to receive either oxytocin or placebo (four weeks of twice daily intranasal administrations), followed by a post-treatment (T1) assessment and a follow-up (T2) assessment four weeks after cessation of the daily administrations. As outlined, for several participants, neural EEG assessments were not acquired at one or more assessment sessions due to physical contact restrictions and closing down of hospital facilities during the COVID-19 pandemic.

As registered at the European Clinical Trial Registry (https://www.clinicaltrialsregister.eu/ctr-search/trial/2018-000769-35/BE) the EEG recordings were part of a larger assessment protocol, evaluating clinical efficacy of OT treatment on distinct autism symptoms questionnaires, including the parent-rated SRS-2 as primary behavioural outcome (J. Constantino & Gruber, 2012), see (Daniels et al., 2022) for a detailed report. In short, no treatment-specific improvements were identified, since both the OT and the placebo group showed significant improvements in social responsiveness immediately post-treatment (T1), as well as at the four-week follow-up session (T2).

Table 1. Demographic characteristics of the trial participants at baseline.

Measures	**ASD (*n* = 68)**	**NT (*n* = 39)**	***p*-value[a]**	**ASD-OT (*n* = 29)**	**ASD-PL (*n* = 32)**	***p*-value[a]**
♂:♀	56:12	31:8	0.95	23:6	27:5	0.91
Age[b] (mean ± SD)	10.08 ± 1.33	9.73 ± 1.26	0.53	10.14 ± 1.30	9.81 ± 1.20	0.32
VIQ[c] (mean ± SD)	107.51 ± 15.59	117.39 ± 12.31	<0.001*	105.10 ± 15.35	109.48 ± 15.88	0.28
PIQ[d] (mean± SD)	102.80 ± 14.38	108.23 ± 12.05	0.04*	104.48 ± 16.46	102.35 ± 13.31	0.59
SRS-2[e] (mean ± SD)	88.07 ± 20.84	22.21 ± 12.67	<0.001*	85.59 ± 21.66	88.22 ± 19.98	0.62
ADOS-2[f] (mean ± SD)	-	-	-	9.5 ± 3.99	9.44 ± 4.3	0.96

[a]*P*-values based on independent, two-sample *t* tests or Chi-square tests. [b]Age expressed in years. [c]Verbal intelligence quotient (IQ) was derived from the subtests Similarities and Vocabulary, Wechsler Intelligence Scale for Children, Fifth Edition, Dutch version (WISC-V-NL; Wechsler, 2018) dPerformance IQ was derived from the subtests Block design and Figure puzzles (WISC-V-NL; Wechsler, 2018). eSocial responsiveness Scale-Children, 2nd edition (SRS-2; Constantino & Gruber, 2012) fPrior to randomization, the Autism Diagnostic Observation Schedule, 2nd edition (ADOS-2; Lord et al., 2012) was administered in the ASD group. *Significant difference at $p < 0.05$ statistical threshold.

OT administration

Participants were randomized to receive OT (Syntocinon®, Sigma-tau) or placebo nasal sprays, consisting of all the ingredients used in the active solution except the OT compound. Sprays were prepared by the University Hospital of Heidelberg (Germany) to look identical in 10 ml brown glass bottles and had a white nasal pump (0.05 ml or 2 IU /puff). Before the start of the study, participants and their parents received clear instructions on how to administer the nasal spray (Guastella et al., 2013). Participants administered the nasal spray twice daily, six puffs (three per nostril) or 12 IU in the morning and six puffs in the afternoon (after school), resulting in a daily dose of 24 IU. On day 28, i.e., the last day before the post-treatment assessment (T1), participants withheld

the afternoon spray, in order to avoid single-dose OT effects. See Suppl. Mat., for more information on OT administration.

Expressive face discrimination paradigm using frequency-tagging EEG

The frequency-tagging EEG assessment entailed periodic presentation of a stream of neutral faces at 6 Hz base rate (Dzhelyova et al., 2017a; Van der Donck et al., 2020), periodically interleaved with an oddball stimulus displaying an expressive face (happy or fearful, in separate runs), every fifth image (i.e., the oddball frequency: 6Hz/5 = 1.2 Hz, see **Fig. 2**). No diagnostic-group or treatment-related differences were found on the base rate (6 Hz) responses (all $p > 0.23$; see Suppl. Mat., **Fig. S2-S5**). To prevent expression discrimination based on low-level visual features, we continuously changed the identity of the faces (i.e., every image). A single run comprised a 2-second fade-in, presentation of the images for 60 seconds through sinusoidal contrast modulation (0-100%), ending with a 2-second fade-out. Accordingly, in each run participants were presented 72 times (60 sec x 1.2 Hz) with the emotional facial expression, which was 'happy' in half of the runs (4 runs), and 'fearful' in the other half (4 runs). Half of the runs consisted entirely of male faces and the other half entirely of female faces, resulting in two runs per unique combination of emotional expression and gender. The order of the runs was randomized across subjects. Stimuli were presented via a custom-made java application. Stimuli consisted of fourteen individual faces (7 male, 7 female), selected from the Radboud-face-database (Langner et al., 2010). Each face stimulus was a full-frontal, coloured image, with a neutral, fearful or happy expression, at a size of 210x290 pixels, against a grey background (RGB = 153, 153, 153; alpha = 255). Luminance and contrast of the stimuli were equalized. In line with Van der Donck et al. (Van der Donck et al., 2019, 2020, 2022) participants performed an orthogonal task, pressing a button whenever a black fixation-cross at the eye-region of the face turned red.

EEG signals were recorded using an ActiveTwo BIOSEMI system, with 64 Ag/AgCl scalp-electrodes at 512 Hz. Two additional electrodes functioned as reference and ground electrodes (Common-Mode-Sense-active-electrode and Driven-Right-Leg-passive-electrode). There was no limit on the impedance of the electrodes and the electrode function was controlled throughout the clinical trial based on the BIOSEMI guidelines (https://www.biosemi.com/faq/check_electrodes.htm). Electrodes were attached on a BIOSEMI 64-electrode headcap.

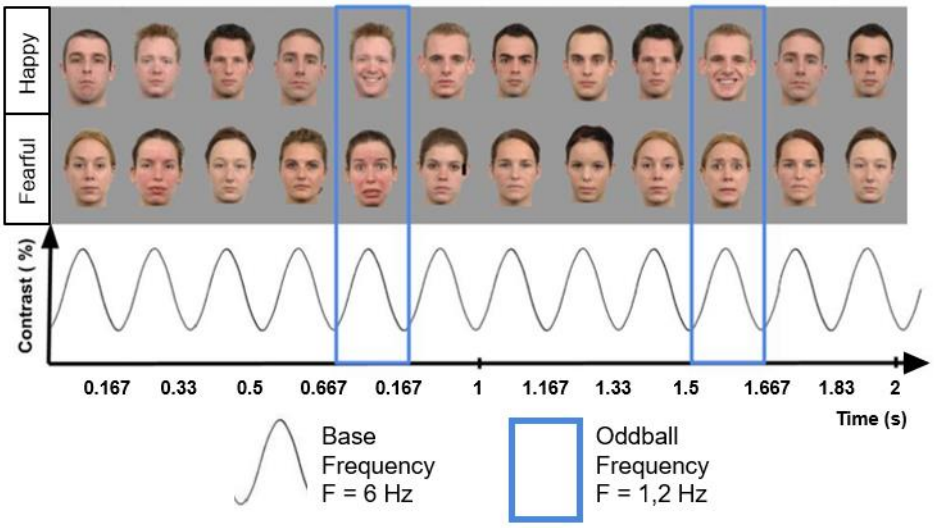

Figure 2. Frequency-tagging EEG oddball paradigm. Neutral faces are presented sequentially at a fast 6 Hz base rate, periodically interleaved with an expressive face (fearful or happy) every fifth image (1.2 Hz oddball rate). The identity of the faces changed between images.

Data handling and statistical analysis

Raw EEG data were pre-processed using letswave6 (http://www.nocions.org/letswave/), a MATLAB-based toolbox (MATLAB R2020b, MathWorks). See Suppl. Mat. for information on EEG analysis. Fast Fourier transformation (FFT) was applied on the pre-processed EEG data, resulting in amplitude spectra in the frequency domain. To obtain a measure of selective neural sensitivity for facial expressions, baseline-subtracted (BS) amplitudes were calculated at the oddball frequency and its harmonics (i.e. n*F/5, e.g. second harmonic: 2*6/5 = 2.4 Hz, etc.), by subtracting the average amplitude of the 20 surrounding bins from the amplitude of the bin of interest (10 bins on each side, excluding the immediately adjacent bin and the two highest bins). The BS amplitudes, summed across harmonics for each expression, were assessed using linear mixed models (LMM) with the 'Afex' package (version 0.28-1) in R (version 4.1.0) (Singmann et al., 2020). To account for the repeated measures, the factor *subject* was included as random factor in all LMMs. Bonferroni-corrected post-hoc tests were performed using the 'emmeans' package (version 1.6.0) in R (Lenth et al., 2019). Assumptions of normality, equal variance and linearity were accounted for.

Assessment of diagnostic-group differences. To assess diagnostic-group-related differences in neural sensitivity, oddball BS amplitudes were subjected to an LMM with *group* (ASD, NT) as a fixed between-subject factor and the factors *ROI* (left occipito-

temporal region or LOT: channels P7, P9 and PO7 and right occipito-temporal region or ROT: channels P8, P10 and PO8, see **Fig. S1**) and facial *expression* (fearful, happy) as fixed within-subject factors. Additionally, exploratory LMM analyses including *gender* as between-subject variable and *IQ* as covariate were performed.

Assessment of OT treatment effects. To assess effects of OT treatment, a modified intention-to-treat approach was applied, including all randomized participants who completed the baseline (T0) session and at least one post or follow-up session. For each assessment session at T1 or T2, change-from-baseline scores (CFB, i.e., subtracting T0 scores)* in oddball BS amplitudes were calculated and subjected to an LMM including the between-subject fixed factor *treatment* (OT, PL), and the factors *ROI* (LOT, ROT) and *expression* (fearful, happy) as fixed within-subject variables. To further investigate within-group pre-to-post changes, additional LMM analyses were performed, separately within each treatment group (OT or PL) examining changes from baseline to post (fixed within-subject factor 'time point': T0, T1); and from post to the follow-up session (T1, T2).

*Note: we are aware that the established term "baseline" refers to different concepts in the frequency-tagging EEG literature and in the clinical trial literature. Here, *change from baseline* (CFB) refers to subtracting the first time point (T0) of the time point of interest (T1 or T2), and *baseline-subtracted* (BS) amplitudes refers to the subtraction of the average amplitude of the 20 surrounding frequencies from the amplitude of the frequency of interest.

Results

Diagnostic-group differences in implicit facial expression discrimination As seen in Figure 4, both facial expressions did elicit clear expression-discrimination EEG responses at the oddball frequency and its harmonics (see SNR spectra in **Fig. 3**, bottom part) mostly centred over right occipito-temporal (ROT) sites (see heat map topographies in **Fig. 3**). Examination of diagnostic-group differences yielded a highly significant main effect of *group* ($F(1, 98.69) = 8.27$, $p = 0.005$), indicating higher responses in the NT control group ($M_{NT} = 0.90$ µV) compared to the ASD group ($M_{ASD} = 0.66$ µV) (see **Fig. 3**, top part). Also, a significant main effect of *ROI* was found, indicating higher responses in the ROT ($M_{ROT} = 0.87$ µV) versus LOT region ($M_{LOT} = 0.68$ µV; $F(1, 96.55) = 12.23$, $p < 0.001$). No main effect of *expression,* nor any two- or three-way interaction effects were found (all $p > 0.08$),

indicating that the *group* and *ROI* differences were evident, irrespective of emotional facial expression (fearful or happy). The exploratory LMM analysis, including *gender* as within-subject factor, showed no main effect of *gender* (p = 0.48) nor a *group x gender* interaction effect (p = 0.62), indicating that the identified effects were similar for boys and girls. When IQ was added as covariate the results remained similar, with significantly higher responses in the NT group (F(1, 94.02) = 7.61, p = 0.007) and in the ROT region (F(1, 286.04) = 10.77, p = 0.001).

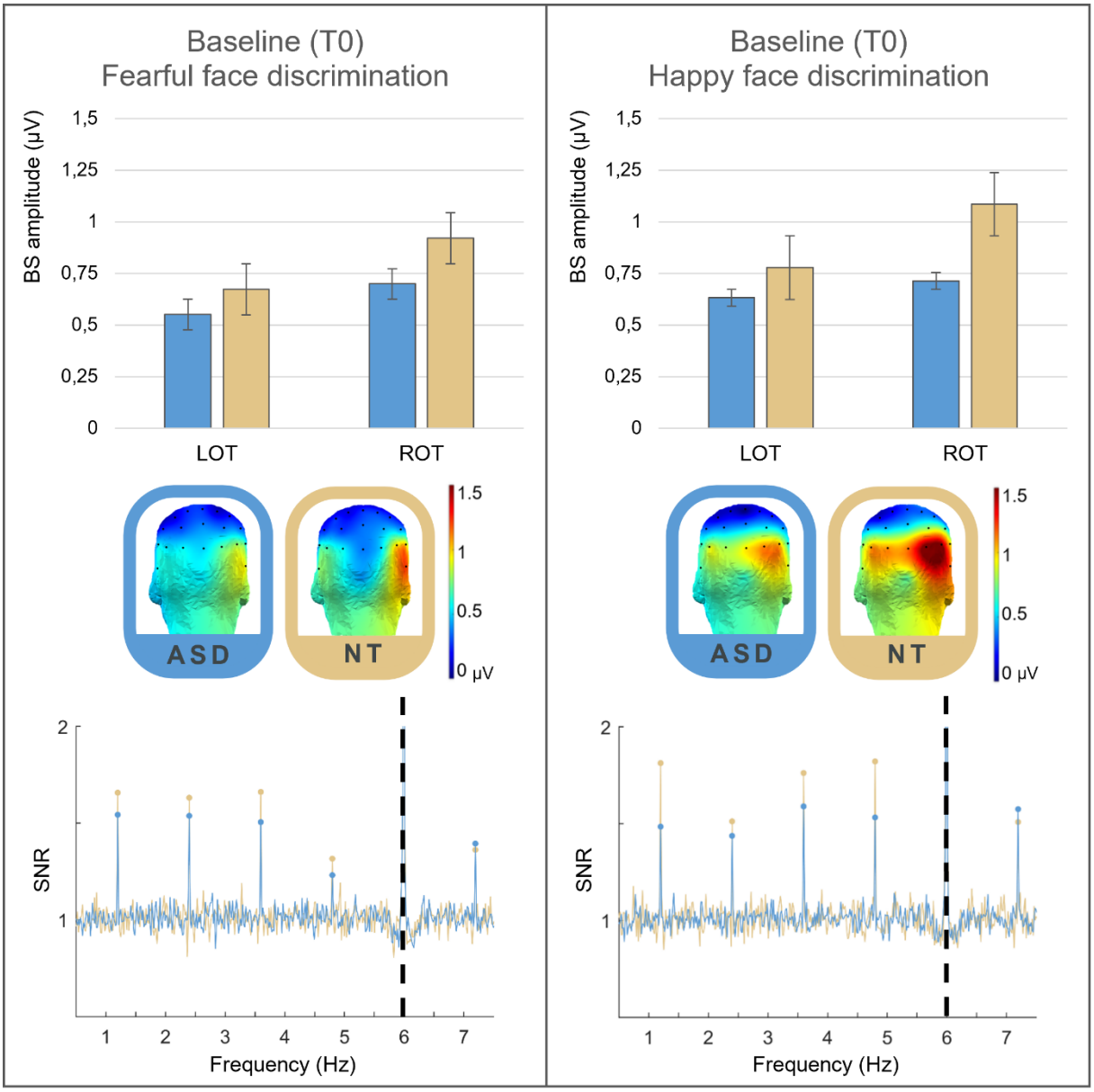

Figure 3. Baseline (T0) ASD versus NT diagnostic group comparison. Top part: Expression-discrimination EEG responses at the oddball frequency (summed BS amplitudes) are shown in bar graphs and scalp topographies, separately for the ASD and NT groups and left and right occipito-temporal (LOT and ROT) regions. Error bars denote standard errors of the mean. Bottom part: SNR spectra show the BS amplitudes per frequency-bin, averaged across LOT and ROT regions; the significant first five harmonics (until 7.2 Hz) are displayed; the dashed line indicates the 6 Hz base rate response. A main effect of group (ASD < NT, p = 0.005) and a main effect of ROI (LOT < ROT, p = 0.001) was found.

OT treatment effects on implicit facial expression discrimination in ASD

Post-treatment CFB assessment (T1). A significant main effect of treatment (F(1, 55.5) = 8.03, p = 0.006) was revealed for pre-to-post changes in BS amplitudes, recorded post-treatment (T1), indicating a significantly *higher* pre-to-post increase in neural sensitivity in the placebo group, compared to the OT group, see **Fig. 4A and 4C**. None of the other main (all p > 0.15) or interaction effects (all p > 0.58) reached significance.

Direct examination of pre-to-post changes, separately in each treatment group, confirmed that only in the placebo group, a significant increment (F(1, 30.82) = 5.33, p = 0.028) in neural sensitivity was evident from the baseline (T0) session (M_{T0} = 0.67 μV) to the post-treatment (T1) session (M_{T1} = 0.78 μV). In the OT group, on the other hand, amplitude scores did not significantly change from the baseline (T0) session (M_{T0} = 0.66 μV) to the post-treatment (T1) session (M_{T1} = 0.59 μV; F(1, 129.30) = 1.99, p = 0.16).

Four-week follow-up CFB assessment (T2). While no significant main effect was found (F(1, 48.21) = 1.11, p = 0.30), a significant *treatment* x *ROI* interaction (F(1, 94.55) = 6.26, p = 0.014) indicated that the treatment-related effect -evident immediately post-treatment (T1)- persisted in the pre-to-post changes at follow-up (T2), but only for the ROT region (t(86.1) = 2.19, p = 0.03) not for the LOT region (t(84.2) = 0.39, p = 0.70), see **Fig. 4B and 4C**. None of the other main (all p > 0.11) or interaction effects (all p > 0.14) reached significance.

Further, exploration of how amplitudes changed from assessment session T1 to T2, separately within each treatment group, showed a significant *time point x ROI* interaction effect (F(1, 268.12) = 8.6, p = 0.004) in the placebo group. Specifically, a further increment in neural sensitivity was evident from T1 (M_{T1xROT} = 0.11 μV) to T2 (M_{T2xROT} = 0.28 μV), but only for the ROT region (t(268) = 2.29, p = 0.02), not for the LOT region (t(269) = -1.86, p = 0.065). Notably, also the OT group displayed a trend level increase in neural sensitivity (F(1, 24.4) = 3.99, p = 0.057) from T1 (M_{T1} = -0.06 μV) to T2 (M_{T2} = 0.05 μV), see **Fig. 4C**.

Additionally see **Fig. S6** for a spaghetti plot visualising the OT effects on implicit facial expression discrimination in ASD across both T1 and T2, depicting individual variability. Here EEG responses across LOT and ROT regions were averaged for fearful and happy faces, considering that no significant effects of expression were evident.

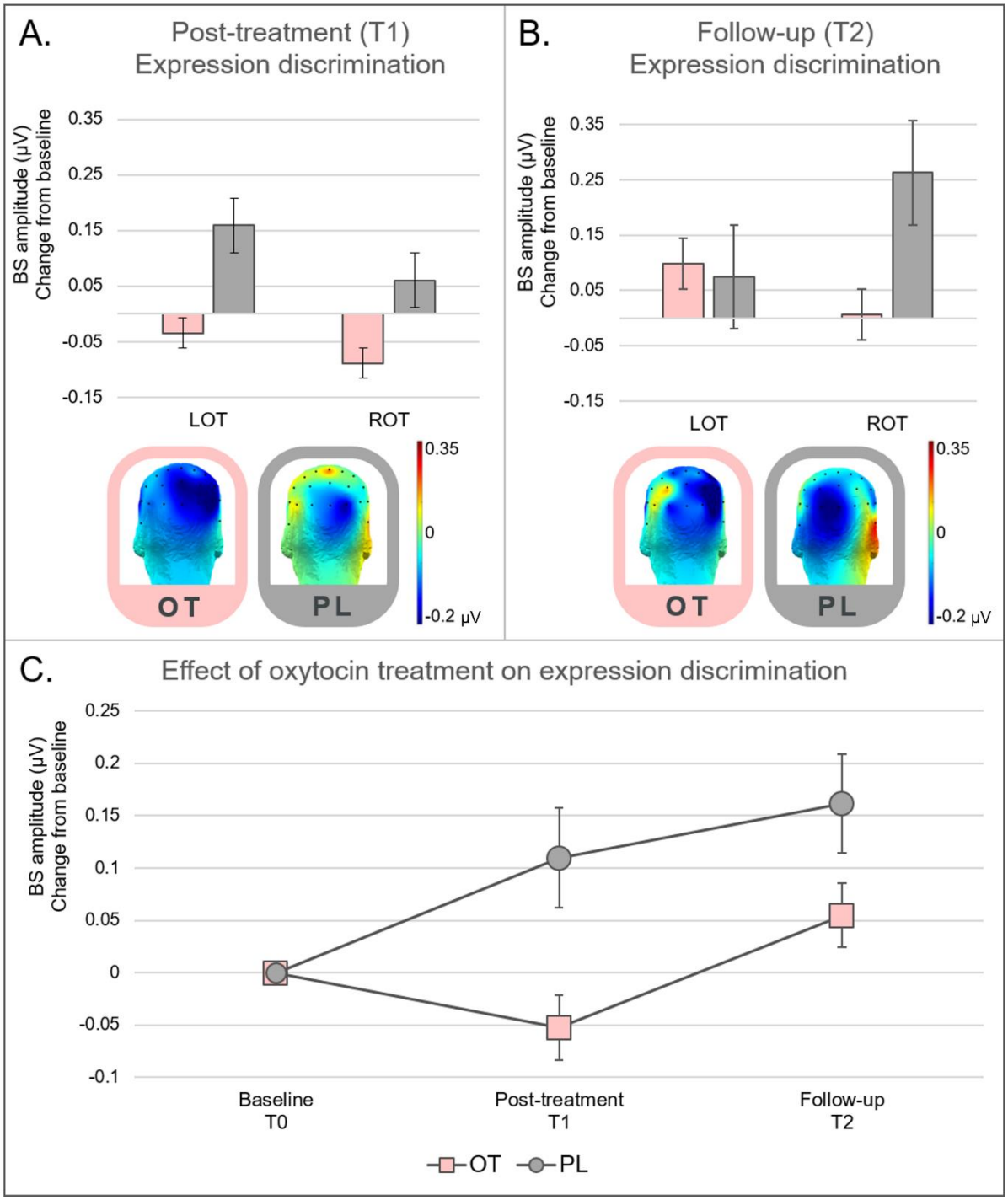

Figure 4. Oxytocin treatment effects at post-treatment (T1) and follow-up (T2). Change from baseline (CFB) in expression-discrimination EEG responses at the oddball frequency (summed BS amplitudes) recorded at the post-treatment (T1) and follow-up session (T2) are visualized separately for the oxytocin (OT) and placebo (PL) group in bar graphs, scalp topographies and a line graph. **Panel A.** visualizes CFB treatment effects separately for left and right occipito-temporal (LOT and ROT) regions at T1. **Panel B.** visualizes CFB treatment effects separately for LOT and ROT regions at T2. **Panel C.** visualizes CFB treatment effects across LOT and ROT regions for baseline (T0), T1 and T2. EEG responses were averaged for fearful and happy faces, considering that no significant effects of expression were evident. Error bars reflect standard errors of the mean. A main effect of treatment was found at T1 (OT < PL, $p = 0.006$), and a treatment x ROI interaction effect at T2 (for ROT: OT < PL, $p = 0.014$).

Control measures: orthogonal task and general visual base rate responses

No diagnostic-group or treatment-related differences on the orthogonal task. Average performance accuracy on the orthogonal task was 91.33%, with no diagnostic-group or treatment-related differences, indicating similar levels of attentiveness to the screen (all $p > 0.24$; see Suppl. Mat.).

No diagnostic-group or treatment-related differences in base rate BS amplitudes. As the 6 Hz base rate responses mainly indexes general low-level visual processing of the faces, no ASD versus NT baseline (T0) differences were expected. This was indeed confirmed ($p = 0.376$; for further details and visualization, see Suppl. Mat. Fig S2)

Additionally, no significant treatment-related (OT vs. PL) differences in general visual base rate processing were found post-treatment (T1), nor at follow-up (T2) (all $p > 0.23$; see Suppl. Mat. Fig S3 and Fig S4, respectively).

No change in base rate BS amplitudes throughout the clinical trial. Lastly, no effect of time point was found post-treatment (T1) nor on follow-up (T2), across expressions (fearful, happy) neither in the OT-group nor the PL-group (all $p > 0.24$; see Fig. S5).

Discussion

The current study evaluated the effect of repeated OT administration on the neural sensitivity for expressive faces in ASD. Pre-treatment, we identified reduced neural sensitivity in children with ASD, compared to NT control children. Importantly, repeated OT administration did not improve this reduced neural sensitivity, indicating no overall effect of OT on enhancing social salience towards expressive faces. Instead, the OT treatment appeared to dampen otherwise typically occurring implicit learning effects, likely reflecting OT's anxiolytic and (social) stress reducing effects towards emotionally-evocative faces.

Reduced neural sensitivity to expressive faces in ASD

Interestingly, prior observations of Van der Donck et al. were consistently replicated and extended, i.e., showing significantly reduced neural sensitivity towards expressive faces (fearful and happy) in boys and girls with ASD compared to NT controls (Van der Donck et al., 2019, 2020). Of note, the similar performance in response time and accuracy on the orthogonal task indicates equal visual attention and task motivation across both groups.

Also, amplitudes in response to the general visual base rate stimulation (6 Hz) were similar across groups, indicating that both NT and ASD children displayed an intact synchronization of neural responses to the periodically flickering stimuli of neutral facial expressions. Accordingly, the diagnostic-group difference was therefore specific to the spontaneous detection and discrimination of the emotional facial expressions as indicated by the reduced neural sensitivity oddball EEG amplitudes in children with ASD, compared to control children.

Repeated OT treatment effects on implicit facial expression discrimination in ASD

Next, we implemented the frequency-tagging EEG paradigm in a randomized, double-blind, placebo-controlled clinical trial, in order to monitor the effect of OT treatment in children with ASD. Strikingly, investigating changes from baseline, we found that only in children with ASD who received the placebo nasal spray, a significant increase in neural response towards expressive faces was evident, both immediately post-treatment and at four weeks follow-up. In children receiving the OT nasal spray, neural sensitivity remained unchanged post-treatment. It has been demonstrated that the positive social context of being enrolled in a clinical trial can motivate social engagement, through the attention of the clinical trial researcher and being supported by loved ones (e.g. parents). This has been proposed to boost the endogenous OT system, resulting in placebo effects (Itskovich et al., 2022). So the placebo effects found in the current and in previous OT studies, could be a result of endogenously released OT. Administration of exogenous OT might create a surplus of available OT and consequently desensitize the OT system. Alternatively, it is possible that the repetitive nature of the clinical trial's sampling caused familiarisation to the facial stimuli through implicit learning (which is intact for social stimuli in ASD (Foti et al., 2015)), making the participants in the placebo group more apperceptive for the subtle socio-communicative cues through increased neural activity. Selectively higher activity in the fusiform face area in response to familiar faces has indeed been demonstrated in ASD (Pierce et al., 2004). Possibly, the multiple-dose OT intervention may have inhibited this spontaneous increase in familiarisation and neural activity in order to soothe the impact of the incoming (stressful) social stimulus. After cessation of the OT treatment at T1, we see an increase in neural sensitivity for the facial expressions at follow-up (T2) in the OT group, comparable to the effect seen in the placebo group.

Accordingly, contrary to the prevailing expectation that OT would heighten and possibly rescue neural sensitivity towards expressive faces in ASD, we see that OT appears to dampen an otherwise typically occurring implicit learning effect in facial expression processing. Similarly, a recent fMRI study, in adults with trait anxiety, also found increased neural reactivity towards negative emotional stimuli in the amygdala, insula, and prefrontal cortex, only after placebo treatment, whereas repeated-intermittent OT administration prevented this increase (Kou et al., 2022). Possibly, OT may attenuate neural responsivity towards (unpleasantly experienced) social stimuli, to reduce social anxiety and promote approach behaviour, in line with the anxiolytic account of OT. Note in this regard that not only the fearful faces may have been experienced as aversive by individuals with ASD, but also the happy faces may have been perceived as such, as they may be inviting for (unwanted) social interaction.

Weighing social salience versus anxiolytic effects

In contrast to the commonly observed behavioural improvements in social salience after a single-dose of OT (Auyeung et al., 2015; Domes et al., 2014; Gordon et al., 2013; Guastella et al., 2010; M. Kanat et al., 2017, but see Van der Donck et al., 2022), its neural underpinnings during expressive face processing are variable, with some studies demonstrating increased (Aoki et al., 2014; Domes et al., 2010, 2014; Gamer et al., 2010) but others decreased (Domes et al., 2007; Gamer et al., 2010; Kirsch, 2005) neural activity (e.g., in the amygdala). On the other hand, the few repeated OT administration studies evaluating chronic *neural* effects, consistently demonstrate an attenuating (anxiolytic) effect, including on the amygdala (Alaerts et al., 2020; Bernaerts, Boets, Steyaert, et al., 2020; Kou et al., 2022). The observed variability in neural response upon single-dose OT administration has been attributed to the variable nature of the stimuli/context that participants are primed with during the acute window of heightened exogenous OT availability (Kou et al., 2022), i.e., positive social contexts/tasks may induce enhanced social salience, whereas negative (social) contexts may primarily induce anxiolytic, stress-reducing effects (Ma et al., 2016). In most prior multiple-dose OT treatment studies, formal standardisation of the context in which the OT treatment is administered is often not implemented, rendering some variability in the neural circuits that can be targeted upon each repeated daily administration. Against this background, it can be speculated that the direction of OT's effects could be impacted depending on whether participants in

multiple-dose administration trials are consistently presented with socially-stimulating stimuli/contexts, versus no explicit social stimulation, or even negatively experienced (social) contexts. Accordingly, also in the current trial, it is possible that standardized stimulation of the children with e.g., facial stimuli during each acute window of the daily OT administration would have facilitated a priming of relevant neural (social) circuits. On the other hand, here, the absence of a specific standardized stimulation may have primarily primed intrinsic neural circuitry towards the brain's default 'resting state', reflective of OT's role in promoting homeostasis and restoration of autonomic balance in the relative absence of acute (social) stimulation (Carter, 2014). Recently, a six-week multiple-dose clinical trial with 3-to-8 year old children with ASD paired each acute OT administration (intermittently administered every other day) with psychosocial stimulation, and showed consistent clinical improvements in autism symptomatology (ADOS-2) as well as enhanced social attention and increased looking time to the eye region (Le et al., 2022). The authors primarily attributed the therapeutic efficacy of this trial to the combination of OT administration with the positive social stimulation, i.e., allowing an optimal capitalisation on OT's role for inducing attentional salience and social sensitivity. Future research is warranted, however, to further explore the differential (neural) impact of combining repeated OT treatment with specific (socially-stimulating) tasks/contexts.

Limitations

While the current study provides important new insights into the neural effects of repeated OT administration in children with ASD during facial expression processing, the following limitations need to be considered. First, the study did not include a single-dose OT measurement, so no direct comparison between acute and chronic OT effects on neural sensitivity could be drawn, which may have been informative for understanding the role of the presented context/stimuli on OT's neural effects, i.e., specifically during the acute window of heigtened exogenous OT availability. Likewise, a more consistent standardization or monitoring of the social context during heigtened exogenous OT availability throughout the long-term trial would have been informative, also to understand possible interindividual variability in neural response patterns.

Further, the current study adopted a dosing scheme of 2x12 IU per day, four weeks long, in accordance with prior studies assessing repeated OT administration effects (Alaerts et

al., 2020; Bernaerts, Boets, Steyaert, et al., 2020), however it cannot be ascertained that a similar pattern of results would have emerged at a different dosing and/or administration duration. As noted above, the study by Le et al. adopted an intermittent dosing scheme (administration every other day) to avoid possible oversaturation of the endogenous OT system (due to desensitization of OT receptors) (Le et al., 2022). Also in terms of dosing, prior single-dose studies have provided indications of inverted U-shaped dose-response curves for eliciting OT efffects (Yamasue et al., 2022). Accordingly, for repeated administration studies, not only the dose and duration of the OT treatment may be important factors to consider, but also the frequency or interval at which doses are administered may be important design-related factors for yielding optimal OT treatment effects.

Conclusion

The ability to quickly and accurately process facial expressions is crucial for efficient social interactions. Individuals with ASD have difficulty processing nonverbal body language, but it is challenging to determine and quantify this in an objective and reliable manner. Here, we validated the robustness of frequency-tagging EEG to assess reduced neural sensitivity towards expressive faces in boys and girls with ASD, compared to age- and gender-matched NT controls. Next, we implemented frequency-tagging EEG as outcome measure in a double-blind placebo-controlled multiple-dose OT clinical trial. Interestingly, only children receiving the placebo nasal spray were shown to display an increase in neural sensitivity upon repeated implicit processing of expressive faces. In the OT group, on the other hand, this implicit learning effect was dampened after the four-week OT treatment. Together, these results argue against the prevailing expectation that OT would heighten and possibly rescue reduced neural sensitivity for socio-communicative cues, as postulated by the social salience hypothesis of OT. On the contrary, repeated OT administration may primarily prime neural circuitry to elicit dampened neural sensitivity towards expressive faces, likely reflecting OT's anxiolytic and (social) stress reducing effects.

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Supplementary Material

Supplementary methods

Participants

Participants of the neurotypical (NT) or autism spectrum disorder (ASD) group were recruited and assessed at baseline (T0). Next, the ASD group was allocated to receive either oxytocin or placebo (four weeks of twice daily intranasal administrations), followed by a post-treatment (T1) assessment and a follow-up (T2) assessment four weeks after cessation of the daily administrations. Main inclusion criteria comprised a clinical diagnosis of ASD (only for children with ASD); age (8-12 years old); IQ above 70; no prior OT treatment and native Dutch speaker. The ASD diagnosis was established by a multidisciplinary team (child psychiatrist and/or expert neuropediatrician, psychologist, speech/language pathologist and/or physiotherapist) based on the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (American Psychiatric

Association, 2013). Main exclusion criteria comprised a history of any neurological disorder (stroke, concussion, epilepsy etc.), any physical disorder (liver, renal, cardiac pathology), significant hearing or vision impairments, or any neuropsychiatric diagnosis (only for NT children). Only premenstrual girls were included. See Suppl. Mat., for additional participant information. Seventeen participants with ASD had a comorbid diagnosis of attention deficit/hyperactivity disorder (ADHD) and one had a comorbid diagnosis of obsessive compulsive disorder (OCD). Thirty of the 68 ASD children followed stable concomitant psychosocial therapy. Twenty of the ASD participants used stimulant medication (e.g., methylphenidate), 13 anti-psychotics (e.g., risperidone), 4 anti-depressants (e.g., sertraline) and 35 used other medication (e.g., sleeping aids, gastro-intestinal medication or nutritional supplements).

EEG analysis

Pre-processing

Raw EEG data were pre-processed using letswave6 (http://www.nocions.org/letswave/), a MATLAB-based toolbox (MATLAB R2020b, MathWorks). EEG-data from each run were cropped into segments of 70 seconds. A Butterworth band-pass filter (fourth order; 0.1-40 Hz) was applied and data were down-sampled to 256 Hz. Data from noisy channels were re-estimated using linear interpolation of the three nearest channels, with a maximum of 5% of the channels to be interpolated per participant. On average, across all participants and time points, 0.4 channels per participant were interpolated. Data were re-referenced to the common average, re-segmented based on the exact number of frequency-bins (avoiding overspill), and averaged in the time domain per expression condition (happy or fearful).

Frequency domain analysis

Fast Fourier transformation (FFT) was applied, resulting in amplitude spectra in the frequency domain. To obtain a measure of neural sensitivity for facial expressions, we calculated baseline-subtracted (BS) amplitudes at the oddball frequency and its harmonics (i.e., n*F/5 = 2.4 Hz, 3.6 Hz, etc.), by subtracting the average amplitude of the 20 surrounding bins from the amplitude of the bin of interest (10 bins on each side, excluding the immediately adjacent bin and the two highest bins). The signal to noise ratio (SNR) was also calculated by dividing the amplitude of the bin of interest by the average amplitude of the 20 surrounding bins, which allows the visualization of small amplitudes,

but with a high SNR (Rossion et al., 2012). Z-scores were computed using the mean and standard deviation of the 20 bins surrounding the bin of interest, to determine how many harmonics of the base rate (6 Hz) and oddball (F/5 = 1.2 Hz) responses should be included in the analyses. Harmonics were included until the Z-score no longer exceeded 1.64 (p < 0.05) for two consecutive harmonics. The oddball response was quantified as the sum of the responses of the first six harmonics (i.e., until 6F/5 = 7.2 Hz), excluding the 6 Hz base rate response. The base rate response was quantified as the sum of the first five harmonics (i.e., until 5F = 30 Hz).

Regions of Interest (ROIs) of the oddball responses

Two regions of interest were defined over left and right occipito-temporal (LOT and ROT regions) sites (**Fig. S1**). These ROIs were a priori identified, based on research using similar frequency-tagging EEG paradigms (Dzhelyova et al., 2017; Van der Donck et al., 2019, 2020, 2022), and a posteriori confirmed to exhibit the highest oddball responses. More specifically, ROI analyses were performed by averaging the summed oddball BS amplitudes in the LOT region over channels P7, P9 and PO7 and in the ROT region over channels P8, P10 and PO8.

Regions of interest (ROIs) of the base rate responses

Three regions of interest (ROI) were defined over LOT and ROT regions and mid-occipital (MO) region to assess base rate responses (**Fig. S1**). These ROIs were a priori identified, based on research using similar frequency-tagging EEG paradigms (Dzhelyova et al., 2017; Van der Donck et al., 2019, 2020, 2022), and a posteriori confirmed to exhibit the highest base rate responses. The base rate ROI analyses were performed by averaging the summed base rate BS amplitudes in the in the LOT region over channels P7, P9 and PO7, in the ROT region over channels P8, P10 and PO8 and in the MO region over channels Iz and Oz. This general visual stimulation response is mainly driven by low-level visual features, which are typically visible in the medial-occipital region.

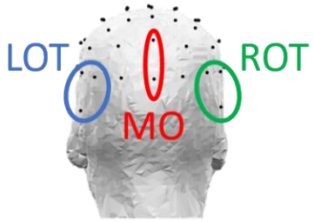

Figure S1. Regions of interest. LOT = left occipito-temporal region, ROT = right occipito-temporal region and MO = medial-occipital region as shown on a scalp topography. Oddball responses were defined over LOT and ROT regions, base rate responses over LOT, ROT and MO regions

OT administration

Nasal spray compliance was monitored via daily diaries, filled in by the parents, that recorded date and time of administration (percentage of compliance; ASD-OT: 97.51 ± 2.43%; ASD-PL: 95.71 ± 5.65 %; $t(58) = 1.59$, $p = 0.12$). Additionally, nasal spray bottles were weighed pre- and post-treatment to calculate and compare the total amount of administered fluid (ASD-OT: 14.62 ± 2.49 ml; ASD-PL: 13.62 ± 2.47 ml; $t(59) = 1.57$, $p = 0.12$). Potential adverse events were also recorded through weekly parent reports and daily parent and child diaries, and revealed minimal and non-treatment specific side effects (see (Daniels et al., 2022).

Supplementary results

Behavioural measure: orthogonal task

No diagnostic-group or treatment-related differences in response time or accuracy

The outcome of the orthogonal task (pressing the button when the black fixation-cross on the nasion turned red) was assessed on response time and accuracy. These results were compared on T0 (ASD vs. NT; ASD-OT vs. ASD-PL), T1 (ASD-OT vs. ASD-PL) and T2 (ASD-OT vs. ASD-PL), to ensure that attentiveness to the screen was similar.

Baseline assessment (T0). For the baseline (T0) group-comparison no difference in response time was found ($M_{ASD} = 0.57$ ms; $M_{NT} = 0.57$ ms; $p = 0.89$), nor in accuracy ($M_{ASD} = 87\%$; $M_{NT} = 90\%$ ms; $p = 0.24$). When comparing ASD-OT vs. ASD-PL on the baseline (T0) (prior to treatment), no differences were found in response time ($M_{OT} = 0.57$ ms; $M_{PL} = 0.57$ ms; $p = 0.63$), nor on accuracy ($M_{OT} = 85$ %; $M_{PL} = 89$ %; $p = 0.4$).

Post-treatment assessment (T1). No treatment-related effects were found immediately post-treatment (T1) for response time ($M_{OT} = 0.58$ ms; $M_{PL} = 0.56$ ms; $p = 0.31$) nor for the accuracy ($M_{OT} = 93\%$; $M_{PL} = 94\%$; $p = 0.75$).

Four-week follow-up assessment (T2). On the follow-up comparison (T2) we also did not find differences in response time ($M_{OT} = 0.61$ ms; $M_{PL} = 0.6$ ms; $p = 0.68$) nor on accuracy ($M_{OT} = 90\%$; $M_{PL} = 91\%$; $p = 0.64$). These results indicate that all participants were equally attentive to the screen on baseline and within each treatment session.

General visual base rate responses

No diagnostic-group differences in base rate BS amplitudes

The general visual response to faces is indexed using the base rate signals of 6 Hz. On the baseline measurement (T0), no base rate group differences between ASD and NT controls were found (($F(1, 103.52) = 0.79$, $p = 0.376$). The LMM did detect a significant main effect of *ROI*, as expected from previous research (Dzhelyova et al., 2017; Van der Donck et al., 2019, 2020), with the strongest neural responses over the medial-occipital (MO) region ($M_{MO} = 5.88$ µV), compared to LOT and ROT ($M_{LOT} = 1.8$ µV; $M_{ROT} = 1.94$ µV; $F(2, 98.14) = 191.74$, $p < 0.001$, see **Fig. S2**), irrespective of facial expression (happy or fearful).

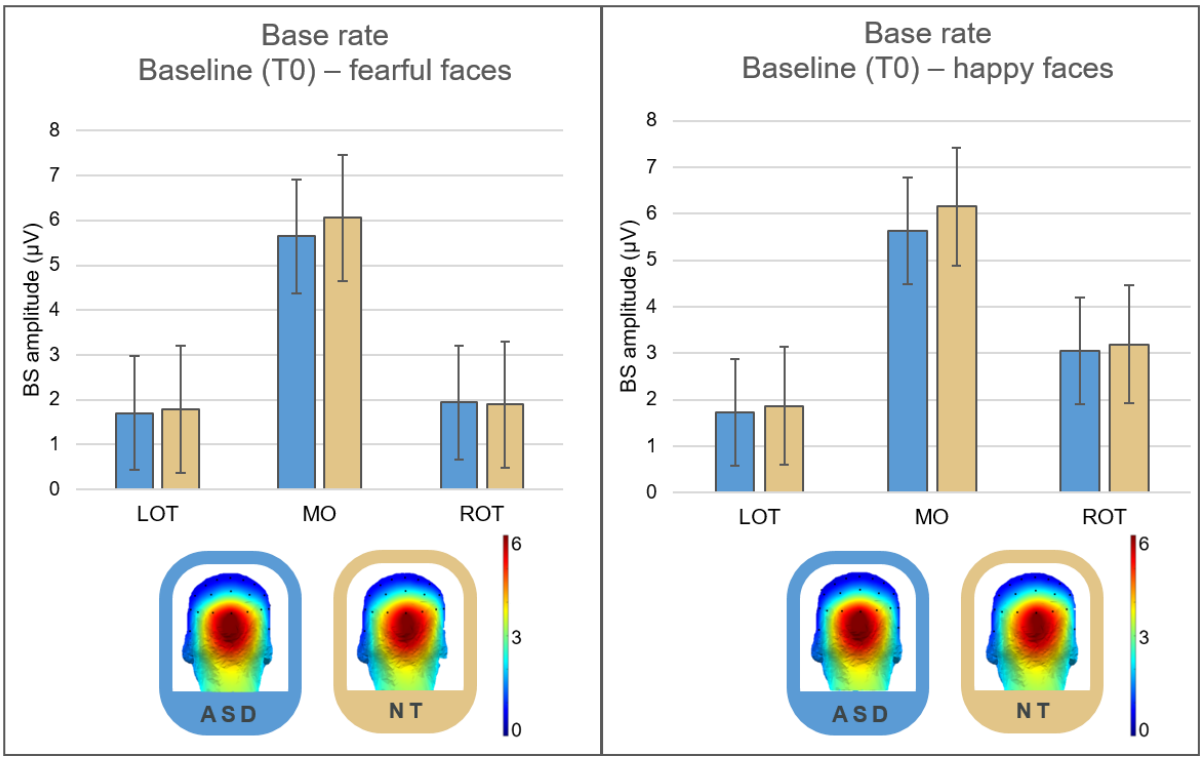

Figure S2. No diagnostic-group differences in base rate BS amplitudes at baseline (T0). EEG responses at the base rate frequency (summed BS amplitudes) are shown in bar graphs and scalp topographies, separately for the ASD and NT groups and left and right occipito-temporal (LOT and ROT) and mid-occipital (MO) regions. Error bars denote standard errors of the mean.

No treatment-related differences in base rate BS amplitudes

Post-treatment assessment (T1). Similar to the T0 assessment, base rate responses recorded at the post session (T1) were not significantly different between OT and PL groups ($F(1, 56.14) = 1.49$; $p = 0.227$). However a significant effect of *ROI* was found, indicating that also here the MO region ($M_{MO} = 6.23$ µV) displayed the strongest base rate response, compared to LOT and ROT ($M_{LOT} = 1.93$ µV; $M_{ROT} = 2.06$ µV; $F(2, 55.03) = 194.5$, $p < 0.001$, see **Fig. S3**), irrespective of facial expression.

Four-week follow-up assessment (T2). Also at T2, no significant *treatment* effect on the base rate was identified ($F(1, 49.19) = 0.67$, $p = 0.418$). Similar to T0 and T1, a main effect of *ROI* was identified, indicating again, maximal base rate responses in the MO region (M_{MO} = 6.14 µV), compared to the LOT and ROT regions (M_{LOT} = 1.95 µV; M_{ROT} = 2.17 µV; $F(2, 97.46) = 195.33$, $p < 0.001$, see **Fig. S4**), irrespective of facial expressions.

To conclude, on all three time points (T0, T1 and T2), the MO region displayed the strongest neural responses to the base rate images, which is in line with prior research.

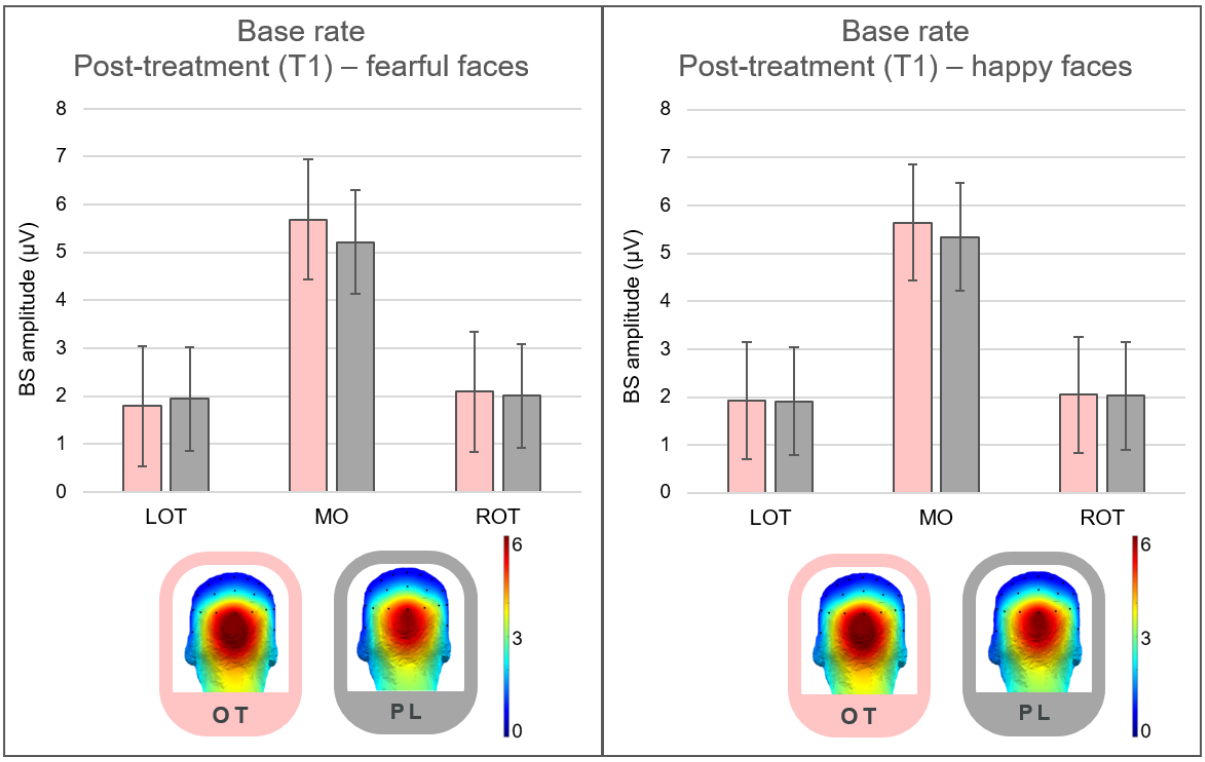

Figure S3. No treatment-related differences in base rate BS amplitudes post-treatment (T1). EEG responses at the base rate frequency (summed BS amplitudes) are shown in bar graphs and scalp topographies, separately for the oxytocin (OT) and placebo (PL) groups and left and right occipito-temporal (LOT and ROT) and mid-occipital (MO) regions. Error bars denote standard errors of the mean.

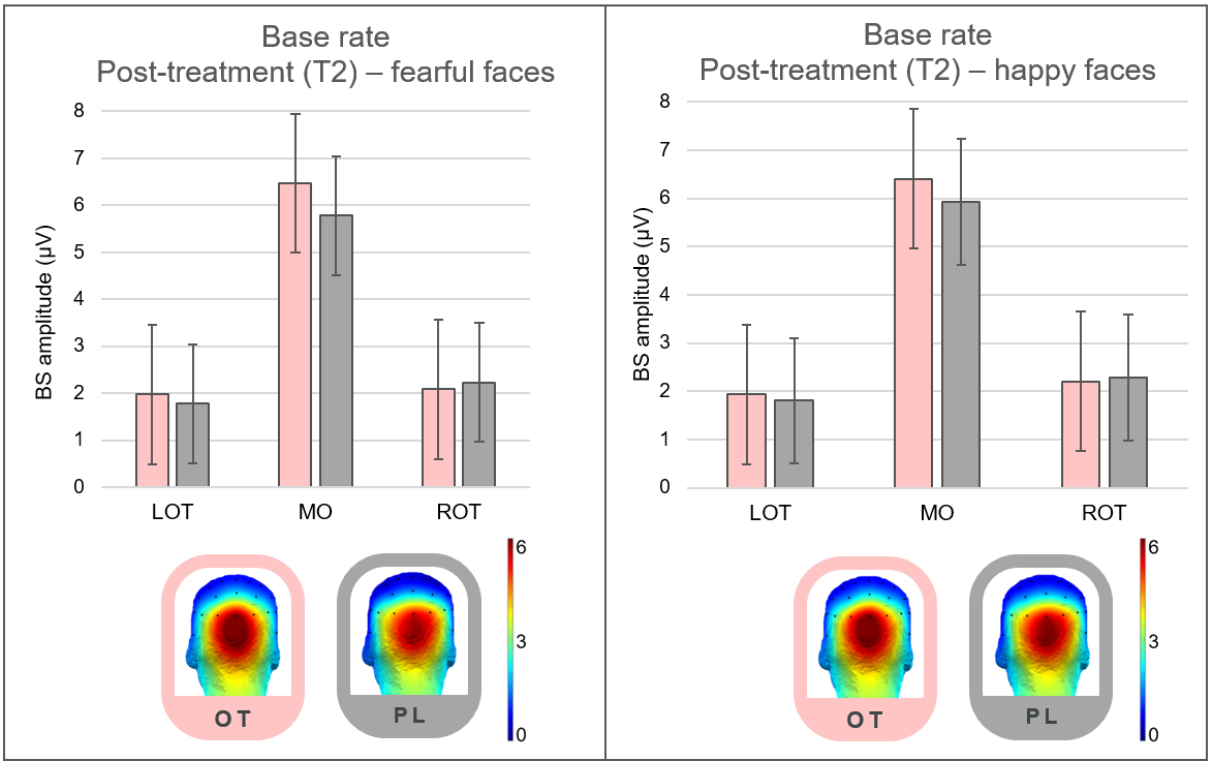

Figure S4. No treatment-related differences in base rate BS amplitudes at follow-up (T2). EEG responses at the base rate frequency (summed BS amplitudes) are shown in bar graphs and scalp topographies, separately for the oxytocin (OT) and placebo (PL) groups and left, right occipito-temporal (LOT and ROT) and mid-occipital (MO) regions. Error bars denote standard errors of the mean.

No change in base rate BS amplitudes throughout the clinical trial.

No effect of *time point* was found post-treatment (T1) nor on follow-up (T2), across expressions (fearful, happy) neither in the OT-group nor the PL-group (all $p > 0.24$; see **Fig. S5)**.

Baseline (T0) to post-treatment (T1) assessment. Explicit testing of changes from T0 to T1 in each of the treatment groups separately, showed no effect of *time point* in the OT-group ($F(1, 322.59) = 0.27$, $p = 0.61$), nor in the PL-group ($F(1, 322.40) = 0.1$, $p = 0.76$; see **Fig. S5**, top part).

Baseline (T0) to follow-up (T2) assessment. Explicit testing of changes from T0 to T2 in each of the treatment groups separately, showed no effect of *time point* in the OT-group ($F(1, 333.38) = 1.38$, $p = 0.24$), nor in the PL-group($F(1, 349.18) = 0.56$, $p = 0.46$; see **Fig. S5**, middle part).

Post-treatment (T1) to follow-up (T2) assessment. Explicit testing of changes from T1 to T2 in each of the treatment groups separately, showed no effect of *time point* in the OT-group ($F(1, 250.36) = 0.01$, $p = 0.95$), nor in the PL-group($F(1, 288.38) = 0.03$, $p = 0.86$; see **Fig. S5**, bottom part).

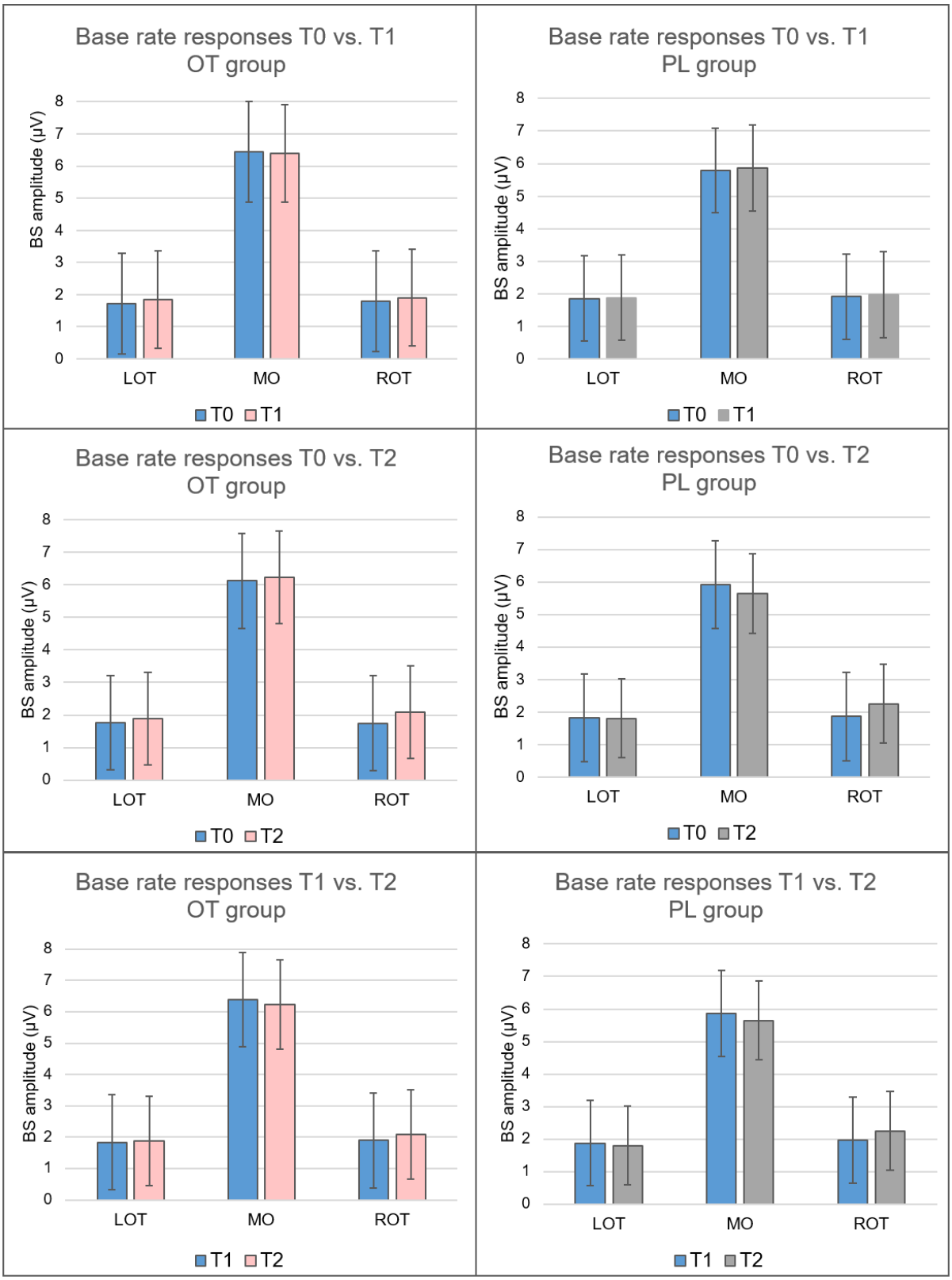

Figure S5. No change in base rate BS amplitudes throughout the clinical trial. EEG responses at the base rate frequency (summed BS amplitudes) are shown in bar graphs, separately for left and right occipito-temporal (LOT and ROT) and mid-occipital (MO) regions. Separate graphs within each treatment group (**left panels** within the oxytocin (OT) and the **right panels** within the placebo (PL) groups) and per time point comparison: **top panels** show the comparison T0 vs. T1, **middle panels** T0 vs. T2 and **bottom panels** T1 vs. T2 and the. Error bars denote standard errors of the mean.

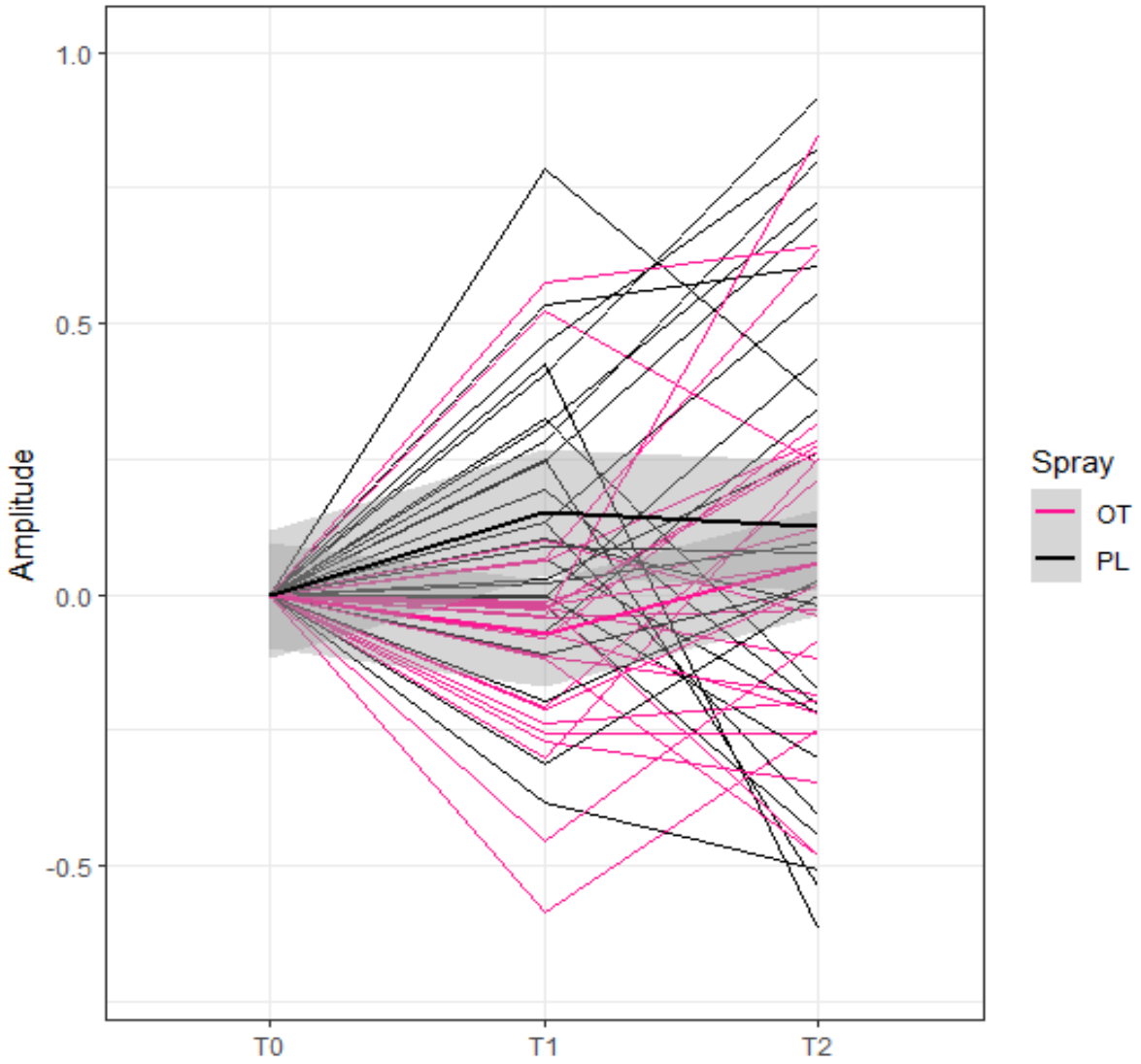

Figure S6. Spaghetti plot of the oxytocin treatment effects at post-treatment (T1) and follow-up (T2). Change from baseline (CFB) in expression-discrimination EEG responses at the oddball frequency (summed BS amplitudes) recorded at the baseline (T0), post-treatment (T1) and follow-up session (T2) are visualized separately for the oxytocin (OT) and placebo (PL) group in a spaghetti plot. EEG responses across LOT and ROT regions were averaged for fearful and happy faces, considering that no significant effects of expression were evident. A main effect of treatment was found at T1 (OT < PL, $p = 0.006$), and a treatment x ROI interaction effect at T2 (for ROT: OT < PL, $p = 0.014$).

Chapter 6 | Effect of repeated intranasal oxytocin administration on fMRI face processing responses in children with ASD: a randomized controlled trial

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Moerkerke, M., Daniels, N., Van der Donck, S., Grytsenko, N., Tang, T., Debbaut, E., Bamps, A., Prinsen, J., Steyaert, J., Alaerts, K., Boets, B. (2022). Effect of repeated intranasal oxytocin administration on face processing in children with ASD: a randomized controlled trial. *In preparation*

Abstract

Background: Difficulties with (non-verbal) social communication, including facial expression processing, constitute a hallmark of Autism Spectrum Disorder (ASD). Intranasal administration of oxytocin (OT) has been considered a potential treatment for improving social deficits in ASD, either by enhancing the salience of social cues or by reducing the social stress and anxiety experienced in social encounters.

Methods: An fMRI fearful face processing paradigm was implemented in a double-blind, placebo-controlled, multiple-dose OT clinical trial in 8-12 year old children with ASD (N_{OT}=20, N_{PL}=24) to evaluate the effect of repeated four-week OT administration on brain activity during face processing. Prior to the intervention, we compared the neural face processing signature of children with ASD with that of matched neurotypical (NT) controls (N=38).

Results: We observed lower activity in ASD compared to NT control children in early visual processing regions, but increased peak activity in the inferior frontal cortex and the left amygdala. The four-week OT treatment in the ASD children did not significantly alter these neural activity patterns. Yet, a weak statistical treatment-specific effect was identified in the left superior temporal sulcus (STS) higher-order visual integration region, indicating reduced STS activity in the OT compared to the placebo group (p_{unc} = 0.03).

Conclusion: These findings indicate (i) atypical face processing fMRI responses in children with ASD compared to matched NT controls, (ii) no OT-induced restoration of neural activity in these altered regions, but (iii) an attenuating effect of repeated OT on STS activity, likely supportive of the anxiolytic account of OT.

Introduction

Autism Spectrum Disorder, also known as ASD, is an early-onset neurodevelopmental condition marked by challenges in social interaction and communication as well as the presence of repetitive and restrictive patterns of behaviours, interests or activities (American Psychiatric Association, 2013). Impairments in non-verbal social communication, such as inferring social meaning from a face, are included in the clinical criteria and may play a key role in the socio-communicative difficulties experienced by individuals with ASD (Aoki et al., 2015). Thus far, no targeted pharmacological interventions have been established to enhance these socio-communicative difficulties.

Functional neuroanatomy of face processing

Accurately and rapidly reading faces and facial expressions is important for fluent social interactions (Rossion, 2014). Our brain extracts the social meaning from a face, generates emotional reactions, activates memories, directs attention and drives social actions through several brain circuits (Elfenbein & Ambady, 2002).The human brain has an innate preference for faces compared to objects (Johnson et al., 1991, 2015) and relies on separate neural mechanisms for processing faces versus objects, as evidenced by the fact that prosopagnosia can exist without the presence of object agnosia (Busigny et al., 2010; Duchaine & Nakayama, 2006; Riddoch et al., 2008). Visual facial information enters the brain via the inferior occipital cortex, which hosts the occipital face area (OFA). This region is involved in accurate face perception, with a preference for parts of faces (e.g. the eyes or mouth) (Pitcher et al., 2011). This supports the idea that the inferior occipital region characterizes low-level facial features and performs early face perception (Haxby et al., 2000). A ventral pathway connects the OFA to the fusiform face area (FFA) (Kanwisher et al., 1997), which primarily processes static features of a face, needed for instance for identity recognition (Axelrod & Yovel, 2015; Goesaert & op de Beeck, 2013). A parallel more dorsal pathway connects OFA with the superior temporal sulcus (STS), a region mainly involved in the dynamic aspects of the face, such as facial expression or eye-gaze (Haxby et al., 2000; Ishai et al., 2005; Puce et al., 1998). Next, an extended face processing network further extracts specific information from the faces. The amygdala is a subcortical region, part of the limbic system, which is mainly involved in the perception of emotional expressions (Adolphs, 2008). Fearful and angry expressions, specifically,

elicit higher amygdala reactivity compared to neutral faces (Zalla & Sperduti, 2013). The anterior temporal cortex is involved in high-level processing of the individual identity of a face, through retrieving semantic knowledge about a perceived identity (Brambati et al., 2010; Jonas et al., 2016). The inferior frontal gyrus plays a part in understanding dynamic features of a face such as gaze and eye movement (Brambati et al., 2010; Duchaine & Yovel, 2015).

Functional neuroanatomy of face processing in ASD

Findings on behavioural deficits in face processing in ASD are generally very mixed (probably due to the use of compensatory approaches) (Rosset et al., 2008; Uljarevic & Hamilton, 2013; van der Geest et al., 2002), but studies employing neuroimaging techniques reveal deficits and atypical processing strategies more convincingly. Neural atypicality's in face processing have frequently been reported in ASD, with reviews mainly pointing towards a pattern of reduced neural activity when looking at faces, particularly in the inferior occipital, fusiform, superior temporal and inferior frontal regions, as well as in the amygdala (di Martino et al., 2009; Kleinhans et al., 2011; Nomi & Uddin, 2015; Philip et al., 2012). In particular when examining expressive face processing, reduced activity has often been observed in the amygdala, fusiform and superior temporal regions in children, adolescents and adults with ASD (Aoki et al., 2015; di Martino et al., 2009; Kleinhans et al., 2011; Nomi & Uddin, 2015; Philip et al., 2012). Conversely, some studies have also demonstrated increased activity in the amygdala in ASD, both towards neutral and expressive faces (Monk et al., 2010; Tottenham et al., 2014; Weng et al., 2011), which has been interpreted as increased emotional arousal in response to an aversively experienced social stimulus. In this regard, Kleinhans et al. (2010) found that higher amygdala activation within the ASD group was related with increased social anxiety.

Effect of intranasal oxytocin administration on fMRI face processing responses

Oxytocin (OT) is an endogenous neuropeptide synthesized in the hypothalamus. It plays an important role in human social behaviour (Heinrichs et al., 2009; Schulze et al., 2011) and acts as a neuromodulator in several brain regions, including the amygdala and other face processing regions (M. Bakermans-Kranenburg & van Ijzendoorn, 2013; Bartz et al., 2011; Jurek & Neumann, 2018; Landgraf & Neumann, 2004). OT can be delivered intranasally and has been shown to improve facial identity and expression recognition

and prosocial behaviour, both in neurotypicals (NT) and in various clinical populations (M. Bakermans-Kranenburg & van Ijzendoorn, 2013; Bate et al., 2015; Van IJzendoorn & Bakermans-Kranenburg, 2012). At a mechanistic level, the social salience hypothesis and the anxiolytic hypothesis are two leading (not mutually exclusive) accounts on how OT can mediate social behaviour accounts.

First, the social salience hypothesis argues that OT primarily enhances attention to and perception of social cues (e.g. faces) by selectively increasing neural activity in the corresponding brain regions. Specifically, research has demonstrated that a single-dose of intranasal OT administration can increase activity in the amygdala, fusiform, superior temporal and inferior frontal regions during expressive face processing in NT adults (Domes et al., 2010; Gamer et al., 2010). In adults with ASD, single-dose OT studies have linked improved facial expression recognition with increased amygdala reactivity (Domes et al., 2013, 2014). Likewise, enhanced activity was found in STS after a single-dose of OT in adults with ASD, while processing emotionally charged point-light displays expressing body language (Bernaerts, Boets, Steyaert, et al., 2020). Note, however, that no differences in STS activity were evident in this same study after a four-week multiple-dose OT regime, indicating differential effects of single- versus multiple-dose OT administration (Bernaerts, Boets, Steyaert, et al., 2020). In children with ASD, single-dose intranasal OT was also shown to increase activity in brain regions related to social attention and perception (i.e. STS, posterior cingulate and premotor cortex) (Gordon et al., 2013).

Secondly, the anxiolytic account of OT highlights its regulating function on (autonomic) stress and (social) anxiety, which may thereby promote social approach behaviour and reduce social avoidance behaviour (Bethlehem et al., 2014; Ma et al., 2016; Quintana et al., 2015; Stoop, 2014). In contrast with the social salience account of OT, previous research has also demonstrated that a single-dose of intranasal OT administration can attenuate amygdala activity during expressive face processing in NT adutls (Domes et al., 2007; Gamer et al., 2010; Kanat et al., 2015; Kirsch, 2005). Moreover, this OT-induced dampening of amygdala reactivity has been shown to be related to decreased social stress and facilitated social interaction (Ma et al., 2016). Likewise, multiple-dose administration of OT in adults with ASD has been shown to dampen amygdala activity and amygdala-frontal connectivity while viewing social stimuli (Alaerts et al., 2020; Bernaerts, Boets, Steyaert, et al., 2020). This reduction in amygdala-frontal

connectivity was interpreted as a reduced need for top-down frontal control once amygdala activity has been attenuated (Alaerts et al., 2020). Together, these studies provide evidence for the attenuating effect of OT on neural activity in response to socially relevant cues (e.g. faces), likely reflecting the neuromodulatory effect of OT in reducing emotional arousal and stress.

In an attempt to reconcile these conflicting findings and theories, it has been postulated that the contrasting neural effects of OT may be due to variability in person-dependent characteristics (such as anxiety levels, diagnosis, gender, etc. (Ma et al., 2016)) or context-dependent variability (e.g., oxytocin can enhance cooperation within a trusting context, but in a threatening one it can decrease such prosocial behaviour (Declerck et al., 2010; Shamay-Tsoory et al., 2009)).

The current study

Using functional magnetic resonance imaging (fMRI) and a double-blind placebo-controlled randomized clinical trial design, we assessed the effect of four weeks of daily intranasal OT administration on neural face processing in children with ASD. In line with previous multiple-dose OT studies (Alaerts et al., 2020; Bernaerts, Boets, Steyaert, et al., 2020), it can be hypothesized that OT administration may induce a general attenuation of arousal and neural responsivity towards faces, in this case we would expect to find a dampening of neural activity. However, in line with the social salience account of OT (and with several single-dose OT studies in ASD, cf. supra), an increase of the salience of the presented faces might also be anticipated, in this case we would expect to find increased neural activity. In addition to the ASD sample, an age and gender matched group of NT control children also performed the fMRI face processing task once (pre-treatment; NT children did not receive any treatment), in order to examine baseline group differences in neural face processing contrasting NT children with those with ASD. In this regard, similar to prior findings, we generally expected to find reduced neural activity towards faces in the classical face regions in the ASD group compared to NT peers (Nomi & Uddin, 2015). Yet, enhanced neural activity, in particular in amygdala, could also be anticipated in ASD (e.g. Monk et al., 2010; Tottenham et al., 2014; Weng et al., 2011; Kleinhans et al., 2010)

Materials & Methods

Clinical trial design

We conducted a single-centre, two-arm, double-blind, randomized, placebo-controlled parallel study at the Leuven University Hospital (Belgium) to assess the effects of four weeks of intranasal OT administration on the face processing circuitry using fMRI (see **Fig. 1** for the CONSORT flow diagram). Children with ASD performed a basic face processing block design fMRI task at baseline (T0) and post-treatment (T1) (24 hours after the last nasal spray administration). Additionally, at baseline (T0) the face processing fMRI task was also administered in a sample of NT control children to compare neural face processing correlates with those in the ASD group. Study procedures and informed consent forms were approved by the Ethics Committee for Biomedical Research at the University of Leuven, KU Leuven (S61358) in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The trial was registered at the European Clinical Trial Registry (EudraCT 2018-000769-35; https://www.clinicaltrialsregister.eu/ctr-search/trial/2018-000769-35/BE). The fMRI recordings were part of a larger assessment protocol, which aimed at evaluating clinical efficacy of OT treatment on several autism symptom questionnaires (see Daniels et al., 2022) and on neural sensitivity towards expressive faces as assessed with EEG (see **chapter 5**). In short, these parallel reports indicate a general but no treatment-specific improvement in autism characteristics (Daniels et al. 2022) and a significant OT-induced dampening of the neural sensitivity towards subtle socio-communicative facial cues (see **chapter 5**).

Participants

ASD participants were recruited through the Leuven Autism Expertise Centre, KU Leuven, which started in July 2019 and ended in January 2021. Neurotypical children were recruited through elementary schools. For all children the parent-rated Social Responsiveness Scale-Children, 2nd edition (SRS-2; (J. Constantino & Gruber, 2012) and verbal and performance intelligence quotients (IQ; WISC-V-NL; (Wechsler, 2018) were acquired. The Autism Diagnostic Observation Schedule, 2nd edition (ADOS-2; (Lord et al., 2012) was also administered in the children with ASD. Diagnostic-groups were matched on age, gender and performance IQ, while verbal IQ was significantly higher in the NT

group compared to the ASD group (**Table 1**). Before the treatment, the PI of the clinical trial randomly allocated the ASD children to receive OT or placebo (PL), and matched both groups as best as possible on age, gender and IQ. There were no significant differences between randomized treatment groups in terms of age, gender, IQ and ASD symptomatology (**Table 1**). See Suppl. Mat., for more participant information.

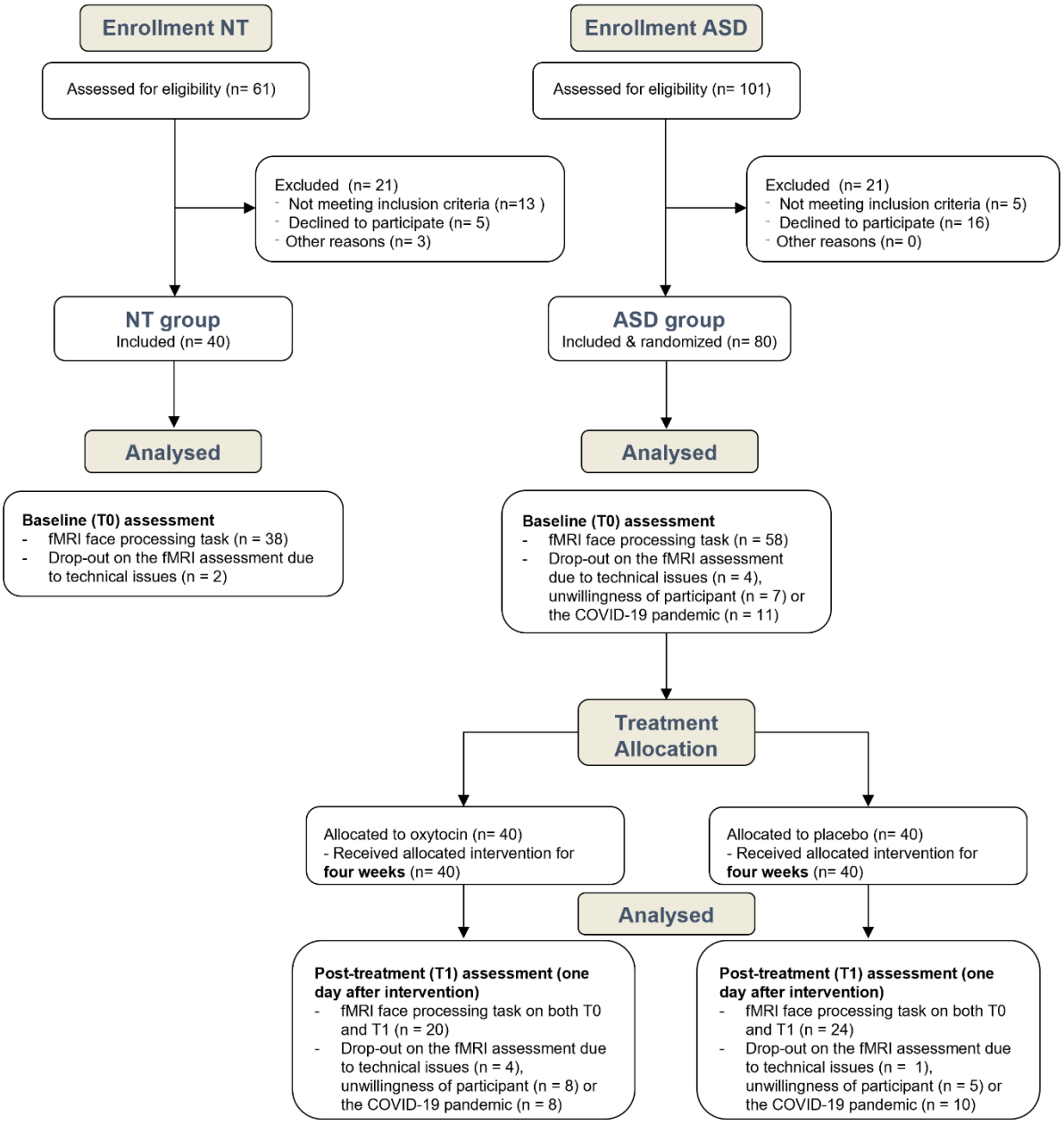

Figure 1. CONSORT flow diagram. Participants of the neurotypical (NT) or autism spectrum disorder (ASD) group were recruited and assessed at baseline (T0). Next, the ASD group was allocated to receive either oxytocin or placebo (four weeks of twice daily intranasal administrations), followed by a post-treatment (T1) assessment. As outlined, for several participants, fMRI recordings were not acquired at one or both assessment sessions due to physical contact restrictions and closing down of hospital facilities during the COVID-19 pandemic, due to technical issues or because children refused to participate in the relatively invasive MRI part of the study.

Table 1. **Demographic characteristics of the trial participants at baseline.**

Measures	ASD (*n* = 58)	NT (*n* = 38)	*P*-value[a]	ASD-OT (*n* = 20)	ASD-PL (*n* = 24)	*P*-value[a]
♂:♀	46:12	30:8	0.99	18:2	21:3	0.95
Age[b] (mean ± SD)	9.93 ± 1.26	9.79 ± 1.28	0.59	10.20 ± 1.36	9.88 ± 1.26	0.47
VIQ[c] (mean ± SD)	108.78 ± 12.24	117.76 ± 15.22	<0.01*	104.25 ± 16.05	111.61 ± 16.61	0.15
PIQ[d] (mean± SD)	104.40 ± 13.77	107.76 ± 12.46	0.22	101.60 ± 13.47	103.43 ± 12.01	0.64
SRS-2[e] (mean ± SD)	88.66 ± 20.97	21.03 ± 12.267	<0.001*	88.65 ± 22.53	85.63 ± 20.78	0.65
ADOS-2[f] (mean ± SD)	-	-	-	9.22 ± 3.41	9.16 ± 4.13	0.96

[a]*P*-values based on independent, two-sample *t* tests or Chi-square tests. [b]Age expressed in years. [c]Verbal intelligence quotient (IQ) was derived from the subtests Similarities and Vocabulary, Wechsler Intelligence Scale for Children, Fifth Edition, Dutch version (WISC-V-NL; Wechsler, 2018). [d]Performance IQ was derived from the subtests Block design and Figure puzzles (WISC-V-NL; Wechsler, 2018). [e]Social responsiveness Scale-Children, 2nd edition (SRS-2; J. Constantino & Gruber, 2012). [f]Prior to randomization, the Autism Diagnostic Observation Schedule, 2nd edition (ADOS-2; Lord et al., 2012) was administered in the ASD group. *Significant difference at $p < 0.05$ statistical threshold.

OT administration

The ASD participants administered OT (Syntocinon®, Sigma-tau) or placebo nasal sprays. Placebo sprays contained all the ingredients as the active solution, except for the OT compound. Sprays were prepared by the University Hospital of Heidelberg (Germany) to look identical in 10 ml brown glass bottles and had a white nasal pump (0.05 ml or 2 IU /puff). Before the start of the study, participants and their parents received clear instructions on how to administer the nasal spray (Guastella et al., 2013). Participants administered the nasal spray twice daily, six puffs (three per nostril) or 12 IU in the morning and six puffs in the afternoon (after school), resulting in a daily dose of 24 IU. On day 28, i.e. the last day before the post-treatment assessment (T1), participants withheld the afternoon spray, in order to avoid single-dose OT effects. Potential adverse events were recorded through weekly parent reports and daily parent and child diaries, and revealed minimal and non-treatment specific side effects (see Daniels et al., 2022).

Face processing fMRI

Block-design fMRI task

Participants looked at consecutively presented series of (scrambled) faces while performing a gender discrimination task. Blocks of neutral (N), fearful (F) and scrambled (S) faces were alternately projected (**Fig. 2**). Each block lasted 15.75 seconds and comprised of 21 faces (from seven different identities) that were each presented for 0.75 seconds each. A whole run lasted 236.25 seconds, and each participant completed two

runs. A fixation cross (fix), was shown for 15.75 seconds at the beginning of the trial, after six face blocks, and at the end of the trial. Two runs were semi-randomly created, with the following sequences: fix-F-N-S-N-F-S-fix-S-F-N-S-N-F-fix and fix-S-F-N-S-N-F-fix-F-N-S-N-F-S-fix. Male and female faces were shown in separate runs. Combining sequence order and gender resulted in four possible sequences, which were randomly presented using Matlab R2018b (MATLAB and Statistics Toolbox Release 2018b, The MathWorks, Inc., Natick, Massachusetts, United States). During the run with female faces, a male face appeared randomly 2 or 3 times per block, and vice versa for the run with male faces. In order to ensure focus on the screen and attention to the face characteristics, participants were instructed to press a button with the thumb of their dominant hand whenever this gender switch occurred. During the scrambled condition the scrambled faces randomly (2 or 3 times per block) changed to a plain face-silhouette, when this happened participants were instructed to press the button as well.

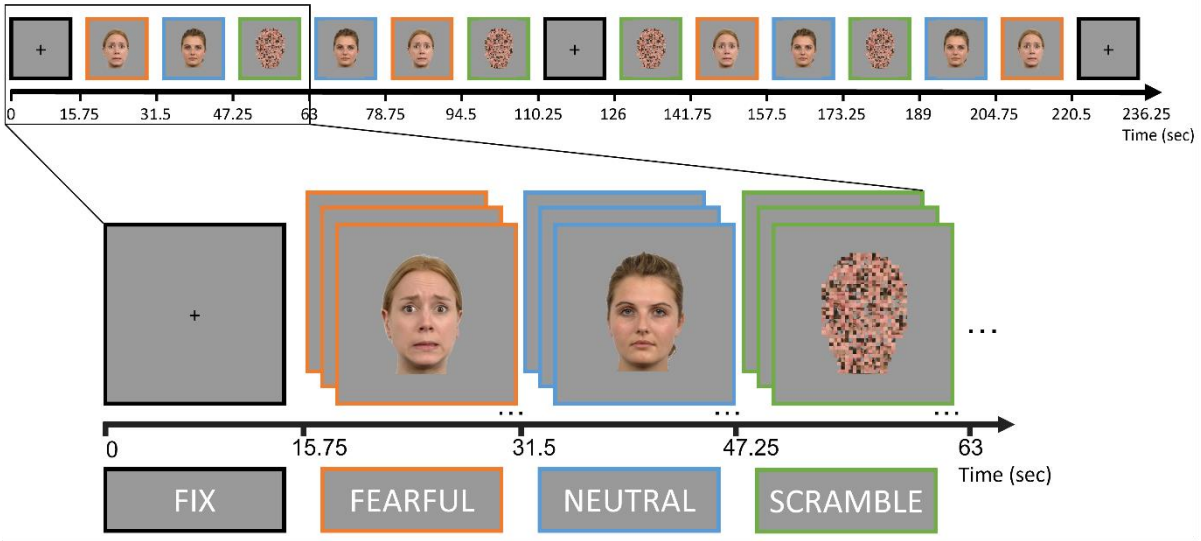

Figure 2. The face processing fMRI paradigm. Blocks of series of neutral, fearful and scrambled faces were alternately projected while participants performed a gender discrimination task. A fixation cross (fix) was shown for 15.75 seconds at the beginning of the trial, after six face blocks, and at the end of the trial. Each block lasted 15.75 seconds, a whole run lasted 236.25 seconds. Twenty-one faces (from seven different identities) were presented for 0.75 seconds each in one block. Male and female faces were shown in separate trials, counterbalanced across runs.

Stimuli

Seven male and seven female identities were chosen from the Radboud Faces Database (RaFD) (Langner et al., 2010). For each identity, a face with a neutral and a fearful facial expression was included. All fearful facial expressions were reliably rated as "fearful" (91.4% agreement (Langner et al., 2010)). The scrambled faces (with a grid size of 10, rendering 30 x 45 grids) were created based on seven, arbitrarily chosen, facial identities

(since the other conditions also included 7 different identities), three with a fearful and four with a neutral facial expression, using an online tool (www.webmorph.org). All images were 5.5 x 8 cm and placed on an 8 x 12 cm grey background. The visual angle of the face stimuli extended 4.50 x 6.54 degrees.

fMRI data acquisition

Structural and functional MRI images were obtained using a 3 Tesla Philips Ingenia CX MR scanner (Best, The Netherlands). A 32-channel head coil placed over the head of the participant contained a mirror so the participant could look at a screen placed behind the MRI. fMRI series were acquired using a BOLD sensitive echo planar imaging sequence (TR/TE 1500/30 ms, 80° flip angle, 228 x 228 mm^2 field of view, 48 axial slices, multiband slice order, 2.75 mm tick, in plane voxel size 2.75 mm^2). Structural scans were collected using a standard T1-weighted pulse sequence (TR/TE 9.6/4.6 ms, 8° flip angle, 250 x 250 mm^2 field of view and voxel size 0.97 x 0.97 x 1.2 mm).

fMRI data analysis

MRI data analyses were performed using SPM 12 software (Statistical Parametric Mapping, Matlab, The MathWorks, Inc., Natick, Massachusetts, United States).

Preprocessing

First, the structural images were manually positioned according to the anterior commissure - posterior commissure line. Second, the functional images were slice timing corrected and realigned to the first functional image. The six head motion parameters (three translational and three rotational), obtained during this process were used as confounds in the general linear model. Third, individual scans displaying a framewise displacement (FD) exceeding 0.9 mm were scrubbed using Artifact Detection Tools (ART, there was no limit to the number of scrubbed volumes). Fourth, the structural images were co-registered to the functional images. Fifth, structural and functional images were normalized to MNI space, resampling the scans to a voxel size of 2.5 x 2.5 x 2.5 mm. Lastly, the images were smoothed with a Gaussian kernel with a full-width half maximum (FWHM) of 8 mm to increase the signal to noise ratio.

Regions of interest

We performed targeted analyses in predefined regions of interest (ROI), chosen a priori based on the face processing neuroimaging literature. Specifically, we used the ROIs built

by Hendriks et al., which were applied in a unique multi-method fMRI study on neural face processing in adults with and without ASD (Hendriks et al., 2021). Seven brain regions involved in the core and extended face processing network were included: the inferior occipital cortex (including the OFA), the posterior temporal cortex (including the FFA), and the superior temporal cortex (including the STS), the amygdala, the anterior temporal cortex, the inferior frontal cortex and primary visual cortex V1, see **Fig. 3**. Thirteen ROIs were used since regions were split into a left and right sided ROI except for V1. These ROIs were created by Hendriks et al., based on anatomical masks from the WFU PickAtlas' 'aal' (Wake Forrest University PickAtlas, http://fmri.wfubmc.edu/cms/software) and functionally restricted by calculating the intersection with face-responsive voxels from a whole-brain second level "faces versus fixation" contrast, thresholded at $p < 0.005$, across all their participants (n=52) (Hendriks et al., 2021).

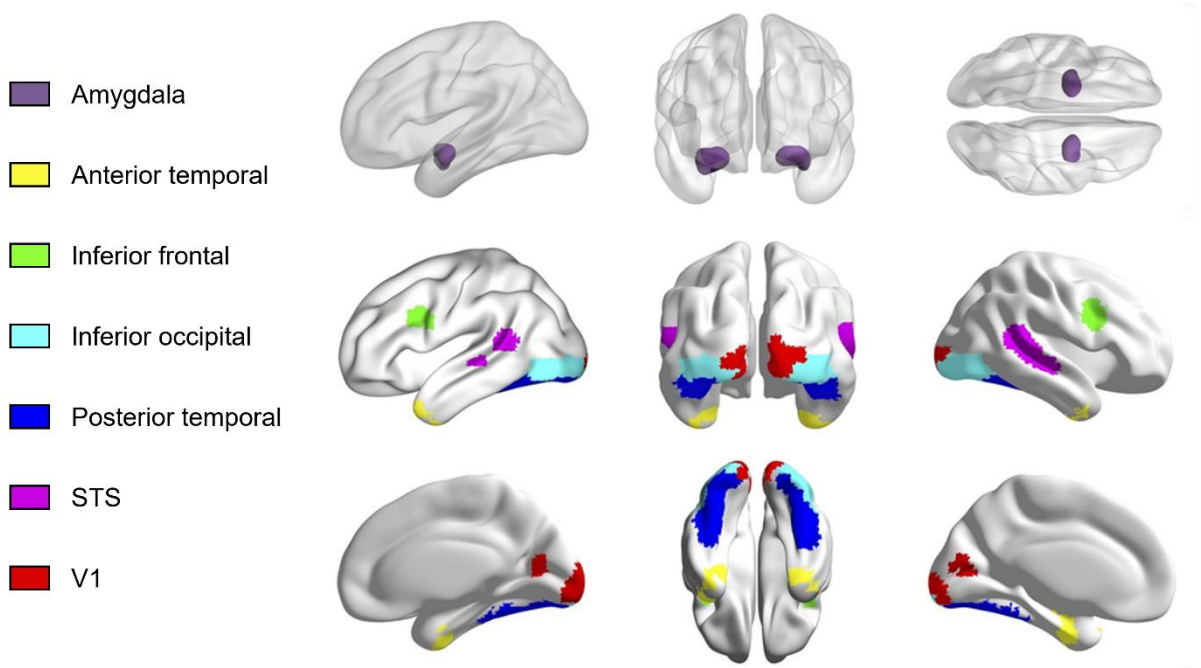

Figure 3. Regions of interest. The top row displays the subcortical region (amygdala) and the middle and bottom row show the six cortical regions of interest used in this study. These ROIs were created based on anatomical masks and were subsequently functionally restricted with voxels active during face processing. Figure reprinted from (Hendriks et al., 2021).

Statistical analysis

At subject level, a first-level general linear model (GLM) was built, based on the onset and duration of each stimulus block (condition). The GLM contained the following variables: two runs with each four conditions (fearful, neutral, scramble and fixation) and additionally six head-motion parameters. Estimation of the GLM resulted in beta-values

for each condition, which were used in the subsequent second-level group comparison. We conducted an ROI-based univariate analysis using custom code in MATLAB to examine diagnosis- or treatment-related differences in the processing of 'faces' (neutral and fearful combined) versus the fixation condition. In line with previous research (Hendriks et al., 2021), we calculated, for every participant, the average beta-value per ROI for the FACESvsFIX contrast. Additional exploratory analyses were performed, examining the effect of neutral and fearful expressions separately (NEUTRALvsFIX, FEARFULvsFIX, NEUTRALvsSCRAMBLED and FEARFULvsSCRAMBLED), see Suppl. Mat., Tables S1-4 for the diagnostic-group comparison and Tables S5-8 for the treatment effects for these contrasts. Further, to account for inter-individual variability in regional activation patterns, we also calculated the peak voxel activity per ROI for the FACESvsFIX contrast of each participant. For all group comparisons, values exceeding two standard deviations from the mean (i.e., the mean beta-value per group), were considered as outliers and were removed. Activity values were then used in two-sample *t*-tests to test for diagnosis-related differences. We corrected for multiple comparisons (i.e. the 7 ROIs) by controlling the false discovery rate (FDR) with $q < 0.05$ (Benjamini & Hochberg, 1995).

To examine treatment-related group differences, change from baseline scores (CFB) were calculated, i.e. we subtracted the beta-values for the activity per ROI at T0 from the beta-values for the activity per ROI at T1. To target exactly the same peak-region in T1 as in T0, for every participant, we created small 3 mm spheres surrounding the voxel with the peak-activity at T0. These spheres were then intersected with the original ROI, to ensure that all included voxels belong to the correct region. The peak-CFB scores were calculated by subtracting the average beta-values at T0 within these spheres, from those at T1. These values were then used in two-sample *t*-tests to test for treatment differences, again corrected for multiple comparisons by controlling the false discovery rate (FDR) with $q < 0.05$ (Benjamini & Hochberg, 1995).

Results

Diagnostic-group comparison for fMRI face processing responses

Diagnostic-group comparison of <u>average</u> ROI activity. Comparison of the average ROI activity between ASD and NT yielded no significant effect of diagnostic-group after correction for multiple-comparisons (all $p_{FDR} > 0.14$; **see Table 2 and Fig. 4**). Only at an uncorrected level, higher activity in right inferior frontal cortex ($t(88) = 2.02$, $p_{unc} = 0.04$) and lower activity in primary visual cortex (V1) ($t(88) = -2.61$, $p_{unc} = 0.01$) were evident in children with ASD, compared to the NT group. On average across the ROIs, 3.4 outliers were removed in the ASD group and 1.8 in the NT group.

Table 2. **Diagnostic-group comparison of average ROI activity during face processing.**

Average ROI activity FACES vs. FIX contrast	ASD Mean beta value	NT Mean beta value	*t* value	P_{unc}	P_{FDR}
Left amygdala	0.10	0.05	0.93	0.36	0.51
Right amygdala	0.09	0.06	0.45	0.65	0.77
Left anterior temporal	-0.03	0.04	-1.59	0.12	0.50
Right anterior temporal	-0.01	0.05	-1.19	0.24	0.51
Left inferior frontal	0.06	0.03	0.97	0.34	0.51
Right inferior frontal	0.27	0.16	2.02	**0.04***	0.31
Left inferior occipital	0.15	0.22	-1.36	0.18	0.51
Right inferior occipital	0.24	0.23	0.27	0.79	0.85
Left posterior temporal	0.23	0.22	0.18	0.86	0.86
Right posterior temporal	0.24	0.28	-0.75	0.46	0.60
Left STS	0.01	0.05	-0.93	0.36	0.51
Right STS	0.06	0.10	-1.21	0.23	0.51
V1	-0.03	0.10	-2.61	**0.01***	0.14

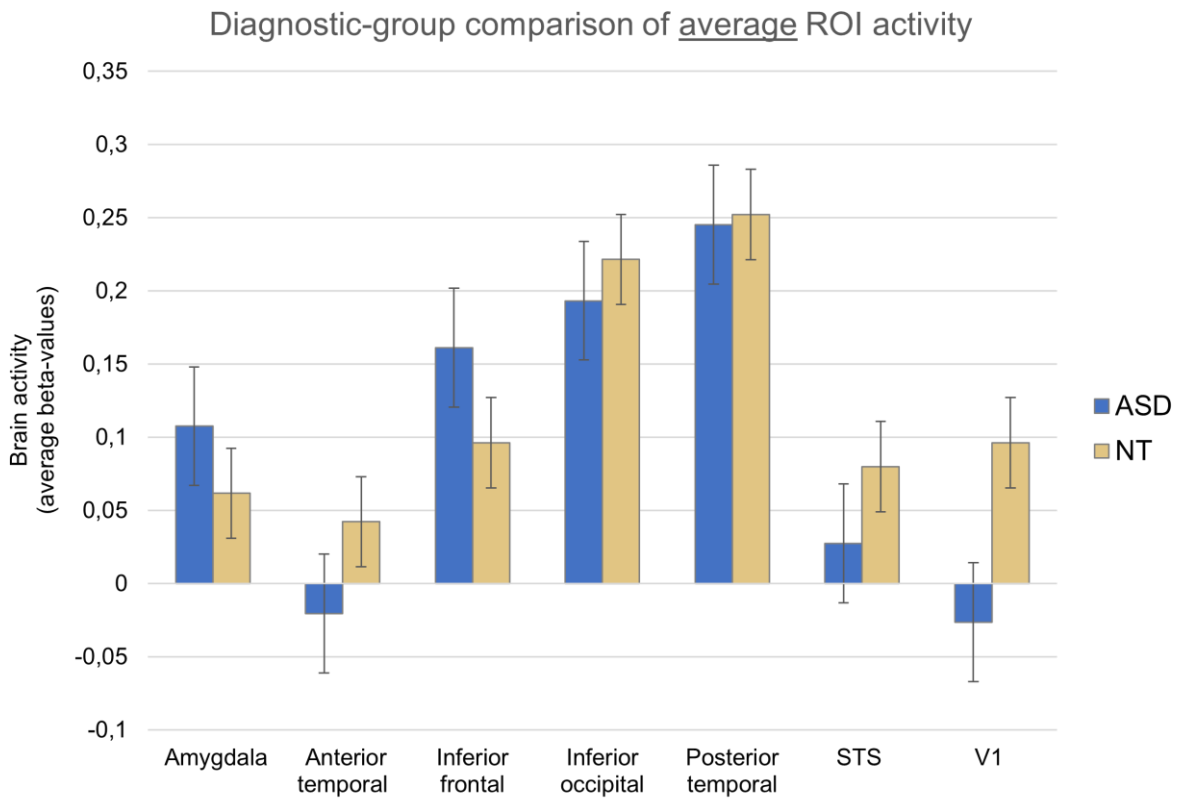

Figure 4. Diagnostic-group comparison of average ROI activity during face processing. fMRI face processing responses (average beta-values) are shown in bar graphs, for the ASD and NT groups and for all included ROIs. To simplify the visual representation we averaged the data across left and right homologue areas, although statistics were calculated for separate ROIs in each hemisphere. Error bars denote standard errors of the mean. Uncorrected effects of group were found in the right interior frontal (NT < ASD, $p = 0.04$) and in the primary visual cortex (V1) (ASD < NT, $p = 0.01$).

Diagnostic-group comparison of peak ROI activity. To account for inter-individual variability in regional brain activity, we also examined diagnosis-related differences in peak-voxel activity. Comparison of these peak ROI values between ASD and NT yielded highly significant group effects (**see Table 3 and Fig. 5**). First, analogous to the average-ROI analysis, we found significantly decreased neural activity in the V1 region ($t(90) = -4.0$, $p_{FDR} < 0.001$) as well as lower neural activity in left inferior occipital cortex ($t(89) = -6.10$, $p_{FDR} < 0.001$) in the ASD, compared to the NT group. Second, and again similar to the average-ROI analysis, children with ASD displayed significantly higher activity in right ($t(90) = 2.61$, $p_{FDR} = 0.03$) as well as left inferior frontal cortex ($t(94) = 2.92$, $p_{FDR} = 0.02$). Furthermore, significantly higher activity in left amygdala was evident in the ASD compared to the NT group ($t(89) = 2.64$, $p_{FDR} = 0.03$). On average across the ROIs, 2.5 outliers were removed in the ASD group and 1.75 in the NT group.

Table 3. Diagnostic-group comparison of peak ROI activity during face processing.

Peak ROI activity FACES vs. FIX contrast	ASD Mean beta value	NT Mean beta value	*t* value	P_{unc}	P_{FDR}
Left amygdala	0.60	0.40	2.64	**< 0.001***	**0.03***
Right amygdala	0.55	0.46	1.39	0.17	0.24
Left anterior temporal	0.31	0.31	-0.07	0.94	0.94
Right anterior temporal	0.18	0.27	-1.69	0.10	0.18
Left inferior frontal	0.31	0.16	2.92	**< 0.01***	**0.02***
Right inferior frontal	0.64	0.45	2.61	**0.01**	**0.03***
Left inferior occipital	0.26	0.66	-6.10	**< 0.001***	**< 0.001***
Right inferior occipital	0.58	0.71	-1.71	0.09	0.18
Left posterior temporal	0.91	0.90	0.09	0.93	0.94
Right posterior temporal	1.02	1.11	-0.78	0.44	0.57
Left STS	0.16	0.24	-1.54	0.13	0.21
Right STS	0.45	0.48	-0.47	0.64	0.75
V1	0.24	0.47	-4.0	**< 0.001***	**< 0.001***

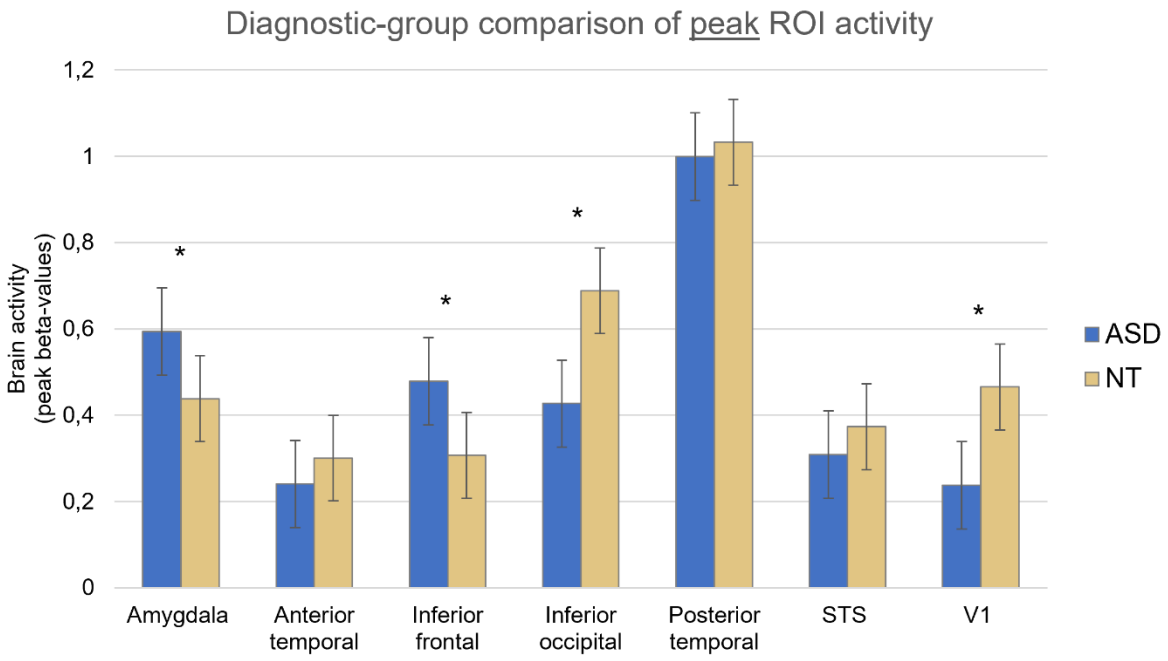

Figure 5. Diagnostic-group comparison of peak ROI activity during face processing. fMRI face processing responses (peak beta-values) are shown in bar graphs, for the ASD and NT groups and for all included ROIs. To simplify the visual representation we averaged the data across left and right homologue areas, although statistics were calculated for separate ROIs in each hemisphere. Error bars denote standard errors of the mean. Significant effects of group were found in the left (NT < ASD, $p = 0.02$) and right interior frontal cortex (NT < ASD, $p = 0.03$), the left amygdala (NT < ASD, $p = 0.03$), the left inferior occipital cortex (ASD < NT, $p = 0.03$) and in the primary visual cortex (V1) (ASD < NT, $p = 0.001$). Asterisks indicate significant group differences.

OT treatment effects on fMRI face processing responses in ASD

We calculated CFB scores by subtracting average beta-values on T0 from the average beta-values on T1. No significant effect of treatment was found (all $p_{FDR} > 0.46$; **see Table 4 and Fig. 6**). However, left STS activity was decreased in the OT group, compared to the placebo group ($t(38) = -2.19$, $p_{unc} = 0.03$), but this effect disappeared upon multiple comparison correction. On average across the ROIs, 0.9 outliers were removed in the OT group and 1.2 in the PL group.

To target exactly the same peak-region in T1 as in T0, we created small, 3 mm spheres surrounding the T0 peak voxel per ROI and calculated the CFB scores at T1. Also here, only the left STS had lower activity after OT compared to placebo ($t(36) = -2.16$, $p_{unc} = 0.04$), but again, this effect disappeared upon multiple comparison correction ($p_{FDR} = 0.51$). On average across the ROIs, 1.2 outliers were removed in the OT group and 1.5 in the PL group.

Table 4. **Treatment effect on the average CFB ROI activity during face processing.**

Average ROI activity FACES vs. FIX contrast	Oxytocin Mean CFB beta value	Placebo Mean CFB beta value	*t* value	P_{unc}	P_{FDR}
Left amygdala	-0.11	-0.05	-0.53	0.60	0.87
Right amygdala	-0.10	-0.02	-0.73	0.47	0.76
Left anterior temporal	0.02	-0.02	0.46	0.67	0.87
Right anterior temporal	-0.01	-0.12	1.11	0.27	0.76
Left inferior frontal	-0.09	-0.02	-0.81	0.43	0.76
Right inferior frontal	-0.13	-0.12	-0.06	0.95	0.95
Left inferior occipital	-0.01	0.10	-1.05	0.30	0.76
Right inferior occipital	-0.06	0.03	-1.08	0.29	0.76
Left posterior temporal	-0.15	0.01	-1.42	0.16	0.76
Right posterior temporal	-0.03	-0.04	0.10	0.92	0.95
Left STS	-0.14	0.06	-2.19	**0.03***	0.46
Right STS	-0.02	0.07	-0.88	0.40	0.76
V1	0.13	0.12	0.11	0.95	0.95

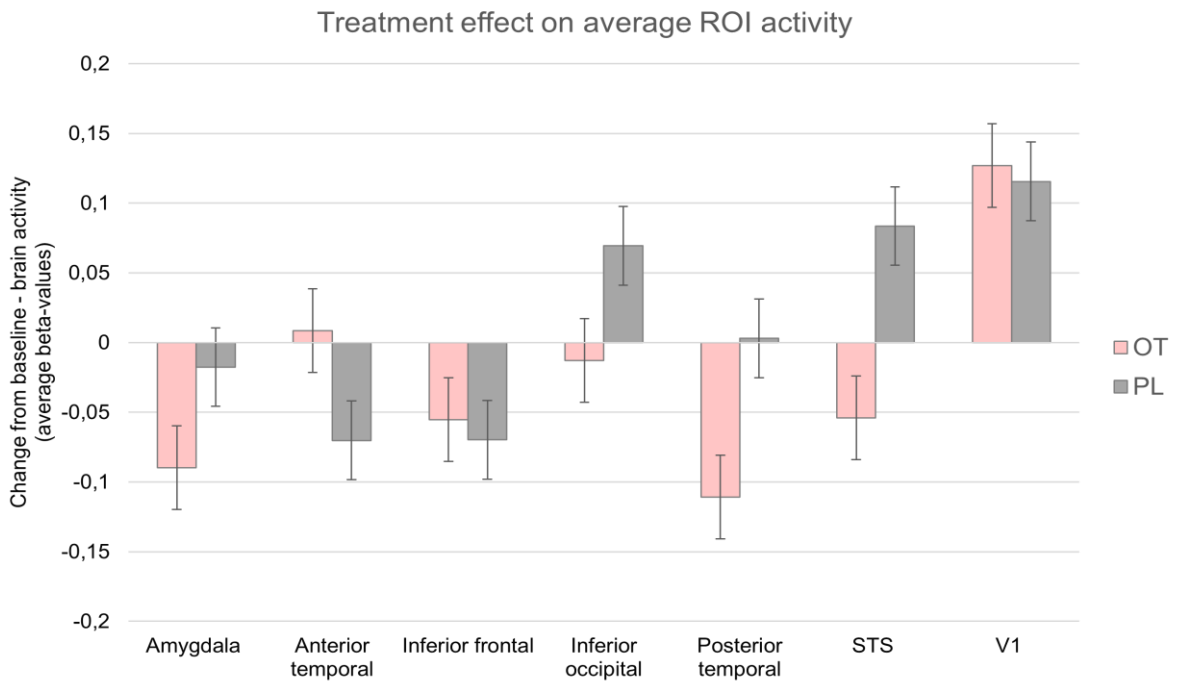

Figure 6. Treatment effect on the average change-from-baseline ROI activity during face processing. Change from baseline fMRI face processing responses (average CFB beta-values) are shown in bar graphs, for the OT and PL groups and for all included ROIs. To simplify the visual representation we averaged the data across left and right homologue areas, although statistics were calculated for separate ROIs in each hemisphere. Error bars denote standard errors of the mean. An uncorrected effect of treatment was found in the left STS (OT < PL, $p = 0.03$).

Control measures: orthogonal task and head motion

Average performance accuracy on the orthogonal task was 81.99%, with no significant diagnostic-group (ASD = 78.69%, NT = 84,76%; $t(86) = 1.70$, $p = 0.09$) or treatment-related differences (ASD_OT = 85.61%, ASD_PL = 83,46%; $t(34) = 0.68$, $p = 0.50$).

Average FD head motion did not differ between diagnostic-groups ($t(94) = 1.2$, $p = 0.23$) nor did we observe treatment-related differences ($t(42) = 0.42$, $p = 0.68$).

Discussion

The present study compared fMRI face processing responses (i.e., neural activity) of children with ASD with those of matched NT controls, and subsequently investigated the effect of repeated OT administration on these responses in ASD. A targeted ROI-based approach was applied, pinpointing neural activity in a predefined set of brain regions encompassing the broader face processing network. While fully controlling for multiple comparisons across this broad set of regions, no significant diagnostic-group differences in average activity were identified in children with ASD versus NT, possibly due to the

large inter-individual variability and because the large size of the ROIs might mask group differences in more regional activity. In an effort to take this into account, we also calculated the peak activity per ROI per participant, and we compared the ASD versus NT groups on peak voxel activity. This analysis yielded significantly reduced neural activity in ASD compared to NT in primary visual and inferior occipital cortex, as well as significantly increased activity in ASD in inferior frontal cortex and left amygdala. Crucially, repeated OT administration did not significantly affect activity in these brain regions. Yet, at an uncorrected level, we see a dampening effect of OT on average and peak STS activity, compared to placebo. Taken together, these findings provide suggestive evidence for an attenuating effect of repeated OT on fMRI face processing responses in children with ASD, which may be in line with the anxiolytic account of OT.

Diagnostic-group comparison in fMRI face processing responses

Despite previous research generally reporting reduced activation of face processing brain regions in individuals with ASD, we did not find significant diagnostic-group differences in the average ROI analysis. Interestingly, the prior study of Hendriks et al., (2021), applying an identical average-ROI analysis in adults with ASD, neither revealed significant group differences. Yet, when applying a more lenient uncorrected statistical threshold, Hendriks et al., also identified reduced activity in the primary visual cortex (V1) in ASD, identical to the current findings. Using this same uncorrected threshold, we also demonstrated significantly higher neural activity in the inferior frontal cortex in ASD. As inter-individual variability in regional activation patterns across these relatively large ROIs might mask more subtle group differences, we also turned towards an individual-peak level analysis. Here, we again demonstrated significantly lower V1 activity in ASD, as well as in the inferior occipital cortex. The latter finding is in line with the general notion of lower neural face processing activity in ASD, even though this has typically been observed in higher-level secondary association cortices or social brain regions (Humphreys et al., 2008; Suzanne Scherf et al., 2010). In contrast, in low-level primary sensory areas (such as V1) *increased* activity has often been reported in ASD (Samson et al., 2012), which has been interpreted as an increased mobilisation of low-level resources for compensatory (perceptual) strategies in ASD (Mottron et al., 2006; Sapey-Triomphe et al., 2020). The peak level analysis also revealed increased inferior frontal activity in

ASD compared to NT, which might indicate an increased recruitment of cognitive control resources as the face processing task required selective attention for interpreting the gender of the face stimuli. When given explicit instructions to infer facial features, individuals with ASD often exhibit atypical brain responses (Nomi & Uddin, 2015). Herrington et al., for instance, similarly demonstrated increased prefrontal cortex activity when attending faces, in children with ASD compared to NT controls, which may serve as a compensatory mechanism for impaired visual information processing in ASD (Herrington et al., 2015). Lastly, we also found increased activity in left amygdala in ASD compared to NT, which is in line with prior ASD studies (Monk et al., 2010; Tottenham et al., 2014; Weng et al., 2011). Notably, Kleinhans et al., revealed a positive relation between amygdala reactivity in ASD and social anxiety (Kleinhans et al., 2011), suggesting that face processing might be experienced as aversive in children with ASD and that amygdala hyper-reactivity might be an index of this. Weng and colleagues have demonstrated increased amygdala activity in ASD during the identification of genders, comparable to the orthogonal task of the current study (Weng et al., 2011).

The observation of atypical fMRI neural activity patterns during face processing in children with ASD aligns with the EEG findings of our parallel report in the same sample of children (see **chapter 5**). There, we applied frequency-tagging EEG and observed highly significantly reduced oddball discrimination responses along occipito-temporal brain areas in children with ASD versus NT, indexing a reduced neural sensitivity for quickly discriminating subtle changes in facial expressions (contrasting both fearful and happy faces with a series of neutral facial expressions; see also Van der Donck et al, 2019; 2020). Thus, while both methodological approaches converge on demonstrating atypical neural face processing in ASD (in particular, reduced responses in occipitotemporal areas), the findings of the frequency-tagging EEG can more unambiguously be interpreted as evidence for deficient and reduced neural sensitivity for important socio-communicative cues. An exploration of possible correlations between face specific fMRI activity and EEG neural sensitivity for facial expressions did not reveal robust association patterns (data not shown in the thesis).

OT treatment effects on fMRI face processing responses in ASD

Next, we implemented the fMRI face processing task in a randomized, double-blind, placebo-controlled clinical trial, in order to monitor the effect of OT treatment in children

with ASD. In general, we observed very few treatment-specific differences. Only while loosening the correction for multiple testing, we did observe lower activity in left STS in the OT group compared to the placebo group. Importantly, this effect was observed both in the analysis where we investigated average activity across the entire ROI and in the analysis where we compared peak activity, which may strengthen the reliability of this finding. At a functional level, changes in STS activity might relate to changes in facial expression processing ability, especially because STS has classically been designated as the core face processing region involved in processing the dynamic aspects of faces, such as expressions (Haxby & Gobbini, 2011; Muukkonen & Salmela, 2022).

The observation of OT-induced reduction in STS activity is in line with prior studies investigating the effects of OT in individuals with ASD. For instance, Aoki et al., (2014) demonstrated reduced STS activity after a single-dose of OT in adults with ASD when they had to infer others' emotions (Aoki et al., 2014). Likewise, Andari et al., (2016) reported diminished activity in the middle temporal cortex during a social ball-tossing game, upon single-dose OT administration in adults with ASD (Andari et al., 2016). In contrast, other single-dose OT studies have also reported *increased* activity in STS in ASD (Bernaerts, Boets, Steyaert, et al., 2020; Gordon et al., 2013). For example, Bernaerts et al., (2020) found increased activity in STS in adults with ASD, during the processing of point-light biological motion, after a single dose of OT, however, no consistent long-term changes in STS activity were induced after a four-week multiple-dose treatment (Bernaerts, Boets, Steyaert, et al., 2020). Note, however, that this same study did observe consistent and long-term reductions in amygdala activity after a four-week OT treatment (Bernaerts, Boets, Steyaert, et al., 2020).

While we did not administer any behavioural face processing tasks throughout the clinical trial, in our parallel report on this same study we describe changes in neural sensitivity for subtle facial expression cues as assessed by frequency-tagging EEG (**chapter 5**). Interestingly, in the EEG data, selective neural sensitivity for facial expressions, as indexed by occipito-temporal oddball responses, significantly increased in children with ASD after receiving placebo, but this effect was dampened after receiving the four-week OT treatment (**chapter 5**). Strikingly, visual inspection of the current change-from-baseline STS activity pattern (Fig. 6) reveals a similar increase after placebo and decrease after OT treatment. Thus, together, the EEG and fMRI findings converge on demonstrating evidence for a generally attenuating effect of OT on an otherwise naturally occurring

learning and tuning effect on face processing seen in the placebo group. While this conclusion conflicts with the social salience account of OT, it may corroborate the anxiolytic and social stress reduction theory of OT, as possibly aversive facial stimuli may be processed in a more attenuated manner. Anecdotal support for this hypothesis can also be found in the correlations between STS and amygdala activity in the ASD group (not elaborated in this thesis).

A recent systematic review summarized the literature on effects of intranasal OT administration on fMRI responses in ASD (Fathabadipour et al., 2022). While the authors acknowledge that OT administration can alter brain activity in individuals with ASD, they also emphasize that this largely depends on the type of task and thus the actual context of OT administration (Fathabadipour et al., 2022; Ford & Young, 2021). In other words, performing a social task within the time window of actively circulating OT administration might boost the circuitry involved in this task, whereas these circuitries might not be affected by OT alone (in isolation). This might explain why some single-dose studies demonstrate increased neural activity in response to faces (in line with the social salience hypothesis), as the brain is concurrently primed by a social task. On the other hand, the current and previous multiple-dose OT studies did not incorporate a priming of the brain with either a social task or a positive social context while exogenous OT was administered and circulating, and therefore they may not have boosted the related neural circuitry, thus not resulting in enhanced neural activity. A unique recent multiple-dose clinical trial in children with ASD did explicitly combine OT administration with psychosocial stimulation, and did demonstrate enhanced social attention (increased looking at the eye region of a face) and consistent clinical improvements in autism symptoms (Le et al., 2022). The latter finding is especially interesting since our (and previous) fMRI results do not indicate restoration of normal (NT) brain activation in ASD, as no baseline diagnostic-group differences were found in STS, so the neural mechanisms of social improvements after OT treatment are still uncertain (Fathabadipour et al., 2022). This calls for further exploration of the impact of combining multiple-dose OT treatment with targeted socially-stimulating tasks in order to uncover the underlying neural mechanisms of the resulting behavioural/clinical effects.

Limitations

Although the current study reveals important insights on the neural effects of repeated OT administration on face processing responses in children with ASD, some limitations should be addressed. First, it is important to highlight that the identified treatment effect of lower STS activity in the OT group compared to the placebo group did not survive multiple-testing correction. Although this gives a tentative first indication of an oxytocin-induced anxiolytic effect, additional measures should further solidify this hypothesis. Second, while fMRI has a good spatial resolution, due to its low temporal resolution we administered a fairly basic and slow blocked face processing paradigm, which might not yield high sensitivity to reveal diagnostic-related and treatment-related group differences in the processing of subtle fast-changing facial cues. This may partially explain why the frequency-tagging EEG technique, with its high temporal resolution, did convincingly show group differences and treatment effects in this same clinical trial (**chapter 5**). Note that a recent multi-method face processing fMRI study, applying a large series of univariate, multivariate, adaptation and functional connectivity analysis approaches, also revealed fairly mild functional differences while contrasting adults with and without ASD (Hendriks et al., 2021). Third, we used anatomically defined ROIs, which were functionally restricted on the basis of adult fMRI contrasts to ensure that only face-selective voxels were included. Yet, adult functional neuroanatomy of face processing may be divergent from children with ASD. Fourth, besides averaging beta values in the ROI analysis, we exploratively applied the small volume correction function within SPM, yet no voxels survived the threshold. Fifth, considering the study design, we did not include an acute single-dose OT assessment, so no direct comparison between single and multiple-dose OT effects on neural face processing could be made. This would have been particularly relevant to directly assess whether face processing during active OT administration would have been enhanced, as predicted by the social salience account (Ford & Young, 2021; Shamay-Tsoory & Abu-Akel, 2016). Sixth, standardization or monitoring of the social context during exogenous OT administrations throughout the long-term trial would have been informative, especially to understand possible interindividual variability in fMRI response patterns. Lastly, another statistical approach might be more suitable for evaluating differences in randomised groups (e.g. Senn et al., 1991).

Conclusion

Being able to accurately read emotions of someone's face is crucial for fluent social interaction, which is often altered in individuals with ASD. The underlying neural correlates of face processing have been extensively examined using fMRI, allowing to quantify brain activity with great spatial resolution. Here, we used an fMRI face processing task to assess and compare the neural face processing signature of children with ASD with that of matched NT controls. First, we observed lower activity in ASD in early visual processing regions, suggesting a reduced sensitivity for faces. Secondly, children with ASD had increased peak activity in the inferior frontal cortex, possibly due to a greater semantic processing demand, as well as increased left amygdala activity, perhaps because faces may be perceived as aversive for children with ASD. Next, we implemented this fMRI face processing task in a double-blind, placebo-controlled, multiple-dose OT clinical trial in children with ASD, to evaluate the impact of four weeks of OT administration on face processing brain activity. While applying multiple-comparison correction for the number of interrogated ROIs, no significant treatment effects were found. Yet, uncorrected for multiple-comparison, we did observe lower left STS activity in the OT group, compared to the placebo group, possibly pointing to an OT-induced attenuating effect, similar as we observed in parallel EEG findings. To conclude, these findings confirm (i) atypical face processing fMRI responses in children with ASD compared to matched NT controls, (ii) no OT-induced restoration of neural activity in these altered regions, (iii) but a tentative attenuating effect of OT on STS activity, likely supportive of the anxiolytic account of OT.

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Supplementary Material

Supplementary methods

Participants

Forty neurotypical children (NT) and eighty children with autism spectrum disorder (ASD) were recruited and assessed at baseline (T0). Only the ASD children were allocated to receive either oxytocin or placebo (four weeks of twice daily intranasal administrations), followed by a post-treatment (T1) assessment. Main inclusion criteria comprised a clinical diagnosis of ASD (only for children with ASD); age (8-12 years old); IQ above 70; no prior oxytocin treatment and native Dutch speaker. The ASD diagnosis was established by a multidisciplinary team (child psychiatrist and/or expert neuropediatrician, psychologist, speech/language pathologist and/or physiotherapist) based on the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (American Psychiatric Association, 2013). Main exclusion criteria comprised a history of any neurological disorder (stroke, concussion, epilepsy etc.), any physical disorder (liver, renal, cardiac pathology), significant hearing or vision impairments, or any neuropsychiatric diagnosis (only for NT children). Only premenstrual girls were included. Fifteen participants with ASD had a comorbid diagnosis of attention deficit/hyperactivity disorder (ADHD). Thirty of the 58 ASD children followed stable concomitant psychosocial therapy. Eighteen of the ASD participants used stimulant medication (e.g. methylphenidate), 10 anti-psychotics (e.g. risperidone), 3 anti-depressants (e.g. sertraline) and 25 used other medication (e.g. sleeping aids, gastro-intestinal medication or nutritional supplements).

Supplementary results

Expression specific contrasts

The primary aim of the current study was investigating whether ASD children differed from NT controls in fMRI face processing responses, and to evaluate the effect of repeated OT administrations on these fMRI responses. Therefore, the main contrast was FACESvsFIX (i.e., the neutral and fearful face conditions combined vs fixation), in line with previous research (Hendriks et al., 2021). Although four additional contrasts were made, in order to evaluate the effect of neutral or fearful expressions separately: NEUTRALvsFIX, FEARFULvsFIX, NEUT RALvsSCRAMBLED and FEARFULvsSCRAMBLED.

Diagnostic-group comparison on fMRI face processing responses for expression specific contrasts

For the diagnostic-group comparison, we only found higher fMRI responses in the left inferior frontal cortex in ASD compared to the NT controls for the NEUTRALvsFIX contrast ($t(87) = 7.83$, $p_{FDR} < 0.01$, **see Table S1**). For all other contrast, no significant group differences were found (all $p_{FDR} > 0.3$, **see Tables S2-4**).

OT treatment effects on fMRI face processing responses in ASD for expression specific contrasts

Considering the treatment effects, fMRI face processing responses were not significantly different between the OT and PL groups (all $p_{FDR} > 0.6$, **see Tables S5-8**) on either of the expression specific contrasts.

Table S1. Diagnostic-group comparison of average ROI activity for the NEUTRALvsFIX contrast.

Average ROI activity NEUTRAL vs. FIX contrast	ASD Mean beta value	NT Mean beta value	*t* value	P_{unc}	P_{FDR}
Left amygdala	0.10	0.05	0.61	0.54	0.78
Right amygdala	0.08	0.11	-0.51	0.61	0.80
Left anterior temporal	-0.02	0.02	-0.92	0.36	0.67
Right anterior temporal	-0.02	0.05	-1.39	0.17	0.67
Left inferior frontal	0.38	0.01	7.83	<0.01	**0.01***
Right inferior frontal	0.27	0.18	1.27	0.21	0.67
Left inferior occipital	0.28	0.19	1.51	0.14	0.67
Right inferior occipital	0.24	0.24	0.04	0.97	0.97
Left posterior temporal	0.26	0.24	0.30	0.77	0.91
Right posterior temporal	0.27	0.31	-0.66	0.51	0.78
Left STS	0.02	0.02	0.04	0.97	0.97
Right STS	0.07	0.11	-1.05	0.30	0.67
V1	-0.01	0.07	-1.03	0.31	0.67

Table S2. Diagnostic-group comparison of average ROI activity for the FEARFULvsFIX contrast.

Average ROI activity FEARFUL vs. FIX contrast	ASD Mean beta value	NT Mean beta value	*t* value	P_{unc}	P_{FDR}
Left amygdala	0.12	0.08	0.77	0.45	0.80
Right amygdala	0.08	0.07	0.34	0.74	0.80
Left anterior temporal	-0.04	0.04	-1.81	0.07	0.30
Right anterior temporal	-0.02	0.06	-1.70	0.09	0.30
Left inferior frontal	0.04	0.03	0.39	0.70	0.80
Right inferior frontal	0.27	0.16	1.97	0.05	0.30
Left inferior occipital	0.16	0.24	-1.53	0.13	0.34
Right inferior occipital	0.26	0.24	0.50	0.62	0.80
Left posterior temporal	0.21	0.19	0.44	0.66	0.80
Right posterior temporal	0.26	0.25	0.17	0.87	0.87
Left STS	0.01	0.05	-0.93	0.36	0.77
Right STS	0.07	0.09	-0.42	0.68	0.80
V1	-0.04	0.08	-2.41	0.02	0.24

Table S3. Diagnostic-group comparison of average ROI activity for the NEUTRALvsSCRAMBLE contrast.

Average ROI activity NEUTRAL vs SCRAMBLE contrast	ASD Mean beta value	NT Mean beta value	*t* value	P_{unc}	P_{FDR}
Left amygdala	0.06	-0.04	1.94	0.56	0.60
Right amygdala	0.04	0.07	-0.48	0.63	0.90
Left anterior temporal	0.08	0.001	1.70	0.09	0.60
Right anterior temporal	0.001	0.02	-0.71	0.48	0.88
Left inferior frontal	0.03	0.03	0.02	0.99	0.99
Right inferior frontal	0.07	0.12	-0.97	0.34	0.87
Left inferior occipital	0.04	-0.02	1.49	0.14	0.61
Right inferior occipital	0.03	-0.02	1.07	0.29	0.87
Left posterior temporal	0.09	0.05	0.77	0.45	0.88
Right posterior temporal	0.08	0.08	-0.08	0.93	0.99
Left STS	0.03	0.05	-0.61	0.54	0.88
Right STS	0.07	0.09	-0.40	0.69	0.90
V1	0.04	0.06	-0.21	0.83	0.99

Table S4. Diagnostic-group comparison of average ROI activity for the FEARFULvsSCRAMBLE contrast.

Average ROI activity FAERFUL vs SCRAMBLE contrast	ASD Mean beta value	NT Mean beta value	*t* value	P_{unc}	P_{FDR}
Left amygdala	0.06	0.02	0.96	0.34	0.56
Right amygdala	0.01	0.02	-0.26	0.80	0.87
Left anterior temporal	0.01	0.04	-0.77	0.45	0.58
Right anterior temporal	0.01	0.04	-0.78	0.44	0.58
Left inferior frontal	0.01	0.04	-1.24	0.22	0.56
Right inferior frontal	0.05	0.05	0.07	0.94	0.94
Left inferior occipital	0.06	0.00	1.39	0.17	0.56
Right inferior occipital	0.03	-0.05	1.66	0.10	0.56
Left posterior temporal	0.03	-0.02	1.09	0.28	0.56
Right posterior temporal	0.05	-0.05	1.93	0.06	0.56
Left STS	0.04	0.02	0.30	0.77	0.87
Right STS	0.05	0.00	0.99	0.32	0.56
V1	0.04	-0.02	1.09	0.28	0.56

Table S5. Treatment effect on the average change-from-baseline ROI activity for the NEUTRALvsFIX contrast.

Average ROI activity NEUTRAL vs. FIX contrast	Oxytocin Mean beta value	Placebo Mean beta value	*t* value	P_{unc}	P_{FDR}
Left amygdala	-0.24	0.06	-1.68	0.10	0.65
Right amygdala	-0.01	-0.07	0.43	0.67	0.80
Left anterior temporal	0.02	-0.04	0.56	0.58	0.80
Right anterior temporal	0.06	-0.21	1.93	0.06	0.65
Left inferior frontal	-0.27	-0.15	-1.03	0.31	0.80
Right inferior frontal	-0.04	-0.10	0.54	0.59	0.80
Left inferior occipital	-0.04	-0.07	0.34	0.74	0.80
Right inferior occipital	0.08	-0.01	0.81	0.42	0.80
Left posterior temporal	0.03	-0.08	0.79	0.44	0.80
Right posterior temporal	0.02	0.02	<0.001	1.00	1.00
Left STS	-0.05	0.03	-0.73	0.47	0.80
Right STS	0.02	0.06	-0.35	0.73	0.80
V1	0.25	0.19	0.47	0.64	0.80

Table S6. Treatment effect on the average change-from-baseline ROI activity for the FEARFULvsFIX contrast.

Average ROI activity FEARFUL vs. FIX contrast	Oxytocin Mean beta value	Placebo Mean beta value	*t* value	P_{unc}	P_{FDR}
Left amygdala	-0.10	-0.13	0.20	0.84	0.91
Right amygdala	-0.13	-0.09	-0.38	0.71	0.91
Left anterior temporal	0.03	-0.07	1.29	0.21	0.91
Right anterior temporal	-0.05	-0.08	0.25	0.81	0.91
Left inferior frontal	-0.09	-0.04	-0.55	0.59	0.91
Right inferior frontal	-0.18	-0.18	-0.01	0.99	0.99
Left inferior occipital	-0.01	0.03	-0.35	0.73	0.91
Right inferior occipital	-0.12	-0.07	-0.42	0.68	0.91
Left posterior temporal	-0.15	-0.01	-1.07	0.29	0.91
Right posterior temporal	-0.09	-0.14	0.36	0.72	0.91
Left STS	-0.05	0.001	-0.44	0.66	0.91
Right STS	-0.09	0.01	-1.11	0.27	0.91
V1	0.02	0.21	-1.78	0.08	0.91

Table S7. Treatment effect on the average change-from-baseline ROI activity for the NEUTRALvsSCRAMBLE contrast.

Average ROI activity NEUTRAL vs SCRAMBLE contrast	Oxytocin Mean beta value	Placebo Mean beta value	*t* value	P_{unc}	P_{FDR}
Left amygdala	0.01	-0.02	0.38	0.71	0.93
Right amygdala	0.04	-0.04	0.68	0.50	0.93
Left anterior temporal	-0.01	-0.11	0.92	0.36	0.93
Right anterior temporal	0.09	0.00	1.23	0.23	0.93
Left inferior frontal	0.01	0.01	<0.01	1.00	1.00
Right inferior frontal	0.05	0.10	-0.40	0.69	0.93
Left inferior occipital	0.00	0.01	-0.18	0.86	0.93
Right inferior occipital	-0.01	0.03	-0.39	0.70	0.93
Left posterior temporal	0.00	0.02	-0.20	0.84	0.93
Right posterior temporal	-0.04	-0.01	-0.38	0.71	0.93
Left STS	0.04	0.02	0.19	0.85	0.93
Right STS	0.01	0.09	-0.96	0.34	0.93
V1	0.09	0.15	-0.48	0.63	0.93

Table S8. Treatment effect on the average change-from-baseline ROI activity for the FEARFULvsSCRAMBLE contrast.

Average ROI activity FEARFUL vs SCRAMBLE contrast	Oxytocin Mean beta value	Placebo Mean beta value	*t* value	P_{unc}	P_{FDR}
Left amygdala	0.03	-0.06	0.92	0.36	0.95
Right amygdala	0.03	-0.01	0.39	0.70	0.95
Left anterior temporal	0.07	0.001	1.00	0.32	0.95
Right anterior temporal	0.03	0.001	0.31	0.76	0.95
Left inferior frontal	-0.02	-0.01	-0.29	0.77	0.95
Right inferior frontal	-0.02	-0.12	0.94	0.35	0.95
Left inferior occipital	-0.09	-0.07	-0.14	0.89	0.95
Right inferior occipital	-0.04	-0.03	-0.08	0.94	0.95
Left posterior temporal	-0.06	0.02	-0.64	0.52	0.95
Right posterior temporal	0.01	-0.07	0.71	0.48	0.95
Left STS	0.03	-0.09	1.34	0.19	0.95
Right STS	-0.01	0.00	-0.11	0.91	0.95
V1	-0.02	-0.01	-0.07	0.95	0.95

Chapter 7 | General discussion

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by impairments in social communication and interaction, and by the presence of restricted, stereotyped and repetitive interests and behaviours (American Psychiatric Association, 2013). At this time, there is no pharmacological treatment available for ASD, however there is exciting support that the neuropeptide oxytocin might have the potential to alleviate social deficits in ASD (Yamasue et al., 2018; Yamasue & Domes, 2017). Oxytocin functions as a hormone in the body (induces childbirth and lactation (Zingg & Laporte, 2003)) and functions in the brain as a neuromodulator, involved in socio-cognitive functioning and behaviours (Fineberg & Ross, 2017; Marlin & Froemke, 2017). A more in-depth understanding how oxytocin impacts on social behaviour is of the utmost importance. Accordingly, this PhD thesis provides an overview of the literature on the endogenous oxytocin system in ASD, and the relation between variations in oxytocin receptor gene epigenetics and ASD characteristics. Furthermore, the thesis reports the behavioural and neural results of a randomized, placebo-controlled, double-blind, parallel, multiple-dose intranasal oxytocin clinical trial performed in children with ASD.

Summary of the main findings

Chapter 2 comprises a systematic review and meta-analysis on endogenous oxytocin concentrations in individuals with ASD compared to matched controls, covering a wide age-range from childhood to adulthood. We included 18 studies comprising a total of 1422 participants. Primarily, the analysis revealed that endogenous oxytocin levels are lower in children with ASD, but, interestingly, this group difference seems to disappear in adolescents and adults with ASD. Secondly, evaluating the effect of sex revealed trend-level lower oxytocin levels in males, but no group difference in females. However, the male group was larger than the female group, which could partially play a role here. Lastly, lower oxytocin levels in ASD were mainly found in studies using blood plasma samples, but not in studies utilizing saliva samples. Of note, both tissue types did show considerable and comparable effect sizes, and no tissue type subgroup differences could statistically be determined.

In **chapter 3**, we systematically reviewed the literature on the association of oxytocin receptor gene (*OXTR*) DNA methylation with ASD characteristics and related social dimensions. Twelve articles investigating *OXTR* DNA methylation in relation to ASD, social perception/cognition and social anxiety were included. We observed higher methylation

levels in adults with ASD, and found an association between higher methylation and more autism traits in adults, and with increased brain activity (but decreased connectivity) during social tasks, indicative for a greater need for neural resources. Conversely, lower methylation levels were associated with more social anxiety. Interestingly, two studies evaluating *OXTR* DNA methylation in children with ASD also found *reduced* methylation, especially in boys.

Chapter 4 describes the behavioural results from a randomized, placebo-controlled, double-blind, parallel, multiple-dose intranasal oxytocin clinical trial performed in 77 children with ASD (n=61 boys, n=16 girls) aged 8-12 years. Oxytocin administrations (12 IU) were administered twice daily for four weeks, in 8-to-12-year-old boys and girls with ASD (n=38 oxytocin, n=39 placebo). The double-blind phase of the clinical trial was followed with a single-blind phase, where all ASD children received oxytocin. Improvements on social responsiveness, as measured with the Social Responsiveness Scale (SRS-2; Constantino & Gruber, 2012), were found in the oxytocin group but also in the placebo group. Thus, no treatment-specific improvements could be concluded. However, important moderator effects were identified, providing important indications that clinical efficacy can be augmented when oxytocin administration is paired with targeted psychosocial therapy. Specifically, greater benefits were found in children receiving concomitant psychosocial treatment in combination with oxytocin, than those who received oxytocin alone. Additionally, when the parents believed that their child received the actual treatment, the perceived oxytocin effect was also larger. Lastly, the children who initially received placebo during the double-blind phase and continued with oxytocin in the single-blind phase, displayed a significant additional improvement, larger than the earlier placebo-induced improvements.

Chapter 5 describes the neural results from the relatively novel, robust and implicit frequency-tagging EEG technique, which was applied in the randomized, placebo-controlled, double-blind, parallel, multiple-dose oxytocin clinical trial (four weeks, 12 IU, twice daily). Specifically, frequency-tagging EEG was used to examine the impact of multiple-dose oxytocin on neural sensitivity towards happy and fearful facial expressions in children with ASD (n=29 oxytocin, n=32 placebo), immediately post-treatment and after a four-week follow-up. Prior to any intervention (at baseline) we compared the neural sensitivity towards facial expressions in children with ASD to that of age- and gender-matched neurotypical children (n=39). First, children with ASD exhibited reduced

neural sensitivity towards expressive faces compared to neurotypical children. Secondly, upon placebo nasal spray administration, children with ASD displayed a significant increase in neural sensitivity at the post and follow-up sessions, likely reflecting an implicit learning effect. Strikingly, neural sensitivity remained unaffected after the four-week oxytocin treatment, suggesting a dampening of an otherwise typically occurring implicit learning effect, likely reflecting oxytocin's anxiolytic effects towards emotionally-evocative faces. Interestingly, at the follow-up session (four weeks after cessation of the oxytocin administrations) an increase in neural sensitivity was found, comparable to that in the placebo-group.

Chapter 6 extends the neurobiological findings of chapter 5 with results from a face processing fMRI task. While frequency-tagging EEG proved to be a robust indicator of (changes in) socio-communicative sensitivity, it does not inform us about the underlying neural mechanisms of aberrant face processing in ASD, or the effect of repeated oxytocin administration. Therefore, using a comparable fMRI task with identical face stimuli as the EEG experiment, we compared the neural face processing signature in children with ASD (n=58) to matched neurotypical controls (n=38). Subsequently, the effect of a multiple-dose oxytocin therapy on fMRI face processing responses was evaluated in the ASD sample (n=20 oxytocin, n=24 placebo). First, we observed atypical face processing fMRI responses in children with ASD compared to matched neurotypical controls, in early visual brain regions, inferior frontal cortex and in the left amygdala. Secondly repeated oxytocin administration in ASD did not restore neural activity in these altered regions, however, a tentative attenuating effect of oxytocin on the STS (a core facial expression processing brain region) was found, likely supportive of the anxiolytic account of oxytocin.

Reflection and future directions

The oxytocinergic system in autism

In **chapter 2 and 3** we aimed to gain a better understanding of the endogenous oxytocin system in ASD. The obtained results of lower oxytocin concentrations in children, but not adolescents and adults with ASD (**chapter 2**), extend the earlier meta-analysis of Rutigliano et al., where no significant differences were found between adults with and without ASD (Rutigliano et al., 2016). When investigating this potential developmental

effect more closely we mainly see diminished oxytocin concentrations in young children with ASD (<9 years old) (Abdulamir et al., 2016; Alabdali et al., 2014; Feldman et al., 2014; Fujisawa et al., 2014; Husarova et al., 2016; J. D. Jacobson et al., 2014; Zhang et al., 2016). Studies in older children, adolescents and adults mostly report no differences, and one adult study even found significantly higher oxytocin concentrations in the ASD population (Althaus et al., 2016), substantiating a potential developmental effect. These results either indicate a normalisation (increase) of oxytocin levels after childhood in ASD or a reduction in neurotypical controls, after a (possible) peak in early childhood. In support of the latter, Freeman at al., (2018) demonstrated such an early-life (2-5 years old) peak in oxytocin receptor density in the ventral pallidum (part of the reward system in the brain) in neurotypical children, but not in children with ASD (Freeman et al., 2018). The lack of such a peak in early-life, when this reward area becomes maximally sensitive to oxytocin binding, may impact the neurotypical social development and may result in social symptoms in ASD.

Two studies reported blood plasma levels of *OXTR* DNA methylation in ASD, within a similar age range (**chapter 3**). Elagoz et al., observed hypomethylation in 2-8 years-olds with ASD, indicative of increased oxytocin receptor expression, compared to controls (Elagoz Yuksel et al., 2016). Siu et al., also showed hypomethylation in children/adolescents with ASD (2-18 year-olds) in their general analysis, however they note that a substantial proportion of their ASD sample (i.e. 35 out of 248 children) had outlying *OXTR* methylation values and 76% of these outliers exhibited hypermethylation, which was not found in the control group, suggesting that hypermethylation of *OXTR* may be specific to a particular subgroup of ASD (Siu et al., 2021). Moreover, this hypermethylation was strongly associated with lower IQ and greater social problems in ASD (Siu et al., 2021). More convincing evidence of ASD-related hypermethylation comes from *OXTR* methylation studies in adults (Andari et al., 2020; Gregory et al., 2009). For instance, Andari et al., reported higher *OXTR* methylation levels in adults with ASD and a strong association with clinical symptom severity (Andari et al., 2020). Hypermethylation of *OXTR* was also observed in relation to hampered social perception and cognition in neurotypical populations. Specifically, hypermethylation appeared to be associated with increased activity in multiple brain regions involved in social cognition and emotional face processing, reflecting an increased need for neural resources (Jack et al., 2012; Krol et al., 2019; Puglia et al., 2015, 2018). Conversely, lower *OXTR* methylation appears to be

associated with more social anxiety in neurotypicals (Puglia et al., 2018; Ziegler et al., 2015), contradicting the intuitive hypothesis that more methylation would be associated with higher anxiety scores. Possibly, a hypomethylated status of *OXTR*, thus high oxytocin receptor expression, may induce an increased sensitivity towards social situations, thereby turning social stimuli extremely saliently, possibly resulting in elevated levels of social anxiety.

Taking together, a preliminary developmental pattern of the oxytocin system starts to emerge. Speculatively, an initial early-life *OXTR* hypomethylation in ASD, and thus a possible oversensitive oxytocin system, may have caused young children to experience social interactions as intrusive, which they may counter by developing a desensitised oxytocin system, by decreasing circulating oxytocin levels in childhood and gradually increasing *OXTR* methylation, so lowering oxytocin receptor expression, as observed in adulthood. This, in turn, may result in the development of social problems, found in ASD. However future research is warranted to jointly monitor these two oxytocin system markers in typical and atypical development, and elucidate the interplay among them. Of course, other biochemicals should also be held into account, for instance cortisol, a stress hormone known to interact with the oxytocin system (within the current clinical trial salvatory cortisol levels were obtained, these analyses are currently in progress).

Biomarkers of autism

Despite substantial progress in identifying the biological underpinnings of ASD, diagnosis and treatment monitoring rely primarily on subjective evaluation of behaviour, as no objective quantitative biomarker exists for ASD (at this time). Developing diagnostic biomarkers (i.e., objectively indicating whether a person has ASD or not) has long been a goal in the field of ASD research (McPartland, 2016). However, biomarkers can serve multiple purposes, for instance stratify individuals into subgroups or monitor treatment outcomes. To fulfil these goals, electrophysiological methods are often put forward, as they are widely applicable, low in cost and have high sensitivity and objectivity (McPartland, 2016). Meeting these requirements, frequency-tagging EEG was recently proposed as a highly reliable and implicit technique to pinpoint individual-subject face processing sensitivity (Rossion et al., 2015). The main principle of frequency-tagging EEG is that the frequency of the electrophysiological response on the human scalp corresponds exactly with the frequency of the visual stimulation (Norcia et al., 2015), thereby allowing

to “tag” streams of particular stimulus categories, such as faces or expressive faces. **Chapter 5** describes the results from this frequency-tagging EEG technique where we show that children with ASD exhibited reduced neural sensitivity towards expressive faces compared to neurotypical children. These results validate findings from pioneering studies, which used frequency-tagging EEG to demonstrate reduced sensitivity towards rapid changes in facial identity (Vettori et al., 2019) and facial expression (Van der Donck et al., 2019, 2020) in boys with ASD. Besides diagnostic-group differences, chapter 5 also describes differences in therapeutic outcome upon oxytocin versus placebo administration. Specifically, four weeks of placebo nasal spray administration increased the neural sensitivity towards expressive faces in children with ASD as measured with frequency-tagging EEG, immediately post-treatment and at follow-up, a month later. Remarkably, neural sensitivity remained unaffected after the four-week oxytocin treatment, suggesting an attenuation of an otherwise naturally occurring implicit learning effect. Interestingly, at the follow-up session (one month after the oxytocin administrations ended) we detected an increase in neural sensitivity, comparable to that in the placebo-group.

Altogether, these results highlight the robustness of frequency-tagging EEG to pinpoint subtle inter-individual differences in facial expression discrimination that may remain concealed while using explicit behavioural tasks. Importantly, we do not contend that facial expression processing difficulties, and thus the reduced frequency-tagged EEG responses, are specific for individuals with ASD, as also other psychiatric disorders are characterized by similar face processing impairments (e.g. schizophrenia, frontotemporal dementia, 22q11del syndrome,…). Thus, as a diagnostic biomarker, the frequency-tagging EEG approach might offer strong sensitivity, but it may lack specificity within a broader psychiatric population. The present results also validate the potential use of frequency-tagging EEG as a therapeutic biomarker, measuring responses pre- and post-treatment, as we have demonstrated its ability to discriminate between oxytocin and placebo effects. Ultimately, it could potentially serve in a clinical setting, for instance as a prognostic biomarker in children with ASD, for monitoring clinical evolution and adequately adapting a particular therapy. Although, future research is necessary to explore how this technique could be effectively translated into a clinically useful tool.

Clinical implications of oxytocin treatment

Chapters 4, 5 and 6 comprise the results from a randomized, placebo-controlled, double-blind, parallel, multiple-dose intranasal oxytocin clinical trial in children with ASD. Primarily, we used the Social Responsiveness Scale (SRS-2; J. Constantino & Gruber, 2012), a rating scale designed to measure autistic traits, to monitor the behavioural effects of repeated oxytocin administrations in children with ASD (**chapter 4**). The trial revealed no significant treatment-specific effects since both the oxytocin and placebo groups displayed improvements, both immediately after the treatment and at follow-up, four weeks later. These findings are in line with previous multiple-dose oxytocin trials in children with ASD (Fastman et al., 2021; Guastella et al., 2015; Sikich et al., 2021). Sikich et al., for example, included a large cohort of 277 children with ASD and also reported no significant treatment-specific effects on the SRS-2 and on the Aberrant Behavior Checklist modified Social Withdrawal subscale (a caregiver-rated questionnaire assessing social withdrawal) (Sikich et al., 2021). Conversely, two trials did find beneficial outcomes on the SRS after multiple-dose oxytocin treatment compared to placebo (Parker et al., 2017; Yatawara et al., 2016), resulting in a need to identify factors contributing to the variability in study results. Several factors, such as dosing scheme, heterogeneity of participant characteristics, trial design or (social) context in which oxytocin is administered, have been put forward as moderators. An important result from the current study relates to the trial design; namely children who crossed over from placebo in the first phase to the actual oxytocin treatment in the second phase exhibited a significant further improvement in social responsiveness (SRS-2) well beyond the placebo-induced improvement seen in the first phase (**chapter 4**). Similar trial designs, putting a blinded placebo phase before the actual treatment, have been implemented before as an effective method to control for placebo effects and to better identify real therapeutic effects (Yatawara et al., 2016). The current finding of further improvement from a blinded placebo phase to the active treatment, corroborates this idea. A second important moderating factor regards the context in which oxytocin is administered. Previous single-dose studies observed that acute effects of oxytocin can be modulated by contextual factors, for instance stress-reducing effects of oxytocin are most pronounced within a supportive context (i.e., social support from a friend) (Heinrichs et al., 2003) and cooperation and trust are mainly enhanced towards in-group members (de Dreu et al., 2010; Mikolajczak et al., 2010). Against this background, it has been hypothesised that

oxytocin may open a 'window of opportunity' to promote prosocial behaviour, but to realize its full potential it should be administered within a socially stimulating environment, for example with simultaneous behavioural therapy assisting social skill development and enhancing prosocial behaviour (Ford & Young, 2021; Geschwind, 2021). In accordance with this idea, our study revealed larger improvements on the SRS-2 in children receiving concomitant psychosocial therapy in combination with oxytocin, than those who received oxytocin alone (**chapter 4**). Additionally, when the parents believed that their child received the actual treatment, the oxytocin effect was larger. These results indicate optimal treatment outcome when children receive oxytocin within a positive, supporting environment.

The importance of the context of oxytocin administration is further supported by neuroimaging studies, specifically through the discrepancies observed between acute, single-dose versus chronic, multiple-dose effects. For instance, single-dose oxytocin studies often reveal increased neural activity (e.g., in amygdala and social brain circuitry) while performing social tasks, potentially reflecting an increased salience towards social stimuli as a result of the increased availability of circulating oxytocin (Aoki et al., 2014; Domes et al., 2010, 2014; Gamer et al., 2010). In contrast, multiple-dose oxytocin studies evaluating chronic neural effects, consistently demonstrate an attenuating effect on brain activity (Alaerts et al., 2020; Bernaerts, Boets, Steyaert, et al., 2020; Kou et al., 2022). The study of Bernaerts et al., for instance, investigated neural processing of emotionally charged point-light displays expressing body language in adults with ASD and found increased activity in the STS upon a single-dose of oxytocin but this effect disappeared after a four-week multiple-dose treatment (Bernaerts, Boets, Steyaert, et al., 2020). The current study extends these findings in children with ASD, by showing lower STS activation during face processing after four weeks of repeated oxytocin administration compared to placebo (significant if uncontrolled for multiple comparisons, **chapter 6**). Furthermore, our frequency-tagging EEG data in children with ASD revealed increased occipitotemporal activity during expressive face processing in the placebo group, but in the oxytocin group, neural sensitivity was not affected from the baseline to the post session, probably reflecting a dampening of an otherwise typically occurring implicit learning effect (**chapter 5**). Accordingly, it is possible that the absence of a specific standardized social stimulation during oxytocin administration, e.g. presenting facial stimuli during the acute window of actively circulating exogenous oxytocin, may primarily

prime and consolidate intrinsic neural circuitry towards the brain's default 'resting state', reflective of oxytocin's role in promoting homeostasis and restoration of autonomic balance in the relative absence of acute (social) stimulation (Carter, 2014). This is consistent with the anxiolytic account of oxytocin, which proposes that oxytocin improves social functioning by attenuating (social) stress (Bartz et al., 2011; Heinrichs & Domes, 2008; Shamay-Tsoory & Abu-Akel, 2016), through dampening activity in threat-processing brain regions and thereby reducing related somatic anxiety responses (Veening & Olivier, 2013). On the other hand, the presence of a social context (e.g., presenting facial stimuli), during the period of heightened oxytocin concentrations after exogenous administration, can facilitate priming of relevant neural (social) circuits and increase neural activity (Domes et al., 2010, 2014). This is in accordance with the social salience hypothesis of oxytocin, which states that oxytocin may improve attention to and perception of social cues by prioritizing the allocation of neural resources to the processing of these social cues (Shamay-Tsoory & Abu-Akel, 2016). In support of this notion, a recent multiple-dose clinical trial in young children with ASD combined oxytocin administration with concurrent psychosocial stimulation, and revealed reliable clinical improvements in autism symptomatology (ADOS-2) as well as improved social attention and increased looking time to the eye region (Le et al., 2022).

In conclusion, although no treatment-specific behavioural improvements were found in the current study, key moderators of clinical efficacy were identified. First, considering the clinical trial design, children who received the placebo treatment first and later crossed over to receive oxytocin in the second phase, displayed a significant further improvement in social responsiveness. This observation partially allows us to discriminate between the placebo effect and actual treatment effect. Secondly, larger improvements were revealed when oxytocin administration was combined with targeted psychosocial therapy that stimulates socio-communicative behaviours. Additionally, the oxytocin effect was larger when the parents were convinced their child received the actual treatment. These findings highlight the importance of social stimulation and social context when administering oxytocin. So, depending on the clinical goal, oxytocin administration could be combined with a particular social skills treatment to increase sensitivity towards related social cues. Alternatively, based on the current findings of oxytocin-induced attenuated neural responses in the absence of concurrent social stimulation, we can hypothesize that this is related to a reduction in social anxiety,

possibly promoting a general increase in prosocial behaviour. However, future research is necessary to further investigate the differential (neural) impact of combining oxytocin treatment with or without specific socially-stimulating tasks/contexts. More so, if we want to aim for a more personalized approach, it is important that future research looks at which combinations are most optimal for which individual characteristics.

Ultimately, a major question is to what extent do the findings observed in this thesis reflect a desired outcome for the ASD individual? Apparently, multiple-dose oxytocin administration (without any social context) might reduce the neural sensitivity for automatic, implicit and fine-grained facial expression discrimination. On a positive note, one may contend that it blurs and removes the sharper edges of the social interaction and promotes a more relaxed and positive baseline attitude and neural physiology. This pattern might echo what people do on a Friday evening or at a reception, where they drink some alcohol to feel more at ease and to manoeuvre social encounters more fluently. In a moderate amount and on an infrequent basis, this behaviour as well may open a window of opportunity to enlarge their social network and explore new social skills. Combining oxytocin with social training and social stimulation might offer the additional advantage that it could also sharpen and enhance social skills and social sensitivity, which -combined with a more relaxed and approach oriented social attitude- might support daily life social functioning for individuals with ASD.

Methodological Limitations and Implications

The following section summarizes the main limitations of the research in this thesis that should be taken into consideration with respect to the generalizability of the results.

First, considering the systematic reviews of chapter 2 and 3, the main limitation was the considerable methodological heterogeneity between the included studies, specifically in terms of (i) phenotypic characterization (including symptomatology of ASD), (ii) the included age range of the participants, (iii) the distribution of sex within the samples, (iv) the tissue samples used for assessing oxytocin concentrations or *OXTR* DNA methylation levels, and (v) the wide range of analysis approaches. The high variability made direct comparison difficult, nevertheless initial patterns of results emerged for both oxytocin concentrations and for *OXTR* DNA methylation levels.

Second, the clinical trial did not include a single-dose oxytocin administration assessment, so no direct comparison between acute and long-term oxytocin effects could be made, which would have been informative for understanding the role of the social context on oxytocin's behavioural and neural effects, i.e., specifically during the acute window of heigtened exogenous oxytocin availability. In a similar vein, a consistent standardization and/or monitoring of the social context during the intranasal administrations throughout the trial would have been useful to inform us on possible interindividual variability in treatment outcomes.

Third, we adopted a dosing scheme of 2x 12 IU per day, four weeks long, similar as previous multiple-dose oxytocin studies (Alaerts et al., 2020; Bernaerts, Boets, Steyaert, et al., 2020). However, it cannot be determined whether the results would have exhibited a similar pattern with a different dosage and/or duration of administration. Nowadays, some studies adopt an intermittent dosing scheme (administration every other day) to avoid possible oversaturation of the endogenous oxytocin system (due to desensitization of oxytocin receptors) (Le et al., 2022) and some single-dose studies have identified inverted U-shaped dose-response curves of oxytocin (Yamasue et al., 2022). So, not only dose and duration of the oxytocin treatment are important to consider in multiple-dose studies, but also the frequency or interval at which doses are administered may be important design-related factors for yielding optimal oxytocin treatment effects.

Fourth, although not uncommon in the ASD literature, the diagnostic groups differed on IQ (especially verbal IQ) in the neuroimaging studies (chapters 5 and 6). However, in the EEG analyses no influence of IQ was found on the neural responses when added as a covariate. Furthermore, it should be noted that the IQ scores in ASD are not below the general population mean (i.e., 100), rather the NT control group show above-average scores.

Fifth, considering the fairly basic blocked fMRI design, it is probable that more subtle and informative group differences in neural activity during face processing may have been detected while using a more advanced fMRI design and analysis approach. Yet, Hendriks et al (2021) applied a sophisticated facial identity and expression processing paradigm and a broad range of state-of-the-art analysis approaches (including univariate, multivariate, adaptation and functional connectivity analyses), but did not reveal spectacular group differences in adults with ASD as compared to matched NT controls. In this regard, I initially designed and piloted a frequency-tagging facial expression fMRI

paradigm, similar to the categorical face processing design described by (Gao et al., 2018). However, after extensive pilot testing (n=12 controls) and in-depth analyses, we decided that this paradigm did not fulfil its envisaged promises, and we therefore opted to use a basic and standard face processing fMRI paradigm within the clinical trial. With regard to the EEG method: While it convincingly reveals group differences and treatment effects, due to its intrinsic low spatial resolution, it does not allow to pinpoint neural sources, leading to an unspecific attribution of the findings to a large brain area (i.e., occipitotemporal regions).

Conclusion

This PhD thesis presents a literature overview that provides evidence for an altered development of the endogenous oxytocin system in ASD, specifically lower oxytocin levels are found in children with ASD, but not adolescents and adults (**chapter 2**) and higher methylation levels of the oxytocin receptor gene are found in adults with ASD and are associated with ASD characteristics in neurotypical adults (**chapter 3**). This thesis also reports the behavioural and neural results from a randomized, placebo-controlled, double-blind, parallel, multiple-dose intranasal oxytocin clinical trial performed in children with ASD (**chapters 4, 5 and 6**). First, no treatment-specific behavioural improvements in ASD could be concluded, however, it was identified that clinical efficacy of oxytocin administration was enhanced when combined with targeted psychosocial therapy (**chapter 4**). Secondly, children with ASD exhibited reduced neural sensitivity towards expressive faces compared to neurotypical children as measured with frequency-tagging EEG, validating the ability of this technique to pinpoint socio-communicative sensitivity differences in children with versus without ASD (**chapter 5**). Thirdly, after four weeks of placebo nasal spray administrations in children with ASD we observed a significant increase in frequency-tagging EEG responses towards expressive faces, but strikingly, neural activity remained unaffected after the four-week oxytocin treatment, suggesting a dampening of an otherwise typically occurring implicit learning effect, likely reflecting oxytocin's anxiolytic effects towards expressive faces (**chapter 5**). Lastly, repeated oxytocin administration in ASD only had a tentatively attenuating effect on STS activity (a core face processing brain region), which together with the EEG data is supportive of the anxiolytic account of oxytocin (**chapter 6**).

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Appendices | Dutch Summary, Personal Contribution, Conflict of Interest, PhD Portfolio, About the Author, Acknowledgements

Dutch Summary

Autisme Spectrum Stoornis (ASS) is een neurologische ontwikkelingsstoornis die gekenmerkt wordt door beperkingen in sociale communicatie en interactie, en door de aanwezigheid van beperkte, stereotype en repetitieve interesses en gedragingen (American Psychiatric Association, 2013). Op dit moment is er geen farmacologische behandeling beschikbaar voor ASS, maar initiële studies tonen aan dat de neuropeptide *oxytocine* het potentieel zou kunnen hebben om sociale beperkingen bij ASS te verlichten (Yamasue et al., 2018; Yamasue & Domes, 2017). Oxytocine functioneert als een hormoon in het lichaam (induceert bevalling en lactatie (Zingg & Laporte, 2003)) en functioneert in de hersenen als een neuromodulator, betrokken bij sociaal-cognitieve functies en gedragingen (Fineberg & Ross, 2017; Marlin & Froemke, 2017). Inzicht in hoe oxytocine sociaal gedrag beïnvloedt is daarom van het grootste belang. Zodoende biedt deze doctoraatsthesis een overzicht van de literatuur over het endogene oxytocinesysteem in ASS, en de relatie tussen variaties in de epigenetica van het oxytocine receptor gen en ASS-kenmerken. Verder rapporteert dit proefschrift de gedrags- en neurale resultaten van een gerandomiseerde, placebogecontroleerde, dubbelblinde, parallelle, meervoudige dosis intranasale oxytocine klinische studie uitgevoerd bij kinderen met ASS.

Hoofdstuk 2 omvat een systematisch overzicht en een meta-analyse van de verschillen in endogene oxytocineconcentraties bij personen met ASS in vergelijking met een controle groep, over een breed leeftijdsbereik van kindertijd tot volwassenheid. Wij includeerden 18 studies met in totaal 1422 deelnemers. In de eerste plaats bleek uit de analyse dat de endogene oxytocinespiegel lager ligt bij kinderen met ASS, maar interessant genoeg lijkt dit effect te verdwijnen bij adolescenten en volwassenen met ASS. Ten tweede bleek uit de evaluatie van het effect van het geslacht dat de oxytocinespiegels bij mannen trendmatig lager zijn, maar dat er geen verschil is tussen de groepen bij vrouwen. De mannelijke groep was echter groter dan de vrouwelijke groep, wat hier gedeeltelijk een rol zou kunnen spelen. Ten slotte werden lagere oxytocinespiegels bij ASS vooral gevonden in studies op bloedplasma stalen, maar niet in studies met speekselstalen. Merk wel op dat beide weefseltypes aanzienlijke en vergelijkbare effectgroottes hadden, en dat er geen verschillen tussen weefseltype-subgroepen konden worden vastgesteld.

In **hoofdstuk 3** onderzochten we systematisch de literatuur over de associatie van oxytocine receptor gen (*OXTR*) DNA methylatie met ASS kenmerken en gerelateerde sociale dimensies. Twaalf artikelen die *OXTR* DNA methylatie onderzochten in relatie tot ASS, sociale perceptie/cognitie en sociale angst werden geïncludeerd. Wij zagen hogere methylatie niveaus bij volwassenen met ASS en vonden een associatie tussen hogere methylatie en meer autismekenmerken bij volwassenen, en verhoogde hersenactiviteit (maar verminderde connectiviteit) tijdens sociale experimenten, indicatief voor een grotere behoefte aan neurale bronnen. Omgekeerd werden lagere methylatie niveaus in verband gebracht met meer sociale angst. Interessant genoeg vonden twee studies die de *OXTR* DNA methylatie evalueerden bij kinderen met ASS verminderde methylatie, vooral bij jongens.

Hoofdstuk 4 beschrijft de gedragsresultaten van een gerandomiseerd, placebogecontroleerd, dubbelblind, parallel, meervoudige dosissen intranasaal oxytocine klinisch onderzoek uitgevoerd bij 77 kinderen met ASS (n=61 jongens, n=16 meisjes) in de leeftijd van 8-12 jaar. Oxytocinetoedieningen (12 IE) werden tweemaal daags toegediend gedurende vier weken, bij 8- tot 12-jarige jongens en meisjes met ASS (n=38 oxytocine, n=39 placebo). De dubbelblinde fase van de klinische proef werd gevolgd door een enkelblinde fase, waarin alle ASD-kinderen oxytocine kregen. Verbeteringen op sociale responsiviteit, zoals gemeten met de Social Responsiveness Scale (SRS-2; J. Constantino & Gruber, 2012), werden gevonden in de oxytocinegroep maar ook in de placebogroep. Er konden dus geen behandeling-specifieke verbeteringen worden geconcludeerd. Er werden echter belangrijke moderator effecten geïdentificeerd, die belangrijke aanwijzingen geven dat de klinische effectiviteit kan worden vergroot wanneer oxytocine toediening gepaard gaat met gerichte gedragstherapie. Er werden met name grotere verbeteringen gevonden bij kinderen die gelijktijdig een psychosociale behandeling kregen in combinatie met oxytocine, dan kinderen die alleen oxytocine kregen. Bovendien was het oxytocine-effect groter wanneer de ouders geloofden dat hun kind de eigenlijke behandeling kreeg. Ten slotte vertoonden de kinderen die in de dubbelblinde fase placebo kregen en vervolgens in de enkelblinde fase oxytocine kregen, een significante extra verbetering, die groter was dan de eerdere verbeteringen door placebo.

Hoofdstuk 5 bevat de neurale resultaten van de relatief nieuwe, robuuste en impliciete frequency-tagging EEG techniek, die werd toegepast in een gerandomiseerde,

placebogecontroleerde, dubbelblinde, parallelle klinische studie met meervoudige doses oxytocine (vier weken, 12 IE, tweemaal daags). Specifiek werd frequency-tagging EEG gebruikt om het effect te onderzoeken van meervoudige dosis oxytocine op neurale gevoeligheid voor blije en angstige gezichtsuitdrukkingen bij kinderen met ASS (n=29 oxytocine, n=32 placebo), onmiddellijk na de behandeling en na een follow-up van vier weken. Voorafgaand aan de interventie (op baseline) vergeleken we de neurale gevoeligheid voor gezichtsuitdrukkingen bij kinderen met ASS met die van leeftijds- en geslachts-gematchte neurotypische kinderen (n=39). Ten eerste vertoonden kinderen met ASS een verminderde neurale gevoeligheid voor expressieve gezichten in vergelijking met neurotypische kinderen. Ten tweede vertoonden kinderen met ASS na toediening van placebo neusspray een significante toename in neurale gevoeligheid bij de post- en follow-up sessies, wat waarschijnlijk een impliciet leereffect weerspiegelt. Opvallend is dat de neurale gevoeligheid onaangetast bleef na de vier weken behandeling met oxytocine, wat wijst op een demping van een anders typisch optredend impliciet leereffect, waarschijnlijk als gevolg van de angst-remmende effecten van oxytocine op emotioneel-evocatieve gezichten. Interessant genoeg zien we bij de follow-up sessie (vier weken na stopzetting van de oxytocine toediening) een toename in neurale gevoeligheid, vergelijkbaar met die in de placebogroep.

Hoofdstuk 6 breidt de neurobiologische bevindingen van hoofdstuk 5 uit met resultaten van een fMRI-taak voor gezichtsverwerking. Hoewel frequency-tagging EEG een robuuste indicator bleek te zijn van sociaal-communicatieve gevoeligheid, informeert het ons niet over de onderliggende neurale mechanismen van afwijkende gezichtsverwerking in ASS, of het effect van herhaalde toediening van oxytocine. Daarom vergeleken we met een vergelijkbare fMRI taak de neurale gezichtsverwerking bij kinderen met ASS (n=58) met gematchte neurotypische controles (n=38). Vervolgens werd het effect van een meervoudige dosis oxytocine therapie op de fMRI gezichtsverwerking geëvalueerd in ASS (n=20 oxytocine, n=24 placebo). Ten eerste zagen we atypische gezichtsverwerking fMRI responsen bij kinderen met ASS in vergelijking met gematchte neurotypische controles, in vroege visuele hersengebieden, inferieure frontale cortex en in de linker amygdala. Ten tweede herstelde herhaalde toediening van oxytocine bij ASS de neurale activiteit in deze veranderde gebieden niet, maar er werd wel een tentatief dempend effect van oxytocine op de STS gevonden, wat waarschijnlijk de angst-remmende hypothese van oxytocine ondersteunt.

Personal Contribution

The work presented in this thesis is the result of several scientific collaborations. Matthijs Moerkerke together with Bart Boets, Kaat Alaerts, Jean Steyaert and Nicky Daniels conceptualised and set up the clinical trial. Matthijs Moerkerke and Nicky Daniels (i) set up the logistics of the trial (in collaboration with Annelies Bamps), (ii) collected all the data (in collaboration with Annelies Bamps, Stephanie Van der Donck, Tiffany Tang, Jellina Prinsen, Edward Debbaut), (ii) performed all data analyses and manuscript preparation (with several contributors for each chapter as listed below). Bart Boets, Kaat Alaerts and Jean Steyaert provided senior mentorship on all articles in this thesis.

Chapter 2:

Author contributions: Matthijs Moerkerke: Conceptualization, Methodology, Investigation, Data curation, Validation, Writing - Original Draft, Writing - Review & Editing, Visualisation, Project administration; Mathieu Peeters: Conceptualization, Methodology, Investigation, Data curation, Writing - Original Draft, Visualisation. Lyssa de Vries: Methodology, Investigation, Writing - Review & Editing, Visualisation; Nicky Daniels: Validation, Writing - Review & Editing; Jean Steyaert: Supervision, Validation, Writing - Review & Editing, Funding acquisition; Kaat Alaerts: Supervision, Validation, Writing- Reviewing and Editing, Funding acquisition; Bart Boets: Supervision, Conceptualization, Methodology, Validation, Writing- Reviewing and Editing, Funding acquisition.

Chapter 3:

Author contributions: Matthijs Moerkerke: Conceptualization, Methodology, Investigation, Validation, Writing - Review & Editing, Visualisation, Project administration; Marie-Laure Bonte: Conceptualization, Methodology, Investigation, Data curation, Writing - Original Draft, Visualisation. Nicky Daniels: Validation, Writing - Review & Editing; Jean Steyaert: Supervision, Validation, Writing - Review & Editing, Funding acquisition; Viktoria Chubar: Validation, Writing - Review & Editing; Kaat Alaerts: Supervision, Validation, Writing- Reviewing and Editing, Funding acquisition; Bart Boets: Supervision, Conceptualization, Methodology, Validation, Writing- Reviewing and Editing, Funding acquisition.

Chapter 4:

Author contributions: Nicky Daniels: Investigation, Project administration, Formal analysis, Writing - Original Draft; Matthijs Moerkerke: Investigation, Writing – Review & Editing; Jean Steyaert: Conceptualization, Resources, Writing – Review & Editing; Annelies Bamps: Project administration, Resources; Edward Debbaut: Investigation, Resources, Writing – Review & Editing; Jellina Prinsen: Investigation, Writing – Review & Editing; Tiffany Tang: Investigation, Writing – Review & Editing; Stephanie Van der Donck: Investigation, Writing – Review & Editing; Bart Boets: Conceptualization, Writing – Review & Editing, Funding acquisition, Supervision; Kaat Alaerts: Conceptualization, Project administration, Formal analysis, Writing - Original Draft, Funding acquisition, Supervision.

Chapter 5:

Author Contributions: Matthijs Moerkerke: conceptualization, methodology, investigation, data curation, validation, writing—original draft, writing—review and editing, visualization, project administration. Nicky Daniels: conceptualization, investigation, data curation, validation, writing—review and editing, project administration. Stephanie Van der Donck.: methodology, investigation, data curation, validation, writing—review and editing, visualization. Laura Tibermont: methodology, investigation, data curation, validation, writing—review and editing, visualization. Tiffany Tang: methodology, investigation, data curation, validation, writing—review and editing. Edward Debbaut: methodology, investigation, data curation, validation, writing—review and editing; Annelies Bamps.: data curation, validation, writing—review and editing. Jellina Prinsen: investigation, data curation, validation, writing—review and editing. Jean Steyaert: supervision, validation, writing—review and editing, funding acquisition. Kaat Alaerts: supervision, methodology, validation, writing—review and editing, funding acquisition. Bart Boets: supervision, conceptualization, methodology, validation, writing—review and editing, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Chapter 6: Matthijs Moerkerke: conceptualization, methodology, investigation, data curation, validation, writing—original draft, writing—review and editing, visualization, project administration. Nicky Daniels: conceptualization, methodology, investigation,

data curation, validation, writing—review and editing, project administration. Stephanie Van der Donck.: methodology, investigation, data curation, validation, writing—review and editing, visualization. Tiffany Tang: methodology, investigation, data curation, validation, writing—review and editing. Edward Debbaut: methodology, investigation, data curation, validation, writing—review and editing; Annelies Bamps.: data curation, validation, writing—review and editing. Jellina Prinsen: investigation, data curation, validation, writing—review and editing. Jean Steyaert: supervision, validation, writing—review and editing, funding acquisition. Kaat Alaerts: supervision, conceptualization, methodology, validation, writing—review and editing, funding acquisition. Bart Boets: supervision, conceptualization, methodology, validation, writing—review and editing, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest Statement

The authors, including Matthijs Moerkerke, Nicky Daniels, Stephanie Van der Donck, Laura Tibermont, Tiffany Tang, Edward Debbaut, Annelies Bamps, Jellina Prinsen, Mathieu Peeters, Lyssa de Vries, Marie-Laure Bonte, Viktoria Chubar, Jean Steyaert, Kaat Alaerts and Bart Boets, declare no conflict of interest.

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PhD Portfolio

Journal Articles

Peer reviewed publications used in the thesis manuscript

Moerkerke, M., Peeters, M., de Vries, L., Daniels, N., Steyaert, J., Alaerts, K., Boets, B. (2021). Endogenous Oxytocin Levels in Autism-A Meta-Analysis. *Brain Sci* , *11* (11). doi: 10.3390/brainsci11111545 Open Access

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Peer reviewed publications outside this thesis manuscript

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Supervision of Master thesis

- Biomedical Sciences master thesis: 3
- Medicine master thesis: 2
- Psychology master thesis: 1

Supervision of internships

- Biomedical Sciences: 6
- Medicine: 8
- Psychology: 3

Membership

- Member of Belgian Association for Psychological Sciences (BAPS) (2022-…)
- Member of KU Leuven Child and Youth Institute (L-C&Y) (2021-…)
- Member of International Society for Autism Research (INSAR) (2018-…)
- Member of Leuven Brain Institute (LBI) (2019-…)
- Member of Leuven Autism Research (LAuRes) (2017-…)
- Member of ADHDynamisch (2015-2017)

About the Author

Matthijs Moerkerke was born on June 15th 1992 in Brugge, Belgium. His passion for science was already clear when he enrolled in the Bachelors program Biomedical Sciences in 2010 at the KU Leuven, campus Kulak Kortrijk. Subsequently, he continued with the Master's program clinical Biomedical Sciences and completed his master thesis "Assessing decisional impulsivity in children with ADHD" under supervision of prof. dr. Marina Danckaerts at the Center for Developmental Psychiatry at the KU Leuven. As daily supervisor, dr. Jeroen Van Dessel introduced him to various radiological techniques, which triggered Matthijs' interest in neuroimaging. Therefore, after obtaining his master's degree (cum laude), Matthijs completed the postgraduate studies in Advanced Medical Imaging, summa cum laude, at the KU Leuven in 2017. Within this postgraduate program he performed an internship and thesis titled "Understanding the role of the amygdala in attention-deficit/hyperactivity disorder: association between brain structure, function and delay aversion". After graduating, he remained involved in the program and became backup ombudsman of the postgraduate Advanced Medical Imaging.

In October 2017, Matthijs started his PhD project at the Center for Developmental Psychiatry at the KU Leuven, under supervision of prof. dr. Bart Boets, prof. dr. Kaat Alaerts and prof. dr. Jean Steyaert. He was involved in a large clinical study on the effects of oxytocin administration in children with autism spectrum disorder (MOX study). For his PhD thesis, he investigated the literature on the endogenous oxytocinergic system in autism and investigated the behavioural and neural effects of multiple-dose oxytocin treatment in children with autism. Furthermore he investigated the ability of a novel neuroimaging technique to pinpoint socio-communicative sensitivity differences in children with versus without autism. The results are described in this PhD thesis and published in scientific international journals.

During his PhD, he got the opportunity to present his work at several international conferences, such as at INSAR in Rotterdam (The Netherlands) and twice at an oxytocin workshop in Sicily (Italy), for which he obtained a FWO travel grant. He also received a FWO travel grant to attend a workshop on state-of-the-art MRI-analysis software (Freesurfer) at the Martinos Center for Biomedical Imaging (MIT-Harvard), Boston (USA). Matthijs recently received a postdoctoral fellowship (PDM) that he will take up in 2023 to continue his research within the Center for Developmental Psychiatry at KU Leuven.

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