Title: Amygdala–hippocampal connectivity is associated with endogenous levels of oxytocin and can be altered by exogenously administered oxytocin in adults with autism

Running title: Oxytocin modulates Amygdala–hippocampal connectivity

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Abstract

Background. Oxytocin (OT) plays a pivotal role in interpersonal bonding, affiliation and trust, and its intranasal administration is increasingly considered as a potential treatment for autism spectrum disorders (ASD).

Methods. We here explored whether variations in endogenous, salivary OT concentration are related to inter-individual differences in core autism symptoms and expressions of attachment in 38 male adults with ASD. Further, resting-state fMRI was adopted to specifically explore whether inter-individual differences are reflected in the intrinsic network organization of key regions of the central oxytocinergic system.

Results. Positive correlations were identified between peripheral OT and expressions of secure attachment (State adult attachment measure; Inventory of peer attachment), but no significant relationships were identified with scales assessing core autism symptom domains (Social responsiveness scale; Repetitive behavior scale).

At the neural level, higher levels of endogenous OT were associated with lower degrees of inter-regional functional coupling between amygdala and hippocampal regions. Interestingly, a single dose of exogenously administered OT induced a further reduction in amygdala-hippocampal connectivity, indicating that a higher availability of OT can alter the degree of amygdala-hippocampal connectivity.

Conclusion. The identified associations between the oxytocinergic system, expressions of secure attachment and amygdala-hippocampal pathways are anticipated to be of relevance for understanding the role of OT in modulating appropriate neural and physiological responses to stress and restoring homeostasis.

European Clinical Trial Registry (Eudract 2014-000586-45)

Introduction

The neuropeptide 'oxytocin' (OT) is produced by the paraventricular and supraoptic nuclei of the hypothalamus and is known to play a pivotal role in a variety of complex social behaviors, including interpersonal bonding, trust and affiliative and cooperative behavior (for reviews see [1-3]). The past decade, intranasal administration of OT has gained increasing interest as a possible treatment for targeting the socio-communicative difficulties characteristic of autism spectrum disorders (ASD).

Initial single-dose administration studies consistently demonstrated behavioral improvements on various social tasks in patients with ASD [4-8]. Also several multiple-dose administration studies showed improvements in the social domain after 5 or 6 weeks of continual OT administration in adults [9;10] or in 4- or 5-week trials in children with ASD [11], even though other multiple-dose studies failed to replicate positive outcomes after 4-day [12] or 8-week trials [13] in children and adolescents with ASD. Interestingly, in a recent study by Parker et al. (2017), examining the effect of a course of 4 weeks of continual OT treatment in children with ASD, it was shown that pretreatment blood OT concentrations predicted treatment response, therefore indicating that individuals with pretreatment OT signaling deficits may benefit the most from OT treatment [14].

To date, controversy exists however with respect to the association between ASD diagnosis and endogenous OT levels, with initial studies demonstrating *lower* OT levels in children with ASD compared to control groups [15-17], while other studies showed no differential effect of diagnosis [18;19] or even higher OT levels in adults with ASD [20]. Beyond diagnosis, more consistent associations have been demonstrated between endogenous levels of OT in plasma and maternal or paternal bonding behaviors, infant social engagement, attachment-related thoughts and reduced psychological distress [21-24].

While evidence with respect to associations of endogenous OT levels with behavioral manifestations of interpersonal bonding, attachment and trust is increasingly emerging, research investigating whether and how inter-individual variations in endogenous OT are related to variations in the central oxytocinergic brain system is relatively sparse. Mielke et al. (2018) recently explored associations between plasma OT and brain morphology of the hypothalamus (where OT is produced) and other brain regions of the central oxytocinergic system (amygdala, hippocampus, nucleus caudatus, nucleus accumbens) and showed that *lower* levels of OT were associated with *larger* grey matter volume of the amygdala and hypothalamus in women with early-life maltreatment [25]. Similarly, Andari et al.

(2014) also identified an association between *lower* OT levels and *higher* grey matter volume in right amygdala as well as right hippocampus [26]. While these structural neuroimaging studies provided initial evidence on a link between endogenous OT levels and *structural* variations in the central OT system (the amygdala in particular), it remains unclear whether and how endogenous OT levels are related to *functional* variations of the central OT circuitry.

To fill this gap, the current study explored whether endogenous (salivary) OT levels in male adults with ASD are related to variations in resting-state functional connectivity (FC) between key regions of the central oxytocinergic system (amygdala, hippocampus, nucleus caudatus, nucleus accumbens, hypothalamus). Resting-state functional magnetic resonance imaging (fMRI) data were acquired (as opposed to task-evoked fMRI), since resting-state functional connectivity is postulated to reflect more *intrinsic* (*trait-like*) individual differences in circuitry integrity, whereas task-evoked changes are anticipated to reflect more transient (*state-like*) changes in brain network characteristics in response to specific task demands. Importantly, for the inter-regional connections that intrinsically showed a significant association with endogenous levels of OT (at baseline), we next specifically explored whether a single dose of exogenously administered OT would be able to yield an alteration in the intrinsic pattern of inter-regional functional coupling (post-administration). Aside the neurophysiological characterizations, we also assessed associations between endogenous OT levels and the degree of core autism symptoms (social responsiveness and repetitive behaviors). Furthermore, and considering prior evidence in neurotypicals of an association between endogenous OT levels and expressions of inter-personal bonding and attachment (e.g. [23]), we also explored whether similar associations between endogenous OT and inter-individual differences in self-expressed state and trait attachment are evident in patients with ASD.

Methods

General study design and participants

The principal aim of the current study was to explore associations between endogenous oxytocin (OT) levels and neural (resting-state fMRI functional connectivity) and behavioral measures (inter-individual variations in core autism symptoms and attachment) in patients with autism spectrum disorder (ASD). For the inter-regional connections that intrinsically showed a significant association with endogenous levels of OT (at baseline), we specifically explored whether a single-dose of exogenously administered OT would be able to yield an alteration in the intrinsic pattern of inter-regional functional coupling.

Forty participants with ASD were recruited from the Autism Expertise Centre at the Leuven University Hospital to participate in this double-blind, randomized, placebo-controlled trial with parallel design (22 oxytocin, 18 placebo) (**Supplementary Figure 1**). As visualized in the CONSORT diagram, data of one participant of the OT group and one participant of the placebo (PL) group were not included in the final MRI analyses due to insufficient data or low data quality (excessive in-scanner head motion: 98.5% of images with framewise displacement exceeding >0.5 mm). Written informed consent was obtained from all participants prior to the study. Consent forms and study design were approved by the UZ / KU Leuven Ethics Committee for Biomedical Research (S56327) in accordance to The Code of Ethics of the World Medical Association (Declaration of Helsinki). The trial was registered with the European Clinical Trial Registry (Eudract 2014-000586-45) and the Belgian Federal Agency for Medicines and Health products.

Diagnosis with autistic disorder was made by a multidisciplinary team (child psychiatrist and/or expert neuropediatrician, psychologist, speech/language pathologist and/or physiotherapist) based on the strict criteria of the DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders). Prior to the study, the ADOS (Autism Diagnostic Observation Schedule) [27] and estimates of intelligence (6-subtest short-version of the Wechsler Adult Intelligence Scale-IV - Dutch version) were acquired from all participants (**Table 1**). Detailed information on sample size and eligibility criteria is provided in **Supplementary methods** and **Supplementary table 1**.

All assessments took place in the University Hospital of Leuven. Prior to the MRI scanning session, participants completed self-report questionnaires assessing social responsiveness (Social

Responsiveness Scale (SRS) [28;29] and repetitive behavior (Repetitive Behavior Scale - Revised (RBS-R) [30]. The State Adult Attachment Measure (SAAM)) [31] and the Inventory of Parent and Peer Attachment (IPPA)) [32] were also acquired to assess inter-individual variations in state and trait attachment, respectively (See **Supplementary Methods** for a detailed description of the adopted questionnaires).

Table 1

Demographic and clinical characteristics of participants randomized to receive oxytocin or placebo.

	Oxytocin N = 21	Placebo N = 17	t-value	p
Age	24.76 ± 4.85	24.06 ± 5.54	0.42	0.68
Handedness	16 R/ 5 L	15 R/ 2 L		
IQ				
Total IQ	101.76 ± 12.52	107.29 ± 18.91	-1.08	0.29
VIQ	105.57 ± 9.27	111.35 ± 12.99	-1.60	0.12
PIQ	104.76 ± 18.35	104.41 ± 21.88	0.05	0.96
ADOS				
Total	7.19 ± 4.312	7.59 ± 3.89	-0.29	0.77
Communication	2.14 ± 1.35	2.24 ± 1.44	-0.20	0.84
Social interaction	5.05 ± 3.41	5.35 ± 3.14	-0.28	0.78
RRB	1.19 ± 1.29	1.06 ± 0.9	0.36	0.72
			Pearson Chi-square	**p**
Use of psychostimulant medication*	5	2	0.91	0.34
Comorbidity*	7	2	2.42	0.12

Mean ± standard deviation. R: right; L: left; IQ: intelligence quotient; VIQ: verbal IQ; PIQ: Performance IQ; ADOS: Autism Diagnostic Observation Schedule; RRB: restricted and repetitive behavior.
*Detailed information on medication use and comorbidities is provided in **Supplementary Table 1**

Nasal spray administration

Participants were randomly assigned to receive a single dose of oxytocin (OT) or placebo (PL) based on a computer-generated randomized order. Except for the manager of randomization and masking of drug administration, all patients and research staff conducting the trial were blind to treatment allocation. OT (Syntocinon®, Sigma-tau) and placebo (saline natrium-chloride solution) were administered in amber 15 ml glass bottles with metered pump (ACA Pharma). A fixed dose of 24 IU was adopted which is in accordance with most studies of intranasal OT in adults [3]. Each puff per nostril contained 4

international units (IU) of OT. Participants received clear instructions to self-administer the nasal spray (3 puffs/ nostril; 24 IU of OT) [33;34].

Assessment of endogenous salivary oxytocin

Saliva samples were collected using the absorbent device technique (Saliva cotton swabs) right before and approximately 60-70 min after nasal spray administration (single-dose of OT or PL). Prior to collection, subjects were oral resting (no eating, chewing gum, smoking, drinking) for approximately 45 min. Salivary OT levels were determined via the commercial enzyme immunoassay Oxytocin ELISA kit of Enzo Life Sciences, Inc (for more detailed information, see **Supplementary Methods** and **Supplementary Figure 2**).

MRI data acquisition and analysis

Functional magnetic resonance imaging (fMRI) was performed pre- and post-nasal spray administration. In healthy humans, the impact of a single dose of intranasal OT on social cognition is commonly evaluated using a 30–45 min wait-time before the experimental task (see [3;35]). Accordingly, post-MRI scanning was initiated approximately 30 min after nasal spray administration.

A 3.0 Tesla MR-scanner was used to acquire (i) anatomical images and (ii) 7-min resting-state fMRI scans during which participants were instructed to relax (but not sleep), keep their eyes open while staring at a white cross and think of nothing in particular. More detailed information on the scanning parameters, MRI data preprocessing and head motion analyses is provided in **Supplementary Methods** and **Supplementary Figure 3**.

A recent structural neuroimaging study explored the relationship between endogenous OT levels and brain morphology of the hypothalamus (where OT is produced) and other brain regions of the central oxytocinergic system (amygdala, hippocampus, nucleus caudatus, nucleus accumbens) [25]. In accordance to this prior study, we here performed between-subject regression analyses to explore the relationship between endogenous OT levels and region-to-region functional connectivity between a priori defined regions-of-interest (ROIs) centered over the hypothalamus; bilateral amygdala, hippocampus, nucleus caudatus, and nucleus accumbens (**Supplementary Figure 4**). All ROIs (n=9) were defined from the subcortical FSL Harvard-Oxford atlas, except for the hypothalamus, which was

defined with a sphere centered on MNI coordinates [xyz: 0,-4,-8] using a 12-mm radius [36]. For each participant, mean time-series were extracted by averaging across all voxels in each ROI-region and bivariate correlation coefficients were computed between each pair of ROIs. The resultant correlation values were Fisher z-transformed. As implemented in the CONN-toolbox, a seed-level correction (false-discovery-rate (FDR)) was applied to correct for multiple comparisons. In addition to the hypothesis-driven analysis, we also performed a whole-brain regression analysis to identify associations between endogenous OT levels and region-to-region functional connectivity between all regions of the cortical (n=91) and subcortical (n=15) Harvard-Oxford atlas (FDR-corrected for multiple comparisons).

Statistical analysis

Between-subject regression analyses were performed to identify relationships between endogenous OT levels and behavioral/ neural (connectivity) measures.

Since negative associations were identified between endogenous OT levels and amygdala-hippocampal connectivity (at baseline), we specifically explored whether a single-dose administration of OT would further *reduce* amygdala-hippocampus connectivity. To do so, pre-to-post difference scores were calculated for each amygdala-hippocampal connection and difference scores were subjected to a linear mixed-effect model with the random factor 'subject' (n=38), and the fixed factors 'treatment' (OT, PL), 'amygdala ROI' (left, right') and 'hippocampus ROI' (left, right).

Results

Relationship between salivary OT and behavior

Between-subject regression analyses revealed a significant relationship between endogenous salivary OT levels and self-reported attachment toward peers (Inventory of Parent and Peer Attachment (IPPA); subscale 'Peers') (β=.62; t(32)=4.46; $p_{uncorrected}$=.0001; $p_{Bonferroni}$<.001), indicating that participants with *higher* endogenous OT levels experience *more secure* attachment towards peers (**Figure 1A**). No significant associations were identified for the 'Mother' (β =.05) and 'Father' (β =-.09) IPPA subscales.

For the State Adult Attachment Measure (SAAM), a similar positive relationship was revealed between feelings of secure attachment (SAAM 'Security' subscale) and endogenous OT levels (β=.52; t(32)=3.40; $p_{uncorrected}$=.005; $p_{Bonferroni}$<.05) (**Figure 1A**). Also for the SAAM 'Avoidance' subscale, a tentative negative relationship was revealed (β=-.30; t(32)=-1.77; $p_{uncorrected}$=.09), indicating that participants with lower OT levels reported more feelings of 'attachment avoidance' (**Figure 1A**). No significant association was identified for the 'Anxiety' subscale (β=.14; p>.05). Also no significant associations were identified between endogenous levels of OT and the behavioral scales assessing autism symptoms (ADOS: β=-.04; SRS: β=-.16; RBS: β=.06, all p >.05).

Exploration of relationships between the different behavioral scales indicated that higher SRS-total scores (more impairment in social responsiveness) were significantly associated with reduced feelings of secure attachment (IPPA) towards peers (r=-.47; $p_{uncorrected}$=.004), mother (r=-.55; $p_{uncorrected}$=.001) and father (r=-.39; $p_{uncorrected}$=.025); but not with reports of state attachment (SAAM) (security: r=-.16; avoidance: r=.24; anxiety: r=.04; all $p_{uncorrected}$>.05). Notably, exploratory analyses also showed that as a group, the patients with ASD showed higher SRS-scores (t(72)=-5.99; $p_{uncorrected}$< .001), higher SAAM attachment avoidance (t(72)=-2.95; $p_{uncorrected}$=.021), reduced SAAM attachment security (t(72)=2.44; $p_{uncorrected}$=.017), and a trend towards reduced feelings of secure attachment towards peers (IPPA) (t(72)=1.63; $p_{uncorrected}$=.10) when compared to behavioral characterizations previously obtained from neurotypical individuals (mean age=21.1, S.D.=2.6) (data adopted from [34]).

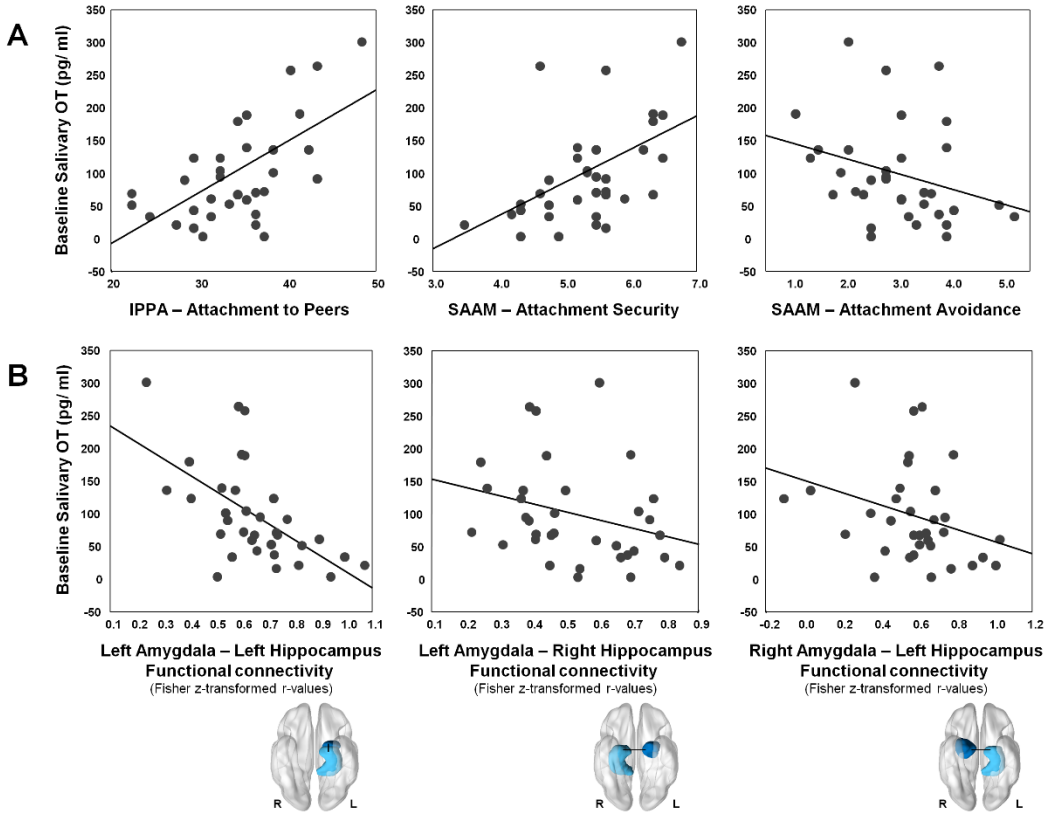

Figure 1

Association of endogenous OT levels with behavior and resting-state functional connectivity

(A) Higher levels of endogenous OT were associated with expressions of secure attachment assessed with the Inventory of Parent and Peer Attachment (IPPA, 'Peers' subscale) and the State Adult Attachment Measure (SAAM, 'Security' subscale). An inverse relationship was shown between endogenous OT levels and SAAM 'attachment avoidance'.

(B) At the neural level, higher levels of endogenous OT were associated with lower degrees of inter-regional functional connectivity between amygdala and hippocampal regions. L: left; R: right.

Relationship between salivary OT and functional connectivity

Between-subject regression analysis identified a significant relationship between endogenous OT levels and functional connectivity between left amygdala and left hippocampus (β=-.60; t(32)=-4.13; p=.0001; p_{FDR}=.001), indicating that participants with *higher* OT levels display *reduced* amygdala-

hippocampal connectivity (**Figure 1B**). At a $p_{uncorrected}<0.05$ threshold, similar associations were identified between endogenous OT levels and connectivity between left amygdala and right hippocampus (β=-.29; t(32)=-1.71; $p_{uncorrected}=.048$) and connectivity between right amygdala and left hippocampus (β=-.30; t(32)=-1.75; $p_{uncorrected}=.044$) (**Figure 1B**). No significant associations were revealed for connectivity with or between the other regions of interest (nucleus caudatus, nucleus accumbens, hypothalamus) (all $p_{uncorrected}>.05$). Note that similar associations with amygdala-hippocampal connectivity were identified when regressions were performed with age and total IQ as covariates.

Whole-brain regression analysis consistently identified the negative association between endogenous OT levels and the left amygdala – left hippocampus connection and additionally identified a similar negative association for connectivity between left amygdala and left anterior parahippocampal gyrus (β=-.55; t(32)=-3.68; $p_{uncorrected}=.0004$; $p_{FDR}=.044$).

Considering the identified association between OT levels and amygdala-hippocampal connectivity (β=-.60), as well as the aforementioned association between OT levels and inter-individual variance in attachment security (IPPA (β=.62); SAAM (β=.52)), we here specifically explored whether variance in amygdala-hippocampal connectivity would also be associated with inter-individual variation attachment security. Regression analyses identified a negative relationships between the extent of left amygdala – left hippocampus connectivity and attachment security (IPPA-peers: β=-.45; t(32)=-2.83; p=.008) (SAAM: β=-.42; t(32)=-2.61; p=.01), indicating that participants with *higher* attachment security also show *lower* levels of amygdala-hippocampal connectivity.

Effect of exogenous OT administration on amygdala-hippocampal connectivity

As expected, after nasal spray administration, salivary OT concentrations were significantly augmented in the OT group, not in the PL group (F(1,28)=35.58; p<.0001) (**Figure 2A**).

Linear mixed-effect analyses, exploring whether a single-dose of OT could further reduce amygdala-hippocampal connectivity, revealed a tentative effect of 'treatment' (across connections) (F(1,36)=2.00; p=.08 (one-sided); $\eta^2=.05$), as well as a trend-level 'treatment x amygdala ROI' interaction (F(1,36)=2.14; p =.07 (one-sided)), indicating that a single-dose of OT significantly reduced connectivity of right amygdala to hippocampal regions (p=.008, Bonferroni post-hoc test), but not connectivity of left amygdala to hippocampal regions (p>.05) (**Figure 2B**). Note that at a whole-brain

level, we revealed no differential changes in functional connectivity from pre-to–post-nasal spray administration in the OT versus the PL group (none of the effects survived correction for multiple comparisons, all $p_{FDR}>0.05$).

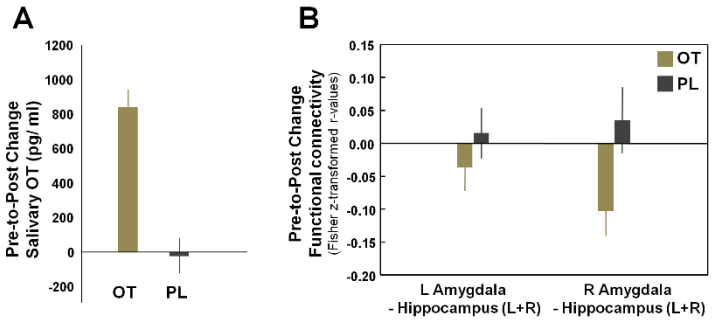

Figure 2

Effect of exogenous OT administration on amygdala-hippocampal connectivity

(A) Change in salivary oxytocin (OT) after nasal spray administration.

(B) The effect of nasal spray administration on functional connectivity of left and right amygdala to hippocampal regions is visualized separately for the oxytocin (OT) and placebo (PL) groups. L: left; R: right.

Discussion

The current study revealed that in adult men with ASD, *higher* levels of salivary oxytocin (OT) are associated with *higher* expressions of secure attachment but *lower* degrees of inter-regional functional coupling between amygdala and hippocampal regions. Notably, administration of a single-dose of OT was able to induce a further *reduction* in the degree of functional connectivity between these core components of the central oxytocinergic system.

Previous fMRI studies have examined the effects of acute stressors on changes in functional connectivity and identified transient increases in amygdala–hippocampal coupling to be associated with dampened cortisol responses both to immediate stress [37] and in the prolonged aftermath of stress [38]. Considering that cortisol has been implicated in enhancing hippocampus-mediated inhibition of the hypothalamic–pituitary–adrenal (HPA) axis [39], these prior findings of Kiem and Vaisvaser provided evidence that *stronger* amygdala–hippocampal coupling is associated with a *decreased* efficiency of the HPA axis to re-establish homeostasis after (stress-induced) perturbation. Interestingly, Fan et al. (2015) demonstrated that state-dependent increases in amygdala-hippocampal connectivity upon stressors were more pronounced in participants with higher reports of early-life maltreatment, and notably, that this positive association was moderated after the administration of intranasal OT [40]. In the current study, we extend these findings by showing that *intrinsic* (trait-like) levels of functional coupling between amygdala and hippocampus were higher in individuals with *lower* endogenous levels of salivary OT, and that both variables were associated with variations in secure attachment (i.e., more insecure attachment in individuals with higher connectivity/ lower OT levels). With respect to the association with OT levels, it is currently unclear, whether the observed variations in amygdala-hippocampal interactions were instrumental to the observed inter-individual differences in OT levels (e.g., inhibition/ facilitation of central release of OT by enhanced/ reduced amygdala-hippocampal connectivity), or inversely, whether a higher availability of OT per se was instrumental to the observed variations in amygdala-hippocampal interactions. In favor of the first interpretation, several anatomical and electrophysiological studies provided indications that the anterior hippocampus can exert an influence on peripheral OT secretion [41;42], although note that the exact influence of hippocampal pathways on central OT release is unclear. Conversely, and in line with the latter interpretation, we here showed that a single-dose of exogenously administered OT was able to induce a *reduction* in amygdala-hippocampal coupling (particularly for connectivity of right amygdala to hippocampal regions). While only peripheral OT levels

were assessed, previous studies in humans [43], non-human primates [44-46] and rodents [47] consistently demonstrated elevated OT levels in the central nervous system after exogenous administration of OT [48]. These and our observations therefore indicate that an elevation of OT levels due to nasal administration was instrumental and causally related to the observed changes in the degree of amygdala-hippocampal connectivity. While the exact pathways in the human brain are unknown, animal research identified axonal fibers containing OT both in amygdala and hippocampal regions [49], suggesting a fast and focal pathway of OT-dependent neuromodulatory regulation in these regions. A recent meta-analysis of human neuroimaging studies identified OT-induced modulations in task-based brain activity of the amygdala, the parahippocampal gyrus as well as the superior temporal sulcus and caudate (see [50] for a recent review). To date however, only a limited number of studies explored the effects of intranasal OT on 'intrinsic' resting-state fMRI functional connectivity and while a majority of studies reported *enhanced* amygdala resting-state connectivity (predominantly with anterior cingulate cortex and prefrontal cortex) [51-56], also a number of studies reported *decreased* amygdala connectivity [52;54-57], or no effect [53;58;59] (see [60] for a recent review).

In terms of behavioral manifestations, higher endogenous OT levels and lower amygdala-hippocampal connectivity were shown to be associated with increased feelings of secure attachment (IPPA, SAAM). These observations are largely consistent with the implicated role of the OT system in parental bonding behaviors and attachment-related thoughts [22-24] and are also in line with the notion that early-life stress may impact on the oxytocinergic brain system. Indeed, research in rodents showed that the endogenous secretion of OT and the expression of OT receptors in the amygdala are modulated by the amount and quality of maternal care [61].

No significant associations were revealed between endogenous OT levels and the degree of core autism symptoms (social responsiveness, repetitive behaviors), which is in line with a prior report demonstrating no associations between baseline OT levels and expressions of autistic traits in the typical population [35]. While to some extent, impairments in social responsiveness were shown to be associated with reduced feelings of parent and peer attachment (IPPA), our data indicate that as a construct, inter-individual variance in attachment style (state and trait attachment security) may be more sensitive for predicting aberrant oxytocinergic signaling, as opposed to core autism symptoms. In the present study, salivary OT levels were only assessed for ASD patients (not for a control group), and it was therefore not possible to establish whether, on average, OT levels were lower in the current ASD

patient sample compared to a sample of neurotypical individuals; as demonstrated before in previous studies [15-17] (although note several exceptions [18-20]). Exploratory analyses showed however that in terms of behavioral characterizations, patients with ASD significantly differentiated from neurotypical individuals on the assessments of attachment style (SAAM attachment security, SAAM attachment avoidance and a trend for IPPA trait attachment towards peers), as well as – and as to be expected - in terms of social responsiveness (SRS). Prior trials investigating the efficacy of long-term (multiple-dose) OT treatment in patients with ASD mostly included evaluations of core autism symptoms (e.g. SRS, RBS, ADOS), with some studies reporting treatment-induced improvements [9-11;14], while others failed to show beneficial effects [12;13]. In view of the current identification of a tight association between attachment-related constructs and the oxytocinergic system in patients with ASD, as well as results from a prior study from our lab, reporting significant improvements in attachment avoidance and peer attachment in neurotypicals after a 2-week course of continual OT treatment [34], we anticipate that it would be of high relevance for future OT trials with ASD patients to continue to include characterizations of attachment style for evaluating treatment outcome. Indeed, since inter-individual variance in attachment style has been highlighted as a potential modulator of OT treatment effects [2;62], it is anticipated that a thorough characterization of attachment style in patients with ASD will aid in explaining inter-individual differences in treatment responses and/ or delineating patient populations that may benefit the most from a course of OT treatment.

Although our study provides new insights regarding the association of endogenous OT levels with behavioral manifestations and intrinsic functional couplings between key nodes of the central OT system, several limitations need to be considered. First, while core autism symptoms were assessed based on observational scales (ADOS) and self-report questionnaires (SRS, RBS), variations in attachment were solely assessed based on self-report questionnaires (SAAM, IPPA). Further, some variation existed in the time point of collection of the salivary samples for assessing OT concentration, and a tentative negative relationship was revealed indicating slightly higher concentrations for the participants tested in the morning, compared to the participants tested in the afternoon/ evening (Supplementary Figure 2). Note however that all the identified associations remained significant after correction for the timing of salivary collection, indicating that the reported relationships persisted over and above variations in OT concentration related to the time point of collection. Further, while a recent study demonstrated moderate to strong correlations between salivary and central (cerebrospinal fluid)

OT levels [63], it should be noted that it remains currently unclear how peripheral OT measures are functionally related to central OT activity. Finally, and as stated before, we also note the lack of a control group of neurotypical individuals as well as the relatively small sample size of included patients with ASD as a limitation of the current study. As such, future research may be warranted to further explore whether similar associations between endogenous OT levels and amygdala-hippocampal circuits are evident in neurotypical or other neuropsychiatric populations.

In summary, we found that higher levels of endogenous OT are associated with lower levels of functional connectivity between two core nodes of the central oxytocinergic system (amygdala and hippocampal regions) and that both variables were associated with expressions of secure attachment, but not core autism symptoms, in male adults with ASD. Notably, the observation that a single-dose of OT was able to induce a reduction in amygdala-hippocampal connectivity indicates that a higher availability of OT causally affected the degree of amygdala-hippocampal connectivity. Together, these findings are in line with the growing body of research highlighting the amygdala and hippocampal regions (and their intrinsic interaction) as key neural loci of the central OT system. In line with prior findings evidencing a link between amygdala-hippocampal connectivity and central responses to stress (i.e., capacity of the HPA axis to restore homeostasis after perturbation) [37;38;40], the identified associations between the oxytocinergic system and amygdala-hippocampal pathways are anticipated to be of relevance for understanding the role of OT in modulating appropriate neural and physiological responses to stress and restoring homeostasis. Since an increased vulnerability to cumulative stress can hugely impact well-being and induce deteriorations in several domains (e.g., social functioning), future research is urged to further explore, both from a neural and behavioral perspective, the role of the endogenous oxytocinergic system in adequate stress responses and maintenance of homeostasis as well as to characterize the effect of exogenously administered OT on these processes.

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Supplementary Information

Amygdala–hippocampal connectivity is associated with endogenous levels of oxytocin and can be altered by exogenously administered oxytocin in adults with autism

Supplementary Methods

Participants

Sample size. To date, several studies explored the effect of single-dose OT administration on changes in task-related fMRI brain activity in patients with ASD [1;5;8;15]. In one prior randomized placebo-controlled trial, a cross-over design was adopted to explore the effect of multiple-dose OT treatment (six weeks of daily doses) on resting-state fMRI functional connectivity in 20 patients with ASD and significant effects (large-size) were reported for a total of 17 patients who completed the OT/ PL cross-over treatment [16]. Considering this prior cross-over study assessing the effects of OT on resting-state fMRI functional connectivity in patients with ASD and the lack of prior studies using parallel designs, the current sample size was set at a comparable sample size.

Inclusion-/ exclusion-criteria. Inclusion criteria comprised clinical diagnosis of autistic disorder; gender (male); and age (18-35 years old). Exclusion criteria for participation comprised any neurological disorder (e.g., stroke, epilepsy, concussion); demonstrated genetic disorder; or any contraindication for magnetic resonance imaging (MRI). Current psychoactive medication use and the presence of comorbid psychiatric disorders were screened (**Table 1 and Supplementary Table S1**). Handedness was assessed based on self-report.

Questionnaires

The *Social Responsiveness Scale* (SRS) [4;11] uses a four-point Likert-scale and comprises of four subscales examining social communication (22 items), social awareness (19 items), social motivation (11 items) and rigidity/repetitiveness (12 items).

The *Repetitive Behavior Scale - Revised* (RBS-R) [9] (43 items) examines a heterogeneous set of repetitive behaviors using a four-point Likert-scale. The RBS comprises six subscales examining stereotypic behavior (6 items), self-injurious behavior (8 items), compulsive behavior (8 items), ritualistic behavior (6 items), sameness behavior (11 items) and restricted interests behavior (4 items).

The *State Adult Attachment Measure* (SAAM) [7] (21 items) comprises three subscales examining attachment security (e.g., “I feel like I have someone to rely on”) (7 items); attachment anxiety (e.g., “I feel a strong need to be unconditionally loved right now”) (7 items); and attachment avoidance (e.g., “If someone tried to get close to me, I would try to keep my distance”) (7 items) using a seven-point Likert-scale.

The *Inventory of Parent and Peer Attachment* (IPPA) [2] (100 items) examines trait attachment to (i) mother (25 items); (ii) father (25 items); (iii) peers (25 items); and (iv) an important person of choice (25 items) using a four-point Likert-scale. The IPPA assesses attachment security along three dimensions: degree of mutual trust, quality of communication and extent of anger and alienation.

Note that the patients included in the present study also participated in a larger clinical trial assessing the (neural and behavioral) effects of multiple-dose treatment with OT (manuscripts in preparation). In the context of this larger project, the following behavioral assessments were additionally obtained from the participants: (i) a questionnaire assessing 'quality of life' and (ii) a side-effect questionnaire assessing changes in mood states (not part of the current report).

Assessment of endogenous salivary oxytocin

Samples were collected using the absorbent device technique (Saliva cotton swabs). Prior to collection, subjects were oral resting (no eating, chewing gum, smoking, drinking) for approximately 45 min. Participants were asked to chew a cotton swab for approximately 60 sec until it was saturated with saliva. After collection, swabs were placed in a sterile conical tube and placed in a −20°C freezer for storage. Samples were centrifuged at 4°C for 15 min at 17.000 rpm and salivary OT levels were determined via the commercial enzyme immunoassay Oxytocin ELISA kit of Enzo Life Sciences, Inc. as used in previous studies [6;10;13]. All sample extraction and concentration procedures were conducted in accordance with the official kit manual. Measurements were performed on duplicate samples (100 µl, undiluted), and the concentrations of samples were calculated according to the relevant standard curve. Concentrations below the detection limit (15 pg/ml) were set to a value half of the detection limit. The intra-assay and inter-assay coefficients of the assay are lower than 13.3% and 20.9%, respectively. Note that for 6 participants (out of 40), baseline salivary OT data were not included in the final analyses due to: (i) drop-out based on MRI (n=2); (ii) saliva sample not available (n=1); (iii) insufficient saliva (n=1); (iv) data identified as extreme outliers (n=2) (i.e., more than 3 interquartile ranges (Q3-Q1) below the first quartile (Q1) or above the third quartile (Q3)). Salivary OT data, collected post-nasal spray administration were not available for 4 additional participants. While regression analyses are reported without inclusion of the extreme outliers, it should be noted that all the identified associations remained significant when Spearman-rank order correlation analyses were performed with inclusion of the outliers. For the majority of participants, the saliva collections took place between 17-22h. To assess potential variations in baseline salivary OT due to diurnal variations, Pearson correlation analyses were performed between the time of collection and baseline OT concentrations. A tentative negative relationship was revealed (n= 34; r= -.34; p= .051), indicating slightly higher concentrations for the participants tested in the morning, compared to the participants tested in the afternoon/ evening (**Supplementary Figure S2**). While regression analyses are reported without correction for timing of saliva collection, it should be noted that all the identified associations remained significant when controlled for timing of saliva collection.

Neural assessment

MRI data acquisition. A 3.0 Tesla Philips Achieva Ds MR-scanner with a 32-channel phased-array head-coil was used to acquire anatomical images and 7-min resting-state fMRI scans during which participants were instructed to relax (but not sleep), keep their eyes open while staring at a white cross and think of nothing in particular. Note that since participants were recruited to participate in a larger

clinical trial assessing the (neural and behavioral) effects of multiple-dose treatment with OT (manuscripts in preparation), the fMRI scanning protocol additionally included two other scan modalities (not part of the current report): (i) task-based fMRI scanning and (ii) diffusion tensor imaging; both performed after acquisition of the resting state scan.

MRI scanning parameters. Anatomical imaging consisted of a high-resolution structural volume acquired using a coronal three-dimensional turbo field echo T1-weighted sequence with the following parameters: 182 contiguous coronal slices covering the whole brain and brainstem, slice thickness = 1.2 mm; repetition time (TR) = 9.4 ms; echo time (TE) = 4.6 ms; matrix size = 208 x 207; field-of-view (FOV) = 250 x 250 mm; in-plane pixel size = 1.2 x 1.2 mm^2; acquisition time = 1 min 43 s. Resting-state fMRI images were acquired using a T2*-weighted gradient-echo echo planar imaging (GE-EPI) sequence with the following parameters: TR = 2500 ms; TE = 30 ms; matrix size = 80 x 78, FOV = 200 x 200 mm; flip angle 90°; slice thickness = 2.7 mm, slice gap = 0.4 mm; axial slices = 45; 162 functional volumes; acquisition time = 7 min.

MRI data preprocessing. SPM-12 (Wellcome Department of Cognitive Neurology, London, UK) and the CONN functional connectivity toolbox 16.b [17] were used for image preprocessing and statistical analyses implemented in Matlab R2015b (Mathworks). Resting-state fMRI images were spatially realigned, normalized to the standard EPI-template of the Montreal Neurological Institute (MNI-152) and resampled into 3-mm isotropic voxels. Realignment parameters were modeled as regressors of no-interest and white matter and cerebrospinal fluid were removed as confounds following the implemented CompCor-strategy [3] in the CONN toolbox. Residual time-series of the resting-state images were then band-pass filtered (0.009<f<0.08Hz). No global signal regression was applied.

Head motion. Given the potential confounding effects of micro-movements on resting-state functional connectivity [12;14], all reported analyses were performed on 'scrubbed' data [12], i.e., censoring frames displaying frame-wise displacement (FD) exceeding >0.5 mm or frame-wise changes in brain image intensity exceeding >0.5 Δ%BOLD. No group- (OT, PL) or session-related differences were revealed in mean FD or the percentage of scrubbed frames (**Supplementary Figure S3**).

Supplementary Table S1

Detailed information on comorbidities and medication use for participants of the oxytocin and placebo group.

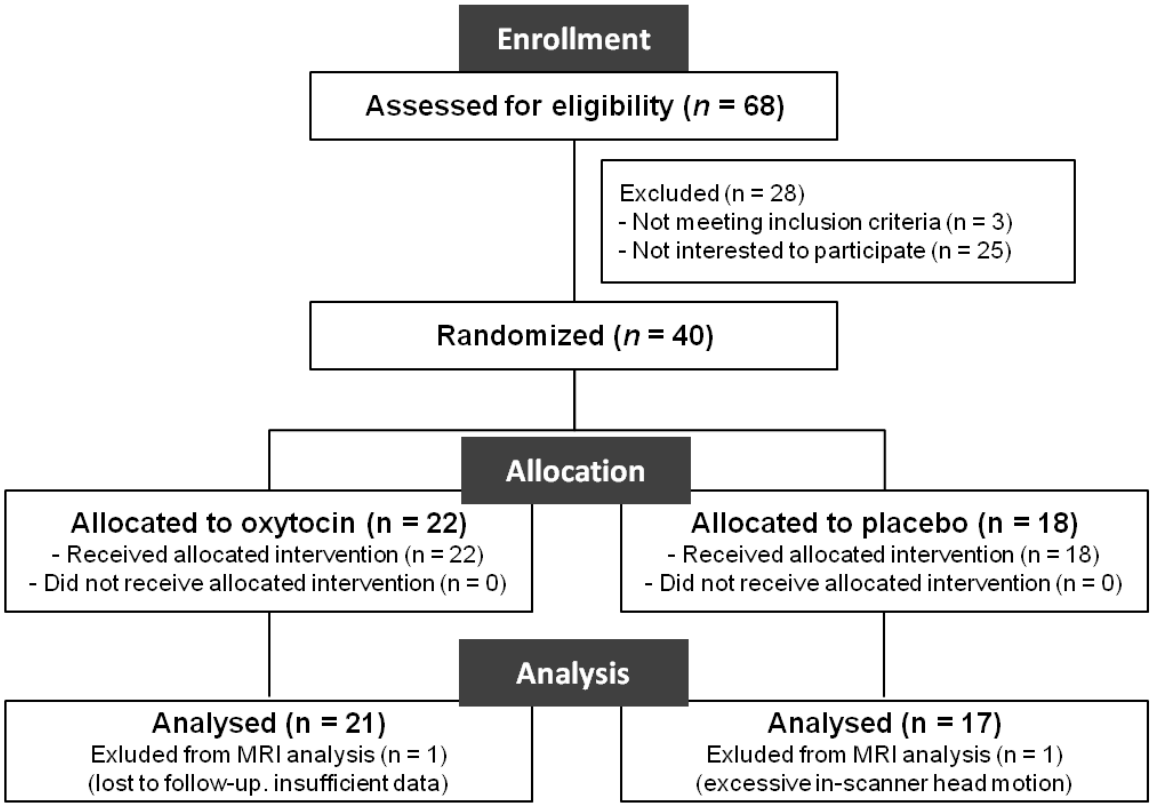
Comorbidities were screened through self-report (with the explicit mentioning of examples in the screening interview including e.g., ADHD, depression, dyscalculia, dyslexia). Current psychoactive medication use was defined as use within three months before study enrollment.

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

ADHD: attention deficit hyperactivity disorder; ADD: attention deficit disorder

Supplementary Figure S1

CONSORT flow diagram of participants in the double-blind, randomized placebo-controlled trial assessing the effect of a single-dose of oxytocin (or placebo) on resting-state fMRI functional connectivity.

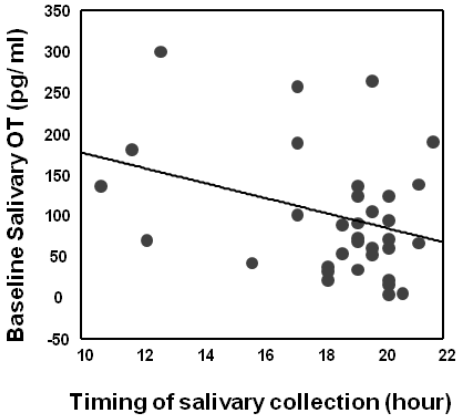


Supplementary Figure S2

Diurnal variations in salivary OT levels at baseline.

To assess potential variations in baseline salivary OT due to diurnal variations, Pearson correlation analyses were performed between the time of collection and baseline OT concentrations.

A tentative negative relationship was revealed (n= 34; r= -.34; p= .051), indicating slightly higher concentrations for the participants tested in the morning, compared to the participants tested in the afternoon/ evening.



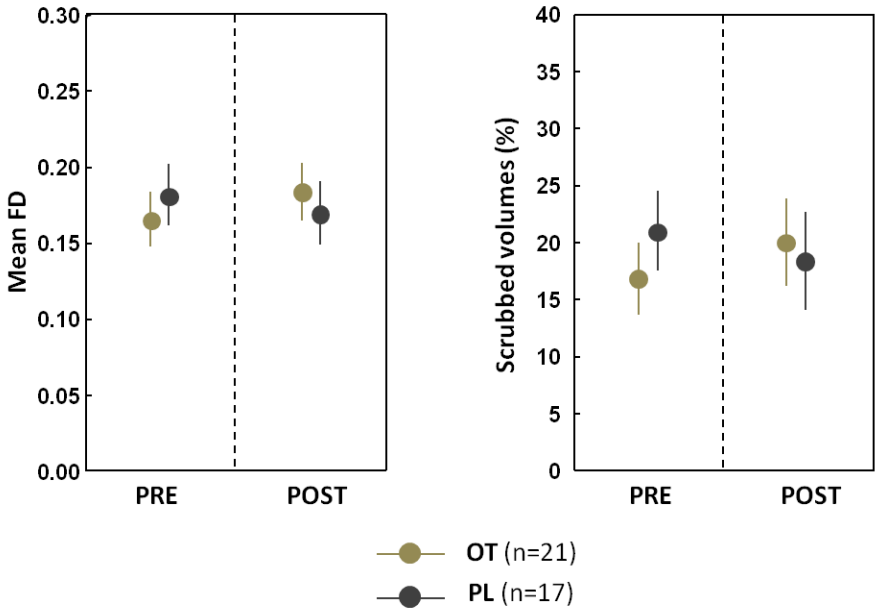
Supplementary Figure S3

Head motion analysis of the resting-state fMRI scans.

For all participants, head motion (mean frame-wise displacement (mean FD)) of the resting-state fMRI scans was assessed at baseline (pre) and after a single-dose of nasal spray administration (post).

Across sessions, mean FD scores were not significantly different between treatment groups (oxytocin (OT), placebo (PL)) ($F(1, 36)= .003$; $p= .96$). Also no significant effect of 'session' ($F(1, 36)= .07$; $p= .78$) or 'session x treatment' interaction ($F(1, 36)=1.64$; $p= .20$) was revealed.

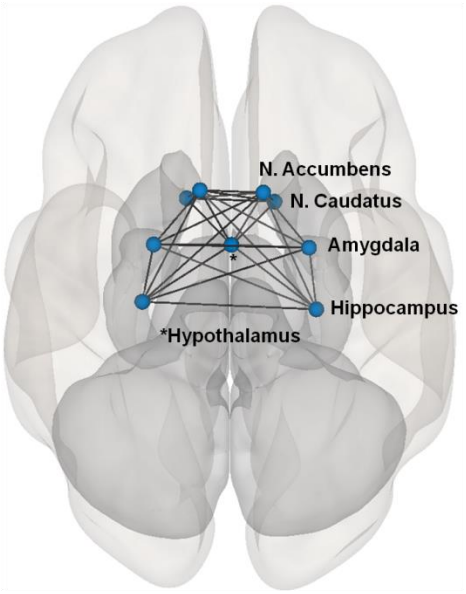
Also for the percentage of scrubbed frames, no significant effect of 'treatment' ($F(1, 36)= .08$; $p= .77$), 'session' ($F(1, 36)= .01$; $p= .92$) or 'session x treatment' interaction ($F(1, 36)=1.09$; $p= .30$) was revealed.



Supplementary Figure S4

Visualization of the regions-of-interest.

Regions-of-interest (n=9) in bilateral amygdala, hippocampus, nucleus caudatus, and nucleus accumbens were defined from the subcortical FSL Harvard-Oxford Atlas maximum likelihood cortical atlas (HarvardOxford-cort-maxprob-thr25-1mm.nii). The hypothalamus was defined with a sphere centered on MNI coordinates [xyz: 0,-4,-8] using a 12 mm radius. Lines denote inter-regional functional connectivity at baseline, assessed across participants (one-sample t-test, $p_{FDR} < 0.05$, seed-level correction) (inferior view).



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