Plasma oxytocin concentrations and *OXTR* polymorphisms predict social impairments in children with and without autism spectrum disorder

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The neuropeptide oxytocin (OXT) and its receptor (*OXTR*) regulate social functioning in animals and humans. Initial clinical research suggests that dysregulated plasma OXT concentrations and/or *OXTR* SNPs may be biomarkers of social impairments in autism spectrum disorder (ASD). We do not know, however, whether OXT dysregulation is unique to ASD or whether OXT biology influences social functioning more generally, thus contributing to, but not causing, ASD phenotypes. To distinguish between these possibilities, we tested in a child ASD cohort, which included unaffected siblings and unrelated neurotypical controls (ages 3–12 y; $n = 193$), whether plasma OXT concentrations and *OXTR* SNPs (*i*) interact to produce ASD phenotypes, (*ii*) exert differential phenotypic effects in ASD vs. non-ASD children, or (*iii*) have similar phenotypic effects independent of disease status. In the largest cohort tested to date, we found no evidence to support the OXT deficit hypothesis of ASD. Rather, OXT concentrations strongly and positively predicted theory of mind and social communication performance in all groups. Furthermore, OXT concentrations showed significant heritability between ASD-discordant siblings ($h^2 = 85.5\%$); a heritability estimate on par with that of height in humans. Finally, carriers of the "G" allele of rs53576 showed impaired affect recognition performance and carriers of the "A" allele of rs2254298 exhibited greater global social impairments in all groups. These findings indicate that OXT biology is not uniquely associated with ASD, but instead exerts independent, additive, and highly heritable influences on individual differences in human social functioning, including the severe social impairments which characterize ASD.

The neuropeptide oxytocin (OXT) and its receptor (*OXTR*) regulate affiliative motivation, social bonding, and social recognition in animals (1, 2). Preclinical research likewise has shown that individual differences in OXT biology (e.g., OXT concentrations, *OXTR* distribution in key brain regions) are associated with individual differences in social functioning (3–6). At the extreme, experimental manipulations that diminish OXT peptide and/or receptor signaling produce a variety of social deficits in animal models (7, 8).

Translation of this animal research to neurotypical populations has confirmed that OXT administration enhances social functioning in humans (9, 10). Individual differences in endogenous OXT concentrations are also positively correlated with social behavior measures (11, 12), such that lower OXT concentrations are frequently associated with diminished social functioning even within neurotypical cohorts. This research has led to the hypothesis that abnormalities in OXT peptide biology may be directly related to social impairments observed in clinical populations, particularly in people with autism spectrum disorder (ASD), who exhibit core deficits in social interactions and preferences, eye contact, facial recognition, empathy, and social communication.

Several studies have begun to explore the so-called OXT deficit hypothesis of ASD. Three studies reported that plasma OXT concentrations are lower in individuals with ASD compared with control participants (13–15). A fourth study reported no overall group differences (16), and a fifth study reported that plasma OXT concentrations are higher in individuals with ASD compared with control participants (17). Relationships between plasma OXT concentrations and measures of social functioning in these small samples were either not analyzed (14, 15) or not found (17) or were evident only in subsets of individuals (16). Additionally, one study paradoxically reported inverse relationships between plasma OXT concentrations and social competence in ASD (a negative relationship) and control (a positive relationship) children (13). It is important to note that these studies had various limitations, including small study cohorts, use of normative range data for control plasma OXT concentrations instead of individual matched blood samples collected from neurotypical study participants, dilution rather than manufacturer-recommended extraction of plasma samples before OXT assay, use of nonstandard methods or unknown methods by which to diagnose ASD, and/or use of inappropriate statistical methods. The important question as to whether low plasma OXT concentrations are a biomarker of ASD and/or social impairments therefore remains unanswered.

The broader autism phenotype (18, 19), in which unaffected family members of ASD probands show subclinical impairments in social and other aspects of behavioral functioning, is thought to have a strong biological basis. Social impairments in family members (particularly in similar-aged siblings) may therefore reflect the heritability of the underlying physiology (i.e., OXT) critical to social functioning. This physiology may not be unique

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Significance

The neuropeptide oxytocin (OXT) is critically involved in mammalian social functioning, and initial clinical research suggests that OXT biology may be altered in individuals with autism spectrum disorder (ASD). Here we provide important evidence that blood OXT concentrations are highly heritable within families, yet also strongly predict social functioning in ASD children, their unaffected siblings, and healthy control children. These findings also extend to OXT receptor genotypes which are significantly associated with differences in social functioning independent of disease status. These findings indicate that dysregulated OXT biology is not uniquely associated with ASD social phenotypes as widely theorized, but instead variation in OXT biology contributes to important individual differences in human social functioning, including the severe social impairments which characterize ASD.

to ASD, but instead individual differences in OXT concentrations may contribute to individual differences in social functioning, including the milder social impairments documented in unaffected siblings and the more severe social deficits found in ASD probands. Whether or not plasma OXT concentrations are heritable, however, remains unknown, as no prior study has examined plasma OXT concentrations in ASD-discordant siblings.

In addition to functional variability in OXT peptide concentrations, preliminary research suggests that genetic variability in the *OXTR* gene may be a risk factor for ASD. Association studies have shown that several SNPs and their haplotypes in the *OXTR* gene increase risk for ASD (20–23). Genome-wide scans likewise indicate that the 3p25 region, which contains the *OXTR* gene (24), may be associated with ASD (25–28). A separate body of research has repeatedly implicated two intronic *OXTR* gene SNPs, rs2254298 and rs53576, in social functioning in both neurotypical and neuropsychiatric populations (29–32). These studies provide suggestive evidence that *OXTR* SNPs may be associated with human social functioning, but it is not clear whether these genetic variants specifically influence ASD social phenotypes or whether they influence social functioning independent of disease status. It is also unknown whether these genetic findings are dependent or independent of OXT peptide concentrations, as no prior study has concomitantly evaluated OXT concentrations and *OXTR* SNPs in the same study population, inclusive of individuals with and without ASD.

The first aim of this study was to test the prevalent but not well-interrogated OXT deficit hypothesis of ASD by investigating whether plasma OXT concentrations are lower in individuals with ASD and whether this relationship is affected by sex and/or *OXTR* SNPs. The second aim of this study was to test for the first time, to our knowledge, in the same statistical model whether plasma OXT concentrations and *OXTR* SNPs (*i*) interact to produce ASD social phenotypes, (*ii*) exert differential social phenotypic effects in ASD vs. non-ASD individuals, or (*iii*) have similar social phenotypic effects for all individuals independent of disease status.

Results

Study participants were 79 children with ASD, 52 unaffected siblings, and 62 unrelated neurotypical control children, ages 3–12 y. Although all cases met criteria for ASD, expert clinical opinion and scores on the diagnostic instruments [i.e., the Autism Diagnostic Interview-Revised (ADI-R) (33, 34) and the Autism Diagnostic Observation Schedule-Generic (ADOS-G)] (35, 36) were also used to test whether OXT biology differed in children with classic autism (n = 47) vs. those with pervasive developmental disorder-not otherwise specified (PDD-NOS) (n = 32)]. A social phenotypic characterization—using the Social Responsiveness Scale (SRS) Total Score (37, 38); NEPSY-II Social and Perception Domain: Affect Recognition and Theory of Mind Tasks (39); and three measures from the Vineland Adaptive Behavior Scales, Second Edition (VABS-2) (40)—was obtained on, and blood samples for OXT quantification and *OXTR* genotyping were collected from, all participants. As expected, children with ASD exhibited impaired social functioning compared with children without ASD. Participant characteristics are presented in Table S1.

Testing the OXT Deficit Hypothesis of ASD: Are Plasma OXT Concentrations Lower in Participants with ASD and Are They Influenced by Sex, *OXTR* SNPs, or Their Interactions?

We used a nested design to test for differences between participant groups, and between symptomatic (i.e., autistic and PDD-NOS) and nonsymptomatic (i.e., sibling and neurotypical control) individuals on average in the same analysis. Despite statistically controlling (i.e., blocking) for possible extraneous sources of variability [i.e., age, ethnicity, blood sample collection time, and full-scale intelligence quotient (IQ), none of which were significant themselves], we found no significant main effects (Fig. 1) or interaction effects (i.e., with sex or *OXTR* SNPs) for plasma OXT concentrations in this

model. Thus, these data, collected from the largest cohort tested to date, did not support the OXT deficit hypothesis of ASD, which was previously advanced on the basis of findings from several smaller-scale pilot studies (e.g., refs. 13 and 14).

Testing the Role of OXT Biology in Social Impairments: Are Plasma OXT Concentrations and *OXTR* Allelic SNPs' Effects on Social Phenotypes Dependent or Independent of Disease Status?

We next tested for the effects of plasma OXT concentrations, the *OXTR* SNPs, group (nested within symptomatic), and their interactions on social phenotypes (while blocking for sex, age, ethnicity, blood sample collection time, and full-scale IQ). We found no evidence that plasma OXT concentrations and *OXTR* SNPs either interact directly to produce ASD social phenotypes or exert differential social phenotypic effects in ASD vs. non-ASD individuals. Rather, all findings converged to uniformly support the third hypothesis: Plasma OXT concentrations and *OXTR* SNPs exert independent, additive, and highly heritable influences on individual differences in human social functioning for children in all groups. (For clarity, we present only the phenotypes with significant findings below.)

Plasma OXT Concentrations and Social Functioning.

Plasma OXT concentrations positively predicted NEPSY theory of mind scores ($F_{1,168} = 4.6514$; $P = 0.0327$), with lower plasma OXT concentrations associated with impaired theory of mind performance. This effect persisted when children below the age of 5 y (i.e., those that may not have yet achieved theory of mind) were excluded from the analysis ($F_{1,115} = 4.6169$; $P = 0.0338$). This relationship was consistent for all groups, with no significant difference observed in the relationship between the four groups [group within symptomatic × OXT interaction: $F_{2,168} = 0.3910$; P = not significant (ns)] or between symptomatic and nonsymptomatic individuals on average (symptomatic × OXT interaction: $F_{1,168} = 0.9846$; P = ns). Symptomatic individuals scored worse on average than nonsymptomatic individuals on the theory of mind measure ($F_{1,168} = 7.6402$; $P = 0.0065$), but the subdivision into individual groups did not predict score (i.e., autistic and PDD-NOS individuals did not differ; siblings and neurotypical control individuals did not differ: $F_{2,168} = 1.6248$; P = ns) (Fig. 2*A*).

The same pattern of results was observed for the Vineland Communication Domain. Plasma OXT concentrations positively
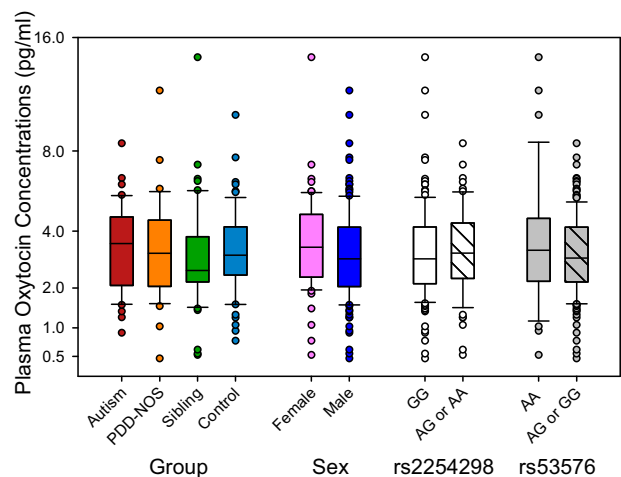

Fig. 1. Plasma OXT concentrations do not significantly differ by group, sex, or *OXTR* SNPs. Group: n = 47 autistic, n = 32 PDD-NOS, n = 52 sibling, and n = 62 neurotypical control children. Sex: n = 62 females and n = 131 males. rs2254928 genotype: n = 127 GG and n = 66 AG or AA. rs53576 genotype: n = 35 AA and n = 158 GG or AG. Box plots are presented as least-squares-mean (LSM) ± SEM and each LSM is partialed for other data in the analysis.

predicted social communication scores ($F_{1,164} = 7.7567$; $P = 0.0060$), with lower plasma OXT concentrations associated with greater communication impairments. This effect was consistent for all groups, with no significant difference observed in the relationship between the four groups (group within symptomatic × OXT interaction: $F_{2,164} = 1.7381$; $P = $ ns) or between ASD and non-ASD individuals on average (symptomatic × OXT interaction: $F_{1,164} = 1.9905$; $P = $ ns). Symptomatic individuals scored worse on average than nonsymptomatic individuals ($F_{1,164} = 46.0306$; $P < 0.0001$) but the subdivision into individual groups did not predict score ($F_{2,164} = 1.0683$; $P = $ ns) (Fig. 2B).

Testing Heritability in ASD-Discordant Siblings: Are the Observed Individual Differences in Plasma OXT Concentrations and the Two Phenotypic Measures They Predict Heritable? We next calculated narrow-sense heritabilities (h^2) (41) for plasma OXT concentrations, NEPSY theory of mind scores, and the Vineland Communication Domain scores for families in which we had data for two individuals. Plasma OXT concentrations were highly heritable ($h^2 = 85.5\%$; $F_{44,51} = 2.619$; $P = 0.0005$) (Fig. 3A), corresponding to a mean correlation between family members of 0.427. By comparison, height in humans has a narrow-sense heritability of ~80% (42). NEPSY theory of mind scores showed moderate heritability ($h^2 = 55.1\%$; $F_{34,38} = 1.812$; $P = 0.0382$) (Fig. 3B), whereas Vineland Communication Domain scores did not show significant heritability ($h^2 = 1.1\%$; $F_{44,51} = 1.012$; $P = $ ns).

OXTR SNPs and Social Functioning. Carriers of the "G" allele (i.e., AG or GG) of rs53576 performed worse on the NEPSY affect recognition task ($F_{1,170} = 4.8577$; $P = 0.0291$) than those with two "A" alleles (Fig. 4A). This effect was consistent for all groups,

Fig. 3. Narrow-sense heritability is estimated as a function of within-family consistency. Individual values are shown for (A) plasma OXT concentrations ($h^2 = 85.5\%$; $n = 47$ families) and (B) the NEPSY theory of mind score ($h^2 = 55.1\%$; $n = 37$ families). To illustrate consistency of values within families, members of the same family are plotted at the same point on the x axis and joined by a vertical line.

with no significant difference observed in the relationship between groups (group within symptomatic × rs53576 interaction: $F_{2,170} = 2.5277$; $P = $ ns) or between symptomatic and nonsymptomatic individuals on average (symptomatic × rs53576 interaction: $F_{1,170} = 3.3463$; $P = $ ns). Further, symptomatic individuals did not score worse on average than nonsymptomatic individuals ($F_{1,170} = 0.1105$; $P = $ ns) nor did the subdivision into the individual groups predict score ($F_{2,170} = 0.6376$; $P = $ ns).

Carriers of the A allele (i.e., AG or AA) of rs2254298 exhibited greater global social impairments as measured by the SRS Total Score ($F_{1,167} = 5.2313$; $P = 0.0234$) compared with those with two G alleles (Fig. 4B). This effect was consistent for all groups, with no significant difference observed in the relationship between the four groups (group within symptomatic × rs2254298 interaction: $F_{2,167} = 1.5253$; $P = $ ns) or between symptomatic and nonsymptomatic individuals on average (symptomatic × rs2254298 interaction: $F_{2,167} = 0.9676$; $P = $ ns). Symptomatic individuals scored worse on average than nonsymptomatic individuals ($F_{1,167} = 220.9639$; $P < 0.0001$) but the subdivision into individual groups did not predict score ($F_{2,167} = 1.8836$; $P = $ ns).

OXT Biology and ASD Social Phenotypes. ADI-R scores were only determined for participants with ASD. Carriers with the A allele of rs2254298 scored worse on the Social A1 total score (failure to use nonverbal behaviors to regulate social interaction) ($F_{1,61} = 4.9899$; $P = 0.0292$) than those with two G alleles. This effect was consistent for both ASD groups (group × rs2254298 interaction: $F_{1,61} = 0.0004$; $P = $ ns), and ASD subgroup did not further predict the A1 total score ($F_{1,61} = 1.0614$; $P = $ ns). Finally, the relationship between this OXTR genotype and the ADI-R score remains significant even in a simple two-sample t test (i.e., AA/AG vs. GG) in which all of the blocking factors (i.e., sex, age, ethnicity, blood sample collection time, and full-scale IQ) are removed ($t = 2.25$; $n = 77$; $P = 0.0276$ df = 75). There were no significant effects for plasma OXT concentrations or the rs53576 SNP.
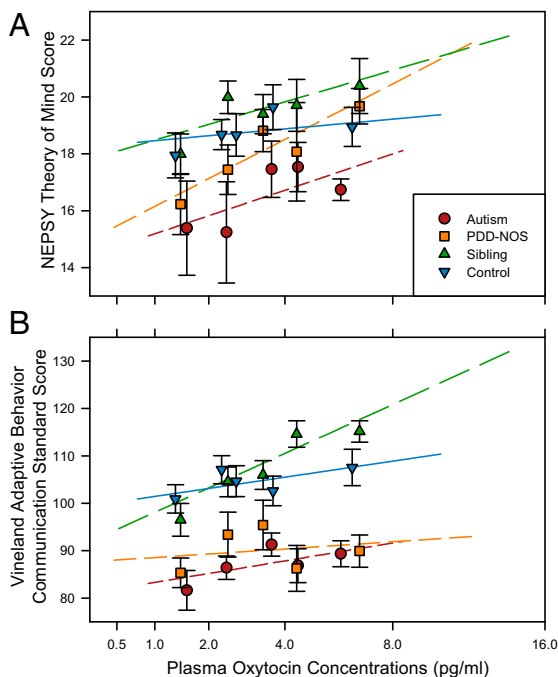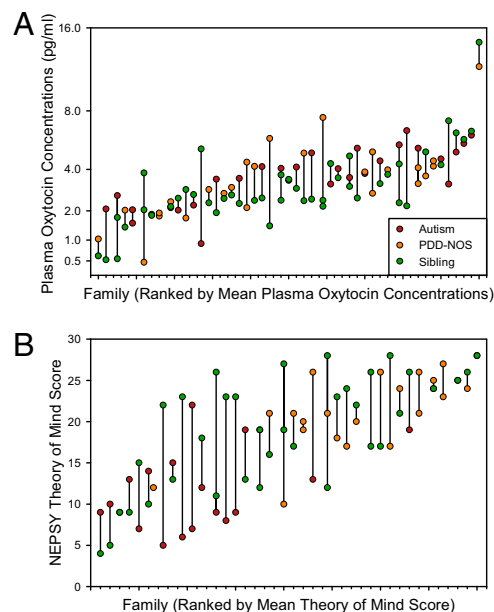
Fig. 2. Plasma OXT concentrations predict (A) NEPSY theory of mind and (B) Vineland Communication Domain scores independent of group. Groups and sample sizes are detailed in Fig. 1. In A and B the intercepts (i.e., the mean phenotypic scores) differ significantly by group ($P < 0.05$), whereas the slopes of the lines do not. The overall regression line averaged across groups is significant. The phenotypic data corrected for other variables in the analysis is shown, and each line is plotted between the full extents of the data. Data points are presented as LSM ± SEM for each quintile of the data, ordered by OXT concentrations within each group.
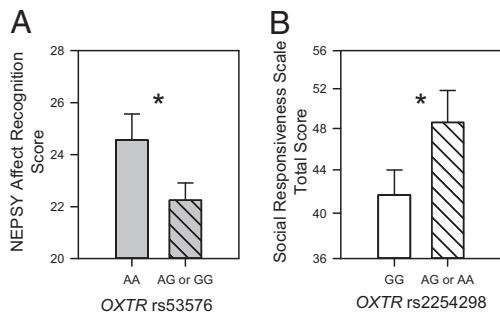
Fig. 4. *OXTR* SNPs predict social functioning independent of group. Groups and sample sizes are detailed in Fig. 1. For each genotype, the study risk allele is shown as a hatched bar. (*A*) Carriers with the G allele (*n* = 158) vs. those with the AA genotype (*n* = 35) of rs53576 show diminished affect recognition. (*B*) Carriers with the A allele (*n* = 66) vs. those with the GG genotype (*n* = 127) of rs2254928 show greater social impairments on the reverse-scored SRS. Data points are presented as LSM ± SEM, with the LSM partialed for all nondepicted variables. The asterisk represents Bonferroni-corrected post hoc comparison with *P* < 0.05.

Discussion

Recent studies (reviewed in refs. 1 and 2) have suggested a potentially important role for OXT biology in the social phenotypes of ASD, but they also present a challenge for the field. This is because existing data cannot distinguish between two critically different scenarios: Plasma OXT concentrations and/or *OXTR* SNPs are either biomarkers of social impairments unique to individuals with ASD or exert similar influences on social functioning, contributing to but not causing, ASD social phenotypes. To resolve this issue we collected the first dataset, to our knowledge, in which plasma OXT concentrations and *OXTR* SNPs were concomitantly investigated in a cohort of children with ASD, their unaffected siblings, and unrelated neurotypical control children using gold standard diagnostic methods and state-of-the-art quantification techniques.

Our study found no empirical evidence to support the prevalent but previously not well-interrogated OXT deficit hypothesis of ASD (study aim 1). Rather, we found that independent of group, that (*i*) plasma OXT concentrations strongly and positively predicted theory of mind and social communication performance, (*ii*) carriers of the G allele of rs53576 showed impaired affect recognition performance, and (*iii*) carriers of the A allele of rs2254298 exhibited greater global social impairments. Together these results paint an intriguing and novel picture with respect to the three hypotheses delineated in study aim 2. First, diminished plasma OXT concentrations and these two *OXTR* SNPs are not specific risk factors for ASD. Second, these biological measures do not exert differential effects in ASD vs. non-ASD individuals. Third, these OXT measures instead exert similar effects on social functioning for all children, such that they contribute to the variation in severity of ASD phenotypes, but in the same way they contribute to variation in social functioning in neurotypical individuals or unaffected siblings. [A very recent report lends support that these findings may even extend to other *OXTR* genotypes (i.e., rs237887) (43).] These OXT measures therefore appear to be biomarkers of social functioning in a universal sense, and at the lower tail of the distribution we find evidence for impaired social functioning, which is evident in clinical diagnostic assessments (i.e., ASD individuals who are carriers of the A allele of rs2254298 showed greater impairments on measures of reciprocal social interactions on the ADI-R).

The pattern of narrow-sense heritabilities is also consistent with the interpretation of our findings. Although there were no differences between ASD-discordant siblings in plasma OXT concentrations, plasma OXT concentrations were highly heritable (h^2 = 85.5%), on par with height in the general population

(42). We also examined heritability for the two behavioral measures that plasma OXT concentrations positively predicted. Theory of mind ability was moderately heritable (h^2 = 55.1%), whereas social communication ability did not show evidence of heritability (h^2 = 1.1%). This pattern of results suggests the intriguing idea that the heritability of plasma OXT concentrations may be driving, at least in part, social functioning within families. Thus, as plasma OXT concentrations are highly heritable, OXT-associated traits should be inherently correlated within families. The degree to which this is the case will depend on the degree to which nonfamilial environmental effects mitigate the impact of endogenous OXT signaling. Thus, whereas theory of mind is a more innate perceptual cognitive skill, but communication skills can be learned, the moderate heritability of theory of mind and the lack of heritability for social communication are consistent with this notion.

Our study also investigated the two *OXTR* SNPs (i.e., rs2254298 and rs53576) most frequently implicated in human social functioning (44, 45). Of particular interest is our finding that the minor allele of rs2254298 predicted global social impairments on the SRS and diagnostic severity on the ADI-R. In contrast, the major allele of rs53576 predicted impaired affect recognition performance on the NEPSY. The scientific literature has yielded inconsistent evidence regarding the risk alleles for each *OXTR* SNP (at least as defined by participant performance on various behavioral and/or biological assessments; see refs. 44 and 45 for reviews) and their associations with autism (21, 22, 46). These variable findings are likely influenced by numerous study-specific differences in sample size and dependent variable measurements. The demographic composition of the study participants may also play an important role (47, 48), as different genetic or ethnic backgrounds may have other functionally significant SNPs in linkage disequilibrium with these two *OXTR* SNPs. This range of findings also underscores an important point: The functional significance of these two intronic variants remains unknown. A better understanding of how these SNPs influence OXTR transcription or translation might explain the observed study-to-study variability.

This increased scientific interest in endogenous OXT biology in humans has been accompanied by numerous pharmacological reports demonstrating that intranasal OXT administration enhances social functioning in both neurotypical participants as well as those with ASD (9, 10, 14, 49). The fact that individuals with and without ASD showed the same relationship between plasma OXT concentrations and social phenotypes in our study gives credence to the idea that OXT treatment might be an effective pharmacotherapeutic for the core social features of ASD. If so, Fig. 2*A* hints at an exciting possibility: The ASD individuals with the highest plasma OXT concentrations scored as well on the theory of mind task as the neurotypical control individuals with the lowest plasma OXT concentrations. OXT dysregulation may not be the underlying cause of ASD, but OXT circuitry could be a promising therapeutic target nonetheless. Similar to the serotonin deficiency hypothesis of major depression (50), dysregulation in the OXT system might merely be a manifestation of other upstream neural deficiencies that are more prevalent in ASD, but that may also affect nonsymptomatic individuals to impair social functioning. If intranasal OXT treatment could enhance social functioning to the highest level seen in the ASD individuals we studied, then it might be able to restore social functioning to within (at least the lower end of) the range observed in the neurotypical control participants.

As with all studies, ours is not without limitations. Due to the invasive nature of sample collection, we were able to draw only one blood sample per participant and were unable to assess OXT concentrations in a matrix more proximal to the brain: cerebrospinal fluid (CSF). Although it remains unclear how blood measures of OXT are related to release of OXT in the brain, it is conceivable that individuals with ASD may differ in their relative allocation of OXT to these two compartments, as has been seen in a monkey model of impaired social functioning (5). If so, CSF

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OXT concentrations might be a more specific biomarker of ASD, whereas (as we found here) plasma OXT concentrations might be a general biomarker of social functioning (perhaps as an indirect measure of tonic hypothalamic OXT release). With regard to our plasma OXT heritability analysis, it should be noted that as every sibling dyad contained one ASD individual, these findings merit replication in sibling dyads from the general population. We were also unable to extend our heritability analysis to assess transmission of the two *OXTR* SNPs investigated in this study, as parental DNA samples were not available to perform this analysis. Finally, our study included an unequal distribution of males and females as well as a mixed ethnic sample. We controlled (i.e., blocked) for both potential extraneous sources of variability in our model. This conservative statistical approach ensured that any significant effects for sex or an allele could not be driven by either of these variables, and therefore were consistent across the sexes and different ethnic groups represented in the study.

In summary, we found no empirical support for the OXT deficit hypothesis of ASD, nor did plasma OXT concentrations differ by sex, *OXTR* SNPs, or their interactions. Plasma OXT concentrations did, however, strongly and positively predict theory of mind and social communication performance for children in all groups. Furthermore, plasma OXT concentrations and theory of mind performance were significantly heritable for ASD-discordant siblings, suggesting the intriguing idea that plasma OXT concentrations may contribute to similar social functioning abilities in families. Carriers of the G allele of rs53576 showed impaired affect recognition performance and carriers of the A allele of rs2254298 exhibited greater global social impairments for children in all groups. Taken together, these findings indicate that plasma OXT concentrations and *OXTR* SNPs are not uniquely associated with ASD, but instead exert independent, additive, and highly heritable influences on individual differences in human social functioning, including the severe social impairments which characterize ASD. Impairments in social cognition are also recognized as core features of many neuropsychiatric disorders (e.g., schizophrenia, bipolar disorder, borderline personality disorder), and for which OXT deficits have been hypothesized, but not yet well interrogated. Research is now required to test whether OXT dysregulation occurs in these patient populations, or whether, like ASD, OXT biology contributes to but does not cause the social features of these disorders.

Materials and Methods

Participants. This study was approved by the Stanford University Institutional Review Board and all participants and their families provided informed consent before initiation of study procedures. Assent was also obtained from children 7 y of age and older when appropriate. Seventy-nine children with ASD (*N* = 17 female, 62 male), 52 unaffected siblings (*N* = 23 female, 29 male), and 62 neurotypical control children (*N* = 22 female, 40 male) between the ages of 3 and 12 y participated in this study. Participant characteristics are presented in Table S1. Children with ASD and their siblings were primarily recruited through the Autism and Developmental Disorders Research Registry and by flyers posted in the Autism and Developmental Disorders Clinic at Stanford University. Unrelated control participants were recruited through advertisements posted online (e.g., Parent Listservs, www.craigslist.org) or hardcopy in the surrounding community (e.g., pediatrician offices, shopping malls). All participants were (*i*) prepubertal, (*ii*) in good medical health, and (*iii*) willing to provide a blood sample. Participants with ASD were included if they had a full-scale IQ of 50 and above. Control participants and siblings were included if they had an IQ in the average range. Cognitive functioning was determined using the Stanford-Binet manual (51). Diagnostic methods and exclusion criteria are described in *SI Materials and Methods*.

Social Phenotyping. Social phenotyping included the following three instruments. The SRS is a norm-referenced questionnaire that measures social behavior (e.g., social awareness, social cognition, social communication, social motivation, and autistic mannerisms) in both clinical and nonclinical populations. This parent-report measure includes the SRS Total Score, which was

used in this study. The psychometric properties of the Total Score have been tested in younger and older participants, and are continuously distributed within each group (37, 38). The NEPSY-II (39) is a widely used and validated norm-referenced measure of child neurocognitive abilities and includes affect recognition and theory of mind, the two measures tested here. The VABS-2 (40) is a well-validated parent interview that measures adaptive behavioral functioning, particularly in socially relevant domains. We assessed socialization, communication, and daily living skills in this study. We also examined ADI-R Social Domain Scores in children with ASD.

Blood Sampling and Plasma OXT Quantification. Descriptions of whole blood collection and plasma OXT quantification are provided in *SI Materials and Methods*.

OXTR Genotyping. DNA was extracted from whole blood using standard laboratory procedures. OXTR genotyping is described in *SI Materials and Methods*. In the study population, the *OXTR* SNP rs2254298 allelic frequencies were 19.4% for the A variant and 80.6% for the G variant. The *OXTR* SNP rs53576 allelic frequencies were 40.9% for the A variant and 59.1% for the G variant. Tables S2 and S3 provide *OXTR* allelic information. Allelic frequencies for both *OXTR* SNPs were in Hardy–Weinberg equilibrium: rs2254298 (χ^2 = 0.621; df = 2; *n* = 193; *P* = 0.733) and rs53576 (χ^2 = 0.629; df = 2; *n* = 193; *P* = 0.731). Initial statistical analyses testing for allelic dosing effects for rs2254298 [AA (*n* = 9; 4.7%), AG (*n* = 57, 29.5%), GG (*n* = 127; 65.8%)] and rs53576 [AA (*n* = 35, 18.1%), AG (*n* = 88; 45.6%), GG (*n* = 70; 36.3%)] revealed the A allele to be the risk allele for rs2254298 and G to be the risk allele for rs53576 for the phenotypic variables of interest. We therefore combined the AA and AG groups for rs2254298 and the AG and GG groups for rs53576 in subsequent analyses. Finally, our San Francisco Bay Area sample was of mixed ethnicity. Given reports of ethnic differences in *OXTR* allelic frequency and effects in several studies (47, 48), we included ethnicity as a statistical covariate for all analyses. This conservative approach ensured that any significant effects for an allele could not be driven by ethnicity, and that any observed effects would be consistent across the different ethnic groups represented in the study.

Statistical Analyses. All analyses were performed as general linear models (GLMs) in JMP Version 10 (SAS Institute Inc.). The first study aim used a GLM to test for differences in mean plasma OXT concentrations by group, sex, and the two *OXTR* SNPs (while blocking for age, ethnicity, blood sample collection time, and full-scale IQ). We represented groups by subdividing (nesting) this variable into symptomatic (autistic and PDD-NOS) vs. nonsymptomatic (sibling and neurotypical control) individuals. This allowed us to test explicitly whether any overall difference was due to differences between symptomatic and nonsymptomatic individuals in general or a particular group. (This approach is far more powerful than resorting to post hoc multiple comparisons between groups.) Second-order interactions between group, sex, and the two *OXTR* genotypes were also tested. Even so, no influences of any of these experimental factors on plasma OXT concentrations were found, and therefore did not require follow-up comparisons. This lack of colinearity with plasma OXT concentrations is optimal for the approach taken for the second study aim, as confounding explanations due to colinearity can be ruled out. To test the second aim, we adopted an epidemiological approach to these data, analyzing each social behavior measure in a GLM that examined all of the experimental factors at the same time, thereby controlling for noise from both the experimental and blocking factors. The same model was used for each behavioral measure. Experimental factors were plasma OXT concentrations, *OXTR* rs53576 genotype, *OXTR* rs2254298 genotype, and group, represented as group-nested-within-symptomatic. Second order interactions between all experimental factors were included. Sex, age, ethnicity, blood sample collection time, and full-scale IQ were included as blocking factors. A major advantage of this approach is that it further maximizes power by avoiding issues of multiple comparisons: The individual terms within a factorial analysis of this kind represent distinct causal hypotheses (and so we limited the model to include only experimental factors about which we had causal hypotheses). Similarly, we limited the phenotypes examined and picked one to test each of our distinct phenotypic hypotheses.

The assumptions of GLM (normality of error, homogeneity of variance, and linearity) were examined graphically and suitable transformations applied as required (52). Plasma OXT concentrations were square-root transformed for all analyses to correct for a skewed distribution and associated nonlinearity in the analyses. Only the NEPSY theory of mind score showed evidence of nonlinearity after these corrections, which was resolved by including a quadratic effect of age (a quadratic age effect was then tested for all

phenotypes and excluded, as it was not significant). Significant interactions were examined with post hoc Bonferroni-corrected orthogonal planned contrasts as required to understand the significance of a factor made up of several levels or when comparing the mean value of groups (52).

Narrow-sense heritability was calculated using the intraclass correlation method (41). Ideally one would calculate heritability from multigenerational data. However, given the considerable changes in autism prevalence in the current generation, this was not a practical approach here. Narrow-sense heritability, in contrast, uses phenotypic data from one generation. This method is the most conservative available for estimating heritability from sibling data, as it excludes dominance, epistatic interactions, and other components which would inflate heritability estimates. Only families where data were available for two individuals were included. A restricted maximum likelihood mixed model was constructed, similar to that used for the main analyses. Thus, the environmental or epistatic effects of sex, age, ethnicity, blood sample collection time, full-scale IQ, and group within symptomatic were controlled by including these factors in the model and estimating the variance components for within-family error and between-

family additive genetic effects. These variance components lead directly to an intraclass correlation estimate for family members, and thus to estimates of heritability, and also an approximate F ratio test. Confidence intervals for narrow-sense heritability can be approximated but are typically excessively inflated. The intraclass correlation estimate therefore was tested here instead.

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PSYCHOLOGICAL AND COGNITIVE SCIENCES

Supporting Information

Parker et al. 10.1073/pnas.1402236111

SI Materials and Methods

Participants. Children with a diagnostic history of autism spectrum disorder (ASD) underwent a comprehensive diagnostic evaluation to determine the accuracy of the previous diagnosis based on *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision* (DSM-IV-TR) criteria (1), which was confirmed with research diagnostic methods. These diagnostic methods included the Autism Diagnostic Interview-Revised (ADI-R) (2, 3) and the Autism Diagnostic Observation Schedule-Generic (ADOS-G) (4, 5). The ADI-R and the ADOS-G were administered by assessors trained by a research-reliable clinician, and all assessors attained standard interrater reliability within the project. Although all participants met DSM-IV-TR criteria for ASD, expert clinical opinion and scores on the ADI-R and ADOS-G were also used to characterize children with ASD as having autism or pervasive developmental disorder-not otherwise specified (PDD-NOS). Children with ASD who met the DSM-IV-TR criteria diagnosis of autistic disorder and scored above the cutoff for autism on ADI-R and ADOS-G were categorized as having autism. Children with ASD who met the DSM-IV-TR criteria diagnosis of autistic disorder and scored above the cutoff for autism on ADI-R but in the autism spectrum range on the ADOS-G were categorized as having PDD-NOS.

Exclusion criteria included (*i*) a genetic, metabolic, or infectious etiology for ASD on the basis of medical history, neurologic history, and available laboratory testing for inborn errors of metabolism and chromosomal analysis; and (*ii*) a DSM-IV-TR diagnosis of any severe mental disorder such as schizophrenia and bipolar disorder. Participants taking psychotropic medications were included as long as their medications were stable for at least 2 wk before the blood draw. Siblings of children with ASD were required to have no evidence of ASD on the basis of behavioral scales [i.e., the Social Responsiveness Scale (SRS)], clinical evaluation, and, if needed, research diagnostic assessments. They were also required to have no present or past history of any severe neuropsychiatric disorder such as schizophrenia or bipolar disorder on the basis of a clinical psychiatric evaluation and information obtained from behavioral scales. Neurotypical control children were required to (*i*) be free of neurological disorders in the present or past on the basis of history; (*ii*) be free of psychiatric disorders in the present or past on the basis of information obtained from behavioral scales, a clinical psychiatric evaluation, and if needed, the Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Aged Children (6); (*iii*) have no historical evidence of significant difficulty during the mother's pregnancy, labor, or delivery or in the immediate neonatal period or abnormal developmental milestones based on neurological history; and (*iv*) have no sibling diagnosed with ASD.

Blood Sampling and Plasma Oxytocin Quantification. Blood samples were collected between 10:00 AM and 2:00 PM from participants to control for potential circadian rhythmicity in plasma oxytocin (OXT) concentrations (7). The average collection latency was 114 ± 5.3 s (mean $\pm$ SEM) and did not differ by group ($F_{3,127} = 1.2242$; $P = 0.3037$). Fifteen milliliters of whole blood were drawn from each child's antecubital region by a trained phlebotomist using standard protocols at the Lucile Packard Children's Hospital (Stanford University, Palo Alto, CA) outpatient laboratory facility. Blood was collected into chilled EDTA-treated vacutainer tubes and placed on wet ice. Whole blood was promptly centrifuged ($1,300 \times g$ at 4 °C for 10 min) and the

plasma fraction transferred and aliquotted into polypropylene tubes and flash-frozen on dry ice and stored in a −80 °C freezer.

OXT concentrations were quantified using a commercially available enzyme immunoassay (Enzo Life Sciences, Inc.). This kit is highly specific and exclusively recognizes OXT and not related peptides. Per Enzo Life Sciences literature, the cross-reactivity with vasopressin is 0.6% and the limit of assay sensitivity where the curve is no longer linear is 10 pg/mL. Based on the recommendation of the technological division of Enzo Life Sciences and published evidence (8, 9), plasma samples were extracted to remove any matrix (macromolecules in plasma) interference effects.

Sample extraction procedures were initiated by thawing plasma samples in an ice bath. Waters Sep-Pak C18 columns (Waters Corp.) were conditioned with 1 mL HPLC-grade methanol followed by 1 mL molecular biology-grade water. Each 1-mL plasma sample was drawn through the column by vacuum on a Supelco SPE vacuum manifold (Sigma-Aldrich Group). The columns were washed with 1 mL wash buffer (89:10:1, water:acetonitrile:TFA) followed by 1 mL elution buffer (80:20, acetonitrile:water). Elutes were evaporated at room temperature using compressed nitrogen and reconstituted in 225 μL assay buffer before quantification to provide a sufficient sample volume to run samples in duplicate wells (100 μL per well). The program used to calculate the final picograms-per-milliliter concentration of OXT allowed for extrapolation based on the starting sample volume. This approach has been shown to be a sound method for increasing the concentration of OXT in each well (8, 10, 11) and ensures that each sample falls within the linear portion of the standard curve when initially read.

Samples were assayed in duplicate with a tunable microplate reader (Molecular Devices) for a 96-well format according to the manufacturer's instructions. All assays were performed by a technician who had no knowledge of phenotypic, diagnostic, or genetic data. Intra- and interassay coefficients of variation were 7.4% and 9.1%, respectively.

***OXT* Receptor Genotyping.** The OXT receptor gene *OXTR* SNP rs2254298 was amplified using the primers 3′-GCCCACCA-TGCTCTCCACATC and 5′-GCTGGACTCAGGAGGAAT-AGGGAC. The PCR reactions were carried out in a final volume of 15 mL consisting of 50 ng genomic DNA, 50 ng each of sense and antisense primers, 7.5 mL Taq PCR Master mix (Qiagen; catalog no. 201445), and 10% (vol/vol) DMSO. The PCR conditions included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min, with a final extension of 10 min at 72 °C. The PCR product of 340 bp was digested with BamHI (New England Biolabs; catalog no. R0136L) at 37 °C for 3 h. The G allele was 340 bp and the A allele yielded 110 and 230 bp. For rs53576 genotyping primers 5′-TGA AAG CAG AGG TTG TGT GGA CAG G-3′ and 5′-AAC GCC CAC CCC AGT TTC TTC-3′ and the same PCR conditions were used; 7.5 μL 307 bp PCR products were digested at 65 °C for 3 h with 5 U of the restriction enzyme BsrI (New England Biolabs; catalog no. R0527L). The products were electrophoresed through 10% polyacrylamide gel (19:1, acrylamide:bis-acrylamide) at 150 V for 40 min. A 10-bp marker was used to measure fragment size. The A allele yielded 164, 136, and 8 bp. The G allele yielded 164, 101, 34, and 8 bp. All genotypes were scored by technicians who had no knowledge of phenotypic or diagnostic data.

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Table S1. Participant characteristics

Participants	N	Sex Female	Sex Male	Ethnicity Caucasian	Ethnicity Asian	Ethnicity Other	Age, y*	Full-scale IQ*	Blood collection time, min; ns
ASD									
Autism	47	9	38	25	13	9	7.60 ± 0.37[a,b]	71.58 ± 3.51[a]	12:41 PM ± 11.29
PDD-NOS	32	8	24	22	4	6	9.10 ± 0.41[a]	96.63 ± 4.18[b]	12:09 PM ± 13.59
Sibling	52	23	29	25	18	9	7.79 ± 0.41[a,b]	108.08 ± 1.84[c]	12:34 PM ± 9.32
Control	62	22	40	45	6	11	7.11 ± 0.37[b]	115.34 ± 1.21[c]	12:29 PM ± 6.93

χ^2 was used to test whether the distribution of individuals to different groups differed by sex and by ethnicity. Overall, weak significant effects ($0.05 > P > 0.01$) where found for each. However, post hoc tests failed to find any group that showed a significant difference from expected. ns, not significant. For age, full-scale IQ, and blood collection time, differences between groups were tested with a simple general linear model. The values are expressed in mean ± SEM. *$P < 0.05$. Values with the same superscript within the same column of the table do not differ according to Tukey's post hoc test.

Table S2. Participant allele frequencies of *OXTR* genotypes rs2254298 and rs53576 by group

Participants	N	rs2254298 GG	rs2254298 AG	rs2254298 AA	rs53576 GG	rs53576 AG	rs53576 AA
ASD							
Autism	47	n = 33 (70%)	n = 12 (26%)	n = 2 (4%)	n = 13 (28%)	n = 28 (59%)	n = 6 (13%)
PDD-NOS	32	n = 21 (66%)	n = 9 (28%)	n = 2 (6%)	n = 11 (34%)	n = 14 (44%)	n = 7 (22%)
Sibling	52	n = 33 (63%)	n = 15 (29%)	n = 4 (8%)	n = 22 (42%)	n = 19 (37%)	n = 11 (21%)
Control	62	n = 40 (64%)	n = 21 (34%)	n = 1 (2%)	n = 24 (39%)	n = 27 (43%)	n = 11 (18%)

Allelic frequencies for both *OXTR* SNPs were in Hardy–Weinberg equilibrium (*Materials and Methods,* OXTR *Genotyping*).

Table S3. Participant allele frequencies of *OXTR* genotypes rs2254298 and rs53576 by ethnicity

Participants	N	rs2254298 GG	rs2254298 AG	rs2254298 AA	rs53576 GG	rs53576 AG	rs53576 AA
Caucasian	117	n = 79 (67%)	n = 36 (31%)	n = 2 (2%)	n = 47 (40%)	n = 54 (46%)	n = 16 (14%)
Asian	41	n = 26 (63%)	n = 9 (22%)	n = 6 (15%)	n = 14 (34%)	n = 11 (27%)	n = 16 (39%)
Other	35	n = 22 (63%)	n = 12 (34%)	n = 1 (3%)	n = 9 (26%)	n = 23 (66%)	n = 3 (8%)

Fisher's exact test was used to test whether allele frequencies for each *OXTR* genotype differed by ethnicity as reported in other studies. Significant effects were found for both rs2254298 ($P = 0.0318$) and rs53576 ($P = 0.0008$). However, ethnicity did not significantly influence any experimental outcomes, consistent with multiple other *OXTR* genotype studies using mixed ethnic samples.