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Biomarker discovery for disease status and symptom severity in children with autism

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ABSTRACT

Autism spectrum disorder (ASD) is characterized by social impairments and repetitive behaviors, and affects 1 in 68 US children. Despite ASD's societal impact, its disease mechanisms remain poorly understood. Recent preclinical ASD biomarker discovery research has implicated the neuropeptides oxytocin (OXT) and arginine vasopressin (AVP), and their receptors, *OXTR* and *AVPR1A*, in animal models. Efforts to translate these findings to individuals with ASD have typically involved evaluating single neuropeptide measures as biomarkers of ASD and/or behavioral functioning. Given that ASD is a heterogeneous disorder, and unidimensional ASD biomarker studies have been challenging to reproduce, here we employed a multidimensional neuropeptide biomarker analysis to more powerfully interrogate disease status and symptom severity in a well characterized child cohort comprised of ASD patients and neurotypical controls. These blood-based neuropeptide measures, considered as a whole, correctly predicted disease status for 57 out of 68 (i.e., 84%) participants. Further analysis revealed that a composite measure of *OXTR* and *AVPR1A* gene expression was the key driver of group classification, and that children with ASD had lower neuropeptide receptor mRNA levels compared to controls. Lower neuropeptide receptor mRNA levels also predicted greater symptom severity for core ASD features (i.e., social impairments and stereotyped behaviors), but were unrelated to intellectual impairment, an associated feature of ASD.

Findings from this research highlight the value of assessing multiple related biological measures, and their relative contributions, in the same study, and suggest that low blood neuropeptide receptor availability may be a promising biomarker of disease presence and symptom severity in ASD.

1. Introduction

Autism spectrum disorder (ASD) is neurodevelopmental disorder characterized by deficits in social communication and interaction, as well as restricted, repetitive patterns of behavior, interests, or activities (American Psychiatric Association, 2013). ASD is clinically heterogeneous (e.g., cognitive capabilities range significantly) and ASD impacts an estimated 1 in 68 US children (Christensen et al., 2016), with severe health, quality of life, and financial consequences for patients, families and/or society. ASD is currently diagnosed on the basis of behavioral criteria because its underlying disease mechanisms remain poorly understood. Consequently, there are no blood-based diagnostic tools to detect, or approved medications to treat, ASD's core features. Research that identifies robust biological substrates of disease status and symptomology in ASD patients is therefore urgently needed.

Neurobiological systems that are critical for social functioning are arguably the most promising signaling pathways for ASD biomarker and therapeutic target discovery. Two such candidates are the oxytocin (OXT) and arginine vasopressin (AVP) signaling pathways. OXT and AVP are primarily synthesized in the hypothalamus and released into both the brain via distributed neural pathways and systemic circulation via the posterior pituitary (Landgraf and Neumann, 2004). OXT and AVP are nearly structurally identical nonapeptides and likely evolved due to duplication of a common ancestral gene (Donaldson and Young, 2008). OXT has a single receptor (*OXTR*), whereas AVP has three receptors (*AVPR1A*, *AVPR1B* and *AVPR2*), with AVP's prosocial effects mediated through *AVPR1A* (Bielsky et al., 2004; Young et al., 1999). It is well established that OXT and AVP are critical for the expression of normative social behavior (e.g., social bond formation, social motivation, social decision making, social learning and memory) (Hammock and Young, 2006; Meyer-Lindenberg et al., 2011; Parker and Lee, 2001). Targeted disruption of OXT and AVP ligand-receptor signaling

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through pharmacologic or genetic manipulation also produces social deficits (Bielsky et al., 2004; Ferguson et al., 2000; Takayanagi et al., 2005) and repetitive behaviors (Sala et al., 2011) in various rodent species. Studies of rodent models of human syndromes with high ASD penetrance likewise have reported social impairments and diminished hypothalamic OXT and/or AVP producing cell numbers [e.g., Fragile-X Syndrome, Prader Willi Syndrome, and cortical dysplasia and focal epilepsy syndrome as modeled using *Cntnap2* knockout mice (Francis et al., 2014; Peñagarikano et al., 2015)], highlighting the potential significance of dysregulated OXT and AVP signaling in ASD.

On the basis of these promising preclinical findings, clinical investigators have begun to investigate a role for the OXT and AVP signaling pathways in idiopathic ASD (Zhang et al., 2017). Several studies have shown that blood OXT and AVP concentrations each positively predict social cognition abilities in children with ASD (Carson et al., 2015; Parker et al., 2014; Zhang et al., 2016), such that individuals with the lowest neuropeptide levels exhibit the greatest social impairments. Human genetic association studies have also shown that several single nucleotide polymorphisms and haplotypes in the *OXTR* and *AVPR1A* genes increase risk for ASD (Kim et al., 2002; Wermter et al., 2010; Wu et al., 2005; Yirmiya et al., 2006), and are associated with restricted, repetitive behaviors (Francis et al., 2016; Harrison et al., 2015). Although preliminary, these findings suggest that variation in OXT and AVP biology may be associated with ASD susceptibility, but much remains unknown.

With few exceptions (Miller et al., 2013; Parker et al., 2014; Zhang et al., 2016) the majority of prior research studies have evaluated single neuropeptide measures as biomarkers of ASD and/or behavioral functioning. Given that idiopathic ASD is a heterogeneous disorder, and unidimensional ASD biomarker studies have repeatedly met with challenges in reproducibility (Walsh et al., 2011), there is a clear and important need to assimilate unidimensional neuropeptide measures into a multidimensional biomarker analysis to more powerfully interrogate disease status and symptom severity in ASD patients. The goals of the present study therefore were three-fold. First, we tested in the same study population whether four blood-based neuropeptide measures (i.e., OXT and AVP peptide concentrations; *OXTR* and *AVPR1A* gene expression) correctly classified study participants as ASD vs. control. Second, we evaluated whether these blood neuropeptide measures differed between children with ASD and control children. Finally, we tested whether the neuropeptide measures predicted symptom severity for core ASD features (i.e., social impairments and repetitive behaviors) but not associated features (i.e., intellectual impairment) in a well characterized child cohort.

2. Materials and methods

2.1. Participant recruitment and eligibility criteria

This study was approved by the Stanford University School of Medicine Institutional Review Board. All participants' parents provided informed consent prior to initiation of study procedures. Assent was also obtained from participants when the child was deemed intellectually capable of understanding the study. Forty-four children with ASD (N = 7 F, 37 M), and 24 unrelated neurotypical control children (N = 6 F, 18 M) between the ages of 6–12 years participated in this study. Participant demographic characteristics are presented in Table 1. Children with ASD were primarily recruited through the Autism Research Registry at Stanford University, by flyers posted in the Stanford University Autism and Developmental Disorders Clinic, or at special events (e.g., Bay Area Autism Speaks Walk). Control participants were recruited through advertisements posted online (e.g., parent listservs) or hardcopy in the surrounding community (e.g., pediatrician offices).

Children with a diagnostic history of ASD underwent a comprehensive diagnostic evaluation to determine the accuracy of their previous diagnosis based on DSM-IV-TR (American Psychiatric Association, 2000) or DSM-5 criteria (American Psychiatric Association, 2013), which was confirmed with research diagnostic methods. These diagnostic methods included the Autism Diagnostic Instrument-Revised (ADI-R) (Lord et al., 1994) and the Autism Diagnostic Observation Schedule, Second Edition (ADOS-2) (Lord et al., 2012). The ADI-R and the ADOS-2 were administered by assessors trained by a research reliable clinician, and administration was reviewed for both initial and ongoing administration and coding reliability.

All participants were: 1) pre-pubertal; 2) in good medical health; and 3) willing to provide a blood sample. Participants with ASD were included if they had a Full-Scale IQ of 50 and above. Control participants were included if they had a Full-Scale IQ in or above the average range. Cognitive functioning was determined using the Stanford Binet Scales of Intelligence, 5th Edition (Roid, 2003). Exclusion criteria for children with ASD included: 1) a genetic etiology for ASD (e.g., Fragile X Syndrome); 2) a DSM-IV-TR or DSM-5 diagnosis of any severe mental disorder (e.g., schizophrenia, schizoaffective disorder, bipolar disorder), or 3) significant illness (e.g., serious liver, renal, or cardiac pathology). Participants taking medications were included as long as their medications were stable (i.e., for at least four weeks) before the blood draw. Control children were required to: 1) be free of neurological and psychiatric disorders in the present or past on the basis of medical history and 2) have no sibling diagnosed with ASD.

2.2. Behavioral phenotyping

The core behavioral features of ASD (i.e., social impairments and restricted, repetitive behaviors) were assessed using two instruments. 1) The SRS (Constantino et al., 2003) is a norm-referenced questionnaire that measures social behavior in both clinical and non-clinical populations. The SRS Total Score is a sensitive measure (i.e., it strongly correlates with DSM criterion scores) with high reliability. 2) The Repetitive Behaviors Scale − Revised (RBS-R) (Lam and Aman, 2007) assesses a wide range of restricted and repetitive behaviors. The RBS-R includes six subscales (Stereotyped Behavior, Self-injurious Behavior, Compulsive Behavior, Ritualistic Behavior, Sameness Behavior, and Restricted Behavior), for which the psychometric validity is established (Lam and Aman, 2007).

2.3. Blood sample collection and processing procedures

Twenty mL of whole blood was drawn from the child's antecubital region by a pediatric phlebotomist at Lucile Packard Children's Hospital outpatient laboratory within two weeks of behavioral phenotyping. It has been shown that circulating levels of OXT and AVP have modest

Table 1
Participant characteristics. Fisher's exact test was used to test whether the distribution of individuals to different groups differed by sex and ethnicity. For age, full-scale IQ, and blood collection time, differences between groups were tested using a simple one-way general linear model (* = $p < 0.05$). The values are reported as mean ± standard error.

		Sex		Ethnicity*					
Group	*N*	Female	Male	Caucasian	Asian	Other	Age (years)	Full-scale IQ*	Blood collection time (min)*
Autism	44	7	37	12	12	20	8.54 ± 0.33	74.15 ± 3.98	14:04 PM ± 15.75
Control	24	6	18	16	3	5	8.71 ± 0.41	116.12 ± 2.57	12:32 PM ± 20.00

daily rhythms of secretion, and that they both rise during the period of sleep (Forsling, 2000; Trudel and Bourque, 2010). Blood samples were therefore collected during daytime hours (i.e., between 10 AM and 5 PM) to reduce circadian effects on our biological measures of interest. Whole blood was collected into chilled EDTA-treated vacutainer tubes and immediately placed on wet ice. These samples were then promptly centrifuged (1600*g* at 4 °C for 15 min), the plasma fraction aliquoted into polypropylene tubes, and flash-frozen on dry ice. Whole blood was also collected into PAXgene RNA tubes (Qiagen, CA) and processed per manufacturer's instructions. All samples were then stored at −80 °C until quantification.

2.4. Quantification procedures

OXT and AVP are primarily synthesized in the hypothalamus and released into systemic circulation by the posterior pituitary. The gold standard by which to measure these neuropeptide concentrations in blood is therefore to use immunoassay; in our case, we used enzyme-linked immunosorbent assays (ELISA). However, *OXTR* and *AVPR1A* are expressed in body tissues (Thibonnier et al., 2001), including in blood lymphocytes (Yamaguchi et al., 2004). We therefore used the gold standard for quantifying gene expression, qPCR, to assess blood mRNA levels of these neuropeptide receptors.

2.5. Quantification of plasma OXT and AVP concentrations

Plasma OXT and AVP concentrations were quantified using commercially available enzyme immunoassay kits (Enzo Life Sciences, Inc., NY). These kits are highly specific and exclusively recognize OXT and AVP, respectively, and not related peptides (i.e., the OXT cross-reactivity with AVP is 0.6% and the AVP cross-reactivity with OXT is < 0.001%). A technician blinded to experimental conditions performed sample preparation and OXT and AVP quantification following established procedures (Carson et al., 2015; Parker et al., 2014). Briefly, plasma samples (1000 µL/participant) for each peptide were extracted per manufacturer's instructions and evaporated using compressed nitrogen. Each evaporated sample was reconstituted in 250 µL of assay buffer prior to OXT and AVP quantification to provide sufficient sample volume to run each participant's sample in duplicate wells (100 µL/well). This practice ensured that the plated samples contained high enough peptide quantities to be read above the limit of detection (15 pg/mL for OXT and 2.84 pg/mL for AVP). Samples were assayed with a tunable microplate reader (Molecular Devices, CA) for 96-well format per manufacturer's instructions. Intra- and inter-assay coefficients of variation were below 10% for both analytes.

2.6. Quantification of OXTR and AVPR1A gene expression levels

Total RNA was isolated and purified using a PAXgene blood RNA kit from blood stabilized in PAXgene RNA tubes (Qiagen, CA). RNA integrity was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, CA), and consistently found to have RIN values (RNA integrity numbers) greater than 9.5. The first strand cDNA synthesis reaction was carried out with QuantiTect reverse transcription kit (Qiagen, CA), with a starting RNA quantity of 1 µg in a 20 µL final volume. The primer sequence information for *OXTR* and *AVPR1A* genes was obtained from published studies and was designed as follows: *OXTR* forward 5′-CTGAACATCCCGAGGAACTG-3′ and reverse 5′-CTCT GAGCCACTGCAAATGA-3′, (Gregory et al., 2009); *AVPR1A* forward 5′-CTTTTGTGATCGTGACGGCTTA-3′ and reverse 5′-TGATGGTAGGGT TTTCCGATTC-3′ (Wang et al., 2008). Two housekeeping genes, hypoxanthine phosphoribosyltransferase 1 [HPRT1; forward 5′-GGACAG GACTGAACGTCTTGC-3′ and reverse 5′-ATAGCCCCCCTTGAGCA CAC-3′ (Wang et al., 2008)] and ubiquitin C [UBC; forward 5′-GCTGC TCATAAGACTCGGCC-3′ and reverse 5′- GTCACCCAAGTCCCGTC CTA-3′ (Wang et al., 2008)] were selected for normalization using geNorm. qPCR was performed on the StepOnePlus Real-Time PCR System (Life Technologies, CA) with SYBR Green (Thermo Fisher Scientific, MA). cDNA was PCR amplified in triplicate and Ct values from each sample were obtained using StepOnePlus software. The relative expression of each gene was calculated based on the ΔΔCt value, where the results were normalized to the average Ct value of HPRT1 and UBC (Lossie et al., 2012).

2.7. Statistical analyses

Data were managed using REDCap (Harris et al., 2009) and analyzed using JMP Pro 13 for Windows (SAS Institute Inc., NC). All analyses included N = 44 ASD children, and (where appropriate) N = 24 neurotypical control children. A logistic regression model, implemented as a Restricted Maximum Likelihood Generalized Linear Model (REML-GLIM), was used to assess whether blood neuropeptide measures (i.e., OXT and AVP peptide concentrations, expression of *OXTR* and *AVPR1A* genes) predict disease status of children with and without ASD. Age, time of blood collection, ethnicity, and sex were included as control variables (or 'stratifiers') in the initial model (Table S1). This model showed over-specification and quasi-complete separation, indicating collinearity between predictors, and an artefactually over-precise classification prone to false positive results (Paul, 1999). Since *OXTR* and *AVPR1A* gene expression was highly correlated, we first considered using Principle Components Analysis (PCA) to yield orthogonal components for analysis. This neatly illustrated the collinearity of the gene expression measures, which loaded onto a single factor with loadings (correlation coefficients) of 0.8058 and 0.7049 for *OXTR* and *AVPR1A* respectively. However, there were differences in the component structure when the ASD and control groups were processed separately. This precluded using a PCA to process the data from the two groups together (Howitt and Cramer, 2005; Miller et al., 2006). Given that OXT and AVP differ by only two amino acids, and their receptors likewise have a high degree of structural similarity, there is a substantial amount of documented crosstalk between these neuropeptides ligands at their receptors (Sala et al., 2011; Schorscher-Petcu et al., 2010; Song et al., 2014). Therefore, we calculated the total neuropeptide gene expression as the sum of the *OXTR* and *AVPR1A* gene expression to capture correlated expression of the two genes, and differential neuropeptide receptor gene expression as the difference between *OXTR* and *AVPR1A* gene expression to capture relative up or down regulation of these receptors. As plasma OXT and AVP concentrations were uncorrelated, we included them separately in the logistic regression model. The resulting model was robust, showing no evidence of over-specification or quasi-complete separation. Plasma AVP concentration was log-transformed in these and all other analyses to correct a skewed distribution. We confirmed the predictive power of total gene expression by running a single factor logistic regression (i.e., excluding all blocking factors and other biomarkers).

A Least Squares General Linear Model (LS-GLM), with the same control variables as those included in the logistic regression model, was used to test whether the neuropeptide measures differed between children with ASD and neurotypical controls. Each neuropeptide measure (total neuropeptide receptor gene expression, differential neuropeptide receptor gene expression, plasma AVP concentration, and plasma OXT concentration) was tested in turn, with the other three neuropeptide measures and IQ included in the model to ensure that any observed differences for a given neuropeptide measure were not better explained by group differences in the other neuropeptide measures or IQ. The assumptions of LS-GLM (homogeneity of variance, normality of error, and linearity) were tested post-hoc (Grafen and Hails, 2002).

Finally, we used an LS-GLM with the same control variables as before to test whether the neuropeptide measures predicted core behavioral phenotypes in children with ASD. We included each of the four neuropeptide measures and IQ in the model; as before, this excluded the possibility that a neuropeptide measure is significant merely due to IQ,

and it allowed us to test each neuropeptide measure in the context of the others in a single model. To assess social impairments, we used the SRS Total Raw Score (instead of the sex-normalized *T*-score, which has lower resolution). However, given that psychometric validity for the RBS-R Total Score is not well established, we also performed the same analyses on each RBS-R subscale, but corrected our critical *p*-value to 0.0083, to protect against multiple comparisons and to achieve the same family-level significance as the total score. Finally, we tested whether the neuropeptide measures predicted IQ (i.e., cognitive ability) to test for core vs. associated ASD feature specificity (thus, in this model, IQ was removed as a control variable). As before, the assumptions of LS-GLM were tested post-hoc (Grafen and Hails, 2002).

3. Results

3.1. Participants

Participant demographic characteristics are presented in Table 1. Ethnicity and blood collection time unexpectedly differed between children with and without ASD. To eliminate the possibility that these confounding effects could generate false positive or false negative results (Grafen and Hails, 2002), we adopted the standard epidemiological approach to this problem, and included these variables in the statistical models as blocking factors. Less surprisingly, IQ also differed between groups. The effect of IQ in our analyses was addressed as described above.

3.2. Biomarker prediction of disease status

The logistic regression model correctly predicted disease status for 57 out of 68 (i.e., 84%) of the participants. Low levels of total neuropeptide receptor gene expression (i.e., sum of the *OXTR* and *AVPR1A* gene expression) predicted disease status (Likelihood Ratio Chi-square = 17.16; P < 0.0001; Fig. 1). Low plasma OXT concentration also predicted disease status (LR Chi-sq = 4.700; P = 0.0302). However, OXT concentration was only significant in statistical models that included gene expression measures, indicating that OXT concentration serves as a moderator explaining additional variation, rather than being directly predictive. Differential neuropeptide receptor gene expression (LR Chi-sq = 3.600; P = 0.0578), and plasma AVP concentration (LR Chi-sq = 0.1023; P = 0.7491) did not significantly predict disease status. In fact, a simple logistic regression, containing only total gene expression, no stratifying (blocking) factors, and no other biomarkers, still significantly predicted disease status (LR Chi-sq = 4.265; P = 0.0389), confirming that other biomarkers and stratifiers in model serve to explain additional noise around this central biological signal.

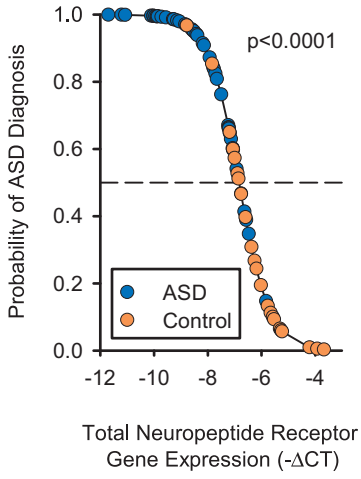

Fig. 1. Total neuropeptide receptor gene expression predicts disease status in children with and without ASD. The effect of total neuropeptide receptor gene expression (i.e., the sum of the *OXTR* −ΔCT and the *AVPR1A* −ΔCT) on predicted (line) and observed (colored circles) group is plotted, adjusted for the other terms in the model, and normalized to its original mean and standard deviation. Children with ASD plotted above, and Control children plotted beneath, the dashed line are correctly classified.

3.3. Total neuropeptide receptor gene expression differs between ASD and control children

Total neuropeptide receptor gene expression was significantly lower in children with ASD ($F_{1,57}$ = 8.5263; P = 0.0050; Fig. 2a). Differential neuropeptide receptor gene expression ($F_{1,57}$ = 1.416; P = 0.2391; Fig. 2b), plasma AVP ($F_{1,57}$ = 0.3883; P = 0.5357; Fig. 2c), and plasma OXT concentrations ($F_{1,57}$ = 0. 6760; P = 0. 4144; Fig. 2d) did not differ significantly by disease status, strengthening our previous interpretation that OXT is a moderator of gene expression.

3.4. Total neuropeptide receptor gene expression predicts core, but not associated, features of ASD

Low levels of total neuropeptide receptor gene expression predicted greater social impairments as measured by the SRS Total (Raw) Score ($F_{1,33}$ = 6.533; P = 0.0154; Fig. 3a). We found no significant effect of the other neuropeptide measures on social functioning (P > 0.05). Low levels of total neuropeptide receptor gene expression also predicted greater severity of stereotypies as measured by the RBS-R Stereotyped Behavior Subscale ($F_{1,33}$ = 8.899; P = 0.0053; Fig. 3b). None of the other neuropeptide measures significantly predicted stereotyped behavior, nor were any significant results found in the other subscales for any neuropeptide measure. Finally, neuropeptide receptor gene expression did not predict level of intellectual functioning as measured by IQ ($F_{1,34}$ = 0.0190; P = 0.8913; Fig. 3c), thereby demonstrating more predictive specificity for core ASD features. Finally, none of the other neuropeptide measures predicted IQ either (P > 0.05).

4. Discussion

This study was the first to our knowledge to employ a multidimensional biomarker approach to investigate the roles of blood OXT and AVP peptides, and expression of their receptors (i.e., *OXTR* and *AVPR1A*), in predicting disease status and symptom severity in individuals with ASD. Considered collectively, these neuropeptide measures correctly classified 84% of study participants as ASD or control. Further analyses revealed that total neuropeptide receptor gene expression was the key driver of group classification, and that it was lower in children with ASD compared to control children. Moreover, total neuropeptide receptor gene expression was strongly associated with ASD symptom severity, such that lower levels of total neuropeptide receptor gene expression predicted greater social impairments and stereotyped behavior in children with ASD. Although there was a significant group difference in IQ, total neuropeptide receptor gene expression was unrelated to intellectual functioning, indicating that neuropeptide receptor gene expression is specific to core features of ASD.

Findings from the present research highlight the value of assessing related biological measures, and their relative contributions, in the same study. We found no evidence of group differences in blood OXT or AVP concentrations, consistent with some previous reports (Carson et al., 2015; Miller et al., 2013; Parker et al., 2014), and neither peptide was found to predict symptom severity. Inclusion of these peptide measures did improve the statistical model's accuracy to correctly classify participants as ASD or control, suggesting that blood OXT and AVP concentrations may serve as moderators explaining additional variation in, rather than being directly predictive of, disease status. In contrast, total *OXTR* and *AVPR1A* gene expression levels robustly predicted disease status, differed between ASD and control children, and predicted symptom severity in children with ASD. This effect was

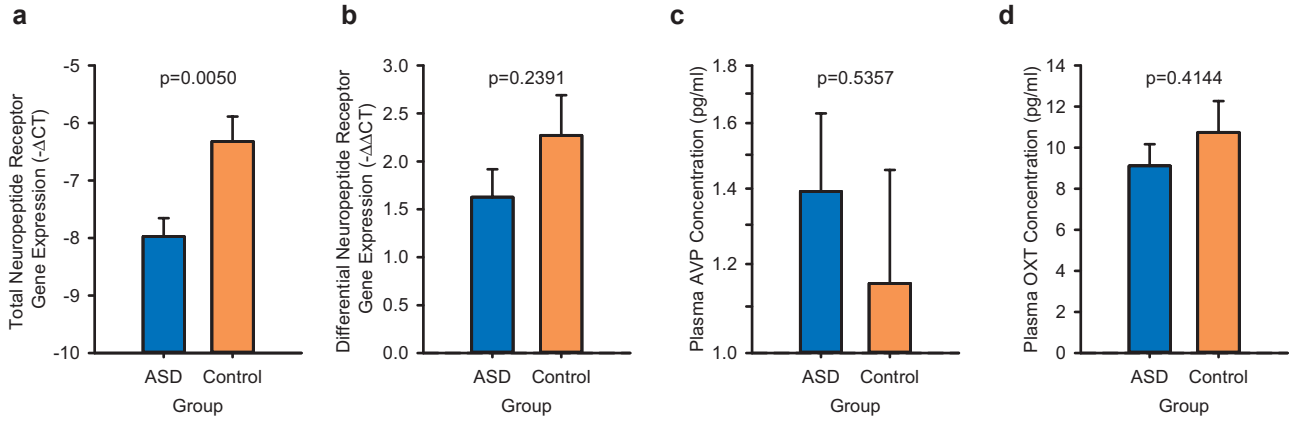

Fig. 2. Group comparisons are presented for the blood neuropeptide measures. Each of the four neuropeptide measures was evaluated in turn, controlling for the same blocking variables as well as the other three neuropeptide measures, in the model. Only total neuropeptide receptor gene expression differed significantly between the ASD and control groups. Data are plotted as LSM +/− SE (i.e., adjusted for the other variables in the model).

specific to overall receptor levels, as we found no evidence in any of the analyses for differential neuropeptide receptor gene expression effects when relative expression was contrasted within participants. Although genetic association studies have previously linked structural variation in the *OXTR* (Harrison et al., 2015) and *AVPR1A* (Francis et al., 2016) genes to social impairments and repetitive behaviors in ASD, to our knowledge, this is the first study to implicate the expression of these genes in ASD diagnosis and phenotype.

Biomarker discovery research in ASD has been largely restricted to peripheral tissue, such as blood, due to its ease in collection. Blood-based biomarkers can be valuable in the development of diagnostic tools to detect disease, or useful as companion diagnostics to identify patients most likely to benefit from specific pharmacotherapies. Exactly how representative of disease biology peripheral measures are for brain disorders such as ASD, however, remains largely unexplored. Nevertheless, one small study on *OXTR* regulation in ASD patients provides potential insight. In this study, individuals with ASD had significantly increased *OXTR* gene methylation levels in postmortem temporal cortical tissue compared to controls, and hypermethylation of the *OXTR* gene correlated with reduced *OXTR* mRNA levels (Gregory et al., 2009). Increased *OXTR* gene methylation levels were also observed in peripheral blood samples in an independent cohort of individuals with ASD compared to controls. Thus, like other genes (Kaminsky et al., 2012), *OXTR* methylation patterns, and hence *OXTR* gene expression, may be relatively conserved across tissue types, and *OXTR* methylation/expression in blood may be a useful surrogate for *OXTR* methylation/expression in the brain. If the same holds true for *AVPR1A*, a global decrease in total neuropeptide receptor gene expression may alter brain protein levels in a manner that functionally contributes to the observed relationship in the present study between peripheral gene expression and core features of ASD.

Intranasally administered OXT and AVP are currently being evaluated as potential pharmacotherapies to improve social abilities in individuals with ASD. Evidence from published OXT treatment trials in ASD patients have been equivocal, with several studies reporting efficacy (Watanabe et al., 2015; Yatawara et al., 2016), whereas others have found no improvement in the trial's primary outcome measure (Guastella et al., 2015; Munesue et al., 2016). Many OXT treatment trials have documented significant variability in responses to OXT, highlighting the need to identify specific factors that contribute to treatment efficacy. Because ASD is a heterogeneous disorder, one possible explanation for these ambiguous OXT treatment trial outcomes is that individual differences in *OXTR* and/or *AVPR1A* availability may influence the ability of a patient to respond, or not respond, to neuropeptide treatment. This observation has been recently reported by our group whereby inclusion of pretreatment neuropeptide measures in the

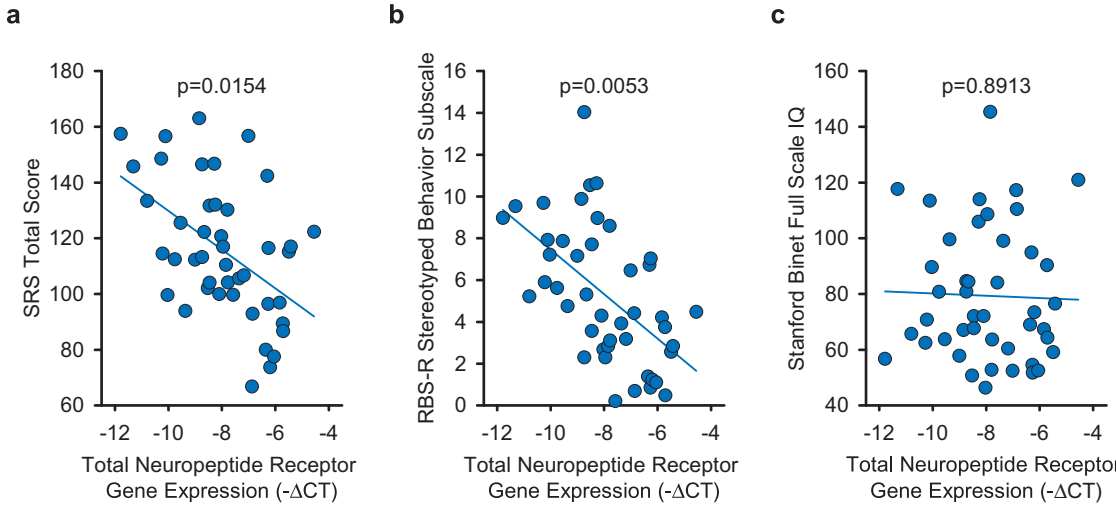

Fig. 3. Total neuropeptide receptor gene expression predicts symptom severity for core, but not associated, features of ASD. a) Social impairments, as measured by the SRS Total (Raw) Score, and b) Stereotypies, as measured by the RBS-R Stereotyped Behavior Subscale, are most severe in ASD children with the lowest levels of total neuropeptide receptor gene expression. c) Cognitive ability, as measured by the Stanford Binet IQ test, is unrelated to total neuropeptide receptor gene expression. Data are plotted adjusted for the other variables in the analysis.

statistical model was critical to accurately assessing OXT treatment efficacy (Parker et al., 2017). Hence, a careful evaluation of pre-treatment *OXTR* and *AVPR1A* gene expression levels to predict treatment efficacy in neuropeptide treatment trials is needed and may lead to use of neuropeptide gene expression as a tool to identify patients most likely to benefit from neuropeptide pharmacotherapy.

There are several limitations of the present study that merit consideration. First, our sample was male-biased and not statistically powered to detect sex differences or sex-by-group interactions in our analyses. Second, we collected only one blood sample per participant (due to the invasive nature of venipuncture, particularly in children), which limited our ability to assess within-individual consistency of our biological measures. Third, some of our study participants were not medication-free. Although their medications were stable (i.e. for at least four weeks) before blood collection, it is possible that our findings were influenced by their medication status. Fourth, down-regulation of *OXTR* and *AVPR1A* gene expression would almost certainly impact peripheral processes, as *OXTR* and *AVPR1A* are expressed in a variety of tissues throughout the body (Thibonnier et al., 2001). Because our study was designed to identify biomarkers of core ASD symptoms, the functional significance of this peripheral receptor down-regulation remains to be explored, as does its disease specificity (i.e., discerning ASD from other neurodevelopmental disorders or neuropsychiatric disorders, i.e., anxiety). Additionally, our study participants were comprised of children already diagnosed with ASD. Future studies should test whether infants at familial risk for developing ASD demonstrate lower total blood neuropeptide receptor gene expression prior to the onset of behavioral symptoms, or coincident with it. Finally, our behavioral phenotyping measures relied on parent report. Although we used gold-standard instruments, these measures were nevertheless subjective in nature.

In conclusion, this study was the first to assimilate unidimensional neuropeptide measures into a multidimensional biomarker analysis to more powerfully interrogate disease status and symptom severity in individuals with ASD. Total neuropeptide receptor gene expression was identified as the key driver of group classification, and low neuropeptide receptor gene expression predicted greater social impairments and stereotyped behaviors in children with ASD. Findings from the present study suggest that low blood neuropeptide receptor availability may be a promising biomarker of disease presence and symptom severity in individuals with ASD.

Contributions

OO and KJP designed the study. OO, AYH, and KJP secured funding to underwrite scientific data collection. AYH oversaw patient recruitment and enrollment. OO, LPJ, RAL, RDS, and JMP contributed to clinical, behavioral, and/or biological data acquisition. OO quantified neuropeptide receptor gene expression. RDS quantified neuropeptide concentrations. OO, KJP, and JPG conducted statistical analyses and interpreted the study data. OO and KJP wrote the first draft of the manuscript. All authors read, significantly edited, and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.psyneuen.2017.12.022.

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