



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 pre-clinical 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 \pm standard error.

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

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