Psychoneuroendocrinology (2014) 40, 159—169

Available online at www.sciencedirect.com

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Evidence for alterations in stimulatory G proteins and oxytocin levels in children with autism

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Received 6 December 2012; received in revised form 3 November 2013; accepted 18 November 2013

KEYWORDS
Autism;
G protein;
Oxytocin;
Signaling;
Autism in early childhood;
ADOS;
ADI-R

Summary The neurotransmitter oxytocin plays an important role in social affiliation. Low oxytocin levels and defects in the oxytocin receptor have been reported in childhood autism. However, little is known about oxytocin's post-receptor signaling pathways in autism. Oxytocin signals via stimulatory and inhibitory G proteins. c-fos mRNA expression has been used as a marker of OT signaling as well as of G protein signaling. Herein, we hypothesized that oxytocin and its signaling pathways would be altered in children with autism. We measured plasma oxytocin levels by ELISA, G-protein and c-fos mRNA by PCR, and G proteins by immunoblot in cultured peripheral blood mononuclear cells (PBMCs) in children with autism and in age-matched controls. Males with autism displayed elevated oxytocin levels compared to controls ($p < 0.05$). Children with autism displayed significantly higher mRNA for stimulatory G proteins compared to controls ($p < 0.05$). Oxytocin levels correlated strongly positively with c-fos mRNA levels, but only in control participants ($p < 0.01$). Oxytocin, G-protein, and c-fos mRNA levels correlated inversely with measures of social and emotional behaviors, but only in control participants. These data suggest that children with autism may exhibit a dysregulation in oxytocin and/or its signaling pathways.

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0306-4530/$ — see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.psyneuen.2013.11.014

Introduction

Autism spectrum disorder (hereafter referred to as ''autism'' or ASD), is defined by deficits in social interaction and language and the presence of stereotypic and obsessive behaviors. The prevalence is currently estimated to be one in 88 children (CDC, 2012). Autism is recognized as a complex genetic disorder involving multiple interacting genes and gene/environment interactions. Currently no genetic or biochemical markers for autism exist (Ring et al., 2008; Rapin and Tuchman, 2008; Abrahams and Geschwind, 2008).

A substantial body of literature implicates abnormalities in neuropeptides and neurotransmitters in autism (McDougle et al., 2005; Lam et al., 2006). Perhaps the neuropeptide of greatest interest has been oxytocin (OT), as it is known to play important roles in social behavior in both animals and humans (Insel, 2010; Winslow and Insel, 2004; Meyer-Lindenberg, 2008) A frequently-referenced report demonstrated lower plasma oxytocin levels in patients with autism compared to typically developing controls (Modahl et al., 1998). A subsequent study by those same authors pointed to faulty processing of the oxytocin prohormone to the active oxytocin peptide (Green et al., 2001). More recently, epigenetic abnormalities in the oxytocin *receptor* gene (OTR) have been implicated in autism (Gregory et al., 2009), raising the possibility of an element of oxytocin resistance in autism. The discovery of abnormalities in oxytocin and its receptor in autism has led to testing of exogenous oxytocin as a treatment in autism. These studies demonstrated that intravenous administration of oxytocin to patients with autism led to decreased repetitive behaviors (Hollander et al., 2003) and to improved affective language (Hollander et al., 2007). Recent studies show that intranasal administration of oxytocin leads to improved recognition of emotion in participants with autism (Guastella et al., 2010). These promising observations have not yet led to widespread clinical use of oxytocin in autism. Questions remain about dosing, routes, and even the mechanisms of action of oxytocin on behavior, given the suspected low permeability of the blood—brain barrier to oxytocin and the short half life of oxytocin in the plasma (Churchland and Winkielman, 2012; Green and Hollander, 2010).

Despite the growing literature on oxytocin and behavioral disorders, only a few studies have looked beyond oxytoxin and its receptor to oxytocin post-receptor signaling cascades. Oxytocin exerts its actions largely via the stimulatory G protein, Gαq (Gould and Manji, 2002; Gimpl and Fahrenholz, 2001). Like many other Gαq/11-coupled receptor ligands, which signal via the protein kinase C (PKC) pathway, oxytocin can also exert actions via other G protein pathways, including Gαs and the protein kinase A (PKA) pathways as well as via inhibitory G protein pathways (Viero et al., 2010). G proteins are the major cell surface mediators for a myriad of neurotransmitters, chemokines, cytokines, leukotrienes, and vasoactive peptides.

Stimulatory G protein pathways can amplify the signals of a small number of cell surface molecules by activating multiple intracellular cascades. In fact, oxytocin has been shown to modulate anxiety via pathways downstream from G protein signals, including mitogen-activated protein kinase (MEK1/2) pathways and extracellular signal-related kinase 1/2 (ERK1/2) pathways in the central nervous system (Blume et al., 2008; Jurek et al., 2012).

G protein signaling has been a subject of investigation in several other neuropsychiatric conditions. In fact, G protein levels in peripheral blood mononuclear cells (PBMCs) correlate with severity of disease and with response to therapy in depression, bipolar disease, and schizophrenia (Schreiber and Avissar, 2003; Catapano and Manji, 2007; Gladkevich et al., 2004). However, the potential roles of G proteins in autism remain understudied.

Herein, we hypothesized that children with autism would exhibit alterations in plasma oxytocin levels as well as in its downstream mediators. Because immune cells bear oxytocin receptors (Maccio et al., 2010) and because OT signals via G proteins (Gravati et al., 2010), we sought alterations in G proteins and these downstream mediators in peripheral blood mononuclear cells (PBMCs) from patients with autism.

We measured plasma oxytocin levels, G protein mRNA by polymerase chain reaction (PCR), and G proteins by immunoblot. Because c-fos mRNA expression in the central nervous system can be downregulated by administration of oxytocin (Windle et al., 1997, 2004) and because its expression has been used as a downstream marker of stimulatory G protein pathways (Liu et al., 2002), we also measured c-fos mRNA induction in PBMCs in the presence and absence of oxytocin. Finally, we hypothesized that oxytocin levels and G protein expression would correlate with behavioral measures, particularly social measures reported to be abnormal in autism.

Methods

Participants

Children between four and six years old were recruited from patient flow at the Center for Child Health and Development (CCHD) at the University of Kansas Medical Center (KUMC) and in the surrounding community from 2005 to 2010. Participants included 78 children, 37 with autism and 41 typically developing controls (TDC). Children with autism met criteria for Autistic Disorder (AD) or Pervasive Developmental Disorder, Not Otherwise Specified (PDD-NOS) as evidenced by scores above the cut-off on the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 1994) and the Autism Diagnostic Observation Schedule (Lord et al., 2000) and based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV, TR) criteria (American Psychiatric Association, 2000). Only children with ''essential autism'' were included for this study, as children with autism were excluded for short stature, microcephaly, or definite dysmorphology and if they were unable to point to pictures to complete cognitive assessments. Children in the TDC group had no history of mental health, developmental, or learning disability as reported by parents; and scored within normal limits on the Behavior Assessment System for Children, Second Edition (BASC-2) Parent Report Form (Reynolds and Kamphaus, 2004). TDC were excluded if they had first degree relatives with autism. Exclusion criteria for all participants included prematurity, known chronic medical conditions, use of psychotropic or immune-altering medications within 6 months, illness the day of the study visits, and immunization

Table 1 Participant demographics.

Participant characteristic	ASD group			TDC group		
	Male	Female	Total	Male	Female	Total
Sample size (*N*)	25	12	37	24	17	41
Mean age (SD)	4.78 (.65)	4.63 (.55)	4.73 (.61)	4.90 (.64)	4.76 (.58)	4.85 (.61)
Race (*N*)						
Caucasian	19	11	30	20	13	33
African-American	2	0	2	1	3	4
Hispanic	3	0	3	3	0	3
Other	1	1	2	0	1	1
ADI-R						
Social	17.04 (6.34)	21.92 (5.23)	18.62 (6.37)			
Communication	15.80 (3.75)	17.67 (3.60)	16.41 (3.76)			
Repetitive behaviors	6.44 (1.87)	7.33 (3.14)	6.73 (2.35)			
Mean* DQ (SD)	84.07 (25.78)	69.64 (17.45)	79.39 (24.14)	111.29 (13.99)	119.81 (10.61)	114.70 (13.29)
DSM-IV, TR diagnosis						
Autistic Disorder	20	11	31			
PDD-NOS	5	1	6			

* *Note*: Developmental Quotient (DQ) mean is 100, with a SD of 15.

within 6 weeks prior to blood draws. Table 1 shows the participant demographics.

Power analysis

Based on oxytocin levels and standard deviations previously reported in autism, and using a two-group *T* test of equal means and a two-sided test at an alpha of 0.05, we estimated that 24 participants with autism and 24 controls would yield an 89% power to detect this two-fold change.

Study visit procedures

Potential participants completed screening interviews via telephone. Study procedures included two study visits (Fig. 1). The first visit included a complete medical history, physical exam and specific eligibility measures depending on study condition. Parents of participants in the autism group completed the Autism Diagnostic Observation Schedule (ADOS, Lord et al., 2000) and Autism Diagnostic Interview-Revised (ADI-R, Lord et al., 1994). Members of an experienced autism diagnostic team confirmed the diagnosis of Autistic Disorder or PDD-NOS based on these measures and DSM-IV criteria. Each participant in the TDC group completed a medical history, physical exam and the Behavior Assessment System for Children, Second Edition (BASC-2). Participants were excluded if scores were not within normal limits on any BASC-2 subscale. Eligible participants completed a second study visit, which included a cognitive measure, the Vineland Social-Emotional Early Childhood Scales (Vineland SEEC) (Sparrow et al., 1998), and the blood draw. Each participant received a $25.00 gift card for participation and written summary of the cognitive and behavioral measures.

Venipuncture

20 to 40 cc of blood was drawn after a 2 hour fast at the General Clinical Research Center at KUMC between 10 am and 12 am, as previously described (Modahl et al., 1998). An aliquot was collected on ice from which plasma was separated and stored at −80 °C for later oxytocin measurement.

Autism measures

Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 2000)

We used the ADOS as part of the diagnostic work-up and as an outcome measure to examine differences within the ASD group. The ADOS is a semi-structured observation schedule that measures the quality of social and communication behaviors, developed for use in the diagnosis of ASDs. The ADOS consists of four modules designed for use at language levels ranging from no expressive language to verbal fluency. Ratings are completed after administration and can be used to formulate diagnoses using the diagnostic algorithm provided for each module. Ten children completed Module 1 (no speech up to and including simple phrases, but no consistent phrase speech) and 27 children completed Module 2 (phrase speech up to fluent speech). The ADOS has good inter-rater reliability (Module 1 inter-rater agreement $\geq$80% on all items; Module 2 mean percent agreement $\geq$89%) and high sensitivity and specificity for distinguishing ASD from other disorders as well as between ASDs (sensitivity ranged from 94 to 100 and specificity from 73 to 95).

Autism Diagnostic Interview-Revised (ADI-R; Rutter et al., 2003)

The ADI-R is a standardized, semi-structured interview, which has been shown to effectively discriminate between

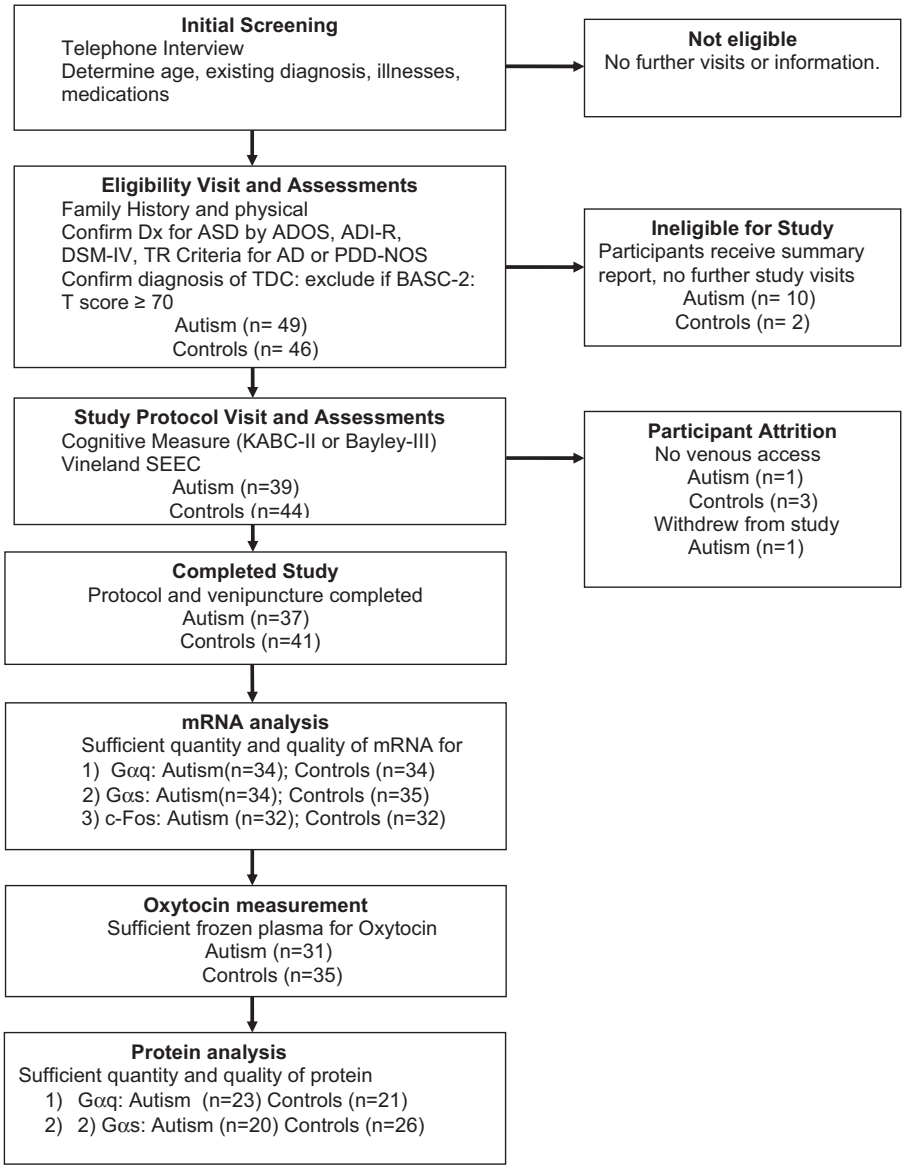

Figure 1 Study flow chart.

people with intellectual disabilities who have autism and those who do not have autism. Items examine severity of possible symptoms in the areas that correspond to the DSM-IV, TR symptoms for ASD. Cut off scores for autism in each of these areas are well defined. The reliability of the ADI-R is good, with individual algorithm items range from $r = 0.63$ to 0.89. Alpha coefficients calculated for internal consistency for the domains range from 0.69 to 0.95.

Developmental Quotient

We calculated a Developmental Quotient (DQ) for each participant (DQ = developmental level/chronological age in months $\times$ 100) based on the KABC-II Mental Processing Index (standard score) or the age equivalent from the Bayley-III, below:

Kauffman Assessment Battery for Children-Second Edition (KABC-II). The KABC-II is a norm-referenced assessment of cognitive abilities using sequential and simultaneous processing based on neuropsychological theory. The normative sample for the KABC-II closely matches 2001 population data and has good reliability and validity (Kaufman AS, 2004). Internal consistency coefficients average .85 to .87 across age ranges. The KABC-II shows good construct validity as evidenced by strong correlations with other measures of cognitive functioning. Standard scores for composites from 85 to 115 are considered average. We selected this test to minimize penalty for language impairment as many children with ASD have impaired language. Sixty-eight children completed the KABC-II (autism = 27, TDC = 40). All children completed the Face Recognition and Hand Movements subtests from the KABC-II to examine relationships between plasma oxytocin and facial recognition and imitation.

Bayley Scales of Infant and Toddler Development-Third Edition (Bayley-III). Children in the ASD group who were unable to complete the KABC-II completed the Cognitive portion of the Bayley-III ($N = 10$). The Bayley-III is a

norm-referenced assessment of development in infants and young children (birth to 42 months) (Bayley, 2006). We calculated a developmental age for children out of the test age range to compute the DQ as described above.

Vineland Social-Emotional Early Childhood Scales (Vineland SEEC, Sparrow et al., 1998)

Caregivers of children in both groups completed the Vineland SEEC (Sparrow et al., 1998) via semi-structured interviews. The Vineland SEEC is derived from the Socialization Domain of the Vineland Adaptive Behavior Scales (VABS) Expanded Form (Sparrow et al., 1984) and examines social and emotional functioning for children from birth to 5 years, 11 months. The Vineland SEEC provides information on three subscales and yields a total composite score. Subscales include the Interpersonal Relationships scale (Vineland-IR; interactions with others), the Play and Leisure Time scale (Vineland-Play; assessing play and enjoyment of leisure time), the Coping Skills scale (Vineland-Cope; assessing responsibility and sensitivity to others), and the Composite scale (Vineland-Comp), which is a total of items from all three scales. The Vineland SEEC has good reliability and validity as evidenced by the specificity of age range, adequate internal consistency (.80 to .87) and relationship with other measures of social-emotional functioning (Sparrow et al., 1998). Standard scores from 85 to 115 are considered average.

Oxytocin Enzyme Immunoassay (EIA)

Oxytocin was assayed using an extraction step that has been shown to reduce the possibility of cross-reactivity with oxytocin degradation products and other potential cross-reacting molecules (Szeto et al., 2011). A 1:1 mixture of sample and 0.1% trifluoroacetic acid (TFA) was centrifuged at 17,000 $\times$ *g* for 15 min at 4 °C. Supernatants were added to Hypersep C18 columns (Fisher Scientific, Pittsburgh, PA) on a vacuum manifold and eluted off with 60:40 acetonitrile: 0.1% TFA. Samples were evaporated and stored at −20 °C until reconstitution. Plasma oxytocin was assayed using Oxytocin Enzyme Immunoassay kits (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions, in duplicate. Samples were read at 405 nm with a correction between 570 and 590 nm on a PowerWave-I microplate spectrophotometer (Bio-Tek, Winooski, Vermont). Standard curves and concentrations were calculated using KC-4 software v.3.0 (Bio-Tek), Excel, and SigmaPlot 8.0. The lower limit of detection of the oxytocin assay was .05 pg/ml. The intra-assay coefficient of variation is 9.1%, and the inter-assay coefficient of variation is 14.5%. This assay kit is 100% specific for oxytocin with a 0.6% cross-reactivity with a sulfated form of vasopression and <.2% cross-reactivity with TRH, somatostatin, enkephalin, and VIP (Enzo Life Sciences, Farmingdale, NY).

PBMC isolation, hormone treatment, and cell culture

PBMCs were isolated from heparinized blood using Histopaque 1077 according to the manufacturers' directions (Sigma-Aldrich, St. Louis, MO). Viability, as determined by trypan blue, was >95%. PBMCs were suspended in RPMI with 1% Penicillin/Streptomycin and 10% charcoal stripped, delipidated fetal calf serum (FCS, Invitrogen, Carlsbad, CA) and plated with vehicle or oxytocin (10^{-7} M to 10^{-9} M) in RPMI + 10% charcoal stripped, delipidated FCS and incubated at 37 °C in 5% CO_2 with vehicle or oxytocin. Samples were incubated in either phenol red containing medium or phenol red-free medium. PBMCs from a subset of participants also underwent short term (40 min) culture with a 10^{-6} M concentration of PMA, which signals via the Gαq pathway, or with 10^{-6} M forskolin, which classically signals via the cAMP pathway. After incubation, cell pellets were resuspended in Buffer RLT Plus from the RNeasy® Mini Plus Kit (QIAgen, Valencia, CA) and stored at −80 °C.

RNA isolation and quantitation and reverse transcription and one-step real time PCR

Samples were placed in QIAshredder columns and processed according to the manufacturer's directions. The homogenized lysate was transferred to a genomic DNA (gDNA) eliminator spin column and centrifuged. DNA in a 70% ethanol mixture was transferred to RNeasy spin columns according to the manufacturer's directions (QIAGEN, Valencia, CA). RNA was examined by spectrophotometry and quantitated using the Ribogreen assay (Invitrogen, Carlsbad, CA). Ribogreen reagent was added to samples and standards, and samples were read on a Bio-Tek FL 800 fluorescent plate reader. RNA was diluted in nuclease-free water. DNA was digested using DNase 1, and residual DNase activity was quenched with EDTA. Reverse transcription was then performed using the SuperScript II Reverse Transcription Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One-step real-time RT-PCR was performed using the SYBR Green PCR kit (Bio-Rad, Hercules, CA). G protein and c-fos sequences were obtained from the Gene Bank database. Human GNAQ primers were based on the sequence NM_002072: Gαq sense: 5′-GAT GTT CGT GGA CCT GAA CC-3′; Gαq antisense: 5′-CAA CTG GAC GAT GGT GTC CT-3′. The following primers were constructed using the GenBank sequence for GNAS NM_ 000516: Gαs sense: 5′-TCT ACC GGG CCA CGC ACC GC-3′; Gαs antisense: 5′-GCA GGA TCC TCA TCT GCT TC-3′. Human c-fos primers were based on the sequence NM_005252: c-fos sense: 5′-CGA GCC CTT TGA TGA CTT CCT-3′; c-fos antisense: 5′-GGA GCG GGC TGT CTC AGA-3′. BLAST searches were performed using the National Center for Biotechnology Information's BLAST WWW Server. Commercially available primers were used for the housekeeping gene, GAPDH (Clontech, Mountain View, CA). PCR was performed on the iCycler (Bio-Rad, Hercules, CA). The following parameters were used for the RT-PCR program for all genes: 95 °C 3 min; 35 cycles of 95 °C 20 s, 56 °C 20 s, 72 °C for 20 s; 95 °C 1 min; and 55 °C 7 min. Data were calculated as the delta Ct (Ct) ratio of the gene of interest compared to the delta Ct of the housekeeping gene (GAPDH). That ratio was also normalized as a percent of an internal standard control sample, prepared from a pool of PBMCs from healthy adult males and females. Dissociation curves were performed for each primer pair, and all primer pairs yielded a single peak with no detectable contaminating or primer/dimer peaks.

Protein quantitation and immunoblot analysis

Protein concentrations were determined by the Bradford method utilizing a Coomassie protein assay kit (Pierce Biotechnology, Rockland, IL). A standard curve using bovine serum albumin was established from 0 to 2000 μg/ml. Sample (5 μl) was added to 96 well plates in duplicate. Coomassie reagent (250 μl) was added to each well and incubated for 10 min at RT. The absorbance was measured at 595 nm on a Power WaveX plate reader (Bio-Tek, Winooksi, VT).

Total membrane protein (200 μg per well) was electrophoresed in 12% Tris—HCl gels and transferred to nitrocellulose membranes (Biorad, Hercules, CA) by electroblotting. Membranes were blocked in Tris-buffered saline with .1% Tween-20 (TBST) and nonfat dry milk, and incubated with affinity-purified polyclonal antibodies specific to each G protein (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, membranes were incubated for one hour in TBST with horseradish peroxidase conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA). Bands were visualized by chemiluminescence via Western blot luminal reagent (Santa Cruz Biotechnology, Santa Cruz, CA) by exposure to Classic BX autoradiography film (MidSci, St. Louis, MO). The membranes were then stripped with western blot stripping buffer (Fisher Scientific, Hanover Park, IL) for 30 min at 37 °C and re-probed for actin protein as an internal control, using a monoclonal primary antibody (MP Biomedicals, Santa Ana, CA). Bands were quantitated using a densitometer employing ImageQuant 5.2 software (Molecular Dynamics, Foster City, CA).

PCR sequencing

Gαs and Gαq PCR fragments were treated with ExoSAP-IT (USB, Cleveland, OH) and subsequently sequenced using BigDye terminator v3.1 cycle sequencing chemistry and a 3730 capillary DNA analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis

We utilized 2-way ANOVAs with a diagnosis factor with two-levels (autism versus controls) and gender with two levels (male and female). Homogeneity of variance was evaluated using Levene's test for all outcomes of interest. Levene's test values indicated that the assumption of equality of variances had been violated for three of the outcomes. Tabachnick and Fidell (2001) indicate that if the ratio of largest cell variance to smallest cell variance is 4 to 1 or less, then Levene's test F values as large as 10 are acceptable (Tabachnick and Fidell, 2001). Our values fit this criterion, so we proceeded with the analyses using a more stringent alpha criterion of $p = .025$ as recommended. We used Spearman rank-order correlations to evaluate strength of associations because of concerns about the normality of the data and outliers. When making within-subject comparisons of c-fos mRNA levels under multiple conditions, Wilcoxon Signed Ranks tests were used.

Results

Plasma oxytocin levels

Sufficient plasma was available in 66 participants. A 2 × 2 ANOVA was conducted to determine whether there were significant differences across diagnosis (autism versus control) and gender (male versus female). The diagnosis by gender interaction was not significant $F(1,62) = 1.85$, $p = .18$, eta squared = .028. The effect of diagnosis was not significant $F(1,62) = 2.53$, $p = .12$, eta squared = .038. The effect of gender was also not significant $F(1,62) = .54$, $p = .47$, eta squared = .008. Because the most frequently referenced study on oxytocin in autism was done in males only (Modahl et al., 1998), we performed a one-way ANOVA to examine to effect of diagnosis for males only. Males in the autism group exhibited higher oxytocin values $F(1,36) = 5.71$, $p = .022$, eta squared = .137 compared to control males. Autistic males exhibited a mean of 24.22 pg/ml, whereas males in the control group had a mean of 18.57 pg/ml. Plasma OT levels for females were more similar across control and OT groups. Means and standard deviations can be seen in Fig. 2.

Gαq and Gαs mRNA

Sufficient quantities of mRNA were available for G protein PCR analyses after overnight culture of PBMCs from 69 participants. The 2 × 2 ANOVA revealed a significant effect of diagnosis on Gαq mRNA $F(1,64) = 10.07$, $p = .002$, eta squared = .128. Participants with autism exhibited higher G*q mRNA levels (mean = 142.06%, SD = 144.06%) compared to control participants (mean = 70.75%, SD = 57.14%). The effect was moderate to large. The main effect of gender was not statistically significant, and the interaction was not statistically significant.

For Gαs mRNA, there were significant main effects for both diagnosis $F(1,65) = 9.82$, $p = .003$, eta squared = .108 and gender $F(1,65) = 12.78$, $p = .001$, eta squared = .141. This represents a large effect. The interaction between diagnosis and gender was not significant ($p = .088$).

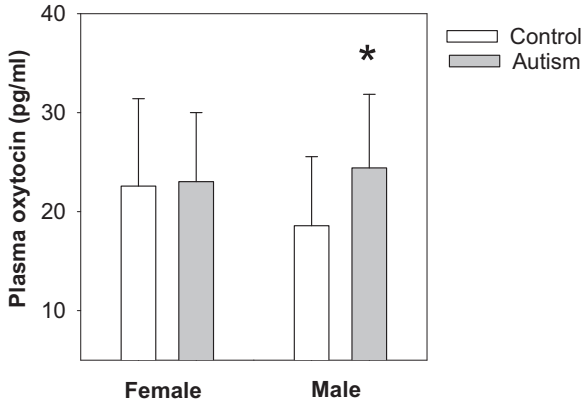

Figure 2 Vertical bar chart of plasma oxytocin levels in control participants and in participants with autism. Error bars show standard deviations. * Males with autism exhibited significantly higher oxytocin levels compared to control males ($p = .022$).

Table 2 Means and standard deviations by diagnosis and gender.

mRNA or protein (% of control)	Control Males	Control Females	Autism Males	Autism Females	All Controls	All Autism
Gαq mRNA	74.10 (24.08)	65.97 (28.79)	115.65 (22.46)	197.29 (32.48)	70.75 (57.14)	142.06** (144.06)
Gαs mRNA	64.01 (21.55)	108.45 (24.89)	97.81 (20.10)	226.00 (29.06)	83.06 (74.30)	139.29** (129.72)
Gαq protein	266.03 (48.90)	224.37 (62.33)	126.84* (41.55)	448.80 (78.84)	250.15 (208.52)	196.83 (189.23)
Gαs protein	214.00 (87.46)	352.82 (110.63)	354.11 (97.03)	231.60 (132.22)	267.39 (347.43)	311.23 (348.63)
c-fos mRNA	45.37 (14.49)	55.80 (16.43)	73.64 (13.11)	143.07* (19.45)	49.94 (45.50)	95.33** (79.65)

* Significantly different from male controls at the .05 level.
** Significantly different from all controls at the .005 level.

Participants with autism exhibited higher levels of Gαs mRNA (mean = 139.29% SD = 129.72) compared to controls (mean = 83.06, SD = 74.30). Females exhibited higher mean levels (mean = 158.18, SD = 155.98) compared to males (mean = 82.09, SD = 47.69). Mean for each diagnosis and gender combination can be seen in Table 2. Both Gαq mRNA and Gαs mRNA are outcomes where the assumption of homogeneity of variance was violated, but the observed p-values were significant even given the more stringent criterion of $p = .025$.

G protein immunoblot

Sufficient quantities and sufficient quality of protein were available from cultured cells on 44 participants for Gαq and for 46 participants for Gαs protein. For Gαq protein, the 2 × 2 ANOVA indicated that there was a significant interaction between diagnosis and gender $F(1,40) = 9.30$, $p = .004$, eta squared = .168 and a large effect. Follow-up tests to examine the effect of diagnosis within each gender group revealed that for males, there were significant differences in Gαq protein between the autism and control groups $F(1,29) = 4.83$, $p = .036$, eta squared = 142 with control males having higher means than males with autism. For females, the mean difference was not statistically significant, largely as a result of the small sample size $F(1,13) = 4.68$, $p = .053$, but the effect size was quite large eta squared = .298. The direction of the effect was opposite of that in males, as females with autism exhibited higher mean levels than control females. Means and standard deviations can be seen in Table 2. There was also a significant main effect of gender $F(1,40) = 5.53$, $p = .024$, eta squared = .100. The female mean (mean = 310.69, SD = 208.09) was higher than that of the males (mean = 185.21, SD = 184.82). The effect for diagnosis was not statistically significant $p = .48$.

The 2 × 2 ANOVA indicated no significant interaction between diagnosis and gender ($p = .23$) and no significant main effects for gender ($p = .94$) or diagnosis ($p = .93$) for Gαs protein (Table 2).

c-fos mRNA and responsiveness to oxytocin, forskolin, and PMA

Sufficient quantities of mRNA were available for c-fos PCR analysis after overnight culture of PBMCs from 64 participants. Results from the 2 × 2 ANOVA indicated that there was not a significant interaction between diagnosis and gender $F(1,60) = 3.38$, $p = .07$, eta squared = .04. There were significant main effects for diagnosis $F(1,60) = 12.96$, $p = .001$, eta squared = .075 with autism participants having a higher mean c-fos mRNA levels (mean = 95.33, SD79.65) compared to control participants (mean = 49.94, SD = 45.50). The effect of gender was also significant $F(1,60) = 6.19$, $p = .016$, eta squared = .041 with females having higher mean levels (92.16, SD = 84.16) compared to males (60.92, SD = 54.61). Refer to Table 2 for means and standard deviations for each diagnosis by gender combination.

We also examined c-fos mRNA induction after in vitro exposure to vehicle or to various concentrations of oxytocin ranging from 10^{-7} to 10^{-9} M in a subset of participants. Exposure to oxytocin led to significant reduction in c-fos

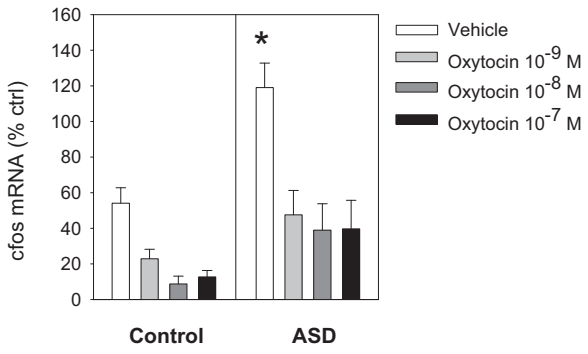

Figure 3 Vertical bar chart of c-fos RNA levels in control participants and participants with autism after overnight exposure to oxytocin. ** Significantly higher than controls. *Exposure to oxytocin leads to a reduction in c-fos mRNA at all concentrations compared to vehicle ($n = 15–20$/group; $p < 02$).

Table 3 Correlations among OT, G proteins, and cFos in Control participants below the diagonal and autism participants above the diagonal.

	OT	Gαq mRNA	Gαq protein	Gαs mRNA	Gαs protein	c-fos mRNA
OT		−.01	−.05	.08	−.50*	.20
Gαq mRNA	.08		.27	.58**	−.50*	.19
Gαq protein	.14	.62**		.37	−.21	.53*
Gαs mRNA	.20	.69**	.48*		−.50*	.31
Gαs protein	−.33	−.41*	−.51*	−.33		−.68**
c-fos mRNA	.51**	.33	.48*	.32	−.10	

* Significant at the .05 level.
** Significant at the .01 level.

mRNA levels in both patients and in controls compared to vehicle (Fig. 3; controls ($n = 15$) 10^{-9} M $p = .019$; 10^{-8} M $p = .004$; and 10^{-7} M $p = 001$; autism ($n = 20$) 10^{-9} M $p = .001$; 10^{-8} M $p = .002$; and 10^{-7}M $p = 004$).

Correlations among oxytocin, G-proteins, c-fos mRNA

Gαq mRNA levels correlated *positively* with Gαq protein, but in the control participants only (Spearman correlation = 27, $p = .22$, $n = 23$ in autism; Spearman correlation = .62, $p = 003$, $n = 21$ in controls; Table 3). Statistically significant *negative* correlations between Gαs mRNA and protein were seen in autism (Spearman correlation = −.50, $p = .02$; $n = 20$; Spearman correlation −.33, $p = .11$, $n = 26$ in controls; Table 3). We found a strong positive correlation between oxytocin levels and c-fos mRNA, a downstream marker of oxytocin responsiveness, but in controls only ($r = .51$; $p = 0.007$). We also found a strong, positive correlation between Gαq mRNA and Gαq protein, but in controls only ($r = .62$; $p = .003$; Table 3).

Correlations between oxytocin/G-proteins and behavior

We found a positive correlation between oxytocin levels and ADOS scores, suggestive of more social impairment with high OT levels in patients with autism. We found strong negative correlations between oxytocin levels and subscores on the Vineland SEEC, but in controls only (Table 4). We also found strong negative correlations between both Gαq and Gαs protein levels and various subscores on the BASC, but in controls only (Table 4).

Discussion

Herein, we describe alterations in plasma oxytocin levels as well as a transcriptional upregulation in oxytocin G-protein signaling pathways in children with autism. PBMCs from children with autism express higher levels of Gαs and Gαq mRNA and higher levels of c-fos mRNA compared to PBMCs from controls. Oxytocin levels correlated directly with c-fos mRNA levels in typically developing controls, but not in participants with autism. This is, to our knowledge, the first evidence of transcriptional alterations in G-protein signaling cascades in autism. We speculate that the higher levels of OT and the transcriptional upregulation of its signaling pathways could reflect a compensatory mechanism for another (unidentified) downstream defect in autism.

We also compared markers of G protein signaling cascades with behavioral measures. High oxytocin levels correlated with greater social impairment (as evidenced by high ADOS scores). Strong inverse correlations were seen between oxytocin and Vineland SEEC subset scores, but only in control participants. Strong inverse correlations were seen between

Table 4 Correlations between OT, G proteins, cFos and behavioral testing in Control and Autism (ASD) participants.

	OT		Gαq protein		Gαs protein		cFos mRNA	
	Control	ASD	Control	ASD	Control	ASD	Control	ASD
Developmental Quotient	−.13	−.14	.27	−.04	.04	−.01	.19	.04
Autism Diagnostic Observation Schedule†		.44*		.35		−.16		.46**
Vineland interpersonal relationships scale	−.58**	−.29	.01	.06	.19	.17	−.34	−.27
Vineland play and leisure time scale	−.56**	−.21	−.07	−.08	−.05	.08	−.40*	−.06
Vineland composite scale	−.59**	−.31	−.04	−.08	.08	.17	−.37*	−.26
BASC — aggression scale	−.08	−.17	−.20	.35	−.40*	−.33	−.36*	.12
BASC-externalizing problems	−.03	−.01	−.04	.29	−.41*	−.36	−.28	.23
BASC — atypicality scale	−.13	.16	−.18	.35	−.47*	−.20	−.10	.52**
BASC — withdrawal scale	−.15	.24	−.55**	.31	.09	−.05	−.38*	.22
BASC — behavioral symptoms index	−.33	.06	−.31	.34	−.25	−.29	−.33	.37*

† ADOS testing was performed in ASD participants only.
* Significant at the .05 level.
** Significant at the .01 level.

both stimulatory G proteins and several BASC subset scores, but, again, only in control participants.

The absence of relationships among oxytocin, signaling markers, and behavioral measures in the autism group could reflect a dissociation between oxytocin and its downstream mediators in this group. Alternatively, the lack of correlation could be explained by an element of resistance to oxytocin in a subset of patients with autism.

Our finding of increased oxytocin levels in males with autism contradicts the most frequently quoted study of oxytocin levels in children with autism. That study demonstrated decreased plasma oxytocin levels in males with autism compared to typically developing males (Modahl et al., 1998). The findings in that study were somewhat paradoxical: although oxytocin levels were lower in autism than in controls, participants with autism who had the highest oxytocin levels were the most socially impaired. In that study, oxytocin levels were measured by RIA rather than by EIA (Modahl et al., 1998) which generally yields lower levels than EIA measurements (Szeto et al., 2011). The oxytocin levels in both patients and controls in that study were substantially lower than oxytocin levels also measured by RIA in other pediatric populations (Alfven et al., 1994; Alfven, 2004). One other published study of plasma oxytocin levels in children with ASD utilized RIA to demonstrate lower levels in children and adolescents with autism (Al-Ayadhi, 2005). Even oxytocin studies utilizing EIA with an extraction step need to be interpreted with caution. given that multiple degradation products and other cross-reacting substances can still be detected by available commercial assays (McCullough et al., 2013).

Several lines of evidence suggest that age, gender, pubertal status, and gonadal steroid exposure also affect oxytocin levels in humans and in animals (Hammock et al., 2012; Parker et al., 2010). CSF levels of OT levels rise with age in female primates of reproductive age but decrease with age in infants (Parker et al., 2010). The participants in our study were restricted in age from 4 to 6 years and were prepubertal. In contrast, Modahl's patients spanned prepubertal and pubertal age ranges. Further, although the participants in our study were receiving no medications, approximately one third of the boys with ASD in Modahl's study were receiving psychotropic medications. Many of these agents, including serotonin reuptake inhibitors, alter oxytocin levels (Singh et al., 2009). A recent study in children challenged the notion of lower oxytocin levels in autism, reporting no differences between autism and controls, but higher levels of oxytocin in females compared to males (Miller et al., 2013).

The adult literature includes several publications demonstrating higher plasma oxytocin levels in adults with autism and/or social anxiety (Jansen et al., 2006; Hoge et al., 2008). Jansen found that higher-functioning adults with autism exhibited increased oxytocin levels compared to controls (Jansen et al., 2006). We speculate that the increased levels of OT reported in some of the literature and observed in our study may be consistent with some degree of resistance to oxytocin or of a dysregulation in OT signaling pathways. If oxytocin is, in fact, important in social affiliation, it seems reasonable to speculate that either oxytocin deficiency or defects in its signaling pathways could be associated with some similar behavioral manifestations.

In Modahl's study, oxytocin levels correlated well with behavioral and developmental scores in control patients but not in autism participants, suggesting the possibility of a dissociation between oxytocin and behavioral measures in autism. Our findings with oxytocin and c-fos may be reminiscent of those results: We found a strong positive correlation between oxytocin and the downstream marker of oxytocin suppression, c-fos mRNA, but in controls only. Similarly, Gαq mRNA correlated well with Gαq protein, but, again, in controls only. The lack of positive correlations between oxytocin and its downstream mediators in autism may be a clue to a disruption in oxytocin regulation or signaling in autism.

There are several other limitations of the current study. First, there is no direct evidence that plasma oxytocin reflects the central nervous system activity of oxytocin. Controversy exists as to whether peripherally derived oxytocin actually crosses the blood brain barrier in biologically relevant quantities (Viero et al., 2010). The recent findings of behavior changes induced by intravenous or intranasal administration of oxytocin provides some evidence that peripheral oxytocin administration does lead to behavioral effects, even if these effects are indirect (Guastella et al., 2010; Hollander et al., 2003). Oxytocin exhibits bidirectional interactions with the hypothalamic pituitary adrenal (HPA) axis, hypothalamic pituitary gonadal axis, the autonomic nervous system, as well as with other neurotransmitters such as dopamine, and serotonin (Ditzen et al., 2013; Windle et al., 2006; Nikolaeva et al., 2007). Thus, peripheral (plasma) levels of oxytocin may be reflective of a complex interplay between the central nervous system and other peripheral organ systems.

It is also not clear whether G protein expression in peripheral blood mononuclear cells necessarily reflects neurological G protein expression. Activation of G protein cascades is highly cell type and tissue specific, and oxytocin activation of signaling pathways in peripheral tissues not necessarily reflect induction of the same cascades in the hypothalamus (Viero et al., 2010). We note that, in other neurodevelopmental/psychiatric diseases, including depression, bipolar disease, and schizophrenia, G protein levels in PBMCs correlate not only with severity of disease but with response to therapy, (Schreiber and Avissar, 2003; Catapano and Manji, 2007; Gladkevich et al., 2004).

Another limitation of the current study is the wide variability in oxytocin measurement and in expression of Gαq and Gαs mRNA. The variability prevented the establishment of cut-offs that could reliably separate autism and controls. Another possible problem in this study was the lack of correlation between mRNA and protein expression, especially in autism participants. This could be related to a variety of factors, including levels of mRNA and/or protein expression at the lower limits of detection, variations in mRNA or protein degradation or stability during processing. On the other hand, our data are consistent with an actual biological abnormality in production, degradation, or stability of mRNA or of protein in autism.

The finding of higher levels of Gαs mRNA may offer another clue to the possibility of epigenetic or imprinting abnormalities in autism. The gene encoding Gαs (GNAS) is one of the best characterized imprinted genes. Its allele-specific and tissue-specific expression is controlled by epigenetics (Weinstein et al., 2002, 2007). Investigators have speculated that epigenetics may play a role in autism (Schanen, 2006; Jones et al., 2008). "Imprinting" abnormalities

are well characterized in a number of syndromes associated with autism, including Rett, Fragile X, Prader-Willi, Angelman, and Beckwith-Wiedemann syndromes (Schanen, 2006; LaSalle, 2007; Eggermann, 2009). The gene mutated in Rett syndrome is methyl-CpG-binding protein 2 (MeCP2), a key mediator in DNA methylation leading to "silencing" of gene expression (LaSalle, 2007).

Recently, a more direct link among epigenetics, autism, and oxytocin was established in a study demonstrating hypermethylation of CpG islands of the promoter region of the oxytocin receptor gene (OTR), which would be expected to be associated with gene silencing (Gregory et al., 2009). Our finding of increased oxytocin levels and the finding of increased G protein subunit expression could both be considered possible compensatory mechanisms for a transcriptional downregulation at the level of and downstream of the oxytocin receptor gene.

In conclusion, this is the first report describing both alterations in plasma oxytocin levels and a transcriptional upregulation of G-proteins and c-fos in autism. Studies linking neurotransmitters and their signaling pathways may help in identifying subgroups of children with autism, and perhaps can clarify pathophysiology. Further studies of neurotransmitters and their signaling pathways should be conducted in larger, well described samples of children with autism.

Role of funding sources

This work was supported in part by a Pilot Grant Award (to KAE) from Cure Autism Now (now merged with Autism Speaks); by the University of Kansas Medical Center GCRC grant M01 RR 023940 NCRR/NIH, the Kansas Intellectual and Developmental Disabilities Research Center (NICHD; 5 P30 HD002528), by funding from the Kansas Technology Enterprise Corporation (KTEC; JDJ and KAE, Co-PIs), and by a grant from Kansas Center for Autism Research and Training (K-CART; JDJ and KAE, Co-PIs). The funding sources did not critique the manuscript.

Conflict of interest

Based on this work, KAE and JDJ have received support through the Kansas Technology Enterprise Corporation and Children's Mercy Hospital and filed a patent (PCT/US09/40282). KAK, KKF, TRJ, CWC, CMS, RMR, and SAS report no biomedical financial interests or conflicts of interest.

Acknowledgements

We thank Judith Miles (Thompson Center, University of Missouri, Columbia) for consultation on the dysmorphology examination, and Nancy Muma (University of Kansas) and Minati Dhar (University of Missouri Kansas City) for technical assistance.

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