



Variant in oxytocin receptor gene is associated with amygdala volume

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Summary The oxytocin system plays a significant role in modulating stress responses in animals and humans; perturbations in this system may contribute to the pathogenesis of psychiatric disorder. Attempts to identify clinically relevant genetic variants in the oxytocin system have yielded associations between polymorphisms of the oxytocin receptor (OXTR) gene and both autism and major depression. To date, however, little is known about how such variants affect brain structures implicated in these disorders. Applying a manual tracing procedure to high-resolution structural magnetic resonance images, amygdala volumes were measured in 51 girls genotyped on OXTR rs2254298(G>A), a single nucleotide polymorphism associated with psychopathology. Results of this study indicate that despite having greater gray matter volume, participants homozygous for the G allele were characterized by smaller volumes of both left and right amygdala than were carriers of the A allele. A subsequent whole-brain voxel-based morphometry analysis revealed additional genotype-mediated volumetric group differences in the posterior brain stem and dorsomedial anterior cingulate cortex. These findings highlight one neurobiological pathway by which oxytocin gene variants may increase risk for psychopathology. Further research is needed to characterize the mechanism by which this polymorphism contributes to anatomical variability and to identify functional correlates of these alterations in regional brain volume.

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1. Introduction

The neuropeptide oxytocin has received considerable attention for its role in modulating stress reactivity and

emotionality. The oxytocin system regulates hypothalamic–pituitary–adrenal (HPA) axis functioning. Research with rodents, for example, indicates that oxytocin buffers both the behavioral and corticosteroid responses to acute stress (e.g., [Windle et al., 1997](#)). In humans, oxytocin is posited to blunt stress reactivity by decreasing the amount of adrenocorticotrophin (ACTH) released in response to corticotrophin releasing hormone (CRH; [Page et al., 1990](#)), thereby diminishing cortisol secretion. In addition, brain oxytocin may directly modulate neural dynamics within the amygdala, a site of high oxytocin receptor concentration ([Veinante and Freund-Mercier, 1997](#)). [Ebner et al. \(2004\)](#) demonstrated that

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levels of oxytocin are increased in the rat amygdala under conditions of emotional and physical stress. Upon release, oxytocin is posited to bind to functional receptors within the central nucleus, enhancing GABAergic transmission and leading to the inhibition of amygdalar output to the hypothalamus and brainstem (Huber et al., 2005), regions critical to the generation of stress and fear responses. Indeed, several functional neuroimaging studies with humans have now demonstrated modulation of amygdala reactivity *in vivo*. Specifically, intranasal administration of oxytocin has been shown to attenuate ordinarily robust amygdala responses to emotional faces and threatening scenes (e.g., Kirsch et al., 2005). Moreover, consistent with the formulation that oxytocin mediates decreases in amygdalar output to brainstem regions, Kirsch et al. (2005) demonstrated that the observed reduction in amygdala reactivity was accompanied by a corresponding decrease in the functional coupling of the amygdala and the brainstem.

Considered collectively, these findings raise the possibility that variations or perturbations within the oxytocin system are involved in the pathogenesis of psychopathology. In fact, there is increasing interest in identifying variants in the genes that code for oxytocin and its receptor, particularly in the context of elucidating heritable risk factors for psychiatric disorders. Among the single nucleotide polymorphisms (SNPs) that have been associated with autism, the G-allele of the rs2254298 polymorphism of the oxytocin receptor [OXTR] gene (located on chromosome 3p25.3) appears to be over-transmitted in certain autistic populations (Jacob et al., 2007) and central to several haplotypes associated with risk for autism (Lerer et al., 2007). Moreover, Costa et al. (2009) have documented an increased frequency of the G allele in individuals diagnosed with unipolar depression.

Given the neuromodulatory functions of oxytocin in the amygdala and the associations between SNPs of the OXTR gene and autism and depression, it is noteworthy that abnormal amygdala structure has been implicated in the pathophysiology of both of these disorders. For example, in a recent meta-analysis, Hamilton et al. (2008) concluded that unmedicated major depression is consistently associated with decreased amygdala volume; several investigators have also reported decreased amygdala volume in adolescents and adults with autism (e.g., Nacewicz et al., 2006). Nacewicz et al. (2006) further describe an association between smaller amygdala volume and social impairment in autistic individuals, supporting the hypothesis that variation in amygdala volume has significant behavioral correlates, if not consequences.

Although researchers are beginning to elucidate the nature of the interaction of oxytocin and amygdala function, we know little about how genetic variation in the oxytocin system may affect amygdala structure. Given the role of oxytocin in diminishing levels of corticosteroids, which with chronic exposure may lead to atrophy of amygdala dendrites (Mitra and Sapolsky, 2008), oxytocin receptor gene polymorphisms may affect amygdala volume beginning at a relatively young age. The present study was designed to explore this formulation. We recruited a healthy adolescent sample, one likely to exhibit the structural brain phenotypes consistent with risk for psychopathology without having yet experienced the onset of a disorder. Because amygdala volume has been shown to increase throughout adolescence in males but to remain stable in females (Giedd et al., 1996), we

restricted our sample to young females. Using a manual tracing procedure, we examined the influence of the (G>A) polymorphism on amygdala volume in this sample. Given that the OXTR rs2254298 G allele has been associated with disorders characterized by decreased amygdala volumes, and that diminished oxytocin system activity may adversely affect amygdala neurons by altering levels of circulating corticosteroids, we hypothesized that participants who carry two copies of the G allele would have smaller amygdalae than would A-allele carriers. In addition, we conducted voxel-based morphometry (VBM) to explore whether the OXTR rs2254298 genotype affects the volume of other regions of the brain that have been found to be associated with stress, fear, and anxiety.

2. Methods

2.1. Sample

Participants were 51 girls between the ages of 10 and 15, recruited with their mothers through advertisements in the local community as part of a larger study on the intergenerational transmission of psychopathology. Given potential interactions between gonadal steroid hormones and oxytocin actions, we obtained self-reports of menarcheal status from the girls. All participants gave informed assent in accordance with the Stanford University Institutional Review Board.

2.2. Assessment of psychopathology

Initial telephone interviews with the participants' mothers were used to exclude girls with learning disabilities or behavior or conduct problems, frequent correlates of autism and related disorders. Trained interviewers assessed the diagnostic status of the girls by administering the Kiddie Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime version (K-SADS-PL; Kaufman et al., 1997). Any girl who received a current or past psychiatric diagnosis was excluded from the study. Nevertheless, to assess possible group differences in subclinical depressive symptomatology, participants completed the short form (10-item) of the Children's Depression Inventory (CDI-5; Kovacs, 1985).

2.3. Genotyping

Participants were genotyped from saliva collected using the Oragene Kit (DNA Genotek Inc.; Ontario, Canada). Amplification of OXTR rs2254298 was performed with the primers 5'-TGA AAG CAG AGG TTG TGT GGA CAG G-3' and the antisense primer 5'-AAC GCC CAC CCC AGT TTC TTC-3' under standard conditions. PCR products were electrophoresed through 10% polyacrylamide gel at 150 V for 40 min. A 10 bp marker was used to measure the fragments' size. The A allele yields 164 bp, 136 bp and 8 bp fragments, whereas the G allele yields 164 bp, 101 bp, 34 and 8 bp fragments.

2.4. MR image acquisition and processing

Anatomical images were obtained on a 1.5T GE scanner using a T1-weighted SPGR sequence (TR = 8.924 ms; TE = 1.792 ms;

flip angle = 15°, in-plane resolution = 0.859 mm², slice thickness = 1.5 mm). Using Statistic Parametric Mapping (SPM8) software (Wellcome Department of Imaging Neuroscience, London, UK; www.fil.ion.ucl.ac.uk/spm), images were checked for scanner artifacts and anatomical anomalies, and manually reoriented in consultation with a canonical template image.

2.5. Amygdala tracing procedure

Images were resampled to 1.0 mm isotropic voxels prior to tracing. Manual tracings of the left and right amygdala were performed in reoriented native space using the Insight Toolkit's SNAP program (www.itksnap.org). Amygdala boundaries were identified consistent with specifications outlined by Sheline et al. (1998). Once initial boundaries were defined, minor adjustments were made in each of the three views to account for individual differences in anatomy. All amygdalae were traced by a single experienced rater (DF) who was blind to participant genotype at the time of tracing. Ten amygdalae were retraced by an independent trained rater to assess inter-rater reliability (intraclass correlation, $r = .83$). Final volumes obtained from ITK-SNAP were divided by total brain volume (gray matter + white matter volume, extracted using image segmentation in SPM8) to yield adjusted amygdala volumes, a technique used to control for differences in head size.

2.6. Statistical analysis

Between-group comparisons of gray and white matter volumes were conducted using independent samples t -tests. Across the entire sample, raw right amygdala volume was correlated significantly with both age ($r = 0.33$, $p = 0.02$) and CDI-S score ($r = 0.26$, $p = 0.06$); therefore, we controlled for these variables in all subsequent analyses. Separate univariate analyses of covariance (ANCOVAs) were conducted to assess the effect of genotype on raw and adjusted amygdala volumes.

2.7. Voxel-based morphometry (VBM)

After volumes were manually reoriented, gray and white matter segmentations were obtained using a version of the unified segmentation in SPM8, described by Ashburner and

Friston (2005). A diffeomorphic image registration technique (DARTEL; Ashburner, 2007) was then used to iteratively build group tissue class templates and estimate the non-linear deformations that best align the individual segmentations to them. Jacobian modulated warped gray and white matter segmented images, produced using the DARTEL tool suite, were smoothed with a Gaussian kernel of 8 mm FWHM. Resulting image voxels measured 1.5 mm³.

Statistics were conducted on smoothed, modulated gray matter segmentations with participants' age and total brain volume entered as covariates. The threshold for resultant t -statistic maps was set at $p < 0.001$, uncorrected. Statistical parametric maps were subsequently corrected for family-wise error ($p < 0.05$) and non-stationary cluster extent, a correction used to account for inhomogeneities in data smoothness (Gaser, <http://dbm.neuro.uni-jena.de/vbm/non-stationary-cluster-extent-correction>).

3. Results

3.1. Participant characteristics

Participant characteristics are presented in Table 1. The sample did not contain any participants with the A/A genotype. Genotype frequencies were in Hardy–Weinberg equilibrium, $\chi^2(2) = 1.76$, $p > 0.05$. There were no differences between the two genotype groups in either age ($t[49] = .165$) or CDI-S scores ($t[49] = .972$), both $ps > 0.05$. Seven participants did not provide data on menarcheal status; the data for the remaining participants yielded no significant group difference ($\chi^2[1] = .39$).

3.2. White and gray matter volume

The two groups did not differ in total white matter volume ($t[49] = 1.59$, $p > 0.05$); however, G-allele homozygotes had greater total gray matter volume than did heterozygotes ($t[49] = 2.70$, $p = 0.01$) (see Table 1).

3.3. Amygdala volume

ANCOVAs yielded a significant effect of OXTR rs2254298 genotype for both raw (R : $F[1,47] = 4.67$, $p = 0.036$; L :

Table 1 Demographic and volumetric data.

OXTR rs2254298 genotype	G/G ($n = 35$)		G/A ($n = 16$)		p
	Mean	S.D.	Mean	S.D.	
Age	13.0	1.5	12.9	1.5	0.87
CDI-S	1.9	2.3	1.3	1.3	0.34
Gray matter volume (cm ³)	826.4	48.4	787.3	46.9	0.01
White matter volume (cm ³)	408.4	39.2	390.2	35.5	0.12
Left amygdala volume (raw, mm ³)	1496	167	1584	140	0.040 ^a
Right amygdala volume (raw, mm ³)	1559	160	1642	154	0.036 ^a
Left amygdala volume (adjusted by total brain volume)	1.22	0.16	1.34	0.13	0.003 ^a
Right amygdala volume (adjusted by total brain volume)	1.27	0.15	1.40	0.15	0.001 ^a
% Post-menarcheal	43.75		33.33		0.53

^a Controlling for age, CDI-S score.

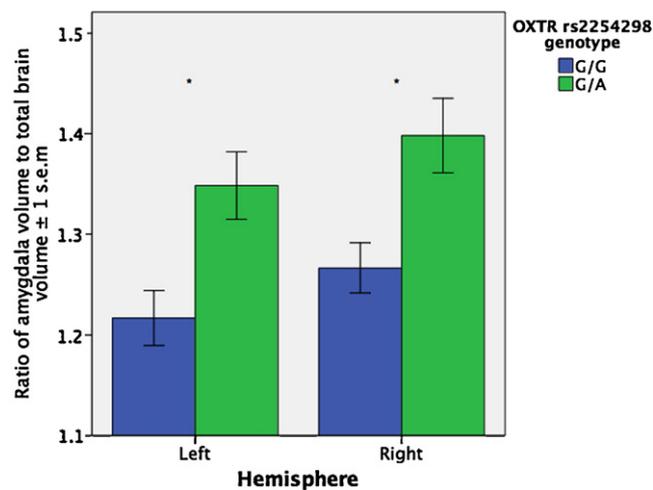


Figure 1 Differences in left and right adjusted amygdala volumes as a function of OXTR rs2254298 genotype. Note: * = Group difference significant at $p < 0.005$.

$F[1,47] = 4.45, p = 0.040$) and adjusted ($R: F[1,47] = 11.59, p = 0.001; L: F[1,47] = 9.67, p = 0.003$) amygdala volumes (see Table 1). Moreover, genotype remained a significant predictor of amygdala volume when gray matter volume was entered into the model as an additional covariate (raw $R: F[1,47] = 6.04, p = 0.018$; raw $L: F[1,47] = 4.23, p = 0.046$; adjusted $R: F[1,47] = 5.31, p = 0.026$; adjusted $L: F[1,47] = 3.66, p = 0.06$). For both hemispheres, G-allele homozygotes had smaller amygdala volumes than did heterozygotes (Fig. 1).

3.4. Exploratory analyses

Because none of the group differences obtained using VBM withstood the conservative family-wise error correction, we present results threshold at $p < 0.001$, corrected at the cluster level for non-stationarity (Fig. 2). Between-genotype comparison of the modulated, smoothed gray matter seg-

mentations revealed increased volume in a region of dorsomedial anterior cingulate cortex (ACC) in G-allele homozygotes (peak $t = 4.93$; MNI $x,y,z = -4, 23, 37$; 491 voxels; non-stationarity adjusted cluster-level $p = 0.032$). Conversely, compared with G-allele homozygotes, G/A heterozygotes had greater volume in the posterior brainstem (peak $t = 4.51$; MNI $x,y,z = -9, -33, -11$; 304 voxels; non-stationarity adjusted cluster-level $p = 0.034$).

4. Discussion

This study provides the first evidence of a neuroanatomical correlate of an oxytocin receptor polymorphism. Using a manual tracing technique, we documented a reduction in total amygdala volume, despite greater overall gray matter volume, in healthy female OXTR rs2254298 G-allele homozygotes relative to G/A heterozygotes. Notably, decreased amygdala volume has also been found to be associated with

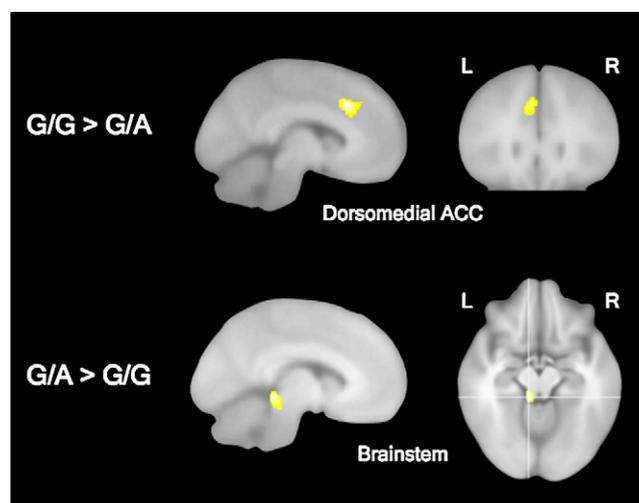


Figure 2 Template sections showing suprathreshold voxels in the comparison of segmented whole brain gray matter estimates of OXTR rs2254298 G/G homozygotes and G/A heterozygotes. Dorsomedial ACC volume was significantly ($p < 0.05$, non-stationarity adjusted cluster-level) greater in G-allele homozygotes, whereas brainstem volume was increased in carriers of the A allele. Notes: OXTR = oxytocin receptor; ACC = anterior cingulate cortex.

both unipolar (e.g., Hamilton et al., 2008) and bipolar (e.g., Kalmar et al., 2009) depression, as well as with autism, though results in this disorder have been mixed (Howard et al., 2000; Nacewicz et al., 2006). Further, the present finding is consistent with documented increases in total gray matter volume in adolescent males with autism spectrum disorder (Hazlett et al., 2006).

Although the relation between the structure and function of the amygdala is not fully understood, mounting evidence suggests that increased size of neuroanatomical structures does not invariably predict increased neural activity; in fact, smaller amygdala volume has been found to be associated with increased neural activation to emotionally valenced stimuli in depressed individuals (Kalmar et al., 2009). Further research is needed to relate the current finding of smaller amygdala volumes in G-allele homozygotes to alterations in metabolism and reactivity. In particular, if the volumetric reductions that characterize G-allele homozygotes are also associated with increased amygdala reactivity, this reactivity may constitute one pathway by which OXTR polymorphisms confer risk for psychopathology. Thus, examining amygdala reactivity in these individuals would allow us to gain a better understanding of the dynamics relating genes, anatomical structure, and neural functioning.

The effect of the OXTR polymorphism on amygdala volume may be mediated, in part, by exposure to corticosteroids. Brown et al. (2007) reported that patients who received at least six months of corticosteroid treatment had smaller amygdalae and, further, that right amygdala volume was inversely correlated with length of treatment. Given that oxytocin is posited to buffer the effects of stress by dampening both HPA-axis and amygdala reactivity, as described above, it is possible that decreased transcription, or sensitivity, of oxytocin receptors leads to greater exposure to cortisol, which in turn affects amygdala volume. Future research could profitably examine whether the effect of OXTR genotype on amygdala volume is mediated by exposure to cumulative stress or cortisol.

Given the documented associations among oxytocin, the amygdala, and the brainstem, it is noteworthy that our VBM analysis revealed decreased volume in a region of caudal midbrain in G-allele homozygotes relative to carriers of the A allele. Interestingly, several lines of research have implicated altered brainstem morphometry in the pathophysiology of autism. Specifically, smaller brainstem volumes have been reported in young autistic children (e.g., Hashimoto et al., 1993). Jou et al. (2008) not only found brainstem volumes to be decreased in a young autistic sample, but also demonstrated that regardless of diagnosis, total brainstem gray matter volume correlated positively with severity of sensory sensitivity, suggesting direct functional consequences of such volumetric reductions.

In our sample we found genotype-mediated differences in a region of brainstem proximal to the periaqueductal gray (PAG). We should note that while the use of a less stringent statistical threshold revealed gray matter differences in more medial regions of the midbrain, the PAG may be of central concern when considering oxytocin-mediated effects on anxiety. This region, implicated in fear behavior and arousal (LeDoux et al., 1988), is the target of heavy reciprocal projections from the central nucleus of the amygdala (Rizvi et al., 1991). Research has demonstrated that activation of the brainstem is signifi-

cantly attenuated following oxytocin administration (Baumgartner et al., 2008), likely through the mediation of activity via these amygdalar projections (Huber et al., 2005). Complementing this indirect influence of oxytocin on brainstem activity, functional oxytocin receptors have been identified within the human central gray, among other brainstem regions (Loup et al., 1989), suggesting that the neuropeptide directly affects brainstem structure and function. Indeed, direct administration of oxytocin into the periaqueductal gray has been shown to attenuate anxiety-related behaviors in post-partum rats (Figueira et al., 2008).

Although we did not have a *priori* hypotheses regarding the effect of rs2254298 genotype on ACC, the finding of increased ACC volume in G-allele homozygotes is interesting for a number of reasons. Investigators have suggested that dorsal ACC is involved in the "felt unpleasantness" of both physical and social pain, perhaps as a consequence of, or precursor to, its role in allocating attentional resources (Eisenberger and Lieberman, 2004). Appropriately, given this formulation, abnormal function or structure in this region has been noted in pediatric anxiety disorder (McClure et al., 2007), obsessive-compulsive disorder (Rosenberg and Keshavan, 1998; Ursu et al., 2003), and disorders along the autism spectrum (Di Martino et al., 2009), each of which has also been associated with the oxytocin system. Importantly, while oxytocin receptor binding has been localized within the rat cingulate cortex, expression in this structure seems to be limited to early development (Tribollet et al., 1989). Although comparable data are not available in humans, it is possible that transient oxytocin actions in the developing brain have lasting and consequential effects, especially when considering genetic variants, present from the earliest stages of development.

We should note four limitations of the current study. First, because our sample was composed exclusively of adolescent females, we could not examine the generalizability of the obtained results to other age groups and to males. Second, the interpretation of our results is limited by the current paucity of information concerning the OXTR rs2254298 polymorphism. It is likely that this SNP is in linkage disequilibrium with one or more unknown functional loci; additional research is required to identify the critical genes that may mediate the observed associations. Third, the group difference in amygdala volume obtained using manual tracing was not corroborated by our VBM analysis. It is likely that our ability to detect differences in amygdala volume using this exploratory procedure was limited by our sample size; studies using VBM that have reported significant group differences in amygdala volume have used larger group sample sizes (e.g., Pezawas et al., 2005). In addition, VBM has been found to be less sensitive than is manual tracing in detecting certain volumetric effects within the amygdala (Good et al., 2002). Finally, given the scanning resolution used in this study, we were not able to localize the observed volumetric differences to particular sub-regions of the amygdala. Nevertheless, we were able to document an overall reduction in amygdala volume in individuals who are homozygous for the OXTR rs2254298 G allele. Scanning protocols targeting the amygdala may be able to localize genotype-mediated volumetric and functional differences to regions of the amygdala known to be particularly dense in OXTR. Examining the effects of this and related polymorphisms on neuroanatomy,

neural function, and HPA-axis activity will increase our understanding of how the oxytocin system affects the stress response and risk for psychopathology.

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Contributors

DJF and IHG designed the study, and DJF and MCC conducted the statistical analyses. DJF wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

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