The Role of the Oxytocin Receptor Gene (OXTR) in Autism Spectrum Disorders (ASD)

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Introduction

Today one out of every 150 people are diagnosed with an ASD. Autism is classified as a pervasive developmental disorder - none other specified (PDD-NOS). Current research has focused on the genetic and genomic factors in ASD (Bakkaloglu, 2008; Buxbaum, 2001). Recent evidence has supported that the oxytocin receptor gene (OXTR) has a influential role in ASD. Oxytocin is a nonopeptide (a nine amino acid peptide) hormone which is known to regulate social behaviors which is why it has become a candidate gene for ASD (Carter, 2006). Research has shown base pair variations known as single nucleotide polymorphisms (SNPs) on the oxytocin receptor gene to be associated with ASD. (Jacob, 2006; Wu, 2004; Yrigollen, 2008).

The objective of this research is to examine the allelic associations between genetic variants (SNPs) and clinical diagnosis in ASD. Probands and their families were made available through Yale Child Study Center; the total sample included 527 participants. Probands and their families were all evaluated with several clinical instruments including: Autism Diagnostic review (ADI), Autism Diagnostic Interview-Revised (ADI-R), Autism Diagnostic Observational Schedule (ADOS), Autism Diagnostic Observational Schedule Generic (ADOS-G), and Vineland Adaptive Behavior Scales. Seven SNPs of the Oxytocin Receptor Gene (OXTR) were chosen to cover the OXTR haplotype blocks. For genotyping purposes samples were sent to the Keck, a core facility of Yale (http://keck.med.yale.edu/); The Illumina bead assay technology was used.
Literature Review

Autism is a neurodevelopmental disorder which affects the main aspects of human behavior. Recent research has revealed that there is a genetic association between OXTR and ASD (Carter, 2006). By genotyping these genes, scientists are slowly beginning to establish an understanding of the biological bases of ASD.

Autism has deficits seen in the areas of social interaction and communication. Social interaction, in part, specifically refers to difficulty in establishing peer relationships. The restricted and repetitive behaviors are also associated with ASD. These include inflexibility to routine as well as preoccupation with inanimate objects. Communication deficits include a delay in spoken language without any other form of communication. There are some cases of ASD where patients are nonverbal, meaning that there is no form of communication; this results in further difficulty with social interaction. ASD subjects also exhibit a lack in peer relationships despite their ability to communicate. This demonstrates the broad spectrum of autism and the different impacts of in these areas (American Psychiatric Association, 2000).

There have been numerous single nucleotide polymorphisms (SNPs) observed in the genotypes of autistic subjects that may be correlated with ASD (Wu et al., 2007). A SNP is defined as a variation in a single base pair that may or may not affect the amino acid for which the gene codes and therefore impacts the protein composed by the amino acid, altering protein function.

Oxytocin is a neuropeptide, specifically a nonapeptide (a nine amino acid peptide) which is synthesized in the paraventricular and supraoptic nucleus and released into the blood stream through axon terminals in the posterior pituitary (Carter, 2006). In Homo
Sapiens, the receptor gene is 17 KB long (Inoue, 1994) The receptor of the oxytocin neuropeptide is a G protein-coupled receptor and is positively coupled with phospholipase C. In mammals oxytocin receptors are expressed at higher levels in early development (Kubota, 1996), and are most prevalent in brain regions that are correlated with social behaviors (Shapiro, 1989). OXTR knockout mice lack ultrasonic vocalizations when socially isolated and exhibit aggressive behaviors when compared to wild type mice (Kubota, 1996).

In 1998, a study observed the plasma oxytocin level in children with autism. It was concluded that children with autism had a lower level of oxytocin in blood when compared to the controls. In addition, lower oxytocin levels were associated with scores. Therefore, the more severe case of autism of the child, the lower the oxytocin plasma level (Modahl, 1998).

Recently a double blind study distributed oxytocin intravenously to 15 adults with ASD. These subjects were then taught certain skills such as intonation comprehension, speech, and social skills. Those who received oxytocin retained the ability they had previously been taught. However, those who received the placebo reverted to base line (Hollander et al., 2007). These results show that if a definite and consistent correlation is found between autism and oxytocin in the future, oxytocin infusions may be a solution to ASD symptoms.

In 2004 Wahl proposed a hypothesis regarding the down regulation of oxytocin mRNA. Wahl hypothesized due to the fact that oxytocin has the ability to cross the blood brain barrier (BBB), when excess oxytocin is administered at birth OT crosses the maternal placenta, downregulating (stopping) mRNA transcription. This may account for
the low levels of plasma oxytocin in children with ASD. This hypothesis has lead to a series of studies genotyping the \textit{OXTR} SNPs in individuals with ASD.

In 2005, Wu et. al genotyped four SNPs on the \textit{OXTR} coding region and found an association between autism and rs2254298 and rs53576 – two specific SNPs on the Oxytocin haploblock. The sample contained individuals diagnosed with ASD of the Chinese Hahn population. Haplotypes constructed with markers revealed a correlation with autism (especially the transmission of rs53576). There was also an over transmission of the G allele and A allele found from parents to offspring. Jacob et al (2007) genotyped the same SNPs within a Caucasian sample. This study concluded that there is an over transmission of the A allele. However, the transmission frequency of the G allele was less than the frequency of the A allele in the Han population. These differences were thought to be a result of the different genetic make up between the populations.

\textbf{Objectives}

This research will focus on seven SNPs on the oxytocin receptor gene to test their correlation with clinical diagnosis of ASD. Association between SNPs occur when there is a non-random association of their alleles due to their proximity on the same chromosome. If there is no association with autism, the SNP will have an equal chance of being transmitted to offspring with ASD (compared to offspring without ASD). If however, there is association, the SNP will be transmitted to individuals with ASD at a higher frequency. DNA from both parents and offspring will be used to test for the transmission of alleles. The allele transmission will determine whether the transmission is nonrandom, therefore indicating whether an association exists. If an allele tends to be
transmitted at a high rate, it would suggest a correlation between the SNP and clinical diagnosis of ASD.

**Hypothesis**

HA= A positive association will appear between clinical diagnosis of ASD and at least one of the SNPs captured in this study. This will be determined if there is calculated $p$ value that is less than .01 for the intensity (presence) of the allele based upon the micro array tests.

H0= None of the seven SNPs captured in this will demonstrate any association with clinical diagnosis of autism. This will be indicated by a calculated $p$ value that is greater than .01 for allele intensity.

**Methods/Materials**

*Participants*

To the proposed hypothesis of OXTR and clinical behaviors, DNA was genotyped from a sample of probands with autism and their families available through the Yale Child Study Center. The sample consisted of 525 participants of which 177 were classified as probands. The participants formed 151 nuclear families. The majority of the sample was Caucasian, The study was approved by the Yale institutional review board (as described in Yrigollen et al, 2008). Some samples were not used as a result of poor or contaminated sample and low DNA or purity yield.

*Genotyping*

(Foster City, California)], was used to identify the seven SNPS captured in this study. The SNPs were chosen to cover a variety of haplotype blocks over the OXTR gene and reveal low minor allele frequencies. DNA was previously extracted from both blood and saliva samples, as well as buccal cells (Yrigollen, 2008). The DNA yield was then concentrated or diluted to 100 nanograms per microliter (plus or minus ten nanograms per microliter).

Invitrogen E-Gels were used to check for samples of low concentration and low molecular weight as well as fragmented or degraded DNA. When quality conditions were satisfied, the samples were run on an Illumina (San Diego, California) micro array using fiber optic bead arrays. The fibers used consist of 50,000 individual fibers fused together into a hexagonally packed matrix and can therefore enclose up to 50,000 beads which are each 3 microns in diameter. Because the assembly of beads into well is a random process the location and the identity of the beads that are included in the array must be decoded after assembly. These arrays are decoded through DNA hybridization techniques. The samples are exposed to a fluorescent marker which has complementary DNA base pairs to those of the SNP sequence. If the sequence is present the fluorescent hybridizes (attaches to) to the SNP revealing the presence of the SNP allele in a specific sample. This ultimately decodes the transmission of SNP allele from parent to offspring.

**Phenotyping**

Probandes were evaluated with several clinical instruments used to diagnose ASD including: Autism Diagnostic review (ADI), Autism Diagnostic Interview- Revised (ADI-R), Autism Diagnostic Observational Schedule (ADOS), Autism Diagnostic Observational Schedule Generic (ADOS-G), and Vineland Adaptive Behavior Scales.
From these clinical instruments a large number of phenotypes were developed. ADI and ADI-R listed “Social Interactions,” “Communication,” “restricted/Repetitive Behaviors,” “onset,” and “ADI-Based Diagnosis”. ADO and ADOS-G listed “Social Skills,” “Communication Skills,” “Stereotyped Behaviors,” and “Imaginative Skills,” and the “ADOS-Based Diagnosis”. The Vineland instrument resulted in “Communication,” “Daily Living Skills,” “Socialization,” and “Motor Skills,” and the “Vineland Composite Score.” (Yrigollen, 2008) Here, however, only the ASD categorical phenotype (i.e., presence or absence of ASD, Clinical Diagnosis) is used.

**Analyses**

For data cleaning and processing, we used R. FBAT (Family Based Association Test) was used (Horvath, Xu, Laird, 2001) for the statistical analyses of genetic association. The analysis was performed under the null hypothesis of “no association in the presence of linkage”; Rejection of the null hypothesis of no association suggests an actual allelic association that goes beyond the conclusion of linkage. The results were consistent with those under the “no association hypothesis”. The empirical $p$ values were calculated.
Results

Figure 1. shows normal genoplots with samples mostly falling in each of the three genotype clusters. Samples represented by black points show a dramatic drop in intensity.
Figure 2. illustrates polar plots that demonstrate the clustering of the genotypes as generated by the seven analyzed SNPS. These plots have been transformed and normalized from the previous genoplots. Each dot represents a transmitted allele which is graphed based on its intensity. Each plot consists of both homozygous and heterozygous alleles.
Figure 1 demonstrates plots of the microarray data genoplots. After the cDNA hybridizes to the SNP, the attached fluorescent will reveal a red, yellow, or green. The red and green color indicate a homozygous allele transmission (a SNP with either GG, AA, or CC). A yellow color is the combination of the red and green alleles, indicating a heterozygous allele transmission (like GA). The heterozygous and homozygous alleles are then visualized as clusters based on intensity. The points on the far right and far left (red and blue) demonstrate homozygous alleles while that in the middle demonstrate heterozygous alleles (purple) and are in expected cluster positions as indicated by ellipses.

Figure 2 demonstrates the micro array cluster results after a transformation to normalize the data. rs1042778 demonstrates both the homozygous alleles of CC and AA, with the presence of the heterozygous allele of CA. rs13087941, rs180789, rs237884, and rs237893 demonstrate homozygous alleles of GG and AA with the heterozygous GA. rs35062132 demonstrates a plot of the homozygous CC along with heterozygous CG. It is interesting to note that there was no presence of an expected homozygous GG. rs4686302 demonstrates the presence of the homozygous allele GG and the heterozygous GA but does not include the presence of AA. A higher intensity indicates a higher presence of the allele (homozygous or heterozygous) in the sample population. $p$ values were calculated based on intensity.

rs4686302 demonstrated homozygous and heterozygous intensities but failed to indicate homozygous (red), indicating that there is no presence of the other homozygous allele. In addition, a fluorescence intensity of 80 was observed in the cluster of heterozygous cluster, a particularly low score indicating little association (see figure 1).
No specific allele indicated a strong fluorescence when the data was normalized, as the cluster was relatively small (see figure 2). rs13087941, revealed all alleles and had a lower fluorescence intensity of 135 for the heterozygous cluster (see figure 1). When the data was normalized the heterozygous cluster was of medium size, with apparent scatter (see figure 2). rs180789 had a fluorescence intensity of 174 for the heterozygous cluster indicating some fluorescence (see figure 1). Its normal polar plot demonstrated a scattered cluster, further demonstrating small intensity (see figure 2).

rs237893 had an extremely high fluorescence intensity of 199 in the heterozygous cluster (see figure 1). The polar plot demonstrated a large cluster, further supporting its high intensity (see figure 2). rs1042778 also demonstrated a high fluorescence intensity of 190 in the heterozygous cluster. However, both the polar plot and the genoplot consist of scatter points along the heterozygous cluster, accounting for its small cluster on the polar plot (see figures 1 and 2). rs237884 demonstrated a fluorescence intensity of 146 in the smaller cluster. Some scatter appeared in addition to a small cluster on the normalized polar plot, indicating low intensity (see figures 1 and 2). rs35062132 demonstrated only one homozygous allele, and lacking any presence of the other (blue) (see figure 1). Interestingly enough, its genoplot indicates and extremely low fluorescence intensity of 24, indicating a low intensity (see figure 1). The polar plot also indicates a small cluster, consistent with the low intensity (see figure 2).

Of the seven investigated SNPs, the FBAT testing revealed significant transmission disequilibrium for rs237893 ($p < .01$, after corrections for multiple comparisons), which showed a consistent statistical association with the categorical phenotype of ASD (Clinical Diagnoses). The remaining six alleles failed to show a
statistically significant intensity. While rs1042778 did not show statistically significant intensities it is important to note its stronger intensity. These results are consistent with the previous findings published elsewhere (Jacob et al., 2007; Wu et al., 2005; Yrigollen et al, 2008) and confirm the presence of statistical associations between the phenotype of ASD and variation in OXTR.

Discussion

Autism might develop as a result of unknown causes of variation. Past literature has supported an association between susceptibility to autism and OXTR (Jacob et al., 2007; Wu et al., 2005; Yrigollen et al, 2008). In this study, seven SNPs –rs4686302, rs13087941, rs180789, rs237893, rs1042778, rs237884, and rs35062132- were investigated to test for an association between clinical diagnosis of autism spectrum disorders and the respective SNPs.

A number of observations are of interest. The extremely low p value for rs237893 demonstrates an association between rs237893 and clinical diagnosis. This suggests that rs237893 has a significant role in the clinical diagnosis of ASD. The association also supports the genetic role of autism, and a hereditability factor, as the microarray analysis took into account the transmission of alleles from parent to offspring. Further analysis regarding allele frequency would be necessary to know the exact rate of heritability.

FBAT testing for rs237893 shows high transmission disequilibrium (excess transmission) of the GA allele. While there is some transmission of the GG and AA allele, however, there is not as much indicated intensity as that of the GA allele which had an extremely high intensity (see figure 2). In addition rs237893 had a fluorescence intensity of 79 and 123 for the respective homozygous clusters. It’s high florescence
intensity of 199 for the heterozygous cluster indicates that the GA allele was transmitted at a high rate from parent to offspring.

The association of rs247893 in clinical diagnosis of ASD contributed to the growing literature supporting the presence of an association between the oxytocin receptor gene and autism. Previously, rs2268493 of the OXTR gene showed association with stereotyped behaviors, diagnosis, and restricted/ repetitive behaviors in ASD (Yrigollen, 2008). Other studies demonstrated association between SNPs on OXTR haploblocks and autism. rs2254298 and rs53576 have also been associated with autism (Jacob, 2007) (Wu, 2005). The association between clinical diagnosis, suggests that the OXTR gene is not only associated with affiliative behaviors of ASD but with clinical diagnosis as well.

Within the study, some other interesting results should be taken into account. First off, both rs4686302 and rs35062132 have no apparent transmission of one homozygous allele. This suggests that the homozygous allele is not transmitted from parent to offspring in ASD subjects. However, a larger study would have to be conducted in order to confirm this observation.

In addition, it is difficult to rule out the possibility of rs1042778 having a role in clinical diagnosis of ASD. Although it did not meet a p value of significance (p < .01), it did have an extremely high florescent intensity or 190 within its heterozygous cluster. This value is extremely close to that of rs237893 which had a florescent intensity of 199. These values are extremely close. It is possible that a study with more participants would have found an association between rs1042778 and clinical diagnosis of ASD. A possible
future study may test for an association between rs1042778 and clinical diagnosis of ASD.

For future research should confirm these findings. A study with a larger sample size may create more accurate findings and confirm any experimental error. Future studies may also address the role of other genes that share a genetic or neurobiological pathway with the oxytocin ligand and its receptor gene, OXTR. Other neurobiological studies suggest vasopressin plays a similar role in autism as oxytocin. Genes of the AVP system have therefore also become a candidate genes for autism research. Given the role of oxytocin in the AVP systems, future research should evaluate the genetic relationships and a possibility in autism (Wu, 2005).

Another interesting factor to consider is rs237893 location on OXTR. rs237893 is located on an intron, meaning that it is cut out from the gene during splicing. Although rs237893 is located in a non-coding region, a genetic contribution of this SNP to ASD cannot be ruled out. rs237893 may be in linkage disequilibrium with an unknown locus for autism (Wu, 2005).

These results provide more evidence to the growing association between allelic variants on OXTR and autism. Not only does the data contribute to the growing number of literatures on ASD but adds to the scientific understanding of a genetic disposition to ASD. However, careful and detailed observations in combination with future research should contribute to the comprehension of ASD and a potential treatment or approach.
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