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ORIGINAL ARTICLE

Association between oxytocin receptor gene polymorphisms and autistic traits as measured by the Autism-Spectrum Quotient in a non-clinical Japanese population

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Abstract

Introduction: Oxytocin is known to facilitate human behavior and social cognition related to autism. Subclinical autistic traits are continuously distributed in the general population. The aim of this study was to explore the association between oxytocin receptor gene (*OXTR*) polymorphisms and autistic traits.

Methods: Participants consisted of 440 healthy Japanese (272 males and 168 females; mean age 40.9 ± 9.7 years). Participants completed the Japanese version of the Autism-Spectrum Quotient (AQ) and donated a whole blood sample. Fifteen *OXTR* single nucleotide polymorphisms (SNPs) were genotyped using TaqMan or by direct sequencing. Single SNP linear regression analysis, permuted 10,000 times, and haplotype linear regression analysis, were conducted for the AQ and its subscale scores.

Results: Three SNPs – rs2268490, rs2301261, and rs1042778 – were excluded from analysis, as the genotype distributions of rs2268490 were not in Hardy-Weinberg equilibrium, and there were less than 10 minor homozygous participants for rs2301261 and rs1042778. This resulted in 12 SNPs being tested. rs62243370, rs62243369, rs2254298, and rs2268491 were associated with *attention switching* subscale in females by the single SNP analysis. However, after performing Bonferroni corrections, statistical significances were eliminated. The *attention switching* subscale was associated with a specific haplotype, comprising rs62243370, rs62243369, rs13316193, rs2254298, rs2268493, and rs2268491 (GGTGTC, corrected $P = 0.0016$) in females.

Discussion: The present study demonstrated a significant association between a specific *OXTR* haplotype and the autistic trait of "strong focus of attention" as measured by the AQ in a non-clinical female Japanese sample.

Introduction

The neuropeptide oxytocin (OXT) is a nine-amino-acid peptide that acts as a hormone and neurotransmitter; it is synthesized in the hypothalamus and released into the bloodstream and the synaptic cleft (Uvnäs-Moberg, 2003). OXT facilitates several reproductive processes: it has an effect on uterine contraction and lactation in the peripheries. It regulates parental attachment behaviors (Bartels & Zeki, 2004; Prichard *et al.*, 2007) and social interactions (Uvnäs-Moberg, 1998; Kosfeld *et al.*, 2005; Donaldson &

Young, 2008) as the neurotransmitter. The impairment of social interaction is a central symptom in autism.

The oxytocin receptor gene (*OXTR*) comprises 19.2 kilobases (kb) on chromosome 3p25, and has been identified as one of the putative regions linked to autism (McCauley *et al.*, 2005; Lauritsen *et al.*, 2006). Several studies have found evidence for the association of *OXTR* polymorphisms and autism. Wu *et al.* (2005) investigated four single nucleotide polymorphisms (SNPs) in *OXTR* in Han-Chinese autism spectrum disorder (ASD) trios, and found rs53576 and rs2254298 were significantly associated. Jacob *et al.* (2007) investigated the same two SNPs in a Caucasian sample, and found an association of rs2254298. Lerer *et al.* (2008) found associations of rs1042778 and a haplotype including rs2254298 with ASD in a Caucasian sample. Yrigollen *et al.* (2008) found that rs2268493 was associated with ASD in a Caucasian sample. Liu *et al.* (2010) investigated 11 SNPs in a Japanese sample, and found associations of rs2268491, rs2254298 and a haplotype including rs2254298 with ASD.

Autism is a neuropsychiatric developmental disorder characterized by a triad of features: (i) difficulties with social interaction; (ii) difficulties with communication; and (iii) the presence of restricted, repetitive and stereotyped patterns of behavior, interests and activities (American Psychiatric Association, 2000). The population prevalence is known to be approximately 40 per 10,000 for autism, and over 100 per 10,000 for ASD, with the male-to-female ratio reported as 4:1 (Baird *et al.*, 2006). It is a highly heritable disorder (Bailey *et al.*, 1995; Pickles *et al.*, 1995), with heritability estimated to be above 90% (Rutter, 2000), although the exact mode of transmission is not known.

Autism manifests along a spectrum (Wing, 1997). ASD encompasses the milder variants, including Asperger's Disorder and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS) (American Academy of Pediatrics, 2001; Johnson *et al.*, 2007). Subclinical autistic traits are continuously distributed in the general population (Baron-Cohen *et al.*, 2001; Constantino & Todd, 2003) as well as in relatives of individuals with autism designated the broader autism phenotype (Bolton *et al.*, 1994; Bailey *et al.*, 1995, 1998; Le Couteur *et al.*, 1996; Piven *et al.*, 1997). The heritability of autistic traits has been demonstrated in the general population (Bishop *et al.*, 2004; Constantino & Todd, 2005; Constantino *et al.*, 2006).

The Autism-Spectrum Quotient (AQ) was developed to evaluate autistic traits (Baron-Cohen *et al.* 2001). It is a self-administered instrument for measuring the degree to which an adult with normal intelligence has certain traits. It consists of 50 statements assessing personal preferences and habits, with a forced-choice format of "definitely agree", "slightly agree", "slightly disagree", and "definitely disagree". Five subscales, comprising 10 questions each, assess five domains associated with the triad of autistic features noted above (Rutter, 1978; Wing & Gould, 1979; American Psychiatric Association, 2000) as well as demonstrated areas of cognitive abnormality in autism. The five domains assessed are *social skill, attention switching, attention to detail, communication,* and *imagination.* AQ scores range from 0 to 50 points with higher scores indicating a greater degree of autistic traits.

OXTR has many polymorphic sites which have been reported to be associated with autism; autistic traits are also known to show heritability, and are observed both in relatives of individuals with autism and in the general population. We therefore postulate that autistic traits may show an association with *OXTR* polymorphisms in the general population. Thus far, one study examined four *OXTR* SNPs, and found no association with autistic traits as measured by the AQ in a Caucasian sample (Chakrabarti *et al.*, 2009). The present study explored associations among a greater number of *OXTR* SNPs and autistic traits in a non-clinical Japanese population. Haplotypes as well as single SNPs were studied.

Methods

Participants

All participants were recruited in 2008 in Kanagawa Prefecture, adjacent to Tokyo, Japan. They comprised 603 genetically unrelated, non-clinical Japanese white-collar workers in a large corporation, representing a high functioning non-clinical adult population. Participants completed the Japanese version of the AQ (Kurita *et al.*, 2003, 2005), and trained clinicians conducted a short structured diagnostic interview, as per the Mini-International Neuropsychiatric Interview (Sheehan *et al.*, 1998), to confirm lifetime diagnoses of affective, anxiety, and psychotic disorders according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) (American Psychiatric Association, 2000). Of the 603 participants, 498 donated a whole blood sample for DNA analysis. Of these 498, five participants were excluded from the study due to ethnic differences (four Chinese and one Russian), and 53 were excluded due to current or past DSM-IV diagnoses: 30 had been diagnosed with major

depressive disorder, 17 with bipolar II disorder, one with dysthymic disorder, one with panic disorder without agoraphobia, two with panic disorder with agoraphobia, and two with agoraphobia without history of panic disorder.

After the above exclusions, the participants of this study comprised 440 high-functioning Japanese (272 males and 168 females). The mean age of participants was 40.9 years (SD = 9.7), with an age range of 23–63 years. Mean ages in males and females were 41.6 (SD = 10.2) and 39.9 (SD = 8.8) years, respectively, with an age range for males of 24–63 years, while for females the range was 23–60 years. No significant differences were observed in demographic data, AQ scores, or AQ subscale scores between the 440 participants and the 163 that were excluded, except for lower scores for *attention switching* ($t = -2.75$, d.f. = 601, $P < 0.01$) and *communication* ($t = -2.24$, d.f. = 601, $P < 0.05$) domains in participants compared to those excluded.

The aim of the present study was clearly explained to all participants, and written informed consent was obtained. The study was approved by the Ethics Committee of the Faculty of Medicine at the University of Tokyo, and conformed to the provisions of the Declaration of Helsinki.

Measurement

The Japanese version of the AQ (Kurita *et al.* 2005) was used to measure autistic traits. The reliability and validity of this version has been confirmed: it demonstrated good reliability (Cronbach's $\alpha = 0.80$ for internal consistency, $r = 0.92$ and mean κ [SD, range] = 0.57 [0.14, 0.28–0.87] for test-retest reliability) and construct validity (Kurita *et al.*, 2003). Subscale scores were calculated by scoring each "slightly agree" and "definitely agree" response as 1, and each "slightly disagree" and "definitely disagree" response as 0. The AQ includes 26 reversed questions, in which a "disagree" response was characteristic for autism. For reversed questions, subscale scores were calculated by scoring each "slightly agree" and "definitely agree" response as 0, and each "slightly disagree" and "definitely disagree" response as 1. Total AQ score was calculated by summing the five subscale scores.

Single nucleotide polymorphism selection and genotyping

Genomic DNA was isolated from leukocytes in whole blood using a Wizard genomic DNA purification kit (Promega Corp., Madison, 2005).

Based on genotype data from the International HapMap Project (International HapMap Consortium, 2003), Haploview 4.2 (Barrett *et al.*, 2005) generated four haplotype blocks plotting 25 tag SNPs with minor allele frequency (mAF) > 5% in *OXTR*. To cover all blocks, seven SNPs whose TaqMan PCR primer and probe sets were available from Assays-On-Demand (http://www3.appliedbiosystems.com) were selected from the 25 SNPs. In addition to these seven SNPs, four SNPs (rs1042778, rs2268493, rs2254298, and rs53576) were selected because they had been reported to be associated with ASD in previous studies (Wu *et al.*, 2005; Jacob *et al.*, 2007; Lerer *et al.* 2008; Yrigollen *et al.*, 2008; Liu *et al.*, 2010). Thus, a total of 11 tag SNPs were selected: rs2301261, rs2268495, rs53576, rs2254298, rs2268493, rs2268491, rs918316, rs2268490, rs237887, rs237885, and rs1042778. These were genotyped using the TaqMan genotyping platform in accordance with the manufacturer's protocol.

Direct sequencing was performed in an 800 b 5′-flanking region of rs2254298, as this SNP had been consistently reported to be associated with autism (Wu *et al.*, 2005; Jacob *et al.*, 2007; Lerer *et al.*, 2008; Liu *et al.*, 2010). The primers were as follows: forward 5′-AGCAGAAACTGTGGGTGTCC-3′ and reverse 5′-CTCTCATCCTCCCTGTGTCC-3′. Sequencing was performed from both 5′ and 3′ ends using the ABI PRISM 3730 Genetic Analyzer in accordance with the manufacturer's protocol (http://www3.appliedbiosystems.com). Four SNPs were genotyped by direct sequencing: rs11131149, rs62243370, rs62243369, and rs13316193. Therefore, a total of 15 SNPs were genotyped in this study (rs2301261, rs2268495, rs53576, rs11131149, rs62243370, rs62243369, rs13316193, rs2254298, rs2268493, rs2268491, rs918316, rs2268490, rs237887, rs237885, and rs1042778).

Statistical analysis

First, distributions of AQ scores by sex were examined by histogram. Gender differences in mean AQ scores were assessed by *t*-test.

Second, the Hardy-Weinberg equilibrium (HWE) for genotype distributions was assessed by χ^2 test using Haploview 4.2.

Third, for single SNP-based quantitative trait association analysis, linear regression analyses by single markers were performed for AQ scores with PLINK version 1.07 (Purcell *et al.*, 2007). To reduce the number of multiple tests, an additive model for minor alleles was conducted and other models were not con-

ducted. Family-wise (SNP-wise) corrected empirical *P*-values were calculated on the basis of 10,000 permutations (Good, 2000) under control of the family-wise error rate (FWER) for each SNP examined (Hochberg and Tamhane, 1987).

Finally, linkage disequilibrium (LD) and haplotype were analyzed (Lewontin, 1964). Haplotype block analysis was conducted using the Gabriel method (Gabriel *et al.*, 2002). Haploview was used to obtain the LD. PLINK was used to implement haplotype-based quantitative trait association analysis for AQ scores. Haplotype distributions for each participant were inferred probabilistically using the standard Expectation-Maximization algorithm (Dempster *et al.* 1977). Corrected *P*-values were calculated using the Bonferroni method, as neither Haploview nor PLINK are able to perform permutation tests for haplotype-based quantitative trait association analysis.

The coefficient of determination (R^2) was obtained through regression analysis. R^2 expresses the contribution ratio, and represents the effect size in regression analysis (Cohen, 1988; Field, 2005). R^2 was categorized approximately as small ($0.01 \leq R^2 < 0.1$), medium ($0.1 \leq R^2 < 0.3$), or large ($0.3 \leq R^2$). Statistical power was estimated using QUANTO version 1.2.4 (Gauderman, 2002). Statistical analyses of mean values and SD, χ^2-tests and *t*-tests for demographic data were conducted with SPSS 16.0.2J for Windows (SPSS, Chicago, 2007). Statistical tests were two-tailed and the significance level was set at $P < 0.05$.

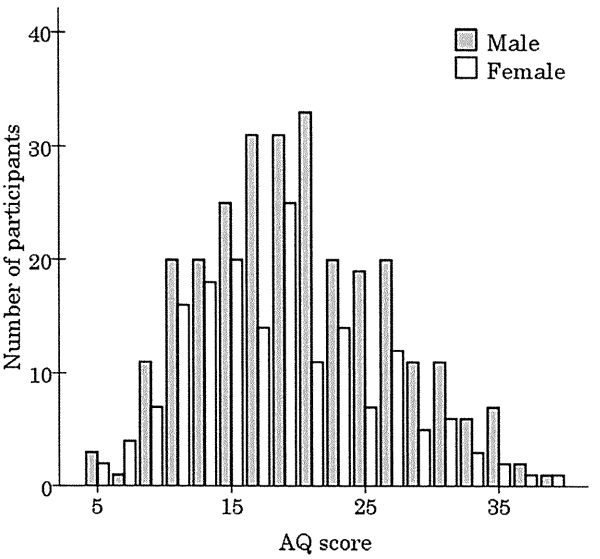

Figure 1 Autism-Spectrum Quotient (AQ) histograms by sex.

Table 1. Mean Autism-Spectrum Quotient (AQ) and subscale scores by sex

	All (n = 440)		Male (n = 272)		Female (n = 168)	
Scale/subscale	Mean	SD	Mean	SD	Mean	SD
AQ	19.1	7.0	19.6	6.9	18.3	6.9
Social skill	4.5	2.8	4.5	2.8	4.4	2.7
Attention switching	4.1	2.0	4.3	2.0	3.8	2.0
Attention to detail	3.8	2.3	3.7	2.2	4.0	2.3
Communication	2.9	2.2	3.0	3.3	2.8	2.2
Imagination	3.8	1.7	4.1	1.7	3.3	1.7

Results

Figure 1 shows histograms of total AQ scores by sex. Total AQ scores were continuously distributed in both males and females. Mean scores for the AQ and its five subscales are presented in Table 1. While the difference in the mean total AQ score between males and females did not reach the level of statistical significance, mean scores of males were significantly higher than those of females for the subscales of *attention switching* ($t = 2.23$, d.f. = 438, $P < 0.05$) and *imagination* ($t = 4.90$, d.f. = 438, $P < 0.001$).

All 15 SNPs are listed in Table 2. All the SNPs had genotyping call rates of >0.95, and concordance for a duplicate sample was >0.99. The genotype distribution of SNP rs2268490 significantly deviated from HWE ($P = 0.009$), and there were less than 10 minor homozygous participants for rs2301261 and rs1042778. Therefore, these three SNPs were excluded from all subsequent analyses, and the 12 remaining SNPs were tested.

Based on single SNP linear regression analysis, the *attention switching* subscale showed a SNP-wise empirically significant association with single SNPs (Table 3). However, after making Bonferroni corrections for each subscale and gender subgroup, none of these SNPs exhibited significant associations. None of the AQ scale, other subscales, or other SNPs showed SNP-wise empirically significant associations (Supporting Table S1).

Figure 2 shows Pairwise LD results for the 12 nonexcluded SNPs. A two-haplotype-block structure was found. Block 1 was 2 kb-long and consisted of six SNPs: rs62243370, rs62243369, rs13316193, rs2254298, rs2268493, and rs2268491. Block 2 was 1 kb-long and consisted of two SNPs: rs237887 and rs237885. Block 1 included four specific haplotypes, and block 2 included three, with frequencies > 0.01 (Table 4). Total frequencies of the four haplotypes in block 1, and three haplotypes in block 2, were estimated to be 100.0% and 98.5%, respectively. Based on haplotype linear regression analyses with Bonfer-

Table 2. *OXTR* SNP genotype frequency

SNP	Position	mAF	Genotype	Frequency	*n*	Total	*P*-HWE
rs2301261	8785896	0.082	GG / GA / AA	0.843 / 0.150 / 0.007	370 / 66 / 3	439	1.00
rs2268495	8782535	0.236	GG / GA / AA	0.576 / 0.376 / 0.048	253 / 165 / 21	439	0.46
rs53576	8779371	0.366	AA / AG / GG	0.395 / 0.477 / 0.128	173 / 209 / 56	438	0.65
rs11131149	8777852	0.169	GG / GA / AA	0.704 / 0.253 / 0.043	295 / 106 / 18	419	0.06
rs62243370	8777812	0.264	GG / GA / AA	0.534 / 0.404 / 0.062	226 / 171 / 26	423	0.49
rs62243369	8777807	0.266	GG / GA / AA	0.530 / 0.409 / 0.061	224 / 173 / 26	423	0.41
rs13316193	8777745	0.169	TT / TC / CC	0.704 / 0.253 / 0.043	295 / 106 / 18	419	0.06
rs2254298	8777228	0.261	GG / GA / AA	0.536 / 0.405 / 0.059	236 / 178 / 26	440	0.39
rs2268493	8775840	0.156	TT / TC / CC	0.722 / 0.244 / 0.034	317 / 107 / 15	439	0.17
rs2268491	8775398	0.261	CC / CT / TT	0.540 / 0.399 / 0.061	237 / 175 / 27	439	0.58
rs918316	8773181	0.221	TT / TC / CC	0.606 / 0.346 / 0.048	264 / 151 / 21	436	1.00
rs2268490	8772085	0.407	CC / CT / TT	0.320 / 0.546 / 0.134	139 / 237 / 58	434	0.009
rs237887	8772042	0.440	GG / GA / AA	0.312 / 0.497 / 0.191	137 / 218 / 84	439	0.97
rs237885	8770543	0.290	TT / TG / GG	0.506 / 0.409 / 0.085	220 / 178 / 37	435	0.98
rs1042778	8769545	0.102	GG / GT / TT	0.805 / 0.186 / 0.009	350 / 81 / 4	435	1.00

mAF, minor allelic frequency; *OXTR*, oxytocin receptor gene; *P*-HWE, *P*-value of Hardy-Weinberg equilibrium; SNP, single nucleotide polymorphism.

Table 3. Results of single SNP linear regression analysis for *attention switching*

	All			Male			Female		
SNP	*n*	β	*P*†	*n*	β	*P*†	*n*	β	*P*†
rs62243370	423	−0.46	0.030	262	−0.15	–	161	−0.92	0.004
rs62243369	423	−0.43	0.047	262	−0.14	–	161	−0.90	0.005
rs2254298	440	−0.41	0.061	272	−0.12	–	168	−0.80	0.014
rs2268491	439	−0.42	0.046	272	−0.16	–	167	−0.77	0.018

†Family-wise (SNP-wise) corrected empirical *P*-value on the basis of 10,000 permutations. *P*-values < 0.1 are indicated.
None of the Autism-Spectrum Quotient (AQ) scale, its other subscales, or other SNPs showed SNP-wise empirically significant associations. After using the Bonferroni method to perform multiplicity corrections for each subscale and gender subgroup, none of the SNPs exhibited significant associations.
β, regression coefficient; SNP, single nucleotide polymorphism.

roni multiplicity corrections for each haplotype, block, subscale, and gender subgroup, including each individual SNP analysis, *attention switching* showed a significant association with a haplotype GGTGTC (frequency = 54.9%), in block 1 in females (corrected $P = 0.0016$, $R^2 = 0.12$) (Table 4). The statistical power of the haplotype was estimated at 0.82 for $n = 168$ females, frequency = 0.549, mean *attention switching* score (SD) = 3.83 (2.02), and two-tailed nominal $P < 0.000146$ (=0.05/ ([12 SNPs + 7 haplotypes] × [1 scale + 5 subscales] × [1 group + 2 subgroups]). Haplotype analysis did not show Bonferroni-corrected significant associations for the AQ or other subscales (Supporting Table S2). No significant associations were observed for any specific haplotypes in block 2.

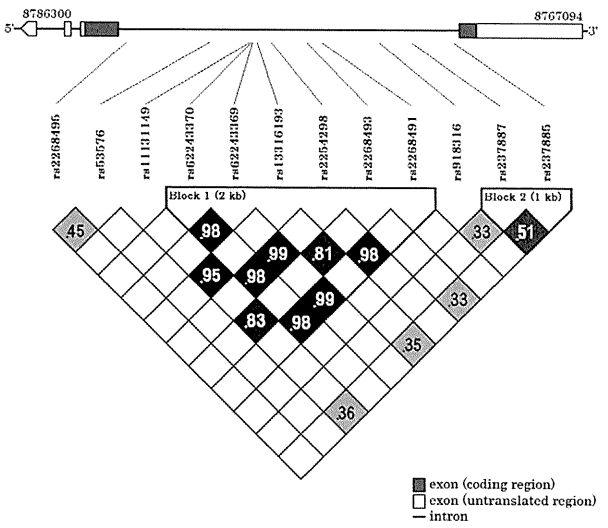

Figure 2 Linkage disequilibrium (LD) plot of the 12 single nucleotide polymorphisms (SNPs). Inter-SNP r^2 of >0.30 is displayed for each pair. D' is >0.8 for all pairs of SNPs in haplotype blocks.

Discussion

OXTR SNPs, including rs62243370, rs62243369, rs2254298, and rs2268491, which exhibited high

Table 4. Results of haplotype linear regression analysis for *attention switching*

		All				Male				Female			
Block†	Haplotype	Freq	β	Nom-*P*	Cor-*P*	Freq	β	Nom-*P*	Cor-*P*	Freq	β	Nom-*P*	Cor-*P*
1	GGTGTC	0.568	0.43	0.002	–	0.580	0.05	–	–	0.549	0.95	4.5×10^{-6}	0.0016**
	AATATT	0.252	−0.46	0.005	–	0.246	−0.20	–	–	0.261	−0.89	0.001	–
	GGCGCC	0.150	−0.30	–	–	0.139	0.01	–	–	0.167	−0.71	0.02	–
	GGCGTC	0.015	1.09	0.08	–	0.019	1.16	0.09	–	0.000	NA	NA	NA
2	GT	0.559	0.04	–	–	0.548	−0.11	–	–	0.577	0.35	–	–
	AG	0.290	−0.05	–	–	0.292	0.11	–	–	0.288	−0.30	–	–
	AT	0.151	−0.01	–	–	0.161	0.03	–	–	0.135	−0.17	–	–

**Corrected $P < 0.01$.

†Block 1, rs62243370/rs62243369/rs13316193/rs2254298/rs2268493/rs2268491; Block 2, rs237887/rs237885.

P-values < 0.1 are indicated. None of the Autism-Spectrum Quotient (AQ) or its other subscales showed significant associations after Bonferroni corrections.

β, regression coefficient; Cor-*P*, Bonferroni-corrected *P*-value for multiplicity, for each haplotype, block, subscale, and gender subgroup, including each single SNP analysis; NA, not applicable; Nom-*P*, nominal *P*-value.

inter-SNP r^2 to each other in haplotype block 1 (Figure 2), were associated with *attention switching* in females by the single SNP analysis (Table 3). These findings implied that participants with more major alleles of these SNPs tend to show higher *attention switching*. The single SNP analysis demonstrated an association between the AQ subscale and rs2254298 which was reported to be associated with ASD in previous studies (Wu *et al.*, 2005; Jacob *et al.*, 2007; Lerer *et al.* 2008; Liu *et al.*, 2010). However, after performing Bonferroni corrections, statistical significance was eliminated for all four SNPs.

The six-SNP haplotype block 1, consisting of the four SNPs noted immediately above and two others, showed a Bonferroni-corrected significant association with *attention switching* (Table 4), with medium effect size and power > 0.8. Females with haplotype GGTGTC in block 1 tended to show higher *attention switching*. This haplotype block included rs2254298, which was reported to be associated with ASD.

So far, one study reported no association between *OXTR* SNPs (rs2228485, rs237902, rs237898, and rs237885) and AQ score in a Caucasian sample (Chakrabarti *et al.* 2009). With regard to rs237885, the present study replicated this result, with the Japanese sample showing no association. The reason that their study did not find any associations might be attributable to differences in ethnicity, methods of analysis and/or SNPs. They did not analyze haplotypes, did not differentiate between sex, and did not investigate AQ subscales. Their SNPs did not overlap with our SNPs, except for rs237885. Nor did they examine any SNPs in block 1 which showed a significance in the present study.

Our results indicate that association may differ according to sex (Carter, 2007). The reason for this difference is not clear. Uhl-Bronner *et al.* (2005) reported gender differences in OXT-binding site expression in the forebrains and spinal cords of rats. Similar differences may exist in the human brain, and may contribute to the regulatory central actions of OXT in human behavior and cognition. This could be related with the present result in gender difference.

This study provided evidence that *OXTR* polymorphism is associated with poor attention switching (or strong focus of attention), which is included in the category of restricted and repetitive interests and behaviors in the autism-specific triad. It has been reported that OXT administration in patients with ASD has led to a reduction in repetitive behaviors (Hollander *et al.*, 2003) and improved social cognition (Hollander *et al.*, 2007). The OXT mechanism in the brain may have a substantial influence on the autism features of restricted and repetitive activities and behavior, as well as difficulty in social interaction.

Limitations to this study include the fact that participants were employees of a major corporation, and were therefore not necessarily representative of the general community: our study participants likely represent a high-functioning segment of the population, and AQ score distribution could be different in the general population. Another limitation is that some SNPs had less than 10 minor homozygous participants, and were therefore excluded from analysis. Due to the small sample size, all selected SNPs could not be examined. A larger sample is needed to detect effects of more SNPs.

In conclusion, the present study demonstrated that one specific *OXTR* haplotype, consisting of rs62243370, rs62243369, rs13316193, rs2254298, rs2268493, and rs2268491, was associated with the

autistic trait of strong focus of attention with medium effect size and power = 0.82 in a non-clinical Japanese female population.

Supporting information cited in this article is available online.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Results of single nucleotide polymorphisms (SNPs) linear regression analysis
Table S2. Results of haplotype linear regression analysis

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Association Between the Oxytocin Receptor Gene and Amygdalar Volume in Healthy Adults

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Background: Recent studies have suggested that oxytocin affects social cognition and behavior mediated by the oxytocin receptor (OXTR) in amygdala in humans as well as in experimental animals. Genetic studies have revealed a link between the OXTR gene and the susceptibility to autism spectrum disorders (ASD), especially in the social dysfunctional feature of ASD.

Methods: We examined the relationship between amygdala volume measured with manual tracing methodology and seven single nucleotide polymorphisms and one haplotype-block in *OXTR*, which were previously reported to be associated with ASD, in 208 socially intact Japanese adults with no neuropsychiatric history or current diagnosis.

Results: The rs2254298A allele of *OXTR* was significantly associated with larger bilateral amygdala volume. The rs2254298A allele effect on amygdala volume varied in proportion to the dose of this allele. The larger the number of rs2254298A alleles an individual had, the larger their amygdala volume. Such an association was not observed with hippocampal volume or with global brain volumes, including whole gray, white matter, and cerebrospinal-fluid space. Furthermore, two three–single nucleotide polymorphism haplotypes, including rs2254298G allele, showed significant associations with the smaller bilateral amygdala volume.

Conclusions: The present results suggest that *OXTR* might be associated with the susceptibility to ASD, especially in its aspects of social interaction and communication mediated by a modulation of amygdala development, one of the most distributed brain regions with high density of OXTR. Furthermore, amygdala volume measured with magnetic resonance imaging could be a useful intermediate phenotype to uncover the complex link between *OXTR* and social dysfunction in ASD.

Key Words: Amygdala, autism, MRI, OXTR, oxytocin, social dysfunction

Oxytocin (OXT) affects affiliative and social behavior in addition to various reproductive behaviors in both male and female animals (1-3). Experimental animal studies have suggested that effects of OXT on social memory and recognition are mediated by the amygdala (4,5). Oxytocin shows significant binding in the limbic system, especially in amygdala (6,7), although the distribution of OXT receptors (OXTR) varied among species, at least partly due to interspecies differences in social organization such as the style of group living (8-10).

Rapidly accumulating evidence has demonstrated that OXT can shape the development of social cognition and behavior not only in experimental animals but also in humans (11-15). Although the neural background of these OXT effects has yet to be clarified, functional magnetic resonance imaging (fMRI) studies have reported changes in amygdala activation associated with the effect of OXT administration on human emotional and social functions (16-19). Taken together, recent studies have suggested that the amygdala plays an important role in mediating the social and affiliative effects of OXT in humans as well as in experimental animals.

As in the preceding overview, OXT is associated with normal social interaction, whereas recent studies have also suggested that OXT might play a role in the pathogenesis of autism spectrum disorders (ASD) characterized by significant deficits in social interaction (20). Intravenous (21,22) and intranasal (23) OXT administration has been reported to be associated with clinical improvements of ASD symptoms. Recent genome-wide linkage studies (24-27) and association studies (28-32) have suggested the OXTR gene (*OXTR*) as a plausible candidate gene for ASD. In particular, a recent association study revealed that patients carrying the *OXTR* haplotype associated with autism showed significant impairments in aspects of social interaction and communication compared with noncarriers of the haplotype (32).

Furthermore, neuroimaging studies have suggested a role of the amygdala in the pathophysiology of ASD, especially in its social aspect, both at brain structural and functional levels. Aberrant amygdala activation and its connectivity to the fusiform face area have been reported during face processing by previous fMRI studies (33,34). Most of the previous volumetric MRI studies examining children with ASD have shown larger than normal amygdala volume (35-40), although some studies did not show significant difference in amygdala volume between individuals with ASD and typically developed subjects (41). Conversely, amygdala volume has been reported to be smaller in adults with ASD than in typically developed individuals (42-44). Schumann *et al.* (39) reported enlarged amygdala volume in children but not in adolescents with autism and suggested an abnormal course of early amygdala development in autism. The heritability of abnormal amygdala volume has been indicated by previous studies including siblings of individuals with ASD (45), although a possible contribution of a nongenetic factor to abnormal amygdala volume has also been reported in a monozygotic twin concordant for ASD but discordant for clinical characteristics such as comorbid depression (46). If this evidence is taken collectively, abnormal amygdala volume might be an intermediate phenotype of the genetic factor of ASD.

In the current study, the effects of *OXTR*—which were previously

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reported to be associated with ASD— on the volumes of amygdala and hippocampus were examined in healthy human adults with a manually traced volumetric MRI. On the basis of the previous literature on social cognition and ASD as overviewed in the preceding text, in which an association of ASD with amygdala volume rather than volume of hippocampus was reported, it would be reasonable to predict an association between *OXTR* and amygdala volume.

Methods and Materials

Subjects

The subjects were 208 (143 men and 65 women) Japanese adults (age, mean ± SD: 33.9 ± 11.6 years), consisting of college students, hospital staff, and their acquaintances. Before MRI scanning, the subjects were screened with the Structured Clinical Interview for DSM-IV Axis I Disorder, Nonpatient Edition (SCID-NP) (47) by a trained psychiatrist (HY or MS), to confirm that the subjects had no history of major mental illness. Other exclusion criteria were neurological illness, traumatic brain injury with any known cognitive consequences or loss of consciousness for more than 5 min, a history of substance abuse or addiction, or a family history of an axis I disorder in their first-degree relatives. All were right-handed on the basis of the Edinburgh Inventory (48). The socioeconomic status (SES) and parental SES were assessed with the Hollingshead scale (49). After a thorough explanation of the study to the subjects, written informed consent was obtained from all study participants. The ethical committee of the Faculty of Medicine, University of Tokyo, approved this study (No. 397-1 for MRI project and No. 639-9 for genetic and imaging-genetic-association project).

Image Acquisition and Processing

The method of MRI acquisition of 1.5-mm slices was the same as that described in our previous study (50). The MRI data were obtained with a 1.5-Tesla scanner (General Electric, Signa Horizon Lx version 8.2, GE Medical Systems, Milwaukee, Wisconsin). We used three-dimensional Fourier-transform spoiled-gradient-recalled acquisition with steady state. The repetition time was 35 msec, the echo time was 7 msec with one repetition, the nutation angle was 30 degrees, the field of view was 24 cm, and the matrix was 256 × 256 [192] × 124.

The amygdala and hippocampus gray matter regions of interest (ROIs) were outlined manually by one rater (HI) who was blind to the group status or genotype. For the manual tracing, we used a software package for medical image analysis (3D Slicer; software available at http://www.slicer.org), which enables a simultaneous view of orthogonal planes. As described in the following text, to accurately measure the volume of these structures, we employed a protocol for delineating the anterior boundary of amygdala and the boundary between amygdala and hippocampus. The landmarks to delineate the ROIs were the same as our previous study (51).

Briefly, dentate gyrus, cornu ammonis fields, subiculum, presubiculum, and parasubiculum were referred to as the hippocampus, whereas the fornix, fimbria, and alveus were excluded from the volumetric measurements. The tracing of the hippocampus was performed mainly in the sagittal plane; however, we also edited the tracings in the axial and coronal planes in every slice to delineate the boundary of the ROIs.

In tracing the amygdala boundaries, the initial tracing process involved defining the borders in coronal sections starting with the most caudal level at which the amygdala was visible. At its caudal extent, the amygdala is bordered dorsally by the substantia innominata, laterally by the putamen, and ventrally by the temporal horn of the lateral ventricle. The medial surface of the amygdala abuts the optic tract (Figure 1A). Proceeding rostrally, it is bordered dorsally by fibers of the anterior commissure as well as the substantia innominata. The lateral border is formed by white matter of the temporal lobe. The ventral surface is formed by the temporal horn of the lateral ventricle. However, because the hippocampus often seems to be fused with the ventral surface of the amygdala, a more reliable boundary is the alveus, the white matter that forms the dorsal surface of the hippocampus (Figure 1B). In more rostral sections, the hippocampus decreases in size, and the entorhinal cortex begins to form part of the medial surface of the amygdala. At this point, a thin band of white matter separates the amygdala from the entorhinal cortex (Figure 1C). In most rostral sections, the dorsomedial surface of the amygdala forms a portion of the medial surface of the brain. The amygdala is bordered laterally by white matter of the temporal lobe, ventrally by the temporal horn of the lateral ventricle and by subamygdaloid white matter, and ventromedially by the entorhinal cortex (Figure 1D). At the rostral pole of the amygdala, the outlining rules are very similar to what has just been described in the preceding text. However, the boundaries between gray matter and white matter are more difficult to delineate. Therefore, it was necessary to corroborate the rostral boundary of the amygdala by reviewing the outlines in sagittal images (Figures 1E and 1F).

For interrater reliability, two raters (HI and MAR) blind to group membership, independently drew ROIs. Ten cases were selected at random, and the raters drew ROIs on every slice. The intraclass correlation coefficient was .87/.85 for the left/right amygdala and .93/.94 for the left/right hippocampus, respectively. Intrarater reliability, computed with all the slices from one randomly selected brain and measured by one rater (HI) on two separate occasions (approximately 2 months apart) was $> .95$ for all structures.

Total gray matter, white matter, and cerebrospinal fluid (CSF) volumes were calculated with SPM2 (52). Then, intracranial volume (ICV) was calculated by summing up the total gray matter, white matter, and CSF volumes. To validate this method, the ICVs of an independent sample of MRI scans for 50 adult subjects were measured by both the current procedure and an intensity-based semiautomated segmentation procedure with ANALYZE PC 3.0 (53). Then, we confirmed that the calculated intraclass correlation coefficient for the ICVs was satisfactory (.96).

OXTR Genotyping

Genomic DNA was extracted from peripheral leukocytes with a standard phenol-chloroform method. The DNA was isolated and amplified from blood samples obtained from all subjects. Seven single nucleotide polymorphisms (SNPs) (rs1042778, rs237887, rs918316, rs2268493, rs2254298, rs53576, and rs2268495) were selected, because they had previously been reported to be associated with ASD (28-31,54). These were genotyped with the TaqMan genotyping platform in accordance with the manufacturer's protocol (http://www.appliedbiosystems.com). In addition, haplotype analysis was also conducted with haplotype block, which was reported to be associated with ASD in an Asian sample (54). The present study was performed with an ethnically homogeneous sample (all subjects were of Japanese descent).

Statistical Analyses

One-way analysis of variance (ANOVA) for differences in potential confounding factors, such as age, handedness, self SES, and parental SES, were performed between the three *OXTR* genotype groups. The $\chi 2$ test was used for testing group difference in gender distribution. For testing regional specificity, the volumes of total gray matter, total white matter, CSF, and ICV were also compared between genotypes with one-way ANOVA.

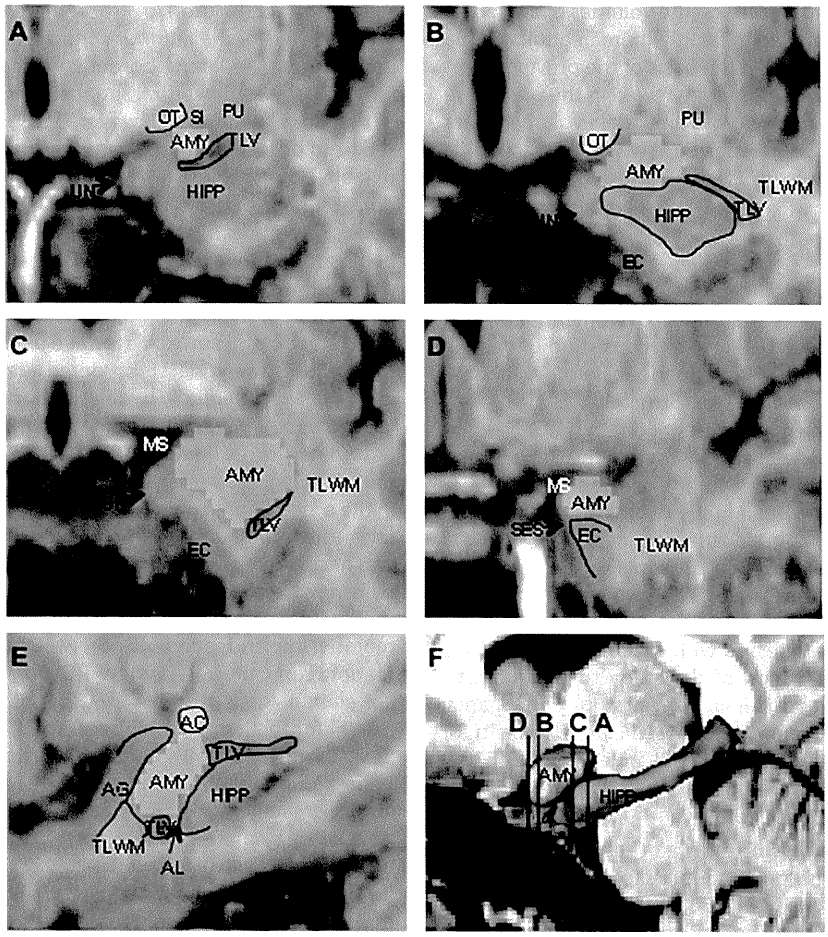

Figure 1. Amygdala (AMY) tracing description: series of coronal images arranged from caudal **(A, B)** to rostral **(C, D)**, sagittal image **(E)** and three-dimensional reconstruction of left AMY and hippocampus (HIPP) **(F)**. The gray matter of left AMY is labeled light green. AC, anterior commissure; AG, ambient gyrus; AL, alveus; IH, inferior horn of the lateral ventricle; MS, medial surface of the brain; OT, optic tract; PU, putamen; SES, semiannular sulcus; SI, substantia innominata; TLV, temporal horn of lateral ventricle; TLWM, temporal lobe white matter; UN, uncus.

To explore the effects of aging on brain morphology, correlations between age and brain volumes (total gray matter, total white matter, CSF, ICV, relative volumes of bilateral amygdala and hippocampus) were assessed with Pearson's correlation coefficient. Statistical significance was set at $p < .05$.

For single SNP-based quantitative trait association analysis, the effects of the *OXTR*s on the manually traced volumes were assessed by repeated-measures ANOVA adopting relative volumes (100 × absolute ROI volume/[ICV]) as the dependent variable, genotype as the between-subject factor, and region (amygdala/hippocampus) and hemisphere (left/right) as the within-subject factors. To correct the multiple testing, Bonferroni correction was implemented on the basis of the number of SNPs analyzed in the *OXTR*. The critical p value was set at .0071 (.05/7 SNPs). Once a significant group × region or group × region × hemisphere interaction was found, follow-up analyses with repeated measures ANOVA separately for each region were performed. Then, in the case of group × hemisphere interaction, post hoc t tests separately for each hemisphere were conducted. We also conducted a separate analysis with gender added as a between-subject factor, because the relationships of gender to both brain morphology and OXT have been reported (55,56). Statistical significance level was set at $p < .05$. Supplementarily, post hoc one-way ANOVA was used to assess the effects of the *OXTR* on the absolute and relative volumes of each region and hemisphere.

PLINK version 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink) (57) was used to implement haplotype-based quantitative trait association analysis for relative amygdala volumes. Haplotype distributions for each participant were inferred probabilistically with the standard Expectation-Maximization algorithm (58). Corrected p values were calculated with the Bonferroni method, because PLINK is not able to perform permutation tests for haplotype-based quantitative trait association analysis. To calculate global p values, we used the permutation test option as provided in UNPHASED (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/) (59). Permutation test correction was performed with 1000 iterations and applied to correction of global p values. Statistical power was estimated with QUANTO version 1.2.4 (http://hydra.usc.edu/gxe/) (60).

Results

Age showed significant correlations with total gray matter volume ($r = -.47, p < .001$), CSF ($r = .45, p < .001$), relative volumes of amygdala (left; $r = -.34$, $p < .001$, right; $r = -.35$, $p < .001$), and

Table 1. Genotype Effects of 7 SNPs in *OXTR* on Amygdala and Hippocampal Volumes

SNPs	Genotype	*n*	P-HWE	*F*-Values			
				Genotype	Genotype × Hemisphere	Genotype × Region	Genotype × Region × Hemisphere
rs1042778[a]	GG/GT/TT	174/32/1	1.00	.05	.94	.28	.56
rs237887	GG/GA/AA	77/98/32	1.00	.46	.61	.39	.09
rs918316[a]	TT/TC/CC	144/55/8	.33	1.59	.04	.40	.39
rs2268493[a]	TT/TC/CC	137/66/4	.34	.05	.07	.01	.24
rs2254298	AA/AG/GG	77/85/44	.09	.44	1.06	5.57[b]	3.20[c]
rs53576	AA/AG/GG	73/98/32	1.00	.62	.40	1.04	.22
rs2268495[a]	GG/GA/AA	112/76/19	.29	4.58[c]	.01	1.36	4.11[c]

SNP, single nucleotide polymorphism; *OXTR*, oxytocin receptor gene; P-HWE, *p* value of Hardy-Weinberg equilibrium.

[a]Because of the sample size of minor allele homozygotes ($n < 20$), we employed 2-group comparison (minor allele carriers vs. major allele homozygotes) instead of 3-group comparison (minor allele homozygotes vs. heterozygotes vs. major allele homozygotes).

[b]Reached significance even after correction for multiple comparisons (Bonferroni corrected $p < .0071$).

[c]$p < .05$.

hippocampus (left; $r = -.26, p < .001$, right; $r = -.31, p < .001$) but not with total white matter volume and ICC ($p > .56$).

All seven SNPs are listed in Table 1. All the SNPs showed call rates of > .95 and *p* values of Hardy–Weinberg equilibrium of > .09. For genotype effects on amygdala and hippocampal volumes, the repeated-measures ANOVAs of single SNPs showed that there was a significant interaction between rs2254298 and region [$F(2,203) = 5.57, p = .004$]. This interaction remained significant after Bonferroni correction for the multiple testing. However, no other genotype effects or interactions remained significant after the correction, including rs2254298 and region and hemisphere interaction [$F(2,203) = 3.20, p = .043$], rs2268495 main effect [$F(1,205) = 4.58, p = .033$], and rs2268495 and region and hemisphere interaction [$F(1,205) = 4.11, p = .044$] (Table 1).

Then, we conducted a survey of demographical characteristics and post hoc analysis of rs2254298. No significant difference was observed in gender, age, handedness, self SES, or parental SES among the three genotype groups in rs2254298 ($p > .30$) (Table 2). Total gray matter, total white matter, CSF, and ICV volume were also not significantly different among the three genotype groups ($p > .83$) (Table 3).

Post hoc repeated measures ANOVA separately for each region showed a significant rs2254298 genotype effect on amygdalar volume [$F(2,203) = 4.22, p = .016$] but not on hippocampal volume [$F(2,203) = .26, p = .77$]. The results of one-way ANOVA bore out these findings (Table 3). Of note, the statistical conclusions about the rs2254298 genotype effect on amygdalar volume reported in the preceding text remained the same when ANOVA with age as the covariate was employed ($p < .043$). There were no significant interactions between gender and genotype, gender and region and genotype, gender and hemisphere and genotype, or gender and region and hemisphere and genotype (*p* values > .22). The statistical conclusion from the main ANOVA is that the subjects carrying A allele of rs2254298 have significantly larger amygdala volume depending on the number of A allele (Figure 2).

The haplotype block was 4 kilobase-pair-long and consisted of three SNPs: rs918316, rs2268493, and rs2254298. The block included six specific haplotypes with frequencies > .05. Total frequencies of the six haplotypes were estimated to 100.0%. A significant evidence for association with relative amygdala volumes was provided by global *p* values (left: $p = .0061$; right: $p = .0029$). On the basis of the haplotype linear regression analyses with Bonferroni correction, two haplotypes (TCG and CTG) showed significant associations with the bilateral amygdala volumes (corrected $p < .05$) (Table 4). In this model, subjects with haplotype TCG or CTG, which carry the "*G*" allele of rs2254298, had a significantly smaller amygdala volume bilaterally. The statistical power was estimated as .75 from the sample size ($n = 208$), CTG frequency (.060), mean and SD of relative amygdala volume (.092 and .018, respectively), β (−.013), and two-tailed nominal *p* (.0042 = .05/12 [six haplotypes × 2 sides (left/right)]).

Discussion

We observed a significant association between the rs2254298A allele of *OXTR*, a candidate genotype to confer risk for ASD, and larger bilateral amygdala volume in a manner depending on allelic dose. Individuals with a larger number of rs2254298A allele tend to have a larger amygdala. In contrast, the genotype showed no significant effect on individual difference in global brain volume or hippocampal volumes.

Previous studies have suggested that *OXTR* shapes dysfunction

Table 2. Clinical and Demographical Characteristics and rs2254298 in *OXTR* of the Study Participants

	A/A ($n = 77$)		A/G ($n = 85$)		G/G ($n = 44$)		
	Mean	SD	Mean	SD	Mean	SD	Group Comparisons
Gender (male/female)	53/24		57/28		32/12		$\chi^2(2) = .44, p = .80$
Age (range)	32.4 (22–65)	10.3	34.3 (22–71)	12.0	35.8 (21–65)	13.2	$F(203) = 1.21, p = .30$
Handedness[a]	95.5	12.5	97.5	6.3	96.5	7.7	$F(198^b) = .91, p = .40$
Socioeconomic Status[c]	1.6	.7	1.6	.6	1.6	.7	$F(203) = .057, p = .95$
Parental Socioeconomic Status[c]	2.3	.9	2.2	.8	2.1	.8	$F(198^b) = .39, p = .68$

OXTR, oxytocin receptor gene.

[a]Determined with Edinburgh Inventory (Oldfield) (48): scores > 0 indicate right-handedness. A score of 100 indicates strong right-handedness.

[b]The degrees of freedom varied, due to unavailability of data in some subjects.

[c]Assessed with the Hollingshead scale (Hollingshead) (49). Higher scores indicate lower educational and/or occupational status.

Table 3. Brain Volumes and rs2254298 in *OXTR*

	A/A (n = 77)		A/G (n = 85)		G/G (n = 44)		One-Way ANOVAs F (p)
	Mean	SD	Mean	SD	Mean	SD	
Volumetric Measures (ml)							
Total gray matter	742.6	80.4	738.6	71.3	734.1	66.5	.19 (.83)
Total white matter	453.5	53.0	450.0	44.3	449.5	53.1	.13 (.87)
Cerebrospinal fluid	378.9	65.2	379.3	71.1	381.9	63.6	.03 (.97)
Intracranial content	1575.0	164.9	1568.0	149.1	1565.5	154.4	.06 (.94)
Amygdala							
Absolute volume (ml)							
Left	1.447	.268	1.403	.284	1.295	.264	4.36 (.014)
Right	1.479	.274	1.454	.307	1.330	.301	3.82 (.023)
Relative volume (%)[a]							
Left	.092	.014	.090	.017	.083	.018	4.03 (.019)
Right	.094	.016	.093	.019	.085	.019	3.70 (.026)
Hippocampus							
Absolute volume (ml)							
Left	2.591	.454	2.641	.334	2.611	.423	.32 (.73)
Right	2.628	.481	2.617	.393	2.669	.403	.22 (.80)
Relative volume (%)[a]							
Left	.165	.027	.169	.021	.167	.025	.55 (.58)
Right	.167	.028	.167	.023	.171	.027	.42 (.66)

OXTR, oxytocin receptor gene; ANOVA, analysis of variance.
[a]Relative volume = absolute volume/intracranial content × 100.

of social cognition and behavior in individuals with ASD (28-32). Oxytocin acts as a neurotransmitter involved in affiliative and social behaviors (61) and could shape the development of social behavior and cognition not only in experimental animals but also in humans (11-15). The present findings further indicate, although indirectly, that the influence of *OXTR* on social cognition and behavior might be mediated by individual differences in amygdala morphological development linked with the genotype.

Experimental animal studies have reported a dense distribution of OXTR in amygdala (6,7), its relationship to social cognition (4), and its interaction with estrogen on social cognition and behavior (62). A small number of recent f-MRI studies have further revealed an effect of intranasal OXT administration on amygdala reactivity to emotional faces in humans (16-19). Our results confirm the link between *OXTR* and amygdala in humans, by indicating that genetically determined OXT function might affect individual differences in amygdala development.

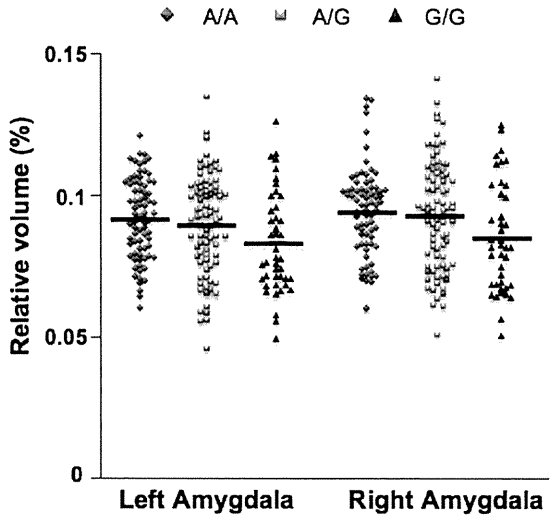

Figure 2. Manually traced amygdalar volume and rs2254298 in *OXTR* genotype. Means and distributions of left and right hemisphere for relative manually traced amygdalar volume (100 × absolute regions of interest volume/intracranial volume) in which individuals with A allele carriers exhibited bilateral enlargement depending on the number of rs2254298A allele compared with individuals with G/G genotype [$F(2,203) = 4.22$, $p = .016$]. Means are represented by solid horizontal lines drawn on each group's distribution.

The present findings might also contribute to uncovering the pathogenesis of ASD, especially its social and emotional aspects associated with OXT and amygdala. Most of the previous literature shows a larger-than-normal amygdala volume in children with autism (35-40) and a smaller-than-normal amygdala volume in adults with ASD (42-44). Previous studies have reported different alleles of rs2254298 in *OXTR* as risk alleles between different populations, although three associatiosn studies have reported a significant role of the rs2254298 SNP in *OXTR* in the development of ASD (28-30). We observed a significant association between amygdala volume and the rs2254298A alleles, such that the larger the number of the alleles an individual has the larger their amygdala is likely to be, and this allele has been found to be a risk allele for ASD in a Chinese sample (28). Previous literature suggested that amygdala volume was initially hypertrophic and sequentially shrunk, due to the excitotoxicity associated with chronic hypersensitivity of amygdala presented as autistic symptom manifestations in individuals with ASD (45). On the basis of their theory, although speculative, the individuals with genetic risk for impaired social reciprocity but without clinical manifestations might have preserved hypertrophic amygdala in adulthood. However, considering the inconsistency of previous studies as to which allele was overtransmitted in individuals with ASD, it is difficult to conclusively determine the direction of the association between a genetic risk for ASD and amygdala volume.

Here we address the limitations and methodological considerations of the present study. First, the *OXTR* effect should be confirmed in a different population, because the previous association studies have suggested a different allele as a candidate allele among different racial populations (28,29). Second, other possible

Table 4. Haplotype Effects in *OXTR* on Amygdala Volume

Haplotype[a]	Freq	Left Amygdala β	Left Amygdala Nom-*p*	Left Amygdala Cor-*p*	Global *p* (*df* = 5)	Right Amygdala β	Right Amygdala Nom-*p*	Right Amygdala Cor-*p*	Global *p* (*df* = 5)
TTA	.361	.0029	.11	1	.0061	.0023	.24	1	.0029
TTG	.288	−.000079	.97	1		.00089	.67	1	
CTA	.112	.0011	.67	1		.0011	.70	1	
TCA	.107	.0058	.043	.52		.0071	.027	.32	
TCG	.072	−.010	.0025	.03		−.011	.0030	.036	
CTG	.060	−.011	.0028	.034		−.013	.00082	.0098	

OXTR, oxytocin receptor gene; β, regression coefficient; Nom-*p*, nominal *p* value; Cor-*p*, Bonferroni corrected *p* value.
[a]Haplotype: rs918316-rs2268493-rs2254298.

roles of the alleles in the pathogenesis of autism should be examined in patient populations in future studies. Third, sexually dimorphic features of OXT (55) as well as of brain morphology (56) are well-known. Therefore, the possible interaction between gender and OXT should be examined in other brain regions in future studies, although no interaction involving the amygdala was found in the current study. Fourth, regional specificity to amygdala should be further examined in future studies. Although hippocampal volume and global brain volume did not show associations with *OXTR*, highly developed and complicated human social behavior medicated by OXT could be associated with highly organized social brain networks involving other regions. Previous studies have shown that the distribution of OXTR was different among species at least partially according to social organization and grouping behavior (8-10,63,64). Therefore, future studies should employ structural analysis throughout brain regions, such as voxel-wise whole brain computational analysis.

The present study showed a significant association between rs2254298A, a plausible candidate gene for ASD, and larger amygdala volume in nonpatient adult population. The *OXTR* might be associated with the susceptibility for ASD especially in its aspects of social interaction and communication through a modulation of amygdala development, one of the most abundant sites of OXTR in the brain. Furthermore, amygdala volume measured with MRI could be an effective intermediate phenotype to uncover the genetic background of social dysfunction in ASD.

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Two genetic variants of *CD38* in subjects with autism spectrum disorder and controls

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ABSTRACT

The neurobiological basis of autism spectrum disorder (ASD) remains poorly understood. Given the role of CD38 in social recognition through oxytocin (OT) release, we hypothesized that CD38 may play a role in the etiology of ASD. Here, we first examined the immunohistochemical expression of CD38 in the hypothalamus of post-mortem brains of non-ASD subjects and found that CD38 was colocalized with OT in secretory neurons. In studies of the association between *CD38* and autism, we analyzed 10 single nucleotide polymorphisms (SNPs) and mutations of *CD38* by re-sequencing DNAs mainly from a case–control study in Japan, and Caucasian cases mainly recruited to the Autism Genetic Resource Exchange (AGRE). The SNPs of *CD38*, rs6449197 ($p < 0.040$) and rs3796863 ($p < 0.005$) showed significant associations with a subset of ASD (IQ > 70; designated as high-functioning autism (HFA)) in the U.S. 104 AGRE family trios, but not with Japanese 188 HFA subjects. A mutation that caused tryptophan to replace arginine at amino acid residue 140 (R140W; (rs1800561, 4693C>T)) was found in 0.6–4.6% of the Japanese population and was associated with ASD in the smaller case–control study. The SNP was clustered in pedigrees in which the fathers and brothers of T-allele-carrier probands had ASD or ASD traits. In this cohort OT plasma levels were lower in subjects with the T allele than in those without. One proband with the T allele who was taking nasal OT spray showed relief of symptoms. The two variant *CD38* poloymorphysms tested may be of interest with regard of the pathophysiology of ASD.

1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disease manifesting in childhood but extending through to adulthood. The disorder is more common than previously supposed, with a worldwide frequency of >0.6% (Honda et al., 2005; Baird et al., 2006; Williams et al., 2006). The region with the maximum reported rate (3% of births) is the Nagoya/Hamamatsu region in Japan (Sumi et al., 2006). ASD can be sporadic or familial and is far more common in males than in females (Zhao et al., 2007). Because ASD is etiologically heterogeneous and forms a continuum, it is likely to involve many genes (Sutcliffe, 2008; Levitt and Campbell, 2009). *De novo* mutations and copy number variations (CNVs) are reported in a small fraction of ASD cases (Sebat et al., 2007; Glessner et al., 2009), but common variants also underlie the disease (Wang et al., 2009), and a unified mechanism for both forms of genetic inheritance has been proposed (Zhao et al., 2007).

Oxytocin (OT) is secreted into the brain by hypothalamic neuronal dendrites and plays important roles in social recognition and memory (Insel and Fernald, 2004; Takayanagi et al., 2005; Donaldson and Young, 2008; Neumann, 2008). This hormone mediates behavioral effects, such as pair bonding, mate guarding, and parental care in rodents (Ferguson et al., 2000; Ludwig and Leng, 2006; Campbell, 2008) and may be involved in romantic love, trust, and fear in humans (Kosfeld et al., 2005; Zeki, 2007; Domes et al., 2007; Ditzen et al., 2009). Recently, evidence has accumulating to suggest that the polymorphisms of multiple OT-related genes are associated with ASD (Wu et al., 2005; Jacob et al., 2007; Ebstein et al., 2009; Gregory et al., 2009; Wermter et al., 2010).

Peripheral or nasal administration of OT facilitates social recognition and trust in healthy humans (Guastella et al., 2008a,b, 2010; Ditzen et al., 2009) and increases eye contact and recognition in autistic subjects (Hollander et al., 2007; Yamasue et al., 2009). Those observations are based mostly on a small number of administrations, and the effects of long-term OT treatment on human social behavior in ASD patients is unknown.

Human CD38 is a type II transmembrane antigen (Malavasi et al., 2008). The *CD38* gene consists of 8 exons on 4p15 (Nakagawara et al., 1995) and spans a genomic stretch of 70.51 kb. The mRNA contains 1227 bases, and single nucleotide polymorphisms (SNPs) have been reported (Nata et al., 1997; Yagui et al., 1998; Ebstein et al., 2009; see Fig. 2A). CD38 has been studied extensively because it is a reliable negative prognostic marker for chronic lymphocytic leukemia (Deaglio et al., 2008). CD38 is expressed in the brain (Lee, 2001; Higashida et al., 2007) and can catalyze the formation of cyclic ADP-ribose (cADPR) from NAD^+ (Lee, 2001; Guse, 2005; Malavasi et al., 2008). cADPR mobilizes Ca^{2+} from intracellular Ca^{2+} stores, thus acting as a second messenger (Lee, 2001). Little was known about the CD38-dependent cADPR/Ca^{2+} signaling pathway in the brain until recent studies in our laboratory. They showed that CD38 regulates OT secretion in the mouse hypothalamus and posterior pituitary, which is critical for mouse social behavior (Jin et al., 2007; Liu et al., 2008). The precise role of CD38 in the human hypothalamus, however, has not been clarified.

As OT seems to be an important factor for the understanding of ASD, we examined the relationships among human *CD38* polymorphisms and mutations in Japanese, Korean, and Caucasian subjects. We identified two functional polymorphisms in subgroups: ASD and high-functioning autism (HFA) based on IQ (>70 classified as HFA). We measured the carrier's serum OT levels and examined each subject for ASD. Here, we also discuss the possibility of treating ASD patients who have a SNP that lowers OT levels by intranasal administration of OT.

2. Materials and methods

2.1. CD38 expression and immunohistochemistry

We measured CD38 mRNA levels by the semi-quantitative or real-time quantitative RT-PCR method (Jin et al., 2007) using commercially available total RNAs from various regions of the human brain. Control human brain tissues were obtained from archival blocks in the Departments of Pathology at the University of California San Francisco and Kanazawa University Graduate School of Medicine. The use of this tissue followed the institutional guidelines established by the Committee on Human Research (CHR) in both universities. The immunofluorescent stainings for human CD38 and OT were performed according to the procedures described previously (Zhang et al., 2007). Briefly, sections of the hypothalamus were treated with antigen retrieval protocol (0.01 M citrate acid buffer pH 6.0, plus heating for 121 °C for 5 min). The sections were then incubated with primary antibody against human CD38 (1:50, sc-7325, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and OT (1:500, AB911, Chemicon, Billerica, MA, USA) overnight at room temperature, followed by washing in Tris-buffered saline (TBS), and incubation with secondary antibodies that were conjugated with Alexa 488 or Alexa 568 (1:300, A11001 and A11011; Invitrogen) for 1 h at room temperature. Images for the co-localization of CD38 and OT in the paraventricular nucleus of the hypothalamus were

Table 1
Data sets for the 4 case–control study cohorts.

Data set[a]	Cohort 1 Subjects, age (range), male/female	Cohort 2 Subjects, age (range), male/female	Cohort 3 Subjects	Cohort 4 Subjects, male/female
Cases (Probands)	29, 22.8 ± 7.6 (12–44), 23/6	301, 11.9 ± 6.7 (3–64), 263/38	16	263, 263/0
Controls	315, 34.1 ± 4.3 (8–75), 171/144	417, 28.6 ± 14.4 (5–65), 229/188	150	–
Trio families	3	334		252
Family member	25, 53.0 ± 4.5 (21–84), 15/10	297, 39.2 ± 15.5 (3–93), 143/154	–	–
HFA subjects	–	188		104
Usage/analysis	Intronic SNP	–	–	Intronic SNP
	Exonic mutation	R140W	R140W	R140W
	Family-based association	HFA association		HFS association
	OT/AVP measurement			

[a] Cohort 1 was from the Kanazawa area, Japan; Cohort 2, the Nagoya/Hamamatsu, Tokyo, and Osaka areas, Japan; Cohort 3, Jeonju, Korea; Cohort 4, study of 252 trio samples from the Autism Genetic Resource Exchange comprising 252 U.S., 7 Russian, and 4 Italian ASD patients. Age ± SEM years (year range).

captured using a Leica confocal microscope (TCS SP, Bannockburn, IL, USA) and imported into the Photoshop software.

2.2. Participants

The participants consisted of cohorts organized by the Osaka-Hamamatsu-Kanazawa University Joint Research Centers for Child Mental Development, the Kanazawa University COE Program, the Core Research for Evolutional Science and Technology Program in Japan, which includes DNA samples collected at the University of Tokyo (Table 1). Three hundred and fifty-seven ASD subjects were recruited from outpatient psychiatry or pediatric clinics of each university hospital. All subjects fullfilled the DSM-IV criteria for autistic disorder. The diagnoses were made by two experienced child psychiatrists through interviews and clinical record reviews, and the subjects had no apparent physical anomalies. We also recruited patients' parents, grandparents, siblings, and other relatives from 322 families. The controls consisted of unrelated healthy Japanese volunteers—315 from the first cohort and 417 from the second. We recruited adult controls mainly from among hospital and facility staff and medical schools, and age-matched children as controls. In the Japanese cohorts, all subjects resided in Kanazawa, Hamamatsu/Nagoya, Tokyo, or Osaka, Japan, and all patients and controls were Japanese with no non-Japanese parents or grandparents. Two experienced child psychiatrists independently confirmed the diagnosis of ASD for most patients by semi-structured behavior observation and interviews with the subjects and their parents. At the interview with the parents, which was helpful in the evaluation of autism-specific behaviors and symptoms, the examiner used one of the following instruments: the Asperger Syndrome Diagnostic Interview (Gillberg et al., 2001), Autism Diagnostic Interview-Revised (ADI-R; Lord et al., 1994), Pervasive Developmental Disorders Autism Society Japan Rating Scale (2006), or Diagnostic Interview for Social and Communication Disorders (Wing et al., 2002).

In addition, for a third case–control study, we recruited 16 male ASD patients and 150 non-ASD male controls from Jeonju University Hospital in Korea. For a fourth study, we recruited 252 families from the Autism Genetic Resource Exchange (http://www.agre.org; AGRE cohort; Geschwind et al., 2001). Additional selection criteria required that (i) there be no possible non-idiopathic autism flags and (ii) all the trios be Whites, with the exclusion of Hispanic and Latino races (Anitha et al., 2008). Seven Russian male patients from Krasnoyarsk State Medical University Hospital, and 4 lymphoblastoid cells from Italian male ASD patients from Trino University Medical Hospital were also included in the study. These subjects met the DSM-IV or ADI-R criteria for autistic disorder.

For the HFA group, the U.S. autistic offspring of 104 trios (patient plus two parents) among the 252 AGRE trios, who had IQ > 70, were considered. In the second cohort, we selected 188 trio families as Japanese HFA cases (Table 1).

Using the Autism-Spectrum Quotient (AQ) (Baron-Cohen et al., 2006; Munesue et al., 2008), we evaluated members of families in which older subjects performed self-evaluation by recalling how they behaved in their 20 s. Subjects of autism traits in 3 kindreds was defined by AQ scores, above the average (>27) but less than the higher level (<32), during interviews by two psychiatrists.

This study was approved by the ethics committees of Kanazawa University, Hamamatsu Medical University, University of Tokyo, Osaka University, RIKEN, and the other participating institutes.

2.3. Marker selection

The genomic structure of *CD38* is based on the University of California, Santa Cruz, March 2006 draft assembly of the human genome (http://www.genome.ucsc.edu). We selected SNPs using information from the International HapMap Project (http://www.hapmap.org) and the National Center for Biotechnology Information (NCBI dbSNP: http://www.ncbi.nlm.nih.gov/SNP). Initially, all the SNPs with MAF > 0.1 were selected. Tags, which

Table 2
Oligonucleotides. Designed for amplification of coding sequences including 60–100 bp of flanking intronic sequences. Primers were followings.

Exon	Up	Down
1	5′-AGGGAAACAGAGAAAAGGCAAGTGU-3′	5′-GGCCAGCTGCTCCTGAAAG-3′
2	5′-GGCATATAATAGATGCTTCC-3′	5′-TGGACCTATGAATTGTTACC-3′
3	5′-GACATGCTAAATTGATCTCAG-3′	5′-CAGCAGAAGTCACTCTGTTC-3′
4	5′-TCCACTATGACTGAACAGCC-3′	5′-AGCACTGACTGAGTAACG-3′
5	5′-CTTAACCAGCTATTGCTAAG-3′	5′-ACTGTGATATTTGCAACAGG-3′
6	5′-TCTGCCTGCTGGTTGTTGAG-3′	5′-TCCTGAGTCAATTTGTTCCC-3′
7	5′-CCCAACAGCCTCTTAACTTT-3′	5′-ATCACCAGAGGTTGCCAT-3′
8	5′-AGCGAATTGGACGACAGATG-3′	5′-CATTGACCTTATTGTGGAGG-3′

Usually, we used the following temperatures: 15 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at annealing temperature at 52 °C, 5 min at 72 °C for extension followed by a final extension of 10 min at 72 °C. SNPs of these samples were examined by the methods described.

Table 3
Sequence information for SNPs1-10 and R140W.

SNP	Sequence	Strand	ON GENE	UCSC No
SNP01 (rs3796878)	TTGCTCTGTTCCCAGGTTGGTCCTCC[A/G]CATACCTCCCACTGCAGGATCCTGG	–	T/C	15,394,757
SNP02 (rs3796875)	GTTTTTCAAGAGTCCTAAGACAAAGA[A/G]GAAAGGAAGAAGCAGAGAAGCCATG	–	T/C	15,396,312
SNP03 (rs6449197)	CAGGTTGAGGAAATTTTATTTCTAAT[C/T]TGCTCAGTGTTTTTTTCATCACAAG	+	C/T	15,424,020
SNP04 (rs11574927)	AAAATTGTGTACCCCAATTCAGTAGT[A/G]AAACTACTACCGGGAACATCGGGAA	+	A/G	15,449,341
SNP05 (rs10805347)	ATTAACATTTCAGAATTTTATGATCT[A/G]ATATTATGGTTCAAGCACTTGAAAC	+	A/G	15,449,937
SNP06 (rs3796863)	GGGAGGGGAGCTATCCATGCCACCTG[A/C]TGGTCAAAAAAACAGCAGGAGCAGC	–	T/G	15,459,084
SNP07 (rs1130169)	TGTACCCTTCCT ACAGATAGTCAAAC[C/T]ATAAACTTCATGGTCATGGGTCATG	+	C/T	15,459,783
SNP08 (rs13137313)	AAATAAACCATATGTGTTGAACAAAG[A/G]ATTAATAAATTAATTTGAGACTCAA	+	A/G	15,461.066
SNP09 (rs3733593)	ATCTTGAACAAAATCGCCTAACCTTTC[C/T]GAACTTCAACTTCCTTGCCACTCCT	+	C/T	15,461,202
SNP10 (rs3733593)	CTGCCTCCGAATTCCATAGTTTCCAC[C/T]GCCTTGCGCTACTTGCATTCTCTGATT	–	G/A	15,463,823
R140W (rs1800561)	TGGCCCATCAGTTCACACAGGTCCAG[C/T]GGGACATGTTCACCCTGGAGGACAC	+	C/T	15,435,656

could capture the common allelic variants with $r^2 > 0.8$ by pairwise tagging, were picked from this set using Haploview v4.0 (http://www.broad.mit.edu/mpg/haploview).

2.4. Genetic analysis

We isolated genomic DNA from venous blood samples using the standard phenol/chloroform method (Easy-DNA kit, Invitrogen, Carlsbad, CA). We amplified *CD38* exons (Tables 2 and 3) and flanking introns (Table 3) by PCR (Taq PCR Core Kit, Qiagen, Hilden, Germany). We used Assay-on-Demand SNP genotyping products (Applied Biosystems, Foster City, CA) to score SNPs based on the TaqMan assay method described previously (Anitha et al., 2008). An ABI 7900 Sequence Detection System (SDS) was used to determine genotypes and analyses were performed with SDS v2.0 software (Applied Biosystems). Fig. 2A shows the SNPs and mutations analyzed in this study and their locations.

2.5. Enzyme immunoassay for OT and vasopressin

Blood samples for measuring OT and vasopressin concentrations were collected in two hospitals in the Kanazawa area between 10:00 and 12:00 or 15:00 and18:00 o'clock from subjects who had been asked to fast for the previous 2 h. Qualified lab technicians drew 10 ml of blood from an arm vein into heparinized tubes in less than 15 min. The samples were centrifuged at 0 °C at 2600 × *g* for 15 min and the plasma was separated off, divided into 2 tubes, and stored at −80 °C. We performed the peptide assay for OT and vasopressin (AVP) as described previously (Jin et al., 2007; Liu et al., 2008).

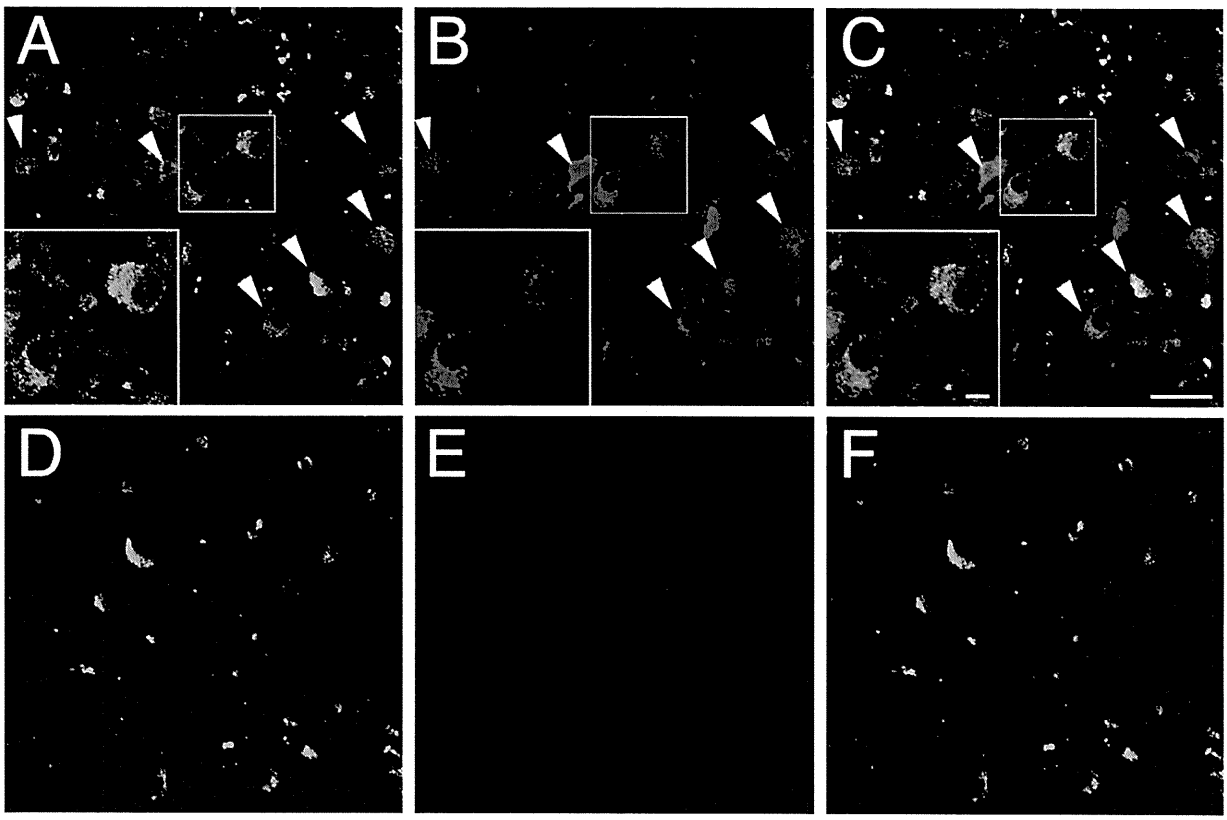

Fig. 1. Immunohistochemical analysis of CD38 (A, D) and oxytocin (B, E) in the human brain. Cell montages of panels were taken from the paraventricular nucleus (PVN) in the hypothalamus (A–C) and insular cortex (D–F) of autopsy subjects from the USA. Arrowheads indicate extensive colabeling with OT. The insets in panels are enlarged images of neurons showing coexpression of CD38 and OT. Scale bars: 40 μm in C and 8 μm in insert.