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Research Article

Evidence for the involvement of genetic variation in the *oxytocin receptor* gene (*OXTR*) in the etiology of autistic disorders on high-functioning level†

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KEYWORDS

autism spectrum disorders (ASD) • Asperger syndrome • single nucleotide polymorphism (SNP) • association • transmission disequilibrium

ABSTRACT

An increasing number of animal studies advert to a substantial role of the neuropeptide oxytocin in the regulation of social attachment and affiliation. Furthermore, animal studies showed anxiety and stress-reduced effects of oxytocin. First human studies confirm these findings in animal studies and implicate a crucial role of oxytocin in human social attachment behavior and in social interactions. Thus, the oxytocin system might be involved in the impairment of social interaction and attachment in autism spectrum disorders (ASD). The human *oxytocin receptor* gene (*OXTR*) represents a plausible candidate gene for the etiology of ASD. To analyze whether genetic variants in the *OXTR* gene are associated with ASD we performed family-based single-marker and haplotype association analyses with 22 single nucleotide polymorphisms (SNPs) in the *OXTR* and its 5′ region in 100 families with autistic disorders on high-functioning level (Asperger syndrome (AS), high-functioning autism (HFA), and atypical autism (AA)). Single-marker and haplotype association analyses revealed nominally significant associations of one single SNP and one haplotype with autism, respectively. Furthermore, employing a "reverse phenotyping" approach, patients carrying the haplotype associated with autism showed nominally significant impairments in comparison to noncarriers of the haplotype in items of the Autism Diagnostic Interview-Revised algorithm describing aspects of social interaction and communication. In conclusion, our

results implicate that genetic variation in the *OXTR* gene might be relevant in the etiology of autism on high-functioning level. © 2009 Wiley-Liss, Inc.

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ARTICLE TEXT

INTRODUCTION

In the last decade, a rapidly growing number of studies suggested a key role of the evolutionary conserved neuropeptide oxytocin in the regulation of affiliative behavior and social bonding in nonhuman mammals [reviewed in Lim and Young, [2006]]. The first few studies in humans support the findings of animal studies implicating a strong evidence for a central role of oxytocin in social attachment and affiliation in humans as well [reviewed in Bartz and Hollander, [2006]; Hammock and Young, [2006]; Heinrichs and Domes, [2008]]. Recently, Kosfeld et al. [2005] published the remarkable finding, that intranasal administered oxytocin causes a substantial increase in trust among humans, suggesting that oxytocin increases the prosocial approach behavior.

Because of the intensified investigation of the role of the oxytocin in social behavior in the last years there is strong evidence for a substantial role of oxytocin in the etiology of autism spectrum disorders (ASD). ASD are a family of neurodevelopmental disorders characterized by early-onset delays and deviance in the development of social, communicative skills and restricted, stereotyped pattern of interests and activities [Volkmar et al., [2004]]. Children with autism fail to acquire an intuitive understanding of mental states, e.g., by reading the language of eyes or facial expressions contributing to social communication impairments [Hill and Frith, [2003]]. First few studies supported the hypothesis that oxytocin might be involved in the impairment of social and behavioral skills in autism [reviewed in Bartz and Hollander, [2008]]. In healthy male volunteers after intranasal administration oxytocin improves the ability to infer the mental state of other from social cues of the eye region [Domes et al., [2007a]]. In a functional magnetic resonance imaging (fMRI) study conducted with healthy males intranasal application of oxytocin reduced amygdala activation and coupling of the amygdala to brainstem regions by viewing fear-inducing visual stimuli whereas the depression of amygdala activation was more pronounced for threatening and angry faces than scenes [Kirsch et al., [2005]]. In a further fMRI study oxytocin reduced right-sided amygdala responses to fearful, angry, and happy faces after intranasal application of oxytocin in healthy male volunteers suggesting a modulatory role of oxytocin on amygdala responses to facial expressions irrespective of their valence [Domes et al., [2007b]]. After intranasal administration of oxytocin 52 healthy male volunteers showed an increased number of fixations and total gaze time toward the eye region of 24 neutral human faces [Guastella et al., [2008]]. Oxytocin increases retention of social cognition in autism suggesting that oxytocin might facilitate social information processing in those with autism [Hollander et al., [2007]].

Furthermore, there is evidence for an implication of the role of the *oxytocin receptor* gene (*OXTR*) in ASD. Oxytocin receptor-deficient mice display pervasive social deficits suggesting a critical role of *OXTR* in regulating several aspects of social behavior [Takayanagi et al., [2005]]. In haploinsufficient (+/-) reeler mice *OXTR* expression is reduced in brain cortical region wherefore the authors hypothesized that down-regulation of reelin may contribute through associated reductions in the oxytocin receptors to the deficiencies in social behavior that are characteristic of autism [Liu et al., [2005]]. In a combined analysis of genome scans from the Autism Genetic Resource Exchange (AGRE) and Finnish autism sample the most promising shared chromosome locus on 3p24-26 with a nonparametric linkage (NPL) score of 2.20 was identified [Ylisaukko-oja et al., [2006]]. The *OXTR* gene is located only 29 kb distally to the marker D3S3691 with the highest NPL score in the combined sample. However, sequencing of the entire polypeptide coding sequence and the flanking splice sites in 22 probands with autism failed to identify clearly disease-related mutations [Ylisaukko-oja et al., [2006]]. In a further genome-wide screen suggestive linkage (LOD score 2.22) was found for the same marker D3S3691 [McCauley et al., [2005]]. Linkage was also reported for the marker D3S3680 [Shao et al., [2002]]. Additionally, genome-wide association analyses revealed the most significant finding for marker D3S3594 [Lauritsen et al., [2006]]. In a sample of 195 Chinese Han families with ASD two out of four analyzed polymorphisms covering 10 kb of DNA (rs2254298, rs53576) were associated with autism [Wu et al., [2005]]. However, these two single nucleotide polymorphisms (SNPs) are not located in a coding region and their functional relevance remains unclear, they might be in linkage disequilibrium with an unknown causal locus for autism [Wu et al., [2005]]. The haplotype analyses in the study by Wu et al. demonstrated evidence for global association and indicated that a number of haplotypes, especially those involving rs53576, were significantly associated with autism [Wu et al., [2005]]. A replication study analyzed both SNPs in a sample of 57 Caucasian trios and demonstrated significant transmission disequilibrium only for rs2254298 [Jacob et al., [2007]]. In contrast to the Chinese study, they reported an overtransmission of the G allele to the probands whereas in the Chinese Han sample the A allele was overtransmitted. This finding was explained by genetic differences between populations and by different susceptibility variants in *OXTR*. In a further association study the entire *OXTR* gene region including 18 tagged SNPs was analyzed and a significant association with single SNPs and haplotypes was detected with ASD. Interestingly, in each significant haplotype the SNP rs2254298 was found [Lerer et al., [2008]]. Very recently, association with ASD was detected for one (rs2268493) of three genotyped SNPs in intron 3 of the *OXTR* gene [Yrigollen et al., [2008]].

This study was aimed at investigating the potential role of genetic variants of the functional and positional candidate gene *OXTR* in

ASD. We performed family-based single-marker and haplotype association analyses with 22 SNPs in the 3′- and 5′-UTRs and in the 5′, intronic and coding regions of the *OXTR* in 100 families with autistic disorders on high-functioning level (Asperger syndrome (AS), high-functioning autism (HFA), and atypical autism (AA)).

MATERIALS AND METHODS

Subjects

A sample of 100 individuals (95 male, 5 female) with ASD (51 AS, 44 HFA, and 5 AA) (age range: 6-24, mean 12.2 ± 4.7) was investigated. The children and their parents or caregivers gave their written informed consent after having been informed about the details and the purpose of this study. The study was approved by the Ethics Committee of the University Hospital Marburg.

The autistic children were diagnosed by experienced clinicians according to the standard criteria of ICD-10 [WHO, [1993]] and underwent an extensive psychiatric examination at the Department of Child and Adolescent Psychiatry, University Hospital Marburg. The expression of autistic symptoms was further assessed by the Autism Diagnostic Observation Scale (ADOS-G) [Lord et al., [2000]] and an autism specific parent interview, ADI-R [Le Couteur et al., [2003]] (German version: [Bölte et al., [2006]]). The ADI-R is a semistructured interview consisting of three major domains: social interaction, nonverbal and/or verbal communication, and restricted, repetitive behaviors and interests. Each of the 3 domains has 4 subdomains for a total of 12 subdomains. A cutoff point for each of the three domains provides a reliable diagnostic algorithm based on ICD-10 and DSM-IV criteria. The IQ was assessed by the Wechsler Scales (WISC-III/WAIS-R) (IQ: mean 100.2 ± 19.4).

Molecular Genetic Methods

DNA was isolated from peripheral white blood cells using standard protocols as described previously [Hinney et al., [1997]]. Twenty-two SNPs were selected from the UCSC database (http://genome.ucsc.edu/) and the HapMap Browser (http://www.hapmap.org/cgi-perl/gbrowse/hapmap_B35/) for genotyping. All 22 SNPs cover a region of 41.139 bp with an average distance of 1.870 bp whereas 16 SNPs in the 17.645 bp long transcript of the *OXTR* had an average distance of 1.103 bp. The SNPs are listed in Table II.

For the genotyping of the SNPs rs237851, rs6791619, rs888566, rs2270465, rs180789, rs2268498, rs237915, rs237913, rs2228485, rs237902, rs237897, rs237894, rs53576, rs237890, rs13316193, rs2254298, and rs4686301 polymerase chain reaction based restriction fragment length polymorphisms (PCR-RFLP) were performed (see Table I). Tetra-primer ARMS-PCR [Ye et al., [2001]] was used for genotyping of SNPs rs2270465, rs2301260, rs2268495, rs237895, rs237893, and rs237884. rs2270465 was genotyped by both PCR-RFLP and tetra-primer ARMS-PCR, because the Hardy-Weinberg equilibrium (HWE) for this SNP showed significant heterozygote excess with the affected children. The genotypes of rs2270465 in all 100 patients and 200 parents were identical by using both methods. Positive controls for the variant alleles were run on each gel. For validity of the genotypes, allele determinations were rated independently by at least two experienced individuals. Discrepancies were resolved unambiguously either by reaching consensus or by re-typing.

Table I. Primers and Enzymes for Genotyping of Sequence Variants in the *OXTR*

No.	Variant	Method	Primer	Enzyme	Products
1	rs237851	RFLP	Ors237851-F: 5′-TCACGGTGATTCAGAGTCCA-3′	*Bpi* I[a]	G: 314, 137
			Ors237851-R: 5′-GCCATCACTGTTGGAAGGAT-3′		T: 451
2	rs6791619	RFLP	Ors6791619-F: 5′-ACACACCACACAAGCCACAG-3′	*EcoR* I[a]	G: 265, 123
			Ors6791619-R: 5′-TCTTTTGATGCTAATGGAAAAGG-3′		A: 388
3	rs888566	RFLP	Ors888566-F: 5′-TGAATGGTGCACACACAATG-3′	*BceA* I[b]	A: 450
			Ors888566-R: 5′-TCTCCCTCCTTCTCTCCACA-3′		G: 247, 203
4	rs2270465	RFLP	Ors2270465-F: 5′-ACACGGAGAATGCAATGGTT-3′	*Hinf* I[a]	C: 265, 225, 9

			Ors2270465-R: 5′-TACCTTTCAGGGAGGCCTTT-3′		G: 265, 204, 21, 9
5	rs180789	RFLP	Ors180789-F: 5′-GCAGAGGACCTTTGGACTTG-3′	*Fok* I[b]	T: 390, 130, 30
			Ors180789-R: 5′-CCCCAAAATATGGGATGGA-3′		C: 316, 130, 74, 30
6	rs2268498	RFLP	Ors2268498-F: 5′-TAGGCTGTCTCACGGGCTAC-3′	*Bsl* I[b]	A: 262, 131, 54, 1
			Ors2268498-R: 5′-TCGGCCTCGAAAATTACAGA-3′		G: 226, 131, 54, 36, 1
7	rs2301260	Tetra-ARMS-PCR	Ors2301260-Fi: 5′-CTGCGCGGCTGGCCTCGACC-3′		C: 600, 303
			Ors2301260-Ri: 5′-ATCGCACGGGTCCGCTAGGGGTCA-3′		T: 600, 341
			Ors2301260-Fo: 5′-GCCGCTACATCAAGCTGGAGGTGTGGG-3′		
			Ors2301260-Ro: 5′-TGACTCCCCCCGGGGAAGTTGCAC-3′		
8	rs237915	RFLP	Ors237915-F: 5′-AAGCAGGTGCTGTGGAAGTT-3′	*BseR* I[b]	A: 409, 79
			Ors237915-R: 5′-CCAGGAACCCAACTCATCTG-3′		G: 280, 129, 79
9	rs237913	RFLP	Ors237913-F: 5′-GCTGAACATCCCGAGGAACT-3′	*BseX* I[b]	G: 375
			Ors237913-R: 5′- GGCCTTCGAGCCCTTTAC-3′		T: 204, 171
10	rs2228485	RFLP	Ors2228484-F: 5′-TGCTGTGTCTCATCCTGCTC-3′	*Bsm* I[b]	T: 223, 41
			Ors2228484-R: 5′-TGAGCAGCAGCAGGTAGGT-3′		C: 264
11	rs237902	RFLP	Ors237902-F: 5′-ATGTTCGCCTCCACCTACC-3′	*Tsp509* I[b]	C: 527
			Ors237902-R: 5′-CTCCACATCTGCACGAAGAA-3′		T: 321, 206
12	rs237897	RFLP	Ors237897-F: 5′-CCAAGCTCAAGCTCCTTCAA-3′	*Ssi* I[a]	T: 501
			Ors237897-R: 5′-TAGGTCGCCCTCTTCTATGC-3′		C: 261, 240
13	rs2268495	Tetra-ARMS-PCR	Ors2268495-Fi: 5′-GGCCAAGACACTGGTTATTCAATATTTCCC-3′		A: 343, 188
			Ors2268495-Ri: 5′-GTTGTTACCAGGGGCCACAGGTGTGCA-3′		G: 343, 209
			Ors2268495-Fo: 5′-CAGTGCCAGCTGCTTTCATTCAGTTTGG-3′		
			Ors2268495-Ro: 5′-ACTTCCACTACTGGCTTTTGCCCAACCC-3′		
14	rs237895	Tetra-ARMS-PCR	Ors237895-Fi: 5′-GTCAGAGGCACAGCTGACGTTTCATA-3′		C: 318, 209

			Ors237895-Ri: 5′-CATGTGTCCTTCATAAGAACCCTGGAAC-3′		T: 318, 166
			Ors237895-Fo: 5′-TTTATGGAACCCTCCCAGTTAGCTCAGT-3′		
			Ors237895-Ro: 5′-TTTCTTGATAGTTGGTGTTCGAGCTCCT-3′		
15	rs237894	RFLP	Ors237894-F: 5′-GGCAGGGAAAGAAGAGGACT-3′	*BsmA* I[b]	C: 222, 132
			Ors237894-R: 5′-CTGAGCTCACAGCCACTCTG-3′		G: 205, 132, 17
16	rs237893	Tetra-ARMS-PCR	Ors237893-Fi: 5′-AGAGGTGGGGCACACATGGGTCTTTT-3′		T: 490, 303
			Ors237893-Ri: 5′-TCCCTGGGGTTTTCATGATAAAGCATGG-3′		C: 490, 241
			Ors237893-Fo: 5′-TCGGAAAAGTGGAATTAATGCACAGCCC-3′		
			Ors237893-Ro: 5′-TTTGGCCACCACTGCACATTACTTTTGG-3′		
17	rs53576	RFLP	Ors53576-F: 5′-GCTGGACTCAGGAGGAATAGGGAC-3′	*Cfr13* I[a]	C: 216, 61, 57, 6
			Ors53576-R: 5′-GCCCACCATGCTCTCCACATC-3′		T: 277, 57, 6
18	rs237890	RFLP	Ors237890-F: 5′-CTGGCTGCATCACACTGTCT-3′	*Dde* I[b]	G: 141, 109, 72, 52, 19
			Ors237890-R: 5′-CTCCCTTCACCAAAGCTGAG-3′		C: 250, 72, 52, 19
19	rs13316193	RFLP	Ors13316193-F: 5′-CCTCTCATCCTCCCTGTGTC-3′	*Ssi* I[a]	A: 242, 212, 44
			Ors13316193-R: 5′-CAAAGGTGCAAAGACAGCAA-3′		G: 454, 44
20	rs2254298	RFLP	Ors2254298-F: 5′-AACGCCCACCCCAGTTTCTTC-3′	*Dde* I[b]	C: 237, 70
			Ors2254298-R: 5′-TGAAAGCAGAGGTTGTGTGGACAGG-3′		T: 207, 70, 30
21	rs4686301	RFLP	Ors4686301-F: 5′-CCAGGACTTTGCATCTGGA-3′	*Hha* I[b]	G: 318, 108, 80
			Ors4686301-R: 5′-AATGTGGTCCACCACTGACA-3′		A: 426, 80
22	rs237884	Tetra-ARMS-PCR	Ors237884-Fi: 5′-ATCCTTATGAAAATCATAGCTGGCGTC-3′		T: 296, 145
			Ors237884-Ri: 5′-TTTGGGACTAGCTTATCAATTTCTGTACAA-3′		C: 296, 208
			Ors237884-Fo: 5′-ATAAATGGAAAGACATCCTGTGTTCATG-3′		
			Ors237884-Ro: 5′-TTCTGCTCCGTATGTCTATCCTTAAACC-3′		

RFLP, restriction fragment length polymorphism analyses; Tetra-ARMS-PCR, Tetra-primer amplification refractory mutation system analysis.

[a] MBI Fermentas, St.-Leon-Rot, Germany.

[b] **All enzymes were purchased by NEB, New England Biolabs, Frankfurt am Main, Germany.**

The primers and restriction endonucleases are listed in Table I.

Statistical Analyses

The distribution of genotypes at each of the 22 SNP markers was checked for departure from HWE, separately for the affected children and for their parents, by a χ^2 test with one degree of freedom as implemented in the program FINETTI (Wienker, TF, unpublished data, https://finetti.meb.uni-bonn.de/). However, while tests of HWE are commonly used in statistical genetics to examine genotyping errors, it has been shown that the power of tests of HWE to detect systematic errors can be low [Leal, [2005]; Cox and Kraft, [2006]]. Therefore we concentrated on minimizing genotyping errors, for instance by using positive controls (see Molecular Genetic Methods Section). Additionally, checking for Mendelian inheritance revealed no inconsistencies in the families.

Haplotype-based association analysis was performed using a TDT-like test for multiple markers proposed by Zhao et al. [2000] as implemented in the program FAMHAP [Knapp and Becker, [2003]; Becker and Knapp, [2004a]]. For a given marker combination, the global statistic that jointly considers all haplotypes (zhao) was considered, as well as the maximum over the single-haplotype TDT statistics (zhaomax). Since it is not known prior to the analysis which markers are involved in an association with the trait, the option "allcombi" of FAMHAP was used to perform separate tests for all possible marker combinations. This includes the TDT calculated for every single SNP (i.e., combinations which consist of only one marker). Simulations were performed to determine nominal *P*-values, as well as a corrected global *P*-value for the marker combination showing the strongest association, which accounts for the multiple tests, i.e., two test statistics and many marker combinations [Becker and Knapp, [2004b]]. Because of the substantial memory requirements, only combinations with up to four markers were initially taken into account for association testing (however, all 22 SNPs were still used for estimation of the underlying haplotype frequencies as well as individual haplotype reconstruction), and 50,000 replicates were simulated for *P*-value calculation. As a second step, the computations were repeated for combinations with up to six markers (zhaomax option only), with 10,000 simulated replicates, on a larger computational resource that provided the required 50 Gb of main memory.

Regarding the combination of four markers, which yielded the strongest evidence of association in the primary analysis (rs237851-rs6791619-rs53576-rs237884, see Results Section), we investigated whether an intermediate phenotype exists that is more closely correlated with the existence of the risk-conferring haplotype than ASD per se. To this end, we determined which quantitative phenotypic items, that underlie the autism phenotype definition, are best discriminated by the existence versus nonexistence of the risk-conferring haplotype (11 and 89 children, respectively). The data of the ADI-R were selected, because the data reflect consistently the time period "age of 4-5 or ever" (behaviors developed by age 4-5 years or throughout the individual's life) for all participants. In contrast the "current" ratings of the ADI-R represent data for the actual expression of autistic symptoms on different age levels (6-24 in our sample). The 12 subdomain items of the ADI-R were chosen for applying Student's *t*-test to identify differences between the groups (existence vs. nonexistence of the risk-conferring haplotype).

Of the four variables which showed nominal significance on the 5% level (mentioned below), the sum for each child was taken to form a new quantitative trait for a subsequent explorative association analysis. This was done by applying Student's *t*-test to investigate differences in the phenotype between carriers and noncarriers of the aforementioned haplotype.

RESULTS

Regarding the test for departure from HWE, in the parents as well as in the affected children all SNPs (see Fig. 1) yielded *P*-values > 0.05, except for rs2270465 that showed significant heterozygote excess in the affected children ($P = 0.029$) but not in the parents. Furthermore, in the single-marker association analysis (i.e., regarding "marker combinations" consisting of only one SNP) rs2270465 was the only marker for which the test of transmitted versus nontransmitted alleles yielded a nominally significant *P*-value of 0.01854 (transmission rate for the minor G-allele 62.4%; see Fig. 1 and Table II). However, the result did not remain significant after correction for multiple testing. Still, the deviation from HWE at rs2270465, occurring only in the affecteds, most likely does not reflect problems with genotyping quality (see Material and Methods Section; rs2270465 was genotyped by two methods and revealed identical genotypes for all 100 trios). Rather, the deviation from HWE may be explained by selective effects arising from association with the trait. Looking at combinations with up to four markers, the strongest evidence for association was obtained for the combination rs237851-rs6791619-rs53576-rs237884 (see Fig. 1). Here, haplotype T-G-T-T yielded a maxTDT statistic (zhaomax) of 11.0 and a simulated nominal *P*-value of 0.00726. The corresponding overall significance corrected for multiple testing (family-wise type I error rate) is $P = 0.82$. Interestingly, this four-marker combination does not include rs2270465 that yielded the best single-marker result. The above-mentioned haplotype occurs in 11 out of the 100 trios, with one copy per family, and is transmitted in all 11 cases to the affected child (9 paternal and 2 maternal transmissions). The computationally intensive analysis for combinations of up to six markers using the "zhaomax" option resulted in the same maxTDT statistic of 11.0, obtained for rs237851-rs6791619-rs53576-rs237884, when adding either one or two markers of the set (rs888566, rs2301260, rs2228485, rs237902, rs237897, rs2268495, rs237895, rs237894, rs237893, rs13316193, and rs4686301) to the original marker combination. For all the resulting 66 additional five- or six-marker combinations, the simulated nominal *P*-values ranged between 0.0039 and 0.0095, i.e., there was only a marginal improvement. The fact that the results for these new combinations are similar to the one obtained for the haplotype T-G-T-T at rs237851-rs6791619-rs53576-rs237884 can be explained by the strong linkage disequilibrium, as measured in D' units, especially for all markers between rs2228485 and rs53576 (Fig. 2). Hence, the four markers rs237851, rs6791619, rs53576, and rs237884 act as tagging SNPs, which capture the full association with the trait. Since, however, rs2270465 is not

included in this combination (nominal *P* = 0.12 for rs237851-rs6791619-rs2270465-rs53576-rs237884), this marker seems to have an independent effect on the risk to develop ASD.

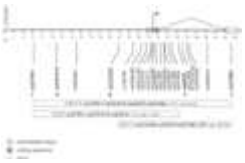

Figure 1. Top: Schematic representation of the oxytocin receptor (*OXTR*) gene with the 22 single-nucleotide polymorphisms (SNPs) analyzed in the current study. The underlined SNP indicates the SNP showing nominally significant association with autism on high-functioning level in the single-marker association analysis. The bold SNPs indicate SNPs of the marker combination (rs237851-rs6791619-rs53576-rs237884) yielding the strongest evidence of association (haplotype T-G-T-T). Bottom: Mainly observed nominally significant haplotypes. D3S3691: marker with the highest NPL score in the combined analysis of the primary genome scan data of two autism samples [Ylisaukko-oja et al., [2006]].
[Normal View 37K | Magnified View 75K]

Table II. Genotypes and TDT Results for 22 *OXTR* SNPs in 100 Trios With Asperger Syndrome, High-Functioning Autism, and Atypical Autism

No.	SNPs[ab]	Alleles[c]	Location	Genotypes (%)[d]	Allele frequency[de]	Transmission rate[f]	Nominal *P*-value[g]
1	rs237851	-24850C > T	5′ region	C/C 24 (24.0)	C: 0.505 (0.525)	0.54	0.513
				C/T 53 (53.0)			
				T/T 23 (23.0)	T: 0.495 (0.475)		
2	rs6791619	-19999G > A	5′ region	G/G 47 (47.0)	G: 0.680 (0.675)	0.49	0.920
				G/A 42 (42.0)			
				A/A 11 (11.0)	A: 0.320 (0.325)		
3	rs888566	-15868A > G	5′ region	A/A 57 (57.0)	A: 0.755 (0.7575)	0.51	1.0
				A/G 37 (37.0)			
				G/G 6 (6.0)	G: 0.245 (0.2425)		
4	**rs2270465**	**-7103C > G**	**5′ region**	**C/C 33 (33.0)**	**C: 0.620 (0.6775)**	**0.62**	**0.0185**
				C/G 58 (58.0)			
				G/G 9 (9.0)	**G: 0.380 (0.3225)**		
5	rs180789	-4054T > C	5′ region	T/T 53 (53.0)	T: 0.730 (0.720)	0.48	0.752
				T/C 40 (40.0)			
				C/C 7 (7.0)	C: 0.270 (0.280)		
6	rs2268498	-2538A > G	5′ region	A/A 29 (29.0)	A: 0.550 (0.550)	0.50	1.0
				A/G 52 (52.0)			
				G/G 19 (19.0)	G: 0.450 (0.450)		
7	rs2301260	-1356C > T	5′UTR, exon 1	C/C 86 (86.0)	C: 0.930 (0.9225)	0.45	0.715
				C/T 14 (14.0)			
				T/T 0 (0)	T: 0.070 (0.0775)		

8	rs237915	-438A > G	Intron 1	A/A 55 (55.0)	A: 0.740 (0.720)	0.46	0.464
				A/G 38 (38.0)			
				G/G 7 (7.0)	G: 0.260 (0.280)		
9	rs237913	-177G > T	Intron 2	G/G 55 (55.0)	G: 0.750 (0.725)	0.44	0.328
				G/T 40 (40.0)			
				T/T 5 (5.0)	T: 0.250 (0.275)		
10	rs2228485	171T > C	Exon 3, CDS	T/T 54 (54.0)	T: 0.740 (0.745)	0.51	0.906
				T/C 40 (40.0)			
				C/C 6 (6.0)	C: 0.260 (0.255)		
11	rs237902	690C > T	Exon 3, CDS	C/C 50 (50.0)	C: 0.710 (0.685)	0.45	0.351
				C/T 42 (42.0)			
				T/T 8 (8.0)	T: 0.290 (0.315)		
12	rs237897	1589T > C	Intron 3	T/T 13 (13.0)	T: 0.415 (0.400)	0.53	0.529
				T/C 57 (57.0)			
				C/C 30 (30.0)	C: 0.585 (0.600)		
13	rs2268495	2339G > A	Intron 3	G/G 60 (60.0)	G: 0.765 (0.7625)	0.49	1.0
				G/A 33 (33.0)			
				A/A 7 (7.0)	A: 0.235 (0.2375)		
14	rs237895	2451C > T	Intron 3	C/C 31 (31.0)	C: 0.575 (0.595)	0.54	0.457
				C/T 53 (53.0)			
				T/T 16 (16.0)	T: 0.425 (0.405)		
15	rs237894	3343C > G	Intron 3	C/C 57 (57.0)	C: 0.750 (0.730)	0.45	0.453
				C/G 36 (36.0)			
				G/G 7 (7.0)	G: 0.250 (0.270)		
16	rs237893	3924T > C	Intron 3	T/T 34 (34.0)	T: 0.570 (0.5525)	0.47	0.553
				T/C 46 (46.0)			
				C/C 20 (20.0)	C: 0.430 (0.4475)		
17	rs53576	5503C > T	Intron 3	C/C 40 (40.0)	C: 0.645 (0.6775)	0.57	0.217
				C/T 49 (49.0)			
				T/T 11 (11.0)	T: 0.355 (0.3225)		
18	rs237890	6217G > C	Intron 3	G/G 32 (32.0)	G: 0.570 (0.5775)	0.52	0.837
				G/C 50 (50.0)			
				C/C 18 (18.0)	C: 0.430 (0.4225)		

19	rs13316193	7131A > G	Intron 3	A/A 39 (39.0)	A: 0.610 (0.605)	0.49	0.923
				A/G 44 (44.0)			
				G/G 17 (17.0)	G: 0.390 (0.395)		
20	rs2254298	7646C > T	Intron 3	C/C 85 (85.0)	C: 0.925 (0.925)	0.50	1.0
				C/T 15 (15.0)			
				T/T 0 (0)	T: 0.075 (0.075)		
21	rs4686301	11288G > A	Intron 3	G/G 47 (47.0)	G: 0.705 (0.7025)	0.49	1.0
				G/A 47 (47.0)			
				A/A 6 (6.0)	A: 0.295 (0.2975)		
22	rs237884	16289T > C	3′UTR, exon 4	T/T 56 (56.0)	T: 0.755 (0.755)	0.50	1.0
				T/C 39 (39.0)			
				C/C 5 (5.0)	C: 0.245 (0.245)		

UTR, untranslated region; CDS, coding sequence.
Underlined: Alleles forming the T-G-T-T haplotype at rs237851-rs6791619-rs53576-rs237884.
Bold values represents SNP with significant p-value ($p < 0.05$).

[a] All SNPs were tested for Hardy-Weinberg equilibrium (all but rs2270465 [in index patients] $P \geq 0.05$).

[b] Numbers are given according to genomic entry NT_022517.17 and the *OXTR* translation start codon (nt + 1 is the A of ATG, which is located in exon 3). SNP alleles correspond to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/).

[c] Major/minor.

[d] Frequencies in the index patients with Asperger syndrome, high-functioning autism, and atypical autism.

[e] Frequencies in parents of the index patients with Asperger syndrome, high-functioning autism, and atypical autism.

[f] Of the minor allele.

[g] Of the simulation-based TDT implemented in FAMHAP (single-marker results).

Figure 2. Linkage disequilibrium between polymorphisms in the oxytocin receptor (*OXTR*) gene in the 200 unrelated parents of 100 individuals with autistic disorders on high-functioning level. Haploview http://www.broad.mit.edu/mpg/haploview/[Barrett et al., [2005]]. Linkage disequilibrium was measured in $R^2 \times 100$; the higher the R^2, the darker the field. Number of polymorphisms correspond to numbers in Table II. [Normal View 35K | Magnified View 68K]

In order to search for an intermediate phenotype that more closely reflects genetic variation than ASD per se, we formed two different groups of 11 children who carry the T-G-T-T haplotype at rs237851-rs6791619-rs53576-rs237884 and the remaining 89 children who do not. The analysis of the 12 subdomain scores showed nominally significant differences between the two groups for the items A1 (failure to use nonverbal behaviors to regulate social interaction), A2 (failure to develop peer relationships), A4 (lack of socio-emotional reciprocity), and B2 (relative failure to initiate or sustain conversational interchange) (see Table III).

Table III. Comparison (*t*-Test) in 12 Items and 3 Sum-Items of the ADI-R Algorithm in 11 Children Who Carry the T-G-T-T Haplotype at rs237851-rs6791619-rs53576-rs237884 (HTC) and in 89 Children Who do Not Carry This Haplotype (NC) (High Scores Reflect Greater Severity of Symptoms)

Item	Mean NC	Mean HTC	SD NC	SD HTC	*P**
A: Qualitative abnormalities in reciprocal social interaction	18.26	22.27	5.91	5.97	0.057
A1: Failure to use nonverbal behaviors to regulate social interaction	**3.65**	**4.73**	**1.72**	**1.55**	**0.052**
A2: Failure to develop peer relationships	**6.09**	**7.55**	**1.87**	**1.57**	**0.013**
A3: Lack of shared enjoyment	3.43	3.45	1.86	1.97	0.966
A4: Lack of socio-emotional reciprocity and modulation to context	**5.17**	**6.45**	**2.20**	**1.81**	**0.048**
B: Qualitative impairments of communication and language	14.35	15.64	4.75	6.20	0.519
B1: Delay in or lack of spoken language, not compensated by gesture	3.36	4.09	2.28	2.70	0.765
B2: Relative failure to initiate or sustain conversational interchange	**2.95**	**3.64**	**1.10**	**0.67**	**0.010**
B3: Stereotyped and repetitive of language and/or idiosyncratic use of words or phrases	3.30	3.00	1.88	2.24	0.674
B4: Lack of varied spontaneous make-believe play of social imitative play	4.38	4.91	1.52	1.64	0.331
C: Restricted, repetitive, and stereotyped behaviors and interests	5.66	6.36	2.60	3.17	0.495
C1: Total, encompassing, or circumscribed pattern of interest	1.98	1.64	1.06	0.50	0.085
C2: Total, apparently compulsive adherence to nonfunctional routines or rituals	1.57	1.82	1.19	1.40	0.589
C3: Total, stereotyped, and repetitive motor mannerisms	1.03	1.54	1.00	1.44	0.276
C4: Total, preoccupations with part of objects or nonfunctional elements or materials	1.08	1.36	0.96	1.29	0.491

HTC: Haplotype carriers - 11 children who carry the T-G-T-T haplotype at rs237851-rs6791619-rs53576-rs237884.

NC: Noncarriers - 89 children who do not carry the haplotype.

***P*: *t*-test.**

Bold values represents subdomain scores with nominally significant differences between the two groups (HTC and NC).

The sum of these four variables for each child, with means 22.36 and 17.87 in haplotype carriers and noncarriers, respectively, was used as new quantitative phenotype. The exploratory association test, looking for differences in this phenotype between carriers and noncarriers, yielded a *P*-value of 0.0099 ($t = 2.63$, df = 98, mean difference between the two groups: 4.50 score units).

These group differences were also found in the data concerning the "current" time period of the ADI-R in a very similar pattern: A1 ($P = 0.009$), A4 ($P = 0.014$), and B2 ($P = 0.052$) differ between the groups, as well as B1 (delay in or lack of spoken language, not compensated by gesture, $P = 0.019$). In sum, the domain of social interaction (A1-A4, $P = 0.012$) and communication (B1-B4, $P = 0.019$) also differ between the groups. Surprisingly, in the data of the ADOS no differences were found in the domains of social interaction and communication.

DISCUSSION

Animal studies and first studies in humans provide evidence for a substantial role of the oxytocin in social attachment and affiliation. Thus, the oxytocin and oxytocin receptor might be involved in the impairment of social interaction and attachment in ASD. To analyze whether genetic variation in the *oxytocin receptor* gene is implicated in autism, we genotyped 22 SNPs in a genetic region of 41 kb ranging from 3′UTR to 23.4 kb of the 5′ region in 100 families with autistic disorders on high-functioning level.

Only one SNP rs2270465 in the 5′ region of the *OXTR* gene was associated (nominally significant *P*-value of 0.01854) with autistic disorders on high-functioning level in the single-marker association analysis, although this result did not remain significant after correction for multiple testing. For all other 21 SNPs we found no association. This is in contrast to four recently published studies [Wu et al., [2005]; Jacob et al., [2007]; Lerer et al., [2008]; Yrigollen et al., [2008]]. In Chinese Han families with ASD two intronic SNPs (rs2254298, rs53576) were associated with autism [Wu et al., [2005]], whereas in 57 Caucasian trios an association was detected only for rs2254298 [Jacob et al., [2007]]. In our sample we cannot replicate these findings (rs2254298 $P = 1.0$; rs53576 $P = 0.22$). However, rs53576 is one component of the four-marker combination providing the haplotype T-G-T-T that displays a nominally significant association with autism in our sample. In a study of Israeli families with ASD, single-marker association analysis detected two further intronic SNPs (rs2268494, rs1042778) in nominal association with ASD [Lerer et al. [2008]], both SNPs were not genotyped in our sample. One intronic SNP (rs2268493, not genotyped in our sample either) was associated with ASD, with the phenotypic subdimensions communication skills and stereotyped behaviors [Yrigollen et al., [2008]]. Ylisaukko-oja et al. [2006] sequenced the entire protein coding sequence including splice sites of the *OXTR* gene and detected genetic variants (rs2228485,

rs4686302, rs237902, A238T) but none of these provided evidence for association. In the current study, for both coding SNPs rs2228485 and rs237902 we did not find evidence for transmission distortion. However, none of the published association studies included SNPs in the 5′ region of the *OXTR*, whereby a comparison of the detected nominal association for rs2270465 in our sample with other studies is not possible. SNP rs2270465 is located 5.7 kb upstream of the transcription start site of *OXTR*. So far no functional relevance is definitely known for this SNP. At the position of rs2270465 (c.-5675C > G) an A-allele is highly conserved in animal species (rhesus, macaque, mouse, dog, horse, armadillo, and opossum) (UCSC database: http://genome.ucsc.edu). rs2270465 (c.-5675C > G) occurs only in chimp and human (UCSC database, HAPMAP data). The infrequent G-allele (allele frequency in our sample: 0.38 in autistic patients and 0.32 in their parents) is more frequently transmitted by parents to the autistic patients. These data and moreover the deviation from HWE at rs2270465 in the affecteds suggest an accumulation of the G-allele in autistic patients. By reason that we found genotyping errors to be unlikely, we hypothesize that the deviation of HWE may be explained by selective effects arising from association with the trait. Furthermore, the highly conserved region around rs2270465 implicates a high probability of functional relevance for this SNP.

Further evidence for functional relevance of rs2270465 provides the position of the C > G exchange two bases distally to the core sequence TTCN$_3$GAA of the conserved transcription factor binding site for STAT5A (signal transducer and activator of transcription 5A; UCSC Genome browser http://genome.ucsc.edu/; in silico analysis using Transfac database and Patch software at www.gene-regulation.com). Because flanking nucleotides may affect the binding affinity of STAT5A to the two palindromic half-sites TTC$\cdots$GAA [Ehret et al., [2001]] an influence of rs2270465 on the binding affinity of STAT5A is conceivable. Thus the modification of this transcription factor-binding site may alter the expression of the *OXTR* gene and therefore contribute to the susceptibility to ASD. To clarify a role of rs2270465 in the etiology of ASD the independent confirmation of association in further autism samples, preferably in samples with autistic disorders on high-functioning level, are necessary.

Haplotype-based association studies for marker combinations with up to four markers revealed numerous haplotypes with simulated nominally significant *P*-values, although the results were no longer significant after correction for multiple testing. The best result was obtained for haplotype T-G-T-T of the marker combination rs237851-rs6791619-rs53576-rs237884 (see Fig. 1). This haplotype comprises the whole analyzed region in our study covering 41 kb, and therefore, a restriction to a narrow region possibly carrying a causal sequence variant associated with autism is not possible. Interestingly, among the nominally significant haplotypes we found predominantly two haplotypes including either rs237851-rs6791619-rs53576 (T-G-T; 11 transmitted vs. 1 nontransmitted) or rs2270465-rs53576-rs237884 (G-T-T; 28 transmitted vs. 10 nontransmitted) (see Fig. 1). The haplotype G-T-T includes rs2270465 with the only nominally significant *P*-value in the single marker association. Furthermore, both haplotypes T-G-T and G-T-T as well as the best haplotype T-G-T-T comprise the T-allele of the SNP rs53576 which is associated with autism and involved in haplotypes significantly associated with autism in the Chinese Han population [Wu et al., [2005]]. Additionally, specific haplotypes associated with ASD scores were described [Lerer et al., [2008]]. These haplotypes significantly associated with ASD clustered close to the exon 3/intron 3 boundary, a region also comprised by haplotypes G-T-T and T-G-T-T in our study. In contrast to the finding that the longer haplotypes associated with ASD included rs237897 (intron 3) as their first 5′ SNP [Lerer et al., [2008]], all nominally significant haplotypes associated with autism extended into the 5′ region of the *OXTR* in the current study. On the basis of the extension of the most significant haplotype over the whole *OXTR* gene and its 5′ region, we hypothesize that decisive regulatory elements in the noncoding region or in the 5′ region of the *OXTR* gene are located on this haplotype or are in linkage disequilibrium with rs2270465.

In our exploratory association analysis we detected a nominally significant difference between the 11 carriers of the T-G-T-T haplotype at rs237851-rs6791619-rs53576-rs237884 and the 89 noncarriers for the sum of 4 items of the 12 subdomain scores of the ADI-R for the early development (A1, A2, A4, and B2) (see Table III) as well as for the current time period. The applied *t*-test should clearly be regarded as explorative, since the selection of the constituent items was based on a large difference between haplotype carriers and noncarriers. However, this observation is notably interesting because by analyzing all 12 subdomain scores of the ADI-R only 4 items showed nominally significant differences between haplotype carriers and noncarriers, for the early development as well as for current symptoms. These subdomains of the ADI-R are of high relevance for socio-emotional aspects of interpersonal relationships. Behaviors like "eye contact," "social smile," "offers comfort," "group play," "interests in children," "reciprocal conversation," "social vocalization/chat," "quality of social overtures," and "appropriateness of social responses" are included. These are core symptoms of autistic disorders. By contrast, analysis of items of the ADOS-G (current symptoms) algorithm revealed no differences. This may have a reason in the fact that the ADOS takes data for a short time period of observation (mostly about 1 hr) and group differences within the autistic spectrum get lost. For substantiation of these observations of genotype-phenotype association, replications in independent samples are necessarily warranted.

Applying the approach of "reverse phenotyping" [Schulze and McMahon, [2004]] we detected a subgroup with a more specifically defined phenotype of autism on high-functioning level in light of the genotype (haplotype) data. This phenotype is characterized by a particular impairment of social interaction and communication that are important for regulation of affiliate behavior and social bonding. Interestingly, the identified differences are part of the social interaction and the communication domain. This corroborates an earlier finding that these behavior domains are interrelated [van Lang et al., [2006]; Georgiades et al., [2007]; Kamp-Becker et al., [2009]]. These findings are notable in light of the hypothesis that the majority of genes relevant to ASD may be specific for core symptoms and a minority of genes may overlap between domains [Happe et al., [2006]]. This implicates recruitment and application of more homogenous samples/subsamples with specific autistic traits for the elucidation of genetic causes of ASD. Moreover, Skuse [2007] hypothesize that the mental retardation of the majority of autistic patients considerably hamper the detection of candidate genes for ASD and that genetic homogeneity could be easier to obtain in high-functioning autistic individuals with normal-range intelligence and good structural language skills. Under consideration of these aspects our sample, composed of children with ASD

with a normal-range intelligence (IQ ≥ 70) and clear autistic symptoms surveyed with standardized methods, has a potential in the way of elucidation of genes underlying the susceptibility to ASD.

In conclusion, despite many open questions concerning the complex regulation of the oxytocin receptor, a crucial role of the oxytocin receptor in prosocial behavior, affiliation, and social communication is as far as possible assured and supported by a number of animal [Liu et al., [2005]; Takayanagi et al., [2005]; Lim and Young, [2006]] and human [Kirsch et al., [2005]; Domes et al., [2007a],[b]; Hollander et al., [2007]; Bartz and Hollander, [2008]; Guastella et al., [2008]] studies. Genome wide linkage studies [Shao et al., [2002]; McCauley et al., [2005]; Lauritsen et al., [2006]; Ylisaukko-oja et al., [2006]] suggested the *OXTR* gene as a plausible candidate gene for ASD. First association studies including our current study provide evidence for association of genetic variants and haplotypes in the *OXTR* with ASD [Wu et al., [2005]; Jacob et al., [2007]; Lerer et al., [2008]; Yrigollen et al., [2008]]. For replication of these association findings, further genetic analyses of the *OXTR* genomic region including their 5′ region are implicitly warranted in preferably genetically homogenous autistic samples like subgroups of ASD with different endophenotypes or samples with HFA. Furthermore, the intensive investigation of the oxytocin and oxytocin receptor provides the potential for the development of new therapeutic strategies for the treatment of disorders that are associated with deficits in social interactions like autism. After successful application of the "trust" hormone oxytocin as a nasal spray in human studies [Kirsch et al., [2005]; Kosfeld et al., [2005]; Domes et al., [2007a],[b]; Guastella et al., [2008]], clinical trials for investigation of potential of the intranasal oxytocin in the treatment of autism were initiated [Opar, [2008]].

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