The hyperserotonemia of autism spectrum disorders

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Chapter 3

Serotonin Transporter Intron 2 Polymorphism Associated With Rigid-Compulsive Behaviors in Dutch Individuals With Pervasive Developmental Disorder

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Abstract

Two putatively functional polymorphisms of the serotonin transporter gene (HTT, SLC6A4) were examined for associations with risk for pervasive developmental disorders (PDDs) and specific autism phenotypes. Dutch patients diagnosed with PDD (n = 125, age range 5–20 years, DSM-IV-TR based criteria, ADI-R and ADOS behavioral assessments) and their parents (n = 230) were genotyped for promoter ins/del (5-HTTLPR) and intron 2 variable number of tandem repeats (VNTR) alleles. Using the transmission disequilibrium test (TDT), no disorder-specific preferential transmission of promoter (long and short) or intron 2 (10- and 12-repeat) alleles was observed. However, multivariate analysis of continuous autism-related behavioral measures revealed that subjects with intron 2 12/12 genotype were significantly more impaired in the rigid-compulsive domain (p = 0.008). Quantitative TDT (QTDT) analysis also showed significant association of the intron 2 VNTR 12-repeat allele with rigidcompulsive behavior (p = 0.015). These results suggest that intron 2 VNTR alleles or nearby polymorphisms in linkage disequilibrium may play a role in specific aspects of the behavioral phenotype of autism.

Introduction

The serotonin transporter gene (HTT, SLC6A4) is of special interest in autism given the platelet hyperserotonemia of autism (Mulder et al., 2004), the treatment effects of serotonergic agents (McDougle et al., 2000), the role of serotonin (5-hydroxytryptamine, 5-HT) in neurodevelopment (Whitaker-Azmitia, 2001) and prior reports of genetic associations with disorder risk and specific autism phenotypes (Lauritsen and Ewald, 2001; Anderson, 2002; Kim et al., 2002). Over 20 HTT polymorphisms have been identified and two are of particular interest in neuropsychiatry given their apparent effects on serotonin transporter expression and functioning. One of the two functional variants is a 44-bp insertion/deletion polymorphism in the promoter region of the gene (5-HTTLPR) with long and short alleles, L and S; the other a variable number of tandem repeats (VNTR) polymorphism in the second intron with 9, 10, or 12 copies of the 17-bp repeat sequence. Most (Lesch et al., 1996; Hanna et al., 1998; Greenberg et al., 1999; Nobile et al., 1999; Anderson et al., 2002), but not all (Kaiser et al., 2002), functional studies of the 5-HTTLPR have found allelic effects on 5-HT uptake. In a transfection experiment, the intron 2 VNTR has been reported to affect transporter expression in the mouse hindbrain during embryonic development (MacKenzie and Quinn, 1999).

Possible linkage and association of HTT and its variants with autism have been examined by genome scanning, familybased, and case-control studies. Although initial genome scans in autism did not find significant linkage at 17q11-12, a chromosomal region that includes the SLC6A4 locus (IMGSAC, 1998; Barrett et al., 1999; Risch et al., 1999), the three most recent reports have all reported significant linkage at 17q11-12 (IMGSAC, 2001; McCauley et al., 2004; Yonan et al., 2003). Studies examining transmission of the promoter alleles in autism have produced inconsistent findings, with preferential L allele (Klauck et al., 1997; Yirmiya et al., 2001), preferential S allele (Cook et al., 1997; Kim et al., 2002; McCauley et al., 2004; Conroy et al., 2004) or no preferential transmission (Maestrini et al., 1999; Persico et al., 2000; Tordjman et al., 2001; Persico et al., 2002; Betancur et al., 2002) having been reported. Several of the studies also explored the transmission of intron 2 VNTR alleles, with most reporting an absence of preferential transmission (Cook et al., 1997; Klauck et al., 1997; Maestrini et al., 1999; Betancur et al., 2002). However, Kim et al. (2002) did observe preferential transmission of the 12-repeat allele. In studies examining combinatorial effects, two groups have found preferential

transmission of an S-12 haplotype consisting of the 5-HTTLPR short allele and the intron 2 VNTR 12- repeat allele (Cook et al., 1997; Kim et al., 2002) and another has reported preferential transmission of a three variant haplotype that included the S-allele and the intron 2 VNTR 12- repeat allele (Conroy et al., 2004). Preferential transmission of a L-12 haplotype has also been reported (Klauck et al., 1997).

Much less attention has been paid to possible linkage or association with specific components or domains of autism related behavior. Tordjman et al. (2001) used Autism Diagnostic Interview-Revised (ADI-R; Lord et al., 1994) domain scores to construct severely or mild/moderately handicapped subgroups and found preferential transmission of the L-allele in the mild/moderately social and communication handicapped group. In a more recent study of multiplex families, McCauley et al. (2004) reported their highest genome-wide LOD score (3.62 at 17q11.2) in a subset of families with high ADI-Rderived rigid-compulsive factor scores.

In this study, we aimed to re-examine possible associations between 5-HTTLPR and intron 2 VNTR alleles and disorder risk, and between the alleles and domain severity. The associations were examined in a large, relatively homogeneous group of well-characterized Northern Dutch subjects.

Method

Subjects and Assessment

Children and young adults with pervasive developmental disorders (PDD) and their parents were recruited through an epidemiological survey carried out in the north of the Netherlands, and through an Autism Outpatient Clinic affiliated with the Child and Adolescent Psychiatry Center of Groningen. The age range for subjects included in the study was 5-20 years, corresponding to the school-age range used as inclusion criterion in the epidemiological survey. Subjects were excluded from the study if they had a known genetic condition or severe peri/prenatal problems. All subjects were of Northern Dutch descent.

Subjects were assessed using the Autism Diagnostic Interview-Revised (ADI-R; Lord et al., 1994) and the Autism Diagnostic Observation Schedule-G (ADOS-G; Lord et al., 2000) administered by trained examiners. Subjects were diagnosed and their intellectual functioning evaluated as previously described (Mulder et al., 2004). Behavioral expression in the social-communication and stereotyped domains was

assessed using factor scores based on items from the ADI-R as per Tadevosyan-Leyfer et al. (2003). As described, 6 factors were obtained of which the factors 'social intent' and 'rigid-compulsive' most closely represented these two domains (Tadevosyan-Leyfer et al., 2003). The 'spoken language' factor was also included in our analyses, since this factor contains items measuring communicative aspects of autism.

DNA was collected by means of mouth-swaps from 125 patients and their parents and/or siblings. In five cases where attributed parentage was inconsistent with child and parent genotypes, typings were repeated and the families subsequently excluded. From the remaining 120 families, 110 were trios of 2 parents and one patient, 7 trios consisted of a mother, patient and an unaffected sibling and 3 were duo's of a mother and a patient. Group characteristics for the 120 patients are presented in Table 3.1.

Table 3.1. Clinical Characteristics of the Sample (mean ± SD)

	Autism	***Asperger***	***PDD-NOS***	***total group***	***Statistic$_{df}$; p***
N	51	4	65	120	
Age, yrs	11.0 ± 4.0	11.6 ± 1.7	11.7 ± 3.7	11.4 ± 3.8	$F_{2,117}$=0.52; .60
IQ	44 ± 25	111 ± 13	52 ± 29	52 ± 29	$F_{2,117}$=11.8; <.001[a]
Gender, n, M/F	44/7	3/1	48/17	95/25	
Gender, % male	86.3%	75%	73.8%	79.2%	X^2_2=2.72; .26
ADI-R Social Intent	28.8 ± 9.0	19.5 ± 6.8	21.1 ± 9.5	24.3 ± 9.9	$F_{2,117}$=10.4 ; <.001[b]
ADI-R Spoken Language	18.7 ± 12.0	6.5 ± 2.7	10.8 ± 9.9	14.0 ± 11.4	$F_{2,117}$ =8.89; <.001[c]
ADI-R Compulsions	6.4 ± 4.2	5.3 ± 4.0	5.1 ± 4.1	5.7 ± 4.1	$F_{2,117}$=1.42; .25

NOTE: a. Posthoc Tukey's HSD: Autism & PDD-NOS vs Asperger, p<.001. b. Posthoc Tukey's HSD: Autism vs PDD-NOS, p<.001. c. Posthoc Tukey's HSD: Autism vs PDD-NOS, p<.001.

DNA Analyses

DNA was extracted from cheekcells obtained by mouth-swap using the Epicentre MasterAmp™ DNA Extraction Solution Kit (BiozymTC, Landgraaf, the Netherlands). The intron 2 VNTR and the promoter variant were analyzed using the method of Cook et al. (1997) with slight modification. Primers were synthesized on an Applied Biosystems 380B DNA synthesizer at the General Clinical Laboratory of the University Medical Center Groningen.

Statistical Analyses

Possible diagnostic group differences in demographic variables and behavioral ratings were analyzed using analysis of variance (ANOVA) or X^2 test where applicable. The X^2 test was performed to test for deviations from the Hardy–Weinberg equilibrium. The transmission disequilibrium test (TDT) for family-based association analysis (Spielman et al., 1993) was used to test association of any allele with pervasive developmental disorder (PDD), autism or PDD-NOS. Possible association of 5-HTTLPR–intron 2 VNTR haplotypes with risk was examined by TDT analysis, cell size considerations precluded such analyses in behaviorally-defined subgroups. Haplotypes were constructed by hand. The ADI-R-derived domain scores, 'social intent,' 'spoken language,' and 'rigid-compulsive' behaviors (Tadevosyan-Leyfer et al., 2003), observed across genotypes were normally distributed and were compared using one-way ANOVA. When an ANOVA was significant, post-hoc Tukey HSD tests were performed to test the differences between the genotype groups. When the genotype comparisons revealed significant or trends to significant differences, association patterns of the ADI-R-derived domains with the HTT alleles were further evaluated with a family-based quantitative transmission disequilibrium test (QTDT, Abecasis et al., 2000a and 2000b).

In order to compare directly our results with prior reports, we also performed subgroup TDT analyses in the social/communicative and the rigid/compulsive domains using the same analytic approaches reported by Tordjman et al. (2001) and McCauley et al. (2003).

The α-value was set at $p = 0.05$ for all analyses and the p values presented are uncorrected. Bonferroni correction factors of 2 (number of polymorphisms tested) and 3 (number of domains) could be applied to P values obtained for the risk analyses and the behavioral domain analyses, respectively.

Results

Risk Association Analyses

The observed genotype distribution did not deviate significantly from that expected according to the Hardy-Weinberg equilibrium in the parents and siblings for both the 5-HTTLPR and intron 2 VNTR polymorphisms. However, there tended to be a greater number of S/L probands than predicted by H-W considerations (S/S, S/L, and L/L: 17, 72, and 30 observed versus 24, 59, and 37 predicted; $X^2_2 = 6.013$, p =

0.05). The family-based TDT did not indicate preferential transmission of 5-HTTLPR or intron 2 VNTR alleles in the combined PDD group. In total, 54 S-alleles were transmitted versus 51 L-alleles (TDT $X^2_1 = 0.086$, $p = 0.77$). For the intron 2 VNTR, 2 of 7 9-repeat alleles (TDT $X^2_1 = 1.286$, $p = 0.26$), 67 of 125 10-repeat alleles (TDT $X^2_1 = 0.648$, $p = .42$), and 58 of 122 12-repeat alleles (TDT $X^2_1 = 0.295$, $p = 0.59$) were transmitted. Preferential transmission was also not observed when the autism and PDD-NOS groups were examined separately (data not given).

The transmission of 5-HTTLPR-intron 2 VNTR haplotypes, S/10, S/12, L/10, and L/12 was examined. TDT analysis indicated that S/10 (16 out of 32) and S/12 (29 out of 58) haplotypes were not preferentially transmitted; however, trends to increased transmission of the L/10 (36 out of 57, TDT $X^2_1 = 3.947$, $p = .047$) and reduced transmission of L/12 were observed (20 out of 52, TDT $X^2_1 = 2.769$, $p = 0.096$).

Behavioral Domain Association Analyses

Genotype comparisons. Promoter alleles: groups defined by HTT promoter genotype (S/S, L/S, and L/L: n=19, 72, 30, respectively) did not differ significantly in terms of mean ADI-R factor scores for 'social intent' ($F_{2,118} = 1.01$, $p = 0.37$) and 'spoken language' ($F_{2,118} = 0.129$, $p = 0.88$). The 'rigid-compulsive' factor scores showed a trend towards a genotype effect ($F_{2,118} = 2.13$, $p = 0.12$), with the highest mean (± SD) scores being observed in the S/S subgroup (S/S, L/S, and L/L scores 7.35 ± 3.53, 5.13 ± 3.82, 5.73 ± 4.71, respectively).

Intron 2 VNTR alleles: neither the ADI-R factor 'social intent' ($F_{2,117} = 0.637$, $p = 0.53$) nor 'spoken language' ($F_{2,117} = 1.481$, $p = 0.23$) factor scores differed across intron 2 VNTR genotypes. However, as seen in Figure 3.1, the 'rigid-compulsive' factor scores were significantly dependent upon genotype, with the highest severity scores observed for the 12/12 genotype, intermediate severity scores seen for the 10/12 genotype, and lowest severity scores seen in the 10/10 group ($F_{2,117} = 5.068$, $p = 0.008$, post-hoc Tukey's HSD: 10/10 versus 12/12, $p = 0.022$, 10/12 versus 12/12, $p = 0.003$). Genotypes containing the 9-repeat allele were not included in these analyses, since only two subjects carried this allele (one 9/10 genotype and one 9/12 genotype).

Family-based QTDT analyses. Possible association of the 'rigid-compulsive' factor with 5-HTTLPR or intron 2 VNTR alleles was further evaluated by performing

QTDT with each polymorphism. Results of these two QTDT's revealed no significant association of either of the promoter alleles (F_{118} = 0.00, p = 1.0), but a significant association of the 12-repeat allele of the intron 2 polymorphism with the 'rigid-compulsive' factor was found (F_{119} = 6.16, p = 0.015).

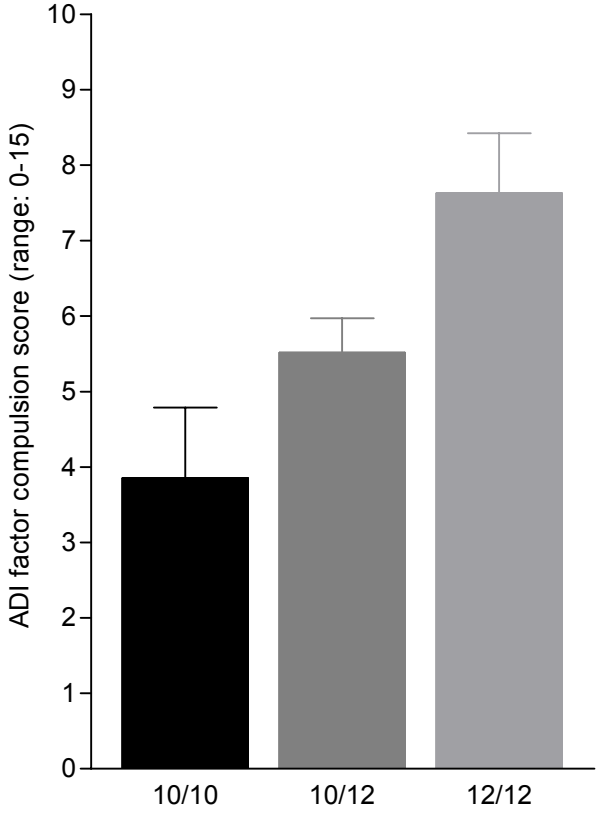

Figure 3.1: Autism Diagnostic Interview-Revised (ADI-R) rigid-compulsive factor score (mean and SE) per HTT intron 2 variable number of tandem repeats (VNTR) genotype (10/10 (n=20), 10/12 (n=74), 12/12 (n=24)). The rigid-compulsive score ranges from 0 to 15. Differences between genotype groups were significant ($F_{2,117}$=5.068, P=0.008, post-hoc Tukey's HSD: 10.10 vs. 12/12, P=0.022, 10/12 vs. 12/12, P=0.003).

Subset TDT Analyses. Following the method of Tordjman et al. (2001), the transmission of alleles was examined in subsets defined by subjects' combined social/communication severity score. TDT in the mild/moderately impaired group showed that neither 5-HTTLPR allele was preferentially transmitted (L-allele transmitted: 35, not-transmitted: 40, TDT X^2_1 = 0.333, p = .56). TDT analysis was not performed in the severely impaired group, as the group only contained 10 subjects. Also when domain scores were compared across genotype groups using the same method as Tordjman et al (2001) no significant differences were found (average soc-comm domain scores were 1.59, 1.59, 1.47 in the S/S, L/S and L/L genotype groups, respectively; Kruskal-Wallis X^2 = 0.515, p = .77).

Analyses of possible preferential transmission of 5-HTTLPR and intron 2 VNTR alleles was also performed in subsets defined by a median-split of probands based on 'rigid-compulsive' factor scores. The 'high' and 'low rigid-compulsive' subsets so

formed had mean (± SD) factor scores of 2.2 ± 1.7 and 9.0 ± 2.8, respectively. No preferential transmission was observed for 5-HTTLPR alleles; and also the 12-repeat intron 2 allele was found not to be preferentially transmitted in the 'high compulsions' subset (L-alleles–transmitted: 25, not transmitted: 24, TDT X^2_1 = 0.020, p = .89 / 12 repeat alleles–transmitted: 29, not transmitted: 27, TDT X^2_1 = 0.071, p = .79).

Discussion

This study revealed no significant associations between PDD disorder risk and either 5-HTTLPR or intron 2 VNTR alleles, and this was true for the autism and PDD-NOS subgroups as well. These findings are not inconsistent with the accumulated prior studies. Of the 11 family-based studies of the 5-HTTLPR, 5 showed no association, 2 showed an association to the L-allele, and 4 to the S-allele of the HTT promoter. Five of the six studies examining the intron 2 polymorphism reported no association with autism.

There was also no strong indication that 5-HTTLPR-intron 2 VNTR haplotypes were preferentially transmitted. While two prior studies (Kim et al., 2002; Conroy et al., 2004) have found nominal or trend level significant preferential transmission of the short/12 repeat haplotype, we observed no tendency (29 transmissions observed; 29 expected) for this haplotype to be transmitted to a greater extend that expected by chance.

The intron 2 VNTR 12-repeat allele was associated with severity of rigid-compulsive behaviors, both when using a genotype comparison approach and a QTDT analysis. On one hand, the results are consistent with McCauley et al.'s (2004) recent report of increased 17q11.2 linkage scores in the high rigid-compulsive subset. However, these investigators failed to find any preferential transmission of SLC6A4 markers in the rigid-compulsive subset. Their more recent examination of intron 2 VNTR allele transmission in the rigid-compulsive subset indicated that these alleles were also not preferentially transmitted (personal communication, J. S. Sutcliffe). In addition, in their sample, intron 2 VNTR genotypes were not significantly associated with rigid-compulsive factor scores and a QTDT analysis did not find significant association with rigid-compulsive factor scores.

Further examination of possible preferential transmission of intron 2 alleles and related haplotypes appears warranted. It is possible that the association we observed is due to specific patterns of linkage that may be present in the Northern Dutch

sample that was studied. Relative isolates might offer special advantages when attempting to identify the underlying genetic basis for the inconsistent, but intriguing, findings that have been reported for the HTT gene and autism. Although the genetic determinants of 'rigid-compulsive' behavior in autism are far from clear, the area appears to be critical and may provide general insight into the genetic control of obsessive-compulsive behaviors. Our and others' interest in the role of the HTT in compulsive and social behavior has recently increased due the reported association of a gain-of-function mutation in the HTT with obsessive-compulsive behavior and social problems (Ozaki et al., 2003). It can be noted that the apparent functional effect of the 12-repeat allele is to increase transporter expression in the developing mammalian brainstem (MacKenzie and Quinn, 1999), an effect that is at least superficially consistent with the physiology of the gain-of-function mutation. It can also be pointed out that both the gain-of-function mutation and the platelet hyperserotonemia of autism involve a presumed or apparent increase of the intracellular pool of serotonin relative to the extracellular compartment. This speculation concerning disposition or compartmentalization of serotonin is quite tenuous as there is no direct evidence of altered extracellular serotonin associated with the gain-of-function mutation or with autism, and the genetic and biochemical basis of the platelet hyperserotonemia of autism (and its relationship to central serotonin) is unclear.

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