

Genetic and expression analyses reveal elevated expression of syntaxin 1A (*STX1A*) in high functioning autism

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Abstract

Autism is a pervasive developmental disorder diagnosed in early childhood. Abnormalities of serotonergic neurotransmission have been reported in autism. Serotonin transporter (5-HTT), which modulates serotonin levels, is a major therapeutic target in autism. Therefore, factors that regulate 5-HTT expression might be implicated in autism. One candidate 5-HTT-regulatory protein is the presynaptic protein, syntaxin 1A (*STX1A*). We examined the association of *STX1A* with autism in a trio association study using DNA samples from 249 AGRE trios with autistic probands. Only male probands were selected, since autism is more prevalent among males. The probands of 102 trios had IQ > 70, and were considered as high functioning autism (HFA). In transmission disequilibrium test (TDT) analysis, rs2293485 ($p = 0.034$) and rs4717806 ($p = 0.033$) showed nominal associations with HFA; modest haplotype association was also observed. The SNPs that showed associations were related to early developmental abnormalities (ADI-R_D). We further compared *STX1A* mRNA expression in the lymphocytes of drug-naive HFA patients ($n = 12$) and age- and sex-matched controls ($n = 13$). *STX1A* expression in the HFA group was significantly higher ($p = 0.001$) than that of controls. Thus, we suggest a possible role of *STX1A* in the pathogenesis of HFA. During early childhood, there is a period of high brain serotonin synthesis that is disrupted in autistic children; *STX1A* might influence the serotonergic system during this stage of neurodevelopment, as implied by the association with ADI-R_D.

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Introduction

Autism is a pervasive developmental disorder diagnosed in early childhood, characterized by social and

communication deficits in combination with restricted and repetitive behaviours and interests (Kanner, 1943). Based on converging evidence from diverse studies, the serotonin (5-HT) system can be considered as a logical candidate in autism pathology. The 5-HT system has been found to be developmentally dysregulated in autism (Anderson, 1994). 5-HT acts as a trophic and differentiation factor (Lavdas et al., 1997), in addition to its role as a neurotransmitter. Hyper-serotonaemia has been observed in the platelets of

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subjects with autism (Ciaranello, 1982; Hanley et al., 1977).

Several studies have reported the association of serotonin transporter (5-HTT) polymorphisms with autism (Betancur et al., 2002; Cook et al., 1997; Klauck et al., 1997; Tordjman et al., 2001; Yirmiya et al., 2001). 5-HTT effects the recycling of 5-HT by mediating its presynaptic reuptake on termination of serotonergic neurotransmission; it is also the major target of selective serotonin reuptake inhibitors (SSRIs) (Moore et al., 2004), which have been proven to be efficient in reducing compulsive and stereotyped behaviours and hyperactivity in autism patients (McDougle et al., 1996). 5-HTT (SLC6A4) is located in 17q11.2, which has been implicated as an autism linkage region; however, other studies have reported different results with stronger linkage findings (Freitag, 2007). 5-HTT gene promoter variants have been suggested to exert a detectable, albeit small effect on 5-HT blood levels in autistic individuals (Lesch et al., 1996); however, as suggested by Persico et al. (2002) enhanced 5-HT blood levels in autism patients, but not in first-degree relatives with the promoter variant, indicate interactive contributions to hyperserotonemia both by 5-HTT variants and by other autism-related loci. Thus, factors that regulate the cell surface expression of 5-HTT might also have a crucial role in the regulation of serotonergic neurotransmission, and therefore, in the pathology of autism.

One candidate 5-HTT-regulatory protein is the presynaptic protein, syntaxin 1A (STX1A); this 35-kDa membrane protein belonging to the syntaxin family (Bennett et al., 1992, 1993; Inoue et al., 1992) was originally identified as a neuron-specific antigen (Barnstable et al., 1985). Together with the synaptosome-associated protein 25 kDa (SNAP-25) and the vesicle-associated membrane protein (VAMP), STX1A forms the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, which is vital for chemical neurotransmission (Rothman, 1994; Sollner et al., 1993).

In-vitro studies have shown that STX1A interacts with 5-HTT, regulating the subcellular localization and expression of 5-HTT (Haase et al., 2001; Quick, 2002). *STX1A* gene is located at 7q11.23; recently, speech delay and autism spectrum behaviours have been found to be associated with duplication of 7q11.23 (Berg et al., 2007; Depienne et al., 2007).

In this trio association study, we examined the SNP and haplotype associations of *STX1A* with autism; we also examined the association of *STX1A* SNPs with ADI-R phenotypic data. Further, we compared the expression of *STX1A* mRNA in the peripheral blood

lymphocytes (PBL) of drug-naive autistic patients and control subjects, since lymphocytes are considered as a convenient and accessible alternative to brain samples for biochemical and genetic investigations of the functions of central nervous system (CNS) (Gladkevich et al., 2004). We also examined the relationship between mRNA expression and genotypes in the lymphocyte samples.

Method

Association study

Subjects

This study was approved by the Ethics Committee of Hamamatsu University School of Medicine. We obtained DNA samples from trio families recruited to the Autism Genetic Resource Exchange [AGRE (<http://www.agre.org>); Geschwind et al., 2001]; 249 trio families with a male proband diagnosed for autism according to Autism Diagnostic Interview – Revised (ADI-R; Lord et al., 1994) and Autism Diagnostic Observation Schedule (ADOS; Lord et al., 1989), were selected. Out of the 249 trios, 235 trios were derived from multiplex families; only male probands [age 9.65 ± 4.87 yr (mean \pm s.d.)] were selected, since autism has a strong gender bias with a 4:1 (male: female) gender ratio. Additional selection criteria required that (i) there be no possible non-idiopathic autism flag and (ii) all the trios be Caucasian. ADI-R data was available for all the 249 affected individuals; however, ADOS testing was available for only 163, and intelligence quotient (IQ) testing for only 112 out of the 249 autistic individuals. The autistic probands of 102 trios had IQ > 70, and were considered as the high functioning autism (HFA) group.

We had taken care to exclude the commonly known genetic causes of autism (e.g. fragile X syndrome), and other neurological disorders such as tuberous sclerosis and neurofibromatosis.

Mutation screening and marker selection

The genomic structure of *STX1A* is based on the UCSC May 2004 draft assembly of human genome (<http://www.genome.ucsc.edu>). *STX1A* consists of 10 exons spanning a genomic stretch of 20.42 kb (mRNA 2064 bases) (Figure 1). In 20 unrelated autism samples selected randomly from among the AGRE probands, all the exons, splice boundaries, and 5'- and 3'-flanking regions of the gene were screened for polymorphisms by direct sequencing of the respective PCR products. Primer sets used for PCR amplification are listed in

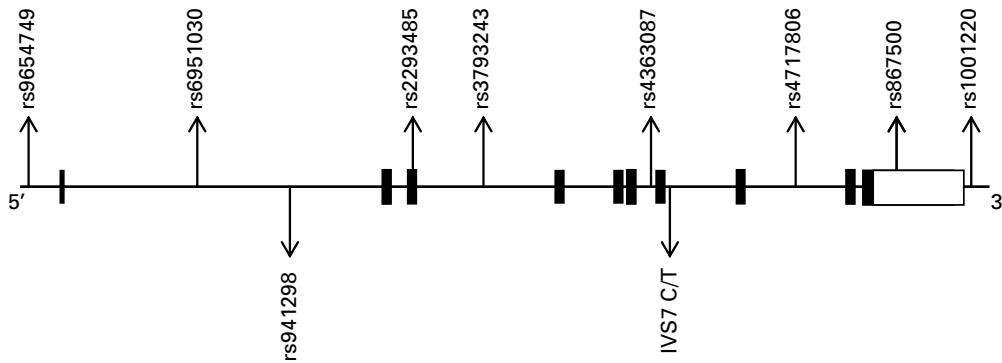


Figure 1. Genomic structure of *STX1A* and locations of SNPs. Exons are indicated by boxes, with translated regions in closed boxes, and untranslated regions in open boxes; SNP positions are denoted by arrows.

Table 1. PCR was performed with an initial denaturation at 95 °C for 12 min, followed by 35 cycles at 94 °C for 30 s, 55–60 °C (optimized for each primer pair) for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min, using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). Direct sequencing of PCR products was done using the BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems) in ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Additional SNPs were selected using the information from International HapMap Project (<http://www.hapmap.org>) and National Centre for Biotechnology Information (NCBI dbSNP: <http://www.ncbi.nlm.nih.gov/SNP>). On the basis of their genomic locations and minor allele frequencies (MAF >0.1) in the Caucasian population, 10 SNPs were chosen for our analysis, to span the *STX1A* gene as evenly as possible. The SNPs used in the study, and their locations are shown in Table 2.

Genotyping

Assay-on-demand/Assay-by-design SNP genotyping products (Applied Biosystems) were used to score SNPs, based on the TaqMan assay method (Ranade et al., 2001). Genotypes were determined using the ABI 7900 Sequence Detection System (SDS; Applied Biosystems), and analysed using SDS version 2.0 software (Applied Biosystems).

Statistical analysis

PedCheck program version 1.1 (<http://www.watson.hgen.pitt.edu>) was used to identify and eliminate all Mendelian inheritance inconsistencies in the trio genotype data. All the SNPs were tested for

Hardy–Weinberg Equilibrium (HWE) using the Haploview software version 3.2 (<http://www.broad.mit.edu/mpg/haploview>). Markers were tested for association by transmission disequilibrium test (TDT), using the TDTPHASE program of the UNPHASED software package version 2.403 (<http://portal.litbio.org>); expectation maximization (EM) algorithm was used to resolve uncertain haplotypes, to infer missing genotypes and to provide maximum-likelihood estimation of frequencies.

One-way analysis of variance [ANOVA; GraphPad Prism, version 4.00 (GraphPad Software, San Diego, CA, USA)] was used to examine the variability in the distribution of ADI-R phenotypic data [ADI-R_A (social interaction), ADI-R_BV (verbal communication), ADI-R_C (restricted, repetitive and stereotyped patterns of behaviour), ADI-R_D (developmental abnormality prior to 36 months)] across the homozygous and heterozygous genotypes of SNPs that showed associations in single SNP TDT.

Linkage disequilibrium (LD) plot was constructed using the r^2 correlation coefficient (Devlin and Risch, 1995) pair-wise LD values between markers were estimated using Haploview software. Subsequently, associations of haplotypes (frequency >0.01) belonging to the various haploblocks of *STX1A* were also examined using Haploview.

Gene expression analysis

Subjects

We obtained blood samples from 12 drug-naive autistic patients (age 21.4 ± 2.31 yr) and 13 age-matched (22.3 ± 1.93 yr) healthy controls. All the patients and controls were males, and of Japanese origin; written informed consent was obtained from all the participants.

Table 1. PCR primers used for the mutation screening of *STX1A* gene

Region	Primer	Sequence	Product size (bp)
Promoter 1	P1-F	5'-GTGGCGAGCAACTCTAGTCC-3'	256
	P1-R	5'-GAAGCCCTGGGGTCTCGT-3'	
Promoter 2	P2-F	5'-AGAGGAGGCAGCAGGGTCT-3'	292
	P2-R	5'-GGTTCGGTCCTTCATGCTC-3'	
Exon 1	E1-F	5'-GTCGCGCATGCGGGGCTCAC-3'	190
	E1-R	5'-CGGCCACCATCCTGGCCGCG-3'	
Exon 2	E2-F	5'-GCACCTGCGTGTGTACAGGT-3'	238
	E2-R	5'-GCAGGTGTGAAAGAGCACTG-3'	
Exon 3	E3-F	5'-CCTACTCTGGGCCATCTCTG-3'	297
	E3-R	5'-AGACACTCCTAAGCCCACCA-3'	
Exon 4	E4-F	5'-GGCTGAGCCTGCACATCA-3'	227
	E4-R	5'-CTCAGGGCGAGCAAAACC-3'	
Exons 5 & 6	E5, 6-F	5'-GGTGGATAGATGCTGGGTGT-3'	429
	E5, 6-R	5'-GCACTCAGCAGCAGACTCG-3'	
Exon 7	E7-F	5'-CCAATGCTGCTGCTGAAC-3'	203
	E7-R	5'-CTAGGGGTCATGGACGTAGG-3'	
Exon 8	E8-F	5'-GCTTGGGAGGAAGCCTCAG-3'	338
	E8-R	5'-CAGAAAGGGCGAGGTTAGTG-3'	
Exon 9	E9-F	5'-CTCAGGGTGCTTTCTGAAG-3'	295
	E9-R	5'-GAAGGGAGAGAGGTGGGATG-3'	
Exon 10	E10-F	5'-TCCTCAGCCTTTGCCATAGT-3'	299
	E10-R	5'-ATGGCAGAGAAGGGAGCAT-3'	
Exon 10 UTR1	E10U-1F	5'-AAACTGCCACTCCACTCCAG-3'	294
	E10U-1R	5'-AGGAAGGGTGGCCTGTGT-3'	
Exon 10 UTR2	E10U-2F	5'-CAGTGTGCACAGCGAGGA-3'	274
	E10U-2R	5'-GCAGTGATCAGCGACTGAGA-3'	
Exon 10 UTR3	E10U-3F	5'-CACATGGTGCTGCTTTTCAG-3'	300
	E10U-3R	5'-GACTGACTGCAGCCTGCTT-3'	

UTR, Untranslated region.

The autistic patients were diagnosed according to ADI-R (Lord et al., 1994), by trained and certified psychiatrists (K.J.T., A.S.). All patients met the autism criteria of DSM-IV (APA, 1994) and ICD-10 (WHO, 1992). The patients underwent screening, and were excluded if they had any major medical or psychiatric conditions. We had taken care to exclude the commonly known genetic causes of autism (e.g. fragile X syndrome), and other neurological disorders such as tuberous sclerosis and neurofibromatosis. All the patients were of the HFA type; the average IQ scores were measured by the Wechsler Adult Intelligence Scale – Revised (WAIS-R; Wechsler, 1981, Japanese version).

We used the following instruments for the assessment of autistic symptoms. Comorbid anxiety and depressive symptoms were assessed using the Hamilton Anxiety Rating Scale (HAMA; Hamilton, 1959, Japanese version) and the Hamilton Depression Rating Scale (HAMD; Hamilton, 1960, Japanese ver-

sion), respectively. Obsessional/repetitive behaviours were clinically rated using the Yale–Brown Obsessive–Compulsive Scale (YBOCS; Goodman et al., 1989a, b, Japanese version). Additional aggression symptoms were also assessed using the aggression questionnaire (AQ; Buss and Perry, 1992, translation in Japanese). A faux pas detection task was used to measure theory of mind (Baron-Cohen et al., 1999, Stone et al., 2003, translation in Japanese). All the evaluations were conducted by a trained research psychiatrist (K.N.).

All the controls were free of medications, and underwent screening to exclude neurological, developmental, or psychiatric disorders and mental retardation; none of them met any of the relevant criteria of DSM-IV. The average IQ of the control subjects (measured by WAIS-R, Wechsler, 1981, Japanese version) did not differ significantly from that of the HFA patients.

Table 2. Single SNP transmission disequilibrium test (TDT) results of *STX1A* SNPs

Marker	dbSNP ID	Variation ^a	Location	Minor allele frequency ^b	HFA trios (IQ > 70)		249 trios	
					T (%) ^c	<i>p</i> value ^d	T (%) ^c	<i>p</i> value ^d
SNP01	rs9654749	C/T	-6425	0.127	50.61	0.538	50.71	0.242
SNP02	rs6951030	T/G	IVS1 + 691	0.183	50.48	0.681	49.58	0.575
SNP03	rs941298	G/A	IVS1 + 8669	0.343	46.62	0.054	49.84	0.893
SNP04	rs2293485	C/T	204 (Exon 3) D68D	0.429	45.37	0.034	49.47	0.699
SNP05	rs3793243	G/A	IVS3 + 1572	0.403	45.99	0.053	49.10	0.491
SNP06	rs4363087	T/C	IVS6 + 282	0.389	46.5	0.075	49.65	0.782
SNP07	Intron 7	C/T	IVS7 + 52	0.451	52.97	0.191	50.55	0.701
SNP08	rs4717806	T/A	IVS8 + 698	0.339	46.3	0.033	49.69	0.788
SNP09	rs867500	G/C	1164 (Exon 10 UTR)	0.342	46.57	0.050	49.85	0.894
SNP10	rs1001220	T/C	+2877	0.389	46.15	0.050	49.53	0.714
Global <i>p</i> ^e					0.124			

HFA, High functioning autism; T, transmitted; UTR, untranslated region.

^a Common allele is listed first.

^b Based on the parental genotypes of 249 trios.

^c T% of common allele is listed.

^d Computed on the basis of likelihood ratio test; significant *p* values (<0.05) are indicated in bold italics.

^e 10 000 permutations.

Lymphocyte RNA

Peripheral blood (20 ml) was drawn from the cubital vein into EDTA-containing plastic syringes. Lymphocytes were isolated from blood samples by the Ficoll-Paque gradient method, and total RNA was extracted using RNeasy spin kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. RNA samples were quantified by analysing the absorbance at 260 nm in a UV spectrophotometer. Complementary DNA (cDNA) was synthesized by first-strand reverse transcriptase (RT) reaction using Random Primer and M-MLV reverse transcriptase (Invitrogen, CA, USA).

We had maintained similar conditions during the collection and processing of all the samples since circadian regulation of *STX1A* has been reported (Allaman-Pillet et al., 2004). The blood samples were collected at around 10:00 hours on the days assigned for sample collection. Lymphocyte isolation and RNA extraction were done immediately thereafter, under standard conditions.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Real-time qRT-PCR analysis was performed using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). TaqMan primer/probes for *STX1A* and for glyceraldehyde-3-phosphate dehydrogenase

(*GAPDH*) that served as the endogenous reference, were purchased from Applied Biosystems (Assay-on-Demand™ gene expression products Hs00195648 and Hs99999905, respectively). All reactions were performed in duplicate, according to the manufacturer's protocol. A comparative threshold cycle (C_T) method validation experiment was performed to check if the efficiencies of target and reference amplifications were approximately equal (the slope of the log input amount vs. $\Delta C_T < 0.1$). One sample was randomly chosen as the calibrator and was amplified in each plate to correct for the experimental differences among consecutive PCR runs. The amounts of *STX1A* mRNA were normalized to the endogenous reference and expressed relative to the calibrator as $2^{-\Delta\Delta C_T}$ (comparative C_T method).

Statistical analysis

Statistical calculations were performed using SPSS statistical package, version 11.0.1 (SPSS Co. Ltd, Tokyo, Japan) and GraphPad Prism. The difference in *STX1A* expression between groups was analysed using *t* test. Correlation between the various clinical features of HFA group and *STX1A* expression was examined by Spearman's rank correlation test. Further, regression analysis was carried out to check the effect of comorbidities, including obsession-compulsion (YBOCS scores) and aggression, on *STX1A* expression.

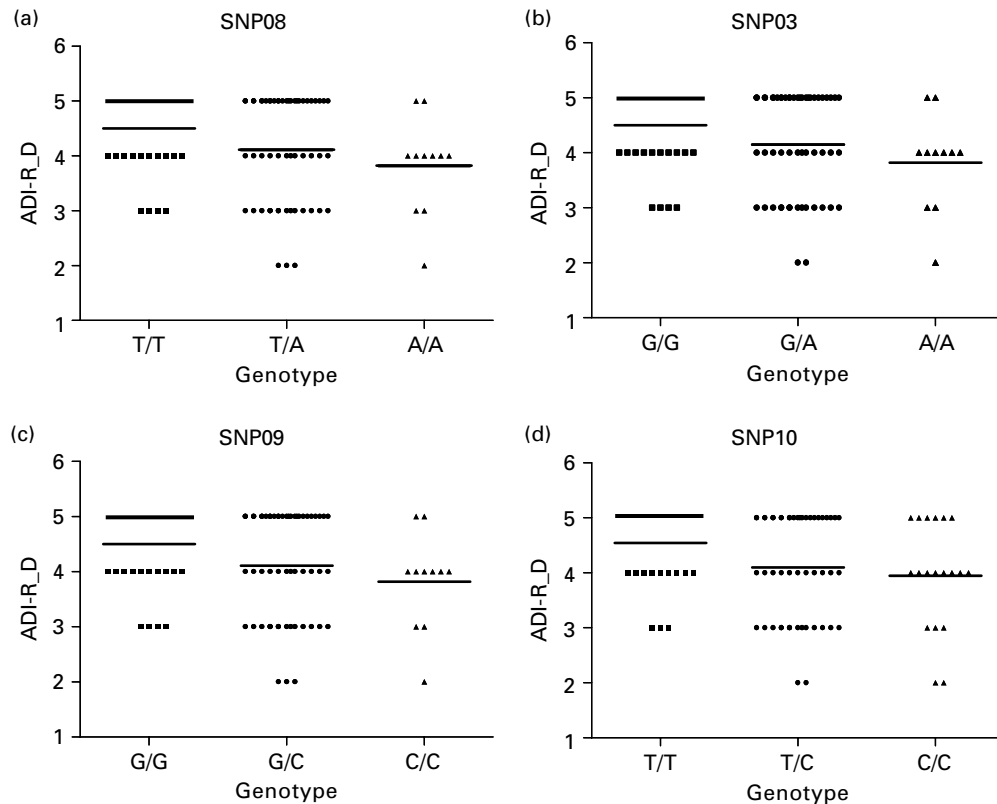


Figure 2. Comparison of the distribution of ADI-R_D scores across the homozygous and heterozygous genotypes of (a) SNP08 ($p=0.036$), (b) SNP03 ($p=0.04$), (c) SNP09 ($p=0.036$), (d) SNP10 ($p=0.029$), in the probands of high functioning autism (HFA) trios.

Relationship between mRNA expression and genotypes

All the samples from the mRNA expression study were genotyped for the SNPs that showed associations in the trio association study. Using the Kruskal–Wallis test, we examined the relationship between genotypes and mRNA expression.

Results

Association study

Mutation screening

No new mutations were observed in the coding or regulatory regions of the gene, other than the SNPs already reported.

Single SNP TDT

Mendelian inheritance inconsistencies were not observed for any of the SNPs. More than 98% of the genotypes were scored for each SNP; none of the SNPs showed deviation from HWE.

TDT was performed separately for the whole set of 249 trios and for the HFA subgroup of 102 trios; the

results are shown in Table 2. In the TDT of HFA trios, SNP04 ($p=0.034$, OR 1.54, 95% CI 1.03–2.29) and SNP08 ($p=0.033$, OR 0.63, 95% CI 0.41–0.96) showed nominal associations. Overtransmission was observed for the minor allele T (56.07%) of SNP04 (rs941298; exon 3, D68D) and for the minor allele A (57.69%) of SNP08 (rs4717806). SNP03 ($p=0.054$), SNP05 ($p=0.053$), SNP09 ($p=0.050$) and SNP10 ($p=0.050$) showed tendencies for association with HFA. The global p value, however, was not significant ($p=0.124$).

None of the SNPs showed significant association in the TDT of 249 trios.

Association with ADI-R phenotypic data

We examined the associations of ADI-R phenotypic data (ADI-R_A, ADI-R_BV, ADI-R_C, and ADI-R_D) with the SNPs that showed nominal associations in single SNP TDT. In the HFA trios, significant association with ADI-R_D scores was observed for SNP08 ($p=0.036$); ADI-R_D was lower in the A/A group of this SNP (Figure 2a). We also examined the associations of SNPs that showed tendency for association in single SNP TDT. SNP03 ($p=0.040$), SNP09 ($p=0.036$)

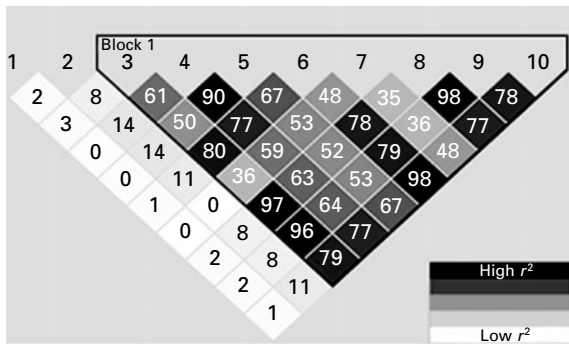


Figure 3. Haplotype block structure of *STX1A* based on r^2 values calculated from 102 high functioning autism (HFA) trios.

and SNP10 ($p=0.029$) showed significant associations with ADI-R_D; the scores were lower in the A/A group of SNP03, in the C/C group of SNP09 and in the C/C group of SNP10 (Figure 2b–d, respectively).

After adjusting for IQ effects with univariate analysis of variance, there were only tendencies for association with ADI-R_D (SNP08, $p=0.070$; SNP03, $p=0.070$; SNP09, $p=0.070$; SNP10, $p=0.055$).

No associations were observed with other phenotypic data. In the whole group of 249 trios, there were no significant associations with any of the ADI-R scores.

LD analysis

LD analysis identified a single haploblock across the *STX1A* gene, comprising SNP03 to SNP10 (Figure 3). The LD pattern observed in our study is similar to that reported for the 30 CEU (CEPH) trios in the HapMap project.

Haplotype TDT

The results of haplotype association analysis are shown in Table 3. The haplotype ATACCTCC showed a nominal association in the HFA subgroup of 102 trios ($p=0.044$); however, this was not significant after the permutation test (10 000 permutations). None of the haplotypes showed any significant association in the 249 trios.

Lymphocyte gene expression analysis

Descriptive clinical data of the autism samples is provided in Table 4. *STX1A* expression in the drug-naive HFA group (0.38 ± 0.39) was found to be significantly higher ($t = -4.37$, d.f. = 14, $p = 0.001$) than that of the control group (0.17 ± 0.06) (Figure 4). No significant

correlation was observed between any of the clinical features of HFA group and *STX1A* expression (Table 4). Further, regression analysis showed that there is no effect of YBOCS/aggression on *STX1A* expression.

Relationship between mRNA expression and genotypes

By Kruskal–Wallis test, the relationship between genotypes and *STX1A* mRNA expression was examined for SNP04 and SNP08, which showed nominal associations with HFA in TDT analysis. SNP08 showed a tendency for gene dose effect, with the following trend: expression of A/A genotype ($\text{mean} \pm \text{s.d.}$) (0.243 ± 0.162) < expression of A/T genotype (0.293 ± 0.143) < expression of T/T genotype (0.462 ± 0.023); however, the difference was not statistically significant.

Discussion

In this study, *STX1A* mRNA expression was significantly higher in the lymphocytes of drug-naive HFA patients compared with controls. Lymphocytes are considered as excellent neural probes for studying neuropsychiatric disorders due to (i) altered lymphocyte functions in neuropsychiatric conditions, (ii) expression of neuroactive proteins and processes in lymphocytes, and (iii) similarities of hormonal effects on the nervous system processes and lymphocyte physiology (reviewed by Gladkevich et al., 2004). In the present study, there was no significant correlation between *STX1A* expression and any of the clinical features of the HFA group; therefore, altered *STX1A* expression may be implicated in the general pathophysiology of HFA, rather than in the symptoms of the disease. The possibilities of using the enhanced expression of *STX1A* as a peripheral marker in the diagnosis of HFA may be explored. However, our study is limited by small sample size; therefore, replication in a larger sample size is warranted.

Lymphoblastoid cells lines have been reported as viable tools for identifying the genes associated with autism (Baron et al., 2006; Hu et al., 2006; Nishimura et al., 2007). There is a close association between the state of the immune system, particularly lymphocytes, and major psychiatric disorders including autism (Ashwood et al., 2006; Krause et al., 2002). *STX1A* is a neuron-specific antigen (Barnstable et al., 1985), which, in association with SNAP-25 and VAMP, forms the SNARE complex that is vital for chemical neurotransmission (Rothman, 1994; Sollner et al., 1993). In a

Table 3. Haplotype associations of SNPs belonging to the single LD block of *STX1A* in 102 HFA trios

Block	Haplotype ^a	Frequency	<i>p</i> value ^b	Permutation <i>p</i> value ^c
Block 1 (SNP03–SNP10)	GCGTTAGT	0.445	0.195	0.777
	ATACCTCC	0.292	0.044	0.161
	GCGTCAGT	0.118	0.275	0.852
	GTATCAGT	0.055	0.819	1.000
	GTACCAGC	0.048	1.000	1.000
	ATGCCTCC	0.025	0.527	1.000

LD, Linkage disequilibrium; HFA, high functioning autism.

^a All possible combinations of haplotypes with frequency >0.01.

^b Significant *p* values (<0.05) are indicated in bold italics.

^c 10 000 permutations.

Table 4. Descriptive data on autism samples and their correlation with *STX1A* mRNA expression

Clinical feature	Mean ± s.d.	Range	Correlation with <i>STX1A</i> mRNA expression	
			Spearman's ρ	<i>p</i> value
HAMD	2.25 ± 4.31	0–15	–0.093	0.773
HAMA	3.58 ± 3.29	0–11	0.042	0.896
YBOCS	10.42 ± 6.43	2–28	0.372	0.234
Aggression	50.75 ± 13.4	34–69	0.014	0.966
Eye movement	18.33 ± 2.02	15–22	–0.117	0.718
Faux Pas test	26.25 ± 7.58	8–34	–0.573	0.052
Full-scale IQ	99 ± 18.9	71–140	0.384	0.244
Verbal IQ	97.09 ± 17.2	71–131	0.279	0.407
Performance IQ	102.55 ± 18	76–137	0.327	0.326

HAMD, Hamilton Depression Rating Scale; HAMA, Hamilton Anxiety Rating Scale; YBOCS, Yale–Brown Obsessive–Compulsive Scale.

process similar to the exocytosis of neurotransmitters, *STX1A* and other SNARE proteins are involved in a process of exocytosis of lytic granules, mediating target cell death in the PBLs that play a pivotal role in the body's defence against infection (Hong, 2005). The presence of sympathetic fibres in lymphoid tissues suggests a direct contact area for neural signalling cascade with the immune cells (Felten et al., 1987). Thus, lymphocyte *STX1A* expression might be indicative of CNS expression. In animal models, *STX1A* expression has been shown to be more or less constant after the embryonic and immediate postnatal stages (Biranowska et al., 2002; Shimohama et al., 1998). Therefore, the expression pattern of *STX1A* in our study subjects with an average age of 22 yr might be

comparable to the *STX1A* expression during their first years of life, which is the most critical period for the formation of neural wiring patterns.

In the trio association study, nominal SNP and haplotype associations were observed with HFA. None of the SNP and haplotype associations will remain significant after multiple testing correction; however, it is difficult to apply a Bonferroni correction in this study, since strong LD was observed between SNP03 and SNP10. Further, single genes are likely to have only small individual effects in complex disorders like autism. Among the two SNPs that showed nominal associations with HFA, rs2293485 is a synonymous exonic SNP, while rs4717806 is an intronic SNP. The other SNPs that showed tendencies for

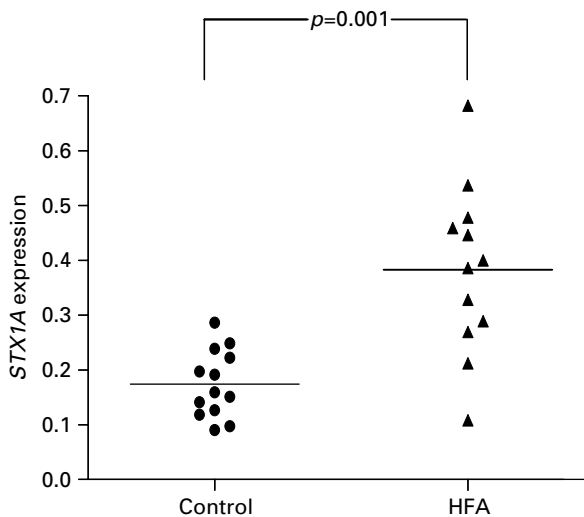


Figure 4. *t* test comparison of *STX1A* mRNA levels in the lymphocytes from control subjects and drug-naive high functioning autism (HFA) patients. Expression level of *STX1A* is normalized against that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Horizontal bars indicate means. A significant difference in *STX1A* expression was observed between the two groups ($p=0.001$).

association are also intronic SNPs. Therefore, the associated SNPs may not have a direct influence on *STX1A* expression. It is possible that the expression is regulated by additional *trans*-acting factors in a network of genes involving *STX1A*.

Considering sample homogeneity, we had selected predominantly high-functioning trio samples, where all the subjects were strictly diagnosed with autistic disorder. Since mental retardation consists of various aetiologies, HFA samples can be considered to be relatively free from non-specific environmental or genetic risks for their impairments. We had used all available AGRE cases that satisfied our strict selection criteria at the time of subject selection.

We observed an association of ADI-R_D scores with the SNPs that showed nominal associations or tendencies for association with HFA. This observation in HFA patients who had been diagnosed for autism is interesting, since ADI-R_D reflects developmental abnormalities observed in autism prior to 36 months; delayed speech, unusual socio-emotional reactions and poor attentions to and exploration of the environment, are among the first clinically noticeable behavioural symptoms of autism during the second and third years of life (Dahlgren and Gillberg, 1989; De Giacomo and Fombonne, 1998). Since autism is a pervasive developmental disorder, neuroanatomical, neurochemical and neurobiological abnormalities

should precede the appearance of clinical symptoms. The importance of 5-HT in several aspects of prenatal and postnatal brain development that are related to autism is well documented. Humans undergo a period of high brain 5-HT synthesis capacity during early childhood, which is disrupted in autistic children (Chugani et al., 1999). Thus, *STX1A* might be involved in the pathogenesis of autism by influencing the serotonergic system, during the early stages of brain development. However, after adjustment for IQ, there were only tendencies for association with ADI-R_D.

There was no relation with other ADI-R subscores (ADI-R_A, ADI-R_BV, ADI-R_C), which reflect the severity of autism.

During the postnatal stages of neurodevelopment, the expression of 5-HTT might be a prerequisite for the neurodevelopmental functions exerted by 5-HT. In vitro studies have shown that 5-HTT expression is regulated by *STX1A* (Haase et al., 2001; Quick, 2002). In cultured cells, *STX1A* has been found to interact with 5-HTT, modulating the cell-surface expression of 5-HTT (Haase et al., 2001). Cells transfected with *STX1A* cDNA showed a decrease in the number of 5-HTT molecules expressed on the cell surface; the decrease in 5-HTT expression was suggested to be caused either by an inhibition of the recycling of previously internalized 5-HTT or by a blocking of the trafficking of newly synthesized 5-HTT to the plasma membrane. Studies using thalamocortical neuronal cultures also have shown that *STX1A* affects the localization and cell-surface expression of 5-HTT (Quick, 2002). Thus, *STX1A* might be suggested to influence the serotonergic system by modulating the expression of 5-HTT.

An alternate mode of action of *STX1A* may be through its interaction with the glutamatergic system (Fan et al., 2006; Yu et al., 2006). Glutamate, the major excitatory neurotransmitter in the brain, plays a vital role in brain development, affecting neuronal migration, neuronal differentiation, axon genesis, and neuronal survival (Coyle et al., 2002). *STX1A* promotes the endocytic sorting of the glutamate transporter EAAC1, leading to inhibition of glutamate transport (Yu et al., 2006). Several studies have implicated abnormalities of glutamatergic neurotransmission in the pathophysiology of autism (reviewed by McDougle et al., 2005). Glutamate-related genes have also been shown to be important in the pathology of autism (Szatmari et al., 2007).

In conclusion, we suggest a possible role of *STX1A* in the pathogenesis of HFA; this is the first report of an association between *STX1A* and HFA. In autism, mental retardation implies the involvement of more

risk factors and genes; this might be the reason that the autism group, as a whole, was not associated with *STX1A*. The association of *STX1A* with the HFA group may thus, indicate a protective role against mental retardation. However, considering our sample size, it may be too premature to draw such a conclusion; the elevated expression of *STX1A* might also be secondary to other metabolic changes in HFA. Therefore, further studies into the functional impact of *STX1A* in HFA are warranted.

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Statement of Interest

None.

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