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BRIEF REPORT

Brief Report: Glutamate Transporter Gene (*SLC1A1*) Single Nucleotide Polymorphism (rs301430) and Repetitive Behaviors and Anxiety in Children with Autism Spectrum Disorder

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Abstract Investigated association of single nucleotide polymorphism (SNP) rs301430 in glutamate transporter gene (*SLC1A1*) with severity of repetitive behaviors (obsessive–compulsive behaviors, tics) and anxiety in children with autism spectrum disorder (ASD). Mothers and/or teachers completed a validated DSM-IV-referenced rating scale for 67 children with autism spectrum disorder. Although analyses were not significant for repetitive behaviors, youths homozygous for the high expressing C allele had more severe anxiety than carriers of the T allele. Allelic variation in *SLC1A1* may be a biomarker for or modifier of anxiety symptom severity in children with ASD, but study findings are best conceptualized as tentative pending replication with larger independent samples.

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Obsessive–compulsive behaviors (OCB) and anxiety are common co-occurring features of autism spectrum disorders (ASD) (e.g., Gadow et al. 2005; White et al. 2009), and the three groups of syndromes evidence overlapping symptoms and family histories in ASD and non ASD samples (e.g., Bolton et al. 1998; Cath et al. 2008; Comings 1990; Cullen et al. 2008; Gadow et al. 2002; Piven and Palmer 1999). Nevertheless, relatively few studies have actually examined whether common gene variants purportedly associated with obsessive–compulsive disorder (OCD) may actually be biomarkers for or potential modifiers of these symptoms in children with ASD. The clinical significance of this effort is underscored by the facts that repetitive behaviors are stressful for parents (e.g., Lecavalier et al. 2006) and singled out as being a source of concern (Fong et al. 1993); have an adverse effect on quality of life (Bishop et al. 2007); and are highly stable over time (Billstedt et al. 2007; Lecavalier et al. 2006), high priorities for intervention (Matson and Dempsey 2009) and associated with poorer long-term outcome (e.g., Billstedt et al. 2007; Howlin et al. 2004). The repetitive behaviors associated with ASD include a complex assortment of vocalizations, motor movements, and cognitions whose nosology is not well-established (e.g., Carcani-Rathwell et al. 2006; Lam et al. 2008; Mandy and Skuse 2008) and not easily differentiated from the classic features of OCD (e.g., Wood et al. 2008; Zandt et al. 2007), which is also true for other neurobehavioral syndromes that are characterized by repetitive behaviors and associated with OCB such as tic disorders (e.g., Grados et al. 2008).

Although a number of studies have examined the molecular genetics of ASD repetitive behaviors (e.g., Coon et al. 2005; Mulder et al. 2005; Sacco et al. 2007; Sakurai et al. 2008; Sutcliffe et al. 2005), few have actually evaluated relations of purported OCD candidate genes with measures of OCB behaviors or with other anxiety-related behaviors. The glutamate transporter gene (*SLC1A1*), which encodes the neuronal glutamate transporter excitatory amino acid carrier 1, is highly expressed in regions of the brain that are implicated in the pathogenesis of OCD (Arnold et al. 2006; Brune et al. 2008; Dickel et al. 2006; Stewart et al. 2007; Wendland et al. 2009). The glutamate transporter regulates neurotransmission by removing glutamate from the extracellular space thus terminating synaptic transmission. It is also involved in the biosynthesis of γ-aminobutyric acid (GABA), which may also be involved in the etiology of OCD. Dickel et al. (2006) examined nine single nucleotide polymorphisms (SNPs) in *SLC1A1* and found two SNPs associated with early onset OCD, one of which was a coding synonymous SNP in exon 10 (rs301430). TDT analyses indicated overtransmission of the lower-frequency C allele. In a similar vein, Stewart et al. (2007) found that a haplotype, which included rs301430, was associated with early-onset OCD in a family-based association study. In a more recent study Wendland et al. (2009) reported on three *SLC1A1* SNPs previously found to be associated with OCD, one of which was rs301430. This SNP was found to be associated with mRNA levels in post mortem brain tissue, and alleles were shown to have a differential effect on reporter gene expression in human and rat cell lines. The T allele evidenced approximately 50% reduced expression compared with the C allele. Others, however, have not found differences in allele frequencies between child-onset OCD cases and controls for rs301430 (Wang et al. 2009). In one of the few studies to investigate its potential role in ASD pathogenesis, Brune et al. (2008) found preliminary evidence for differential transmission of a haplotype that included rs301430.

Based on findings for *SLC1A1* SNP rs301430 in nonASD patients with OCD, we examined whether rs301430 was a biomarker for OCB severity in children with ASD. Owing to the aforementioned nosological uncertainties about movement disorders within the ASD clinical phenotype, we also examined tics. As we and others have found that common polymorphisms of genes such as *DAT1* (Comings et al. 1996; Gadow et al. 2008a; Rowe et al. 1998), *BDNF* (Gadow et al. 2009b) and *DRD4* (Gadow et al. 2009a) are associated with both movement and anxiety symptoms, a secondary objective was to assess possible relations with anxiety as well. However, as there are no published data about these symptoms and rs301430 in ASD samples, our analyses are exploratory (i.e., hypothesis generating, not hypothesis confirming).

Materials and Methods

Participants

Participants in this study were recruited from referrals to a university hospital developmental disabilities specialty clinic located on Long Island, New York. All families with at least one child with a confirmed diagnosis of ASD were contacted by mail for participation in a genetic study. A total of 92 individuals were initially recruited, but to maximize homogeneity, the study sample ($N = 67$) was limited to individuals who were children (4–14 years old) when the diagnostic and behavioral evaluations were conducted. Child participants were excluded from the study if a Rett MECP2 or a Fragile X mutation was discovered. Demographic characteristics were as follows: age ($M = 6.9$; SD = 2.6), gender (87% male), ethnicity (96% Caucasian), IQ ($M = 79.2$; SD = 23.2), socioeconomic status (SES) assessed with Hollingshead's (1975) index of occupational and educational social status ($M = 42.4$; SD = 11.4), single-parent household (1%), and psychotropic medication use (24%). Mothers' and teachers' ratings of psychiatric symptoms were available for 62/57 of the children, respectively. This study was approved by a university Institutional Review Board, informed consent was obtained, and appropriate measures were taken to protect patient (and rater) confidentiality.

Procedure

Diagnoses of ASD were confirmed by an expert diagnostician and based on five sources of information about ASD symptoms to verify DSM-IV criteria: (a) comprehensive developmental history, (b) clinician interview with child and caregiver(s), (c) direct observations of the child, (d) review of validated ASD rating scales including the Child Symptom Inventory-4 (CSI-4) (Gadow et al. 2008b), and (e) prior evaluations and, additionally ($n = 49$), with (f) the Autism Diagnostic Observation Schedule (Lord et al. 2000) and/or Autism Diagnostic Interview-Revised (Rutter et al. 2003).

Prior to scheduling their initial clinic evaluation, the parents of potential participants were mailed a packet of materials including behavior rating scales, background information questionnaire, and permission for release of school reports, psycho-educational, and special education evaluation records. Rating scales included parent and teacher versions of the CSI-4, which in most cases were completed by the child's mother. Child's genotype status was determined using DNA isolated from peripheral blood cells and polymerase chain reaction (PCR).

Genotyping

SNP analysis was performed for the *SLC1A1* SNP rs301430 genotypes with high-resolution melting. PCR was carried out in a 10 μl volume containing the following primers: SLC1A1SNPF-ACAATCAACATGGATGGGAC and SLC1A1SNPR-CGCTGCCACTGCTTCATA. Each amplification was overlaid with mineral oil and contained 20 ng of DNA, 0.25 μM of each primer, and 1X Light Scanner Master Mix (Idaho Technology Inc). Reaction conditions were: an initial denaturation at 95°C for 2 min, followed by 45 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, with a final extension step (72°C for 10 min) and a final 'hold' at 4°C. Melt analysis was performed between 70 and 98°C with a Light Scanner (Idaho Technology, Inc) (Zhou et al. 2004), and SNP status determined using the Small Amplicon Module. One individual with each of the three expected genotypes (C/C, C/T, T/T) was sequenced to confirm correct genotype calling, and these samples were included in the melt analysis (data not shown). Genotype analyses were conducted by an investigator who was blind to the behavioral characteristics of the study sample.

Measures

The CSI-4 (Gadow and Sprafkin 1986, 2002) is a behavior rating scale that assesses the behavioral symptoms of a broad range of psychiatric syndromes and has both parent and teacher versions. Individual items bear one-to-one correspondence with DSM-IV symptoms (i.e., high content validity). To assess symptom severity, items are scored (never = 0, sometimes = 1, often = 2, and very often = 3) and summed separately for each symptom dimension. In the present study, analyses pertained to two symptom domains: repetitive behaviors (obsessive–compulsive behavior, tics) and anxiety (separation, generalized, social). The findings of numerous studies indicate that the CSI-4 demonstrates satisfactory psychometric properties in community-based normative, clinic-referred non-ASD, and ASD samples (see Gadow and Sprafkin 2008). Moreover, confirmatory factor analysis in large a ($N = 730$) sample of children with diagnosed ASD supports the construct validity of DSM-IV syndromes (Lecavalier et al. 2009) as well as the tripartite model of ASD symptom domains (Lecavalier et al. 2009). As with almost all behavior rating scales, mother and teacher ratings evidence modest convergence, which likely in part reflects important gene *x* environment interactions. In other words, settings (e.g., home, school) vary considerably in the demands they place on children resulting in setting-specific phenotypic variation as well as source-specific (e.g., mother, teacher) perceptions of deviance. The complexity of these relations is compounded by the fact that the same "susceptibility" allele may be adaptive in one context but maladaptive in another (see Belsky et al. 2009).

Statistical Analyses

Chi-square tests (categorical variables), correlations (continuous variables), and ANOVAs (combined categorical and continuous variables) of demographic characteristics with genotype groups and the dependent variables were examined to identify potential covariates for subsequent analyses. Next, we conducted MANOVAs comparing genotype group differences in the two domains of symptoms to control for multiple comparisons thereby reducing the risk of Type 1 error. Subsequent univariate analyses were examined for evidence of gene-behavior relations. Follow-up pairwise comparisons were examined to identify specific differences between genotype groups. Analyses were conducted separately for the two informants and the two domains of behaviors: repetitive behaviors (OCB, tics) and anxiety. For the latter, data were available for separation anxiety (mothers' ratings), generalized anxiety (mothers' and teachers' ratings), and social phobia (teachers' ratings). We calculated partial eta-squared (ηp^2) to gauge the magnitude of group differences and to address in part the inherent limitations of significance testing (Cohen 1994; Feise 2002; Perneger 1998; Rothman 1990; Zhang et al. 1997). A rule of thumb for determining the magnitude of ηp^2 suggests the following: 0.01–0.06 = small, 0.06–0.14 = moderate, and >0.14 = large (Cohen 1988).

Results

Child *SLC1A1* rs301430 allele frequencies were C (32%) and T (68%) and genotype frequencies were as follows: C/C ($n = 5$; 8%), C/T ($n = 33$; 49%), and T/T ($n = 29$; 43%), which does not deviate from Hardy–Weinberg equilibrium ($X^2 = 1.34$, $p = .29$). Comparisons between genotype groups did not evidence statistically significant differences in child's age, gender, ethnicity, IQ level, psychotropic medication, special education, family's SES, or whether the mother was a single-parent. Importantly, genotype groups did not differ significantly in severity of any of the three ASD domains (communication deficits, socialization deficits, perseverative behaviors) as assessed with the CSI-4.

OCB and Tic Behaviors

MANOVAs did not indicate multivariate effects for repetitive behaviors (OCB, tics) for either mothers' or

Table 1 Group differences in severity of psychiatric symptoms for *SLC1A1* genotypes

Variable (CSI-4)[a]	C/C Mean (SD)	C/T Mean (SD)	C/T Mean (SD)	F	$p =$	ηp^2
Parent ratings						
Generalized anxiety	4.8 (2.9)	2.4 (2.4)	2.9 (3.0)	1.53	0.23	0.05
Separation anxiety[a]	8.4 (7.5)	2.2 (2.9)	2.3 (3.1)	7.91	0.001	0.22
Tics	2.6 (1.9)	1.3 (1.5)	1.3 (1.5)	1.69	0.19	0.06
OCB	1.4 (1.1)	0.8 (1.0)	1.3 (1.5)	1.22	0.30	0.06
Teacher ratings						
Generalized anxiety	5.6 (2.6)	2.3 (2.1)	2.6 (2.2)	3.48	0.04	0.12
Social phobia[b]	3.0 (2.4)	1.9 (1.7)	1.3 (1.6)	2.12	0.13	0.08
Tics	2.0 (2.3)	1.9 (2.0)	2.3 (2.3)	0.33	0.72	0.01
OCB	2.8 (2.2)	1.0 (1.4)	1.5 (1.8)	2.64	0.08	0.09

[a] Included in the parent version of the CSI-4 only

[b] Owing to modifications in the item content of the CSI-4, we were unable to directly compare parent and teacher ratings of social phobia

CSI-4 child symptom inventory-4, *OCB* obsessive–compulsive behavior

teachers' ratings. Therefore, follow-up univariate analyses were not conducted as part of our planned analyses. Nevertheless, owing to the possibility of Type 2 error with an uncommon genotype, we present the results of these analyses in Table 1 to include effect sizes so readers can better evaluate the likelihood of this risk.

Anxiety Behaviors

The MANOVA for mothers' ratings of anxiety was significant ($F = 3.78$, $p = .006$) as was the univariate analysis for separation anxiety ($F = 7.71$, $p = .001$, $\eta p^2 = .22$) but not generalized anxiety ($p = .23$; $\eta p^2 = .12$) (Table 1). Planned comparisons indicated that children homozygous for the C allele had more severe separation anxiety than either the C/T ($p < .001$) or T/T ($p < .001$) groups (see Fig. 1). The multivariate effect was marginally significant for teachers' ratings of anxiety ($F = 2.25$, $p = .07$) and univariate analyses were significant for generalized anxiety ($F = 3.48$, $p = .04$, $\eta p^2 = .12$) but not social phobia ($p = .13$; $\eta p^2 = .08$). Planned comparisons indicated C/C group had more severe symptoms of generalized anxiety than the C/T ($p = .02$) or the T/T ($p = .01$) groups.

Discussion

The notion that common gene polymorphisms may be biomarkers for and possibly modifiers of co-occurring

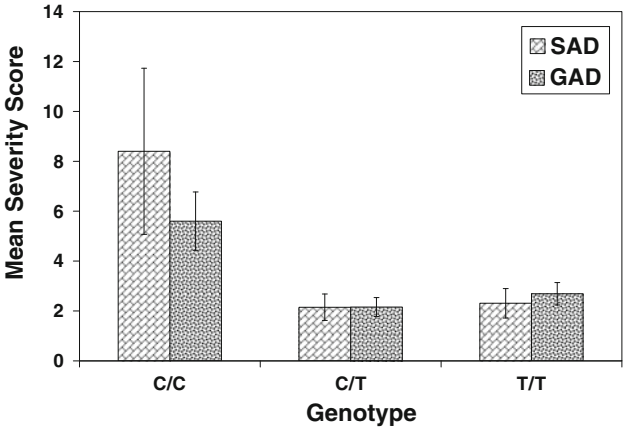

Fig. 1 Child *SLC1A1* rs301430 genotype and mothers' and teachers' ratings of separation anxiety disorder (SAD) and generalized anxiety disorder (GAD) symptoms, respectively. Results are presented as $M \pm SEM$

behavioral disturbances within the ASD clinical phenotype as well as severity of ASD symptoms finds support in a growing number of studies (e.g., Brune et al. 2006; Cohen et al. 2003; Gadow et al. 2008a, b, 2009a, b; Mulder et al. 2005; Roohi et al. 2009; Tordjman et al. 2001). Not unexpectedly, the results of these same studies also provide evidence of polygeny, pleiotropy, epistasis, and contextual variability (informant specificity), consistent with what is known about the genetic architecture of physical and behavioral characteristics (Flint and Mackay 2009) as well as commonalities with similar traits in other neurobehavioral syndromes. The present study provides tentative evidence for an association of the C/C genotype with separation anxiety and generalized anxiety in children with ASD. To the best of our knowledge, this is the first report of a potential genetic biomarker for separation anxiety in the ASD clinical phenotype. Similar to other common candidate genes that we have studied, the magnitude of genotype group differences was in the moderate range. An association with different types of anxiety suggests that the *SLC1A1* rs301430 polymorphism may be a more general risk factor for trait anxiety in this clinical phenotype. Importantly, these relations were still evident after controlling for ASD symptom severity and intellectual ability and therefore less likely to be linked to the etiology of ASD. Unfortunately, owing to limited research in this area to include the molecular mechanisms by which the rs301430 polymorphism influences gene expression, it is premature to construct a model for a potential role of the C/C genotype in the pathogenesis of anxiety in ASD.

Unexpectedly, allelic variation was not associated with either OCB or tic severity, which is nevertheless consistent with our finding of equivalent severity of perseverative ASD behaviors in the three genotype groups. Our prior

research with other genes, however, has found unique gene-behavior linkage for each type of movement disturbance even when associations with perseverative behavior scores were nonsignificant (Gadow et al. 2008a; Gadow et al. 2009a, b). In other words, evidence clearly supports the notion that repetitive behaviors are multi-factorial both within (Carcani-Rathwell et al. 2006) and between (Moss et al. 2009) neurodevelopmental syndromes. Moreover, the marginally significant ($p = .08$) main effect of genotype for teacher ratings of OCB suggests that further research is warranted. It is noteworthy that the C/C group received the highest mean OCB ratings as was the case for generalized anxiety.

Although our results do not provide compelling support for dysfunctional glutamatergic neurotransmission in the etiology of OCB in children with ASD, they are subject to several qualifications. The percentage of youths with the C/C genotype was small, and though consistent with nonASD samples, increased the risk of a Type 2 error. Moreover, gene-behavior relations may have been more evident had we examined a wider array of SNPs or conducted haplotype analyses (e.g., Brune et al. 2008; Dickel et al. 2006; Wendland et al. 2009; Stewart et al. 2007). For example, it is possible that other loci, some of which in linkage disequilibrium with rs301430, would provide stronger evidence of or better explanation for an association with OCB severity. Although the study sample was primarily Caucasian, this does not rule out population stratification as a possible confound. For these and other reasons, our findings must be considered tentative pending replication in larger independent samples.

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