Published in final edited form as:

J Dev Phys Disabil. 2018 June ; 30(3): 355–371. doi:10.1007/s10882-018-9590-4.

Testing genetic modifiers of behavior and response to atomoxetine in autism spectrum disorder with ADHD

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Abstract

Background—Frequent non-pathogenic genetic variants may act as moderators of phenotypic severity for complex disorders such as autism spectrum disorder (ASD). We previously identified polymorphisms affecting mRNA expression of candidate genes, including tryptophan hydroxylase 2 (*TPH2*), dopamine beta hydroxylase (*DBH*), and dopamine transporter (*DAT*, *SLC6A3*).

Method—We compare genotypes and (1) clinical response to atomoxetine, (2) scores from the Autism Diagnostic Interview-Revised (ADI-R), and (3) severity of Attention Deficit Hyperactivity Disorder (ADHD) symptoms in a cohort of patients with ASD from multiple study sites.

Results—There was no association between CYP2D6 metabolizer status and atomoxetine response. *TPH2* rs7305115 genotype was associated with ADI-R Restrictive/Repetitive Behavior score ($p=0.03$). *DBH* rs1611115 genotype was associated with ADI-R Social score ($p=0.002$) and Restrictive/Repetitive Behavior score ($p=0.04$). The *DAT* intron 8 5/6 repeat was associated with ADHD symptoms (ABC Hyperactivity $p=0.01$ and SNAP ADHD $p=0.03$), replicating a previous finding.

Conclusions—We find associations between ASD phenotypes and regulatory variants in catecholamine biosynthesis genes. This work may help guide future genetics studies related to ASD.

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Compliance with Ethical Standards:

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Authors' Conflicts of Interest

Dr. Elizabeth Barrie declares that she has no conflict of interest.

Ms. Emily Hansen-Kiss, LGC declares that she has no conflict of interest.

Keywords

autism spectrum disorder (ASD); genetics; restrictive and repetitive behavior; atomoxetine (Strattera); tryptophan hydroxylase 2 (*TPH2*); dopamine transporter (*DAT*, *SLC6A3*); attention deficit/hyperactivity disorder (ADHD)

INTRODUCTION

The diagnostic criteria for Autism Spectrum Disorder (ASD) include persistent impairment of social communication and interaction across multiple contexts, as well as the presence of restricted, repetitive patterns of behavior, interests, or activities with age of onset below 3 years old (American Psychiatric Association [APA], 2013). ASD is a developmental disorder, often with a life-long impact on a child's ability to function in school, at home and in the community. In addition, many children with ASD have comorbid psychiatric disorders. Simonoff et al. (2008) conducted psychiatric diagnoses in a representative sample of 10–14-year-olds and found a 70% rate of comorbid psychiatric conditions, with attention-deficit/hyperactivity disorder (ADHD) occurring second-most commonly at 28% of the sample. Not surprisingly, psychiatric conditions result in increased prescription rates of psychotropic medication. One cross-sectional study showed that the use of psychotropic drugs grew with age, ranging from 10% in 3–5-year-olds to 64% in 12–17-year-olds (Coury et al., 2012). In addition to psychotropic drugs, several evidence-based treatments have been developed to treat ASD, educational, and comorbid behavioral symptoms in this population; these include early intensive behavioral intervention, parent training, and social skills training (Koegel, Koegel, & McNerney, 2001; Corsello, 2005; Virués-Ortega, 2010; Warren et al., 2011).

The diagnosis of ASD is often supported by the Autism Diagnostic Interview—Revised version (ADI-R) (Lord et al., 1994), which is a structured interview asking about developmental history and current behavior. In the genetic analyses that follow, we use three ADI-R subscales: “Qualitative Abnormalities in Reciprocal Social Interaction,” “Qualitative Abnormalities in Communication,” and “Restricted, Repetitive, and Stereotyped Patterns of Behavior” subsequently referred to as “Social,” “Communication” and “Restrictive/Repetitive Behavior” scores. These three components of ASD are of interest to genetic researchers, as they each represent a continuum of severity, potentially modified by genetic factors. In addition, there are behavioral comorbidities that are also frequent in the context of ASD, such as inattention, impulsiveness, and overactivity. Indeed, these ADHD symptoms are so common in ASD that clinicians, using the Diagnostic and Statistical Manual—Fourth Edition (Text Revision) (DSM-IV-TR; APA, 2000) or the International Classification of Diseases-9 (WHO, 1996), were not permitted to diagnose ADHD in the presence of ASD, although this restriction was removed in DSM-5 and ICD-10.

The current study was designed to determine whether genetic variants contribute to clinical features and drug response in children and adolescents with ASD and with severity of co-occurring ADHD symptoms. Our objectives were to answer the following questions. 1. Are there genetic variants associated with clinical response to atomoxetine treatment in children with ASD and ADHD symptoms? 2. Are specific genotypes associated with the three

phenotypes of ASD as assessed on the ADI-R (deficits in social interactions or communication; or restrictive/repetitive behavior)? 3. Are specific genotypes associated with the variable levels of ADHD symptoms that are so often encountered in ASD?

Comparing cohorts composed of participants with and without ASD, with no further stratification, may be of limited value because ASD features are often highly variable within a clinical sample. Likewise, control groups may also have phenotypic variability. Therefore, more quantitative components of ASD can provide greater accuracy in matching different magnitudes of these characteristics with genetic modifiers. We have taken advantage of data from several studies conducted over the past decade that included individuals with ASD [“pervasive developmental disorders” in the parlance of DSM-IV-TR (APA, 2000), which preceded the term ASD used today] and usually comorbid behavior problems or psychopathology.

Atomoxetine response

A subset of participants were treated with the norepinephrine reuptake inhibitor atomoxetine, which reduces ADHD symptoms in 26–46% of children who have ASD with co-occurring ADHD (Handen et al., 2015; Harfterkamp et al., 2012). Atomoxetine is metabolized by CYP2D6, so we used a *CYP2D6* genotyping panel to test for an effect on ADHD-symptom reduction by classifying subject metabolizer status.

Genes and ASD

Numerous studies have implicated candidate genes in ASD (Abrahams & Geschwind, 2010; Li et al., 2012; Miles, 2011). Of particular interest are functional SNPs (single nucleotide polymorphism, see Glossary of Genetic Terms) in genes that regulate dopamine, norepinephrine and serotonin levels (Lam et al., 2006) such as norepinephrine transporters (*NET* rs5569), serotonin receptors (*HTR2A* rs6311) and catecholamine degradation (*COMT* rs4680). High levels of serotonin in whole blood have been associated with greater symptom severity of ASD (Gabriele et al., 2014) and changes in brain levels of serotonin are also preliminarily linked to ASD (Muller et al., 2016). It is not clear whether this is compensatory or a biomarker resulting from a causal factor. Genetic modifiers of serotonin levels therefore could potentially be detected via changes in the Social or Communication scores of the ADI-R.

Here our SNP selection was based on two principles. These SNPs have demonstrated an effect at the mRNA level in previous work and they have been associated with mental health disorders in at least one other study (Lim et al., 2007; Zhang et al., 2007; Retz et al., 2008; Moyer et al., 2011; Pinsonneault et al., 2011; Bray et al., 2013; Barrie et al., 2014; Smith et al., 2014).

TPH2 and Restrictive and Repetitive Behavior

Tryptophan hydroxylase isoform 2 (TPH2) is expressed in serotonergic neurons and encodes a protein that catalyzes the rate-limiting step in the synthesis of serotonin. We proposed that *TPH2* is not a risk factor for ASD *per se* but a modifier of associated restrictive and repetitive behaviors. Two SNPs (rs4341581 and rs11179000) had been associated with high

repetitive and stereotyped behavior scores from the ADI-R in Caucasians (Coon et al., 2005) and Koreans (Yang et al., 2012); but the results failed to replicate in subsequent studies (Sacco et al., 2007; Ramoz et al., 2006) including a large Japanese sample (Egawa et al., 2012). A different SNP in *TPH2*, rs4570625 was associated with early onset obsessive-compulsive disorder, an “endophenotype” of ASD (Mössner et al., 2006). Mothers preferentially transmitted the *A* allele of rs7305115, located on a haplotype with rs4570625 and rs11179001 to ASD probands (Singh et al., 2013). The *A* allele of rs7305115 generates an exonic splicing enhancer site, resulting in increased TPH2 expression levels (Lim et al., 2007).

DBH and Social/Communication Abnormalities of ASD

Dopamine beta-hydroxylase (*DBH*) is the rate-limiting enzyme in the conversion of dopamine to norepinephrine. Dopamine plays a key role in the reward pathway, attention, and psychomotor tasks that are affected in ASD and ADHD (Lam et al., 2006). The promoter SNP rs1611115 (−1021C/T) has been associated with lower serum DBH levels, in the general population (Zabetian et al., 2001; Garland et al., 2007; Mustapic et al., 2007; Bhaduri et al., 2008). Previous studies failed to find associations between genetic variants in *DBH* and ASD (Tassone et al., 2011; Herault et al., 1993); however, *DBH* may act as a disorder modifier. Maternal rs1611115 genotype may be tied to offspring’s development of ASD (Robinson et al., 2001). We have studied genetic variants in *DBH*, showing that rs1611115 and a SNP at a splicing junction profoundly affect DBH activity in sympathetic peripheral neurons, but showing little effect in the CNS (Barrie et al., 2014). Nevertheless, *DBH* variants have been implicated in CNS disorders, including ASD phenotypes (Robinson et al., 2001). In addition to changes in catecholamine metabolism in the brain, large changes in circulating dopamine and norepinephrine levels could have indirect effects on the CNS.

The dopamine transporter DAT and ADHD symptoms

A widely accepted hypothesis, supported by therapeutic effects of dopaminergic (and noradrenergic) drugs is that a link in the etiologic chain for ADHD is a deficiency of dopamine (and norepinephrine) receptors, reuptake transporters, or both. We have previously identified variants that affect expression of DAT mRNA and influence risk for bipolar disorder (Pinsonneault et al., 2011). Moreover, we have reported an interaction between the *DAT* intron 5/6 repeat and a *DRD2* splicing variant, with a strong effect on cocaine-abuse-related death (Moyer et al., 2011), possibly a result of a “dopamine storm” exacerbated by these genetic variants. Therefore, both *DAT* and *DRD2* variants were included in the present study. In addition to this, we have reported that a *DAT* intron 8 repeat was associated with parent-rated hyperactivity and *DRD2* SNP rs2283265 was associated with teacher-rated inattention in a sample of children with ASD and ADHD (Gadow, Pinsonneault, Perlman & Sadee, 2014).

Given the major role that genetic variation plays in gene expression and mRNA processing, we suspected that polymorphisms that alter the expression of ASD candidate genes in dopamine, norepinephrine, and serotonin pathways may be relevant to core symptoms of ASD. Here we describe their associations with ASD severity as measured by a standardized

diagnostic interview, the ADI-R. We also compare differences in ADHD symptoms and genetic variants.

METHODS

Sample Characteristics

Subjects in the initial discovery cohort (Samples #1–3) were 86 youth (71 boys and 15 girls), ages 5 to 14 years, with a mental age of at least 18 months at time of ADI-R administration and diagnosis. All were diagnosed with an ASD [most commonly autistic disorder, but also including children with pervasive developmental disorder not otherwise specified (PDD-NOS) or Asperger's disorder]. Diagnosis was made by a research reliable psychologist based on the DSM-IV-TR (APA, 2000) with the ADI-R administered upon entry into the study. Any other pharmacological treatments, aside from the described interventions, were suspended for Samples #1, 2, and 3 upon entry into the study. Because allele frequencies vary among world populations, potentially confounding results, we restricted our analyses to Caucasian participants, as our cohorts were primarily of European descent. The subjects were a subset of participants in three studies conducted by our research group who had genetic data available.

1. Sample 1. Children with Hyperactivity and Autism Research Treatment Study (CHARTS)—

CHARTS was a 10-week randomized double-blind, placebo-controlled, parallel groups study of atomoxetine (Strattera) and parent training for children with ASD and ADHD (Handen et al., 2015). The sample comprised participants from three sites (total with genetic material, N=59, female n=11). All children in this cohort had ADHD symptoms.

2. Sample 2. Cholesterol in Autism Spectrum Disorders: Characterization and Treatment <https://clinicaltrials.gov/ct2/show/NCT00965068?term=cholesterol+autism&rank=1>—

This study examined the efficacy and safety of using a cholesterol supplement for improving core ASD features in children with concurrent hypocholesterolemia. Participants were randomly assigned to a powdered cholesterol supplementation or placebo with supplements given daily for 12 weeks (total with genetic material, N=15, female n=4). As no behavioral symptoms were required, beyond ASD, subjects in this sample had relatively low ADHD scores on the Swanson, Nolan, and Pelham (SNAP) and Aberrant Behavior Checklist (ABC) described below.

3. Sample 3—Other participants (total with genetic material, N=12, no females) were captured opportunistically with the plan to include them in the comparisons of ASD phenotypes and ADHD severity that follow.

4. Sample 4—Finally, a sample of 88 additional participants from The Central Ohio Registry for Autism (CORA) at Nationwide Children's Hospital served as a replication cohort for cross validation. Families were recruited as previously reported (Cottrell et al., 2011; Smith et al., 2014). Caucasian participants with ADI-R scores and genetic material were used for the current analyses (74 boys, 14 girls, N=88). The ADI-R was typically administered during research participation and not during subjects' clinical diagnostic

process by primary care practitioners and/or psychologists. Therefore, the ages at time of ADI-R administration ranged from 2 to 21 years, but age at *ASD diagnosis* was often earlier than the time of ADI-R administration, especially for older children in the sample. Checklists for assessing severity of ADHD symptoms were not available for this sample.

Measures of ASD and ADHD symptoms

ADI-R—The ADI-R is a well-established diagnostic interview for ASD. We used raw scores from the three scales in the ADI-R diagnostic algorithm: Social (range of possible raw scores: 0–30), Communication (range: 0–26 for verbal subjects, 0–14 for nonverbal subjects), and Restricted/Repetitive Behavior (range: 0–12). The ADI-R Communication scale includes 13 items for verbal children but only 7 (overlapping) items for nonverbal children. Across our samples, there were 7 nonverbal children. To enable comparisons between all children on the Communication scale, we computed a mean-item severity score (mean of 13 and 7 items, respectively), which assumed that all 13 items were of equal importance. (In sensitivity analyses, we removed the seven nonverbal children and the results for Communication did not differ.)

ADHD symptoms—Participants were rated by their parents on the 16-item Aberrant Behavior Checklist Hyperactivity subscale (ABC; Aman & Singh, 2017; Aman, Singh, Stewart, & Field, 1985) and on the 18-item Swanson, Nolan, and Pelham (SNAP) ADHD rating subscale (<http://www.myadhd.com/snap-iv-6160-18sampl.html>). Both the ABC and the SNAP are rated on a 0–3 Likert scale, with higher scores indicating greater severity. By convention, we analyzed the mean SNAP ADHD item scores and total ABC Hyperactivity subscale scores. Subjects in the atomoxetine (CHARTS) trial were classified as responsive to treatment if given a rating of “much improved” or “very much improved” for ADHD symptoms by the blinded evaluator and a >30% decrease on the Parent SNAP-IV. Furthermore, we used the SNAP ADHD and the ABC Hyperactivity subscales to quantify severity of ADHD symptoms within samples #1–3.

Sample preparation

Genomic DNA was isolated and SNPs genotyped as previously reported (Smith et al., 2014). Briefly, genomic DNA was extracted from either whole blood or saliva. For whole blood, cells were lysed with a sucrose Triton solution, pellet digested with SDS and proteinase K, followed by NaCl protein precipitation and DNA purified with ethanol precipitation. Saliva was collected in an Oragene kit, treated with RNase A, and purified with the QIAamp Kit (Qiagen, Alencia, CA).

Genotyping Assays

Fluorescent restriction fragment length polymorphism was used if the SNP of interest was included in a restriction enzyme cut site (*TPH2* rs7305115, *HTR2A* rs6311, *DRD2* rs2283265, *NET* rs5569). Genomic DNA was PCR amplified using a fluorescently labeled primer and the amplicons digested with the appropriate restriction enzymes. The resulting fragments were analyzed directly by capillary electrophoresis on an ABI3730 (Life Technologies, Carlsbad, CA). Similarly, a fluorescent assay was used to amplify an intron 8 repeat in *DAT*, without enzymatic digestion. *DBH* rs1611115 was genotyped with an allele-

specific melting curve analysis (Papp et al., 2003). *COMT* rs4680 and *DAT* rs27072 were genotyped using TaqMan probes (Life Technologies) on an ABI 7500 Real-Time PCR System (Life Technologies) according to manufacturer's instructions. Primer sequences available upon request.

Metabolizer Status classification—A *CYP2D6* genotyping panel was used to genotype the *CYP2D6* alleles. Metabolizer status was determined by *CYP2D6* allele combinations following CPIC (Clinical Pharmacogenomics Implementation Consortium) guidelines (<https://cpicpgx.org/guidelines/guideline-for-codeine-and-cyp2d6/>).

Statistical Analysis

Genetic data were analyzed with R programming environment v 3.1.3 or SVS Golden Helix SNP & Variation Suite v 8.3.4, a genetic statistical analysis software package that includes genetic association, and regression analysis capabilities. Sex was included as a covariate in the linear regression. As the Restricted/Repetitive Behavior scores were not normally distributed, a Kendall correlation was used. Analyses were restricted to Caucasians (sample numbers previously cited were Caucasian participants only). Plots and corresponding statistics were generated by GraphPad Prism v3.03.

RESULTS

Atomoxetine Response

The potential relationship between atomoxetine response and genetic variants was examined. Of the overall cohort of 86, a subset ($n=57$) were treated with atomoxetine (Caucasian $n=46$, African American $n=6$, Multiracial/Biracial $n=5$, Female $n=10$). Several *CYP2D6* markers were used to determine metabolizer status (Table 1). Following drug administration, we determined whether ADHD or noncompliance symptoms were resolved (see Handen et al., 2015). There was no significant effect of metabolizer status on ADHD response ($p=0.22$) or compliance response ($p=0.88$), but we report the number and percentage response for each metabolizer subgroup (Table 2).

ADI-R/ASD Phenotype Analyses

We tested for an association between an ASD characteristic and each SNP of interest. The ADI-R Social and Communication domains were only moderately correlated with each other ($R^2 = 0.38$) and were normally distributed; hence, they were analyzed separately and handled as continuous variables. The Restrictive/Repetitive Behavior score showed low correlation with the other scales and thus was analyzed separately; while the scores were not normally distributed, results were similar in analyses using parametric and non-parametric statistics. Results are reported in Table 3, with statistically significant associations in bold. Next we evaluated the direction of effect for the significant results, to determine whether these SNPs were protective or conveying risk.

A significant association was observed between Restrictive/Repetitive Behavior and the *TPH2* SNP rs7305115 ($p=0.03$, $\beta = -1.1$, $N=82$ female $n=15$) (Figure 1). The *A* allele was associated with lower symptom severity. We used a recessive model and combined the *GA*

and *AA* groups, for comparison to *GG* (wild-type). Patients homozygous for the major allele (*GG*) had significantly higher scores than those with the minor allele. The difference between mean scores was 1.6 ± 0.6 (on a scale 0–12).

An additional significant association occurred between the *DBH* SNP rs1611115 and the Social ($p=0.002$, $\beta=4.2$) and Restrictive/Repetitive Behavior scores ($p=0.04$, $\beta=1.1$), ($N=72$ for all, females $n=10$) (Figure 2). Carriers of the *T* allele had higher scores for both ADI-R components compared to wild-type.

There was a significant association between the intron 8 repeat in *DAT* and scores on the ABC Hyperactivity subscale ($N=80$, females $n=15$, $p=0.01$, $\beta=-4.6$) (Figure 3). Presence of the 5-copy repeat was associated with lower ABC hyperactivity scores (i.e., more consistent with scores from non-ADHD children) than the 6-copy repeat. There also was a significant association between the intron 8 repeat in *DAT* and mean scores on SNAP ($N=69$, females $n=15$, $p=0.03$, $\beta=-0.24$). Presence of the 5 copy repeat was associated with lower SNAP scores (i.e., fewer ADHD symptoms).

We tested whether these significant results replicated in a second cohort (Sample 4), specifically testing *DBH* rs1611115 with the Social and Restrictive/Repetitive Behavior scores and *TPH2* rs730115 with Restrictive/Repetitive Behavior. The cohort was of a similar size ($N=88$), to Samples 1–3 ($N=86$) combined and the analysis was again restricted to Caucasians. Both SNPs were in Hardy-Weinberg equilibrium (HWE) ($p=0.56$ for *TPH2* and $p=0.13$ for *DBH*). The results for *TPH2* and *DBH* did not replicate for Restrictive/Repetitive Behavior. While there was a significant result for the *TPH2* SNP rs7305115 ($p=0.03$, $\beta=0.71$), with Restrictive/Repetitive Behavior, the effect was in the opposite direction observed in the discovery sample ($\beta=-1.1$). The *A* allele was associated with lower symptom severity in the discovery sample, but increased severity in Sample 4. For *DBH* SNP rs1611115, the associations were not significant for the ADI-R Social score ($p=0.27$, $\beta=-1.1$). This indicates that there may be a genetic effect, but more complicated than a single SNP acting alone. It would be important to consider epistatic interactions; however, this type of analysis would require a larger sample set.

DISCUSSION

There is considerable evidence documenting the negative impact of a diagnosis of ASD on overall adaptive functioning, with many children and adolescents functioning significantly below expectation in adaptive behavior, especially in the areas of communication and socialization (Carter et al., 1998). Furthermore, most children with ASD were in special educational settings (i.e., in special classes or with classroom aides assigned to them), more than half had high ratings for ADHD, and social adjustment was poor (Eaves & Ho, 1997). Not surprisingly, adult outcome has often been poor for youth identified with ASD. One follow-up study found high dependence on families/support services, few close friendships, low rates of permanent employment, poor communication, poor reading and spelling, and persistence of stereotypic behavior and restricted interests into adulthood (Howlin, Goode, Hutton, & Rutter, 2004). Children with ADHD also exhibit significant deficits in attention, usually with maladaptive impulsiveness and overactivity/fidgetiness. Such children often

present with comorbid conditions, such as oppositional defiant disorder (in 54–84% of cases), smoking or substance abuse disorders, learning disabilities, and anxiety disorders (AACAP Work Group on Quality Issues, 2007). Not surprisingly, a comorbid diagnosis of ASD and ADHD, which impacts approximately one-third of children with ASD (with some estimates as high as 80%), is likely to affect adaptive functioning, academic achievement (Rommelse et al., 2010), and other areas of functioning.

We sought to determine genetic associations with the following: (a) the effect of genetics on the efficacy of atomoxetine to reduce ADHD symptoms, (b) whether ASD phenotypes for impaired social and emotional reciprocity, deficits in social communication, or restrictive and repetitive behaviors were associated with certain genetic variants, and (c) whether severity of a prominent co-occurring comorbidity (i.e., ADHD) was associated with certain genetic variants. The selected genetic variants in this study have previously been implicated in biological function such as RNA expression. However, most comparisons failed to show significant genetic correspondence with the ASD symptoms tested. As a result, the relationship may be more complicated than initially thought, as recently described for *HTR2A*, in which rs6311 and SNPs in high linkage disequilibrium are not only associated with sense *HTR2A* mRNA expression, but also expression of its anti-sense transcript, reflecting a complex regulatory network (Ruble et al., 2016). Another possible explanation for a lack of association is that variants may have combined effects that are not evident when analyzed individually. A larger sample size would be required to test the interaction between SNPs, but would be a worthwhile pursuit.

However, several significant observations did reveal associations with ASD phenotypes attributable to frequent regulatory variants in genes relevant to catecholamine biosynthesis and signaling, previously demonstrated to have significant impact on functional expression.

We detected an association of a previously characterized functional SNP in *TPH2*, rs7305115, which enhances *TPH2* expression in raphe nuclei (Lim et al., 2007), with lower symptom severity scores on the ADI-R Restrictive/Repetitive Behavior scores. *TPH2* catalyzes the rate-limiting step in the synthesis of serotonin in the brain. Hence, more *TPH2* (associated with the *A* allele of rs7305115) suggests higher serotonin levels. Functionally, our data suggest that higher serotonin levels were associated with fewer repetitive symptoms. Others have found associations between *TPH2* and OCD (Mössner et al., 2006), a related phenotype. However, the effect of rs7305115 on Restrictive/Repetitive Behavior scores was opposite in our replication cohort; therefore the effect is unknown.

We also observed an association between the *DBH* SNP rs1611115 and the Social and Restrictive/Repetitive Behavior scores, measured separately, although these effects did not replicate in a separate cohort.

The *DAT* intron 8 5/6 repeat (rs3836790) has been shown to affect *DAT* expression in the brain (Pinsonneault et al., 2011). The effect of this variant on ADHD symptoms (6-repeat is associated with higher ABC and SNAP scores) was consistent with an earlier study that demonstrated that the 6-repeat allele was associated with more severe symptoms of parent-

rated hyperactivity than the 5/6 heterozygote (Gadow, Pinsonneault, Perlman & Sadee, 2014).

We acknowledge several limitations in this study. First, our sample size is small. Second, we examined several SNPs and behavioral phenotypes, allowing for multiple comparisons, possibly increasing Type II error. Third, it was not possible to attempt replicating the ADHD effect reported in samples #1–3, because we did not have parent-rated ADHD symptoms in Sample 4. Finally, reliance on indirect data sources such as interviews, questionnaires, and parent report is a limitation, especially the absence of teacher ratings of ADHD, as teacher report is often the gold standard for ADHD diagnosis. Nevertheless, this study provides genetic characterization of ASD symptoms and guides directions for future work. Understanding the pharmacogenetics of atomoxetine response is also of importance. However, a larger sample is needed to provide statistical power for analyzing candidate genetic variants, perhaps enabling development of a predictive marker panel for atomoxetine response.

Even though atomoxetine is metabolized by CYP2D6, we did not find an association between CYP2D6 alleles and atomoxetine response in children with ASD and ADHD symptoms. Therefore, our results do not support routine genetic testing to determine whether or not a trial of atomoxetine is appropriate for these children. We did find an association between *TPH2* rs7305115 genotype and Restrictive/Repetitive Behavior, which is consistent with the well-established finding of elevated blood serotonin levels in patients with ASD (Gabriele et al., 2014.) As such, our finding suggests that continued research on a link between serotonin and repetitive behavior is warranted.

The study addresses the influence of variants in key genes encoding proteins with known relevance to ADHD and atomoxetine therapy. While the number of subjects was small, we tested frequent genetic variants of known biological function, rather than candidate or rare variants as done routinely in many other studies. While the results suggest possible effects on clinical phenotypes and treatment outcomes, these variants taken singly likely account for only a small portion of the estimated large heritability in ASD phenotypes. Large-scale studies suggest that numerous genes contribute to ASD; however, the number of genes affecting atomoxetine treatment outcomes alone may be much smaller, with a larger effect size. The present results support future studies with additional strong candidate genes, with the hypothesis that key genetic variants interact dynamically with each other (epistasis) – an important area to pursue in future research. Such gene-gene-drug interaction studies however will require larger study cohorts even if the combined effect size is large. The current results can serve to guide such future multi-gene studies.

Acknowledgments

The authors are grateful to Gloria Smith for assistance with sample preparation and to the participating families for volunteering for this research.

Funding: This work was supported by grants from: the National Institute of General Medical Sciences (U01GM092655), Center for Clinical and Translational Science at The Ohio State University (ARRA Pilot Project UL1 RR025755 project 60023888), National Institute of Mental Health to Ohio State University (5R01MH079080), University of Pittsburgh (5R01MH079082-05), and University of Rochester (5R01 MH083247), by Eli Lilly and Co., who provided atomoxetine and placebo, the National Center for Research Resources [University of Rochester

CTSA (UL1 RR024160), Ohio State University CTSA (UL1TR001070)] and the National Center for Advancing Translational Sciences of the National Institutes of Health and the United States Air Force Department of Defense (FA7014-09-2-0004 and FA8650-12-2-6359). We are grateful to all the families participating in the CORA registry. The views and opinions expressed in this article are those of the author(s) and do not reflect official policy or position of the National Institutes of Health (NIH), United State Air Force, Department of Defense, or US Government. The use of product(s) and/or manufacturer name(s) is added for clarification only; in no way implies endorsement by the authors, USAF, or DoD of the product(s) or manufacturer(s). The funders have not imposed any restrictions on free access to or publication of the research data.

Dr. Julia K. Pinsonneault received research funding from the Center for Clinical and Translational Science at The Ohio State University.

Dr. Wolfgang Sadec received research funding from the National Institute of General Medical Sciences.

Dr. Jill Hollway has received research contracts from Forest Research Institute, Supernus Pharmaceuticals, Sunovion Pharmaceuticals, Young Living Essential Oils and F. Hoffman-La-Roche.

Dr. Tristram Smith has received research funding from the National Center for Research Resources and the National Institute of Mental Health.

Dr. Benjamin Handen has received research funding from Curemark, Autism Speaks, Eli Lilly and Co., Roche and the National Institute of Mental Health.

Dr. L. Eugene Arnold has received research funding from Curemark, Forest, Lilly, Neuropharm, Novartis, Noven, Shire, Supernus, and YoungLiving (as well as NIH and Autism Speaks), has consulted with Gowlings, Neuropharm, Organon, Pfizer, Sigma Tau, Shire, Tris Pharma, and Waypoint, been on advisory boards for Arbor, Ironshore, Novartis, Noven, Otsuka, Pfizer, Roche, Seaside Therapeutics, Sigma Tau, Shire, and received travel support from Noven.

Dr. Eric Butter has received research funding from CogState, Bracket, Autism Speaks, the Simons Foundation, and Roche.

Dr. Gail E. Herman has received research funding from the National Center for Advancing Translational Sciences of the National Institutes of Health and the United States Air Force Department of Defense

Dr. Michael Aman has received research contracts, consulted with, served on advisory boards, or done investigator training for Aevi Genomic Medicine, AMO Pharma, CogState, Inc.; CogState Clinical Trials, Ltd.; Coronado Biosciences; Forest Research; Hoffman-La Roche; Johnson and Johnson; Lumos Pharma, MedAvante, Inc.; Ovid Therapeutics; ProPhase LLC; and Supernus Pharmaceuticals. He also received research funding from the National Institute of Mental Health and the Ohio State University CTSA.

Glossary of Genetic Terms

Allele	a version of the gene, each inherited from a different parent
Homozygous	a genotype where both alleles are the same at a given SNP
Heterozygous	a genotype of two different alleles at a given SNP, also referred to as carrier
Major allele	the allele that is most frequent in the general population
Minor allele	the allele that is less frequently observed in the population
SNP	single nucleotide polymorphism, also referred to as genetic variant
Wild-type	homozygous for the major allele

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Figure 1.

Box plot illustrating *TPH2* rs7305115 genotype association with ADI-R Restrictive/Repetitive Behavior score. The mean $\pm$ SEM is reported: GG (6.56 ± 0.55 N=30) GA/AA (4.96 ± 0.34 N=52). A significant difference between the groups was found using a two-tailed unpaired *t*-test ($p=0.01$).

Figure 2.

Box plot illustrating *DBH*rs1611115 genotype association with ADI-R Social and Restrictive/Repetitive Behavior scores. For the CC genotype group, N=38 and CT/TT, N=34. A two-tailed unpaired *t*-test revealed significant differences between genotype groups for the Social (5.5 ± 1.5 , $p=0.0006$) and Restrictive/Repetitive Behavior scores (1.5 ± 0.6 , $p=0.02$).

Figure 3.

Box plot illustrating *DAT* intron 8 repeat length association with ABC Hyperactivity or mean SNAP scores. The mean $\pm$ SEM is reported: ABC: 6_6 (32.5 ± 1.3 N=42) 6_5 or 5_5 (26.9 ± 1.7 N=38); SNAP: 6_6 (2.2 ± 0.07 N=35) 6_5 or 5_5 (2.0 ± 0.07 N=34). A significant difference between the group means was found using a two-tailed unpaired *t*-test (ABC: 5.6 ± 2.1 , $p=0.01$; SNAP: 0.24 ± 0.11 , $p=0.03$).

Table 1

Determination of metabolizer status with *CYP2D6* alleles

Nucleotide change	rs5758550	-1584C>G	100C>T	1023C>T/A	1707delT	1846G>A	2549delA	2615_2617delAAG	2850C>T	2988G>A
Star allele			*10	*17	*6	*4	*3	*9	*2	*41
SNP ID	rs5758550	rs1080985	rs1065852	rs28371706	rs5030655	rs3892097	rs35742686	rs5030656	rs16947	rs28371725
Effect of minor allele	enhance		decrease	decrease	no activity	no activity	no activity		decrease	

Table 2

Symptom subtype response to atomoxetine for participants with genetic data and number of participants with each CYP2D6 Metabolizer Status

	Ultra Rapid Metabolizer (UM)	Extensive Metabolizer (EM)	Intermediate Metabolizer (IM)	Poor Metabolizer (PM)
Total	6	25	17	4
Compliance symptom improvement	3 (50%)	7 (28%)	6 (35%)	2 (50%)
No compliance symptom improvement	3	18	11	2
ADHD symptom improvement	3 (50%)	9 (36%)	10 (59%)	3 (75%)
No ADHD symptom improvement	3	16	7	1

Table 3

Correlations between ASD characteristics and SNP genotypes

Gene	SNP	Minor Allele (MA)	MA Frequency	HWE <i>p</i>	ABC Hyperactivity <i>p</i>	SNAP <i>p</i>	Social <i>p</i>	Communication <i>p</i>	Restrictive/Repetitive Behavior <i>p</i>
<i>TPH2</i>	rs73051115	A	0.41	0.55	0.92	0.14	0.15	0.26	0.03
<i>DBH</i>	rs16111115	T	0.27	0.87	0.52	0.14	0.002	0.19	0.04
<i>DAT</i>	Intron8 repeat	5	0.26	0.35	0.01	0.03	0.08	0.85	0.86
<i>DAT</i>	rs27072	T	0.18	0.60	0.08	0.25	0.26	0.79	0.30
<i>DRD2</i>	rs2283265	T	0.10	0.90	0.13	0.28	0.10	0.93	0.95
<i>HTR2A</i>	rs6311	T	0.42	0.29	0.36	0.91	0.82	0.47	0.80
<i>NET</i>	rs5569	T	0.35	0.51	0.50	0.98	0.77	0.79	0.81
<i>COMT</i>	rs4680	G	0.45	0.55	0.91	0.75	0.73	0.88	0.44