## Title

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ORIGINAL ARTICLE

# Positive effects of methylphenidate on hyperactivity are moderated by monoaminergic gene variants in children with autism spectrum disorders

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Methylphenidate (MPH) reduces hyperactive-impulsive symptoms common in children with autism spectrum disorders (ASDs), however, response and tolerability varies widely. We hypothesized monoaminergic gene variants may moderate MPH effects in ASD, as in typically developing children with attention-deficit/hyperactivity disorder. Genotype data were available for 64 children with ASD and hyperactivity who were exposed to MPH during a 1-week safety/tolerability lead-in phase and 58 who went on to be randomized to placebo and three doses of MPH during a 4-week blinded, crossover study. Outcome measures included the Clinical Global Impression-Improvement (CGI-I) scale and the Aberrant Behavior Checklist (ABC-hyperactivity index). A total of 14 subjects discontinued the study because of MPH side effects. Subjects were genotyped for variants in *DRD1–DRD5*, *ADRA2A*, *SLC6A3*, *SLC6A4*, *MAOA* and *MAOB*, and *COMT*. Forty-nine percent of the sample met positive responder criteria. In this modest but relatively homogeneous sample, significant differences by *DRD1* ($P=0.006$), *ADRA2A* ($P<0.02$), *COMT* ($P<0.04$), *DRD3* ($P<0.05$), *DRD4* ($P<0.05$), *SLC6A3* ($P<0.05$) and *SLC6A4* ($P<0.05$) genotypes were found for responders versus non-responders. Variants in *DRD2* ($P<0.001$) and *DRD3* ($P<0.04$) were associated with tolerability in the 14 subjects who discontinued the trial. For this first MPH pharmacogenetic study in children with ASD, multiple monoaminergic gene variants may help explain individual differences in MPH's efficacy and tolerability.

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**Keywords:** autism spectrum disorders; dopamine; genetics; hyperactivity; methylphenidate

## INTRODUCTION

The autism spectrum disorders (ASDs) are a group of neurodevelopmental disorders including autistic disorder, Asperger's disorder and pervasive developmental disorder, not otherwise specified, which share impairment in social communication. Surveys estimate 1 out of 88 to 160 children as having an ASD.[1] Impairment from ASD varies considerably across its core dimensions, but also from associated maladaptive behaviors, such as symptoms resembling attention-deficit/hyperactivity disorder (ADHD).[2–4] Moderate-to-severe hyperactive-impulsive behaviors overlap in one-third or more individuals with ASD. These behaviors are often targeted by pharmacologic and other treatment efforts,[5,6] such as stimulants.

Despite common use of stimulants for ASD,[7,8] variable effects and reduced tolerability was suggested from small early studies,[9–12] but no moderators of response have been reported. In the largest stimulant trial in ASD, the Research Units on Pediatric Psychopharmacology (RUPP) Autism Network (2005) found methylphenidate (MPH) was superior to placebo in reducing ratings of hyperactivity,[13,14] but only half (35/72; 49%) were considered clinical responders. In addition, 19% of subjects were withdrawn because of adverse effects; variability in optimal dose, adverse events and response was striking.[15]

Variability in response to stimulant treatment is well known in typically developing children,[16] and there is interest in individual genetic variation moderating response in the treatment of ADHD.[17,18] However, no reports examining the pharmacogenetics of stimulant response exist for children with ASD. In addition, although a small number of reports have described genetic associations with adverse events and stimulants, none in typical ADHD or subjects with ASD have examined possible genetic associations in those with 'intolerable' side effects leading to dropout.

Investigations in typically developing children have suggested that variants in catecholamine-related genes may be associated with MPH response in ADHD.[17] The majority of these examined the variable number tandem repeat (VNTR) polymorphism located in the 3′ untranslated region of the dopamine transporter gene (*SLC6A3*),[17,19,20] the site of MPH action. A meta-analysis has suggested that the nine-repeat variant may confer reduced efficacy;[21] however, recent reports showed enhanced response of 9/9 homozygotes with a dose-dependent effect.[22] Investigations of stimulant response and the 48-base pair (bp) VNTR polymorphism in exon 3 of the dopamine D4 receptor (*DRD4*) gene[17] have been mixed, with greater efficacy seen in subjects with one or two copies of the four-repeat VNTR genotype

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(*DRD4.4*),[23–25] while others found opposite[26] or no effects.[27–29] Variants such as *DAT1 9/10*, *DRD4.7* and the α-adrenergic receptor 2A (ADRA2A) influence the slope of dose-response relations.[19,22] Few data are available regarding the impact of variation in other dopamine (DA)- receptors *DRD1*, *DRD2*, *DRD3* or *DRD5*. Although data are sparse and conflicting, some reports have associated MPH side effects with variants in *DAT1* and *DRD4* as well.[19,25,30]

Awareness of pharmacogenetic interactions of other neurotransmitter genes with psychostimulant action has grown. Variants in *ADRA2A* influence response of inattentive symptoms to MPH.[22,31,32] Interactions between DA and serotonin (5HT) have also been suggested as integral to MPH's behavioral and locomotor effects.[33] Two studies presented significant moderating effects of the 44-bp insertion–deletion polymorphism in the promoter region of the serotonin transporter gene (*SLC6A4*),[19,34] although one report did not detect an effect.[29] Genetic variants in *COMT* also influence basal transmitter availability,[35] working memory[36] and cognitive response to acute stimulant administration.[37] In ADHD, three reports concur that the Val158Met *COMT* variant moderates MPH effects.[19,38,39]

Variability in existing ADHD pharmacogenetic studies reflect the heterogeneity in methodology and outcomes. MPH dosing differs, there are few randomized, blinded, placebo-controlled studies, and only four assessed outcomes at multiple doses.[19,20,25,31]

The purpose of this preliminary investigation was to thoroughly evaluate for the first time in subjects with ASD whether gene variants in candidate loci would be found to associate with the overall clinical response to MPH. Candidates were suggested as moderators by at least two prior pharmacogenetic studies of MPH (*DRD4*, *SLC6A3*, *SLC6A4*, *COMT* and *ADRA2A*), risk genes for ADHD, known targets of MPH action (*DRD1*, *DRD2* and *DRD5*)[40] or associated with psychomotor functioning and impulsivity (*DRD2* and *DRD3*).[41,42] We hypothesized that gene variants suggested to exert significant effects on MPH response in typically developing children would also influence effects on response and tolerability in a homogeneous sample of children with ASD. However, we noted the multiple findings suggesting dysregulated DA function in ASD,[43–45] which could alter the relations between gene variants and MPH response and tolerability.

## MATERIALS AND METHODS

### Subjects

The details of the recruitment and baseline characteristics of the sample have been described previously.[46] Briefly, subjects were recruited at the five centers that formed the RUPP Autism Network funded by the National Institute of Mental Health (NIMH). The study was approved by each local institutional review board and monitored by a central NIMH data and safety monitoring board. Written informed consent was obtained from a parent or guardian, and assent was given by the child when capable.

To be eligible for the study, subjects from 5 to 14 years were required to meet Diagnostic and Statistical Manual, version-IV criteria for autistic disorder, Asperger's disorder, or PDD-NOS using clinical examination and the Autism Diagnostic Interview-Revised,[47] with diagnoses following Diagnostic and Statistical Manual, version-IV criteria. All subjects had significant symptoms of ADHD (based on the Clinical Global Impression (CGI)-Severity and Swanson, Nolan, and Pelham-IV (SNAP-IV)), were medically healthy, and were not taking any concomitant psychotropic drugs during the treatment phase of the study.

### Study design

Details of the study's design and rationale were published previously.[46] Briefly, it included a 1-week test dose phase of 1-day placebo, and 2 days each of the low, medium and high MPH doses used in the next phase to determine initial MPH tolerability, followed by a 4-week random-order, placebo-controlled, double-blind crossover phase to assess efficacy, and an 8-week open-label continuation phase for subjects showing a positive response to assess the maintenance of beneficial effects.

Subjects who were unable to tolerate MPH during the test dose phase as evidenced by a CGI rating of 'much worse' or 'very much worse' at a low or medium dose of MPH were excluded before being randomized. Subjects who tolerated all three dose levels of MPH during the test dose phase entered into the 4-week double-blind crossover phase. Each subject received placebo and three different doses of MPH in random order, 1-week each.

Low, medium and high doses of MPH were assigned based on body weight to approximate 0.125, 0.25 and 0.5 mg $kg^{-1}$ per dose, administered morning and noon, with an additional half-dose given at 1600 hours. Subjects with moderate or greater side effects to the highest dose level of MPH in the test dose phase received the medium dose during 2 weeks of the double-blind, crossover phase. Clinicians, the patient and the caregiver were blind to treatment assignment during the crossover phase.

Positive response was defined as a rating of 'much improved' or 'very much improved' on the CGI completed by the masked study clinician and a significant reduction on the Aberrant Behavior Checklist (ABC)-hyperactivity subscale (defined as a 25% reduction by both parent and teacher or a 30% reduction by parent or teacher) at any given weekly assessment during the blinded phase, and completion of all weeks of the blinded phase. For the purpose of these responder analyses, subjects who dropped out at any point after entering the double-blind phase ($n = 8$) are grouped with non-responders. For the purpose of tolerability analyses, any subject who dropped out in either the test dose or double-blind phase secondary to adverse events formed the intolerant group ($n = 14$).

### Genotyping

Ten candidate genes were selected for typing. A total of 36 variants were genotyped across the 10 loci. Common (>10%) locus variability was captured across the dopamine receptor genes *DRD1–DRD5*, *ADRA2A*, *MAOA* and *MAOB*, and *SLC6A4*. Several known functional variants were also genotyped, including the Val158Met *COMT* non-synonymous single-nucleotide polymorphism (SNP),[19] and VNTRs in *SLC6A3* (480-bp VNTR in the 3′ untranslated region),[17] *DRD4* (exon 3 48-bp VNTR)[48] and *SLC6A4* (44-bp insertion–deletion in the promoter region (5-HTTLPR) with modifying SNP (rs25531) and intron 2 VNTR (STin2)).[49] To minimize multiple testing of non-independent genotypes, allele frequencies were computed and genotype classifications defined *a priori* to any outcome analyses.

The *SLC6A3* VNTR (*DAT1*) was genotyped using published methods and primers.[50] The *DRD4*-exon 3 VNTR was genotyped with the following modified primers: F—5′-CTACCCTGCCCGCTCATG-3′; R—5′-CCGGTGATCTTG GCACGC-3′ using previously described methods.[51] The *SLC6A4* promoter region gene-linked polymorphic region (5-HTTPLR) short–long variant and intron 2 10/12 VNTR (STin2) were genotyped according to published protocols.[52] For VNTR variants (5-HTTLPR, 5-HTT STin2, *DAT* and *DRD4*), PCR products were electrophoresed in 2% gold agarose (BMA, Rockland, ME, USA) gels in 1x TBE and imaged with ethidium bromide under fluorescent Kodak digital camera (Rochester, NY, USA). Alleles were determined by comparison with molecular weight standards and control individuals with previously determined genotypes. Samples were double-scored by two technicians.

Tag SNPs were selected using Haploview Software[53] publicly available from the HapMap Consortium to capture variability present at ⩾10% with an $r^2 \geqslant 0.8$. SNPs were genotyped using the Life Technologies (Life Technologies, Grand Island, NY, USA) TaqMan platform with Qiagen Type-it Fast SNP Probe PCR Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. All genotypes were in Hardy–Weinberg Equilibrium ($P > 0.05$). Ten percent of the data set was genotyped in duplicate without any conflicting genotypes, and allele frequencies were checked for consistency with those reported by the HapMap Consortium.

Potential functional roles of associated non-coding variants were explored using the online UCSC Genome Browser[54] with ENCODE tracks[55] and the Broad Institute's Haploreg online resource.[56]

### Data analysis

All subjects randomized to the double-blind crossover phase were included in the responder analyses. Descriptive statistics are reported as the mean ± s.d. Before examining allelic effects on response, a general linear model was constructed using the ABC-hyperactivity for each gene variant examined to test for the presence of a positive dose effect (for example, if a treatment effect existed). Dose (low, medium, high or placebo), genotype and the interaction dose by genotype were then entered as predictor variables of parent-rated ABC-hyperactivity scores. The primary analyses were response by genotype compared using $\chi^2$ analyses or Fisher's exact tests. As the *DRD3* gly allele is the minor variant and some prior reports have shown biological differences between ser/ser and ser/gly versus gly/gly genotype groups, we restricted our comparison

accordingly.[57] As some investigators have noted significant moderation of stimulant effects of DRD4 variants in typically developing children specifying genotypes by the presence of 0, 1 or 2 copies of the '7' allele,[19] we also performed a similar three-way grouped analysis for this variant. Based on the fact that the previous literature on the *SLC6A3* VNTR has shown significant effects for 9/9 homozygotes, we analyzed our data according to this model.[22] Statistical significance was set as $P<0.05$, and $P<0.002$ with correction for multiple testing.

## RESULTS

Sixty-six children with autistic disorder ($n=47$), Asperger's disorder ($n=5$) or pervasive developmental disorder, not otherwise specified ($n=14$) completed the test dose phase and entered the double-blind crossover phase. Six subjects discontinued during the test dose phase. Of the 72 subjects who entered the trial, 7 subjects were unable or refused to provide samples for DNA and data were missing for one subject, leaving 64 subjects for genotyping. Sixteen subjects received an additional week of medium dose during the crossover phase because of moderate or greater side effects to the high dose in the test dose phase. Fifty-eight subjects were able to complete all 4 weeks of the crossover with the remainder leaving either because of adverse events ($n=7$) or for other reasons ($n=1$). The mean age of the subjects in this report was 6.90 years (±2.2, range 5.0–13.0); 75.9% were Caucasian, 87.9% were male; the mean intelligence quotient was 65.0 (±33.3 range = 16–135). Neither age, ethnicity nor diagnosis was a significant covariate.

**Table 1.** ABC-hyperactivity ratings and SNAP ratings in genotyped children with ASD ($n=58$) at entry into the double-blind phase

| *Outcome measure* | N | *Mean (± s.d.)* | *Item mean (± s.d.)* |
|---|---|---|---|
| Parent SNAP-IV ADHD (18 items) | 58 | 39.1 ± 7.9 | 2.26 ± 0.43 |
| Parent SNAP-IV ODD (8 items) | 58 | 9.67 ± 6.17 | 1.21 ± 0.78 |
| Parent ABC-hyperactivity rating at baseline (16 items) | 58 | 33.6 ± 7.43 | 2.09 ± 0.47 |
| Teacher SNAP-IV ADHD (18 items) | 54 | 35.74 ± 8.23 | 2.05 ± 0.46 |
| Teacher SNAP-IV ODD (8 items) | 54 | 8.48 ± 4.64 | 1.07 ± 0.59 |
| Teacher ABC-hyperactivity rating (16 items) | 54 | 31.46 ± 8.68 | 1.94 ± 0.55 |

Abbreviations: ABC, Aberrant Behavior Checklist; ADHD, attention-deficit/hyperactivity disorder; ASD, autism spectrum disorder; ODD, oppositional defiant disorder; SNAP-IV, Swanson, Nolan, and Pelham-IV.
Varying sample size (*N*) because of missing teacher data on six subjects.

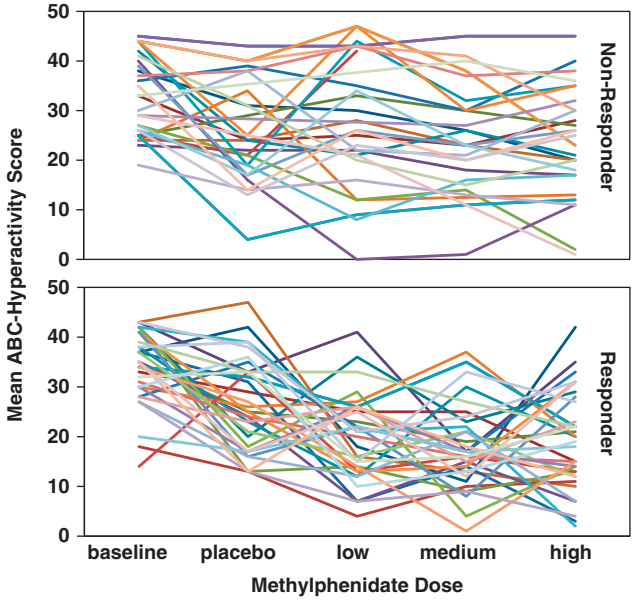


**Figure 1.** Individual parent-rated Aberrant Behavior Checklist (ABC)-hyperactivity scores during double-blind crossover trial. Top panel includes non-responders; bottom panel responders (see text for definition). Individual weekly ratings obtained at baseline and during random-order crossover weeks of methylphenidate treatment.

Table 1 shows parent and teacher rated SNAP-ADHD, SNAP-ODD and ABC-hyperactivity at baseline before entry into the double-blind phase. In general, subjects in the study had moderately severe symptoms of ADHD, and the optimal dose week demonstrated a moderate benefit associated with significantly reduced ABC-hyperactivity ratings and lower CGI-I scores. Analyses of ABC-hyperactivity scores across all four dose assessments showed a main effect for dose for all genotypes (all $P<0.001$). Variability in individual dose-response trajectories and optimal dose was substantial (Figure 1).

After grouping subjects by *a priori* defined responder or non-responder categories, we analyzed the association of genotypes with responder status. Table 2 reports the results of $\chi^2$ tests for genotype by response. Nominally significant responder by genotype effects (Figure 2) were noted for the dopamine receptors *DRD1* rs5326 ($P=0.006$, Figure 2a) and rs4867798 ($P<0.05$, Figure 2b), *DRD3* ser9gly rs6280 ($P<0.05$, Figure 2c) and *DRD4* rs11246226 ($P<0.04$, Figure 2d); the adrenergic receptor *ADRA2A* rs1800544 ($P<0.02$, Figure 2e); the dopamine and serotonin transporters *SLC6A3* VNTR ($P<0.05$, Figure 2f) and *SLC6A4* STin2 VNTR ($P<0.05$, Figure 2g), respectively; and the catabolic enzyme *COMT val/met* rs4680 ($P<0.05$, Figure 2h). No significant effects on response were evident for the *DRD2*, *DRD5* or *MAOA/B* variants tested.

In an effort to further reveal possible interactions between dose condition and genotype, in secondary analyses we tested the relationship between ABC-hyperactivity scores across the four treatment conditions for main effects of dose, genotype and dose by genotype interaction using analysis of covariance with baseline ABC-hyperactivity scores as the covariate. Results (Table 2) showed significant dose by genotype interactions for promoter SNPs in *DRD4* rs11246226 ($P<0.02$) and *SLC6A4* rs12150214 ($P<0.03$). In contrast to previous findings, *SLC6A3* VNTR analysis did not yield dose by genotype effects;[22] however, our power to detect these effects was very low given the small number of subjects with the 9/9 genotype ($n=3$), all of whom were responders.

The 14 subjects who dropped because of intolerability were similar to those completing the study (83% male, 83% Caucasian, mean age 8.6 years). Genetic comparison of those 14 subjects to trial completers revealed a protective effect for carriers of the minor allele at *DRD2* synonymous variant rs6275. Intolerability increased from 4% in minor allele carriers to 23% in those homozygous for the common allele ($P<0.001$), a result surviving correction for multiple testing. The DRD3 variant rs6280 (Ser9Gly) was nominally associated with tolerability, as intolerability increased from 3% in common allele homozygotes to 18% in carriers of the minor allele ($P=0.031$), suggesting that the minor allele is a risk factor for stimulant intolerance.

## DISCUSSION

In this first pharmacogenetic study of MPH in a modest but homogeneous (90% male, 90% stimulant naive) sample of children with ASD, improvement in hyperactive-impulsive symptoms associated with MPH administration was moderated significantly by several gene variants comprising elements of monoaminergic systems for dopamine, norepinephrine and serotonin, all of which have known regulatory effects on motor activity, and higher order cognitive functions.[58–60] Of the 10 candidate genes queried based on prior research, our data supported a contribution of genetic variation in 7 genes (*SLC6A4*,

**Table 2.** MPH responder/non-responder number by genotypes from double-blind crossover trial

| *Gene* | *Variant* | *Alleles* | *Responder % (n)* | *Non-responder % (n)* | *Genotype × responder status P-value* | *Dose × genotype P-value* |
|---|---|---|---|---|---|---|
| DRD1 | rs4867798 | C+ | 67% (22/33) | 33% (11/33) | 0.042 | 0.577 |
| | | TT | 40% (10/25) | 60% (15/25) | | |
| | rs5326 | T+ | 82% (14/17) | 18% (3/17) | 0.006 | 0.215 |
| | | CC | 44% (18/41) | 56% (23/41) | | |
| | rs686 | | | | 0.796 | |
| DRD2 | rs6277 | | | | | |
| | rs6589377 | | | | 0.714 | |
| | rs4938019 | | | | 0.292 | |
| | rs7131056 | | | | 0.505 | |
| | rs1800498 | | | | 0.714 | |
| | rs2283265 | | | | 0.566 | |
| | rs6275 | | | | 0.148 | |
| | rs1800497 | | | | 0.660 | |
| DRD3 | rs6280 | Ser/Ser | 74% (14/19) | 26% (5/19) | 0.044 | 0.569 |
| | (Ser9Gly) | Gly+ | 46% (18/39) | 54% (21/39) | | |
| | rs2134655 | | | | 0.696 | |
| | rs9880168 | | | | 0.259 | |
| | rs7633291 | | | | 0.404 | |
| | rs167771 | | | | 0.795 | |
| | rs3732790 | | | | 0.397 | |
| DRD4 | rs11246226 | AA | 79% (11/14) | 21% (3/14) | 0.038 | 0.013 |
| | | C+ | 48% (21/44) | 52% (23/44) | | |
| | rs3758653 | | | | 0.271 | |
| | Exon 3 VNTR | | | | 0.810 | |
| DRD5 | rs10033951 | | | | 0.930 | |
| SLC6A3 | 3′UTR VNTR | 10+ | 51% (27/53) | 49% (26/53) | 0.049 | 0.316 |
| | | 9/9 | 100% (3/3) | 0% (0/3) | | |
| SLC6A4 | rs12150214 | | | | 0.078 | 0.026 |
| | rs4251417 | | | | 0.577 | |
| | rs11080121 | | | | 0.642 | |
| | 5HTT-LPR | | | | 0.881 | |
| | STin2 VNTR | 10+ | 67% (22/33) | 33% (11/33) | 0.041 | 0.764 |
| | | 12/12 | 39% (9/23) | 61% (14/23) | | |
| ADRA2A | rs1800544 | CC | 71% (20/28) | 29% (8/28) | 0.015 | 0.790 |
| | | G+ | 40% (12/30) | 60% (18/30) | | |
| | rs12246561 | | | | 0.319 | |
| | rs3750625 | | | | 0.546 | |
| MAOA/B | rs1465108 | | | | 0.501 | |
| | rs3810709 | | | | 0.416 | |
| | rs3027399 | | | | 0.073 | |
| | rs10521432 | | | | 0.308 | |
| | rs1799836 | | | | 0.191 | |
| COMT | rs4680 | Met+ | 64% (25/38) | 36% (13/38) | 0.049 | 0.576 |
| | (Val158Met) | Val/Val | 36% (7/19) | 63% (12/19) | | |

Abbreviation: MPH, methylphenidate.
Shaded values indicate significant *P*-values.

*SLC6A3*, *DRD1*, *DRD3*, *DRD4*, *COMT* and *ADRA2A*) to clinical MPH response. Although our sample size did not provide adequate power for multiple comparison correction, the number of positive associations observed is unlikely to occur by chance. An exact binomial calculation for the probability of 7 or more significant hits out of 36 variants, whereas not an exact *P*-value, suggests that many of these associations are in fact true positives ($P<0.002$). These genetic effects may reveal some of the sources of marked variability in clinical outcomes observed in this trial and in clinical experience for this common treatment in ASD; in this trial only half of all subjects responded to moderate-dose MPH.

Our results add support to the previous suggestions of contributions of variants in several genes influencing MPH response in ADHD (*SLC6A4*, *SLC6A3*, *DRD4*, *COMT* and *ADRA2A*) but in some cases suggest new loci not previously implicated as MPH-response modifiers (*SLC6A4* STin2 VNTR and *DRD4* rs11246226). In addition, we also identified new associations with other genes not extensively examined thus far in the ADHD literature (*DRD1* rs5326 and rs4867798, *DRD3* ser9gly rs6280), but rational candidates given their distribution and known cognitive and behavioral pharmacologic effects of agonists and antagonists at these targets. Our findings should encourage other investigations of these variants in larger samples.

Not surprisingly, DA transporter and DA receptor variants showed the strongest associations with treatment response. In contrast to earlier studies,[19,20,61] our data agree with the recent report by Froehlich *et al.*,[22] showing an enhanced MPH response in *SLC6A3* 9/9 homozygotes. DRD1, highly expressed in cortex and striatum, is important in cognitive and motor control,[62] and may be a minor ADHD risk gene.[63,64] *DRD1* was most strongly associated with clinical response with two SNPs (rs5326 and rs4867798; $P=0.006$ and $P=0.043$, respectively) in our sample. *In silico* exploration suggests that both *DRD1* SNPs (rs5326 and rs4867798) may be functional, as they map to areas of open chromatin in the 5′ and 3′ untranslated regions and appear to alter binding sites for the transcriptional repressor ZNF263 and the transcriptional activator POU2F1 respectively. The non-synonymous Ser9Gly variant in *DRD3* was also associated with

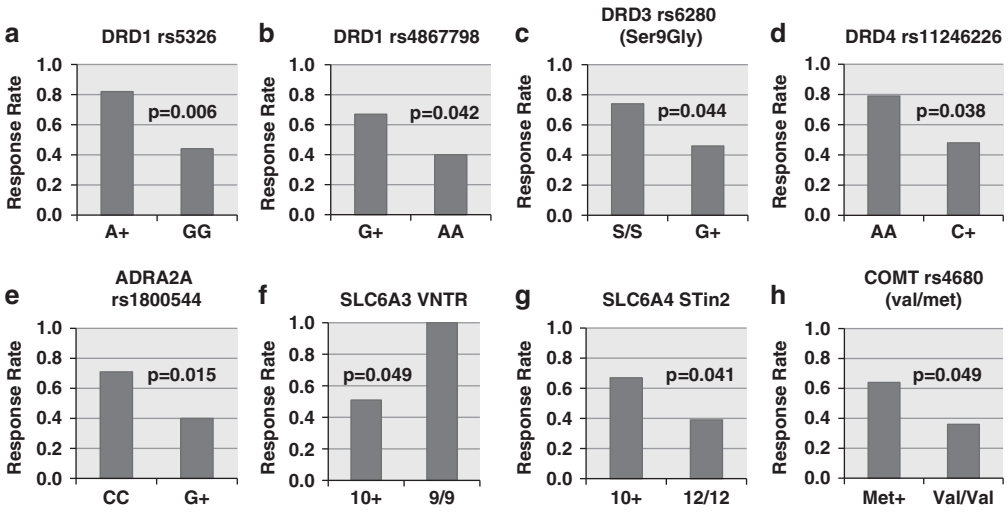


**Figure 2.** Genotype associations with methylphenidate clinical response rates. Positive response defined as Clinical Global Impression-Improvement (CGI-I) rating by blinded study clinician of 'much improved' or 'very much improved' and decrease in parent-rated Aberrant Behavior Checklist-Hyperactivity subscale of $>25\%$ from baseline for any active drug week. $n = 58$ subjects. *P*-values reflect results of $\chi^2$ tests. Panels a–h are plots of genotype group and their respective response rates for: 2a) *DRD1* rs5326; 2b) *DRD1* rs4867798; 2c) *DRD3* rs6280; 2d) *DRD4* rs11246226; 2e) *ADRA2A* rs1800544; 2f) *SLC6A3* VNTR; 2g) *SLC6A4* STin2; 2h) *COMT* rs4680.

MPH response. Ser/ser homozygotes demonstrated greater response than glycine allele carriers. Finally, we observed an association of a promoter SNP (rs11246226) in *DRD4* with both clinical response as well as a genotype by dose interaction. *DRD4* is well known as a minor risk gene for ADHD[65] and the exon 3 VNTR has been extensively studied for associations with cognitive phenotypes and drug response, but studies have not tested effects of other variants at this locus. It is difficult to ascertain the likely sources of differences between our data and some previous studies for effects of the *DRD4* exon 3 VNTR variant in particular, however, we note the literature is quite inconsistent with respect to its associations with response, even in reports from the same group.[19,25] Consistent with other reports, *DRD2* was not found to associate with clinical response to MPH, surprising given the multiple functional variants and stimulant-induced displacement of DRD2 binding following acute MPH.[40] Taken together, our findings of associations between *DRD1*, *DRD3* and *DRD4* and response measures suggest that common variants in the DA pathway do exert influences on the effects of MPH and moderate its DA-mediated impact on behavior.

Despite our modest sample size, the multiple (admittedly nominal) associations with several dopamine receptor subtypes (*DRD1*, *DRD3* and *DRD4*) lead us to speculate that the effects of gene variants in these targets may be amplified by ASD-related dopamine dysregulation. We posit that ASD-related differences in DA function may influence the interaction between gene variants and MPH, as evidence supports increased DA turnover (increased frontal uptake of[18] fluoro-3,4-dihydroxy-5-fluorophenylalanine and increased cerebrospinal fluid homovanillic acid) in ASD as well as increased dopamine transporter binding.[44,45,66] Such ASD-related differences may shift dose-response, enhancing or distorting pharmacogenetic relationships seen in typically developing children. Indeed, the high rate of MPH intolerance, several-fold greater than reports from typically developing children, suggests greater sensitivity to MPH pro-dopaminergic effects in ASD. These data add modest support to the notion that although common gene variants influence psychostimulant response in both typically and atypically developing subjects with hyperactivity, there are some possible differences because of underlying disorder neurobiology.

Our findings also reinforce the emerging view that beneficial effects of stimulants are multifactorial in their mechanism, reflecting drug effects on serotonergic and noradrenergic functions in addition to dopaminergic actions.[59] However, it is possible that identified non-DA gene effects may be also mediated via downstream effects on dopaminergic neurotransmission. For example, *SLC6A4* alleles associated with high expression (LPR *l* and particularly STin2 12 alleles), have been suggested to moderate mood effects of amphetamine in healthy subjects[52] and in typically developing children with ADHD, and LPR *l/l* homozygous subjects showed less improvement in global functioning with MPH.[34] However, opposing effects of the two variants were seen on different outcome variables.[19] Mice lacking *SLC6A3* still demonstrate reductions in activity from MPH, apparently mediated via 5HT.[33] The lower response rates in our 10/12 and 12/12 genotype subjects is in line with the known effects of the 12 allele on higher transporter expression. We also noted a tagging SNP in the first intron of *SLA6A4,* just upstream of the translational start, was associated with differential response at high dose MPH. This SNP, while intronic, is in a linkage disequilibrium block showing multiple enhancer histone marks suggesting an area of active transcriptional regulation.

Similarly, these data also contribute to the growing support for the involvement of the noradrenergic system in stimulant response in ADHD, consistent with direct effects of stimulant-induced NE release[58] and the reciprocal relationship of noradrenergic–dopaminergic pathways.[67] Our data did identify a moderating effect of the *ADRA2A* promoter SNP rs1800544 and clinical response, extending prior observations.[31,32] Online database mining supports a functional effect of the associated variant, as this SNP maps to a site of open chromatin in the *ADRA2A* promoter with histone marks and binding sites for the transcription factors RAD21 and CTCF, which have a role in promoter methylation, and ultimately expression.

Relevant to dopaminergic and noradrenergic transmission, we found support for earlier observations[19,38,39] that variants in *COMT* moderated clinical benefit from MPH. However, contrary to studies in healthy subjects[37] and typical ADHD, in our data set *met*-carriers showed a greater reduction in hyperactivity with MPH treatment than *val/val* homozygotes. Indeed, *met/met* subjects had a 75% (9/12) response rate versus 37% (7/19) for *val/val* homozygotes, a twofold differential. If ASD is associated with reduced tonic dopamine (consequences of increased DAT and increased pre-synaptic uptake), the resulting hypothesized shift in dose-response

could favor *met/met* individuals, as *val/val* subjects may lack sufficient dopamine availability to overcome reduced DA tone despite MPH releasing effects.

Our report is novel in our analyses of possible genetic underpinnings of stimulant intolerance, not heretofore researched in the ADHD pharmacogenetic literature. A *DRD2* variant, rs6275, not associated with response, was significantly associated with MPH intolerability and remained significant after correction. Homozygotes for the common allele at rs6275 showed a sixfold greater rate of intolerance. The non-synonymous Ser9Gly (rs6280) variant in *DRD3* was associated with MPH response and tolerability, as gly allele carriers were highly likely to either be intolerant of MPH and/or be less responsive than ser/ser homozygotes. These variants merit additional investigation in relationship to a range of stimulant-induced adverse effects in much larger samples.

Strengths of our findings include the thorough genetic analyses, use of a rigorous study design including comparisons of placebo versus multiple fixed-dose treatment conditions, blinded evaluations by multiple informants (parents, teachers and study clinicians), application of a careful, multi-informant based and clinically relevant definition of responder status, and recruitment of a largely male, stimulant-naive, homogenous sample. Relative to the only other small ($n \leqslant 13$), controlled studies of stimulants in ASD, our sample is more homogenous with respect to sex, ethnicity, treatment history and diagnosis.[11,12] Capitalizing on the strengths of our design, we were able to examine genetic effects observed under optimal treatment conditions, as determined by blinded assessments and multiple raters.

Limitations of this work include the short duration of MPH treatment observation periods, the modest sample size and the restrictions of genotyping to selected candidate genes. Our observations could be associated with an increased risk of type I statistical error.

In summary, the reduction of hyperactivity by MPH in individuals with ASD may be moderated at the level of individual genetic variability, particularly at loci that influence monoaminergic signaling. ADHD symptoms are common in ASD, and MPH remains the best empirically validated treatment for this target, although variation in response and tolerability is large. Additional studies replicating our observations and identifying other potential individual predictors of response to MPH in individuals with ASD are strongly needed. Improving overall outcomes from long-term intervention efforts for ASD remains a major clinical and public health challenge.

## CONFLICT OF INTEREST

Dr Aman has received consulting fees from Bristol-Myers Squibb, BioMarin, Roche and Supernus. Dr Aman also reports research support from Bristol-Myers Squibb and Johnson and Johnson. Dr Arnold has received research funding from Curemark, Shire and Lilly, and has consulted on advisory boards for AstraZeneca, Biomarin, Novartis, Noven, Seaside Therapeutics and Shire. Dr McCracken reports receiving consulting fees from BioMarin, Novartis and PharmaNet; he also reports research support from Bristol-Myers Squibb, Roche and Seaside Therapeutics. Dr McDougle reports having received consultant fees from Bristol-Myers Squibb, Hoffman-LaRoche and Forest Research Institute; he has also received research support and is on the speakers' bureau of Bristol-Myers Squibb. Dr Scahill reports receiving consultant fees from Brackett, Pfizer, Hoffman, BioMarin; he has also received research support from Pfizer, Shire and Hoffman. The remaining authors declare no conflict of interest.

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