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ORIGINAL ARTICLE

Alpha-2A adrenergic receptor gene variants are associated with increased intra-individual variability in response time

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Intra-individual variability in response time has been proposed as an important endophenotype for attention deficit hyperactivity disorder (ADHD). Here we asked whether intra-individual variability is predicted by common variation in catecholamine genes and whether it mediates the relationship between these gene variants and self-reported ADHD symptoms. A total of 402 non-clinical Australian adults of European descent completed a battery of five cognitive tasks and the Conners' Adult ADHD Rating Scale. Exclusion criteria included the presence of major psychiatric or neurologic illnesses and substance dependency. A total of 21 subjects were excluded due to incomplete data or poor quality cognitive or genotyping data. The final sample comprised 381 subjects (201 males; mean age = 21.2 years, s.d. = 5.1 years). Principal components analysis on variability measures yielded two factors (response selection variability vs selective attention variability). Association of these factors with catecholamine gene variants was tested using single-step linear regressions, with multiple comparisons controlled using permutation analysis. The response selection variability factor was associated with two *ADRA2A* single-nucleotide polymorphisms (SNPs) (rs1800544, rs602618), $p_{corrected} = 0.004$, 0.012, respectively, whereas the selective attention variability factor was associated with a *TH* SNP (rs3842727), $p_{corrected} = 0.024$. A bootstrapping analysis indicated that the response selection variability factor mediated the relationship between the *ADRA2A* SNP rs1800544 and self-reported ADHD symptoms. Thus this study finds evidence that DNA variation in the *ADRA2A* gene may be causally related to ADHD-like behaviors, in part through its influence on intra-individual variability. Evidence was also found for a novel association between a *TH* gene variant and intra-individual variability.

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INTRODUCTION

Increased intra-individual variability in response time (RT) is observed in many neurological and psychiatric conditions and a number of lines of evidence suggest that it may be an important endophenotype for attention deficit hyperactivity disorder (ADHD) that is able to index disease liability. First, differences between ADHD cases and controls in terms of intra-individual variability have been reported across a range of reaction time tasks and are associated with medium-to-large effect sizes in children, adolescents and adults.[1] Second, behavior genetic studies in both ADHD and general population samples have revealed that intra-individual variability is heritable and that this is driven largely by additive genetic influences.[2–4] Third, intra-individual variability and ADHD symptoms appear to share a common genetic basis, satisfying a key criterion of the endophenotype approach, that of bivariate heritability.[5] Yet despite strong evidence for its genetic origin, the precise molecular genetic drivers of intra-individual variability remain unknown. Further, it is not known whether the intra-individual variability endophenotype is better described under a liability-index model or a mediation model.[6,7] That is, whether genetic variants increase risk for both intra-individual variability and ADHD (liability-index model) or whether genetic risk passes directly through the endophenotype (mediation model).

Rather than being a nonspecific marker of general brain pathology with little explanatory value, increased intra-individual variability appears to be intimately linked with the integrity of the prefrontal cortex. For example, human lesion work suggests that lesions in the frontal cortex, but not other more posterior brain regions, are associated with increased intra-individual variability.[8] These data are supported by functional magnetic resonance imaging work in both children and adults that shows robust relationships between intra-individual variability in RT and task-related activations in a distributed network consisting of prefrontal, inferior parietal and caudate regions.[9,10] These lines of evidence have led to the suggestion that increased intra-individual variability may arise from fluctuations in the deployment of top–down attentional control across the duration of a reaction time task.[11,12] Other explanatory accounts posit that increased intra-individual variability may arise from a bottom-up failure to appropriately regulate arousal or from a failure to suppress activity in the default-mode network of the brain.[13–16] Common to each of these explanatory accounts is the suggestion that catecholamine systems may be particularly important in stabilizing moment-to-moment fluctuations in behavior (see Kuntsi and Klein[17] for a review of this literature). Accordingly, pharmacological work in humans has shown that the catecholamine agent methylphenidate (MPH) is able to robustly reduce intra-individual variability in both ADHD[18–20] and non-ADHD participants.[21]

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A number of studies have reported isolated genetic associations between both dopaminergic and noradrenergic candidate genes, such as *SLC6A3*, *DRD4* and *SLC6A2* and intra-individual variability in ADHD samples.[11,22,23] In addition, a quantitative trait linkage analysis using this phenotype in 238 ADHD and 147 control families revealed suggestive linkage to chromosomes 12, 13 and 17,[3] although these linkage signals showed little overlap with findings from linkage scans of ADHD. Clearly, a fuller account of the molecular genetic architecture of intra-individual variability and any causal role in the emergence of ADHD symptoms is required.

Here we sought to advance this literature in a number of important ways. First, we measured intra-individual variability in 402 non-clinical adults across a broad neurocognitive battery and performed principal components analysis (PCA) to reduce the dimensionality of the data and thereby reduce the need for multiple comparison corrections at the level of the phenotype. Second, we performed high-density single-nucleotide polymorphism (SNP) mapping across 22 autosomal catecholamine genes and performed genetic analysis against our PCA-derived intra-individual variability indices. Third, we examined the relationship between self-reported ADHD symptoms and the intra-individual variability indices and determined whether the latter mediated the relationship between genes and self-reported ADHD symptoms.

MATERIALS AND METHODS

Participants

Right-handed non-clinical participants of European descent were recruited from undergraduate participant pools at the University of Queensland, Brisbane, Australia and from the greater Brisbane population via public advertisements from July 2007 to October 2011. Following the Hapmap procedure for defining an ethnic group, the ethnicity criterion was satisfied if the participant self-reported that all four grandparents were of European descent.[24] The ethics committee of The University of Queensland approved the study and all participants gave informed consent before completing a battery of five cognitive tasks and the CAARS-S:L scales.[25] Full details regarding screening and exclusion criteria can be found in the Supplementary Information. Saliva was collected from each participant with Oragene kits (DNAgenotek, Kanata, Ontario, Canada). The final sample included 402 individuals (214 males and 188 females; mean age = 21.2 years, s.d. = 5.2 years). ADHD index scores for this sample ranged between 31 and 75 (mean = 47.8, s.d. = 8.5).

Stimuli and procedures

The following five tasks were presented in counterbalanced order across participants: (1) an Eriksen flanker task[26] in which participants made directional responses (left or right) to a central target arrow while ignoring four flanking distractors; (2) a choice response-time task in which participants were required to make rapid motor responses to two 'go' stimuli (X and O); (3) a stop-signal task[27,28] in which participants made rapid motoric responses to a go-stimuli but also inhibited their response when the go-stimulus was followed by a stop signal (red square presented around go-stimulus on 25% of trials); (4) a spatial competition task that required participants to select targets (Ts) from among competing distractors, with the set-size of stimulus arrays (target plus three or seven distractors) manipulated to tax attentional capacity; (5) an exogenous covert orienting task based on the cued target detection task of Posner *et al.*,[29] that required participants to select (validly, invalidly or neutrally) cued targets (Ts) from among competing distractors. These tasks are hereafter referred to as the Flanker, Go, Stop, Competition and Cueing tasks, respectively. See Figure 1 for the task schematics and the Supplementary Information for the full procedural details.

For each task, we calculated the mean RT (ms) for correct responses and the intra-individual coefficient of variation (ICV, standard deviation of RT (s.d. of RT)/mean RT) for correct responses. As individuals may differ on response-time variability measures simply because they have different processing speeds, the ICV provides a measure of response-time variability that controls for differences in the baseline speeds of processing.[8,9] Although other measures of variability such as s.d. of RT and the ex-Gaussian parameter of tau would also be suitable for analysis (see Kofler *et al.*[1]), our focus on ICV only mimimizes the number of multiple comparisons.

Genetic variant selection

SNPs were selected from autosomal catecholamine genes, namely those that are involved in synthesis, degradation, transport and receptor signalling of dopamine and/or noradrenaline (as identified in the KEGG, (http://www.genome.jp/kegg/pathway.html) and Gene Ontology, (http://www.geneontology.org) metabolic pathway databases). Haplotype tagging SNPs were chosen to provide 5′—3′ coverage of each gene using the HapMap project database of European Caucasians.[24] Selection criteria for tagging SNPs were a minor allele frequency of >0.1 and a correlation to surrounding known variants of $r = 0.9$ ($r^2 = 0.8$). We also included catecholamine SNPs that have been shown to modify the risk for ADHD, SNPs at exon–intron boundaries (300 bp) and SNPs mapped to within 5 kb of the gene including the promoter region and the 5′ and 3′ untranslated regions, which may have regulatory roles. In total, 151 SNPs on 22 genes were selected (see Supplementary Information).

Genotyping

Genotyping of all SNPs was performed by the Australian Genome Research Facility using iPLEX GOLD chemistry with a Sequenom MassArray on an Autoflex Spectrometer.

Genetic association analysis

Permutation methods are considered the gold standard for multiple comparison correction because they provide unbiased control for type 1 error while maintaining statistical power.[30] A special class of permutation methods achieves this while applying multiple comparison correction for correlated samples such as groups of SNPs where correlation arises from linkage disequilibrium.[30] Accordingly, we used such a single-step Monte-Carlo permutation method to test for genetic associations with our cognitive variables (see Supplementary Information for a brief description of our method and Whelan *et al.*[31] for a full description). After correction for the number of SNPs, the family-wise error rate for examining the association between a cognitive variable and all SNPs is $\alpha_{FWE} = 0.05$. As we examined genetic association with two ICV factors (see below), the final family-wise critical value was $p_{crit} = 0.025$. Our sample size of 381 yielded power $>80\%$ to detect a small/medium effect size, $r^2 = 0.06$ for any given SNP with this method of multiple comparison correction.

RESULTS

Genotyping

A quality control process was conducted on all task and genotypic data (see Supplementary Information). The remaining sample comprised 381 subjects (201 males; mean age = 21.2 years, s.d. = 5.1 years).

Principal components analysis

The ICV data for the five tasks (Flanker, Go, Stop, Competition, Cueing) were subjected to PCA using SPSS version 20. The correlation matrix contained many coefficients above 0.3, Bartlett's test of sphericity was significant[32] and the Kaiser–Meyer–Oklin value of 0.69 exceeded the recommended value of 0.6,[33] indicating that there was underlying latent structure in the data that could be exploited by a PCA. Analysis revealed two components with eigenvalues over one. The retention of two components was further supported by a screeplot, which showed a clear break after the second component. The two-component solution explained 66.9% of the variance, with components 1 and 2 explaining 46.0% and 21.0% of the variance, respectively. Oblimin rotation was performed and revealed the presence of a simple structure,[34] with both components showing strong loadings and all variables loading substantially on only one component. ICV from the Flanker, Go and Stop tasks loaded very strongly on component 1 and ICV from the Competition and Cueing tasks loaded very strongly on component 2 (see Table 1 for the pattern matrix). Consideration of these loadings indicated that

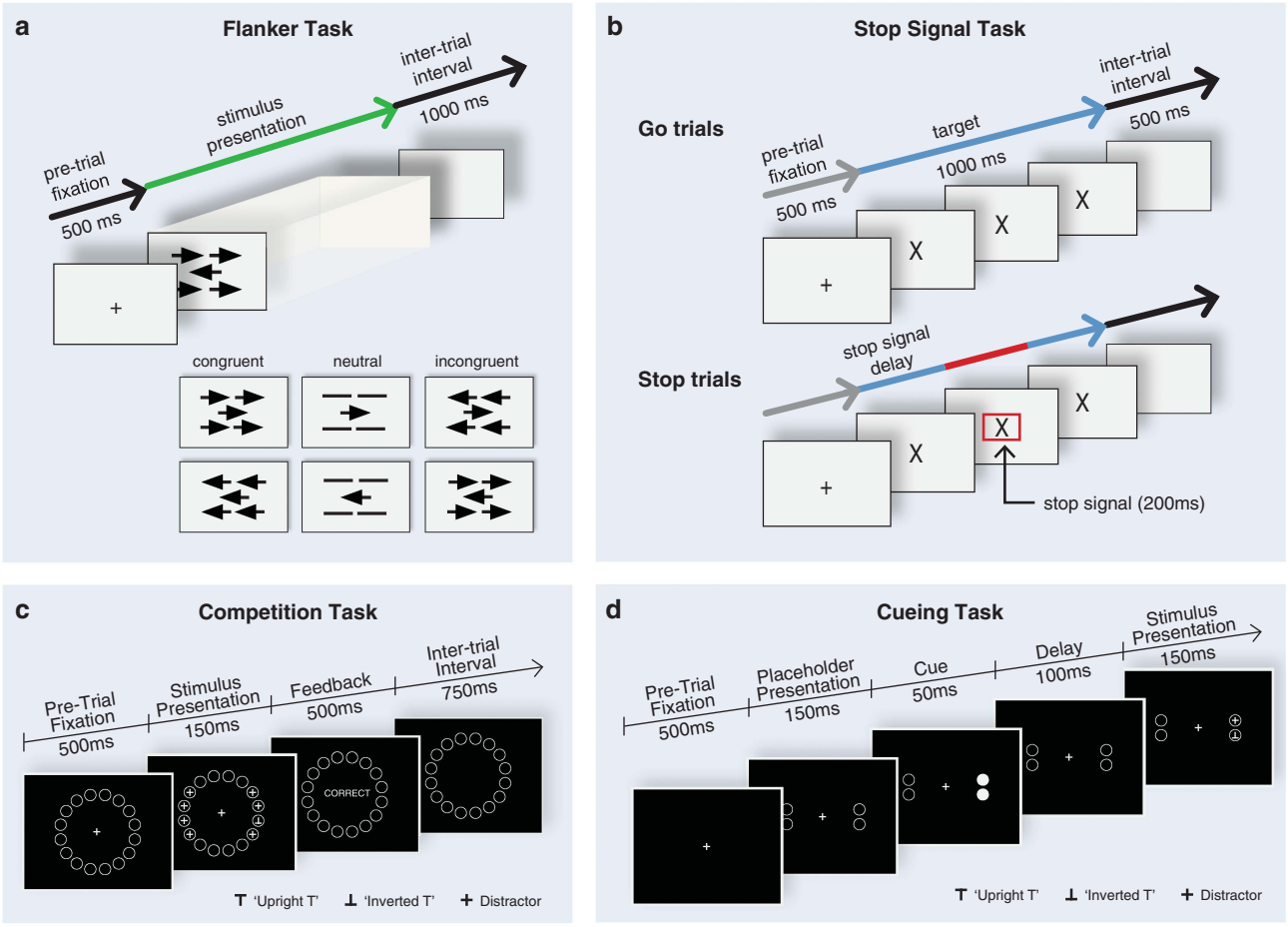

Figure 1. Schematic illustrations of the flanker, stop, competition and cueing tasks. (**a**) Flanker task: participants made a directional response (left or right) to a central target arrow while ignoring four flanking distractors. Relative to the direction of the central target, the set of flankers provided information that was either: congruent, incongruent or neutral (see trial type panel at bottom right of **a**; note that this panel is for illustration purposes only, it was not shown during the task). The timing schematic shows an incongruent trial with a left-pointing target. (**b**) Stop-signal task: on 'go' trials one of two targets was presented and participants responded with a button press (left for 'X', right for 'O'), on 'stop' trials a red square appeared around the target after a given stop-signal delay and the participants attempted to inhibit their response. The timing schematic shows both a go and a stop trial with an 'X' target. Note that the parameters of the GO task are identical to those of the Go trials in panel **b**. (**c**) Competition task: participants indicated the orientation of a target (upright or inverted 'T') while ignoring distractors ('+'). The distractors (3 or 7) appeared in the same visual field as the target (unilateral) or in both visual fields (bilateral). The figure shows a bilateral eight condition during practice (note: feedback on correctness of response was only provided in the practice blocks). (**d**) Cueing task: participants indicated the orientation of a target (upright or inverted 'T') while ignoring distractors ('+'). Each trial was preceded by a cue that was valid, invalid or neutral in regards to the information that it gave about where the target would appear. The figure shows a valid trial.

Table 1. Principal components analysis pattern matrix for ICV data from five task measures[a]

Component	*Task measure*				
	Flanker	*Go*	*Stop*	*Competition*	*Cueing*
1	**0.832**	**0.744**	**0.702**	−0.014	0.035
2	0.137	−0.151	−0.059	**−0.888**	**−0.855**

[a]Rotation converged in 5 iterations. Bold entries highlight the task measures that loaded strongly on a given component.

the first component was best represented as a response selection variability factor; these tasks predominantly required participants to select one response from among competing response alternatives. The second component was best represented as a selective attention variability factor; these tasks required participants to select task-relevant stimuli from task-irrelevant visual distractors, which did not map to a response alternative.

Genetic association analyses

Permutation analyses examined associations between ICV factor scores and the genetic markers. The response selection variability factor (factor 1) showed a significant association with the alpha-2A adrenergic receptor (*ADRA2A*) SNP rs1800544 with ICV scores increasing in an additive manner with each copy of the C allele ($p_{uncorrected} = 6.10 \times 10^{-5}$, $p_{corrected} = 0.004$, semi-partial correlation squared (r^2_{sp}) = 0.042). Likewise, there was a significant association between factor 1 and the *ADRA2A* SNP rs602618, with the ICV increasing additively with each copy of the A allele ($p_{uncorrected} = 1.67 \times 10^{-4}$, $p_{corrected} = 0.012$, $r^2_{sp} = 0.037$) (see Supplementary Information Table 1 for factor 1 results at all SNPs). These findings were replicated at an uncorrected significance level in each of the tasks that loaded on factor 1 (Flanker, Go and Stop), with additive increases in ICV with each copy of the C allele of rs1800544 and with each copy of the A allele of rs602618 (Figure 2) (rs1800544: *p*-values $= 3.09 \times 10^{-4}$, 0.008 and 0.008; rs602618: *P*-values $= 6.69 \times 10^{-4}$, 0.011 and 0.016, respectively). Only for the flanker task did the associations with each of rs1800544 and rs602618 also survive correction for the number of

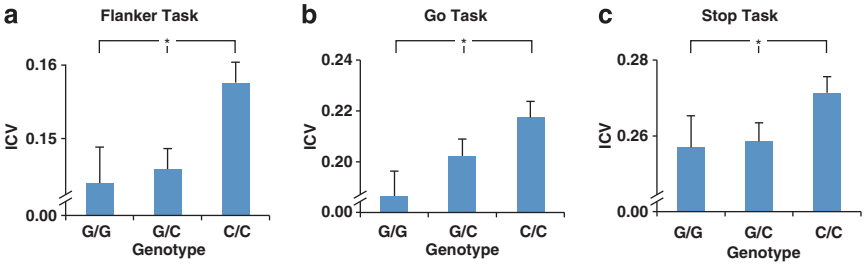

Figure 2. The relationship between genotypic variation at the *ADRA2A* SNP rs1800544 and the intra-individual variability coefficient (ICV) for the behavioral measures that loaded on the response selection variability factor (Flanker, Go and Stop). *Indicates a significant additive effect.

SNPs examined ($p_{corrected} = 0.018$, 0.042, respectively). These results indicated that although all of the measures that loaded on factor 1 (Flanker, Go and Stop) contributed to its strong association with SNPs in *ADRA2A*, the findings for this response selection variability factor were primarily driven by associations with the flanker task data (see Supplementary Information Table 2 for ICV task results at all SNPs). Inspection of linkage disequilibrium (LD) patterns for the associated *ADRA2A* SNPs indicated that rs1800544, lying in the promoter region, and rs602618, lying in the 3′ flanking region, are in near perfect LD ($D' = 0.989$, $r = 0.964$), indicating that the results for *ADRA2A* are likely to reflect a functional variant that lies between these SNPs or within a greater haplotype block.

The selective attention variability factor (factor 2) showed a significant association with a SNP in the tyrosine hydroxylase (*TH*) gene, rs3842727, located in the 3′ untranslated region. ICV scores increased additively with each copy of the A allele ($p_{uncorrected} = 1.30 \times 10^{-4}$, $p_{corrected} = 0.024$, $r^2_{sp} = 0.038$). This finding was replicated at an uncorrected level in each of the measures that loaded on factor 2 ($p_{uncorrected} = 0.003$ and 0.001 for competition and cueing, respectively (see Supplementary Information Table 1 for factor 2 results for all SNPs).

Correlations were calculated between the *ADRA2A* and *TH* SNPs, the ICV factors and the DSM IV inattention, DSM IV hyperactivity/impulsivity and ADHD index subscales of the Conners' Adult ADHD Rating Scale. Increased ICV scores were associated with increases in each of the above ADHD symptom indices. ICV factor 1 was significantly correlated with the ADHD index and showed a trend towards association with the DSM IV inattention measure. Similarly, factor 2 was significantly correlated with the ADHD index and the DSM IV hyperactivity/impulsivity measure. However, only the correlation between the ADHD Index and ICV factor 1 survived the multiple comparison correction (see Table 2). The *ADRA2A* and *TH* SNPs were not correlated with the symptom measures (note, mediation can be established via calculation of the indirect effect without the prior establishment of a total effect (SNP to symptom measure) because: (1) the indirect effect tests the compound pathway of predictor to mediator, mediator to outcome, (2) indirect effects can (and often do) occur in the absence of total effects.[35]).

Finally, we examined the role of intra-individual variability as an endophenotype of psychiatric symptoms in the mediational, rather than the pleiotropic, sense[6] by determining whether ICV factor 1 mediated any association between DNA variation in the *ADRA2A* SNPs and self-reported ADHD symptoms (ADHD index). Given the near perfect LD between the significant *ADRA2A* SNPs, we only examined the mediation model for the SNP with the strongest association with factor 1 (rs1800544). On the basis of extensive simulations, MacKinnon *et al.*,[36,37] recommended bootstrapping of the indirect effect over the Baron and Kenny model,[38] the Sobel test[39] and various other approaches to mediation because it has higher power while maintaining good control over the type I error rate. Therefore, we used Preacher and Hayes[40] SPSS bootstrapping macro to bootstrap the sampling distribution of the indirect effect (where the indirect effect is the reduction in the strength of the gene/symptom association that is due to the ICV). The indirect effect of rs1800544 on the ADHD index through ICV factor1 had a point estimate of 0.307 and a 95% bias-corrected bootstrap confidence interval of 0.072–0.665 (bootstrap estimates based on 100 000 bootstrap samples). That is, at the lower bound of the confidence interval the mediation effect was different to zero. These data therefore show that in a healthy adult population, DNA variation in *ADRA2A* accounts for significant variation in self-reported ADHD symptoms, in part through the effects of the gene on the intermediate phenotype of intra-individual variability.

Table 2. Correlations between ICV factor scores and Conners Adult ADHD Rating Sub-scale scores

Component	*Conners Adult ADHD Rating Sub-scale*		
	DSM IV inattention	*DSM IV hyp/imp*	*ADHD index*
1 Corr.	0.091	0.001	0.138
Sig.	0.080	0.983	0.008[a]
2 Corr.	−0.051	−0.102	−0.110
Sig.	0.332	0.050[b]	0.034[b]

Abbreviations: ADHD, attention deficit hyperactivity disorder; ICV, intra-individual coefficient of variation. DSM IV hyp/imp: DSM IV hyperactivity/impulsivity. Note: the directions of the correlations reported above are not directly interpretable because PCA maximizes the squared correlations between the variables and factors by rotation of the co-ordinate axes. However, examination of the relationship between ICV and symptom scale score for each task reveals that increased ICV was associated with increases in each of the ADHD symptom indices. [a]Significant at corrected level. [b]Significant at uncorrected level.

DISCUSSION

Heightened intra-individual variability in RT is a common finding across a number of different psychiatric and neurological conditions. In ADHD, increased intra-individual variability has been advanced as an important endophenotype that is able to index underlying genetic vulnerability.[17] Here we have shown using a large sample of non-clinical adults that intra-individual variability is predicted by common variation in catecholamine genes and that it mediates the relationship between catecholamine gene variants and self-reported ADHD symptoms.

The current study measured intra-individual variability across a neurocognitive battery and used PCA to derive independent factors for genetic analysis. The PCA yielded a two-component solution indicating a degree of domain specificity for intra-individual variability measured across a range of cognitive tasks. Assessment of the loadings on these factors suggested that they were best characterized as response selection variability and selective attention variability factors, and that these accounted for

46% and 21% of the variance, respectively. A number of past studies have used factor or composite scores derived across a number of reaction time tasks to enhance the familial loading of intra-individual variability in ADHD samples.[3] In contrast to some of these studies, our findings indicate that intra-individual variability may not be task invariant and thus it may be more appropriate to initially test for latent structure among tasks rather than simply aggregating measures across tasks (see Supplementary Information Table 3 for a comparison of genetic association results for the PCA-derived factors vs an aggregated measure).

The relevance of the association with *ADRA2A* reported here to ADHD is underscored by the observation that the response selection variability factor mediated the relationship between *ADRA2A* gene variants and self-reported ADHD symptoms. Interestingly, despite the preliminary evidence for a role of *ADRA2A* in conferring risk to ADHD and its executive dysfunction,[41] no consistent relationship with either the G or C allele of rs1800544 has been found. Indeed, a meta-analysis of 11 studies that examined association between childhood ADHD and rs1800544 genotype found no evidence for a relationship.[42] Thus one possibility highlighted by the current study is that the use of an intra-individual variability endophenotype may clarify the role played by *ADRA2A* in the aetiology of ADHD by providing a cognitive marker that is more proximal to the products of gene expression than the diagnostic category.[43] The support of a mediational, rather than pleiotropic, model of the endophenotype also has translational significance as it suggests that lowering of ADHD liability may follow from treatments that directly target intra-individual variability. Such treatments may include cognitive training and/or medications that influence ICV.

The results for *ADRA2A* and intra-individual variability are also consistent with the known pharmacological action of methylphenidate and its ability to modulate intra-individual variability in both ADHD and non-clinical populations.[18–21] Although MPH is often viewed as an indirect dopamine agonist, its therapeutic benefit is mediated in part by reuptake inhibition of the noradrenaline transporter and downstream effects on α2A receptors in prefrontal cortex.[44] A number of previous studies have also reported that allelic variation in *ADRA2A* is a significant predictor of MPH response in children with ADHD (see Polanczyk *et al.*[45]). Further, Kim *et al.*,[46] have recently shown that MPH-related changes in intra-individual variability measured on the flanker task are significantly predicted by variation in the *ADRA2A* gene in children with ADHD.

Although the functional significance (if any) of rs1800544 that maps to the promoter region of the *ADRA2A* gene (-1291 bp to the first codon) is uncertain, it has been shown that approximately one-third of all promoter variants alter gene expression to a functionally relevant extent.[47] Further, bioinformatics analysis predicts that rs1800544 will change the binding site of the human transcription factor HS$DPOLB_04 (http://brainarray.mbni.med.umich.edu/Brainarray/Database/SearchSNP/snpfunc.aspx). Nevertheless, replication of our result is necessary before functional genomic analyses of the *ADRA2A* polymorphisms.

An association was also found between allelic variation (rs3842727) in the gene encoding TH and the selective attention variability factor. TH is the enzyme responsible for catalysing the conversion of L-tyrosine to L-DOPA and is viewed as the rate-limiting step in catecholamine synthesis.[48] Mutations in *TH* could therefore have profound effects on catecholamine signalling. Indeed an association between the *TH* gene and ADHD is suggested by animal models. Specifically, rats that show the main behavioral traits of ADHD have altered expression of the TH enzyme, with hyper-expression seen in Naples high-excitability rats and hypo-expression in spontaneously hypertensive rats.[49] In humans, a non-significant trend has been reported between ADHD and a microsatellite mapped to intron 1 of the *TH* gene (rs35444567),[50] yet the LD between this marker and rs3842727 is low. To our knowledge, this is the first study to report a significant association between DNA variants in *TH* and intra-individual variability. The association with the selective attention variability factor is however consistent with known contributions of both dopamine and noradrenaline to selective attention.[51,52]

In sum, intra-individual RT variability across the response selection variability factor was found to be associated with common variation in the *ADRA2A* gene and to mediate the relationship between gene variants and self-reported ADHD symptoms. This finding is consistent with the suggestion that MPH is able to reduce intra-individual variability by selectively engaging the α2A receptor[44,53–57] and with a hypothesized role for *ADRA2A* in the aetiology of ADHD.[58] We also report a novel association between intra-individual variability in the selective attention variability factor and variation in the *TH* gene. The consistency of our genetic association results and their relationship to self-reported ADHD symptoms stands in contrast to the inconsistently reported genetic associations with a clinical ADHD phenotype. Our data support the notion that genetic effects may have greater penetrance at the level of an objectively measured endophenotype rather than a broad, subjectively defined clinical phenotype (for example, Catellanos and Tannock[43]). Nevertheless, the need for replication in psychiatric genetics is well recognized and future studies may wish to examine additional measures of variability when determining whether the associations reported herein generalize to population-based cohorts or clinical ADHD samples. Given that increased intra-individual variability is a feature of a number of psychopathological conditions,[1] our data may also have implications for gene discovery beyond ADHD.

CONFLICT OF INTEREST

MAB and LSN have received remuneration for speaking and travel expenses from Lilly Pharmaceuticals. LSN has also received remuneration for speaking and travel expenses from Janssen-Cilag, Astra-Zeneca, Lundbeck and Bristol Meyers Squibb. MAB, CDC, ZH and LSN report no other conflicts of interest. TDRC, OJ, MAVB, B-NK and JW report no conflicts of interest.

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