

Identification and functional characterisation of a novel dopamine beta hydroxylase gene variant associated with ADHD

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Abstract (< 250 words)

Dysregulation in neurotransmitter signalling has been implicated in the aetiology of ADHD. Polymorphisms of the gene encoding dopamine beta hydroxylase (*DBH*), a key player in catecholamine signalling, have been shown to be associated with increased risk for ADHD. Previous genetic studies of ADHD have reported associations with a range of *DBH* gene variants (rs2519152, rs1611115, rs1108580 and rs6271) however small sample sizes have led to inconsistency. Here we conducted TDT analysis in a large ADHD sample of 794 nuclear families to re-examine the relationship between *DBH* and ADHD. Although we did not replicate associations of rs2519152 and rs1611115 with ADHD, we identified a significant association with rs129882 ($p_{\text{corrected}} = 0.02$). Further, gene reporter assays of *DBH* rs129882 showed a significant impact of the ADHD-associated C allele on luciferase expression in a human neuroblastoma cell line, SH-SY5Y. These data demonstrate for the first time that a *DBH* gene variant which confers risk to ADHD is also associated with reduced *in vitro* gene expression.

Introduction

Attention deficit hyperactivity disorder (ADHD) is one of the most commonly diagnosed childhood psychiatric conditions with an estimated worldwide-pooled prevalence rate of 5.3-7.1% (Polanczyk et al., 2007). Affected individuals have significant impairments in attention or hyperactivity/ impulsivity or, more commonly, both. The morbidity associated with ADHD is high, with negative consequences for school and work achievement, family interactions and interpersonal and social functioning (Hoza, 2007; Lee et al., 2008; Merikangas et al., 2010). Convergent evidence from pharmacology, animal models, neuropsychology and brain imaging, suggests that dysregulated catecholamine signalling is a key pathophysiological substrate for ADHD (Arnsten, 2011; Pliszka, 2005). Here we show that a SNP within the 3' untranslated region (3'-UTR) of the gene encoding dopamine beta hydroxylase (*DBH*), a key regulator of catecholamine signalling in the brain, is associated with ADHD and influences gene expression levels in a human neuroblastoma cell line.

Dopamine beta hydroxylase (D β H) is a major enzyme involved in the regulation of the catecholamines, dopamine and noradrenaline. D β H is synthesized and packaged into vesicles of central noradrenergic and adrenergic neurons, peripheral noradrenergic neurons and adrenomedullary neurosecretory cells, where it catalyses the conversion of dopamine to noradrenaline (Weinshilboum, 1978). Its localisation within synaptic vesicles means that D β H is released into the extracellular space along with noradrenaline and any unconverted dopamine for example, during transmitter release. As a result D β H can be measured in cerebrospinal fluid, plasma or serum where it exists as a stable and highly heritable trait (Cubells & Zabetian, 2004). Not surprisingly, plasma D β H levels have been assayed in a range of heritable psychiatric conditions with putative catecholamine disturbance including schizophrenia, psychotic depression, substance abuse and ADHD (Cubells et al., 2002, 2011; Smith et al., 2003; Stallings et al., 2003). Although several early studies reported lower plasma D β H levels in ADHD (Bowden et al 1988; Rogeness et al, 1982), results were influenced by the presence of comorbid conduct disorder, making the specific relationship between plasma D β H and ADHD unclear. Nevertheless, a role for D β H in ADHD remains highly plausible given that frequently prescribed medications for ADHD (e.g. methylphenidate) potentiate catecholamine signalling (Berridge & Arnsten, 2013; Bymaster et al., 2002; Volkow et al., 2001).

The *DBH* gene locus is located in chromosome 9q34 and is 22,985 bases in length. The most frequently reported genetic association between ADHD and *DBH* maps to a single nucleotide polymorphism (SNP) at intron 5 (rs2519152) which is commonly referred to as the ‘Taq1poly’, owing to the use of the restriction endonuclease *TaqI* for genotyping (Daly et al., 1999). Although many subsequent studies have confirmed this association (Bhaduri et al., 2005; Carpentier et al., 2013; Roman et al., 2002) meta-analysis involving 6 studies failed to replicate it (Gizer et al., 2009) and rs2519152 failed to show an association with plasma D β H activity (Mustapic et al., 2014; Zabetian et al., 2001; Zabetian et al., 2003). In contrast to rs6271, a non-synonymous SNP located in exon 11 of the gene, appears to contribute to plasma D β H level, yet does not appear to associate with ADHD (Tang et al., 2006). Another frequently studied SNP in *DBH*, rs1611115 (-1021C/T polymorphism), is associated with plasma D β H activity ($p = 7.2 \times 10^{-51}$) at genome-wide significance level (Mustapic et al., 2014), and has been reported to associate with ADHD in Caucasian adults (Hess et al., 2009), Chinese nuclear families (Zhang et al., 2005) and Korean ADHD children (Kwon & Lim, 2013). Nevertheless, the association with rs1611115 and ADHD was not supported in other studies (Bhaduri & Mukhopadhyay, 2006; Brookes et al., 2006). Thus although inconsistency in the literature exists, the potential biological relevance of D β H to the pathophysiology of ADHD and preliminary evidence of genetic association, suggests that further investigation of this locus in ADHD is warranted.

To clarify the *DBH* association with ADHD and to assess the potential gene regulatory effect of the associated markers, we initially performed fine linkage disequilibrium analysis in a large sample of 794 ADHD nuclear families. We then conducted gene reporter assays in a human neuroblastoma cell line to examine the impact of any ADHD-associated gene variant/s on luciferase expression. We decided *a priori* to perform gene reporter assays on rs1611115 irrespective of its association with ADHD because of its critical influence on D β H enzymatic activity and because there is an absence of previous gene expression work in neural cell lines. Evidence for an association between ADHD and the previously reported *DBH* SNPs (rs2519152 and rs1611115) was not observed, however a significant association was observed with rs129882. Further, gene reporter assays of *DBH* rs129882 (and rs1611115) showed a significant impact of allelic variation on the expression level of the luciferase gene. Specifically, the C allele of the ADHD-associated rs129882 SNP produced a 2-fold decrease in luciferase activity while the C allele of rs1611115 yielded a 1.5 fold increase.

Materials and methods

ADHD sample

Seven hundred and ninety four ADHD nuclear families were ascertained from child psychiatric clinics and schools through Ireland, the United Kingdom (UK) and Australia. Of these families, 201 Irish and 69 Australian families have been included in a previous study by Hawi et al., 2003 and 57 families from the UK were included in Brookes et al (2006)). All families were ethnically Caucasian of European descent (Altshuler et al., 2005) and the ADHD probands were predominantly males (88%) with a mean age of 10.53 years. Clinical assessments were performed by experienced child psychiatrists/psychologists using both gold-standard questionnaires [the Conners' Parent ADHD Rating Scale-Revised: Long Version (CPRS-R:L); all sites] and semi-structured interviews [the Child and Adolescent Psychiatric Assessment (CAPA)(UK and Ireland) or the Anxiety Disorders Interview Schedule for Children (A-DISC) (Australia)]. Exclusion criteria included known neurological conditions, including pervasive developmental disorders and epilepsy (Silverman & Albano, 1996)(Silverman & Albano, 1996).

SNP selection and genotyping

Eleven *DBH* SNPs were included in the analysis with an average coverage of 2.09 Kbp/SNP. Tagging SNPs were selected using HapMap data. The SNPs that have been reported to be functional or associate with ADHD in past studies (rs1611115, rs1108580 and rs6271, rs2519152) (Cubells et al., 2011; Mustapic et al., 2014; Tang et al., 2006; Zabetian et al., 2001; 2003) were included as tags. The remaining tagging SNPs were selected to capture other variants within *DBH* (of minor allele frequency greater than 5%) with linkage disequilibrium (LD) of $r^2 \geq 0.8$.

Genotyping of 5 SNPs (rs2797849, rs1548364, rs2797853, rs6479643, rs77905, rs10761412) was commercially performed at the Australian Genomic Research Facility (AGRF). Sequenom technology was implemented to conduct SNPs genotyping via an initial locus-specific PCR reaction, followed by single base extension of an oligonucleotide that anneals immediately upstream of the polymorphic site of interest. Four other SNPs (rs1611115, rs1108580, rs6271, rs129882) were genotyped using a standard TaqMan® assay (Life Technologies) as recommended by the manufacturers. Finally, rs2519152 was genotyped using restriction fragment length polymorphism as described in (Daly et al., 1999).

Gene reporter assay

Cloning and construct preparation

To investigate the regulatory potential of rs1611115 and any identified ADHD-associated variants, DNA fragments containing the homozygotes (of the ‘associated’ and ‘non-associated’ alleles) were cloned into a luciferase gene reporter system. To mimic their positions within the *DBH* locus, these variants were cloned either upstream of the SV40 promoter or downstream to the Firefly luciferase gene, as appropriate. The resultant construct was then co-transfected with the *Renilla* luciferase control vector into SH-SY5Y, a human neuroblastoma cell line that is known to express *DBH* (Thibault et al., 2000). Co-transfection of Firefly and *Renilla* luciferase reporter vectors allows simultaneous detection and normalization of luminescence signals in the SH-SY5Y cell line.

Cell culture, transfection and luciferase reporter assays

The prepared constructs were transfected into the SH-SY5Y cell line (from Dr Kip Gabriel; Monash University, Australia) and maintained in Dulbecco's modified Eagle medium, GlutaMAX (Gibco, Life Technologies) supplemented with 100 µg/ml each of penicillin and streptomycin (Gibco, Life Technologies) and 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies) at 37°C in 5% CO₂. SH-SY5Y cells were plated at 2 X 10⁴/cm² on transparent black bottomed 24-well plates one day prior to transfection. Cell lines were co-transfected with 100 ng each of Firefly luciferase reporter vectors and 30 ng of *Renilla* luciferase reporter vector, pGL4.74 (Promega) using lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocol. Control transfections were performed using Firefly luciferase reporter vector with no insert. Forty-eight hours post-transfection, the cells were harvested, and the Firefly and *Renilla* luciferase activities were measured using the Dual-Glo® Luciferase Assay System (Promega) by VICTOR™ X Light Luminescence Plate Reader (Perkin-Elmer). For each construct, four independent transfections and triplicate luciferase assays were performed. Relative activity was normalized by the *Renilla* luminescence (as a ratio of firefly to *Renilla*), which accounted for variation in transfection efficiency and cell density.

Statistical Analysis

In order to test for association between the *DBH* SNPs and ADHD while avoiding population stratification issues associated with case control designs, we employed the transmission disequilibrium test (TDT) which uses untransmitted parental alleles as

internal controls. TDT analysis was carried out using the program UNPHASED which implements maximum-likelihood inference on genotype and haplotype effects. UNPHASED also allows for missing data arising from uncertain phase or missing genotypes (Dudbridge, 2008). Statistical comparisons of relative activity (Firefly/*Renilla*) from luciferase reporter assays were conducted in SPSS (IBM SPSS Statistics for Windows (Version 22.0) released 2013). For each *DBH* SNP, we conducted a 4 (independent experiment) x 3 (allelic group) analysis of variance (ANOVA) with bonferroni correction of post hoc tests.

Results

A total of 794 nuclear ADHD families were used to examine the role of *DBH* in ADHD. The observed and expected heterozygosity for all examined SNPs, Hardy-Weinberg equilibrium and minor allele frequencies are presented in Table 1. The observed genotype frequencies for the examined SNPs did not significantly differ from those expected according to Hardy-Weinberg equilibrium. The genotyping success rate ranged between 92-99% except for rs2519152 where the rate was 86%.

The genetic association results across 11 SNPs are presented in Table 2. A significant association of ADHD and rs129882 was observed ($\chi^2 = 9.71$, $p = 0.0018$, $OR = 1.37$). This association remained significant after bonferroni correction for multiple testing. Although there was a slight increase in the frequency of C and G allele transmission of rs2519152 and rs6479643 respectively, neither was significant. Notably, there was no evidence for an ADHD association signal with any of the functional SNPs that have been linked to plasma *DβH* activity (rs1611115, rs1108580 and rs6271). Haplotype analysis using sliding windows of 2, 3, 4 and 5 markers was also performed. However, no significant association stronger than the individual SNP association (rs129882) was observed.

Luciferase reporter assay of DBH rs129882 and rs1611115

We next sought to examine the impact of our ADHD associated *DBH* gene variant, rs129882 on luciferase expression as a *proxy* for *DBH* messenger RNA expression. DNA samples from individuals homozygous for either the C or T allele of rs129882 were selected from CEU HapMap individuals. The genomic region containing the C and T allele was PCR-amplified using Hot Fire DNA polymerase (Integrated Sciences) to create compatible ends for vector-insert ligation. The forward primer was synthesized with a

*Bam*HI restriction site (5' GCGCGGATCCGGAACAGCCCTGCAT 3') and the reverse primer with a *Sal*I restriction site (5' GATCGTCGACACTGAGTCAGCCGGG 3'). The PCR products were cloned downstream to the Firefly luciferase gene of the pGL3-Control vector (Promega), thus approximating the 3' location of this variant within *DBH*. Sequence orientation of the inserts was confirmed by Sanger sequencing at Micromon sequencing facility, Monash University.

Figure 1A displays gene expression (relative activity or luminescence ratio of firefly to *Renilla*) for each of the CC and TT homozygotes of rs129882 as well as the negative control vector. CC homozygosity was associated with a two-fold decrease of gene expression ($p < 0.001$) relative to TT homozygosity and the control vector. Gene expression in the TT homozygotes was also reduced relative to the control vector ($p < 0.001$), suggesting that it also alters luciferase expression but to a lesser extent than CC homozygosity. These data demonstrate for the first time that a *DBH* allele (C allele) which confers risk to ADHD is associated with reduced *in vitro* gene expression.

Although the current study failed to find support for a relationship between rs161115 and ADHD, we note that rs161115 plays a critical role in driving D β H enzymatic activity (Mustapic et al., 2014; Zabetian et al., 2001; Zabetian et al., 2003) and to our knowledge no previous study has examined the impact of the C/T alleles of rs161115 on gene expression in a human neuroblastoma cell line. We therefore also conducted a luciferase reporter assay on this polymorphism. DNA samples from individuals homozygous for either the C or T allele of rs161115 were selected from CEU HapMap individuals. Genomic regions containing homozygous C and T alleles were PCR-amplified with the forward primer containing a *Kpn*I restriction site (5' GATCGGTACCCAGCTGCCCTCAGTC 3') and the reverse primer with a *Xho*I restriction site (5' GCGCCTCGAGAGGGTGAGTGACAGG 3'). PCR products were cloned upstream to the SV40 promoter of Firefly luciferase reporter vector, pGL3-Control (Promega), thereby approximating the genomic location of rs161115 within *DBH*. Sequence orientation of the inserts was confirmed by Sanger sequencing at Micromon sequencing facility, Monash University.

As shown in figure 1B an influence of allelic variation (C/T) in rs161115 on luciferase expression was observed. There was a 1.5-fold increase in relative luciferase activities

associated with the CC homozygous condition relative to the control condition ($p < 0.01$). When compared to the vector control, a slight increase of luminescence for the TT homozygote suggested it has a minimal effect on luciferase expression. This data therefore provide further evidence that the *DBH* promoter variant rs1611115 is functional and supports results from genotype controlled studies of plasma D β H levels, where individuals homozygous for the C allele had higher mean levels of D β H activity relative to heterozygotes or those homozygous for the T allele (Tang et al., 2006).

Discussion

In the present investigation, TDT analysis of 11 *DBH* SNPs was conducted in a combined sample of 794 ADHD families. Although evidence for an association between ADHD and three SNPs previously reported to associate with ADHD (rs2519152, rs1611115 and rs1108580) was not observed, a significant association was observed with rs129882 ($p_{\text{corrected}} = 0.02$) (Table 2). Further, gene reporter assays of *DBH* rs129882 showed a significant impact of allelic variation on the expression level of the luciferase gene. Specifically, the C allele of rs129882 SNP produced a 2-fold decrease in luciferase activity relative to the control vector. We hypothesise that rs129882 may be associated with reduced *DBH* gene expression and that this may represent a novel risk mechanism for ADHD.

Previous genetic studies of ADHD that have tested association with *DBH* gene variants have generally reported evidence of association with rs2519152 (historically known as the TaqI polymorphism). It has been assumed that the inconsistent results for this SNP are attributable, at least in part, to the relatively small sample sizes of the individual studies. However, despite the large combined sample of the current investigation, only a slight (non-significant) increase in the transmission of the C allele of rs2519152 to ADHD cases was observed. Nor was there a significant association signal for rs1108580 in the current study, despite previous evidence of association for this SNP in a case-control design (Bhowmik et al., 2013). A slight non-significant increase in the transmission of the C allele of rs1611115 to ADHD cases was also observed (Table 2). In contrast to the findings for rs2519152, the result for rs1611115 is largely consistent with the literature as the majority of past studies have not found an association with ADHD (Bhaduri & Mukhopadhyay, 2006; Brookes et al., 2006). Nevertheless rs1611115 has been shown to be

a major contributor to plasma D β H activity (Hess et al., 2009; Mustapic et al., 2014; Zabetian et al., 2001; Zabetian et al., 2003) and our findings from gene reporter assays of rs1611115 confirm a regulatory effect of the C/T polymorphism on luciferase expression within a human neuroblastoma cell line. Thus it appears that although rs1611115 is strongly associated with plasma D β H levels and may influence *DBH* gene expression, allelic variation in this SNP does not confer risk to ADHD.

The present study observed a strong association signal mapped to rs129882 at the 3' UTR of *DBH*. It is important to note that this SNP is in linkage disequilibrium ($D'=0.63$, $r^2=0.1$) with rs2797853 which has been reported to associate with ADHD symptoms in a quantitative trait loci genome wide association study (Lasky-Su et al., 2008). Interestingly, rs2797853 in turn is in fairly strong LD ($D'=1$, $r^2=0.49$) with rs2519154 which was reported to associate with ADHD in Han Chinese sample (Guan et al., 2009). It is also notable that a haplotype constructed from rs1611115, rs1108580, rs5320 and rs129882 (C-A-G-C) was reported to associate with Parkinson disease ($p = 0.000005$, OR = 1.76) in a UK sample (Punia et al., 2010) and only rs129882 was associated with disease severity.

It is notable that although a number of *DBH* gene variants have been reliably associated (at GWAS significance levels) with plasma D β H activity, this is not the case for rs129882 (Mustapic et al., 2014). However, the correlation between D β H activity measured in the peripheral nervous system (PNS) and that measured in the central nervous system (CNS) is not known. Thus the possibility remains that a SNP such as rs129882 may not influence D β H activity in the PNS but could do so in the CNS. Indirect support for this possibility comes from *DBH* knockout studies in mice (Thomas et al., 1998). Mice lacking the *DBH* gene show a wide range of dopamine (and noradrenaline) distribution in the CNS and the PNS (Thomas et al., 1998). A greater than 1200-fold difference in dopamine level was detected in the striatum (600 ng dopamine; CNS) of these mice relative to the liver and muscle (0.5 ng dopamine; PNS), suggesting that dopamine levels in the CNS and PNS are uncorrelated in the *DBH* knockout mice.

So how might a SNP residing in the 3' UTR influence the functioning of *DBH* and hence confer risk to ADHD? SNPs in the 5' flanking region of genes are known to interact with transcription factors to initiate transcription (Buckland, 2006). However, binding of transcription factors has also been reported at the 3' UTR of genes. For example, the transcription factor SP1 was reported to regulate *SLC7A1* expression by differential

binding to a DNA motif at the gene's 3' UTR (Yang & Kaye, 2009). The rs129882 SNP is mapped to the 3' UTR region of the *DBH* gene and 3,960 bp upstream of the *DBH* antisense RNA 1 (*DBH-AS1*). Experimental data from the Encyclopedia of DNA Elements (ENCODE), shows that this region is enriched with greater than 20 bound transcription factors (e.g HNF4A, SP1 and POLR2A), histone modification and DNase hypersensitivity sites. Such enrichment of transcriptional regulators suggests that the 3' UTR of *DBH* could play an important role in the recruitment of transcription factors to activate transcription of *DBH-AS1* and consequently interfere with expression. Antisense transcripts have been demonstrated to repress or inactivate transcription of the sense strand by transcriptional interference through chromatin modification (to either disrupt structural conformation of chromatin or affect recruitment of non-histone proteins to DNA) (Pelechano & Steinmetz, 2013). For instance, co-transcriptional interference of antisense transcription has been demonstrated in the zinc-finger E-box-binding homeobox 2 gene that represses mRNA splicing by masking specific splice sites via antisense expression (Beltran et al., 2008). In this context, the 2-fold reduction in luciferase activity produced by the C allele of rs129882 highlights the potential functional importance of this substitution for gene expression. We speculate that the C to T allelic substitution of rs129882 activates the transcription of *DBH-AS1* which subsequently reduces/represses the expression level of *DBH*.

An alternative explanation for the altered expression of the C allele of rs129882 in the neuroblastoma cell SH-SY5Y involves microRNA regulation. DNA variations at 3' UTRs have been documented to impact microRNA targeting and microRNAs could consequently direct translational repression of target genes (Bartel, 2004; Lai, 2002). Gong et al (2012) observed that 43.4% of the SNPs mapped to the 3' UTR's of protein-coding genes either generate or abolish microRNA targets. Further 9.2% of all 3' UTR SNPs were predicted to disrupt and create microRNA sites at the same time (Gong et al 2012). For example, SNAP-25 (known ADHD candidate gene) expression was reported to be regulated by miR-153. Expression control of SNAP-25 via miR-153 resulted in significant change in motor neuron development, neurosecretion, neuron patterning and movement in zebrafish (Wei et al 2013). Interestingly, the *DBH* rs129882 SNP maps within seed regions of miRNAs including hsa-miR-1268, hsa-miR-1268b, hsa-miR-4468 and hsa-miR-585 (by Target Scan 6.2, Lewis, Burge, & Bartel, 2005). Further, hsa-miR-1268b is predominantly expressed in the brain tissue (intragenic microRNA database) and in the neuroblastoma SH-SY5Y cell line (Hinske et al., 2014; Surgucheva et al., 2013). Thus the putative *DBH*-3' UTR binding

of hsa-miR-1268b and its expression in SH-SY5Y cells suggests that the C/T substitution of rs129882 could impact hsa-miR-1268b binding and affect the expression of *DBH*. It is important to note that the molecular mechanism of microRNA regulation of gene expression is subtle and further work is required to clarify the mechanism by which the C/T substitution could influence DBH expression.

In summary, here we report a novel association between a SNP (rs129882) residing in the 3' UTR of *DBH* and ADHD. Although past studies suggest that this SNP does not correlate with plasma D β H activity, our gene reporter assays in a neuronal cell line showed a significant influence of the C allele on luciferase expression, suggesting that this SNP may influence *DBH* gene expression. Reduced *DBH* gene expression would be consistent with decreased conversion of dopamine to noradrenaline and thus with a relative hypoadrenergia in ADHD. Future studies should now examine whether antisense RNA or miRNA regulation via rs129882 could influence DBH expression and be a plausible risk mechanism for ADHD.

Table 1. Observed and expected heterozygosity, genotyping success rate and minor allele frequency of the examined *DBH* markers

SNP	Position	ObsHet	PredHet	HWEp	Geno	MAF
rs1611115	135490336	0.36	0.34	0.18	92	0.22
rs2797849	135491762	0.44	0.44	0.71	98	0.33
rs1108580	135494935	0.49	0.50	0.56	93	0.48
rs1548364	135497563	0.49	0.50	0.47	98	0.48
rs2519152	135499455	0.50	0.50	0.85	86	0.48
rs2797853	135502336	0.43	0.44	0.38	99	0.33
rs6479643	135504489	0.49	0.47	0.31	99	0.38
rs77905	135507918	0.52	0.50	0.23	99	0.50
rs10761412	135509411	0.45	0.47	0.10	98	0.37
rs6271	135512095	0.12	0.13	0.58	94	0.07
rs129882	135513490	0.32	0.33	0.54	95	0.20

ObsHET = Observed heterozygosity, PredHET= Predicted heterozygosity,
HWEp= Hardy Weinberg p value, Geno = Genotyping success rate,
MAF=Minor allele frequency

Table 2: TDT of DBH SNPS in 794 ADHD nuclear families

SNPs	Allele	T	UT	Tf	Uf	TDT	p-value	OR
rs1611115	C	1137	1123	0.78	0.77	0.31	0.58	1.05
rs2797849	G	1000	997	0.67	0.67	0.01	0.92	1.01
rs1108580	A	692	709	0.47	0.48	0.34	0.56	0.95
rs1548364	C	795	767	0.53	0.52	0.90	0.34	1.01
rs2519152	C	663	625	0.50	0.47	1.78	0.18	1.12
rs2797853	A	509	507	0.34	0.34	0.005	0.94	1.01
rs6479643	G	946	912	0.63	0.61	1.37	0.24	1.10
rs77905	T	758	753	0.51	0.50	0.03	0.87	1.01
rs10761412	C	579	557	0.39	0.37	0.60	0.44	1.07
rs6271	C	1375	1376	0.93	0.93	0.001	0.97	0.99
rs129882	C	1199	1127	0.82	0.77	9.71	0.0018*	1.37

T= Transmitted, UT= Untransmitted, Tf=Transmitted frequency,
Uf= Untransmitted frequency, OR=Odds ratio, * significant at corrected levels.

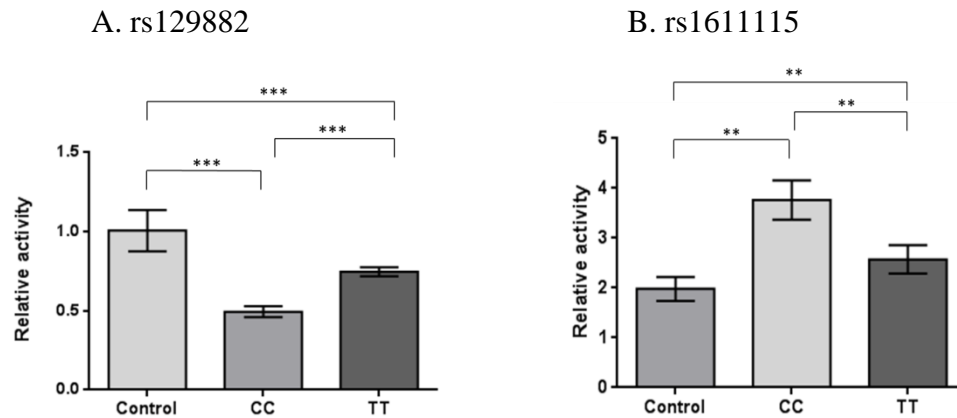


Figure 1. Relative luciferase activities associated with rs129882 and rs161115 of DBH in the human neuroblastoma SH-SY5Y cell line. (A) The homozygous C allele of rs129882 displayed lower relative luciferase activity than the homozygous T allele in SH-SY5Y cells. (B) The homozygous C allele of rs161115 demonstrated higher relative luciferase activity than the homozygous T allele in SH-SY5Y cells. To correct for variation of transfection efficiency and cell density, relative activities were calculated by the ratio of firefly luminescence to *Renilla* luminescence. Data represents mean and standard error of the mean. Four independent transfections and triplicate luciferase assays were performed for each construct. **P < 0.01, ***P < 0.001

References

- Altshuler, D., Brooks, L., Chakravarti, A., Collins, F., Daly, M., & Donnelly, P. (2005). A haplotype map of the human genome. *Nature*, *437*, 1299–1320.
- Arnsten, A. F. T. (2011). Catecholamine influences on dorsolateral prefrontal cortical networks. *Biological Psychiatry*, *69*(12), e89–99. doi:10.1016/j.biopsych.2011.01.027
- Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, *116*(2), 281–97.
- Berridge, C. W., & Arnsten, A. F. T. (2013). Psychostimulants and motivated behavior: arousal and cognition. *Neuroscience and Biobehavioral Reviews*, *37*(9 Pt A), 1976–84. doi:10.1016/j.neubiorev.2012.11.005
- Bhaduri, N., & Mukhopadhyay, K. (2006). Lack of significant association between -1021C->T polymorphism in the dopamine beta hydroxylase gene and attention deficit hyperactivity disorder. *Neuroscience Letters*, *402*(1-2), 12–16.
- Bhaduri, N., Sinha, S., Chattopadhyay, A., Gangopadhyay, P. K., Singh, M., & Mukhopadhyay, K. K. (2005). Analysis of polymorphisms in the dopamine beta hydroxylase gene: association with attention deficit hyperactivity disorder in Indian children. *Indian Pediatrics*, *42*(2), 123–129.
- Bowden, C. L., Deutsch, C. K., & Swanson, J. M. (1988). Plasma dopamine- β -hydroxylase and platelet monoamine oxidase in Attention Deficit Disorder and Conduct Disorder. *Journal of the American Academy of Child & Adolescent Psychiatry*, *27*(2), 171–174.
- Brookes, K., Xu, X., Chen, W., Zhou, K., Neale, B., Lowe, N., ... Asherson, P. (2006). The analysis of 51 genes in DSM-IV combined type attention deficit hyperactivity disorder: association signals in DRD4, DAT1 and 16 other genes. *Molecular Psychiatry*, *11*(10), 934–53. doi:10.1038/sj.mp.4001869
- Buckland, P. R. (2006). The importance and identification of regulatory polymorphisms and their mechanisms of action. *Biochimica et Biophysica Acta*, *1762*(1), 17–28. doi:10.1016/j.bbadis.2005.10.004
- Bymaster, F. P., Katner, J. S., Nelson, D. L., Hemrick-Luecke, S. K., Threlkeld, P. G., Heiligenstein, J. H., ... Perry, K. W. (2002). Atomoxetine increases extracellular levels of norepinephrine and dopamine in prefrontal cortex of rat: a potential mechanism for efficacy in attention deficit/hyperactivity disorder. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *27*(5), 699–711. doi:10.1016/S0893-133X(02)00346-9
- Carpentier, P. J., Arias Vasquez, A., Hoogman, M., Onnink, M., Kan, C. C., Kooij, J. J. S., ... Buitelaar, J. K. (2013). Shared and unique genetic contributions to attention deficit/hyperactivity disorder and substance use disorders: a pilot study of six

candidate genes. *European Neuropsychopharmacology : The Journal of the European College of Neuropsychopharmacology*, 23(6), 448–57.
doi:10.1016/j.euroneuro.2012.07.003

Cubells, J. F., Price, L. H., Meyers, B. S., Anderson, G. M., Zabetian, C. P., Alexopoulos, G. S., ... Gelernter, J. (2002). Genotype-controlled analysis of plasma dopamine beta-hydroxylase activity in psychotic unipolar major depression. *Biological Psychiatry*, 51(5), 358–64.

Cubells, J. F., Sun, X., Li, W., Bonsall, R. W., McGrath, J. A., Avramopoulos, D., ... Elston, R. C. (2011). Linkage analysis of plasma dopamine β -hydroxylase activity in families of patients with schizophrenia. *Human Genetics*, 130(5), 635–43.
doi:10.1007/s00439-011-0989-6

Cubells, J. F., & Zabetian, C. P. (2004). Human genetics of plasma dopamine beta-hydroxylase activity: applications to research in psychiatry and neurology. *Psychopharmacology*, 174(4), 463–76. doi:10.1007/s00213-004-1840-8

Daly, G., Hawi, Z., Fitzgerald, M., & Gill, M. (1999). Mapping susceptibility loci in attention deficit hyperactivity disorder: preferential transmission of parental alleles at DAT1, DBH and DRD5 to affected children. *Molecular Psychiatry*, 4(2), 192–196.
doi:10.1038/sj.mp.4000510

Das Bhowmik, A., Sarkar, K., Ghosh, P., Das, M., Bhaduri, N., Sarkar, K., ... Mukhopadhyay, K. (2013). Significance of Dopaminergic Gene Variants in the Male Biasness of ADHD. *Journal of Attention Disorders*. doi:10.1177/1087054713494004

Dudbridge, F. (2008). Likelihood-based association analysis for nuclear families and unrelated subjects with missing genotype data. *Human Heredity*, 66(2), 87–98.
doi:10.1159/000119108

Gizer, I. R., Ficks, C., & Waldman, I. D. (2009). Candidate gene studies of ADHD: a meta-analytic review. *Human Genetics*, 126(1), 51–90. doi:10.1007/s00439-009-0694-x

Guan, L., Wang, B., Chen, Y., Yang, L., Li, J., Qian, Q., ... Wang, Y. (2009). A high-density single-nucleotide polymorphism screen of 23 candidate genes in attention deficit hyperactivity disorder: suggesting multiple susceptibility genes among Chinese Han population. *Molecular Psychiatry*, 14(5), 546–554.

Hawi, Z., Lowe, N., Kirley, a, Gruenhage, F., Nöthen, M., Greenwood, T., ... Gill, M. (2003). Linkage disequilibrium mapping at DAT1, DRD5 and DBH narrows the search for ADHD susceptibility alleles at these loci. *Molecular Psychiatry*, 8(3), 299–308. doi:10.1038/sj.mp.4001290

Hess, C., Reif, A., Strobel, A., Boreatti-Hümmer, A., Heine, M., Lesch, K.-P., & Jacob, C. P. (2009). A functional dopamine-beta-hydroxylase gene promoter polymorphism is associated with impulsive personality styles, but not with affective disorders. *Journal of Neural Transmission (Vienna, Austria : 1996)*, 116(2), 121–30.
doi:10.1007/s00702-008-0138-0

- Hinske, L., França, G., Torres, H., Ohara, D., Lopes-Ramos, C., Heyn, J., ... Galante, P. (2014). miRIAD – Integrating microRNA Inter- And Intragenic Data. *Database - Oxford*, (in press).
- Hoza, B. (2007). Peer functioning in children with ADHD. *Journal of Pediatric Psychology*, 32(6), 655–63. doi:10.1093/jpepsy/jsm024
- IBM SPSS Statistics for Windows (Version 22.0). (2013).
- Kirley, A., Hawi, Z., Daly, G., McCarron, M., Mullins, C., Millar, N., ... Gill, M. (2002). Dopaminergic system genes in ADHD: toward a biological hypothesis. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, 27(4), 607–19. doi:10.1016/S0893-133X(02)00315-9
- Kwon, H. J., & Lim, M. H. (2013). Association between dopamine Beta-hydroxylase gene polymorphisms and attention-deficit hyperactivity disorder in korean children. *Genetic Testing and Molecular Biomarkers*, 17(7), 529–34. doi:10.1089/gtmb.2013.0072
- Lai, E. C. (2002). Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation. *Nature Genetics*, 30(4), 363–4. doi:10.1038/ng865
- Lasky-Su, J., Neale, B. M., Franke, B., Anney, R. J. L., Zhou, K., Maller, J. B., ... Faraone, S. V. (2008). Genome-wide association scan of quantitative traits for attention deficit hyperactivity disorder identifies novel associations and confirms candidate gene associations. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics : The Official Publication of the International Society of Psychiatric Genetics*, 147B(8), 1345–54. doi:10.1002/ajmg.b.30867
- Lee, S. S., Lahey, B. B., Owens, E. B., & Hinshaw, S. P. (2008). Few preschool boys and girls with ADHD are well-adjusted during adolescence. *Journal of Abnormal Child Psychology*, 36(3), 373–83. doi:10.1007/s10802-007-9184-6
- Lewis, B., Burge, C., & Bartel, D. (2005). Conserved Seed Pairing, Often Flanked by Adenosines, Indicates that Thousands of Human Genes are MicroRNA Targets. *Cell*, 120, 15–20.
- Merikangas, K. R., He, J.-P., Burstein, M., Swanson, S. A., Avenevoli, S., Cui, L., ... Swendsen, J. (2010). Lifetime prevalence of mental disorders in U.S. adolescents: results from the National Comorbidity Survey Replication--Adolescent Supplement (NCS-A). *Journal of the American Academy of Child and Adolescent Psychiatry*, 49(10), 980–9. doi:10.1016/j.jaac.2010.05.017
- Mustapic, M., Maihofer, A. X., Mahata, M., Chen, Y., Baker, D. G., O'Connor, D. T., & Nievergelt, C. M. (2014). The catecholamine biosynthetic enzyme dopamine β -hydroxylase (DBH): first genome-wide search positions trait-determining variants acting additively in the proximal promoter. *Human Molecular Genetics*. doi:10.1093/hmg/ddu332

- Pelechano, V., & Steinmetz, L. M. (2013). Gene regulation by antisense transcription. *Nature Reviews. Genetics*, *14*(12), 880–93. doi:10.1038/nrg3594
- Pliszka, S. R. (2005). The neuropsychopharmacology of attention-deficit/hyperactivity disorder. *Biological Psychiatry*, *57*(11), 1385–90. doi:10.1016/j.biopsych.2004.08.026
- Polanczyk, G., de Lima, M. S., Horta, B. L., Biederman, J., & Rohde, L. A. (2007). The worldwide prevalence of ADHD: a systematic review and metaregression analysis. *The American Journal of Psychiatry*, *164*(6), 942–8. doi:10.1176/appi.ajp.164.6.942
- Punia, S., Das, M., Behari, M., Mishra, B. K., Sahani, A. K., Govindappa, S. T., ... Juyal, R. C. (2010). Role of polymorphisms in dopamine synthesis and metabolism genes and association of DBH haplotypes with Parkinson's disease among North Indians. *Pharmacogenetics and Genomics*, *20*(7), 435–41. doi:10.1097/FPC.0b013e32833ad3bb
- Rogeness, G. A., Hernandez, J. M., Macedo, C. A., & Mitchell, E. L. (1982). Biochemical differences in children with conduct disorder socialized and undersocialized. *American Journal of Psychiatry*, *139*(3), 307–311.
- Roman, T., Schmitz, M., Polanczyk, G. V., Eizirik, M., Rohde, L. A., & Hutz, M. H. (2002). Further evidence for the association between attention- deficit/hyperactivity disorder and the dopamine-beta-hydroxylase gene. *Am J Med Genet*, *114*(2), 154–8.
- Silverman, W., & Albano, A. (1996). *Anxiety Disorders Interview Schedule for Children for DSM-IV: (Child and Parent Versions)*.
- Smith, K. M., Daly, M., Fischer, M., Yiannoutsos, C. T., Bauer, L., Barkley, R., & Navia, B. A. (2003). Association of the dopamine beta hydroxylase gene with attention deficit hyperactivity disorder: genetic analysis of the Milwaukee longitudinal study. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics : The Official Publication of the International Society of Psychiatric Genetics*, *119B*(1), 77–85. doi:10.1002/ajmg.b.20005
- Stallings, M. C., Corley, R. P., Hewitt, J. K., Krauter, K. S., Lessem, J. M., Mikulich, S. K., ... Crowley, T. J. (2003). A genome-wide search for quantitative trait loci influencing substance dependence vulnerability in adolescence. *Drug and Alcohol Dependence*, *70*(3), 295–307.
- Surgucheva, I., Gunewardena, S., Rao, H. S., & Surguchov, A. (2013). Cell-specific post-transcriptional regulation of γ -synuclein gene by micro-RNAs. *PloS One*, *8*(9), e73786. doi:10.1371/journal.pone.0073786
- Tang, Y., Buxbaum, S. G., Waldman, I., Anderson, G. M., Zabetian, C. P., Köhnke, M. D., & Cubells, J. F. (2006). A single nucleotide polymorphism at DBH, possibly associated with attention-deficit/hyperactivity disorder, associates with lower plasma dopamine beta-hydroxylase activity and is in linkage disequilibrium with two putative functional single nucleotide pol. *Biological Psychiatry*, *60*(10), 1034–8. doi:10.1016/j.biopsych.2006.02.017

- Thibault, C., Lai, C., Wilke, N., Duong, B., Olive, M. F., Rahman, S., ... Miles, M. F. (2000). Expression profiling of neural cells reveals specific patterns of ethanol-responsive gene expression. *Molecular Pharmacology*, *58*(6), 1593–600.
- Thomas, S. A., Marck, B. T., Palmiter, R. D., & Matsumoto, A. M. (1998). Restoration of norepinephrine and reversal of phenotypes in mice lacking dopamine beta-hydroxylase. *Journal of Neurochemistry*, *70*(6), 2468–76.
- Volkow, N. D., Wang, G., Fowler, J. S., Logan, J., Gerasimov, M., Maynard, L., ... Franceschi, D. (2001). Therapeutic doses of oral methylphenidate significantly increase extracellular dopamine in the human brain. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *21*(2), RC121.
- Weinshilboum, R. (1978). Human biochemical genetics of plasma dopamine-beta-hydroxylase and erythrocyte catechol-o-methyltransferase. *Hum Genet Suppl.*, *1*, 101–112.
- Yang, Z., & Kaye, D. (2009). Mechanistic insights into the link between a polymorphism of the 3'UTR of the SLC7A1 gene and hypertension. *Hum Mutat*, *30*, 328–333.
- Zabetian, C. P., Anderson, G. M., Buxbaum, S. G., Elston, R. C., Ichinose, H., Nagatsu, T., ... Cubells, J. F. (2001). A quantitative-trait analysis of human plasma-dopamine beta-hydroxylase activity: evidence for a major functional polymorphism at the DBH locus. *American Journal of Human Genetics*, *68*(2), 515–22. doi:10.1086/318198
- Zabetian, C. P., Buxbaum, S. G., Elston, R. C., Köhnke, M. D., Anderson, G. M., Gelernter, J., & Cubells, J. F. (2003). The structure of linkage disequilibrium at the DBH locus strongly influences the magnitude of association between diallelic markers and plasma dopamine beta-hydroxylase activity. *American Journal of Human Genetics*, *72*(6), 1389–1400.
- Zhang, H.-B., Wang, Y.-F., Li, J., Wang, B., & Yang, L. (2005). [Association between dopamine beta hydroxylase gene and attention deficit hyperactivity disorder complicated with disruptive behavior disorder]. *Zhonghua Er Ke Za Zhi. Chinese Journal of Pediatrics*, *43*(1), 26–30.