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Research Article

DOPAMINE BETA HYDROXYLASE: ITS RELEVANCE IN THE ETIOLOGY OF ATTENTION DEFICIT HYPERACTIVITY DISORDER

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Abstract: Attention Deficit Hyperactivity Disorder (ADHD) is a common neurodevelopmental condition characterized by impairing symptoms of inattention, hyperactivity, and impulsivity. Though symptoms of hyperactivity diminish with age, inattention and impulsivity persists through adulthood and often leads to behavioral as well as cognitive deficits. Majority of the patients respond to psychostimulants which forms the first line of therapy for ADHD. Some cases however fail to do so and treatment targeting the norepinephrine (NE) system has been found to be an alternative for them. Dopamine (DA) is metabolized to NE by the enzyme dopamine β-hydroxylase (DβH) and availability of these neurotransmitters in the prefrontal cortex is regulated by DβH. The enzyme is encoded by the DBH gene and polymorphisms in DBH have been found to exert independent influence on the enzymatic activity. We have explored association between DBH and two functional genetic polymorphisms, rs1611115 and rs1108580, in families with ADHD probands and compared with ethnically matched control individuals. Genomic DNA was subjected to PCR amplification followed by restriction fragment length polymorphism analysis. Plasma DβH activity was measured using a photometric assay. Age-wise DβH activity and its correlation with genetic polymorphisms were analyzed in ADHD subjects. Data obtained were subjected to statistical evaluations. Though the genotypes failed to show any statistically significant association individually, strong correlation was observed between DβH activity and the studied SNPs. Statistically significant correlation between the rs1108580 "A" allele and hyperactive/oppositional traits were also noticed. The present investigation thus supports a role of DBH in the etiology of ADHD.

Keywords: ADHD; DBH; genetic polymorphisms; rs1611115; rs1108580.

Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is the most common neurobehavioral disorder with a global prevalence of 5.29% (Polanczyk *et al.*, 2007) and a mean heritability of 0.76 places ADHD among the most heritable childhood onset psychiatric disorders (Faraone *et al.*, 2005). Percentage of school-going children affected with the disorder has been found to be as diverse as 1-2% to 10-20% (Bird, 2002; Faraone *et al.*, 2003). Influence of different environmental factors in the development of the disorder has also been observed (Banerjee *et al.*, 2007).

Although stimulant medications, targeting principally neurotransmitters like dopamine transporter, form the first line of pharmacotherapy for ADHD, α-2 noradrenergic agonists and selective norepinephrine re-uptake inhibitors were also found to provide relief to a number of patients with ADHD (Waxmonsky *et al.*, 2005; Weisler, 2007).

Synthesis of NE from dopamine is catalyzed by dopamine beta hydroxylase (DβH), a copper

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type-II, ascorbate dependent monooxygenase [EC 1.14.17.1] in the noradrenergic neurons (Weinshilboum, 1978). Localized within the synaptic vesicles of central and peripheral noradrenergic neurons, DβH is simultaneously released into the extra-cellular space during the secretion of the synthesized NE (Dunnette and Weinshilboum, 1976). Hence, DβH can be readily detected in the cerebrospinal fluid (from the central nervous system) and plasma (from the peripheral nervous system).

Both cerebrospinal fluid and plasma DbH (plDβH) activity has been reported to be under genetic control (Goldin *et al.*, 1982), albeit with an extensive variation among populations (Weinshilboum *et al.*, 1973). The 12 exons of *DBH* were found to harbor several polymorphisms that affect plDβH level (Hawi *et al.*, 2003; Zhang *et al.*, 2005) and two high-density single-nucleotide polymorphism (SNP) screening studies identified the DBH gene as a potential candidate for ADHD (Lasky-Su *et al.*, 2008; Guan *et al.*, 2009). In particular, a 5' flanking -1021C→T (rs1611115) SNP has been found to have profound genetic effect on the plDβH (Zabetian *et al.*, 2001). Apart from the rs1611115, few other variations have also been found to be positively associated with ADHD in numerous populations, including European-American (EA), African-American, German, Japanese, and Indo-Caucasoid (Tang *et al.*, 2006, 2007; Bhaduri and Mukhopadhyay, 2008; Bhaduri *et al.*, 2010). ADHD probands showing homozygosity for the risk allele of intron-5 TaqI site were shown to have significantly poor sustained attention in comparison to ADHD children not possessing this allele (Bellgrove *et al.*, 2006). Two SNPs -1021C→T (rs1611115) and exon 2 444G/A transition (rs1108580) have also been analyzed to understand the role of DBH in executive functioning and individuals harboring TT/AA genotypes, hypothesized to have lower DβH activity, were found to perform better in decision making tasks (Parasuraman *et al.*, 2012).

During our initial attempt to characterize ADHD genetically for *DBH* polymorphisms, we have failed to notice any significant association of the disorder with the exon 2 444G>a (rs1108580), intron 5 TaqI site (rs2519152) and -1021C→T (rs1611115) in the Indo-Caucasoid population (Bhaduri *et al.* 2005; Bhaduri and Mukhopadhyay 2006). In a follow up study on ADHD, the "G" allele of the rs2519152 showed mild paternal overtransmission and a haplotype consisting of the 13R of DBH-STR, 'T' of rs1611115, and 'A' of both rs1108580 and rs2519152 was found to be associated with low plDβH activity ($P<0.00005$) in both ADHD cases and controls (Bhaduri *et al.*, 2010). Independent effects of the of DBH-STR "13R", rs1611115'T', and 'A' of both rs1108580 and rs2519152, on plDβH activity were also reported earlier in various other populations (Cubells *et al.*, 1998; Zabetian *et al.*, 2001; Tang *et al.*, 2006). In the present investigation, the -1021C→T (rs1611115) and exon 2 444G/A (rs1108580), along with plDβH activity was analyzed in an extended number of subjects and attempts have been made to correlate the DβH activity and genetic polymorphisms with endophenotypic traits of ADHD.

Subjects and Methods

Subjects – ADHD probands (n = 180) and their family members, selected from the out-patient department of Manovikas Kendra Rehabilitation and Research Institute for the Handicapped, Kolkata, were interviewed by child psychiatrist using a behavioral questionnaire modeled after the DSM-IV (APA, 1994). This was followed by psychological evaluations through: (1) Conners' Parents and Teachers Rating Scale-Revised (Conners, 1997) for measurement of oppositional/cognitive/hyperactive traits and (2) Intelligence Quotient assessment by Wechsler Intelligence Scale for children above five years (Wechsler, 1991) and Developmental Quotient by Developmental Screening Test for children below 5 years (Bharat Raj, 1971). Probands suffering from exclusively psychiatric problem, pervasive developmental disorders, and/or mental retardation (IQ ≤ 75) with externalizing behavior mimicking ADHD were excluded from the study. Mean age of ADHD probands was 7.57 years (± 2.32 SD), with 10:1 male to female ratio. Out of the 180 subjects recruited, 137 families were complete parent-proband trios, while 18 families had only one parent and 25 were affected proband only. 19 probands belonged to the inattentive subtype, 21 belonged to the hyperactive/

impulsive subtype; the rest 140 belonged to combined subtype of ADHD. The most common co-morbid conditions observed were learning disorder (35%), while 22% suffered from co-morbid conduct disorder.

Two control groups (healthy volunteers and their children), one for the genetic study (n = 150; mean age 32.3 years ± 7.2 SD; 1:2 male to female ratio) and another age-matched group for analysis of enzymatic activity (n = 32; mean age 8.5 years ± 2.4 SD; 1:1 male to female ratio) were also recruited following evaluation by the same psychometric procedure. Anthropologically all the cases and controls belonged to the Indo-Caucasoid ethnic category. Informed written consent was obtained from all the participants. An Institutional Human Ethical Committee approved the study protocol.

Analysis of genetic polymorphisms – Peripheral blood was collected from ADHD subjects, their parents, and control individuals. Genomic DNA was prepared from leukocytes using the high-salt precipitation protocol (Miller *et al.*, 1988). Two SNPs were included for the study, rs1611115 (-1021C→T) and rs1108580 (exon 2 444G/A) and in the rest part of the text, the polymorphisms will be denoted by their respective rs numbers obtained from the NCBI database (dbSNP: *http://www.ncbi.nlm.nih.gov/SNP/*). Details of primer sequences, PCR amplification protocols and restriction fragment length polymorphism analyses will be available on request.

Association analyses for genetic polymorphisms – Case-control analysis for both alleles and haplotypes was carried out using COCAPHASE, which is part of a suite of programs UNPHASED (Dudbridge, 2003). GENEPOP program (web version 3.4; available at: *http://wbiomed.curtin.edu.au/genepop/*) was utilized for calculating the genotype frequencies of individual markers. Transmission Disequilibrium Test, which is a family-based association study (Spielman *et al.*, 1993), was performed using TDTPHASE (for both alleles and haplotypes) from the UNPHASED suite.

Measurement of plasma DβH activity – Blood collected in chilled heparinized vials was centrifuged at 16,060 g for 10 min; plasma was separated and stored at -20 ºC till further analysis (maximum 8 wks). DβH activity was measured following the protocol of Nagatsu and Udenfriend (1972); detailed analysis protocol has been discussed elsewhere (Bhaduri and Mukhopadhyay, 2008). DβH activity was calculated as nmoles of octopamine/min/ml plasma. Comparative analysis on DβH activity in ADHD subjects and controls was performed by Student's T-test.

Analysis of correlation between DBH polymorphisms and plasma DβH activity – For studying the correlation of plDβH with DBH polymorphisms, ADHD probands and controls were sub-grouped, based on their genotypes, for the two *DBH* polymorphisms. One-way analysis of variance (ANOVA) with Bartlett's test for equal variances was carried out using GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA (*www.graphpad.com*) in order to study the effect of the genotypic variation on the enzymatic activity.

Analysis of association between endophenotypic traits, genotypes, and plDβH activity – Limited number of ADHD probands (N = 45) were analyzed for three major ADHD associated endophenotypes, viz. oppositional behavior, cognitive problem and hyperactivity. Based on the T-score values obtained from the Conner's Rating Scale-Revised (Conner's, 1997), ADHD cases were given scores of nil (T score 61-64=0), low (T score 65-71=1), medium (T score 72-81=2) and high (T score >81 = 3) for the three endophenotypes. These cases were further assorted based on their genotypes and the data obtained was subjected to comparison by Student's T-test.

Results and Discussion

Genetic Association Studies

Genotypes of both rs1611115 and rs1108580 followed the Hardy-Weinberg Equilibrium in all the groups. Case-control and family-based studies for the SNPs yielded no significant difference (Tables 1 and 2). On the other hand, an earlier report on Han Chinese ADHD combined subtype subjects, the 'T' allele of rs1611115 were reported to be preferentially transmitted (Zhang *et al.*, 2005). Later the 'TT' genotype was shown to be

Table 1
Case-control comparison of allelic/haplotypic frequencies for rs1611115 and 1108580

Polymorphism	*Allele/ Haplotype*	*Frequency in Control*	*Frequency in Case*	χ^2, *P-value*	*Frequency in parents*	χ^2, *P-value*
rs1611115	C	0.82	0.81	0.08, 0.77	0.77	2.268, 0.13
	T	0.18	0.19		0.22	
rs1108580	G	0.32	0.31	0.05, 0.8	0.29	0.42, 0.51
	A	0.68	0.69		0.70	
Haplotypes of –	C-G	0.31	0.26	0.51, 0.47	0.27	1.01, 0.3
rs1611115 and	C-A	0.50	0.53	0.1, 0.09	0.49	0.03, 0.86
rs1108580	T-G	0.007	0.04	2.7, 0.75	0.01	0.73, 0.39
	T-A	0.17	0.15	0.001, 0.97	0.20	1.45, 0.22

Table 2
Familial transmission pattern analyzed by Transmission Disequilibrium Test

Polymorphism	*Allele/ Haplotype*	*Frequency of transmitted*	*Frequency of non-transmitted*	*Odds Ratio*	χ^2	*P value*
rs1611115	C	0.84	0.79	0.7	2.3	0.1
	T	0.16	0.21			
rs1108580	G	0.29	0.23	0.7	2.2	0.1
	A	0.71	0.77			
Haplotypes of–	C-G	0.28	0.22	1	1.97	0.16
rs1611115 and	C-A	0.01	0.005	2.3	1.057	0.3
rs1108580	T-G	0.54	0.57	0.7	0.26	0.6
	T-A	0.15	0.20	0.6	1.43	0.2

in strong association with neuropsychological performance (Kieling *et al.*, 2007) and adult ADHD with personality traits (Hess *et al.*, 2009). The present study, however, failed to observe any significant contribution, a difference that could be attributed to ethnic variations.

Though our study on the Indo-Caucasoid population revealed no significant association of individual alleles, a comparison of haplotypes between ADHD cases and controls revealed that a haplotype comprising of the 'C' allele of rs1611115 and 'A' allele of rs1108580 was over-represented in ADHD cases, which failed to reach statistical significance (χ^2 = 0.1; P = 0.8). Similar to this observation, in Irish ADHD subjects a minor over-transmission (P = 0.34) of the 'A' allele was observed and a haplotype comprising of rs1108580 'G' and rs2519152 'A' was reported to be strongly associated (P=0.045) with ADHD (Hawi *et al.*, 2003). Risk of ADHD was observed in presence of the rs1108580 'A' allele in the Czech population also (Kopeckova *et al.*, 2008). The rs1108580 G/A silent mutation in the exon 2 – intron 2 splice junction of *DBH* was earlier hypothesized to have an effect on mRNA splicing (Cubells *et al.*, 1998) and from the above observations further in depth analysis, involving large number of subjects, is warranted to find out whether that the "A" allele has a role to play in the etiology of ADHD.

Plasma DβH activity

Age-wise distribution of plDβH activity in ADHD probands is presented in Table 3. ADHD children (3-4 yrs) showed higher plDβH activity (9.89±1.7) as compared to age-matched normal healthy controls (3.03±0.20) and the difference was statistically significant (p<0.002). The plDβH activity was found to be low in other age groups. This difference was statistically significant for the late teens and adults (15-25 yrs) with ADHD (3.36±0.55) in comparison to children with ADHD (Table 3) and age-matched controls (6.7±0.89). While earlier investigators have reported no significant alteration in DβH activity in different age groups (Weinshilboum *et al.*, 1973; Rapapport

Table 3
Plasma DβH activity observed in ADHD subjects of different ages (Mean ± SEM)

Age (yrs)	*N*	*DβH activity nmoles of octopamine/min/ml plasma*	*P value*
3-4	8	9.895±1.7	—
5-6	21	6.93±0.95	0.07
7-8	19	6.68±0.92	0.06
9-10	11	6.60±1.14	0.06
11-12	8	6.14±1.65	0.06
13-14	10	6.93±0.95	0.07
15-25	5	3.36±0.55	**0.003**

Statistically significant difference is presented in bold.

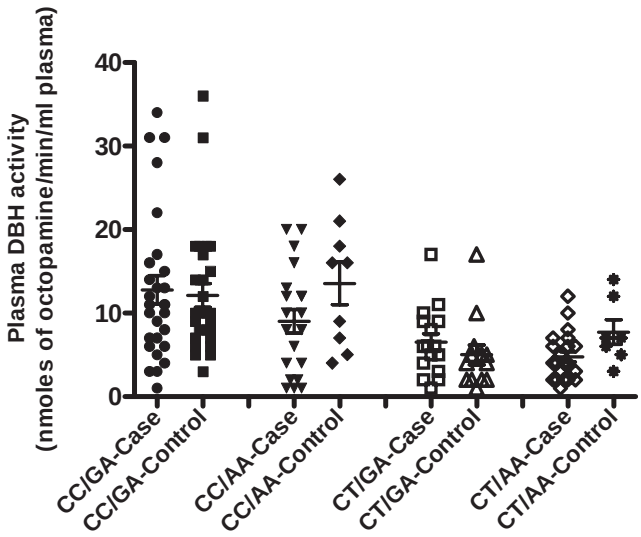

Figure 1: **Genotype-based comparison of plasma DbH activity in ADHD cases and age-matched controls**

et al., 1974), a study on normal healthy controls has revealed significantly lower levels in the 10-14 yrs age group which became stable by the age of 21 (Paclt and Koudelova, 2004). The pattern of distribution of plDβH activity in normal individuals (Paclt and Koudelova, 2004) was comparable with our age-matched control group. On the other hand, in our study, the ADHD subjects showed a significantly low enzymatic activity in late teens/adults. Whether this low activity is a genuine characteristic in association with the disorder or contributed by the small number of samples used for the present study is a matter of speculation and merits further analysis.

Correlation between genetic polymorphisms and plasma DβH activity

Four common genotype combinations between rs1611115 and rs1108580 were plotted in a vertical scatter-plot format (Figure 1). The rare genotype combinations (CC/GG and TT/AA), which occurred in very low frequencies, were omitted from the analysis. Strong negative association of the 'T' allele of rs1611115 with plDβH activity was observed; one-way ANOVA among the eight groups yielded a highly significant P-value of < 0.0001 for the CT genotype. Both case and control group yielded similar result. Although no functional importance of this site is yet known earlier investigators have also reported a very strong influence of this polymorphism on the plDβH where individuals with 'TT' genotype had very low plDbH activity while those homozygous for 'C' had the highest activity (Zabetian *et al.*, 2001; 2003). It may be plausible that rs1611115 is in strong LD with a true functional polymorphism not yet discovered.

The rs1108580 'A' allele was also reported to significantly lower the plDβH activity under homozygous condition in EA mood and anxiety disorder patients (Cubells *et al.*, 1998). A study on EA and German subjects have identified a haplotype block surrounding rs1611115 and all the markers within it were strongly associated with DβH activity; rs1108580 was reported to be a component of this haplotype block (Zabetian *et al.*, 2003). In the present study also, ADHD cases with the AA genotype showed lower DβH activity (Figure 1, CC/AA and CT/AA genotypes). We have also observed independent lowering effect of rs1611115 "T", rs1108580 "A" and rs2519152 "A" alleles in ADHD cases, while rs1611115 "T" exerted its effect on controls alone (Bhaduri *et al.*, 2010). Whether this difference in activity is controlled by the SNPs or haplotype blocks necessitate further investigation on a large cohort of samples.

Association of endophenotypes with DβH alleles and DβH level

Assortment of ADHD subjects based on genotypes and endophenotypes revealed a strong association of rs1108580 "A" allele with lower levels of hyperactivity and oppositional traits (Table 4). While cognitive problem failed to show

Table 4
Association of genotypes with endophenotypic attributes of ADHD

SNP	*Genotypes*	*N*	*Endophenotypes*					
			Oppositional behavior		*Cognitive problems*		*Hyperactivity*	
			Score±SEM	*P value*	*Score±SEM*	*P value*	*Score±SEM*	*P value*
rs1611115	CC	26	1.15±0.19	-	1±0.12	-	1.57±0.20	-
	CT	17	1.11±0.18	0.44	1.411±0.243	0.07	1.76±0.264	0.29
	TT	2	1.12±0.85	0.3	1.5±0.5	0.24	1.5±0.5	0.45
rs1108580	GG	7	1.71±0.28	-	1.14±0.34	-	2.428±0.297	-
	GA	17	1.05±0.21	**0.045**	1.41±0.12	0.25	1.41±0.27	**0.01**
	AA	18	0.94±0.20	**0.024**	1.10±0.20	0.4	1.47±0.20	**0.01**

Statistically significant differences are presented in bold.

any strong association with rs1108580, ADHD probands with rs1611115 CT genotype showed a trend of association (P = 0.07). Both the rs1611115 "T" and rs1108580 "A" alleles have been reported to be associated with low enzymatic activity (Cubells *et al.*, 1998; Bhaduri *et al.*, 2010). An association of rs1611115 with differences in performance on the Stroop Task was also noticed (Ji *et al.*, 2011). Further, individuals with TT/AA genotypes of rs1611115/rs1108580 were reported to have better executive functioning (Parasuraman *et al.*, 2012). In our study subjects also, significantly better behavioral attributes and less hyperactivity was noticed in individuals harboring the rs1108580 "A" allele. On the other hand, we have noticed a decrease in cognitive performance (higher score) in individuals having rs1611115 "CT" genotype though the difference was statistically not significant. Analysis of plDβH activity in association with these traits failed to show any significant difference which could be due to reduction in number of cases in association with wide variation in enzymatic activity in each groups. Therefore, our data on this small number of ADHD probands merits further in depth investigation on the matter.

Conclusion

This is the first study to explore association of DBH gene variants with plDβH activity as well as endophenotypic traits of ADHD. In the present study we have noticed: (1) statistically significant difference between children and adolescents for plDβH activity in both ADHD probands and controls; (2) significantly low plDβH activity in ADHD probands at late teens/adults as compared to age-matched controls; (3) association of rs1611115 "T" and rs1108580 "A" alleles with low plDβH activity; (4) a trend of association for rs1611115 "CT" with cognitive deficit; (5) less behavioral problems as well as hyperactivity in individuals harboring rs1108580 "A" alleles. It may be concluded from these observations that DBH plays an important role in the etiology of ADHD and investigations on the genetic as well as functional attributes of an individual may be helpful before prescribing medications. Major limitation of the present study is the small number of samples used. Extensive research incorporating genetic, environmental as well as demographic variables, in a large cohort of ADHD subjects, would help us to validate the above observations and facilitate in getting a better understanding of the disease etiology.

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Abbreviations

ADHD, Attention Deficit Hyperactivity Disorder; ANOVA, Analysis of Variance; DβH, Dopamine beta hydroxylase; DNA, deoxyribonucleic acid; DSM IV, Diagnostic and Statistical Manual for Mental Disorders, version IV; EA, Europen-American; IQ, Intelligence Quotient; NE, Norepinephrine; plDβH, plasma dopamine-beta hydroxylase; SEM, standard error mean; SNP, single nucleotide polymorphisms.

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