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Association of regulatory variants of dopamine β-hydroxylase with cognition and tardive dyskinesia in schizophrenia subjects

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Abstract

Background—Dopamine-β-hydroxylase (DBH, EC 1.14.17.1), which converts dopamine to norepinephrine, is a candidate gene in neuropsychiatric diseases.

Aim—To assess the effect of regulatory variants in *DBH* on schizophrenia and its endophenotypes –cognition and tardive dyskinesia.

Methods—We tested association of functional variants 19bp *Ins/Del*, rs1989787 and rs1611115 in *DBH* with i) schizophrenia (1236 cases, 1136 controls), ii) tardive dyskinesia (83 positive, 162 negative) and iii) performance functions of cognition (357 cases, 306 controls) estimated by the Penn Computerized Neurocognitive Battery.

Results—A modest haplotypic (*Ins*-C; 19bp *Ins/Del* – rs1989787 C>T; p=0.04) association was observed with schizophrenia. We observed ~39% reduction in activity of 19bp *Del* allele on luciferase assay. Analysis of covariance revealed interactions of tardive dyskinesia status and: i) 19bp *Ins/Del* (genotypic, p=0.04) and ii) rs1989787 and rs1611115 (combined genotypic, p=0.004) on Abnormal Involuntary Movement Scale total score. Association of rs1611115 with positive and negative syndrome scale (PANSS) total score (p=0.05) and allelic/genotypic association with lower positive (p=0.03/0.04), general psychopathology (p=0.01/0.01) PANSS

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Statement of human rights: Ethical approval: all procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (ethical committee at Dr. Ram Manohar Lohia Hospital, no. 18-15/2002-RMLH(HA-I)/3140 dated 5 March 2004 for tardive dyskinesia cohort; no. 18-62/06-RMLH(HA-I)/vol. II/63 dated 30 November 2008 for cognition cohort; no. 18-62/06-RMLH(HA-I)/1088 dated 15 January 2008 for schizophrenia cases and adult controls; no. 18-9/2002-RMLH(HA-I)/5262 dated 9 May 2005 for cord blood samples) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Statement on the welfare of animals: this article does not contain any studies with animals performed by any of the authors.

Supplemental material

Supplemental material for this article is available online.

scales in tardive dyskinesia-positive; and allelic/genotypic (p=0.02/0.05) with higher score of depressive factors in tardive dyskinesia-negative subgroups were observed. Analysis of covariance with continuous variable of cognition showed interaction of health status with: i) rs1989787 on accuracy and efficiency (p=0.03) of abstraction and mental flexibility; ii) rs1611115 on accuracy of working memory and emotion (p=0.05); iii) 19bp *Ins/Del* on processing speed of emotion (p=0.03). Allelic/genotypic association of rs1989787 with spatial ability (p=0.02–0.05) among healthy controls; association of rs1611115 with Global Assessment Scale scores in the past month (p=0.05) among schizophrenia subjects of cognition cohort was also observed.

Conclusions—With modest genotype–phenotype correlations available for *DBH* variants, personalized treatment regimens based on DBH activity for ameliorating tardive dyskinesia and cognitive symptoms may be plausible.

Keywords

Dopamine-β-hydroxylase; regulatory variants; 19bp *Ins/Del* (rs141116007); rs1611115; rs1989787; genetic interaction; schizophrenia; cognition; tardive dyskinesia; dual luciferase reporter assay

Introduction

Notable disturbances in thought process, emotion and loss of perception of reality are characteristic features of schizophrenia, a debilitating neuropsychiatric disorder. Dysfunctional dopaminergic and noradrenergic neurotransmission are implicated in the pathophysiology of schizophrenia (Davis et al., 1991; Klimek et al., 1999). Dopamine-β-hydroxylase (*DBH*) is an oxidoreductive enzyme in the norepinephrine biosynthetic pathway, a subpathway for catecholamine biosynthesis (Lamouroux et al., 1987), and catalyses the conversion of neurotransmitter dopamine to norepinephrine (Kaufman and Friedman, 1965; Levin et al., 1960). Several coding and regulatory variants (minor allele frequency (MAF) >0.05)) in this gene have been reported to confer susceptibility to a wide range of brain disorders including schizophrenia (Cubells and Zabetian, 2004; Gonzalez-Lopez and Vrana, 2019), Attention Deficit Hyperactivity Disorder (Kopeckova et al., 2006) and as expression quantitative trait loci (eQTLs) (Cubells et al., 1998; Mustapic et al., 2014; Zabetian et al., 2001). With specific reference to schizophrenia, there was association of the *Del/Del* genotype of 19bp *Ins/Del* with first episode schizophrenia (Hui et al., 2012); lower immediate memory in first episode schizophrenia (Hui et al., 2013); higher positive and negative syndrome scale (PANSS) score (Hui et al., 2012); higher excited symptoms score in tardive dyskinesia subjects (Hui et al., 2015a); lower attention scores in tardive dyskinesia-negative subjects (Hui et al., 2015b); lower attention scores in chronic schizophrenia (Hui et al., 2016). The two regulatory single nucleotide polymorphisms (SNPs) in this study, rs1989787 (p=0.11, 0.12) and rs1611115 (p=0.67, 0.9), were not associated with schizophrenia in the two genome-wide association studies (GWASs) (Pardinas et al., 2018; Schizophrenia Working Group of the Psychiatric Genomics, 2014). However, such observations are not uncommon considering the polygenic/genetically heterogeneous nature of schizophrenia. It is likely not captured in a GWAS at a genome-wide significance level, but may be relevant for a subset of the cohort. In this context, *DBH* is of considerable pharmacological relevance in schizophrenia/tardive dyskinesia and is also one of the few

genes wherein a few regulatory variants have been demonstrated to be of notable functional relevance, warranting its detailed analysis in schizophrenia and related phenotypes. Needless to say, such studies may be insightful for personalized medicine. A haplotype of 19bp *Ins/Del* and exonic rs1108580 variants was found to be more common among schizophrenia patients not responding to neuroleptics (Yamamoto et al., 2003). In some conditions, low DBH activity could be rate limiting for norepinephrine synthesis, leading to a hike in the dopamine/norepinephrine ratio (Parasuraman et al., 2012), and this was hypothesized to be a risk factor for psychiatric diseases such as schizophrenia (Cubells et al., 1998; Meltzer et al., 1976).

The *Del* allele of 19bp variant was speculated to decrease promoter activity leading to lower DBH levels and enzyme activity (Tang et al., 2007). Furthermore, a single SNP (rs1611115) was found to be the main predictor of plasma DBH activity, accounting for ~29% variation in plasma activity while 19bp *Ins/Del* was shown to account for ~6.5% (Tang et al., 2007). However, the activity status of 19bp *Ins/Del* has not been experimentally demonstrated to date. Another SNP (rs1989787) was also found to be functional and was shown to alter transcription, enzyme secretion and blood pressure (Chen et al., 2011). SNPs in this gene may result in a quantitative or a qualitative change. Defective secretion of DBH due to non-synonymous SNPs was found in DBH/norepinephrine deficiency (Kim et al., 2011) and in the case of rs6271 (Punchaichira et al., 2017) associated with bipolar disorder (Ates et al., 2013).

By targeted sequencing of all the exons and 10kb upstream region of the DBH gene combined with enzyme phenotyping in subjects of Indian origin we have recently demonstrated that rs1611115 accounts for ~31% of the variance in DBH activity (Punchaichira et al., 2016). Though a large number of association studies of *DBH* variants were reported, studies on genetically distinct Indian populations are negligible (Srivastava et al., 2010). Furthermore, contribution of *DBH* variants to tardive dyskinesia, an important iatrogenic disorder in schizophrenia, and to cognition has been poorly investigated. As mentioned above, functional validation of the common 19bp *Ins/Del* regulatory variant and its contribution to enzyme activity in a cellular model has not been deciphered to date. Therefore, in this study we focused on identifying associations, if any, of the three common upstream regulatory variants (19bp *Ins/Del*, rs1989787 and rs1611115) with schizophrenia, tardive dyskinesia and cognition in a schizophrenia cohort of north Indian ancestry. We also performed dual luciferase assays to demonstrate for the first time the functional implications of the 19bp *Ins/Del* variant *in vitro* using the SH-SY5Y cell line, which endogenously expresses DBH (Biedler et al., 1978).

Materials and methods

Recruitment of study subjects

Schizophrenia subjects and healthy controls in this study were recruited as described previously (Kukshal et al., 2013; Tiwari et al., 2005a, 2007). Briefly, subjects with schizophrenia or schizoaffective disorder were diagnosed according to Diagnostic and Statistical Manual of Mental Disorders-IV criteria at the Postgraduate Institute of Medical Education and Research–Dr. Ram Manohar Lohia Hospital, New Delhi. Assessment of all

the participants was done using the Hindi version of the Diagnostic Interview for Genetic Studies and the Family Interview from Genetic Studies (Deshpande et al., 1998; Nurnberger et al., 1994). In addition to healthy adults, cord blood samples collected from anonymous discarded placenta served as controls. Venous blood (5 mL), was collected from participants for genetic analysis. Isolation of genomic DNA was done using phenol chloroform extraction method. Experiments were undertaken after procuring an informed consent from the participants or parents/guardian of this study and this study conforms with the code of Ethics of the World Medical Association Declaration of Helsinki. Further institutional ethical committee clearance was obtained from the participating hospital.

Assessment of tardive dyskinesia

Tardive dyskinesia was assessed in a subset of schizophrenia cases included in this study using the Schooler and Kane criteria (Schooler and Kane, 1982) for items 1 to 7 of the Abnormal Involuntary Movement Scale (AIMS) (Guy, 1976) as described previously (Tiwari et al., 2005b). This cohort comprised tardive dyskinesia-positive (TD+ve) (males n=51 (age=35±12 years); females n=32 (age=32±12 years); AIMS score=6.2±3.4) and tardive dyskinesia negative (TD−ve) (males n=77 (age=30±10 years); females n=85 (age=31±9.2 years); AIMS score=0.7±1.0) samples and is described elsewhere (Tiwari et al., 2005a, 2005b). Two mild or one moderate or higher rating in any of the symptoms was used as a research diagnostic criterion to classify schizophrenia patients as TD+ve or TD−ve. While the AIMS total score was used as continuous variable, tardive dyskinesia status was used as a dichotomous variable for testing association. In addition, PANSS (Kay et al., 1987) was estimated for subjects in the tardive dyskinesia cohort (TD+ve, PANSS total score=61.4±18.5; TD−ve, PANSS total score = 56.4±18.9). Disorganized/concrete (P2+N5+G11), excited (P4+P7+G8+G14) and depressed factors (G2+G3+G6) were derived from the PANSS scores as described elsewhere (Wallwork et al., 2012).

Neurocognitive assessment using Penn Computerized Neurocognitive Battery (PennCNB)

A Hindi version of PennCNB was used for cognitive assessment as described in previous studies (Bhatia et al., 2012; Kukshal et al., 2013). The PennCNB measures neurobehavioural functions of eight domains, namely abstraction and flexibility, attention, working memory, face memory, spatial memory, spatial processing, sensorimotor dexterity, and emotional processing (Gur et al., 2001a, 2001b, 2007). Three performance functions, namely accuracy, processing speed and efficiency were calculated for each domain. PennCNB was administered to 306 adult controls comprising 192 males (age=40±16 years) and 114 females (age=36±11 years) and 357 schizophrenia cases comprising 247 males (age=33±9.4 years) and 110 females (age=32±9.0 years) at the time of recruitment. The scores obtained from the PennCNB repository were transformed to confirm to near normality and were used for association testing. Scale for the Assessment of Positive Symptoms (SAPS, 22±16), Scale for the Assessment of Negative Symptoms (SANS, 45 ±28) (Andreasen and Olsen, 1982) and Global Assessment Scale during the previous month (GAS, 36±14) (Endicott et al., 1976) were estimated for schizophrenia subjects in the cognition cohort.

Genetic analysis

Selection of variants and genotyping

Three variants, namely 19bp *Ins/Del*, rs1989787 and rs1611115, in the 5′ upstream region of *DBH* (Supplementary Figure 1 online) were selected for genotyping. The selection was based on their: i) reported association with a wide range of neuropsychiatric disorders in the literature (Cubells and Zabetian, 2004; Kopeckova et al., 2006); ii) functional evidence or genotype × phenotype correlation for rs1611115 and rs1989787 (Chen et al., 2010, 2011) respectively; and iii) common nature with MAF >0.05. Primers were designed online using Primer 3 (v. 0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/). The restriction fragment length polymorphism (RFLP) patterns for two markers were generated using WatCut (http://watcut.uwaterloo.ca/template.php?act=snp_new). Polymerase chain reaction (PCR)-RFLP method was used to genotype two markers (rs1611115, rs1989787) and the 19bp *Ins/Del* variant was genotyped by size separation of the PCR product on a 4% TAE-agarose gel (primers used for amplification and RFLP details are provided in Supplementary Table 1). PCR amplification was done using 3B DNA polymerase (Biotools B&M Labs, SA, Madrid, Spain) and constituents are shown in Supplementary Table 1. PCR was performed on a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific Waltham, Massachusetts, USA) using the thermal cycling conditions given in Supplementary Table 2. Restriction digestion conditions for markers rs1989787 and rs1611115 are given in Supplementary Table 2. Sanger sequence confirmed DNA samples for each of the three genotypes were used as PCR and digestion controls for each of the 96 well plates for genotyping rs1611115 and rs1989787. Enzyme digested samples were electrophoresed on a 2% TAE-agarose gel, visualized on Gel Doc-It™ Imaging system (Ultra-Violet Products Ltd, Upland, California, USA) and genotypes were called.

Statistical analysis

Deviation, if any, of the three variants from Hardy–Weinberg equilibrium (HWE) were assessed using PLINK 1.07 (Purcell et al., 2007). MAF and linkage disequilibrium measures for these markers were also assessed. The power of the entire sample set was calculated with Quanto v. 1.2.4 software (Gauderman and Morrison, 2006).

Test of association with schizophrenia and tardive dyskinesia

Associations of the three variants with schizophrenia and tardive dyskinesia were tested using chi-squared ($\chi 2$) tests in PLINK 1.07. For analysing the effect of *DBH* genotypes on tardive dyskinesia, a full factorial model under analyses of covariance (ANCOVA) was done with tardive dyskinesia status and *DBH* genotypes as fixed factors and AIMS total score as dependent variable, with age and gender as covariates using IBM® SPSS® Statistics Subscription (IBM Corp., Armonk, New York, USA). Main effects of tardive dyskinesia status and genotype independently and tardive dyskinesia status × genotype on AIMS score were tested in the model. On identification of significant main effect or an interaction with AIMS total score, Fisher's least significant difference (LSD) multiple comparisons were done to assess significantly different groups. Linear regression was done for TD+ve and TD −ve sub cohorts for genotypes of DBH variants with positive, negative, general psychopathology sub scales, total score, disorganized/concrete, excited and depressed

factors of PANSS with age and gender as covariates using gPLINK v. 2.050 separately. If significant associations were found, Fisher's LSD or Bonferroni multiple comparisons were done to assess significantly different groups.

Test of association with cognition

Cognition scores were age adjusted and transformed by skew power transformation ($z = z_{\text{bcn,u}}(y, \lambda, \gamma)$) (Hawkins and Weisberg, 2017), if data were not conforming to normal distribution as implemented in the car package in R (Fox and Weisberg, 2011). Association of the *DBH* variants with cognitive scores in healthy adult controls was done by linear regression with age and gender as covariates using gPLINK v. 2.050. An ANCOVA with full factorial model was performed with health status and *DBH* genotypes as fixed factors; transformed cognition scores corresponding to performance functions (accuracy, processing speed and efficiency) for eight cognitive domains assessed through PennCNB as dependent variable, with gender as covariate using IBM® SPSS® Statistics Subscription (IBM Corp., Armonk, New York, USA). Main effects of health status and genotypes independently and health status × genotype were tested in each model. If a significant main effect or an interaction with cognitive scores was identified, Fisher's LSD multiple comparisons were done to assess significantly different groups. The inverse BCN transformation was used to estimate the marginal means and confidence intervals where $q = 2(\lambda z+1)^{1/\lambda}$ for $\lambda \neq 0$, and $y = (q^2 - \gamma^2)/(2q)$. Linear regression was done for *DBH* variants with SANS, SAPS and GAS scores with age and gender as covariates for schizophrenia subjects in the cognition cohort using gPLINK v. 2.050 separately.

Principal component analysis (PCA)

A Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy and Bartlett's test of sphericity was done to estimate whether PCA needs to be done on the neurocognitive measures. A scree plot with eigenvalue versus component number was done to estimate the number of components which explain maximum amount of variance in the trait. PCA with direct oblimin rotation method was done to reduce neurocognitive measures of accuracy, processing speed and efficiency to their principal components. ANCOVA was performed with health status and *DBH* genotypes as fixed factors and each principal component of accuracy, processing speed and efficiency with gender as covariate.

K-means cluster analysis

A K-means clustering for all the 24 cognitive scores (eight each for accuracy, processing speed and efficiency) with 10 iterations was done using IBM® SPSS® Statistics Subscription to assess the variables that contribute the maximum to differentiate the study cohort into cases and controls.

Functional analysis of 19bp Ins/Del *(rs141116007)*

Functional characterization of this upstream 19bp *Ins/Del* variant was done using *DBH* promoter construct and with the aid of a non-commercial dual luciferase assay in this study. *DBH* promoter region (Supplementary Figure 1) was amplified from genomic DNA of two different individuals previously sequenced (Punchaichira et al., 2016) and identified to be

homozygous for *Ins/Ins* and *Del/Del* respectively. Amplification was performed (5677bp; amplicon I) with primers (*DBH* 19bp incF and Prom 2) having Mlu I HF and Xho I overhangs (Supplementary Table 1) using LA Taq with GC buffer I (Clontech laboratories, Mountain View, California, USA) and appropriate cycling conditions (Supplementary Table 2). PCR product and pGL3 Basic vector (Promega, Madison, Wisconsin, USA) were restriction digested with Mlu I HF and Xho I (New England Biolabs, Ipswich, Massachusetts, USA), gel extracted using Wizard®SV Gel and PCR clean-up system (Promega, Madison, Wisconsin, USA) and ligated into pGL3 Basic vector using T4 DNA ligase (Promega, Madison, Wisconsin, USA) yielding pGL3 *DBH* promoter WT (Supplementary Figure 2). In this construct we identified two unique restriction enzymes (Nhe I and Sac II) that flank the region of 19bp *Ins/Del* polymorphism to be used for subsequent cloning steps. Another amplicon encompassing the region of *Ins/Del* polymorphism (2261bp; amplicon II) was amplified from the second individual who had *Del/Del* genotype of 19bp *Ins/Del* marker using primers Del XIX F and Del XIX R with suitable cycling conditions (Supplementary Table 2). The pGL3 *DBH* promoter WT construct (with amplicon I) and amplicon II were digested with Nhe I HF and Sac II (New England Biolabs, Ipswich, Massachusetts, USA) gel extracted using Wizard®SV Gel and PCR clean-up system (Promega, Madison, Wisconsin, USA) and ligated into the resulting pGL3 Basic vector backbone devoid of the region of *Ins* allele using T4 DNA ligase (Promega, Madison, Wisconsin, USA) resulting in construct pGL3 *DBH* Promoter 19bp *Del*. The scheme for construct creation is given in Figure 1.

Cell culture and transfection

SH-SY5Y cells were grown on DMEM high glucose medium (Life Technologies, Carlsbad, California, USA) supplemented with 10% foetal bovine serum and 1% antibiotic and antimycotic. Cells were confirmed to be mycoplasma free with MycoAlert® mycoplasma detection kit (LT07–418, Lonza, Rockland, ME, USA). SH-SY5Y cells (2×10^4) in antibiotic free media were plated in white flat bottom tissue culture treated 96 well plates (Costar® Assay plate, Corning Incorporated, Kennebunk, Maine, USA). Three independent transfections of 100 ng of pGL3 basic vector, pGL3 *DBH* promoter WT and pGL3 *DBH* promoter 19bp *Del* each were carried out and co-transfected with 30 ng of pRL TK vector using 0.3 μL of Lipofectamine 3000 (Life Technologies, Carlsbad, California, USA) per transfection in four technical replicates. Mock transfection with the transfection reagent alone was also done as above. The cells were incubated for 48 h post transfection until luciferase assay was performed as described below. These experiments were performed three times.

Luciferase assay

The cells were washed with Dulbecco's phosphate-buffered saline and lysed in 1× passive lysis buffer (Promega, Madison, Wisconsin, USA). A non-commercial dual luciferase assay as described previously (Dyer et al., 2000) was adopted for estimating Firefly and Renilla luciferase activity. Enzyme assays were done by injecting 100 μL of luciferase assay buffer into each well and assessed for Firefly luciferase activity followed by injection of Renilla assay buffer (100 μL) for assessing Renilla luciferase activity using the multimode reader (POLARstar Omega, BMG Labtech, Offenburg, Germany). The multimode reader was set

for a continuous reading with 5 s delay followed by a 5 s reading for each luminescence with 0.5 s intervals. An unpaired one tailed *t* test was done to check for significant differences in expression between constructs with 19bp *Ins* and *Del* alleles using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, California, USA).

Results

Demographic data of the study cohort are shown in Table 1. All three variants, namely 19bp *Ins/Del* (rs141116007 Ins>Del, MAF=0.5), rs1989787 (C>T, MAF=0.14) and rs1611115 (C>T, MAF=0.24) were found to be in HWE (p=0.6, 0.09 and 0.5 respectively).

Association with schizophrenia

The study cohort (N = 1236 cases, 1136 controls) had 99.4, 90.5 and 97.7% of power under a log additive mode of inheritance, with genetic effect of 1.3 and disease population risk of 0.01 to detect associations, if any, at each of the three markers. Of note, none of the markers were associated with schizophrenia (19bp *Ins/Del*, χ^2=2.7, p=0.1; rs1989787, χ^2=1.3, p=0.2 and rs1611115, χ^2=0.004, p=0.9). However, a two-marker haplotype *Ins*-C (19bp *Ins/Del* – rs1989787 C>T) showed modest association with schizophrenia (χ^2=4.4, p=0.04).

Association with tardive dyskinesia

No association of any of the markers (19bp *Ins/Del*, χ^2=1.6, p=0.2; rs1989787, χ^2=2.4, p=0.1 and rs1611115, χ^2=0.3, p=0.6) was seen with tardive dyskinesia. However, ANCOVA revealed a significant interaction of tardive dyskinesia status and genotypes of 19bp *Ins/Del* with AIMS total score (F(2, 174)=3.3, p=0.04), but with small effect size (0.20) and marginal means of AIMS total score adjusted for gender and age (Figure 2(a)). Furthermore, a significant interaction between tardive dyskinesia status and genotypes of rs1989787 and rs1611115 (with 83% power) with AIMS total score (Figure 3(a)) was also identified (F(1, 174)= 8.7, p=0.004), with small effect size (0.22). Test of interaction between tardive dyskinesia status and the three markers performed considering only orofacial (1–4 items of AIMS) or limb-truncal (5–7 items of AIMS) scores showed significant effect of tardive dyskinesia status, rs1611115 and rs1989787 interaction on limb-truncal scores only (F(1,174)=8.3, p=0.004).

Allelic association of the T allele of the proximal promoter variant rs1611115 with positive (allelic (β=−1.8, p=0.03)), general psychopathology subscales (allelic (β=−4.27, p=0.01)) and PANSS total score (allelic (β=−6.5, p=0.05)) was identified in the TD+ve sub cohort in the additive model. Genotypic association of the same variant with positive (p=0.04) and general psychopathology (p=0.01) was also identified in the TD+ve sub cohort (Figure 3(b) and (c)). Further, there was allelic association of the T allele of rs1611115 with depressive factors in the TD−ve sub cohort (allelic (β=0.81, p=0.02)). Though there was genotypic association of rs1611115 (p=0.05) with depressive factors, only subjects with the C/T genotype were significantly different from those with the C/C genotype post LSD multiple comparisons (p=0.03).

Association with cognition

Transformations using the recent skew power approach were performed by estimating the λ and γ from the linear model with cognitive domains as dependent variable and health status and *DBH* genotypes as independent variables. In healthy controls, rs1989787C >T was found to be an eQTL for spatial ability$_{accuracy}$ (β=−0.10, p=0.02) and spatial ability$_{efficiency}$ (β=−0.13, p=0.02) in an additive model. This SNP also exhibited genotypic association (Figure 3(d) and (e)) with spatial ability$_{accuracy}$ (p=0.05 and spatial ability$_{efficiency}$ (p=0.03). Transformation parameters (λ and γ) for cognitive domains, where an association was observed, are described as follows. Abstraction and mental flexibility$_{accuracy}$ with $z = z_{bcn,u}$ (y, −0.34, 2.5); abstraction and mental flexibility$_{efficiency}$ with $z = z_{bcn,u}$ (y, −0.53, 3.3); attention$_{accuracy}$ with $z = z_{bcn,u}$ (y, 0.94, 1.0); attention$_{efficiency}$ with $z = z_{bcn,u}$ (y, 0.95, 1.2); working memory$_{accuracy}$ with $z = z_{bcn,u}$ (y, 0.58, 1.9); emotion$_{accuracy}$ with $z = z_{bcn,u}$ (y, 0.31, 2.5) and emotion$_{processing\ speed}$ with $z = z_{bcn,u}$ (y, 0.82, 2.1). A significant effect of the interaction of health status and genotypes of rs1989787 on the abstraction and mental flexibility$_{accuracy}$ (F(2, 608) =3.7, p= 0.03) and abstraction and mental flexibility$_{efficiency}$ (F (2, 601) =3.5, p=0.03) was observed. The effect size of these interactions on these domains was found to be small (0.11 and 0.10 respectively). The T/T genotype of rs1989787 had significantly lower scores of accuracy and efficiency of abstraction and mental flexibility than those with C/C and C/T genotypes in schizophrenia subjects (Figure 2(b) and (c)). Further, there was significant interaction between health status and genotypes of rs1611115 on working memory$_{accuracy}$ (F(2, 583)=3.1, p=0.048), with an effect size of 0.1. A significant interaction of health status and genotypes of rs1611115 on emotion$_{accuracy}$ (F (2, 603) =2.9, p=0.05) was also identified. The effect size of this interaction on emotion$_{accuracy}$ was found to be small (0.10). There was also a significant interaction of health status and genotypes of 19bp *Ins/Del* (rs141116007) on emotion$_{processing\ speed}$ (F(2, 598) =3.4; p=0.03). The effect size of this interaction was small (0.1). Another significant interaction of health status with markers rs1989787 and rs1611115 on attention$_{accuracy}$ (F(1, 529)=4.5; p=0.03), emotion$_{accuracy}$ (F(1, 603)=4.4; p=0.04) and attention efficiency (F(1, 527)=4.1; p=0.04) was identified with a small effect size (0.1). The gender adjusted marginal means for significant interactions of *DBH* variants with health status are presented (Figure 2(b) to (f)). A summary of interactions of *DBH* variants that had an effect on performance functions of cognitive scores of accuracy, processing speed and efficiency is given in Supplementary Tables 3–5 respectively. Further, there was allelic association of the T allele of rs1611115 with GAS during the past month (β=2.7, p=0.05) in schizophrenia subjects of the cognition cohort.

PCA

PCA was conducted on the neurocognitive measures of accuracy, processing speed and efficiency separately with oblique rotation (direct oblimin). Verification of the sampling adequacy for the analysis was done with the KMO measure. The KMO values for the cognitive scores of accuracy, processing speed and efficiency were found to be ⩾0.87, above the acceptable score of 0.5. For the cognitive measures, Bartlett's test of sphericity was assessed (accuracy, $\chi^2(28)=1237.6$, $p<0.001$; processing speed, $\chi^2(28)=1781.3$, $p<0.001$ and efficiency, $\chi^2(28)=1587.4$, $p<0.001$) indicating high correlation between cognitive scores and a PCA could be done on the cognitive measures. Eigenvalues were obtained for

each component by running an initial analysis on data. Only the first component of accuracy, processing speed and efficiency had Eigenvalues over Kaiser's criterion of 1 and explained 46.1%, 53.7% and 53.1% of the variance for accuracy, processing speed and efficiency respectively. The second component explained 11%, 11.5% and 9.4% of variance. The Eigenvalues for the second principal component for the same measures were 0.9, 0.9 and 0.8 respectively. Scree plots showed inflections and the first component explained most of the variance in cognitive scores. Though two components were extracted, because of correlation between first and second components only the first component was retained in the final analysis. The factor loadings after rotation are given in Figure 4. Health had a significant effect on the two principal components of accuracy, processing speed and efficiency ($p<0.05$). The interaction of health and genotypes at rs1989787 was found to have a significant effect on the first principal component of efficiency of the neurocognitive scores ($F(2, 460)=3.0$, $p=0.05$).

K-means cluster analysis

K-means clustering was done in two clusters based on the health status. Convergence of change in cluster centres was achieved by the fourth iteration. The final cluster centres are given in Supplementary Figure 3. A general decrease in all the cognitive scores in subjects with schizophrenia was observed as compared with healthy controls. Of all the cognitive scores, abstraction and mental flexibility$_{efficiency}$, abstraction and mental flexibility$_{accuracy}$, emotion$_{efficiency}$ and working memory$_{efficiency}$ contributed the most in separating subjects into these two clusters.

Functional characterization of 19bp Ins/Del *marker*

A significant 39% decrease in luciferase activity was observed with the construct having 19bp *Del* allele as compared with the wild type ($p=0.03$). This clearly provides the first ever evidence for functional significance of this common variant in *DBH* promoter (Figure 5).

Discussion

Association of coding and regulatory genetic variants at *DBH* locus across brain disorders (Kopeckova et al., 2006), including schizophrenia, have been well reported (Cubells and Zabetian, 2004). Correlation of exonic variants with qualitative (Ishii et al., 1991) and with quantitative enzyme phenotypes (Kim et al., 2011; Punchaichira et al., 2017) has also been documented. Functional characterization of two commonly investigated regulatory variants, namely rs1989787 (Chen et al., 2011) and rs1611115 (Chen et al., 2010), has also been carried out. Of note, association of *19bp Ins/Del*, yet another regulatory variant, has been well reported but not functionally validated. Furthermore, contribution of these three significant regulatory variants to phenotypes such as tardive dyskinesia and cognition are seldom documented. In this study, we investigated the likely contribution of three variants, namely 19bp *Ins/Del*, rs1989787 and rs1611115, in the 5′UTR of the gene to schizophrenia, tardive dyskinesia and cognition. To strengthen our association findings, we also established for the first time the functional significance of the *Ins* and *Del* alleles of 19bp marker based on high and low reporter activity respectively (Figure 5).

Genetic associations

Schizophrenia—None of the three variants were associated with schizophrenia despite the well powered (>90%) study cohort. Only a two-marker haplotype *Ins*-C of 19bp *Ins/Del*-rs1989787 showed modest association (p=0.04).

Tardive dyskinesia—No association of the three markers was observed with tardive dyskinesia *per se*. However, ANCOVA on TD+ve and TD−ve schizophrenia cohorts identified two differential genotypic effects in TD+ve subjects: i) higher AIMS score with 19bp *Ins* allele (Figure 2(a)); and ii) interaction of genotypes at rs1989787 and rs1611115 on AIMS score wherein those with the genotypic combination C/T–C/C(intermediate/high activity) had higher scores than those with C/T–C/T and C/C–C/C (Figure 3(a)). The former could be explained by the high activity of the *Ins* allele of 19bp marker as reported previously (Tang et al., 2007) and indirectly by the high reporter activity demonstrated in our study (Figure 5). The second differential genotypic effect involving rs1989787 and rs1611115 could also be explained by the higher DBH activity reported for their T and C alleles previously (Punchaichira et al., 2016). In addition, subjects with C/C and C/T genotypes of rs1989787 had lower DBH activity compared with those with T/T genotype (Chen et al., 2011) and the C allele of rs1611115 was found to increase plasma DBH activity and epinephrine excretion, and predict a higher basal blood pressure (Chen et al., 2010). These correlations imply that in the TD+ve subjects, higher DBH activity may lead to higher AIMS scores (Figures 2(a) and 3 (a)). Previous studies have also reported that schizophrenic subjects with tardive dyskinesia had higher DBH activity compared with those without tardive dyskinesia (Jeste et al., 1981; Kaufmann et al., 1986; Wagner et al., 1982). Furthermore, among TD+ve subjects those with C/C genotype (high activity) at rs1611115 were found to have higher positive scale and general psychopathology scale of PANSS (Figure 3(b) and (c)). This implies that among TD+ve subjects those with higher DBH activity have more severe disease. However, in the TD−ve cohort the variant allele (T) at this marker was associated with depressive factors.

Cognition—Substantial neurocognitive deficits have been documented in large scale studies of schizophrenia subjects (Greenwood et al., 2007; Heinrichs and Zakzanis, 1998; Toulopoulou et al., 2010). A computerized neurocognitive battery analyses accuracy, response time and efficiency of neurocognitive domains and have been used in genetic studies (Gur et al., 2001a, 2010). In healthy controls, accuracy and efficiency of spatial ability was higher in those with the C/C genotype of rs1989787 as compared with those with T/T genotype (Figure 3(d) and (e)). From this it may be inferred that this SNP is an eQTL for spatial ability$_{\text{accuracy}}$ and spatial ability$_{\text{efficiency}}$. The most notable association of the three *DBH* markers in this study was with different domains of cognition (Figure 2(b) to (f) and Supplementary Tables 3–5). We observed differential cognitive effect of rs1989787 on abstraction and mental flexibility in controls and schizophrenia subjects. The T/T genotypes (high activity) attributed to higher cognitive scores in controls, but to lowest scores among the three genotypes in schizophrenia subjects (Figure 2(b) and (c)). On the other hand, there was allelic association of T allele (low activity) of rs1611115 with increase in GAS scores in the past month among schizophrenia subjects in the cognition cohort. Hence, those with the T allele had lower severity of the disease as compared with those with the C allele. These

results corroborate with the general psychopathology subscale of PANSS in the TD+ve cohort (Figure 3(c)) and suggest that rs1611115 may be an important SNP influencing disease severity.

PCA was done to reduce the dimensionality of cognitive variables. The interaction of health status and rs1989787 was significant with the first principal component of efficiency of cognitive scores. This could be explained in light of the fact that abstraction and mental flexibility$_{efficiency}$ was loaded onto the first component of efficiency (Figure 4(c)) and the interaction was still significant even after multivariate corrections. Clustering analysis confirmed that all the cognitive variables showed a substantial reduction in schizophrenia subjects as compared with healthy controls and cognitive scores of abstraction and mental flexibility$_{efficiency}$, abstraction and mental flexibility$_{accuracy}$, emotion$_{efficiency}$ followed by working memory$_{efficiency}$ contributed the most in segregating schizophrenia subjects from healthy controls. Overall these observations derive notable support from previous studies. An extended promoter haplotype spanning seven common SNPs (six in the promoter region, and one in intron-A: rs1076151, rs1076152, rs1076150, rs1989787, rs1611114, rs1611115, rs2797849) was reported to predict DBH activity. Haplotype 1 (GCTCCTG) decreased DBH activity whereas haplotype 2 (GCCTCCC) increased the activity (Chen et al., 2011). In addition, dopamine and other neurotransmitters have been reported to have implications for cognition in schizophrenia subjects (Braver et al., 1999; Condray and Yao, 2011; Friedman et al., 1999; Sakurai et al., 2013). Of note, one of the limitations of our study is that subjects recruited in this study were not drug naïve. This may be kept in mind while considering genotype–phenotype correlations in cognition reported in this study.

The role of DBH levels in cognition as evidenced above also derives support from the documented role of norepinephrine in cognition (Chamberlain and Robbins, 2013). This is further substantiated by the documented improvement/decline of cognitive scores by augmenting (Gamo et al., 2010; Jentsch et al., 2009; Tzavara et al., 2006) or depleting (Cai et al., 1993; Sontag et al., 2008) norepinephrine levels through a range of pharmacological interventions. In addition, cerebrospinal fluid DBH levels were found to be lower in schizophrenia subjects who became nonpsychotic than in those who remained psychotic following neuroleptic treatment (Sternberg et al., 1982). These observations reiterate the importance of norepinephrine signalling in schizophrenia, tardive dyskinesia and cognition.

Taken together, investigation of the contribution of the three common regulatory variants in *DBH* to tardive dyskinesia and cognition among schizophrenia subjects in this study has provided useful insights with pharmacogenetic implications. However, considering that tardive dyskinesia and cognitive symptoms are observed with higher severity in subjects with high DBH activity, estimation of DBH activity (Punchaichira et al., 2018) prior to use of DBH inhibitors along with neuroleptics may be of therapeutic relevance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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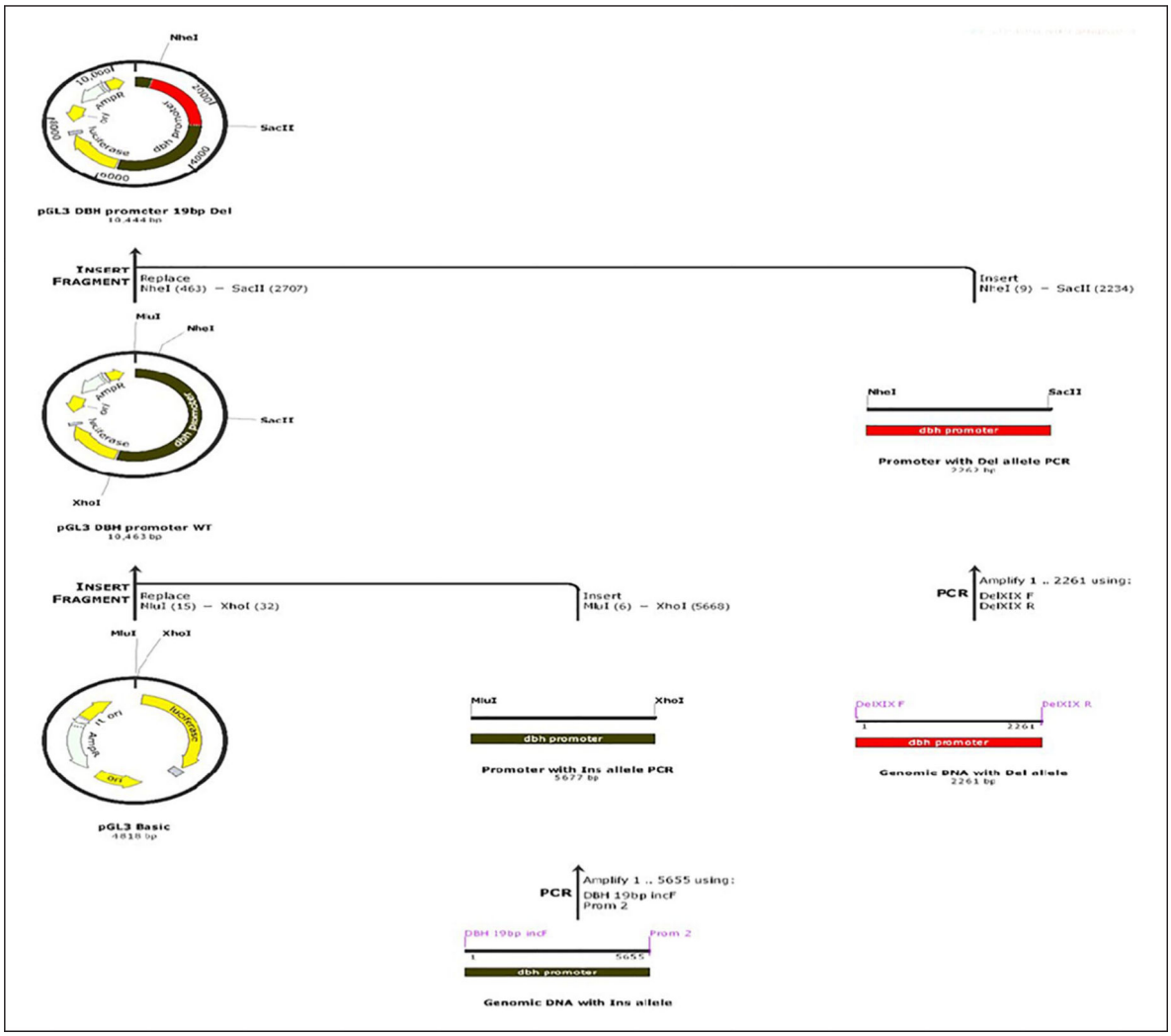

Figure 1:

Scheme of creation of plasmids used for luciferase assay. *DBH Ins* allele was cloned into pGL3 Basic between Mlu I and Xho I to generate pGL3 *DBH* promoter WT. The *Ins* allele was removed by restriction digestion of the region of *Ins* allele with two flanking restriction sites (Nhe I and Sac II) and an amplified fragment with *Del* allele was cloned to generate pGL3 *DBH* promoter 19bp *Del*.

DBH: dopamine-β-hydroxylase; PCR: polymerase chain reaction

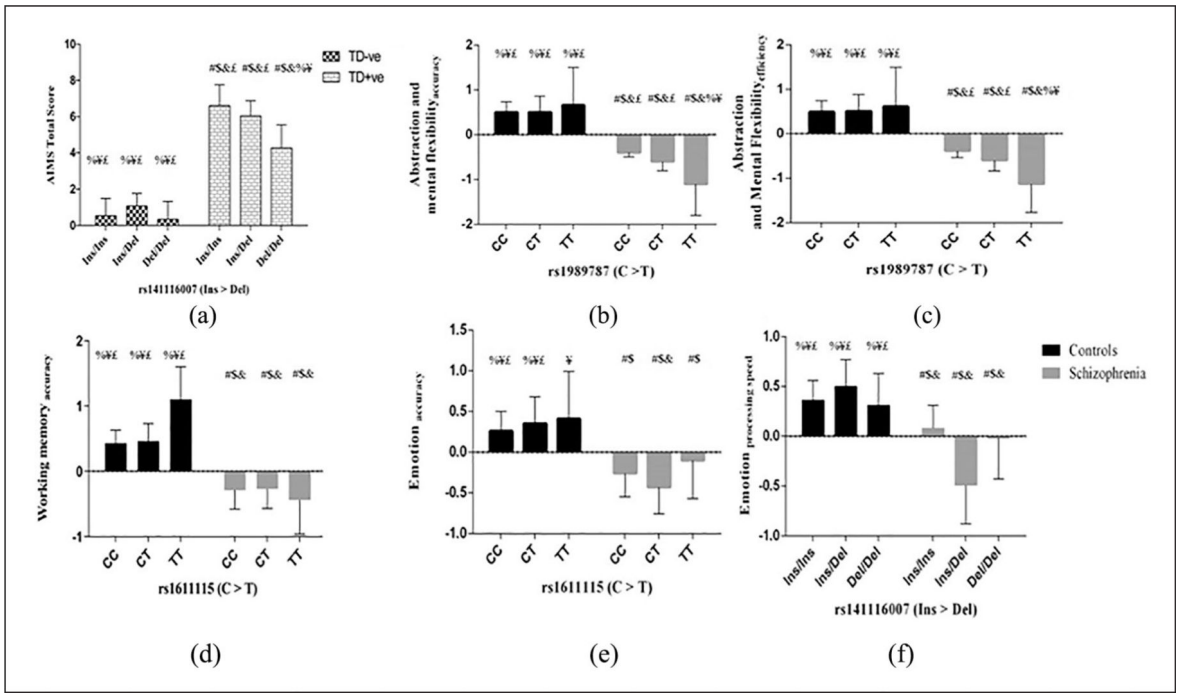

Figure 2:

Estimated marginal means of (a) Abnormal Involuntary Movement Scale (AIMS) total score for the interaction of tardive dyskinesia status and *DBH* genotypes and (b to f) cognitive scores for the interaction of health status and *DBH* genotypes. An analysis of covariance with full factorial model was done to assess the effect of interaction of (a) tardive dyskinesia status and genotypes of 19bp *Ins/Del* on AIMS total score and (b to f) health status and genotypes of rs141116007, rs1989787 and rs1611115 on the transformed cognitive scores. Marginal means of AIMS total score adjusted for age and gender where a significant effect of interaction of tardive dyskinesia status and genotypes (p<0.05) is presented. In the tardive dyskinesia-positive (TD+ve) cohort, subjects with *Ins/Ins* genotype had higher AIMS score than subjects with *Ins/Del* or *Del/Del* genotype. While in the tardive dyskinesia-negative (TD−ve) cohort, subjects with the *Ins/Del* had higher AIMS score than those with *Ins/Ins* and *Del/Del* (a). The inverse BCN transformed marginal means adjusted for gender for the domains that had significant effect of interaction of health status and genotypes (p<0.05) is presented. One single nucleotide polymorphism (SNP; rs1989787) had different effect on accuracy and efficiency of abstraction and mental flexibility depending on health status (b and c). Accuracy and efficiency of abstraction and mental flexibility of the T/T genotype of rs1989787 was significantly lower than those with C/C and C/T genotypes in schizophrenia subjects. Another SNP (rs1611115) had differential effect on accuracy working memory (d) and emotion (e) depending on health status. The *Ins/Del* polymorphism (rs141116007) also had a differential effect on processing speed of emotion (f). Error bars indicate 95% confidence interval. #, $, &, %, ¥ and £ denote significantly different from the first to sixth bars (p<0.05) in the graph after Fisher's least significant difference multiple comparisons. BH: dopamine-β-hydroxylase

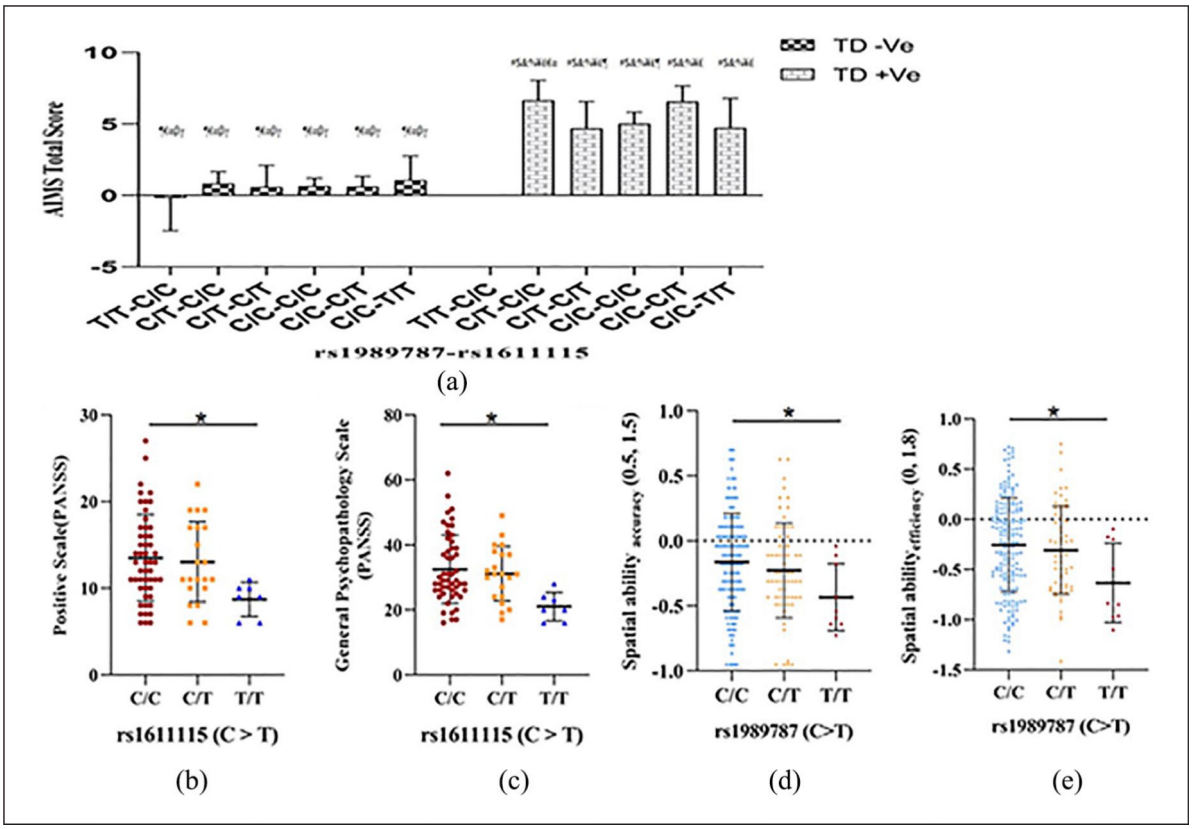

Figure 3:

Effect of (a) interaction of rs1989787 and rs1611115 with tardive dyskinesia status on Abnormal Involuntary Movement Scale (AIMS) total score of tardive dyskinesia in the tardive dyskinesia cohort; rs1611115 on (b) positive scale, (c) general psychopathology subscale scores of the Positive and Negative Syndrome Scale (PANSS) in the tardive dyskinesia-positive (TD+ve) sub cohort, (d) rs1989787 on accuracy and (e) efficiency of spatial ability in healthy subjects. In the (a) interaction of genotypes of rs1989787-rs1611115 with tardive dyskinesia status, the genotypic combination C/T–C/C had higher AIMS tardive dyskinesia scores than C/T–C/T. Further, the genotypic combination C/T–C/C had higher AIMS tardive dyskinesia scores than C/C–C/C. Since the T allele of rs1989787 and the C allele of rs1611115 were earlier associated with higher dopamine-β-hydroxylase (DBH) activity and earlier luciferase reporter assays involving promoter constructs containing these variants have confirmed these, these results show a direct correlation between DBH activity and AIMS tardive dyskinesia scores. Error bars denote 95% confidence interval. #, $, &, %, ¥, £, ¶, €, α, β and γ depict significantly different from first to 11th bars post Fisher's least significant difference (LSD) multiple comparisons. The T/T genotype of rs1611115 had significantly lower (b) positive scale and (c) general psychopathology sub scores of PANSS in TD+ve sub cohort post Bonferroni multiple comparisons. Healthy subjects with (d) C/C genotype of rs1989787 had significantly higher spatial ability$_{accuracy}$ scores than those with the T/T genotype post LSD multiple comparison. Subjects with (e) the T/T genotype had lower spatial ability$_{efficiency}$ scores than

those with C/C genotype in healthy controls post Bonferroni multiple comparisons. (b) to (e) Error bars denote SD.

*denotes significantly different groups ($p<0.05$).

TD−ve: tardive dyskinesia-negative

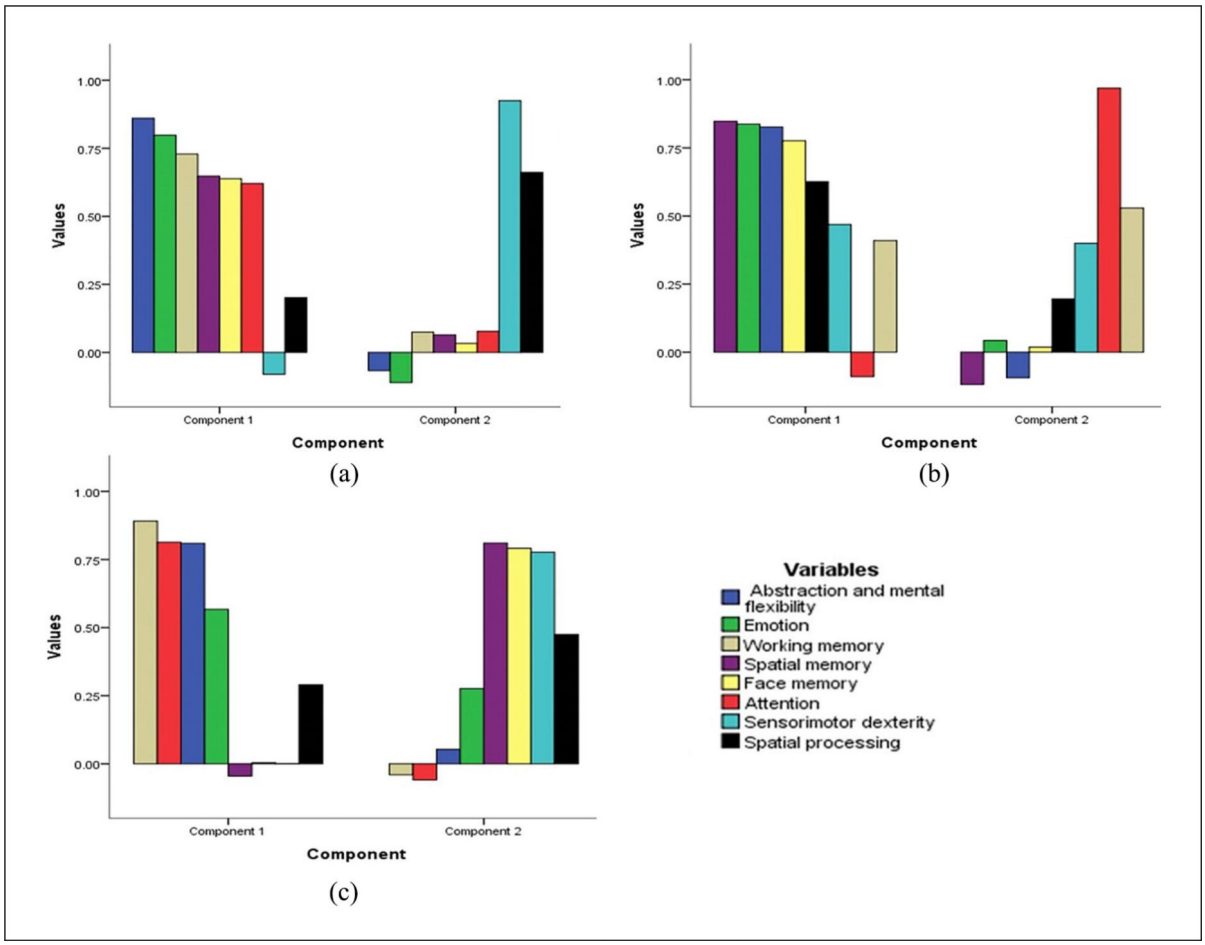

Figure 4.

The factor loadings of individual cognitive domain scores onto the principal components of (a) accuracy, (b) processing speed and (c) efficiency. Notably abstraction and mental flexibility$_{accuracy}$, abstraction and mental flexibility$_{processing\ speed}$ and abstraction and mental flexibility$_{efficiency}$ loaded onto the first component of performance functions of accuracy, processing speed and efficiency respectively. Accuracy, processing speed and efficiency of cognitive scores of abstraction and flexibility, attention, working memory, face memory, spatial memory, spatial processing, sensorimotor dexterity and emotional processing are illustrated.

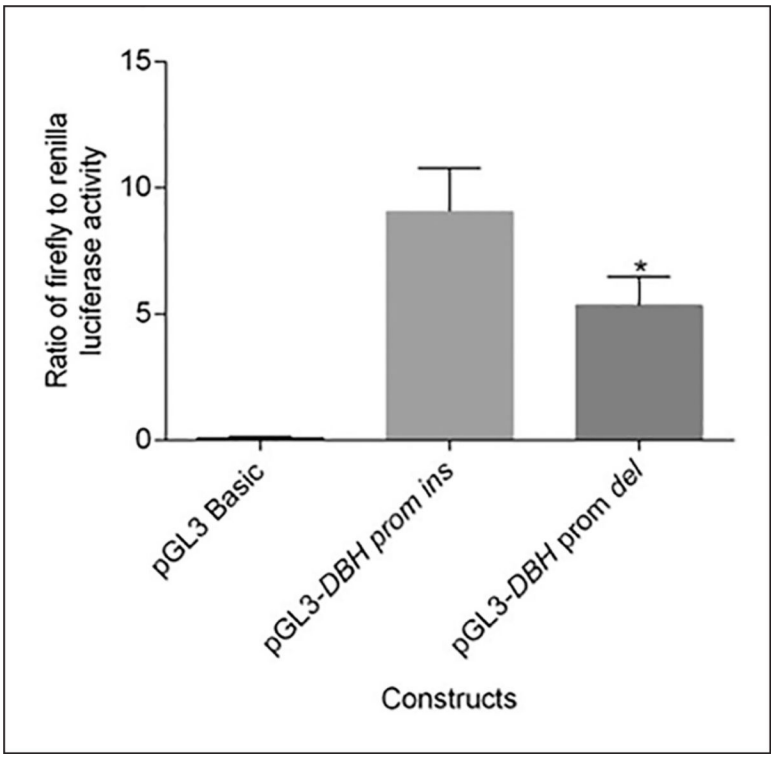

Figure 5:

Differential expression of the *DBH* 19bp *Del* allele as compared with the *Ins* allele. The ratio of Firefly to Renilla luciferase activity was calculated from the luminescence measurements. A one tailed *t*-test as implemented in GraphPad Prism 6.0 was done to check for differences in expression of *Ins* and *Del* alleles. A significant decrease in expression of the *Del* allele as compared with the *Ins* allele was observed (*denotes $p<0.05$).

Table 1.

Study cohort composition.

Gender	Case-control				Cognition samples				TD samples			
	Males		Females		Males		Females		Males		Females	
	Case	Control[a]	Case	Control[a]	Case	Control	Case	Control	TD+ve	TD−ve	TD+ve	TD−ve
Samples (*n*)	682	566	554	570	247	192	110	114	51	77	32	85
Age in years (mean ± SD)	31±9.0	41±14	31±10	35±12	33±9.4	40±16	32±9.0	36±11	35±12	30±10	32±12	31±9.2

[a]Of the controls 507 were cord blood controls.

TD: tardive dyskinesia; TD+ve: TD-positive; TD−ve: TD-negative