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FUNCTIONAL CHARACTERIZATION OF RARE VARIANTS IN HUMAN DOPAMINE RECEPTOR D4 GENE BY GENOTYPE–PHENOTYPE CORRELATIONS

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Abstract—Next generation sequencing technologies have facilitated a notable shift from common disease common variant hypothesis to common disease rare variant, as also witnessed in recent literature on schizophrenia. *Dopamine receptor D4* (*DRD4*), a G-protein-coupled receptor is associated with psychiatric disorders and has high affinity for atypical antipsychotic clozapine. We investigated the functional role of rare genetic variants in *DRD4* which may have implications for translational medicine. CHO-K1 cells independently expressing four rare non-synonymous variants of *DRD4* namely R237L, A281P, S284G located in the third cytosolic loop and V194G, located in the fifth transmembrane domain were generated. Their genotype–phenotype correlations were evaluated using [^{3}H]spiperone binding, G-protein activation and molecular dynamics-simulation studies. A281P and S284G were functionally similar to wild-type (WT). With R237L, potency of dopamine and quinpirole reduced ~sixfold and threefold respectively compared to WT; [^{3}H]spiperone binding studies showed a reduction in total number of binding sites (~40%) but not binding affinity, *in silico* docking studies revealed that binding of both dopamine and spiperone to R237L was structurally similar to WT. Of note, V194G variant failed to inhibit forskolin-stimulated adenylate cyclase activity and phosphorylate extracellular signal-regulated kinase; showed significant reduction in binding affinity (K_d = 2.16 nM) and total number of binding sites (~66%) compared to WT in [^{3}H]spiperone binding studies; and ligand docking studies showed that binding of dopamine and spiperone is superficial due to probable structural alteration. Transmembrane variant V194G in *DRD4.4* results in functional alteration warranting continuing functional analysis of rare variants.

Key words: rare variants, G-protein-coupled receptors, dopamine receptor D4, genotype–phenotype correlations, ligand docking.

INTRODUCTION

Dopamine is a monoamine neurotransmitter, predominantly synthesized in the neurons of four major dopaminergic pathways namely nigrostriatal, mesolimbic, mesocortic and tuberoinfundibular (Anden et al., 1964; Dahlstroem and Fuxe, 1964). Dopamine mediates the signaling in the central nervous system upon binding with dopamine receptors in both pre- and post-synaptic regions (Sokoloff et al., 2006; Rankin et al., 2010). It also plays as a potential intermediate substrate in the biosynthesis of catecholamines such as norepinephrine and epinephrine (Blaschko, 1954). Based on the innervations of dopaminergic neurons, it has been shown to regulate a wide range of neurological and physiological (Missale et al., 1998) functions. Dopamine receptors are important neurotransmitter receptors and belong to the superfamily of G-protein-coupled receptors (GPCRs). This is the largest and most diverse protein family in mammals, involved in signal transduction across membranes (Pierce et al., 2002; Rosenbaum et al., 2009). GPCRs are prototypical members of the family of seven transmembrane domain proteins and include >800 members which are encoded by ~5% of human genes (Zhang et al., 2006). GPCRs regulate multiple physiological processes and therefore have emerged as major targets for the development of novel drug candidates in all clinical areas (Heilker et al., 2009). It is estimated that ~50% of clinically prescribed drugs act as either agonists or antagonists of GPCRs (Schlyer and Horuk, 2006). Dopamine receptors can be classified based on structural similarity, pharmacological profile and biochemical properties as D1-like (D1 and D5) and

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Abbreviations: ADHD, attention deficit/hyperactivity disorder; BCA, bicinchoninic acid; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; DRD3, dopamine receptor D3; DRD4, dopamine receptor D4; EDTA, ethylenediaminetetraacetic acid; EGFP, enhanced green fluorescent protein; ERK 1/2, extracellular signal-regulated kinase; GPCRs, G-protein-coupled receptors; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IFD, Induced Fit Docking; MD, molecular dynamics; MM–GBSA, Molecular mechanics–generalized Born and surface area; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; RIPA, radioimmunoprecipitation assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNPs, single nucleotide polymorphism; TR-FRET, time-resolved fluorescence resonance energy transfer; VNTR, variable number tandem repeat; WT, wild type.

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D2-like receptors (D2, D3 and D4). D1-like receptors positively stimulate the adenylyl cyclase system, whereas the D2-like receptors are inhibitory (Spano et al., 1978). G proteins that contain nucleotide-binding Gα subunit and a heterodimeric Gβγ subunit are primary mediators of the downstream signaling of dopamine receptor activation such as cyclic adenosine monophosphate (cAMP) synthesis and inhibition, activity of calcium channels, inwardly rectifying potassium channels and Mitogen-activated protein kinases (MAPK) activation (Rondou et al., 2010).

All D2-like receptors have been reported to be associated with several common complex traits (Beaulieu and Gainetdinov, 2011). Of these, *Dopamine receptor D4* (*DRD4*) has been widely implicated in major neurological and psychiatric disorders such as Schizophrenia, attention deficit/hyperactivity disorder (ADHD) and Alcoholism (Nanko et al., 1993; Petronis et al., 1995; Licinio, 1996; Muramatsu et al., 1996; Hwu et al., 1998; Rowe et al., 1998). Importance of this receptor was noticed when a sixfold elevated expression of *DRD4* was observed in schizophrenia patients compared to the control group (Seeman et al., 1993) and a high degree (>10-fold) of affinity to atypical neuroleptic drug clozapine compared to other D2-like receptors was reported (Van Tol et al., 1991). Though DRD4 is less abundant than other D2-like receptors, this exceptional binding affinity with clozapine makes it an important therapeutic target. There are 2–11 repeats of 48-bp variable number tandem repeat (VNTR) located in the exon 3 of DRD4 gene. Of all these repeats, four repeats has been reported to be the most prevalent across ethnic groups (Wang et al., 2004) and has also been well studied biochemically. VNTRs in the third exon have shown to alter the ability of the inhibition of cAMP production moderately (Asghari et al., 1995).

To date, several common and rare genetic variants in *DRD4* have been reported (Seaman et al., 1999; Wong et al., 2000; Grady et al., 2003). However, only a few of these are shown to be associated with a wide range of disorders such as Schizophrenia (Catalano et al., 1993; Lung et al., 2002), ADHD (LaHoste et al., 1996; Manor et al., 2002), Tourette's syndrome (Comings et al., 1999), Novelty seeking (Okuyama et al., 2000), Tardive dyskinesia (Srivastava et al., 2006) and Parkinson's disease (Kronenberg et al., 1999). Of these associated markers, seven 48-bp VNTR (7R allele) in exon 3 which codes for the third cytoplasmic loop of this receptor protein has been shown to be mainly associated with Schizophrenia and ADHD (Rowe et al., 1998; Muglia et al., 2000; Lung et al., 2002).

While, single nucleotide polymorphisms (SNPs) in the upstream region of the gene are extensively investigated (Kereszturi et al., 2006), the role of exonic SNPs remains obscure. Most of these exonic SNPs are located in the transmembrane and cytosolic loops of the receptor protein. This prompted us to investigate the role of exonic SNPs which may influence the structure–function relationship of this important GPCR.

The widely acknowledged common disease common variant (CDCV) hypothesis (Wang et al., 2005) which was the rationale of genome-wide association studies (GWAS), had undoubtedly a major impact on understanding the etiology of common complex diseases. However, the bulk of heritable variance still remains unexplained. The main takeaway from these studies is that the genetic component underlying common complex diseases can be attributed to one of the following: (i) a large number of small effect common variants; (ii) a small number of large effect rare variants; or (iii) a combination of genes, environment and epigenetic interactions. Of these, the common disease rare variant (CDRV) hypothesis is exemplified by several recent studies on inflammatory bowel disease (Rivas et al., 2011), multiple sclerosis (Ramagopalan et al., 2011), type 2 diabetes (Bonnefond et al., 2012), Schizophrenia (Gulsuner et al., 2013; Kenny et al., 2013), etc. With this background, we characterized four non-synonymous SNPs (rs1800443, V194G; rs4991150, R237L; rs3889692, A281P; rs34662058, S284G) of human *DRD4.4*, using a combination of *in silico* and *in vitro* tools. Functional characterization of such variants may prove useful for the translation of genetic findings to disease prediction, pharmacogenetic applications and novel drug development.

METHODOLOGY

Functional characterization of the wild type and four rare exonic variants of *DRD4.4* was carried out in this study using *in silico* and *in vitro* approaches which are described below.

Cell lines

CHO-10001 (CHO-K1) cells were grown and maintained in D-MEM/F-12 (1:1) supplemented with 2.4 g/l of sodium bicarbonate, 10% fetal calf serum, 60 μg/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml gentamycin sulfate in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were either grown in T-25 flasks or in 6-well culture plates depending on the experimental needs.

Rationale for selection of DRD4 SNPs

When we initiated our work (Human genome NCBI build 36.3, dbSNP build 129), there were six non-synonymous and six synonymous SNPs reported in the DRD4 gene. Of these, rs4991149 (R237*) was a nonsense variant leading to truncated protein, hence excluded from our study. Of the other five, rs12720386 (D10G) SNP is located at the signal sequences, which may not affect the structure–function relationship, and therefore excluded. However, the localization of green fluorescent protein (GFP) tagged (at C-terminal) protein carrying this variant was carried out and it was seen to be highly localized in the cytoplasm compared to the plasma membrane (unpublished observations).

Of the remaining four non-synonymous SNPs (rs1800443, rs4991150, rs3889692 and rs34662058), rs1800443 was located in the fifth transmembrane domain and near to the serine residue (at 196 position)

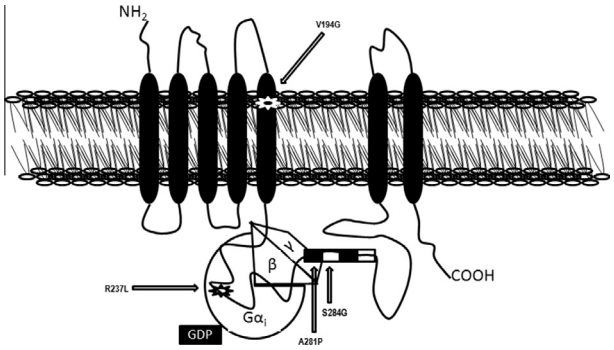

Fig. 1. Depicts a membrane-embedded human dopamine D4 receptor showing the location of the variants.

which is critical for dopamine binding. rs4991150, rs3889692, and rs34662058 SNPs were located in the third cytoplasmic loop of the seven transmembrane domain receptors where the heterotrimeric G-proteins associate themselves to the receptor protein (Fig. 1). Based on these features, these four variants were selected for functional analysis.

Further, rs34662058 and rs4991150 were available in the database but without population frequency. We believed that these SNPs could be rare variants and were therefore included for analysis.

Construction of *DRD4.4* wild-type and four exonic variant clones

DRD4.4 ORF clone in an expression vector (pCMV6huDRD4.4) with a c-myc tag at the C-terminal was obtained from Origene Inc., Rockville, MD, USA. Sequence of the ORF (1.3 kb) was confirmed by polymerase chain reaction (PCR)-sequencing using two sets of primer pairs. Four clones, one each with the four non synonymous variants namely rs1800443, V194G; rs 4991150,R237L; rs3889692, A281P; rs34662058, S284G were constructed using Quick change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) with the wild type (WT) clone as a template (Table 1). The specific nucleotide substitutions in each of the four clones were confirmed by PCR – sequencing using the primers mentioned above.

Generation of stable cell lines

The WT and four variant clones were independently transfected into CHO-K1 cells using Lipofectamine 2000 transfection reagent (Life Technologies, USA) as per the manufacturer's protocol. After 48 h of transfection, cells were trypsinized and challenged with 800 μg/ml of Geniticin (G-418) over 2–4 weeks duration to obtain stable (and clonal) cell lines expressing the WT and variant forms of huDRD4.4 in the respective cell lines.

Expression of the *huDRD4.4* constructs

Expression status of each of the five constructs was checked by western blot analysis. One flask each ($\sim 4 \times 10^6$ cells) of the five stable cell lines generated as mentioned above were washed with phosphate-buffered saline (PBS), harvested and lysed using radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1× Protease inhibitor cocktail (Sigma)]. Total protein in each of the samples thus prepared was estimated using BCA assay kit (Smith et al., 1985). Thirty micrograms of each of the five whole cell homogenates was then separated using 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto Polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) after blocking of the membrane with 5% skimmed milk (Cell Signalling Technology Inc., Danvers, MA, USA). The blot was probed with c-Myc mouse monoclonal antibody (Origene Inc.) for 16 h at 4 °C, followed by goat anti-mouse immunoglobulin G (IgG) conjugated with horse-radish peroxidise (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Detection of c-Myc tagged huDRD4.4 was done by using Supersignal west pico chemiluminescent kit (Pierce, Rockford, IL, USA) as per the manufacturer's protocol.

Localization of WT and variant huDRD4.4 proteins

Immunofluorescence-based localization of the WT and variant DRD4.4 protein was carried out. Cells were seeded in six well plates containing poly-l lysine-coated cover slips. After 36 h, they were washed with PBS and fixed with 4% paraformaldehyde (in 1× PBS). huDRD4.4 expression in the five samples was detected by incubating the fixed cells with anti-huDRD4 antibody (Santa Cruz Biotechnology) at 4 °C for 16 h followed by incubation with alexa fluor® 488 labeled goat anti-mouse secondary antibody (Life Technologies), mounted with VECTASHIELD® Mounting Media with DAPI. Image analysis was done by laser-scanning confocal microscopy (Leica SP5; Ernst-Leitz-Straße, Wetzlar, Germany). Localization of the receptor proteins from the five constructs was also done using transiently expressed WT and variant clones tagged with enhanced green fluorescent protein (EGFP) at the c-terminal region of the protein. Image analysis was done by fluorescence microscopy.

Functional analysis of the WT and variant huDRD4.4 proteins

cAMP assay. Dopamine D2-like receptors reduce cellular cAMP levels via the inhibition of the adenylate cyclase system. It is hypothesized that SNPs in these genes may alter this inhibitory activity. Therefore, cells expressing WT and variant receptor proteins were evaluated for the inhibition of cAMP signals stimulated by forskolin in the presence of agonists namely dopamine and quinpirole. Level of the cytosolic cAMP was determined using homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay (LANCE® Ultra cAMP Detection Kit from

Table 1. Primer sequences for the generation of variant clones

rs1800443, V194G	
Sense	GAGGACCGCGACTACGTGGGCTACTCGTCCGTGTGC
Anti-sense	GCACACGGACGAGTAGCCCACGTAGTCGCGGTCCTC
rs4991150, R237L	
Sense	CGGCCGCGCGCCCCGCCTACCCAGCGGCCCTGGCC
Anti-sense	GGCCAGGGCCGCTGGGTAGGCGGGGCGCGCGGCCG
rs3889692, A281P	
Sense	CCCCGACTGTGCGCCCCCCGCGCCCAGCCTCCCC
Anti-sense	GGGGAGGCTGGGCGCGGGGGGCGCACAGTCGGGG
rs3662058, S284G	
Sense	GCGCCCGCCGCGCCCGGCCTCCCCCAGGACCCCTG
Anti-sense	CAGGGGTCCTGGGGGAGGCCGGGCGCGGCGGGCGC

PerkinElmer Inc.) as per the manufacturer's protocol. Briefly, cells were grown up to ~70% confluence, washed with PBS and lifted using versene solution (PBS + 5 mM EDTA) and centrifuged at 275*g* for 10 min. Cells were then counted and also checked for viability using trypan blue. Cells were then washed in 1× Hank's balanced salt solution (HBSS), centrifuged and resuspended in stimulation buffer (HBSS 1×, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.1% bovine serum albumin (BSA); pH 7.4) with recommended dilution and seeded into white, opaque OptiPlate-96 well microplates (PerkinElmer Inc., Waltham, MA, USA). Cells were incubated with or without quinpirole/dopamine (0.1 nM–10 μM) in the presence and absence of forskolin (10 μM) for 30 min. After incubation with the agonists, Eu-cAMP tracer working solution (donor beads) was added into the well followed by ULight-anti-cAMP working solution (acceptor beads) and incubated for 1 h at room temperature. Competition between the europium (Eu) chelate-labeled cAMP tracer and sample cAMP for binding sites on cAMP-specific monoclonal antibodies labeled with the ULight dye was initiated. In the absence of free cAMP, maximal TR-FRET signal is achieved. Free cAMP produced by stimulated cells competes with the Eu-cAMP tracer for the binding to the ULight-mAb, causing a decrease in TR-FRET signal. Signals were captured using Tecan infinite 200 pro multimode reader (Tecan Group Ltd., Switzerland).

Extracellular signal-regulated kinase (ERK 1/2) phosphorylation assay. DRD4 mediates the activation of ERK1/2 through transactivation of platelet-derived growth factor which may be affected by variant(s) within the gene. This was investigated by mitogen-activated protein kinase (MAPK) phosphorylation assay which was carried out as described earlier (Oak et al., 2001) with minor modifications. Briefly, cells expressing the WT and variant huDRD4.4 receptor proteins were grown in six well plates as mentioned before up to ~80% confluence, rinsed with serum-free D-MEM/F-12 medium and then incubated overnight at 37 °C under the same serum-free conditions. These serum-deprived cells were activated by incubating them with 1 μM dopamine/quinpirole. After incubation for 5 min, activation was terminated by washing the cells with ice-cold PBS, followed by the addition of RIPA lysis buffer. Total cell lysate was resolved on 12.5% SDS–PAGE and transferred onto PVDF membrane. Membrane was incubated with blocking buffer [TBS (20 mM Tris–HCl, 137 mM NaCl, pH 7.6) and 0.1% Tween-20 with 5% BSA and 0.02% NaN3]. Samples were incubated with anti-phospho ERK 1/2 antibody (1:1000 in blocking buffer) (Santa Cruz Biotechnology). The blot was washed three times with TBS-T for 5′ each and then probed with peroxidase–conjugated secondary antibody (1:10,000 anti-mouse-HRP in blocking buffer without NaN3). Phophorylated ERK1/2 was visualized using supersignal west pico chemiluminescent kit (Pierce). Total ERK1/2 was detected by probing the blot with anti ERK1/2 followed by peroxidase–conjugated secondary antibody. Total ERK1/2 was detected by chemiluminescence kit.

Saturation radioligand binding assay. Variants in DRD4 gene may alter the binding affinity and the total number of DRD4 receptor molecules expressed at the plasma membrane. This was determined by saturation radioligand binding assay. Cell membranes from WT and two variants (rs1800443, V194G; rs 4991150, R237L) cell lines were prepared as described earlier (Kalipatnapu et al., 2004) with a few modifications. Confluent cells grown in 162 cm^2 flasks were harvested by treatment with ice-cold 10 mM Tris, 5 mM EDTA, and 0.1 mM PMSF, pH 7.4 buffer. Cells were then homogenized for 20 s at 4 °C at maximum speed with a Polytron homogenizer. The cell lysate was then centrifuged at 500*g* for 10 min at 4 °C and the resulting postnuclear supernatant was centrifuged at 40,000*g* for 30 min at 4 °C. The pellet thus obtained was suspended in 50 mM Tris, pH 7.4 buffer and used for saturation radioligand binding assay. The total protein concentration of cell membranes was determined using the bicinchoninic acid (BCA) assay kit (Smith et al., 1985).

Receptor binding assays were carried out as described earlier (Kalipatnapu et al., 2004; Cummings et al., 2010) with some modifications. Briefly, tubes in duplicate with 50 μg of total protein in one ml of 50 mM Tris buffer, pH 7.4 were used. Tubes were incubated with increasing concentrations (0.05–7.5 nM) of the radiolabeled antagonist [^{3}H]spiperone for 90 min at 25 °C. Nonspecific binding was determined by performing the assay in the presence of 10 μM spiperone. The binding reaction was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B 2.5 cm diameter glass microfiber filters (1.0 μm pore size) which were presoaked in 0.15% (w/v) polyethylenimine for 1 h (Bruns et al., 1983). The filters were then washed three times with 3 ml each of cold water (4 °C) and dried. Retained radioactivity was measured in a Packard Tri-Carb 2900TR liquid scintillation counter using 5 ml of scintillation fluid. The concentration of the bound radioligand (RL^*) was calculated from the equation:

$$RL^* = 10^{-9} \times B/(V \times SA \times 2220)\ M$$

where B is the specific bound radioactivity in disintegrations per minute (dpm), V the assay volume in ml, and SA is the specific activity of the radioligand.

Nonlinear curve fitting and statistical analysis. The dissociation constant (K_d) and maximum binding sites (B_{max}) were calculated by non-linear regression analysis of binding data using Graphpad Prism software version 4.00 (San Diego, CA, USA). The data could be fitted best to a one site ligand binding equation. Data obtained after regression analysis were used to plot graphs with GRAFIT program version 3.09b (Erithacus Software, Surrey, UK). Statistical significance analysis was done by two-tailed, unpaired Student's *t*-test using Graphpad Prism software version 4.00.

In silico molecular dynamics (MD) simulation and ligand docking analysis of WT and variant huDRD4.4 proteins

Molecular dynamic simulations are routinely used to calculate time-dependent behavior of a molecular system which may also provide detailed information on the structural changes upon ligand binding. We hypothesized that the genetic variants in DRD4 may affect conformation of the receptor structure upon ligand binding.

Structure prediction and mutational studies. The three-dimensional model of DRD4.4 was predicted using I-TASSER (Zhang, 2008) which used the crystal structures of human dopamine receptor D3 (DRD3) (PDB #3PBL) and human M2 muscarinic acetylcholine receptor (PDB #3UON) as templates. Five models were generated out of which the top ranked model was selected for further studies. Two of the variants namely, V194G and R237L were generated using Modeller 9.10 (Eswar et al., 2007).

Docking of agonist and antagonist. The 3D-model of WT and two variants were further docked to dopamine (agonist) and spiperone (antagonist) using Induced Fit Docking (IFD) protocol (Sherman et al., 2006; Suite, 2012). Prior to docking, binding site was characterized using Q-SiteFinder (Laurie and Jackson, 2005), CASTp (Dundas et al., 2006) and SiteMap (Schrödinger) (Halgren, 2007) and validated by literature review. The docked poses were ranked according to the IFDScore that accounts for protein–substrate interaction energy and the total energy of the system. Also, binding-free energy estimates were evaluated for the top docked complexes using MM–GBSA calculations. The top-ranked docking pose for each protein–ligand complex was further taken up for refinement using MD simulation.

MD simulation. All atom-MD simulation was carried out on nine systems using Desmond 3.1 (Bowers et al., 2006) (Table 2). MD simulations were carried out in lipid bilayer-explicit solvent environment using Palmitoyl oleoyl phosphatidyl choline (POPC) as lipid bilayer. All the simulations were performed under NPT ensemble with pressure and temperature set to 1.01325 bar and 300 K respectively. The details of the MD simulation are listed in Table 2.

Docking studies with refined structures. Post MD simulations, docking studies using IFD protocol and free energy calculations using MM–GBSA were again carried out in a similar manner as mentioned above by taking the lowest energy frame from the trajectories.

RESULTS

A comparative genotype–phenotype correlations of the WT DRD4.4 and four variants namely rs1800443, V194G; rs4991150, R237L; rs3889692, A281P and rs34662058, S284G were carried out using *in silico* tools and *in vitro* assays in this study. Resequencing of the WT and the four variant constructs confirmed the presence of desired nucleotide substitutions (Fig. 2). CHO-K1 cells stably expressing the WT and the variant receptor proteins were obtained after approximately four weeks after transfection. Clonal lines for each of these five constructs were obtained after another four weeks and were used for all subsequent experiments.

Expression of DRD4.4 variants

Western blot analysis confirmed the expression of the WT and four variant receptor proteins in the respective cell lines (Fig. 3).

Localization of WT and variant huDRD4.4 proteins

Immunofluorescence analysis of cells expressing WT and four variant proteins expressing cells were carried out by using c-Myc monoclonal antibodies (Fig. 4I) and clones tagged with EGFP at the c-terminal end of the receptor proteins (Fig. 4II) confirmed the localization of the five respective receptor proteins on the plasma membrane.

Functional analysis of the WT and variant huDRD4.4 proteins

cAMP assay. TR-FRET-based estimations of intracellular cAMP concentrations were made following treatment of cells expressing WT and variant receptor proteins with dopamine/quinpirole molecules and with forskolin as an inducer. Intracelluar level of cAMP which are amplified 13- to 15-fold upon forskolin stimulation

Table 2. Details of MD simulation on various systems of DRD4.4

System	Ligand	Duration of MD run (ns)
DRD4.4-wild type	Nil	100
DRD4.4-wild type	Dopamine	100
DRD4.4-wild type	Spiperone	100
DRD4.4-V194G	Nil	100
DRD4.4-V194G	Dopamine	100
DRD4.4-V194G	Spiperone	100
DRD4.4-R237L	Nil	120
DRD4.4-R237L	Dopamine	100
DRD4.4-R237L	Spiperone	100

were downregulated on an average of ~80% by dopamine and ~60% by quinpirole in cells expressing the WT receptor protein. On the other hand, notable differences were observed among the variant proteins. In cells expressing the V194G variant protein, an over 500-fold reduction (which is beyond test range to check the relative efficacy) in the potency to lower the cAMP levels was observed compared to the WT receptor upon dopamine and quinpirole stimulation. Cells with R237L variant showed an approximately six- and threefold potency reduction for dopamine and quinpirole respectively (relative efficacy was ~60%) but which was significantly different ($p < 0.01$) compared to the WT protein. However, potency and efficacy values for both the agonists in the other two variants (A281P, S284G) were only two to threefold lower and not significantly different from the WT (Fig. 5A, B; Table 3).

ERK1/2 (Thr202/Tyr204) phosphorylation assay. Efficiency of ERK1/2 phosphorylation could be estimated only transiently (5-min duration for dopamine in this study) since phosphorylation reaches basal level in less than 30 min (Oak et al., 2001). Western blot analysis showed a notable reduction in the phosphorylation level in the cells expressing V194G protein compared to WT protein but no difference was observed with the other three variants (Fig. 6).

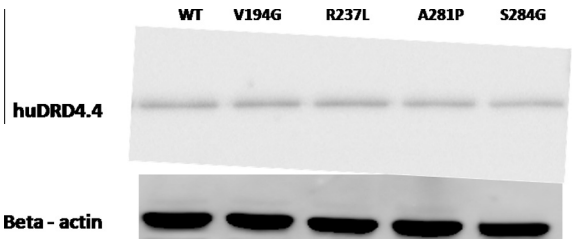

Fig. 3. Western blot analysis of the WT and four variant DRD4.4 receptor proteins.

Radioligand binding analysis

Based on the results of the inhibition of cAMP synthesis and efficiency of ERK 1/2 phosphorylation by the WT and variant receptor proteins, it was evident that only the two variants namely V194G and R237L were functionally important. Therefore, only these two variants were taken forward for radioligand binding assay.

We pharmacologically characterized binding of the selective dopamine receptor antagonist [^{3}H]spiperone to cell membranes of the isolated CHO clones stably expressing the WT and V194G and R237L variant proteins. Representative saturation binding results are shown in Fig. 7. Data for saturation binding were analyzed using Graphpad Prism software and the binding parameters are shown in Table 4. A significant reduction ($p < .001$) in the binding affinity (K_d = 2.16

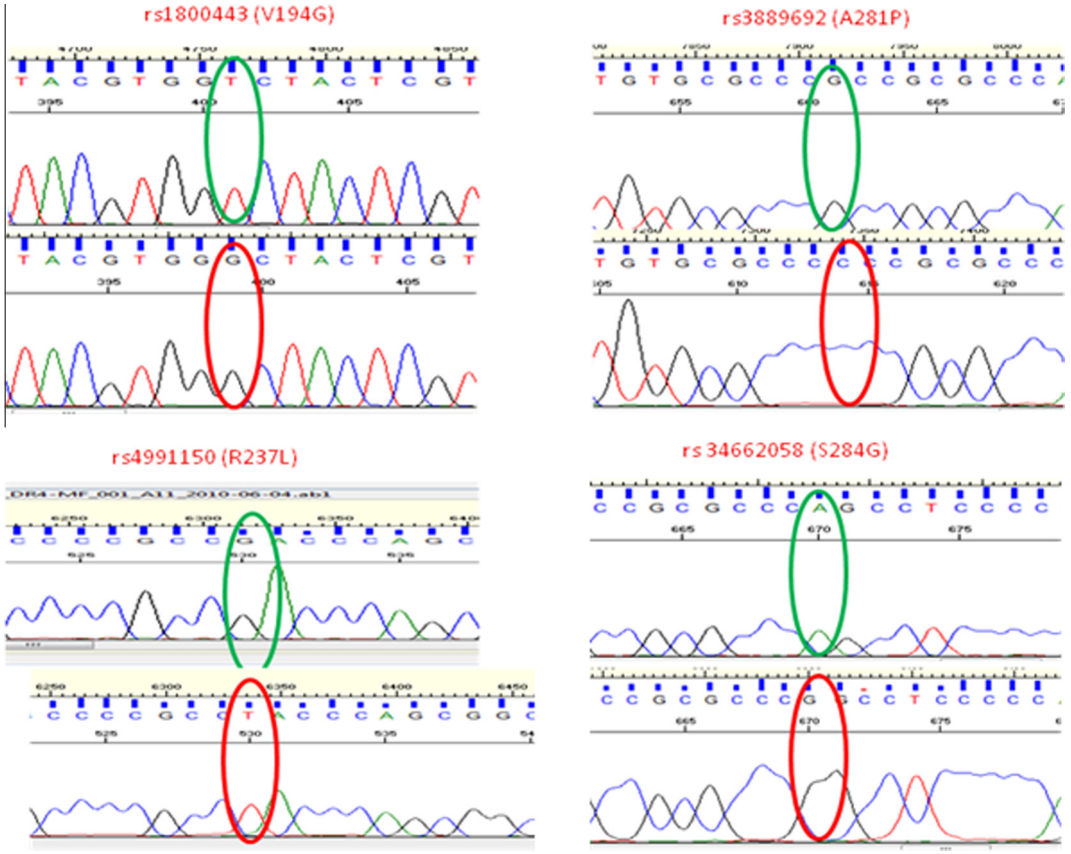

Fig. 2. Confirmation of variant clones.

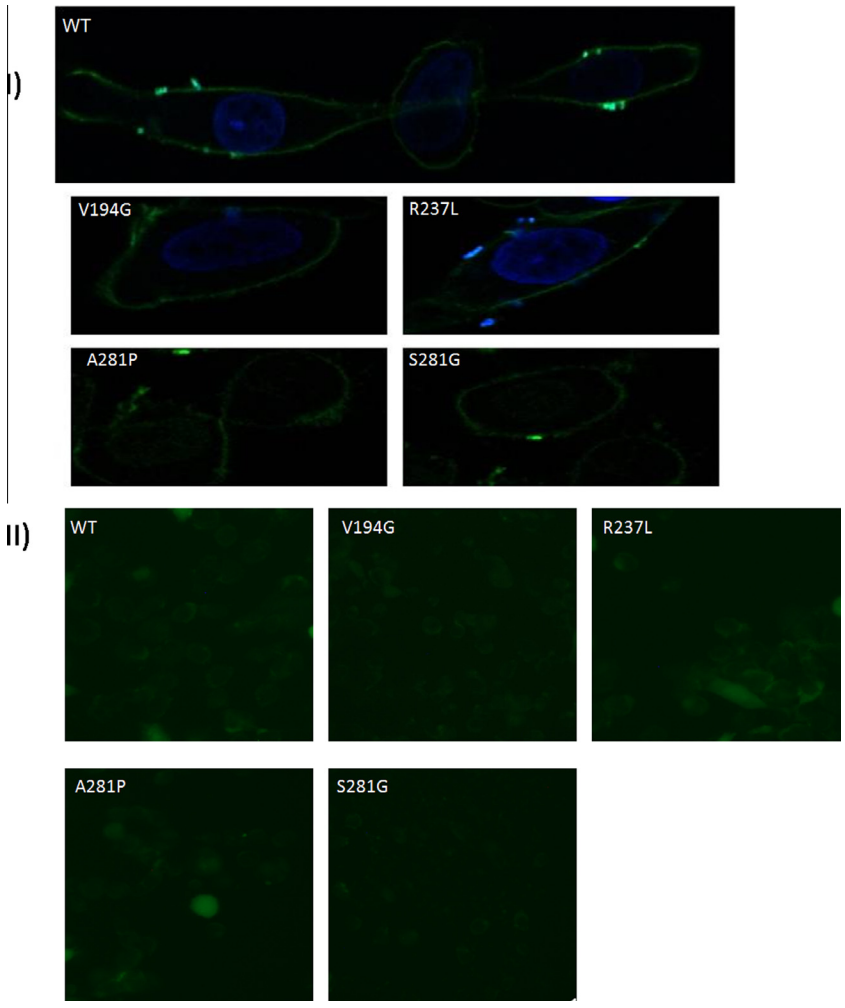

Fig. 4. Localization of DRD4.4 receptors on CHO-K1 cells stably expressing – WT; variants-V194G; R237L; A281P and S284G receptor proteins by using (I) c-Myc mouse monoclonal antibodies and (II) clones with EGFP tagged at the c-terminal end of the protein.

nM) and the total number of binding sites (B_{max}) for [^{3}H]spiperone binding (~66% reduction) was observed for V194G protein compared to the WT. R237L variant protein also exhibited a significant reduction (~40%, $p < 0.01$) in number of total binding sites (B_{max}) but not in the affinity of ligand binding (K_d) (Fig. 7 and Table 4). This significant reduction in the total number of binding sites (B_{max}) of R237L could be due to reduced level of this variant receptor expression at the plasma membrane compared to WT and V194G variant (Fig. 8).

In silico and ligand docking analysis of WT and variant huDRD4.4 proteins

For structure prediction in this study, sequence of DRD4 variant 4R was used as it is more prevalent worldwide, especially in the Asian population (Wang et al., 2004). Due to the lack of information on the crystal structure of DRD4, structure of DRD4.4 was predicted using I-TASSER, a combination of *ab initio* folding and threading method. The top model with a *C*-score of −1.17 was selected for further studies. *C*-score is a confidence score which accounts for quality of predicted models where a model with higher value for *C*-score signifies a high confidence. The predicted model of DRD4.4 was further taken up for the generation of two mutants, namely, V194G and R237L using Modeller9.10. The predicted model of DRD4.4 highlighting the positions of mutated residues is shown in Fig. 9.

Binding site analysis and docking studies with agonist and antagonist

For docking studies, residues around the dopamine and spiperone binding site were taken up for the generation of grid. The protein structure of WT and two mutants were docked to dopamine (agonist) and spiperone (antagonist) using IFD protocol, both before and after MD. The results are tabulated in Table 5. Post MD, dopamine shows a docking score of −10.022 kcal/mol and −8.688 kcal/mol with the WT structure and R237L respectively, which reduced to −6.241 kcal/mol in the case of V194G. Similarly, spiperone shows a docking score of −11.690 kcal/mol and −12.524 kcal/mol with WT and R237L respectively and a low score of

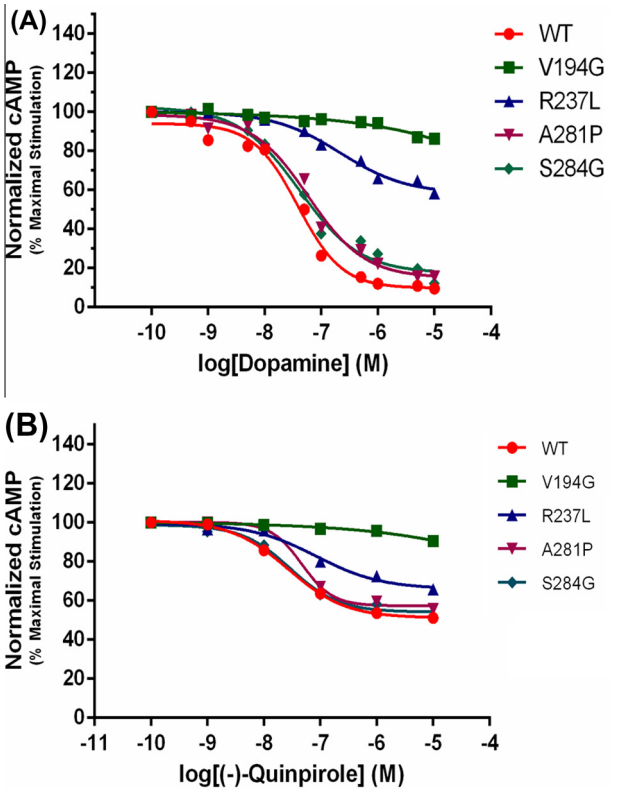

Fig. 5. Representative plot of the inhibition of forskolin-stimulated cAMP by (A) dopamine and (B) quinpirole.

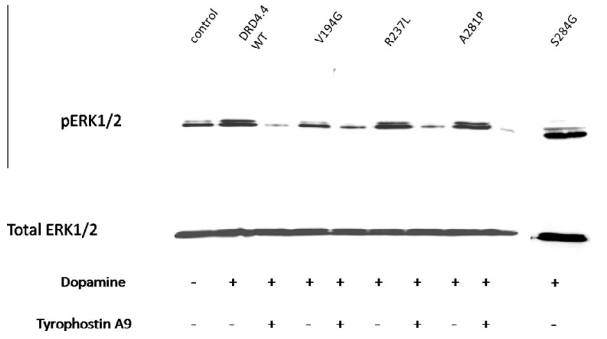

Fig. 6. Extracellular signal-regulated kinase (ERK1/2) phosphorylation profile mediated by DRD4.4 WT and four variant clones-V194G; R237L; A281P and S284G with CHO-K1 cells (with dopamine as a stimulant and tyrophostin A9 as a MAPK inhibitor).

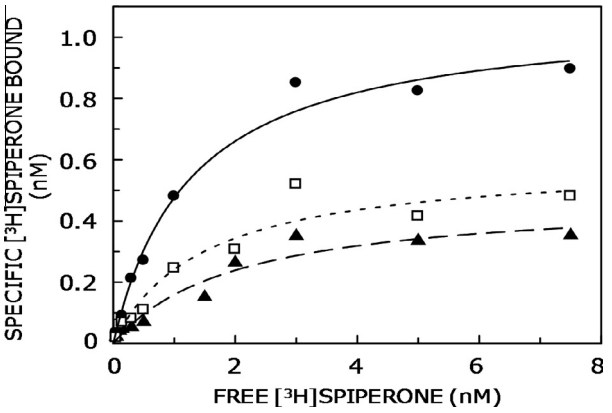

Fig. 7. Representative plot of specific saturation binding analysis/curve of [^{3}H]spiperone to DRD4.4 WT (—●—), DRD4.4 V194G (– –▲– –) and DRD4.4 R237L (—□—) receptors stably expressed in CHO-K1 cells.

−8.643 kcal/mol in the case of V194G. This led to the observation that the ligands bind to the protein structures of WT and R237L with a greater affinity as compared to V194G. The docked protein–ligand complex of DRD4 WT and V194G bound with dopamine and spiperone are represented in Fig. 10.

The non-covalent interactions of dopamine and spiperone with the amino acid residues in the binding site of DRD4.4 WT, DRD4.4-V194G and DRD4.4-R237L are depicted in Fig. 11. The amino acid residues Ser196 and Ser200 form H-bonds with the two OH groups on dopamine in DRD4.4 WT (Fig. 11A) whereas only Ser196 forms a H-bond with the two OH groups on dopamine in the case of R237L. There is an additional H-bond between Asp115 and primary amine in both dopamine-bound DRD4.4 WT and DRD4 R237L (Fig. 11C). On the other hand, only Leu86 forms H-bonds with two OH on dopamine in the case of DRD4.4-V194G (Fig. 11B). The common residues involved in hydrophobic interactions include Val116, Leu187, Tyr192, Val193, Phe362, Phe363 and Ile367 in both DRD4.4 WT and DRD4.4-R237L with many others. On the other hand, in V194G only a few interactions with

Table 3. Potency and relative efficacy of dopamine and quinpirole on DRD4.4 WT and variant receptors as determined by cAMP assays

Receptor	Dopamine		Quinpirole	
	Ec_{50} (nM)	% Inhibition	Ec_{50} (nM)	% Inhibition
DRD4.4 WT	36.36 ± 8.66 (1)	83.09 ± 3.92	28.63 ± 3.54	46.23 ± 3.39
DRD4.4 V194G	~18,000 (~500)[a]	N.D[a]	N.D[a]	N.D[a]
DRD4.4 R237L	215.05 ± 18.08** (6)	41.96 ± 2.57**	89.66 ± 12.60** (3.1)	32.54 ± 1.30**
DRD4.4 A218P	65.66 ± 12.42 (1.8)	81.22 ± 2.07	45.50 ± 5.9 (1.5)	43.96 ± 2.26
DRD4.4 S284G	60.06 ± 18.14 (1.6)	84.57 ± 4.87	26.00 ± 7.39 (0.9)	46.11 ± 5.90

Potencies (EC_{50}, nM) and inhibition efficiencies (%) of both dopamine and quinpirole are shown as mean ± SD of duplicate points from three independent measurements. Inhibition efficiencies expressed in percentage decrease in the intracellular cAMP level related to the level of cAMP generated by 10 μM forskolin minus basal levels of cAMP.

[a] Maximal effect could not be determined since the potency of both dopamine and quinpirole exceeded the range i.e. > 18 μM. Significance was analyzed by two-tailed, unpaired Student's *t*-test using Graphpad Prism software version 6.00 (San Diego, CA).

** Corresponds to $p < 0.01$.

Table 4. Binding parameters[a] of [^{3}H]spiperone with WT and variant DRD4.4 receptors stably expressed in CHO-K1 cells

Receptor	K_d (nM)	B_{max} (pmol/mg)
Wildtype DRD4.4	1.24 ± 0.05	1.04 ± 0.05
DRD4.4-V194G	2.16 ± 0.09**	0.46 ± 0.04**
DRD4.4-R237L	1.42 ± 0.14	0.60 ± 0.03*

[a] Binding parameters were calculated by analyzing saturation binding isotherms with a range (0.05–7.5 nM) of radiolabeled [^{3}H]spiperone using Graphpad Prism software. Data represent the means SE of duplicate points from three independent measurements. Significance relative to the WT receptor was determined by two-tailed, unpaired Student's *t*-test using Graphpad Prism software.

* Correspond to $p < 0.01$.

** Correspond to $p < 0.001$.

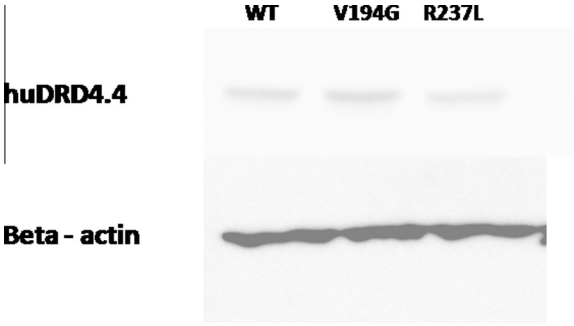

Fig. 8. Expression level of human DDR4.4 receptor WT and two variant clones – V194G, R237L in the plasma membrane using c-Myc mouse monoclonal antibodies by Western blot analysis.

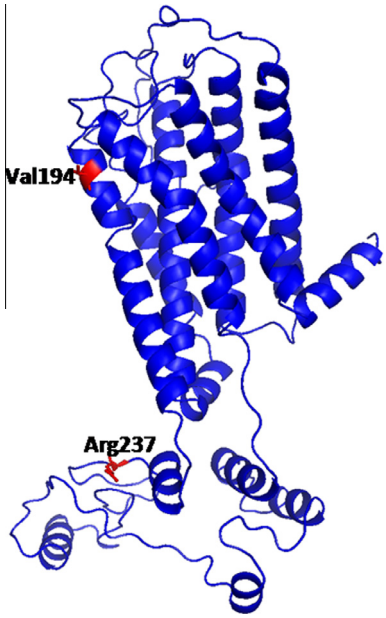

Fig. 9. Predicted structure of Dopamine receptor D4.4. Residues taken up for mutation are highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

residues Met1, Leu12, Val87, Leu90, Leu107, Ala110 and Leu111 were observed.

In the case of spiperone-bound complexes, His366 forms a H-bond with both DRD4.4 WT and DRD4.4-R237L. On the other hand, Leu86 and Trp101 form H-bonds with spiperone in the case of DRD4.4-V194G. Hydrophobic interactions are shown by residues Val116, Cys119, Leu187, Val193, Phe201, Phe362 and Tyr390 in both WT and DRD4.4-R237L, whereas, in the case of DRD4.4-V194G amino acid residues Leu12, Pro89, Leu90, Tyr93, Ala100, Leu102, Leu103, Leu107, Ala110 and Leu111 form hydrophobic interactions. Also, π-stacking interactions are shown by residues Trp101, Trp359 and Phe363 in DRD4.4 WT; by Trp359 in the case of DRD4.4-R237 and by residue Trp101 in the case of DRD4.4-V194G. These observations led to the conclusion that the mutant V194G seems to affect ligand binding with the protein, therefore, the binding of ligand with DRD4.4-V194G seems superficial.

Taken together, these results show that valine (at V194G) is important for structure–function relationship in the DRD4 receptor protein.

DISCUSSION

Conventional candidate gene based and contemporary genome-wide association studies in a wide range of common complex disorders have identified several risk conferring variants. In addition, with the advent of next generation sequencing tools, a large number of rare variants are also being discovered in these disorders, affecting a notable shift from the common disease common variant hypothesis to common disease rare variant hypothesis (Bamshad et al., 2011; Gibson, 2012). An abundance of such rare variants in drug target genes has also been recently reported (Nelson et al., 2012). DRD4 is an important gene from the dopaminergic pathway and several variants in this gene have been shown to be associated with different disorders (Ptacek et al., 2011). However, functional characterization of these variants has been restricted to the VNTR repeats and the upstream region of the gene (Asghari et al., 1995; Kereszturi et al., 2006). This gene has also been shown to be under positive selection with an observed bias toward non-synonymous amino acid changes (Ding et al., 2002). Such variants may contribute to disease etiology and therefore their functional characterization is important.

Quantitative and functional analysis of four variant receptor proteins in comparison to the WT in this study has provided significant insights into the functionally important amino acid residues in this receptor. Of the four variants analyzed, A281P and S284G located within the 4R allele in the 3rd intracellular loop region of DRD4.4, expressed and localized on the plasma membrane of the host cells (Figs. 3 and 4.I, 4.II). They also seem to have no functional implications either in inhibiting cAMP synthesis (Fig. 5A, B and Table 3) or in ERK 1/2 phosphorylation (Fig. 6). This is not unexpected considering that these two SNPs do not fall in the region involved in heterotrimeric G-protein binding in the 3rd intracellular loop. Therefore, these two SNPs were not taken forward for saturation radioligand binding

Table 5. Docking of agonist and antagonist to DRD4.4 (WT, V194G and R237L) using Induced Fit Docking

Structure	Ligand	Pre-MD		Post-MD	
		Glide docking score (kcal/mol)	Binding-free energy estimates (kcal/mol)	Glide docking score (kcal/mol)	Binding-free energy estimates (kcal/mol)
DRD4.4-WT	Dopamine	−8.837	−52.254	−10.022	−68.490
DRD4.4-WT	Spiperone	−10.561	−107.353	−11.690	−120.62
DRD4.4-V194G	Dopamine	−6.022	−37.428	−6.241	−45.53
DRD4.4-V194G	Spiperone	−6.587	−55.874	−8.643	−91.56
DRD4.4-R237L	Dopamine	−8.523	−45.172	−8.688	−46.687
DRD4.4-R237L	Spiperone	−12.184	−87.067	−12.524	−103.61

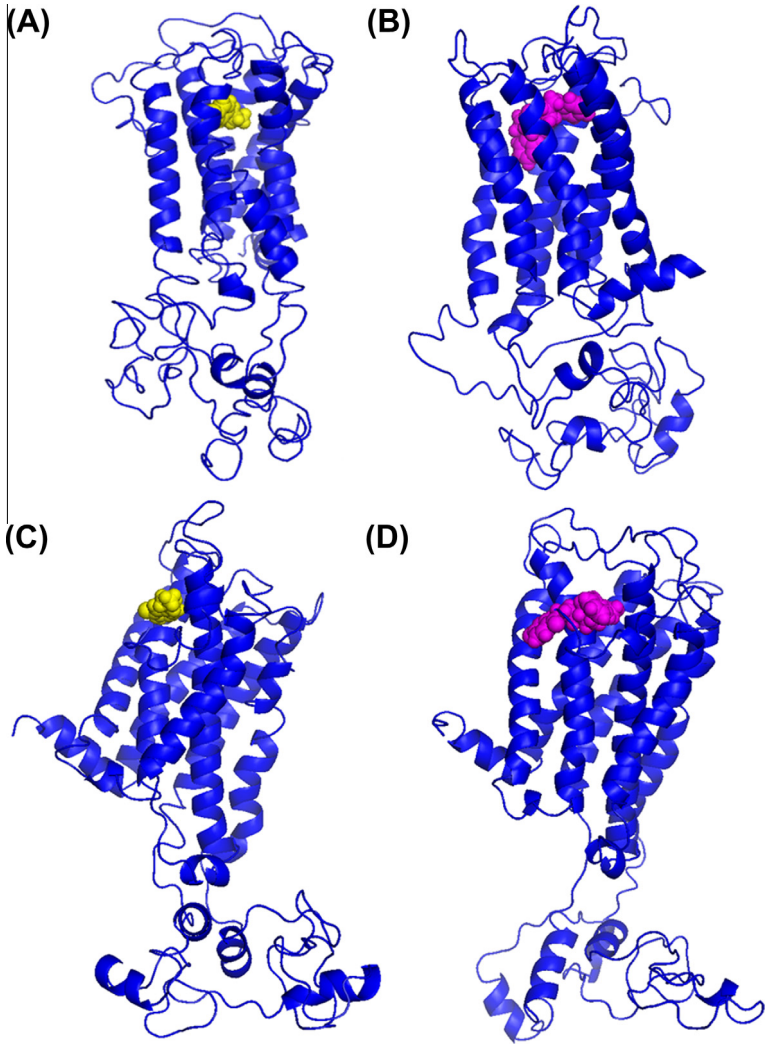

Fig. 10. Structure of DRD4.4 WT docked to (A) dopamine and (B) spiperone post MD and DRD4.4 V194G docked to (C) dopamine and (D) spiperone post MD. Dopamine is represented in yellow and spiperone in magenta. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

analysis and docking studies. As for R237L variant, substitutions at all three nucleotide positions in the codon have been reported (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=1815), with CGA → TGA resulting in a stop codon, CGA → CTA coding for R237L and CGA → CGT leading to synonymous change. Therefore, only R237L variant was considered for further characterization.

Our results demonstrated that leucine at R237L lowers cAMP synthesis marginally (Table 3) but did not alter the ERK1/2 phosphorylation status (Fig. 6). The results of saturation binding analysis shows that the

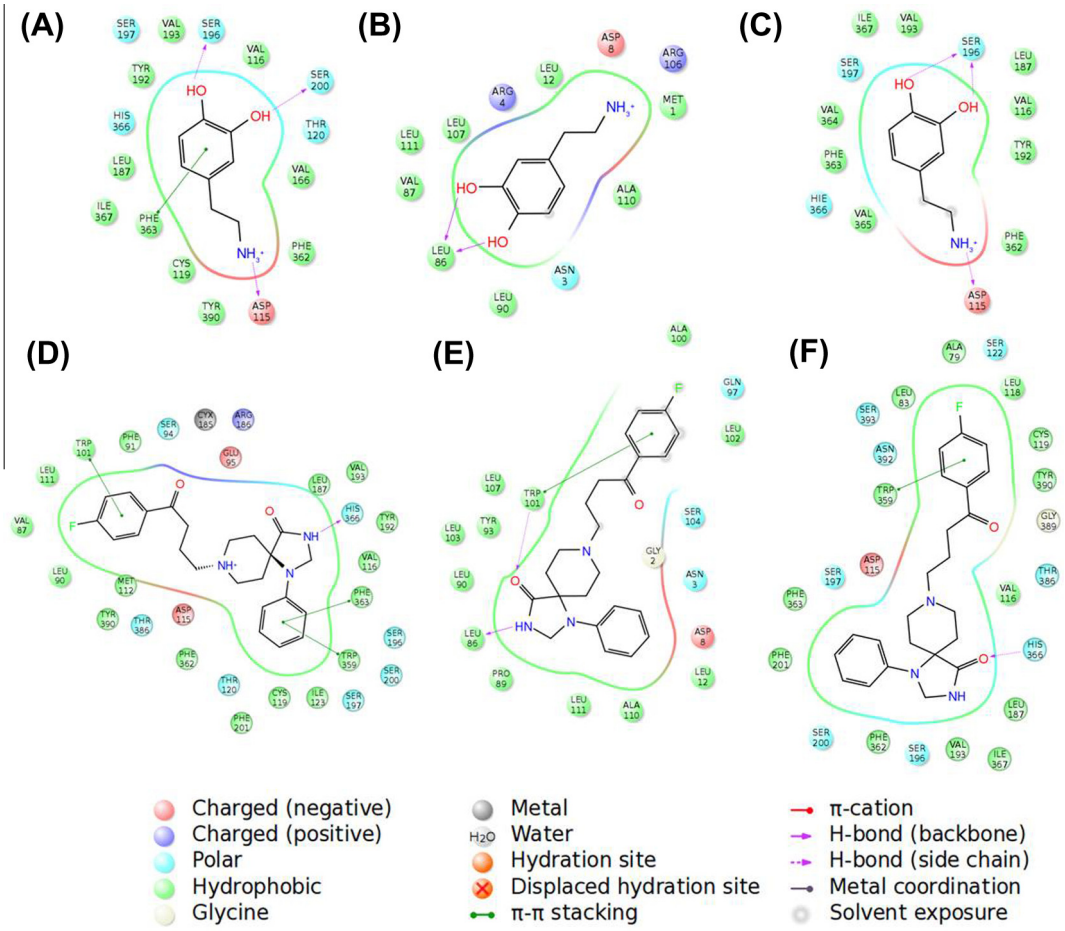

Fig. 11. Ligand-interaction diagram representing protein–ligand interactions of dopamine with (A) WT, (B) V194G and (C) R237L and of spiperone with (D) WT, (E) V194G and (F) R237L.

reduction in ligand binding (Fig. 7 and Table 4) can primarily be attributed to a reduction in the number of total binding sites with no significant change in the affinity of ligand binding. Our in silico analysis (ligand docking) of the spiperone binding to this variant receptor shows that the binding pattern and interaction is unaltered and comparable to WT (Fig. 11 and Table 5). Therefore, reduction in ligand binding sites could be because of limited availability of this variant receptor in the cell membrane, possibly due to defective trafficking of this variant receptor (Fig. 8). The limited availability of the variant receptor may be directly correlated with the reduction in the inhibition of adenylyl cyclase activity (Table 3). However, this expression difference may not suffice to alter the phosphorylation level of ERK which happens through an indirect or downstream signaling. Dissociation of heterotrimeric G protein occurs upon agonist binding with receptor, whereby Gβγ subunits stimulate the mitogen-activated protein kinase pathway (Ras → Raf → MEK → ERK) via transactivating the platelet-derived growth factor receptor β in timeframe consistence (Oak et al., 2001; Gill et al., 2009).

As for the fourth variant receptor protein V194G in this study, this seems to be the most critical variant for ligand binding as demonstrated by the specific binding with [^{3}H]spiperone (Fig. 7). In addition, inhibition of cAMP synthesis (Table 3) and ERK 1/2 phosphorylation (Fig. 6) are also notably influenced by the glycine variant. Ligand docking studies showing the structural rearrangement of this receptor and its ability to bind to both dopamine (agonist) and spiperone (antagonist) (Figs. 10 and 11 and Table 5) convincingly support these observations. This provides a substrate to understand structure–function relationship in this important neurotransmitter receptor which is also a high affinity antipsychotic target.

At this juncture, it may be mentioned that except for the V194G variant, there are no reports on the functional status of the other DRD4 variants analyzed in this study. The V194G variant has been reported to occur in 12.5% of 186 Afro-caribbeans (but not in Caucasians) and was not associated with Schizophrenia (Seeman and Van Tol, 1994). The same group also carried out a limited characterization of this variant by radioligand binding assay and cAMP assays and reported on its insensitivity to dopamine (Liu et al., 1996). Our results are in concordance with their findings. Based on the observed lower affinity of the

glycine variant at 194 amino acid residue to dopamine, it may be assumed that this rare allele should have a protective role in conditions such as Schizophrenia (characterized by excess of dopamine). We tested this by screening 1000 schizophrenia cases and 1000 controls for this specific variation by genotyping using the iplex method (Gabriel et al., 2009). None of the samples showed this substitution and this observation is further explained below. Lower dopamine binding to DRD4 in the central nervous system especially in the prefrontal cortex is known to be associated with the pathophysiology of ADHD (LaHoste et al., 1996). Inability of dopamine binding to the DRD4.4-V194G variant protein as evidenced in our study (Table 2) may thus be hypothesized to lead to severe ADHD condition and by the same mechanism confer a protective effect for schizophrenia. Designing a psychostimulant which mimics dopamine action for this variant DRD4.4 protein may thus be of therapeutic value for ADHD and similar dopamine deficiency disorders. It is likely that continuing such functional investigations of rare genetic variants could hold promise for development of lead molecules. In this context, it seems appropriate to cite an exciting report (Cohen et al., 2006) and also a recent view on the topic of the effect of a rare homozygous mutation in PCSK9 resulting in very low cholesterol levels and its potential for translation into a blockbuster drug (Hall, 2013). Our results based on genotype–phenotype correlations provide a further level of proof that rare variants of large effect may well be distributed with lower minor allele frequencies and thus support the common disease rare variant hypothesis (Bamshad et al., 2011; Gibson, 2012).

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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AUTHOR CONTRIBUTIONS

K.A.M., N.J., L.N. and T.B.K. conceived and designed the study. K.A.M., N.J., M.J. performed the experiments and analyzed the data. K.A.M., N.J., M.J., L.N., A.C. and T.B.K. contributed to writing the manuscript.

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