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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)