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Identification of key residues involved in the activation and signaling properties of dopamine D3 receptor

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ABSTRACT

The dopamine D3 receptor exhibits agonist-dependent tolerance and slow response termination (SRT) signaling properties that distinguish it from the closely-related D2 receptors. While amino acid residues important for D3 receptor ligand *binding* have been identified, the residues involved in activation of D3 receptor signaling and induction of signaling properties have not been determined. In this paper, we used *cis* and *trans* isomers of a novel D3 receptor agonist, 8-OH-PBZI, and site-directed mutagenesis to identify key residues involved in D3 receptor signaling function. Our results show that *trans*-8-OH-PBZI, but not *cis*-8-OH-PBZI, elicit the D3 receptor tolerance and SRT properties. We show that while both agonists require a subset of residues in the orthosteric binding site of D3 receptors for activation of the receptor, the ability of the two isomers to differentially induce tolerance and SRT is mediated by interactions with specific residues in the sixth transmembrane helix and third extracellular loop of the D3 receptor. We also show that unlike *cis*-8-OH-PBZI, which is a partial agonist at the dopamine D2S receptor and full agonist at dopamine D2L receptor, *trans*-8-OH-PBZI is a full agonist at both D2S and D2L receptors. The different effect of the two isomers on D3 receptor signaling properties and D2S receptor activation correlated with differential effects of the isomers on agonist-induced mouse locomotor activity. The two isomers of 8-OH-PBZI represent novel pharmacological tools for *in silico* D3 and D2 receptor homology modeling and for determining the role of D3 receptor tolerance and SRT properties in signaling and behavior.

1. Introduction

The dopamine D3 receptor has the highest affinity for dopamine and some of the ligands used for treating Parkinson's disease and schizophrenia [1,2]. The D3 receptor belongs to the D2-like dopamine receptor subfamily and couples via $G_{i/o}$ G-proteins to downstream effectors such as adenylate cyclase V, G-protein coupled inward rectifier potassium (GIRK) channels, mitogen-activated protein kinases (MAPK) and P/Q-type calcium channels [3–7]. Information obtained from the *antagonist*-bound D3 receptor crystal structure, together with modelling and mutagenesis studies have identified important structural domains and key residues of the D3 receptor involved in ligand binding; however, the residues involved in *agonist-induced activation* of D3 receptor signaling remains to be determined [8–17]. In this paper, we used site-directed mutagenesis to identify residues important for activation of human D3 receptor signaling by three different agonists.

An *agonist*-bound crystal structure of the D3 receptor is not available and, to complicate matters, both in heterologous expression system and *in vivo*, there are agonist-dependent differences in D3 receptor activation which manifests as two characteristic signaling properties distinguishing the D3 receptor from the closely-related dopamine D2 receptor [18,19]. The D3 receptor exhibits a tolerance property wherein repeated activation of the receptor results in a progressive decrease in signaling response. The receptor also exhibits a slow response termination (SRT) property wherein the termination of agonist-induced response, following agonist removal, is very slow compared to the D2 receptors [18]. We have shown that the tolerance and SRT properties are agonist-dependent [20]. While dopamine, classical D2/D3 receptor agonists

Abbreviations: SRT, slow response termination; GPCRs, G-protein coupled receptors; GIRK, G-protein coupled inward rectifier potassium; MAPK, mitogen-activated protein kinases; SES, standard external solution; 30K-ES, standard external solution with 30 mM potassium.

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such as quinpirole, PD128907, 7-OH-DPAT, pramipexole etc. elicit tolerance and SRT, a novel class of atypical D3 receptor agonists which include *cis*-8-OH-PBZI (*cis*-PBZI), ES609 and FAUC73 do not elicit the two D3 receptor signaling properties [20]. Why some D3 receptor agonists elicit tolerance and SRT but others do not, is still not clear; however, we have shown that the D3 receptor adopts a distinct conformation when in a tolerant state [21]. Our working hypothesis is that the agonist-specific tolerance conformation is achieved by specific initial interactions between individual agonists and a subset of residues within and outside the orthosteric binding site of the D3 receptor. In this paper, we test this hypothesis by comparing the acute response and tolerance property of wild-type and mutant D3 receptors using the classical agonist, 7-OH-DPAT, as well as the *cis* and *trans* isomers of 8-OH-PBZI that have different effects on D3 receptor tolerance and SRT properties.

Our previous studies using homology modeling and site-directed mutagenesis have identified different residues in both extracellular and intracellular regions of the D3 receptor involved in mediating tolerance and SRT induced by different agonists [7,22]. For example, we have shown that the PD128907-induced tolerance property might be mediated by an ionic-lock involving the Asp187 residue in extracellular loop 2 and His354 residue in extracellular loop 3 of the human D3 receptor [22]. In contrast, quinpirole-induced tolerance property involves the Cys147 residue in intracellular loop 2 of the human D3 receptor [7]. Identification of residues in the D3 receptor that distinguish a tolerance-eliciting agonist from an agonist that does not elicit tolerance would help understand the D3 receptor conformations generated after agonist binding, receptor activation and inactivation. This would help refine agonist-bound conformational models of the D3 receptor which could be subsequently used to screen chemical libraries to identify different classes of novel D3 receptor agonists.

In addition to being an agonist that does not induce D3 receptor tolerance and SRT, *cis*-PBZI is a partial agonist at dopamine D2S receptor isoform and a full agonist at D2L receptor isoform [20]. The two D2 receptor splice isoforms are different in that the D2L receptor has 29 additional amino acid residues in the third cytoplasmic loop. In this paper, we compared the ability of *cis* and *trans* isomers of 8-OH-PBZI to activate D2S and D2L receptor signaling. Administration of classical tolerance and SRT-eliciting D3 receptor agonists to rodents, induces an initial decrease, followed by an increase in locomotor activity [23–26]. We have hypothesized that the biphasic locomotor response to D3 receptor agonist might be a result of D3 receptor tolerance property wherein the initial inhibitory modulation of D1- and D2 receptor-driven locomotor activity by D3 receptor is attenuated as the D3 receptor undergoes tolerance [19]. In this paper, we tested this hypothesis by comparing the locomotor response elicited by the two isomers of 8-OH-PBZI in *drd3*-EGFP mice. The differential effect of the two isomers of PBZI on D3 and D2 receptor signaling and properties might contribute to the differences in agonist-induced locomotor activity.

2. Materials & methods

2.1. Computational modeling of D3 receptor

The D3 receptor optimized by Platania et al. was employed in our computational modeling [17]. At first, bond orders were assigned, hydrogens were added and subsequently optimized, followed by minimization within the Protein Preparation Wizard routine. The minimization employed the 'Impref' utility, which runs a series of constrained impact minimizations with gradually decreasing strength of the heavy-atom restraining potential. Two minimizations were initially performed. In the first, the torsional potential was turned off to improve hydrogen optimization, whereas the second minimization restored the torsional potential. The RMSD is checked at the end of each round of minimization. If the output structure from a minimization exceeds the specified RMSD threshold of 0.3, relative to the starting structure, the program stops and returns the structure from the previous minimization.

Receptor grid generation was performed with ligand scaling of 0.8 for the van der Waals radii, followed by docking with Glide version 5.7 [28,29]. Ligands were docked and ranked using standard precision, followed by post-docking minimization. The binding pocket was defined within 10 Å from $Ser192^{5.42}$ and $Asp110^{3.32}$. Docking experiments were performed with default settings, while 30 poses per ligand were saved. Poses were visually inspected and analyzed based on scoring and binding interactions. Docking was also performed using GOLD 5.1, with both ChemScore and GoldScore employed as fitness functions [30,31]. Standard default settings were used in all calculations; search efficiency was set to 50%, with GoldScore annealing parameters dependent upon the calculated number of GA operations. All single bonds were treated as rotatable. Early termination was not allowed and the neither option for diverse solutions was selected. The specific stereoisomer of each *cis*/*trans* isomer used in the docking studies is described in the Supplementary data.

2.2. Cell culture and transfection

AtT-20 mouse pituitary cells were cultured and transfected as reported by us previously [18]. Briefly, cells were grown in Ham's F10 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum, 2 mM glutamine and 50 μg/ml of gentamicin (Invitrogen, Carlsbad, CA, USA). AtT-20 cells stably expressing either human D2S, D2L or D3 receptors were cultured in the above medium, supplemented with 500 μg/ml G418. We have previously shown that these three stable cell lines express 854 ± 20, 1032 ± 51, and 2006 ± 75 fmol/mg of human D2S, D2L and D3 receptors, respectively [32]. AtT-20 cells transiently-transfected with wild type, mutant or chimeric receptors were co-transfected with a plasmid containing enhanced green fluorescent protein (EGFP; Clontech, Palo Alto, CA, USA) to identify transfected cells. AtT-20 cells were transfected using the DMRIE-C reagent (Invitrogen). Electrophysiological recordings were made 36–48 h after transfection.

2.3. Generation of chimeric and mutant D3R

The chimeric and mutant human D3 and D2 receptors were generated using a strategy described previously by us [18,22]. Briefly, chimeric receptors and receptors with point mutations were generated by ligating together DNA fragments that were generated using PCR. The PCR was performed with multiple primer pairs that encoded amino acids found at the junction of swapped regions. The cDNA encoding the wild-type and chimeric receptors were subcloned in the pcDNA3 expression plasmid (Invitrogen). All recombinant plasmids were characterized by restriction enzyme analysis and DNA sequencing to verify that no spurious changes were introduced in the entire receptor-coding region. All plasmids used for the transfection studies were purified on two sequential cesium chloride density gradients.

2.4. Synthesis of cis- and trans-8-OH-PBZI

cis-(+/−)-1,2,3a,4,5,9b-Hexahydro-3-*n*-propyl-1*H*-benz[*e*]indole hydrobromide (*cis*-8-OH-PBZI) and *trans*-(+/−)-1,2,3a,4,5,9b-Hexahydro-3-*n*-propyl-1*H*-benz[*e*]indole Hydrobromide (*trans*-8-OH-PBZI) were prepared using previously described methods [33,34]. To determine purity, elemental analyses were performed by Oneida Research Services, Inc, NY and the purity