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Original article

Genetic variants of dopamine D2 receptor impact heterodimerization with dopamine D1 receptor

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ABSTRACT

Background: The human dopamine D2 receptor gene has three polymorphic variants that alter its amino acid sequence: alanine substitution by valine in position 96 (V96A), proline substitution by serine in position 310 (P310S) and serine substitution by cysteine in position 311 (S311C). Their functional role has never been the object of extensive studies, even though there is some evidence that their occurrence correlates with schizophrenia.
Methods: The HEK293 cell line was transfected with dopamine D1 and D2 receptors (or genetic variants of the D2 receptor), coupled to fluorescent proteins which allowed us to measure the extent of dimerization of these receptors, using a highly advanced biophysical approach (FLIM-FRET). Additionally, Fluoro-4 AM was used to examine changes in the level of calcium release after ligand stimulation of cells expressing different combinations of dopamine receptors.
Results: Using FLIM-FRET experiments we have shown that in HEK 293 expressing dopamine receptors, polymorphic mutations in the D2 receptor play a role in dimmer formation with the dopamine D1 receptor. The association level of dopamine receptors is affected by ligand administration, with variable effects depending on polymorphic variant of the D2 dopamine receptor. We have found that the level of heteromer formation is reflected by calcium ion release after ligand stimulation and have observed variations of this effect dependent on the polymorphic variant and the ligand.
Conclusion: The data presented in this paper support the hypothesis on the role of calcium signaling regulated by the D1-D2 heteromer which may be of relevance for schizophrenia etiology.
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Introduction

The classic theory of signaling transduction cascades acting *via* G protein coupled receptors (GPCRs) assumed that a ligand activated monomeric receptor protein causes stimulation or inhibition of the receptor actions which only depended on the kind of compound and receptor protein. Numerous studies provided evidence that signal transduction is much more complex and many proteins are able to interact with membrane receptors affecting signal transduction, and also that GPCRs can act as two-protein or higher order protein complexes [1]. Although experiments which provide evidence for GPCRs oligomerization can be easily misinterpreted and frequently require very critical analysis, at present not only the ability of GPCRs to take on oligomeric forms is being discussed, but more often, its physiological implications as well [2,3].

Dopamine D1 and D2 receptors belong to GPCRs exerting opposite actions on the level of cyclic AMP formation, but plethora of behavioral, biochemical and electrophysiological studies provided evidence that these receptors act in concert [4,5]. It has been shown that a part of central dopamine D1 and D2 receptors are colocalized in rat and human neostriatal neurons [6–13]. This discovery was used in our studies as well as by others in studies applying heterologous expression systems *in vitro*. Experiments using the FRET (Förster Resonance Energy Transfer) phenomenon showed that in HEK 293 cells transiently expressing both receptors, these proteins are in close proximity (which has been interpreted as an ability to form heterodimers) that is

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affected by ligand co-stimulation [14,15]. In recent years, a novel signaling pathway, activated *via* the dopamine D1-D2 receptor heteromer has been identified. This pathway is linked to intracellular calcium release as an effect of D1–D2 heteromer interaction with the Gq protein. As a result, phospholipase C and IP3 (inositol 1,4,5-tris-phosphate) receptors are activated [9,10,16]. The consequences of this signaling pathway are not known, but there are suggestions that it may be involved in the etiopathology of schizophrenia. This presumption comes from the fact that both increased dopamine transmission and abnormal calcium signaling have been linked to this neuropsychiatric disorder [17,18]. The existence of D1–D2 heteromer in neuronal tissues and its coupling with Gq signaling started investigations indicating direct association of calcium signaling and dopamine D1–D2 heteromer with schizophrenia [9,12,19–24].

There are numerous theories of schizophrenia etiopathology and genetics is often taken into consideration [25]. The dopamine D2 receptor is important since it is blocked by typical antipsychotic drugs with high affinity which is correlated with the ability to control psychotic symptoms in schizophrenia. There are three polymorphisms in the D2 dopamine receptor gene found in the human population. Ala for Val substitution in position 96 (V96A) is localized in the second transmembrane domain in the region responsible for ligand binding. Pro310Ser (P310S) and Ser311Cys (S311C) substitutions are located in the third intracellular loop which is probably responsible for G protein coupling, and thus, due to their localization they may induce changes in protein functioning. There is evidence that these polymorphic changes are correlated with schizophrenia, with the strongest evidence for S311C [26–29], although relatively little work has been done on the subject of polymorphism impact on molecular changes in dopamine receptor interactions [30].

In the present study, we have applied a highly advanced biophysical approach (Fluorescence Resonance Energy Transfer measured by Fluorescence Lifetime Imaging Microscopy, FLIM-FRET) to measure dopamine D1-D2 receptor heterodimerization, which allowed us to show that single nucleotide polymorphisms in the D2 dopamine sequence influence its interactions with the dopamine D1 receptor and evoke changes in calcium release from intracellular stores, presumably by Gq protein.

Materials and methods

Materials

HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), *Escherichia coli* DH5 (Dam+) cell line was obtained from Novagen (Darmstadt, Germany). Plasmids pcDNA3.1(+) encoding human D1 and D2 receptors were obtained from the UMRcDNA Resource Center (University of Missouri, Rolla, MO, USA), pEGFP-N1 and pmCherry-N1 vectors were from BD Biosciences, Clontech (Palo Alto, CA, USA), pBUDCE4.1 was from Invitrogen. Molecular biology reagents were from Thermo Fisher Scientific.

Site-directed mutagenesis

Plasmids containing point mutations in coding regions of the dopamine D2 receptor and the plasmid containing a monomeric variant of EGFP protein were prepared in PCR reactions followed by treatment with methylation-specific endonuclease Dpn I and *E. coli* cells transformation, according to the Quik Change Site-Directed Mutagenezis Kit (Stratagene, LA Jolla, CA, USA). The sequences of sense primers were as follows: pcDNA3.1_D2(V96A): GTTGTCTACCTGGAGGCGGTAGGTGAGTGG; pcDNA3.1_D2(P310S): GACTCTCCCCGACTCGTCCCACCATGGTCTCC; pcDNA3.1_D2 (S311C): CTCCCCGACCCGTGCCACCATGGTCTCCAC; pEGFP-N1 (A206K): CACCCAGTCCAAGCTGAGCAAAGACC. The results were verified by DNA sequencing (Genomed S.A., Poland).

Construction of fusion proteins for transient transfection

For confocal microscopy, cDNA of the dopamine D1 and D2 receptor and polymorphic variants of the latter were inserted in-frame into the mammalian expression vector pEGFP-N1 with point mutation A206K or vector pmCherry-N1 [31,32]. The coding sequences were PCR amplified using primers introducing unique restriction sites recognized by NheI and XhoI endonucleases and omitting codon STOP. Following insertion of the D2 receptor genetic variants into the pmCherry-N1 plasmid, the polylinker sequence was extended with additional 35 amino acids (GGSGGGSGGGSGGGSGGGSGGGSGGGSGGGS) in Quik Change Site-Directed reaction to achieve plasma membrane localization.

Construction of plasmids containing two receptors for stable transfection

The pBUDCE4.1 expression vector was used to generate cell lines with stable expression of the dopamine D1 receptor alone or concurrently with genetic variants of the D2 receptor. The D1 dopamine receptor cDNA was inserted into the second multiple cloning site after enzymatic digestion of pcDNA3.1(+)/D1 with KpnI and XhoI endonucleases. The cDNAs encoding genetic variants of human D2 dopamine receptor were amplified with a pair of oligonucleotides introducing unique SalI and XbaI restriction sites and inserted within the first multiple cloning site.

Cell culture and transfection

HEK 293 cells were grown in a minimum essential medium supplemented with 10% fetal bovine serum at 37 °C in a humidified environment containing 5% CO_2. For FLIM-FRET experiments, cells were seeded on glass coverslips at a density 0.2×10^6 cells/coverslip one day before transfection. Cells were transfected transiently with 0.3 μg of DNA or 0.6 μg in case of co-transfection by the calcium phosphate precipitation method [33]. Experiments were performed 48 h later. Stably transfected cells were used for calcium measurements. Cells were seeded on 40 mm plates and transfected by the calcium phosphate precipitation method with 2 μg of pBUDCE4.1 vector encoding dopamine D1 receptor alone or with one of the dopamine D2 receptor polymorphic variants. Selection of stably transfected clones was performed in the presence of 100 μl/ml of zeocin (Invivogen). Clones resistant to antibiotics were analyzed by RT-PCR.

Confocal fluorescence microscopy

All confocal fluorescence microscopy experiments were performed using a Leica TCS SP5 microscope equipped with 60×1.4NA oil-objective. Image acquisition was performed at 37 °C in F-12 medium supplemented in 2% FBS. Images were obtained by sequential excitation at 488 nm for EGFP and 596 nm for mCherry in 512×512 pixel mode using Las AF Lite software. Emission was recorded in a range of 500–550 nm and 650–700 nm for the donor and acceptor, respectively.

FLIM-FRET measurements

The fluorescence donor (mEGFP) was excited at 470 nm using pulsed laser diode at 40 MHz. Fluorescence emission was detected by a single-photon avalanche photodiode with a 525 ± 50 nm filter

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