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Role of silent polymorphisms within the dopamine D_1 receptor associated with schizophrenia on D_1–D_2 receptor hetero-dimerization

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Abstract:
Within the coding region of the dopamine D_1 receptor (D_1R), two synonymous polymorphisms, D_1R^{G198A} and D_1R^{G1263A}, have been identified and postulated to correlate with the schizophrenia phenotype. Binding studies revealed that the density of these genetic variants was much lower than the density of wild type D_1R in the human embryonic kidney (HEK) 293 cell line, used as a model system. From the data obtained using MFOLD software it is apparent that the G198A mutation has a greater impact on the secondary structure of the mRNA, which may affect its translation. However, the G1263A mutation is localized within the serine 421 codon of D_1R, which is predicted to be a potential site of phosphorylation according to the PHOSIDA database.
In order to determine whether the studied synonymous polymorphisms influence the process of dopamine D_1–D_2 receptors hetero-dimerization, we employed fluorescence resonance energy transfer (FRET) technology. The dopamine D_2 receptor (D_2R) was tagged with cyan fluorescence protein and the D_1R and its genetic variants were tagged with yellow fluorescence protein. The degree of D_1–D_2 receptor hetero-dimerization was significantly decreased when genetic variants of D_1R were co-expressed with D_2R. Since the D_1R mutations affected the expression levels of the proteins in the cell membrane without affecting the cellular localization of the receptor proteins, we postulated that the D_1R polymorphisms altered the translation rate and protein structure of the receptor. The altered hetero-dimerization that likely results from the lower expression of these genetic variants of D_1R with D_2R may be partially responsible for the association of both G198A and G1263A polymorphisms with the schizophrenia phenotype.

Keywords:
dopamine D_1 receptor, dopamine D_2 receptor, D_1–D_2 dimerization, silent polymorphism, FRET

Abbreviations: CFP – cyan fluorescent protein, D_1R – D_1 receptor, D_2R – D_2 receptor, GPCRs – G protein-coupled receptors, FRET – fluorescence resonance energy transfer, HEK293 – human embryonic kidney 293 cell line, SNPs – single nucleotide polymorphisms, TCSPC – time-correlated single photon counting measurements, YFP – yellow fluorescent protein

Introduction

As dysfunctions in the brain dopaminergic system are involved in a variety of important disorders including Parkinson's disease, schizophrenia, Tourette syndrome and drug abuse, dopaminergic receptors are often targeted in the treatment of these disorders. In addition to the development of drugs, potential links between dopamine receptor polymorphisms (single- nucleotide sequence variations) and neuropsychiatric diseases have been the subject of extensive research with the advancement of molecular biology techniques. Besides the restriction fragments length polymorphism recognized by EcoR1 [17] and TaqI [27], three other polymorphisms were reported by Liu *et al.* [28], a –48A/G substitution in the 5' untranslated region of exon 2, and 198G/A and 1263G/A substitutions.

Although none of these polymorphisms confer a functional amino acid change, some genetic studies have linked them to schizophrenia [31]. Not many studies have investigated these polymorphisms, as it had been widely assumed that silent (synonymous) mutations do not alter the biological activity or pharmacological profile of the receptor. However, data indicating the role of synonymous mutations in the biosynthesis of proteins are emerging [5, 9, 14, 23, 34, 37, 39]. Thus, we decided to address the question of whether two silent mutations within the coding region of the dopamine D_1 receptor (D_1R) – D_1^{G198A} and D_1^{G1263A} – would influence its binding parameters as well as its hetero-dimerization with the dopamine D_2 receptor (D_2R). The hetero-dimerization issue is important especially in light of the emerging concept of G protein-coupled receptor (GPCR) hetero-dimerization, but also to better understand mechanism of action of therapeutic agents. We have recently shown that at least a subset of dopamine D_1 and D_2 receptors form hetero-dimers, and that specific ligands can affect hetero-dimerization [10, 30]. Also, co-expression of both receptors in the same cell has a pronounced effect on the affinity of antipsychotic drugs [11], indicating that physical interaction of these receptors may be an important issue in the field of drug design.

To better understand D_1–D_2 receptor dimerization processes we have adapted a strategy previously described in our studies [10, 11, 30] that allows the monitoring of the physical interactions between receptor proteins in a single living cell. The investigated receptors were labeled with cyan (CFP, fluorescence donor) and yellow (YFP, fluorescence acceptor) fluorescent proteins and expressed in HEK293 (human embryonic kidney) cells. Based on measurements of fluorescence resonance energy transfer (FRET) occurring between fluorescently tagged proteins, the receptor dimer formation was estimated.

Materials and Methods

Materials

HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell culture reagents were purchased from Gibco (Carlsbad, CA, USA) and Sigma (Poznań, Poland). The bacterial cell line *Escherichia coli* DH5α (Dam+) was obtained from Novagen (Darmstadt, Germany).

All molecular biology reagents were obtained from Fermentas (Vilnius, Lithuania). Oligonucleotides were synthesized by IBB PAN (Warszawa, Poland). The pcDNA3.1(+) plasmids encoding human D_1 and D_2 receptors were obtained from UMRcDNA Resource Center (University of Missouri, Rolla, MO, USA). The pECFP-N1 and pEYFP-N1 vectors were purchased from BD Biosciences, Clontech (Palo Alto, CA, USA).

Construction of genetic variants of the dopamine receptors

Two genetic variants of the D_1R: D_1R^{G198A} and D_1R^{G1263A} were constructed. The appropriate point mutations exchanging the guanine nucleotide to adenine were produced according to the Quik Change Site-Directed Mutagenesis Kit (Stratagene, LA Jolla, CA, USA). PCR-Quik reactions were performed with pcDNA3.1(+)/D_1 as the template and the following sets of oligonucleotide primers each containing the desired mutations: D1G198A: forward (5'-TTA-3'), reverse (5'-TAA-3'); D1G1263A: forward (5'-TCA

-3'), reverse (5'- TGA-3'). Next, the reaction product was treated with the methylation-specific endonuclease DpnI in order to select synthesized DNA containing the introduced mutations. *E. coli* cells were transformed with the mutated plasmids.

Construction of fusion proteins

The cDNAs encoding genetic variants of human D_1Rs were amplified with a universal forward primer for pcDNA3.1(+) and a reverse primer that obliterated the stop codon and introduced a unique restriction site for XhoI. The resulting fragments were inserted in-frame into NheI/XhoI sites of the pEYFP-N1 vector. The construction of fusion proteins D_1 tagged with YFP and D_2 tagged with CFP was described previously [29].

Cell culture and transfection

HEK293 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. Cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% L-glutamine. A day before transient transfection, the cells were seeded into 100 mm dishes (3 × 10^6 cells per dish) for radioligand binding assays, or on cover glass slips in 30 mm dishes (1 × 10^6 cells per dish) for confocal microscopy and fluorescence lifetime measurements. HEK293 cells were transiently transfected with 15 µg of DNA per 100 mm dish or with 2 µg of DNA per glass cover slip using a calcium phosphate precipitation method as described by Sambrook et al. [36]. The ratio of DNA encoding donor to acceptor was 1:1 or 1:2. Forty-eight hours after transfections cells were harvested or microscopic measurements were taken.

Membrane preparation and radioligand binding assay

For binding experiments the transfected HEK293 cells were washed with phosphate-buffered saline (PBS), scraped from the dish in PBS and centrifuged for 5 min at 160 g. The resulting cell pellet was frozen at –30°C. Frozen pellets were resuspended in binding buffer [50 mM Tris-HCl pH 7.4 containing 120 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 1,5 mM $CaCl_2$ and 1 mM EDTA] using an Ultra Turrax homogenizer. The homogenate was centrifuged twice for 15 min at 16,500 g (4°C). Following the modified Lowry's procedure the protein content of membrane preparations was determined, using bovine serum albumin as a standard.

Binding assays were performed in a total volume of 0.5 ml using concentrations of [^{3}H]SCH23390 (SA 85 Ci/mmol, NEN, Boston MA, USA) ranging from 0.12 nM up to 8 nM. Saturation studies were performed on a fresh membrane preparation (final protein concentration 15–20 µg per tube). Nonspecific binding was assessed with 10 µM *cis*-(Z)-flupentixol (Lundbeck, Copenhagen, Denmark). Binding assays were incubated for 2 h at room temperature and terminated by rapid filtration through glass fiber filters (GF/C Whatman). The filters were washed four times with 5 ml of ice-cold wash buffer [50 mM Tris-HCl pH 7.4]. The amount of bound radioactivity was determined by liquid scintillation quantification (Beckman LS 650).

The radioligand binding parameters K_d and B_{max} were estimated with the GraphPad Prism 4.0 curve-fitting program (GraphPad Software, San Diego, CA, USA).

Measurements of FRET by Fluorescence Lifetime Microscopy

To monitor whether the FRET phenomenon occurs between receptor proteins tagged with fluorescent protein CFP (donor) and YFP (acceptor), quantitative methods were used. Measurements of FRET by fluorescence lifetime microscopy in a single living cell expressing the investigated receptors in different combinations (performed on the Nikon Eclipse TE-2000 inverted fluorescence microscope) were performed at 37°C, 48 h after transfection. During each experiment, the fluorescence decay from at least 10 cells was measured. For each combination of receptors at least 4 independent experiments were conducted. Fluorescence intensity decays were analyzed as both mono- and multi-exponentials. Analysis of the reduced χ^2 value and residual distribution has led to the conclusion that best fit parameters were obtained with two exponentials. The analysis parameters were not significantly influenced by an additional third exponential, and the fractional contribution of the additional lifetime approximated zero. Statistical analysis was performed using one-way ANOVA followed by a Dunnett's test for *post-hoc* comparisons. Detailed information on the statistical analysis is described in our previous papers [10, 11, 29, 30].

Confocal microscopy

Analysis of the localization of investigated receptor fusion proteins in HEK293 was performed with confocal microscopy. Images were acquired with two systems: a Leica TCS SP2 (Leica Microsystem, Mannheim, Germany) equipped with an acusto-optical beam splitter and a 100 mW argon laser for excitation at 458 nm (CFP) and 514 nm (YFP) described previously [15], and a BioRad MRC 1024 confocal system (BioRad) interfaced with a Nikon Diapoth 300 (Nikon) inverted microscope. The microscope was equipped with a 60× PlanApo oil-immersion 1.4 NA objective lens and a 100 mW argon ion air-cold laser (ITL). Detailed information is provided in a previous publication [30].

Colocalisation analysis was performed with Image ProPlus 4.5 software. Pearson's correlation coefficient as a measure of the overlap of pixels was also determined. Pearson's correlation coefficient was calculated from randomly selected parts of the image from individual cells cotransfected with different combinations of D_2R tagged with CFP and wild-type or polymorphic variants of D_1R fluorescently tagged with YFP. A subtracted background of each image was the average intensity of the fluorescence signal in a determined individual area of interest (AOI), free of cells. Regions with correlated fluorescence intensities were used for further analysis. For each combination of the proteins, at least 10 individual regions from different, independently transfected cells were analyzed and Pearson's correlation coefficients were calculated. The statistical significance was assessed with one-way ANOVA followed by a Dunnett's test for *post-hoc* comparisons.

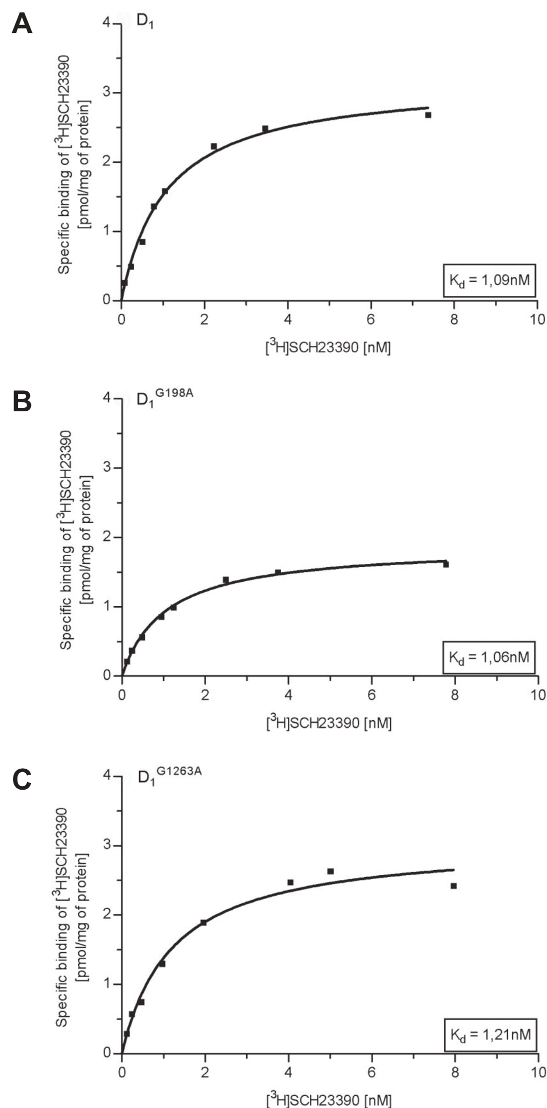

Fig. 1. Saturation binding of [^{3}H]SCH23390 to: (**A**) dopamine D_1 receptor (D_1R) expressed in HEK293 cells, (**B**) a genetic variant D_1R^{G198A} expressed in HEK293 cells, (**C**) a genetic variant D_1R^{G1263A} expressed in HEK293 cells. Nonspecific binding was assessed with 10 μM *cis*-(Z)-flupentixol as described in Materials and Methods. Data are from a single experiment performed in duplicate and are representative of at least three independent experiments

Results

Radioligand binding assay

As evident in Figure 1, both mutants of the dopamine D_1 receptor (D_1R^{G198A} and D_1R^{G1263A}) displayed significantly lower expression compared to wild type D_1R in HEK293 cells.

For both mutants, the receptor density values (B_{max}) were lower than the control value; however, much less of the D_1R^{G198A} than D_1R^{G1263A} was produced in the cells. Despite this, all of these receptors were functionally similar, as evidenced by the similar K_d values estimated for all three genetic variants of D_1R (Tab. 1).

Fluorescence lifetime microscopy studies of dopamine receptor dimerization

To study the physical interaction of D_1R and its mutants with D_2R, we monitored the FRET. FRET occurs between fluorescence donor and acceptor chro-

Tab. 1. Summary of the saturation binding of [^{3}H]SCH23390 to the dopamine D_1 receptor and its genetic variants D_1R^{G198A} and D_1R^{G1263A}

Species	K_d ± SEM [nM]	B_{max} ± SEM [pmol/mg of protein]
D_1R	1.17 ± 0.04	4.19 ± 0.39
D_1R^{G198A}	1.31 ± 0.14	2.18 ± 0.24 *** #
D_1R^{G1263A}	1.10 ± 0.05	3.20 ± 0.20 *

Statistical significance was evaluated by one-way ANOVA followed by a Dunnett's test for *post-hoc* comparisons: *** $p < 0.001$ D_1R *vs.* D_1R^{G198A} and * $p < 0.05$ D_1R *vs.* D_1R^{G1263A}; # $p < 0.05$ D_1R^{G198A} *vs.* D_1R^{G1263A}; (n = 5–9)

mophores if they are located within ca. 10 nm of each other and are arranged in appropriate positions in terms of their transition dipole moments [25]. The details of the technology used for this kind of measurements have been described previously [10, 11, 29, 30].

Here, we utilized the fluorescence lifetime measurements, using time-correlated single photon counting experiments performed on an inverted fluorescence microscope. The FRET phenomenon was observed in a single living cell transiently transfected with the D_1R or its genetic variants and D_2R, tagged with fluorescent proteins. The precise determination of the donor fluorescence lifetime (receptor-CFP) in the presence and absence of acceptor (receptor-YFP) is required for this kind of experiment. An average fluorescence lifetime obtained for HEK293 cells transfected with D_2R-CFP was equal to 2.52 ns, while it was reduced to 2.40 ns when D_2R-CFP was co-expressed with D_1R-YFP. A partial reduction in fluorescence lifetime was similar to the value observed for both polymorphic variants of D_1R: the average values of fluorescence lifetimes were 2.47 and 2.45 ns for D_1R^{G198A}-YFP and D_1R^{G1263A}-YFP, respectively. According to the previously described method, we calculated the fluorescence energy transfer efficiency for D_1R and each polymorphic variant of D_1R. The average FRET efficiency for HEK293 cells expressing D_2R-CFP|D_1R-YFP was 4.71%, while a significant decrease in FRET efficiency for both polymorphic variants was detected, 65% for D_1R^{G198A} and ca. 54% for D_1R^{G1263} (Tab. 2).

Co-localization analysis

Cells co-expressing D_2R with genetic variants of D_1R were visualized by confocal microscopy. These experiments were performed to determine whether the introduced synonymous mutations within the D_1R in any way influenced the cellular localization of the studied receptors. The degree of co-localization of the receptors of interest was quantitatively estimated by the Pearson's correlation coefficient. As evident in Figure 2B, these parameters do not differ, which indicates that there is no change in the localization between the wild type D_1R and its genetic variants (D_1R^{G198A} and D_1R^{G1263A}) with D_2R.

HEK293 cells transiently co-transfected with plasmids encoding the D_2R and D_1R or one of its genetic variants (D_1R^{G198A} and D_1R^{G1263A}) are shown in Figure 2A. The green fluorescent signal (CFP channel) originates from receptors that express CFP, while the red fluorescent signal (YFP channel) originates from receptors tagged with YFP. Merged pictures (with apparent yellow signal indicating overlap of green and red signal) show co-localization.

Tab. 2. Summary of energy transfer measurements by Fluorescence Lifetime Microscopy in HEK293 cells. Excitation at 434 nm and emission was observed through the appropriate filters as described in Materials and Methods. The data obtained from at least 18 single cells represent means ± Std. Dev. Statistical significance was evaluated by one-way ANOVA followed by a Dunnett's test for *post-hoc* comparison: *** $p < 0.001$ D_2R-CFP|D_1R^{G198A}-YFP *vs.* D_2R-CFP|D_1R-YFP and ** $p < 0.01$ D_2R-CFP|D_1R^{G1263A}-YFP *vs.* D_2R-CFP|D_1R-YFP (n = 18–30).

Species	Average lifetime [ns]		FRET efficiency
	τ_D ± SD	τ_{DA} ± SD	E [%]
[a] D_2R-CFP	2.52 ± 0.02	–	–
[b] D_2R-CFP|D_1R-YFP	–	2.40 ± 0.05	4.71
[c] D_2R-CFP|D_1R^{G198A}-YFP	–	2.47 ± 0.05	1.64***
[d] D_2R-CFP|D_1R^{G1263A}-YFP	–	2.45 ± 0.03	2.55**

[a] Measured in cells expressing CFP (cyan fluorescent protein) coupled to the dopamine D_2 receptor (D_2R). [b] Measured in cells co-expressing the dopamine fusion proteins D_2R-CFP and dopamine D_1 receptor (D_1R)-YFP (yellow fluorescent protein). [c] Measured in cells co-expressing the dopamine fusion proteins D_2R-CFP and D_1R^{G198A}-YFP (a genetic variant of the D_1R). [d] Measured in cells co-expressing the dopamine fusion proteins D_2R-CFP and D_1R^{G1263A}-YFP (a genetic variant of D_1R)

Proposed mRNA secondary structure models

The relationship between the mRNA secondary structure and gene expression is widely postulated. Taking this into account, we modeled the effect of synonymous polymorphisms on the D_1R mRNA secondary

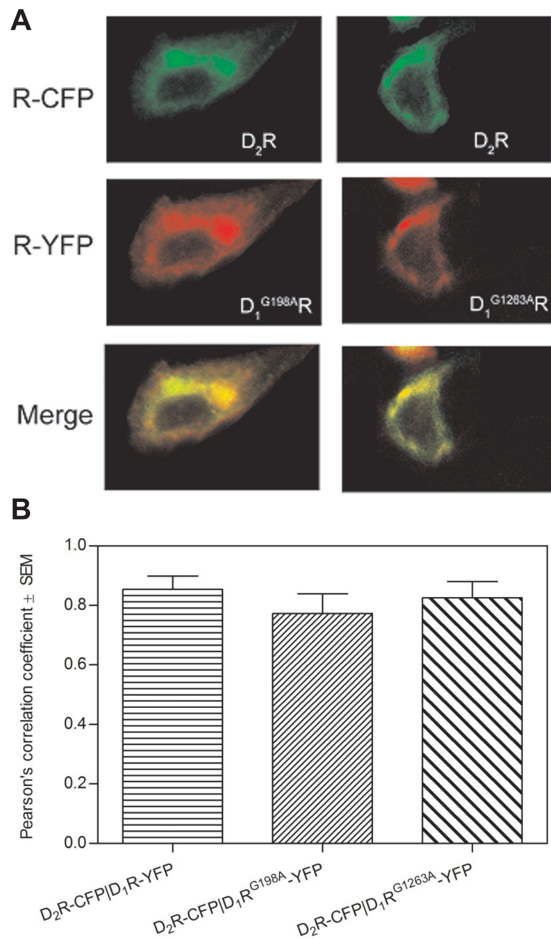

Fig. 2. (**A**) Expression of dopamine D_1 receptor (D_1R) or its genetic variants and dopamine D_2 receptor (D_2R) in HEK293 cells. HEK293 cells were cotransfected with D_2R-CFP and either D_1R-YFP, D_1R^{G198A}-YFP or D_1R^{G1263A}-YFP (green and red colors, respectively). Image overlays show colocalization in each case. (**B**) Pearson's correlation coefficient calculated for HEK293 cells co-transfected with the dopamine D_2R-CFP fusion protein with the D_1R-YFP dopamine receptor fusion protein or its polymorphic variants D_1R^{G198A}-YFP or D_1R^{G1263A}-YFP. Data represent means ± SEM. Statistical significance was evaluated by one-way ANOVA followed by a Dunnett's test for *post-hoc* comparisons: for either D_2R-CFP/D_1R^{G198A}-YFP or D_2R-CFPlD_1R^{G1263A}-YFP *vs.* D_2R-CFPlD_1R-YFP results were not statistically significant

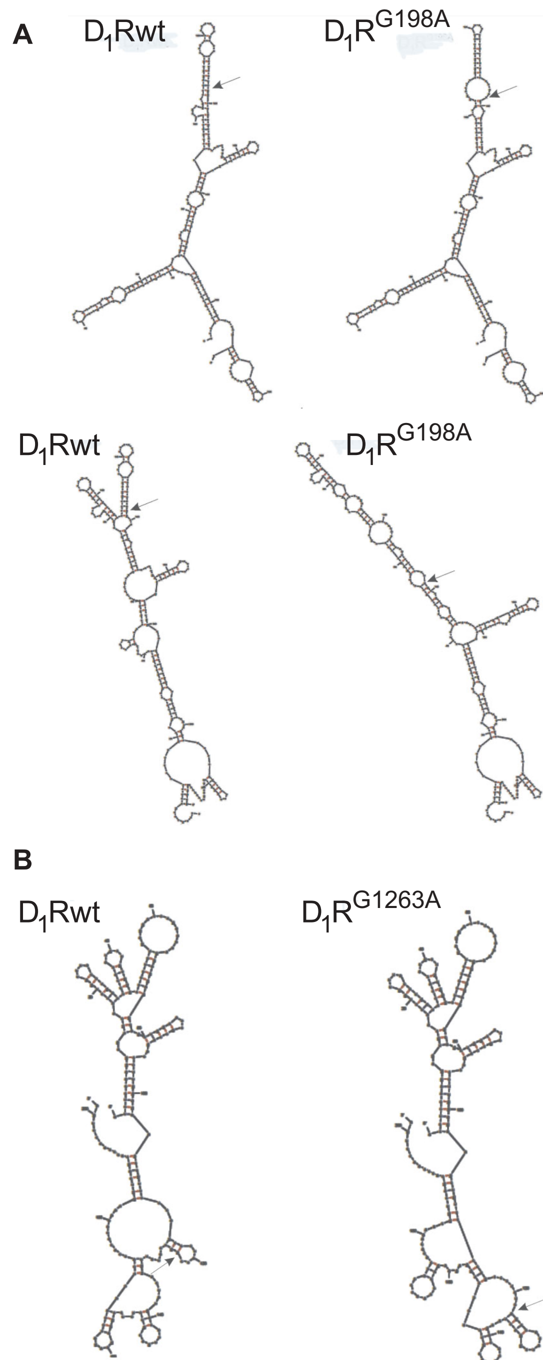

Fig. 3. Examples of the mRNA secondary structures predicted by MFOLD. The polymorphic bases are pointed. Only part of a coding sequence of each modeled structure is shown. (**A**) Structures proposed for D_1R^{G198A}, nucleotides 70–300; (**B**) structures proposed for D_1R^{G1263A}, nucleotides 1140–1341. Fig. 1

structure using MFOLD [45]. The D_1R mRNA sequence carrying separately investigated synonymous SNPs was used for secondary folding structure model building. Only a portion (about 200 base) of each receptor variant was modeled. We obtained 11 different structures for nt 70–300 for both D_1R and D_1R^{G198A}. Each structure shows alterations after replacing G 198 for A. In case of second polymorphism, G1263A, we obtained 6 different structures for nt 1140–1341 of both D_1R and D_1R^{G1263A}, but the introduced mutation altered the secondary structure of the mRNA in only one case. Thus, the first polymorphism induced the more obvious change of folding patterns in comparison to the native sequence of D_1R. In contrast to wild-type D_1R, an exchanged base (D_1R^{G198A}) is lo-

calized mainly within the loop structure. In Figure 3, examples of mRNA secondary structures are shown.

Discussion

The present study addressed whether synonymous mutations within the coding region of the D_1R, which has been reported in some studies to be linked to the schizophrenia phenotype and/or the altered drug response [28], impact parameters characteristic of D_1R function.

Binding studies revealed that the density of D_1R^{G198A} is much lower than the density of wild type D_1R in the heterologous expression model system. The second genetic variant of D_1R, the D_1R^{G1263A}, is also expressed to a lesser degree than wild type D_1R, although our results indicate that it is expressed more efficiently than D_1R^{G198A}. Such results indicate that two polymorphisms within the coding region of D_1R, although designated as "silent" because they do not change the amino acid sequence, are not silent at all since they significantly change the amount of receptor present on the cell membrane. From the data obtained using MFOLD software we could observe the potential impact of the studied SNPs on the secondary structure of the mRNA. The mRNA structure was affected more in the G198A mutant, which is consistent with the experimental data showing a lower level of expression of D_1R on the cell membrane.

The molecular mechanism underlying these observations likely involves the translational stage of receptor biosynthesis. Recently, convincing data have been provided indicating that synonymous mutations can have a profound impact on the kinetics of translation, as the least common codon requires a less abundant tRNA species [23].

Furthermore, Tsai et al. [39] suggested that synonymous mutations cause so-called ribosome stalling. In their elegant discussion, they hypothesize that sufficient translational stalling signals introduced by mutations may lead to formation of an altered protein. The present studies of the physical interaction of D_2R with genetic variants of D_1R partially confirm this suggestion. We did not observe any change in the K_d value, which is commonly regarded as an indicator of the lack of conformational changes within the protein structure, in the domains responsible for ligand binding. However, the interaction with D_2R was altered, as indicated by the FRET experiments. The observed change in the hetero-dimerization of genetic variants of D_1R with D_2R did not result from the altered localization of these receptors, as the mutants co-localized with D_2R in the cell membrane to the same extent as observed for the wild type D_1R. However, one possible explanation is that the less pronounced hetero-dimerization of the studied receptors may result from the lower expression level of the genetic variants of D_1R. In addition to the main question of the present study, our data speak in favor of the biophysical methodology used to monitor the physical interaction of receptor proteins, which is sensitive to changes in their expression level.

However, different molecular mechanisms have been described for the protein-protein interactions that occur during receptor dimerization processes [1, 4, 13, 16, 18, 19, 22, 24, 32, 33, 44]. Electrostatic interactions are also considered effectively engaged in the formation of stable noncovalent complexes between proteins. This kind of interaction may occur between characteristic epitopes, one containing the cationic guanidinium groups (located at the terminus of the arginine side chain) and the other one containing the anionic carboxyl groups of aspartate or glutamate and/or the anionic phosphate group on the phosphorylated residue [2, 40–42]. Motifs composed of a minimum of two adjacent arginine residues, "RR" or "RKR", on one peptide and two adjacent aspartate "DD" or glutamate "EE" residues or/and one phosphorylated serine residue in close proximity of the other, were found to be sufficient to generate stable complexes between two peptides [20, 21, 43]. Moreover, Jackson et al. [21] demonstrated that the interaction involving just one phosphate is almost as stable as the one involving multiple glutamate residues. Additionally, the presence of a phosphorylated residue was shown to cause a significant increase in the stability of noncovalent interactions between epitopes [21]. Simultaneously, the importance of phosphorylation-dephosphorylation events in the modulation of the electrostatic interactions was highlighted [42].

Various reports suggest that the mechanisms involved in hetero-dimerization of some GPCRs are connected with the presence of the motifs described above, *via* neutralization of each other's charge and the formation of salt bridges [3, 12, 26]. Recently, Łukasiewicz et al. [30] demonstrated that electrostatic interactions between an arginine-rich epitope (Arg-

rich) from the third intracellular loop (ic3) of the dopamine D_2 (217RRRRKR222) receptor and two adjacent glutamate residues from the carboxyl terminus of the D_1R (404EE405) participate in a hetero-dimerization process between two dopamine receptors (D_1 and D_2). Similar interactions were also described by Jackson et al. [20] for the D_2R and the cannabinoid CB1 receptors and by Lee et al. [26] for the ionotropic glutamate NMDA (NR1 subunit) and the dopamine D_1 receptors. Moreover, Ciruela et al. [7] used pull-down and mass spectrometry experiments to postulate that the hetero-dimerization of the adenosine A_{2A} and the dopamine D_2 receptors strongly depends on electrostatic interactions between an Arg-rich epitope within the ic3 of D_2R and acidic aspartate epitope from C-tail of the adenosine A_{2A} receptor. They also pointed to an essential role for the phosphorylation of serine 374 (localized in the C-tail sequence of the A_{2A} receptor within the casein kinase 1 (CK1) consensus site) in this process. Moreover, it was shown that in contrast to the CK1 consensus site, two adjacent aspartate residues were not conserved among species, which suggests that the hetero-dimerization process is likely primarily mediated by the phosphorylated epitope [7].

It is well known that phosphorylation of serine residue plays an important role in the regulation of various cellular processes and that mutations of the phosphorylation site of a substrate can lead to the development of some disorders [8]. As the silent polymorphism G1263A investigated in the present study is localized within the serine 421 codon (C-tail) of the D_1R , we addressed whether this serine residue could be phosphorylated.

The PHOSIDA (phosphorylation site database) server designed to identify phosphorylation sites enabled us to predict that the serine 421 residue from the C-tail of the dopamine D_1 receptor is a potential CK1 recognition site since it is localized in an epitope possessing a CK1 consensus site (S-X_3-pS) [15, 35]. CK1 belongs to the superfamily of serine/threonine-specific protein kinases. The widespread distribution of CK1 suggests that it plays an important regulatory role for the protein [6]. Beside numerous biological functions, CK1 was also shown to enhance agonist-dependent phosphorylation of the M3 muscarinic receptor and to mediate the phosphorylation of rhodopsin in response to light stimulation [6, 38].

Since the investigated silent polymorphism (G1263A) can lead to alterations in the translation levels, what may affect structure of proteins, it seemed probable that the phosphorylation may be weakened or impaired. Therefore, the observed decrease of FRET efficiency may be a result of the abrogated hetero-dimerization of both dopamine receptors. This observation may be due to the alteration in the degree of phosphorylation of serine residue 421, which might be not be fully accessible to the appropriate kinase due to possible changes in receptor protein folding. Similarly to Ciruela et al. [7], we point to a potential role for the phosphorylation phenomenon in the direct receptor association, especially in the present investigated case of dopamine D_1–D_2 heterodimerization. It is likely that this process is also mediated by phosphorylation at this epitope.

The role of D_1–D_2 receptor hetero-dimerization has also been indicated as important in the mechanism of action of antipsychotic drugs [11]. Therefore, the data obtained in the present paper as well as the discussion above suggest a molecular mechanism responsible for the association of both G198A and G1263A polymorphic variants of D_1R, with altered response to antipsychotic treatment.

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