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Antagonist Binding Characteristics of the Ser^{311}→Cys Variant of Human Dopamine D_2 Receptor *in Vivo* and *in Vitro*

Tiina Pohjalainen,*[,1] Anibal Cravchik,† Pablo V. Gejman,†[,1] Juha Rinne,‡ Kjell Någren,§ Erkka Syvälahti,* and Jarmo Hietala[¶]

**Department of Pharmacology and Clinical Pharmacology, ‡Department of Neurology, and ¶Department of Psychiatry, University of Turku, Turku FIN-20014, Finland; †Unit of Molecular Clinical Investigation, Clinical Neurogenetics Branch, NIMH, Bethesda, Maryland; and §Turku PET Center, Turku FIN-20520 Finland*

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We report *in vivo* and *in vitro* antagonist binding characteristics of the naturally occurring Ser^{311}→Cys variant of the human D_2 dopamine receptor. Striatal receptor binding characteristics *in vivo* were measured with positron emission tomography and the D_2 antagonist [^{11}C]raclopride. The *in vitro* affinity of raclopride for the Ser^{311}→Cys variant and the wild type receptor was studied in membrane binding assays from stably transfected cell lines. One healthy male carrying the heterozygous Ser^{311}→Cys (TCC→TGC) substitution was identified with denaturing gradient gel electrophoresis and DNA sequencing. The striatal D_2 receptor binding characteristics *in vivo* in this subject were normal. This was supported by the *in vitro* data as the Ki values of raclopride for the Ser^{311}→Cys variant and the wild type receptor were identical. Our data suggest that the Ser^{311}→Cys variant of the human D_2 receptor does not influence antagonist-receptor recognition *in vivo* or *in vitro.*

The neurotransmitter dopamine is involved in the control of movement, hormone secretion, mood and various motivational behaviours such as reward. The effects of dopamine are mediated via specific dopamine receptors, which are members of the G protein-coupled receptor superfamily. Dopamine receptors are currently classified into D_1-like (D_1 and D_5) and D_2-like (D_2, D_3 and D_4) receptors identified by molecular cloning and pharmacological properties (1).

Alterations in dopamine the D_2 receptor function have been suggested to be involved in the biology of many psychiatric disorders, like schizophrenia and alcoholism. The possible genetic basis for such D_2 receptor changes has extensively been explored (2-6). Three relatively rare naturally occurring nucleotide variants predicting an altered amino acid sequence have been identified in the D_2 receptor gene (5,6). The allelic frequencies of the amino acid substitutions in Caucasians are 0.014 for Ser^{311}→Cys and 0.001 for Pro^{310}→Ser substitutions, respectively. Additionally, one subject heterotsygous for Val^{96}→Ala substitution has been detected in a sample of 380 Caucasians (5).

According to phenotype-genotype correlations between the patients and controls, the genetic variation in the most common missense variant, Ser^{311}→Cys, has not been found to play a major role in schizophrenia (5, 7-10) alcoholism (5), panic disorder (11) or bipolar disorder (12), although some positive associations have been reported (13-15). Allelic variants of the human D_2 receptor that result in amino acid substitutions may have different binding, signal transduction or regulation characteristics. Ser^{311}→Cys variant located in the third intracellular loop could affect G-protein recognition and coupling or sequestration (16, 17). In this report, we describe antagonist binding characteristics of the D_2 receptor Ser^{311}→Cys variant *in vitro* and in one healthy subject *in vivo.*

MATERIALS AND METHODS

Subjects. The protocol was approved by the ethical committee of Turku University and University Hospital. A total of forty-nine unrelated Finnish healthy subjects (thirty-three males and sixteen females) giving an informed consent were included in the study. Ages ranged from 19 to 82 years, with a mean of 41 ± 2.5 (± SEM). The subjects were clinically examined and were found to be free of psychiatric and neurologic disorders. All subjects were non-smokers and had a normal CT or 1.5 T MRI scan of the brain.

Polymerase chain reaction and denaturing gradient gel electrophoresis. Blood samples (2 × 10 ml) were collected in EDTA-tubes. Genomic DNA was isolated by standard procedures. DGGE was used to examine exons 2 through 8 of the dopamine D_2 receptor gene for the

[1] Reprints requests to Tiina Pohjalainen or Pablo V. Gejman.

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nucleotide variants in 23 subjects. Exons 3 and 7 were examined in additional 26 subjects. PCRs were performed in a DNA thermal cycler (Perking Elmer) as described earlier (5) with annealing temperatures of 56°C (exon 3), 63°C (exon 2b), 65°C (exon 2L), 68°C (exons 4,5,6 and 7) and 72°C (exon 8). DGGE conditions were as previously reported (5) but only parallel DGGEs were run with gradients of denaturants of 20% to 80% for exons 2,3,4,5 and 8 and 30% to 80% for exon 6 and 0% to 65% for exon 7. The gels were stained in silver. PCR fragments that were shown to contain sequence alterations in DGGE were sequenced using the dideoxy chain termination method (70770 Sequenase version 2.0 sequencing kit, United States Biochemical, Cleveland, Ohio). Single stranded fragments for sequencing were prepared using the lambda exonuclease digestion method (PCR template prep for ssDNA sequencing, Pharmacia, Piscataway, NJ).

Positron emission tomography. Striatal D_2 dopamine receptor density (Bmax) and affinity (Kd) were measured by PET using a [^{11}C]raclopride and an equilibrium model as described earlier (19). Part of the binding characteristics has been previously published (18-20). Analyses of the binding characteristics were blind to genotyping and vice versa. The PET experiments were performed using a whole-body PET scanner (ECAT 931/08-12, CTI, Knoxville, TN, USA) with fifteen slices and with a spatial resolution of 6.1 mm on the plane and an axial resolution of 6.7 mm. Each subject underwent two [^{11}C]raclopride scans (high and low specific activity) within the same day with an average injected dose of 3.0 mCi/scan.

Radioligand competition binding assays. The CHO-K1 cell clones D-12 and C-31, stably transfected with the short form of the human dopamine D_2 receptor and the Cys^{311} variant respectively (16) were used for *in vitro* binding assays. The cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C. The cells were harvested with 5 mM EDTA in Ca^{2+}/Mg^{2+} free EBSS and centrifuged at 300 × g for 8 min, washed with EBSS, resuspended in 5 mM Tris-HCl, pH 7.4 at 4°C, 5 mM $MgCl_2$ and homogenized. Cell membranes were collected by centrifugation twice at 34 000 × g for 20 min with an intermediate resuspension and were finally suspended in binding buffer (50 mM Tris-HCl, 10 mM $MgSO_4$, 1.2 mM EDTA, pH 7.4) at 0.3 mg of protein/ml. Membrane suspension (100 μl) was added to duplicate assay tubes containing 0.3 nM [^{3}H]methylspiperone and increasing concentrations of raclopride (0.1 nM to 0.1 mM) in a final volume of 1 ml. Non-specific binding was defined using 1 mM (+)-butaclamol. The assay tubes were incubated at 37°C for 40 min and the assay was terminated by rapid filtration through GF/C filters pretreated with 0.3% polyethylenimine. The filters were rapidly washed with 3 × 4 ml of 50 mM Tris-HCl, pH 7.4 at 4°C and radioactivity bound to the filters was quantitated by scintillation counting. Radioligand binding data was analyzed with the non-linear least-squares fitting program GraphPad Prism version 1. Inhibition constants (Ki) were calculated from the IC_{50} values by the method of Cheng and Prusoff.

RESULTS

The average values for the striatal Bmax were 26.7 ± 1.0 pmol/ml (mean ± SEM) and for Kd 10.0 ± 0.3 nM for the forty-nine subjects. The average value for Bmax/Kd was 2.70 ± 0.08. The values were calculated as a mean of both hemispheres.

One nucleotide change predicting an amino acid substitution was found in the present study. A 20-year-old male was heterozygous for Ser^{311}→Cys (TCC→TGC) missense substitution in exon 7. He also had His^{313} and Pro^{319} silent variants in the same exon. His striatal Bmax, Bmax/Kd and Kd values were 33.2 pmol/ml, 3.68 and 9.0 nM respectively, which corresponded well with the age-adjusted Bmax and Bmax/Kd as well as Kd values of all subjects (Fig. 1A, B and C).

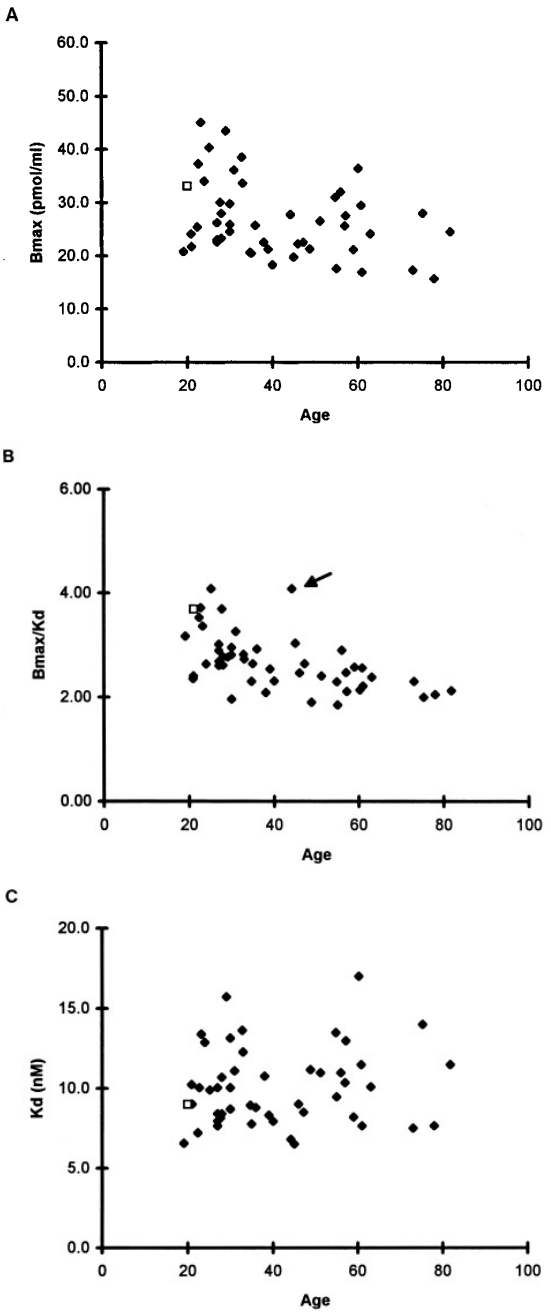

FIG. 1. Striatal D_2 dopamine receptor density (Bmax) and apparent affinity (Kd) were measured using positron emission tomography (PET) and [^{11}C]raclopride in forty-nine healthy volunteers. Striatal D_2 dopamine receptor density declines with age. (A,B) Compared to the age-matched counterparts, the 20-year-old subject possessing the Ser^{311}→Cys variant (drawn as an open square) shows normal Bmax and Bmax/Kd. (B) The 44-year-old male with the Thr^{412} variant is indicated by an arrow. (C) Striatal D_2 dopamine receptor affinity (Kd) of the 20-year old subject possessing the Ser^{311}→Cys variant (drawn as an open square) is also unaltered, compared to the values of all subjects.

Several silent variants were found including two silent Leu^{141} in exon 4 and several His^{313} and Pro^{319}

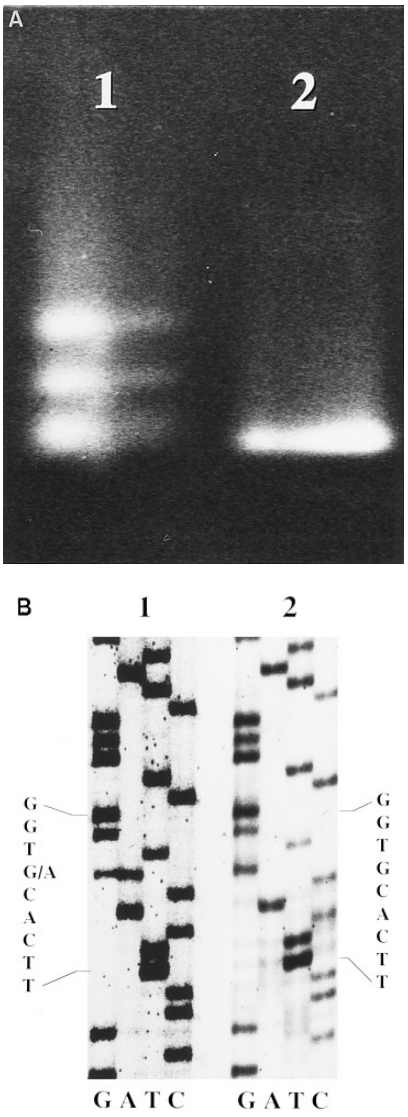

FIG. 2. Identification of the novel silent Thr^{412} variant in exon 8 of D_2 dopamine receptor gene. (A) DGGE analysis of exon 8. **Lane 1:** heterozygous subject, **lane 2:** homozygous wild type. (B) Direct sequencing of the amplified PCR product revealed a heterozygous substitution of ACG to ACA at Thr^{412} in the seventh transmembrane domain of D_2 dopamine receptor. **1:** heterozygous subject at Thr^{412}, **2:** wild type.

variants in exon 7. His^{313} and Pro^{319} variants were not identified in this study. Moreover, a novel silent Thr^{412} (ACG→ACA) variant was found in one individual in exon 8 (Fig. 2), located in the beginning of the seventh transmembrane domain. Variation in exon 8 was investigated from twenty-three individuals. The conversion of ACG to ACA introduces a recognition site for the restriction enzyme NlaIII and abolishes recognition sites for BsaAI and MaeII. None of the silent variants were associated with clear-cut alterations in D_2 dopamine receptor binding characteristics. However, the 44-year old subject with the Thr^{412} variant had a high Bmax/Kd value (4.08) (Fig. 1B). All subjects were heterozygous for the nucleotide variants.

The *in vivo* [^{11}C]raclopride binding data was in agreement with *in vitro* studies using membranes form CHO-K1 cell clones stably expressing the human wild type D_2 receptor and its Ser^{311}→Cys variant. The binding affinity of raclopride for the both D_2 receptor forms was compared performing competition binding assays with [^{3}H]methylspiperone. The raclopride Ki values (mean ± SEM, n = 3) were 7.4 + 0.6 nM for the wild type D_2 receptor and 7.5 ± 1.1 nM for the Ser^{311}→Cys variant.

DISCUSSION

The structural mutations of D_2 dopamine receptor gene were analyzed in forty-nine healthy Finnish subjects. One heterozygous Ser^{311}→Cys substitution in the third intracellular loop of D_2 dopamine receptor was detected whereas we did not find the rarer Val^{96}→Ala or Ser^{310}→Pro missense variants in our study. The healthy volunteer with the heterozygous Ser^{311}→Cys substitution exhibited normal binding density and affinity *in vivo* for the D_2 antagonist [^{11}C]raclopride. Moreover, no significant differences were detected in the binding affinities of raclopride for the Ser^{311}→Cys variant and the human wild type D_2 receptor *in vitro*. Therefore, according to the *in vivo* and *in vitro* results, the genetic variation in Ser^{311} does not seem to affect raclopride binding affinity.

Dopamine D_2 receptor has two isoforms generated by alternate splicing of mRNA (21-23). These are the D_{2S} (short) and the D_{2L} (long), which differ only by the presence of a 29-amino acid insert in the putative third intracellular loop. In human striatum the long form of the D_2 receptor mRNA predominates about 1.5-1.8 fold compared to the short form (24-26). The actual densities of the human D_2 receptor isoforms are unknown but it has been suggested that the D_{2L} is the predominant form of the receptor in the rat striatum (27). A large series of D_2 antagonists including several benzamide derivatives showed similar binding properties between D_{2L} and D_{2S} of the cloned human D_2 receptors expressed in 293 human kidney cells (28) indicating that the presence of the 29-amino acid insert in the third intracellular loop does not affect antagonist binding. Therefore, the short form of the D_2 receptor is adequate forthe characterization of raclopride binding *in vitro*.

The Ser^{311}→Cys substitution is located in the third intracellular loop, which has shown to be essential for appropriate G protein recognition and coupling. In fact, there is evidence indicating that the Ser^{311}→Cys variant of D_2 receptor may be associated with a reduced capacity to inhibit adenylate cyclase (16). The variation in the third intracellular loop has also been shown to

affect sequestration of D_2 receptor (17). If the replacement of a serine by a cysteine in the third intracellular loop of the D_2 receptor affects G-protein coupling or sequestration, this could result in an adaptive response in ligand-receptor recognition *in vivo.* However, this appeared not to be the case in our PET study. Alternatively, it might be that both alleles need to be affected to cause an apparent alteration in ligand binding. It would be interesting, therefore, to measure the binding characteristics for a Cys^{311} homozygote.

In conclusion, our data indicate that the heterozygous Ser^{311}→Cys substitution of the D_2 dopamine receptor is not associated with altered D_2 dopamine receptor binding density or apparent affinity *in vivo.* Furthermore, Ser^{311}→Cys substitution does not produce a change in binding affinity for raclopride *in vitro.*

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