Schizophrenia: Dopamine D_1 Receptor Sequence Is Normal, But Has DNA Polymorphisms

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Genes that regulate dopamine (DA) receptors may underlie the overactive DA system in schizophrenia. Since it is known that there is an abnormally reduced or absent regulation of the DA D_2 receptor by the DA D_1 receptor in the postmortem schizophrenia brain, the human DA D_1 receptor gene was sequenced from genomic deoxyribonucleic acid (DNA) of seven schizophrenic individuals. The tissues from two schizophrenics had previously been found to have a reduced link between DA D_1 and D_2 receptors. The D_1 receptor genes were amplified by the polymerase chain reaction, subcloned, and sequenced. Although three DNA polymorphisms were found, the deduced amino acid sequence of the DA D_1 receptor was normal in these tissues. **[Neuropsychopharmacology 8:131–135, 1993]**

KEY WORDS: *Schizophrenia; Dopamine D_1 receptor; Polymorphism; Polymerase chain reaction*

Patients with schizophrenia may suffer from an overactive dopamine (DA) system in the brain (Wyatt et al. 1989), based on the observations that antipsychotic and neuroleptic medications block brain DA D_2 receptors in direct relation to their clinical potencies (Seeman et al. 1976) and that brain D_2 receptors may be more numerous in schizophrenia (Seeman et al. 1987; Wong et al. 1986, but for a more extensive analysis and discussion, see Farde et al. 1990 and Seeman et al. 1990, 1992).

The activity of the D_2 receptor is normally moderated by its link to the D_1 receptor (Seeman et al. 1989). This D_1–D_2 link, although present in control brain tissues and in Parkinson's-diseased or Alzheimer-diseased brain tissues, is missing or reduced in schizophrenia brain tissue (Seeman et al. 1989). In addition, Memo et al. (1983) found D_1-stimulated adenylate cyclase to be elevated in schizophrenia brain tissue.

These findings justify a search for a possible abnormality in the gene for the human DA D_1 receptor in schizophrenia. The recent cloning of this gene (Sunahara et al. 1990; Dearry et al. 1990; Zhou et al. 1990) permitted us to examine the D_1 receptor sequence in schizophrenia. We report here that the amino acid sequence of the D_1 receptor in schizophrenia was normal in seven subjects, but there were three apparent polymorphisms in the deoxyribonucleic acid (DNA) sequence.

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MATERIALS AND METHODS

Tissues

Post mortem brain samples were obtained at autopsy from five patients who had schizophrenia, and from one control individual, as previously described (See-

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man et al. 1987). The tissues were obtained through the Canadian Brain Tissue Bank (Toronto) and the Lainz Hospital (Vienna). Tissues from schizophrenia Patients T751 and T708 had previously been found to have a reduced link between D_1 and D_2 receptors, being absent in T751 and reduced by 60% in T708 (Seeman et al. 1989). An additional two blood samples were obtained from two schizophrenic outpatients. The diagnosis of schizophrenia met the criteria of International Classification of Disease version 9 (World Health Organization 1979) for tissues from Vienna or DSM-III-R (American Psychiatric Association 1987) for cases from Toronto.

Table 1. Tissues Used for Genomic DNA

Case No.	Sex	Age	Tissue	Criteria
V433-88	F	77	Striatum	ICD-9[a]
V232-81	F	80	Striatum	ICD-9
V185-80	F	56	Striatum	ICD-9
T751	F	85	Striatum	DSM-III-R[b]
T708	F	82	Striatum	DSM-III-R
TB1	M	37	Blood	DSM-III-R
TB2	M	31	Blood	DSM-III-R
T813	M	81	Striatum	Control

[a] International Classification of Diseases, Version 9.
[b] Diagnostic and Statistical Manual, 3rd ed, Revised.

Genomic DNA Preparation

Genomic DNA was extracted from each tissue or blood sample (Ausubel et al. 1988). The brain tissues (weighing 100 mg) were homogenized in 1.2 ml of 10 mmol/L Tris-HCl (pH 8) containing 0.1 mg/ml proteinase K, 100 mmol/L NaCl, 25 mmol/L ethylenediaminetetraacetic acid (EDTA) and 0.5% sodium dodecylsulfate (SDS), followed by overnight incubation at 50°C. Proteins were removed using phenol and chloroform extraction. The genomic DNA was precipitated with ethanol and resuspended in 10 mmol/L Tris-EDTA buffer (pH 8) and stored at 4°C.

Genomic DNA from the blood samples was extracted by the method of Miller et al. (1988). Briefly, 10 ml of blood was mixed with 10 mmol/L Tris-HCl (pH 7.6) in 320 mmol/L sucrose, 1% Triton X-100, 5 mmol/L $MgCl_2$ to a final volume of 40 ml. After centrifugation at 3000 × g for 10 minutes at 4°C, the remaining leukocytes were digested overnight with proteinase K (125 µg/ml) in 3 ml of nucleus-lysing buffer (400 mmol/L NaCl, 2 mmol/L EDTA, 0.5% SDS, 10 mmol/L Tris-HCl, pH 8.2) at 37°C. Proteins were precipitated with 1 ml of 6 mol/L NaCl. The DNA was precipitated with ethanol, dried, and dissolved in 500 µl of 20 mmol/L Tris-EDTA buffer (pH 8) and stored at 4°C.

DNA Amplification by the Polymerase Chain Reaction

Genomic DNA was amplified *in vitro* by the polymerase chain reaction (PCR) using *Taq* polymerase (Cetus, Norwalk, CT) with oligonucleotide primers 758 and 757 (Biotechnology Service Centre, Hospital for Sick Children Research Institute, Toronto). Primer 758 was 5′-**tacggatccAAGCAATCTGGCTGTGCAAAGTGCTGCCTGGT**-3′, where the first 9 nucleotide bases (in lower case) encode a *Bam*HI site, and where the other nucleotides correspond to bases at positions −123 to −92 in the human DA D_1 receptor sequence (Fig. 2). Primer 757 was 5′-**tacggatccCTCACCGTACCTTAGTTTCTTAATAGCAAA**-3′, where the bases in lower case correspond to the *Bam*HI site, and the other bases are complementary to the DNA coding sequence of bases 1403 to 1432 in the D_1 sequence (Fig. 2). The PCR was carried out by the method of Saiki et al. (1988) in tubes containing 200 ng genomic DNA, 1 µg of each primer, 200 µmol each of adenosine 5′-triphosphate, cytidine 5′-triphosphate, guanosine 5′-triphosphate, and thymidine 5′-triphosphate, and 2.5 U of *Taq* polymerase in a final total volume of 100 µl GeneAmp buffer (Perkin-Elmer Cetus Instruments, Norwalk, CT). The conditions for the PCR were as follows: denaturing time of 1.5 minutes at 94°C; annealing time of 3 minutes at 60°C, and elongation time of 4 minutes at 72°C, using a DNA Thermal Cycler (Perkin-Elmer Cetus Instru-

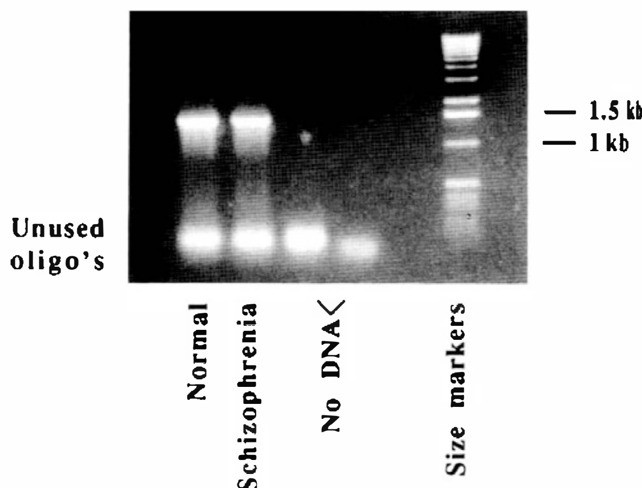

Figure 1. Agarose gel electrophoresis of amplified products. Thirty µl of amplified DNA were run in 1% agarose gel and visualized by ethidium bromide (1 µg/ml). Lane 1 shows size markers. Lanes 2 and 3 show PCR-amplified genomic DNA from control and schizophrenia individuals, respectively. The arrow indicates the 1.5 kb fragment that contains the entire DA D_1 receptor gene.

ments). Following 30 cycles, the samples were given an additional elongation period of 7 minutes at 72°C. An aliquot was removed and analyzed by gel electrophoresis in 1% low-melt agarose containing ethidium bromide.

Subcloning and Sequencing

The PCR products were extracted in phenol and chloroform, precipitated with ethanol and digested with *Bam*HI. The cloning vector plasmid pSP73 (Promega) was digested with *Bam*HI for 40 minutes followed by 30-minute incubation period with alkaline phosphatase. The PCR product and plasmid pSP73 were extracted from the 0.8% low-melt agarose gel following electrophoresis, mixed in the same tube in the presence of T4 DNA ligase, and incubated overnight at room temperature. Transformation was done under standard conditions using AG1 cells (Stratagene) as the host. The DA D_1 gene was screened by digestion of the plasmid DNA (extracted from the colony) with *Bam*HI, generating a fragment of approximately 1.5 kb. The nucleotide sequence of the cloned fragment was obtained by the dideoxy chain termination method (Sanger et al. 1987), using Sequenase V.2 (USB, Cleveland, OH).

RESULTS

The size of the D_1 receptor gene fragment from control and schizophrenia samples were the same (Fig. 1). Dideoxy sequencing confirmed the fragments encoding the D_1 receptor in both control and schizophrenia tissues. There were three DNA polymorphisms (see Fig. 2).

1) The nucleotide at position −48 was an **A** in our earlier work on one human individual (Sunahara et al. 1990), as well as in one sample by Dearry et al. (1990) and in another by Zhou et al. (1990). In patient T708, however, this nucleotide was a **G**. A total, therefore, of 10 humans had an **A**, and only one revealed a **G**.

2) Nucleotide 90 in all cases (except V433-88) revealed an **A** to **G** change. This did not, however, alter the coded amino acid, leucine. Other groups (Dearry et al. 1990; Zhou et al. 1990) also found a **G**. Thus, a total of nine humans have revealed a **G** with two exhibiting an **A**.

3) Nucleotide 222 in all individuals had an **A** to **C** change, but this did not affect the coding of the amino acid, alanine.

DISCUSSION

The results indicate that the amino acid sequence of the DA D_1 receptor from seven schizophrenic individuals was normal, but that there were three apparent polymorphisms in the DNA sequence.

Concerning the DNA polymorphisms, it should be noted that the *Taq* polymerase used in these experiments has an error rate or base misincorporation rate of about 0.1%. Thus, it is possible that the apparent DNA polymorphisms may arise as a PCR-generated artifact. Although various types of PCR procedures may rule out such artifacts, at least two of the three polymorphisms were identical in more than one subject.

Tissues from schizophrenia Patients T751 and T708 had previously been found (Seeman et al. 1989) to have a reduced link between D_1 and D_2 (absent link in T751, and 60% reduction of the link in T708). These schizophrenia patients, however, revealed DNA sequences identical to control individuals.

Since the number of different samples tested here was small, the results do not rule out the existence of an abnormal amino acid sequence in the D_1 dopamine receptor of different patients in a more extensive series of schizophrenia patients. For example, it may be estimated statistically that studying only seven subjects is insufficient to rule out a relevant receptor mutation in many (perhaps one-third) of schizophrenic patients. A more rapid method for screening possible mutations in a larger series of tissues is to use PCR and denaturing gradient gel electrophoresis (Sung et al. 1991; Catalano et al. 1991).

Furthermore, the absence of any changes in the D_1 receptor protein in these seven schizophrenia cases does not preclude possible D_1 receptor abnormalities in schizophrenia, since there may be abnormalities in posttranslational processing of these receptors (phosphorylation and glycosylation, for example).

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Figure 2. The DNA and amino acid sequences of the human DA D_1 receptor (Sunahara et al. 1990). Primers 757 (in italics) and 758 were used to amplify DNA from control and schizophrenia tissues; each primer had a 9-base sequence attached for the *BamHI* restriction enzyme. Three polymorphisms are shown at base positions −48, 90, and 222.

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REFERENCES

American Psychiatric Association (1987): Diagnostic and Statistical Manual of Mental Disorders, ed 3, revised. Washington, DC, American Psychiatric Press

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG,

Smith JA, Struhl K (1988): Current Protocols in Molecular Biology. Vol 1. New York, Wiley Interscience

Catalano M, Nobile M, Novelli E, Marino C, Smeraldi E (1991): Use of PCR and DGGE to detect possible genetic variations in dopamine D_2 receptor gene: A preliminary report. In Racagni G, Brunello N, Fukuda T (eds), Biological Psychiatry, Vol 2. Amsterdam, Excerpta Medica, pp 469-471

Dearry A, Gingrich JA, Falardeau P, Fremeau RT Jr, Bates MD, Caron MG (1990): Molecular cloning and expression of the gene for human D_1 dopamine receptor. Nature 34:72-76

Farde L, Wiesel F-A, Stone-Elander S, Halldin C, Nordström A-L, Hall H, Sedvall G (1990): D_2 dopamine receptors in neuroleptic-naive schizophrenic patients: a positron emission tomography study with [^{11}C]raclopride. Arch Gen Psychiatry 47:213-219

Memo M, Kleinman JE, Hanbauer I (1983): Coupling of dopamine D_1 recognition sites with adenylate cyclase in nuclei accumbens and caudatus of schizophrenics. Science 221:1304-1307

Miller SA, Dykes DD, Polesky HF (1988): A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich H (1988): Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491

Sanger F, Nicklen S, Coulson AR (1977): DNA sequencing with chain termination inhibitors. Proc Natl Acad Sci USA 74:5463-5467

Seeman P, Lee T, Chau-Wong M, Wong K (1976): Antipsychotic drug doses and neuroleptic/dopamine receptors. Nature 261:717-719

Seeman P, Bzowej NH, Guan H-C, Bergeron C, Reynolds GP, Bird ED, Riederer P, Jellinger K, Tourtellotte WW (1987): Human brain D1 and D2 dopamine receptors in schizophrenia, Alzheimer's, Parkinson's and Huntington's diseases. Neuropsychopharmacology 1:5-15

Seeman P, Niznik HB, Guan H-C, Booth G, Ulpian C (1989): Link between D1 and D2 dopamine receptors is reduced in schizophrenia and Huntington diseased brain. Proc Natl Acad Sci USA 86:10156-10160

Seeman P, Niznik HB, Guan H-C (1990): Elevation of dopamine D_2 receptors in schizophrenia is underestimated by radioactive raclopride. Arch Gen Psychiatry 47:1170-1172

Seeman P, Ohara K, Ulpian C, Seeman MV, Jellinger K, Van Tol HHM, Niznik HB (1992): Schizophrenia: Normal sequence in the dopamine D2 receptor region which couples to G proteins. DNA polymorphisms found in D2. Neuropsychopharmacology (In press)

Sunahara RK, Niznik HB, Weiner DM, Stormann TM, Brann MR, Kennedy JL, Gelernter JE, Rozmahel R, Yang Y, Israel Y, Seeman P, O'Dowd BF (1990): Human dopamine D1 receptor encoded by an intronless gene on chromosome 5. Nature 347:80-83

Sung C-H, Davenport CM, Hennessey JC, Maumenee IH, Jacobson SG, Heckenlively JR, Nowakowski R, Fishman G, Gouras P, Nathans J (1991): Rhodopsin mutations in autosomal retinitis pigmentosa. Proc Natl Acad Sci USA 88:6481-6485

Wong DF, Wagner Jr HN, Tune LE, Dannals RF, Pearlson GD, Links JM, Tamminga CA, Broussolle EP, Ravert HT, Wilson AA, Toung JKT, Malat J, Williams JA, O'Tuama LA, Snyder SH, Kuhar MH, Gjedde A (1986). Positron emission tomography reveals elevated D2 dopamine receptors in drug-naive schizophrenics. Science 234: 1558-1563

World Health Organization (1979): Manual of the International Classification of Diseases, Injuries, and Causes of Death. 9th Revision, Clinical Modification. Geneva, World Health Organization

Wyatt RJ, Kirch DG, Delisi LE (1989): Schizophrenia: Biochemical, endocrine and immunological studies. In Kaplan HI, Sadock BJ (eds), Comprehensive Textbook of Psychiatry. Baltimore, Williams & Wilkins, pp 717-732

Zhou Q-Y, Grandy DK, Thambi L, Kushner JA, Van Tol HHM, Cone R, Pribnow D, Salon J, Bunzow JR, Civelli O (1990): Cloning and expression of human and rat D1 dopamine receptors. Nature 347:76-80