Research report

Schizophrenia: elevated mRNA for dopamine D_{2_{Longer}} receptors in frontal cortex

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Abstract

Because dopamine D2 receptors are the primary targets for antipsychotic drugs, including clozapine and quetiapine, and because some studies have found D2 receptors to be elevated in schizophrenia, we examined the mRNA of three forms of the D2 receptor, particularly the new form of the dopamine D2 receptor, D_{2_{Longer}}, in post-mortem brains from patients who died with schizophrenia. Using quantitative competitive RT–PCR (reverse transcriptase–polymerase chain reaction), the D_{2_{Longer}} mRNA was higher in the frontal cortex, compared to control tissues. The mRNA concentration of D_{2_{Long}} and D_{2_{Short}} was also higher in the frontal cortex, compared to control tissues. Although most of the schizophrenia patients had received different antipsychotic drugs for varying periods of time, the mRNA of D_{2_{Longer}}, as well as that for D_{2_{Long}} and D_{2_{Short}}, in such medicated tissues was similar to that in a frontal cortex tissue from a patient who had reliably never received antipsychotic drugs. It is possible, therefore, that the elevation of the mRNAs for D_{2_{Longer}}, D_{2_{Long}} and D_{2_{Short}} in the frontal cortex may be related to the disease of schizophrenia itself. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Disorders of the nervous system

Topic: Neuropsychiatric disorders

Keywords: Dopamine receptor; Psychosis; Antipsychotic drug; Frontal cortex; Caudate nucleus; Putamen

1. Introduction

Although dopamine D2 receptors are the primary targets for antipsychotic drugs, including clozapine and quetiapine [10,18], no DNA abnormality or linkage has been found for D2 receptors in schizophrenia [13,19]. However, because dopamine D2 receptors are elevated in schizophrenia, as detected using ^[14C]methylspiperone (Ref. [21]), ^[125I]iodobenzamide (Refs. [1,17]) or ^[3H]spiperone (Ref. [16]), but not with ^[14C]clonidine (Ref. [4]) (although see Ref. [14]), it is likely that the production of D2 receptors is increased. If so, then the mRNA of D2 receptors would be expected to be elevated in schizophrenia.

Dopamine D2 receptor mRNA expression is homogeneously distributed in the normal human striatum, while that in the normal human neocortex occurs in layers [12] as well as in bands which are perpendicular to the layers [5]. Although no difference has been found in the proportions of two forms of the dopamine D2 receptor, D_{2_{Short}} and D_{2_{Long}}, between control and schizophrenia brain tissues, the caudate nucleus, and the orbital and temporal gyri did reveal an increased expression in the total amount of D_{2_{Short}} and D_{2_{Long}} mRNA [15].

A new form of the dopamine D2 receptor, D_{2_{Longer}}, has recently been discovered in the human brain (GenBank accession number AF176812) [20]. Although the number of D_{2_{Longer}} mRNA transcripts occurred at the low level of several percent of the total number of D2 transcripts sequenced [20], it is known that low or very low levels of mRNA may have considerable biological significance [8]. Therefore, in order to determine whether the D_{2_{Longer}} form was selectively altered in schizophrenia brain, we measured the mRNA expression of all three forms of the dopamine D2 receptor, D_{2_{Short}}, D_{2_{Long}} and D_{2_{Longer}}, in the frontal cortex of control and schizophrenia brains.
2. Materials and methods

2.1. Brain tissues

Human post-mortem brain tissues (frontal cortex, frozen at −70°C) were dissected by, and obtained from, the Canadian Brain Tissue Bank (Toronto, ON, Canada) and the National Neurological Research Specimen Bank (West Los Angeles VA Medical Center, Los Angeles, CA). Although the frontal cortex areas dissected were all within the dorsolateral frontal cortex (Brodman areas 8, 9, 10, 12, 45 and 46), no cytoarchitectonic mapping was done to confirm that these areas were specifically dissected by the brain bank personnel. The tissues remained frozen at −70°C until used for the extraction of total RNA. The diagnosis of schizophrenia was made by two psychiatrists independently examining the case records and using DSM-IV criteria (Diagnosis and Statistics Manual of the American Psychiatric Association). Table 1 summarizes the ages of the individuals, their sex, the post-mortem delay (in h), the cause of death (where known), the main antipsychotic drugs used, and the approximate number of years the patients had been psychotic.

2.2. Extraction of total RNA from tissues

Total RNA was extracted from the frontal cortex tissues using TRIZOL® Reagent and the method specified by the manufacturer’s guidelines (TRIZOL® is a trademark of Molecular Research Center, Inc.; Gibco BRL, Life Technologies, Burlington, Ontario). The tissue (50 mg) was homogenized (Polytron, PT-10 probe, Brinkmann Instruments, Westbury, NY) in 1 ml TRIZOL Reagent. The homogenized samples were incubated at room temperature for 5 min to dissociate nucleoprotein complexes. Chloroform was added (0.2 ml per ml of TRIZOL Reagent). The capped tubes were shaken for 15 s and incubated at 25°C for 2 min. The samples were centrifuged at 12,000×g for 15 min at 4°C. The aqueous upper phase, containing the RNA was transferred to a fresh tube. The RNA was precipitated with isopropyl alcohol (0.5 ml per 1 ml of TRIZOL Reagent), incubated at 25°C for 10 min and centrifuged at 12,000×g for 10 min at 4°C. The RNA pellet was washed once with 75% ethanol (1 ml per ml of TRIZOL Reagent) and air-dried for 5 min and dissolved in RNase-free water at 60°C. The final total RNA concentration was 5 μg RNA per 10 μl of water, which was subsequently further diluted with RNase-free water to 5 μg RNA per 11 μl water for cDNA synthesis.

2.3. First-strand cDNA synthesis

Each total RNA preparation was then used to prepare the cDNAs for each tissue, using the SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis (Cat. No. 18089-011; Gibco BRL, Life Technologies, Burlington, Ontario). The conversion of the mRNA for different forms of D2 in schizophrenia frontal cortex (four measurements for each tissue) is shown in Table 1.

<table>
<thead>
<tr>
<th>Brain</th>
<th>Age</th>
<th>PM. delay</th>
<th>Death</th>
<th>Duration of psychosis</th>
<th>Antipsychotic Rx</th>
<th>Long + Short cDNA per 100 μg RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control tissues:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L451*</td>
<td>25 m</td>
<td>14 h</td>
<td>Drowning</td>
<td>None</td>
<td>None</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>L475</td>
<td>77 f</td>
<td>19 h</td>
<td>Car accident</td>
<td>None</td>
<td>None</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>L480</td>
<td>66 m</td>
<td>19 h</td>
<td>Cardiac</td>
<td>None</td>
<td>None</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>T1214*</td>
<td>92 m</td>
<td>3 h</td>
<td>Cardiac</td>
<td>None</td>
<td>None</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>T1225</td>
<td>31 m</td>
<td>11 h</td>
<td>Car accident</td>
<td>None</td>
<td>None</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>T1367</td>
<td>80 m</td>
<td>16 h</td>
<td>Cancer</td>
<td>None</td>
<td>None</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>L1768</td>
<td>71 m</td>
<td>25 h</td>
<td>Cardiac</td>
<td>None</td>
<td>None</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>average±S.E.:</td>
<td>2.6±0.4</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L477</td>
<td>82 f</td>
<td>16 h</td>
<td>Cardiac</td>
<td>36 y</td>
<td>Fluphenazine*</td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td>L695</td>
<td>30 m</td>
<td>32 h</td>
<td>Cardiac</td>
<td>9 y</td>
<td>Thioridazine*</td>
<td>7.0 ± 0.5</td>
</tr>
<tr>
<td>L707</td>
<td>28 m</td>
<td>41 h</td>
<td>Suicide</td>
<td>6 y</td>
<td>Fluphenazine*</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>T1149</td>
<td>22 m</td>
<td>18 h</td>
<td>Suicide</td>
<td>5 y</td>
<td>Flupenthixol*</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>T1176</td>
<td>19 m</td>
<td>52 h</td>
<td>Suicide</td>
<td>1 y</td>
<td>None</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>T1293</td>
<td>29 m</td>
<td>4 h</td>
<td>Drowning</td>
<td>13 y</td>
<td>Risperidone*</td>
<td>8.8 ± 0.5</td>
</tr>
<tr>
<td>L1755</td>
<td>74 m</td>
<td>14 h</td>
<td>Cancer</td>
<td>&gt;30 y</td>
<td>Trifluoperazine*</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>average±S.E.:</td>
<td>4.9±0.6</td>
</tr>
</tbody>
</table>

*Abbreviations: L indicates brain tissue from the National Neurological Research Specimen Bank in Los Angeles; T indicates brain tissue from the Canadian Brain Tissue Bank in Toronto.
*Antipsychotic medication not known in the last few months before death.
*Antipsychotic medication not known in the last 4 years before death.
*Significantly elevated compared to controls, P<0.05.
the total RNA preparation) into first-strand cDNAs was done as follows. One μl of oligo(dT)12-18 primers (0.5 μg/μl) was added to each 11 μl sample of 5 μg total RNA. The mixture was incubated for 10 min at 70°C to denature the sample and then cooled on ice for 5 min. An aliquot of 7 μl of master mix was added; the master mix contained 2 μl of 10X PCR buffer (200 mM Tris–HCl, pH 8.4; 500 mM KCl; Cat. No. Y020028), 2 μl 25 mM MgCl2, 1 μl of 10 mM dNTPs (10 mM each of dATP, dCTP, dGTP, dTTP) and 2 μl of 0.1 M dithiothreitol. The samples were incubated at 42°C for 5 min. One μl of SUPERSCRIPT™ II RT (200 units/μl) was added and the sample further incubated at 42°C for 50 min. The reaction was terminated at 70°C for 15 min and chilled on ice for 5 min. The RNA was then removed by adding 1 μl of RNase H and incubating at 37°C for 20 min before storing at −20°C until further use. Thus, each sample provided 2 μl of a cDNA preparation.

2.4. Preparation of the standard truncated D2_{longer}

In order to do quantitative competitive PCR, a truncated standard form of the D2_{longer} receptor DNA was added to each PCR tube. Fig. 1 shows the DNA sequence of this standard, where bases 91 to 207 (using the numbering system in Ref. [6]) were deleted from the DNA sequence of D2_{longer} [20]. This truncated standard was prepared by the PCR method, using the forward and reverse primers shown in Fig. 1. Each PCR tube received 18.5 μl of autoclaved, distilled water, 2.5 μl of 10X PCR buffer, 1 μl of the forward primer (10 mM; Fig. 1), 1 μl of the reverse primer (10 mM; Fig. 1), 1 μl of the D2_{longer} DNA (which had been subcloned into the vector pCR®2.1 [Invitrogen, San Diego, CA]) [20], 0.5 μl of 10 mM dNTP mix and 0.5 μl of Advantage® cDNA Polymerase Mix (Catalog No. 8417-1; CLONTECH Laboratories, Inc., Palo Alto, CA), making a total volume of 25 μl. The mixture was preincubated at 94°C for 2 min, followed by 39 thermocycles, each consisting of 30 s at 94°C for denaturation, 45 s at 65°C for annealing, and 60 s at 72°C for extension, followed by a final period of 7 min at 72°C for extension, after which the sample was maintained at 4°C, using a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer Cetus, Foster City, CA). The predicted size of the PCR product was 654 bp. The PCR products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide. The band corresponding to the truncated standard was excised and solubilized with 3 vol of QX I buffer; 12 μl of QX II were added to bind the DNA (QIAEX II DNA Extraction Kit; Cat. No. 20021; QIAGEN, Inc., Santa Clarita, CA). The samples were incubated at 50°C for 10 min and centrifuged. The pellet was washed once with 500 μl QX I to remove agarose and other contaminants, and washed twice with PE buffer to remove salts. The pellet was then air-dried and the DNA eluted twice, using 20 μl of 10 mM Tris–HCl, pH 8.5 (room temperature). A 1/10 dilution of this DNA pellet (of the truncated D2_{longer} standard) was analyzed for its optical density at 260 nm. The average optical density of this diluted standard solution was 0.108, corresponding to

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Fig. 1. DNA sequence of a portion of the truncated D2_{longer} used as an internal standard to measure the amount of D2_{longer}, D2_{short} and D2_{short} in the cDNA preparations from the postmortem tissues, using quantitative RT–PCR. The deleted bases in the truncated standard are shown by the strike-through line. Compared to D2_{longer} there are six extra bases in D2_{short} as shown by the double underline. The bases are numbered according to Grandy et al. [6].
2.5. Quantitative competitive PCR

The cDNA concentrations for D2\textsubscript{Longer}, D2\textsubscript{Long} and D2\textsubscript{Short} were measured by quantitative competitive PCR. Each of the PCR reaction tubes received 1 \(\mu\)l of different dilutions of the standard truncated D2\textsubscript{Longer} cDNA preparation. The PCR reaction co-amplified the cDNA of D2\textsubscript{Longer} and the added standard truncated D2\textsubscript{Longer} DNA simultaneously in the same tube. The synthesis of the cDNA for D2\textsubscript{Longer} competed with the synthesis of the standard truncated D2\textsubscript{Longer} DNA for the added primers. When the PCR products were examined on a gel, two bands were seen, one band corresponding to the MW of D2\textsubscript{Longer} cDNA and the other band corresponding to the lower MW of the truncated D2\textsubscript{Longer} DNA. The two bands would only be identical in fluorescence in the PCR tube where the concentration of the D2\textsubscript{Longer} cDNA matched that of the concentration of the truncated D2\textsubscript{Longer} DNA, thus identifying the concentration for the D2\textsubscript{Longer} cDNA. Thus, using the HotStarTaq\textsuperscript{TM} DNA Polymerase Kit (Catalog No. 203203, QiagenInc., Mississauga, Ontario), each PCR tube contained 15.2 \(\mu\)l of double-filtered water, 2.5 \(\mu\)l of 10X buffer, 2.5 \(\mu\)l of Q solution, 0.5 \(\mu\)l of 10 mM dNTP mix, 1 \(\mu\)l of the forward primer (10 mM; but only the primer portion before the deleted bases in Fig. 1), 1 \(\mu\)l of the reverse primer (10 mM), 1 \(\mu\)l of a 1/10 dilution of the cDNA sample to be quantified, 1 \(\mu\)l of different concentrations of the truncated standard D2\textsubscript{Longer} DNA, and 0.3 \(\mu\)l of the HotStarTaq\textsuperscript{TM} DNA Polymerase. The PCR products were analysed using gel electrophoresis (1.2% agarose) and ethidium bromide. The fluorescent intensities of the bands were measured using a Bio-Rad Gel Doc Imaging System. An example of a typical result is shown in Fig. 2. The primers for the PCR amplification of the D2\textsubscript{Longer} cDNA were 5’-GCC GCA AGC GAG TCA ACA CCA A-3’ (upper primer; position 689 in Ref. [6]) and 5’-GCT GGG TGG GAT GGG GCT GTA-3’ (lower primer; position 916 in Ref. [6]).

3. Results and discussion

Using the method of quantitative competitive RT–PCR (reverse transcriptase-polymerase chain reaction) to measure the mRNA concentrations of D2\textsubscript{Longer}, D2\textsubscript{Long} and D2\textsubscript{Short}, the results for seven control frontal cortices and seven schizophrenia frontal cortices are given in the Table. Compared to control tissues, the D2\textsubscript{Longer} cDNA was higher in the frontal cortex. The amount of the D2\textsubscript{Longer} cDNA was also higher in the frontal cortex, compared to control tissues.

However, there was no selective alteration in the schizophrenia tissues in the concentration of D2\textsubscript{Longer} cDNA compared to the other forms of D2. That is, the D2\textsubscript{Longer} cDNA, as a percent of the D2\textsubscript{Longer} + D2\textsubscript{Short} cDNAs, was 6.4% in control frontal cortices and 4.3% in schizophrenia tissues.

The elevation of D2\textsubscript{Longer} + D2\textsubscript{Short} mRNAs in the frontal cortex found in this study agrees with the elevation found by Roberts et al. [15]. Although not part of the present study, preliminary data on three post-mortem human striata showed that the absolute concentration of either the D2\textsubscript{Longer} mRNA or the D2\textsubscript{Long} mRNA in the striata was approximately two orders of magnitude higher than the corresponding value in the frontal cortex (data not shown). It is known that the D2 receptor density in the human striatum is approximately two orders of magnitude higher than that in the cerebral frontal cortex [3,7,9].

Although only fourteen tissues were examined, there was no indication of a relation between the level of the D2\textsubscript{Longer} mRNA and the post-mortem (death-to-freezing) interval or the age of the patient (Fig. 3).

It is known that virtually all actively psychotic schizophrenia patients are treated with antipsychotic drugs. One of the post-mortem tissues examined was from a 19 year old man who had never taken antipsychotic medication and who had suicided (see Table). Although the elevation of the D2 mRNAs in the frontal cortex of the other six schizophrenia tissues may have resulted from pre-mortem medication with antipsychotic drugs, this is unlikely because the mRNAs for D2 in the never-medicated patient were similar to those for the other six post-mortem tissues. Furthermore, although many studies with animals have
shown that antipsychotic drugs may elevate the density of D2 receptors, most of the studies have not found an elevation in the D2 mRNA [15]. It is possible, therefore, that the elevation of the mRNAs for D2 Longer, D2 Long and D2 Short in the frontal cortex may be related to the disease of schizophrenia itself. Although D2 Longer, is a functional D2 receptor [11], the present findings do not indicate any selective increase of this form of the D2 receptor in schizophrenia brain.

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