Schizophrenia: elevated mRNA for calcium-calmodulin-dependent protein kinase IIβ in frontal cortex

Gabriela Novak, Philip Seeman, Teresa Tallerico

Abstract

Because amphetamine releases two to three times more dopamine in schizophrenia patients than in control subjects, and because calcium-calmodulin-dependent protein kinase IIβ has a key role in the enhanced action of amphetamine-induced dopamine release in rats, the synaptic content of calcium-calmodulin-dependent protein kinase IIβ mRNA was measured (by quantitative competitive RT–PCR; reverse transcriptase–polymerase chain reaction) in seven frontal cerebral cortices of post-mortem brains from patients who had schizophrenia and in seven control tissues. The results indicate that the mRNA of this kinase is elevated in the schizophrenia frontal cortex.

1. Introduction

It has been hypothesized that dopamine neurotransmission is overactive in schizophrenia [15]. Although this hypothesis is supported by the fact that dopamine D2 receptors are the primary targets for antipsychotic drugs, including clozapine and quetiapine [10,13], no DNA abnormalities or linkage have been found for D2 receptors in schizophrenia [11,12]. However, one of the most consistent and reproducible findings in schizophrenia is that an intravenous dose of amphetamine releases two to three times more dopamine in schizophrenia patients than in control subjects [1,4,8,9]. Although there are many biochemical factors which may account for the increased amphetamine-induced release of dopamine in schizophrenia, it is known that there is an increased amphetamine-induced release of dopamine in striatal slices from rats which have been pretreated for 5 days with amphetamine and that this enhanced action of amphetamine depends on calcium-calmodulin-dependent kinase II [7]. It is also known that calcium-calmodulin-dependent kinase II promotes neurotransmitter release [5] by phosphorylating synapsin I which normally inhibits the release of transmitter by linking transmitter vesicles to actin [3,6].

Because the amphetamine-pretreated rat is a useful animal model of psychosis which has long-term changes in the utilization of dopamine in the frontal cortex [14], and because of the critical role of calcium-calmodulin-dependent kinase II in the enhanced action of amphetamine-induced dopamine release in these rats [7], it was possible that the synaptic content of calcium-calmodulin-dependent kinase II, and possibly also its associated mRNA, would be elevated in the frontal cortices of patients who died with schizophrenia. The purpose of the present study, therefore, was to examine whether the mRNA of calcium-calmodulin-dependent protein kinase IIβ was altered in the frontal cerebral cortices of post-mortem brains from patients who had schizophrenia. The results indicate that the
mRNA of this kinase is elevated in the schizophrenia frontal cortex.

2. Materials and methods

2.1. Post-mortem human tissues

Human post-mortem brain frontal cerebral cortices (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 (Brodmann 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. Polaroid photographs of each tissue were provided by the brain bank to indicate the appropriate brain region from which the tissue was removed. 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 hours), the cause of death (where known), the main antipsychotic drugs used, the number of years of antipsychotic use, the number of years the patients had been ill, and the most persistent psychiatric symptom or sign during the psychosis. We used seven control frontal cortices and seven schizophrenia frontal cortices. The total RNA was extracted and used to prepare the first-strand cDNA for each tissue.

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 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 air-dried for 5 min and dissolved in RNase-free water and incubated for 10 min at 60°C. The final total RNA concentration was 5 μg of RNA per 2 to 6 μl of water, which was subsequently further diluted with RNase-free water to 5 μg RNA per 11 μl water for cDNA synthesis. Two ‘housekeeping’ genes, beta-actin and

<table>
<thead>
<tr>
<th>Brain</th>
<th>Age/sex</th>
<th>P.M. delay (h)</th>
<th>Death</th>
<th>Diagnosis; symptoms</th>
<th>Duration of psychosis</th>
<th>Antipsychotic Rx</th>
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<tr>
<td>Control tissues:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T 1214</td>
<td>92/m</td>
<td>3</td>
<td>Tachycardia</td>
<td>Control</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>T 1225</td>
<td>31/m</td>
<td>11</td>
<td>Car accident</td>
<td>Control</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>T 1367</td>
<td>80/m</td>
<td>16</td>
<td>Cancer</td>
<td>Control</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>L 451</td>
<td>25/m</td>
<td>14</td>
<td>Drowning</td>
<td>Control</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>L 475</td>
<td>77/f</td>
<td>19</td>
<td>Car accident</td>
<td>Control</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>L 480</td>
<td>66/m</td>
<td>19</td>
<td>Cardiac infarct</td>
<td>Control</td>
<td></td>
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<tr>
<td>L 1768</td>
<td>71/m</td>
<td>25</td>
<td>Cardiac; cancer</td>
<td>Control</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Schizophrenia tissues:</td>
<td></td>
<td></td>
<td></td>
<td>Schizophrenia symptoms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T 1149</td>
<td>22/m</td>
<td>18</td>
<td>Suicide</td>
<td>Paranoid</td>
<td>5 years</td>
<td>Flupenthixol</td>
</tr>
<tr>
<td>T 1176</td>
<td>19/m</td>
<td>52</td>
<td>Suicide</td>
<td>Depression</td>
<td>1 year</td>
<td>None</td>
</tr>
<tr>
<td>T 1293</td>
<td>29/m</td>
<td>4</td>
<td>Drowning</td>
<td>Hallucinations; depression</td>
<td>13 years</td>
<td>Risperidone</td>
</tr>
<tr>
<td>L 477</td>
<td>82/f</td>
<td>16</td>
<td>Cardiac</td>
<td>Delusions</td>
<td>36 years</td>
<td>Fluphenazine</td>
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<tr>
<td>L 695</td>
<td>30/m</td>
<td>32</td>
<td>Cardiac</td>
<td>Delusions</td>
<td>9 years</td>
<td>Thioridazine</td>
</tr>
<tr>
<td>L 707</td>
<td>28/m</td>
<td>41</td>
<td>Suicide jump</td>
<td>Depression</td>
<td>2 years</td>
<td>Off fluphenazine 4 years</td>
</tr>
<tr>
<td>L 1755</td>
<td>74/m</td>
<td>14</td>
<td>Cancer</td>
<td>Depression</td>
<td>&gt;30 years</td>
<td>Trifluperazine</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.
glucose-6-phosphate dehydrogenase, were used to assess the general quality and quantity of the mRNA from each brain tissue. Tissues which did not reveal abundant amounts of these two proteins (after 30 cycles of amplification by PCR) were not used.

2.4. Preparation of a truncated DNA template for calcium-calmodulin-dependent protein kinase IIβ to serve as an internal standard template

In order to do quantitative competitive PCR, a known amount of a truncated form of the calcium-calmodulin-dependent protein kinase II DNAβ (to serve as an internal standard) was added to each PCR tube. Fig. 1 shows the DNA sequence for calcium-calmodulin-dependent kinase II (GenBank Accession number U50358). A truncated standard of this DNA sequence was prepared by deleting the bases from 77 to 172, using the PCR method and using the Platinum® Taq DNA Polymerase kit (Life Technologies, Burlington, Ontario). Each PCR tube received 16.8 µl of autoclaved, distilled water, 2.5 µl of 10X PCR buffer (Cat. No. Y02028), 1 µl of the forward primer (10 mM; Fig. 1), 1 µl of the reverse primer (10 mM; Fig. 1), 1.2 µl of 25 mM MgCl₂ (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 41°C for 5 min. One microliter of SUPERSCRIPT™ II RT (200 units/µl) was added and the sample further incubated at 42°C for 50 min to permit annealing. 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 209 min before storing at −20°C until further use. Thus, each sample provided 21 µl of a cDNA preparation.

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 mRNAs (in the total RNA preparation) into first-strand cDNAs was done as follows. One microliter of oligo(dT)18±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 41°C for 5 min. One microliter of SUPERSCRIPT™ II RT (200 units/µl) was added and the sample further incubated at 42°C for 50 min to permit annealing. 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 209 min before storing at −20°C until further use. Thus, each sample provided 21 µl of a cDNA preparation.
The concentrations of the calcium-calmodulin-dependent protein kinase IIβ cDNAs in the cDNA preparations were measured by quantitative competitive PCR. Each of the PCR reaction tubes received 1 μl of different dilutions of the truncated DNA standard for calcium-calmodulin-dependent protein kinase IIβ. The PCR reaction co-amplified the unknown calcium-calmodulin-dependent protein kinase IIβ cDNA and the added truncated standard DNA simultaneously in the same tube. The two syntheses of the two DNAs competed with one another for the added primers. When the PCR products were examined on a gel, two bands were seen, one band corresponding to the MW of the calcium-calmodulin-dependent protein kinase IIβ cDNA and the other band corresponding to the lower MW of the truncated DNA standard. The two bands would only be identical in fluorescence in that PCR tube where the concentration of the calcium-calmodulin-dependent protein kinase IIβ cDNA matched that of the concentration of the truncated standard DNA, thus identifying the concentration for the calcium-calmodulin-dependent protein kinase IIβ cDNA. Thus, using the HotStarTaq™ DNA Polymerase Kit (Catalog No. 203203, QiagenInc., Mississauga, Ontario), each PCR tube contained 15.2 μl of double-filtered water, 2.5 μl of 10×buffer, 2.5 μl of Q solution, 0.5 μl of 10 mM dNTP mix, 1 μl of the forward primer (10 mM; but only the primer portion before the deleted bases in Fig. 1), 1 μl of the reverse primer (10 mM), 1 μl of a 1/10 dilution of the cDNA sample to be quantified, 1 μl of different concentrations of the truncated DNA standard, and 0.5 μl of the HotStarTaq™ DNA Polymerase. The PCR products were analyzed 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.

### 3. Results and discussion

The cDNA concentrations of calcium-calmodulin-dependent protein kinase IIβ were measured by quantitative competitive RT–PCR (reverse transcriptase–polymerase chain reaction). The results for the 14 frontal cerebral cortices (seven controls and seven from individuals who had schizophrenia) are given in Table 2.

The cDNA concentrations for calcium-calmodulin-dependent protein kinase IIβ in the control frontal cerebral cortices ranged from 57 to 320 fg cDNA/μg of total RNA, with an average of 173 ± 33 fg/μg RNA. The schizophrenia tissues ranged from 150 to 715 fg cDNA/μg of total RNA with an average of 320 ± 74 fg/μg RNA, an increase of about 85%. Each tissue was measured a minimum of three times, the measurements revealing a reproducibility of 15%. There was no obvious relation between the cDNA level and the age of the patient or the post-mortem delay (Tables 1 and 2).

As a non-specific control for the calcium-calmodulin-dependent protein kinase IIβ cDNA, human beta-actin cDNA was also measured in all the samples. As indicated in Table 2, the average values for beta-actin cDNA were 6.5 ± 1.1 and 6.6 ± 0.7 units for the control samples and the schizophrenia samples, respectively, where the units of beta-actin were expressed as a percentage of the integrated

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**Fig. 2.** Sample result for the method of quantitative competitive RT–PCR to measure the cDNA concentration of calcium-calmodulin-dependent protein kinase IIβ in the post-mortem frontal cortex from schizophrenia brain. Each of the PCR tubes contained the unknown cDNA and a different dilution of the truncated standard (Fig. 1). The PCR thus co-amplified the cDNA and the added standard simultaneously. The synthesis of the cDNA competed with the synthesis of the truncated 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 the calcium-calmodulin-dependent protein kinase IIβ cDNA and the other band corresponding to the lower MW of the truncated DNA standard. When the two bands were identical in fluorescence, this indicated that the concentration of the unknown cDNA was the same as that for the known concentration of the truncated standard. The numbers indicate that the fluorescence intensity of the calcium-calmodulin-dependent protein kinase IIβ cDNA (in frontal cortex 707) matched the truncated standard at a final concentration of between 4.8 and 3.6 × 10⁻⁴ μg of the truncated standard per μg of total RNA. The MW standard scale is shown on the left side.
Table 2  
Messenger RNA for calcium-calmodulin-dependent protein kinase IIβ in schizophrenia frontal cortex (Average of three measurements for each tissue)

<table>
<thead>
<tr>
<th>Brain</th>
<th>Ca-MKIIβ</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fg cDNA/μg RNA</td>
<td>% total</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>451</td>
<td>180</td>
<td>10.0</td>
</tr>
<tr>
<td>475</td>
<td>57</td>
<td>9.6</td>
</tr>
<tr>
<td>480</td>
<td>180</td>
<td>9.2</td>
</tr>
<tr>
<td>1214</td>
<td>180</td>
<td>4.4</td>
</tr>
<tr>
<td>1225</td>
<td>320</td>
<td>3.6</td>
</tr>
<tr>
<td>1367</td>
<td>84</td>
<td>4.2</td>
</tr>
<tr>
<td>1768</td>
<td>210</td>
<td>4.8</td>
</tr>
<tr>
<td>Average</td>
<td>173±33</td>
<td>6.5±1.1</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1293</td>
<td>715</td>
<td>4.1</td>
</tr>
<tr>
<td>707</td>
<td>420</td>
<td>7.4</td>
</tr>
<tr>
<td>1176</td>
<td>300</td>
<td>6.6</td>
</tr>
<tr>
<td>695</td>
<td>260</td>
<td>5.9</td>
</tr>
<tr>
<td>1149</td>
<td>200</td>
<td>5.2</td>
</tr>
<tr>
<td>1755</td>
<td>195</td>
<td>9.7</td>
</tr>
<tr>
<td>477</td>
<td>150</td>
<td>7.5</td>
</tr>
<tr>
<td>Average</td>
<td>320±74</td>
<td>6.6±0.7</td>
</tr>
</tbody>
</table>

* Elevated compared to controls, P<0.05.  
* Because all 14 cDNA samples were amplified by PCR simultaneously (30 cycles), using human beta-actin primers (CLONTECH Laboratories, Inc., Palo Alto), the units of actin are in % of the total imaging intensities of all samples.

It is possible that the elevation of the mRNA for calcium-calmodulin-dependent protein kinase IIβ in the frontal cortex may have resulted from pre-mortem medication with antipsychotic drugs. However, this is unlikely because the mRNA for dopamine D2 receptors in the post-mortem brain striata (caudate nucleus and putamen) examined at the same time were not elevated, indicating that the antipsychotic drug effects on transcription, especially on their primary target of D2 receptors [10,13] were probably weak or absent. For example, data from another extensive study (Tallerico et al., submitted for publication) show that the cDNA for the human dopamine D2 Long receptor in the striatum was 140 fg cDNA per μg of total RNA in control tissues but lower (~100 fg cDNA per μg of total RNA) in schizophrenia tissues (n=4 tissues). Furthermore, in the case of frontal cortex tissue 707, the individual had been off fluphenazine for at least four years before he suicided (Table 1), and this tissue had a high calcium-calmodulin-dependent protein kinase IIβ mRNA of 420 fg cDNA/μg total RNA (Table 2), approximately 140% higher than the average control value.

Although the elevation of calcium-calmodulin-dependent protein kinase IIβ in the frontal cortex is consistent with the increased releasability of dopamine in schizophrenia, it will be necessary to measure other proteins and enzymes involved in the various steps associated with the release of synaptic vesicles, in order to determine whether the elevation of this particular enzyme is uniquely altered in schizophrenia. Although future research on this topic should be done on tissues which are matched for age, sex, post-mortem delay, anatomical identity, cause of death, pre-mortem medications, and pre-mortem drug-free interval, the practical obstacles in obtaining such tissues are overwhelming. While much more research remains to be done, and more tissues need to be analyzed, it appears that the mRNA for calcium-calmodulin-dependent protein kinase IIβ in the frontal cortex is elevated in schizophrenia, a finding compatible with the increased amphetamine-induced release of dopamine in schizophrenia [1,4,8,9].

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