Synapsin II gene expression in the dorsolateral prefrontal cortex of brain specimens from patients with schizophrenia and bipolar disorder: effect of lifetime intake of antipsychotic drugs

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INTRODUCTION

Synapsins are a family of neuronal phosphoproteins that are important for neurotransmitter release, synaptogenesis and the maintenance of synaptic vesicular pools.1,5 There is an increasing body of evidence to support a role for aberrant synaptic processes in the underlying pathophysiology of schizophrenia.2,9,10 In particular, abnormalities in synapsin II gene and protein expression have been shown to occur in patients with schizophrenia.11–13 A microarray study by Mirnics et al.11 demonstrated decreased mRNA concentrations of synapsin II in the prefrontal cortices of patients with schizophrenia. Additionally, research by Chen et al.14 reported positive associations between specific synapsin II gene polymorphisms and schizophrenia.14–16 The synapsin II gene is located on chromosome 3p25, which was suggested to be a region of vulnerability for schizophrenia, thus placing particular significance on the study of this gene.16,17

Previous investigations from our laboratory have also reported that synapsin II concentrations are differentially regulated by the modulation of dopamine receptors.11,18,19 Dopamine receptors, in particular the dopamine D2 receptor, have been strongly implicated in the etiology of schizophrenia.20–24 Our cDNA study revealed that haloperidol, a typical antipsychotic drug and an antagonist of the inhibitory dopamine D2 receptor, increases mRNA expression of synapsin II in the rat striatum.11,18,19 Treatment with the atypical antipsychotic drug olanzapine, on the other hand, increases synapsin II expression in the medial prefrontal cortex of synapsin II knockdown rats.5 Furthermore, treatment with SCH23390, an antagonist of the excitatory dopamine D1 receptor, reduces concentrations of synapsin II, whereas treatment with SKF38393, an agonist of the dopamine D1 receptor, upregulates expression of synapsin II in primary cell cultures.18 Given the importance of the dopamine D2 receptors in schizophrenia, studying the role of dopamine receptors and targeting therapeutics in the context of synapsin II and its expression is fundamental to understanding this debilitating mental disease.

Dysregulated glutamate neurotransmission in the prefrontal cortex has been a highly investigated theory of schizophrenia.25–27 Synapsins are located at the presynaptic site and help tether synaptic vesicles to the cytoskeleton at a reserve pool away from the active site of the synapse. In addition to synaptic proteins, however, vesicular transporters are also present on synaptic vesicles for the uptake and storage of their respective neurotransmitters into the synaptic vesicles for subsequent release into the synaptic cleft.28 Evidence from previous studies have shown that synapsin II reduction in the prefrontal cortex is accompanied by reductions in VGAT (GABA vesicular transporter), VGLUT1 and VGLUT2 (vesicular glutamate transporters).2 Altered prefrontal glutamatergic signaling, as a result of subnormal synapsin II expression, can subsequently disrupt local and long loop pathways and influence the release of neurotransmitters, including GABA and dopamine, in both the cortical and subcortical regions.29,30 This resembles the neurochemical changes which are characteristic of schizophrenia. Recent studies have now also taken the approach of studying schizophrenia as a disease of the synapse and connectivity and presynaptic protein, synapsin II, has accordingly been uniquely positioned at a critical site of impact. Consequently, the study of the synapsin II and its isoforms is imperative to
understanding the pathophysiology of this disease and the mechanisms involved in the therapeutic action of antipsychotic drugs. In order to further assess synapsin II expression, the present study examined synapsin II mRNA expression in the dorsolateral prefrontal cortex of patients with schizophrenia, patients with bipolar disorder and healthy subjects using coded RNA samples obtained from the Stanley Foundation Neuropathology Consortium (SFNC) array collection. This investigation utilized a large post-mortem sample size to accurately model the changes in genetic expression of synapsin II. The specific objectives of this study were to: (a) confirm reductions in synapsin II mRNA concentrations in the dorsolateral prefrontal cortex of patients with schizophrenia; (b) examine whether these reductions occur with both isoforms of synapsin II; (c) evaluate whether these decreases in synapsin II isoform expression occur in schizophrenia and/or bipolar disorder; and (d) ascertain whether lifetime use of antipsychotic drugs affect mRNA expression of synapsin II isoforms in the dorsolateral prefrontal cortex of patients with schizophrenia.

**MATERIALS AND METHODS**

Post-mortem dorsolateral prefrontal cortical RNA samples

DNase-treated dorsolateral prefrontal cortical RNA samples from post-mortem brain specimens, Breddman’s area 46 (in the dorsolateral prefrontal cortex), from healthy subjects (n = 35), patients with schizophrenia (n = 35) and patients with bipolar disorder (n = 35) were donated by the Stanley Array Collection of the Stanley Foundation Brain Collection. The dorsolateral prefrontal cortical regions from which the RNA samples were extracted were independently identified in whole-brain specimens by two neuropathologists employed at the SFNC. Subjects were clinically assessed by two independent psychiatrists, and control subjects were confirmed to be free of psychiatric illness and substance abuse. Patient diagnosis with either schizophrenia or bipolar disorder was established using criteria from the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV-TR). The demographics and patient histories of all the subjects were provided by the SFNC post-analysis and are summarized in Table 1. Analyses of RNA expression were performed with coded samples in order to keep experimenters blind to the diagnostic status of subjects, and the range of clinical variables and diagnostic status were only provided by the SFNC at the time of sample decoding, after the qualitative analysis of the RNA samples was provided by the SFNC. RNA purities were verified using A260/A280 ratio analyses and RNA integrities were confirmed by gel electrophoresis and ethidium bromide staining. These studies were approved by the Human Research Ethics Board of McMaster University, Health Sciences.

Real-time reverse transcriptase–PCR (RT-PCR) quantification of synapsin IIa and IIb mRNA expression

Real-time RT-PCR was performed in triplicates for each sample using a MX-3000P Real-Time RT-PCR machine (Stratagene, La Jolla, CA, USA). The following primers were used to examine synapsin IIa and IIb mRNA concentrations: 5’-CCACAGCTCAAAAGTC-3’ (synapsin IIa forward); 5’- ATCTGAAAGAGGCCTGCGG-3’ (synapsin IIa reverse); 5’-AGACCCCCAAAACACCC- CAG-3’ (synapsin IIb forward); and 5’-CTGTTTGTGGGCCTACTGTG-3’ (synapsin IIb reverse). Respective forward and reverse primers for housekeeping cyclophilin are as follows: 5’- GCA AGA CCA GCA AGA AGA 3’; and 5’- CAG CGA GAG CAC AAA GAT 3’. cDNA was reverse transcribed from the RNA samples. Before real-time RT-PCR analysis, standard curves were established for primer sets. These curves were then used to standardize synapsin IIa and IIb mRNA concentrations among the different patient groups, and template copy numbers were calculated at the end of each reaction. Melting temperature analysis of resulting amplicons confirmed the presence of a single product for each reaction. Real-time RT-PCR conditions were optimized to ensure that the amplification efficiencies remained constant throughout all reactions. Additional controls included the use of no-reverse transcriptase and no-cDNA template. Human cyclophilin mRNA concentrations were also quantified to show no significant alterations from sample to sample in the synapsin IIa and IIb mRNA concentrations obtained for each sample. The following components were used in the reactions described above: 10 µl of Invitrogen Platinum SyberGreenPCR 2X Supermix; 0.4 µl of Rox; 375 nM of forward primer; 375 nM of reverse primer; 5.6 µl of diethylpyrocarbonate water; and 50 ng of cDNA prepared from the RNA samples. The real-time RT-PCR conditions were as follows: 50 ºC for 2 min (1 cycle); 94 ºC for 2 min (1 cycle); 94 ºC for 30 s, 54 ºC for 1 min and 72 ºC for 1 min (40 cycles).

**Statistical analyses**

Data were entered and analyzed with SAS, Version 9.2 (SAS Institute, Cary, NC, USA). The differences in synapsin IIa and IIb mRNA copy number between each patient group (control, bipolar disorder or schizophrenia) were determined using analysis of variance, and pairwise comparisons were conducted post hoc with the Scheffé test, as this is the most conservative approach. Multiple regression was used to examine the impact of lifetime antipsychotic drug use on synapsin IIa and IIb mRNA expression within the schizophrenia patient profile, while controlling for the potential confounding effects of age, brain pH, refrigerator interval, time spent in hospital, post-mortem interval, cause of death, history of substance abuse, alcohol use and smoking status. Diagnostic analyses for

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**Table 1. Summary and overview of patient profiles**

| Variable                        | Normal control (n = 35) | Bipolar disorder (n = 35) | Schizophrenia (n = 35) |
|---------------------------------|------------------------|---------------------------|------------------------|
| Age, mean ± s.d. (range) years  | 44.2 ± 7.6 (31–60)     | 45.3 ± 10.5 (19–64)      | 42.6 ± 8.5 (19–59)     |
| Sex (M/F)                       | 26/9                   | 17/18                     | 26/9                   |
| Race (Caucasian/other)          | 35/0                   | 33/2                      | 34/1                   |
| Age of onset, mean ± s.d. (range) years | 21.5 ± 9.1 (14–48) | 21.3 ± 10.2 (1–45)       | 21.3 ± 6.1 (9–34)      |
| Duration of illness, mean ± s.d. (range) years | 20.1 ± 9.5 (2–45) | 21.3 ± 10.2 (1–45)       | 21.3 ± 6.1 (9–34)      |
| Time in hospital, mean ± s.d. (range) years | 0.5 ± 1.4 (0–8)    | 1.2 ± 2.3 (0–12)         | 1.2 ± 2.3 (0–12)       |
| Alcohol abuse at TOD, n         | 12                     | 11                        | 12                     |
| Drug abuse at TOD, n            | 2                      | 1                         | 2                      |
| Smoking at TOD (yes/no/unknown) | 9/19/7                 | 16/6/13                   | 23/4/8                 |
| Psychotic feature (yes/no/unknown) | 0/35/0               | 21/12/2                   | 35/0/0                 |
| Lifetime antipsychotic use, FE ± s.d. (range) mg | NA                    | 100.035 ± 22.896 (0–130.000) | 85.003 ± 100.335 (50–400.000) |
| Relative brain mass, mean ± s.d. (range) g | 1444 ± 148.4 (1120–1900) | 1394 ± 139.1 (1120–1670) | 1442 ± 107.5 (1170–1630) |
| Right brain, n                  | 19                     | 15                        | 15                     |
| Left brain, n                   | 16                     | 20                        | 20                     |
| PML mean ± s.d. (range) h       | 29.4 ± 12.9 (9–58)     | 37.9 ± 18.4 (12–81)      | 31.4 ± 15.5 (9–80)     |
| Refrigerator interval, mean ± s.d. (range) h | 3.6 ± 2.6 (0–14)  | 10.1 ± 10.4 (1–54)       | 6.0 ± 4.2 (1–19)       |
| Brain pH, mean ± s.d. (range)   | 6.61 ± 0.27 (6.00–7.03) | 6.43 ± 0.30 (5.76–6.97)  | 6.48 ± 0.24 (5.90 ± 6.93) |
| 28S:18S rRNA ratio, mean ± s.d. (range) | 2.18 ± 0.50 (0.90–3.74) | 2.21 ± 0.75 (0.49–4.18)  | 2.13 ± 0.55 (1.18–3.81) |

Abbreviations: FE, female; FE, fluphenazine equivalents; M, male; NA, not applicable; PML, post-mortem interval; TOD, time of death.
the regression analysis indicated that no outliers were influential; as such, all samples were retained in the analysis to maximize statistical power. As the distribution of the synapsin IIa and IIb isoform mRNA expression was negatively skewed, the data were log-transformed. A log-transformation resulted in normally distributed synapsin IIa and IIb data—a requirement for the application of the statistical analyses utilized in this study in order to draw valid inferences from the data. All statistical tests were two-tailed using a confidence level of $\alpha = 0.05$.

RESULTS

Patient data and study variables

Post-mortem dorsolateral prefrontal cortex RNA samples extracted from 35 healthy subjects, 35 patients with schizophrenia and 35 patients with bipolar disorder obtained from the SFNC were evaluated. A summary of the relevant patient information is provided in Table 1 and stratified according to patient’s diagnosis (control, bipolar disorder or schizophrenia).

Synapsin II mRNA expression is significantly decreased in patients with schizophrenia

Representative real-time RT-PCR amplicons are shown in Figures 1 and 2. Results from Figure 1 display reductions in synapsin IIa and IIb mRNA in the dorsolateral prefrontal cortex of a representative patient with schizophrenia, without any changes in the housekeeping gene cyclophilin. This same reduction was not seen in a representative patient with bipolar disorder (Figure 2).

Box plots in Figures 3 and 4 show a comparison of synapsin II mRNA expression among different patient groups. Analysis of variance followed by pairwise comparisons using the post hoc Scheffé test revealed that synapsin IIa mRNA concentrations were significantly decreased in patients with schizophrenia when compared with both controls and patients with bipolar disorder ($F = 15.625, P < 0.002$) (Figure 3). However, analysis of synapsin IIb mRNA data revealed that expression levels of this isoform were significantly reduced in patients with schizophrenia only when compared with normal controls ($F = 3.65, P < 0.030$) (Figure 4). As patients with schizophrenia and with bipolar disorder display similar clinical features,31 we examined whether bipolar disorder individuals also exhibit the same reductions in synapsin II gene expression observed among individuals with schizophrenia. No differences in synapsin II levels in the dorsolateral prefrontal cortex were observed in patients with bipolar disorder when compared with normal control subjects ($P > 0.05$) (Figures 2, 3 and 4).

Effects of antipsychotic drug use on synapsin II mRNA expression

In the present study, multiple regression analysis showed that lifetime antipsychotic drug use was positively associated with synapsin IIa mRNA expression in patients with schizophrenia (beta $5.9 \times 10^{-6}, P < 0.001$) (Figure 5). The confounding factor effects of age, brain pH, refrigerator interval, time spent in hospital, post-mortem interval, cause of death, history of substance abuse, alcohol use and smoking status were controlled for in this analysis. However, analysis of the synapsin IIb isoforms revealed no association between lifetime antipsychotic drug use and mRNA expression levels (beta $3.0 \times 10^{-6}, P < 0.411$) (Figure 6). Furthermore, multiple regression analysis revealed no evidence to suggest a correlation between antipsychotic drug use and mRNA expression in patients with bipolar disorder (data not shown).

DISCUSSION

Patients with schizophrenia consistently display deficits in cognitive abilities, including attention, executive functioning, memory and language.32–34 These cognitive deficiencies have
primarily been associated with a dysfunction of the prefrontal cortex. Accordingly, evidence gathered from various neuropsychological, neuroimaging, histopathological and neurochemical investigations have implicated the dorsolateral prefrontal cortex in the pathophysiology of schizophrenia. However, the physiological mechanisms underlying such disturbances remain unclear. As the synapsin family of phosphoproteins are involved in neurotransmitter release, synapse formation and synapse maintenance, dysfunction of these molecules in the prefrontal cortex may cause aberrant neurotransmitter release and has been suggested to have a role in the pathology of schizophrenia.
et al., antipsychotic drug intake in patients with schizophrenia. Con-
isoform IIa is positively correlated with lifetime cumulative
expression. Factors via multiple regression analysis in our study of synapsin II
alcohol use and smoking status, can affect the integrity of RNA
mortem interval, cause of death, history of substance abuse,
sources. Although potential variables, such as age, brain pH, post-
expression levels comparable with those observed in control
these reductions are specific to schizophrenia, as patients with
the hypothesis that lifetime antipsyhotic drug use would have a
greater effect on synapsin isoform IIa over IIb in the dorsolateral
prefrontal cortex, our results support the hypothesis that lifetime antipsychotic drug use would have a
greater effect on synapsin isoform IIa over IIb in the dorsolateral
prefrontal cortex (Figures 5 and 6).
Furthermore, the present investigation further showed that
these reductions are specific to schizophrenia, as patients with
bipolar disorder exhibit prefrontal cortical synapsin II mRNA expression levels comparable with those observed in control
subjects. Although potential variables, such as age, brain pH, post-
mortem interval, cause of death, history of substance abuse,
alcohol use and smoking status, can affect the integrity of RNA
and gene expression, these were not found to be confounding
factors via multiple regression analysis in our study of synapsin II
expression.
The present study also provides evidence that the synapsin
isoform IIa is positively correlated with lifetime cumulative
antipsychotic drug intake in patients with schizophrenia. Con-
versely, Mirnics et al. demonstrated that antipsychotic agents
had no effect on the prefrontal cortical mRNA concentrations of
these phosphoproteins, contradicting the findings of this study and
of previously published preclinical and human experiments. In the Mirnics et al.’s study, synapsin II gene expression remained unchanged in the prefrontal cortices of monkeys chronically treated with haloperidol. However, the sample size (n = 2) used to obtain those results was small.
Furthermore, haloperidol plasma concentrations administered
were in the lower range (5 ng ml⁻¹) of its therapeutic window
(5–12 ng ml⁻¹), and analyses of individual synapsin II isoforms
were not distinguished in these monkeys, which may explain why
these results contradict the present observations.
Another study by Imai et al. also reported that prefrontal
cortical synapsin II mRNA concentrations were similar between
patients with schizophrenia and healthy controls. However, a
number of technical differences may also account for the
discrepant observations. Firstly, these previous investigations
evaluated prefrontal cortical synapsin II expression in only six
patients with schizophrenia, and such a small sample size may not
be sensitive enough to detect underlying differences. Our current
investigation utilizes a much larger population of patients with
schizophrenia and may consequently provide a more accurate
representation of the general population. Secondly, the investi-
gators did not provide patient information, such as antipsychotic
drug treatment and dosage, both of which have shown to have
significant roles in our findings. Thirdly, the study by Imai et al.
did not distinguish between the synapsin IIa and IIb isoforms.
Our observations support a differential effect on synapsin IIa and IIb isoforms, both in the disease of schizophrenia and in the
treatment with antipsychotic drugs, distinctions which were
paramount in our findings. Results in this extensive study have
taken into account various confounding factors and have utilized
a significantly larger sample size of the population affected with
schizophrenia. As such, we contend that results from this study
may depict a more accurate representation of the role of synapsin
II in this patient population.

Previous studies from other investigators have utilized these
same specimens for analysis of expression and alterations of
various other genes in schizophrenia, including catecholamine-
regulated protein/mortalin, carboxyl-terminal PDZ ligand of
neuronal nitric oxide synthase, tryptophan hydroxylase 2,
Sprouty2 and the human endogenous retrovirus-K10. Despite the apparent strengths in this investigation, there are,
regrettably, some unavoidable shortcomings. Although appro-
priate statistical analyses have been performed in this study to
assess the effect of antipsychotic drugs in schizophrenia and
bipolar disorder, it is not feasible to account for the potential roles
of individual antipsychotic drug use and other additional types of
medications used to treat these disorders. Due to the complexity
of the disorder, patients are often treated with multiple types and/
or classes of drugs. Further, although this study utilized a relatively large sample size, future investigations can further strengthen the results of this study in larger clinical populations to better address the influence of various treatments prescribed for schizophrenia. A characteristic shortcoming of human post-mortem studies in schizophrenia is the lack of availability of patient tissue that has not been exposed to antipsychotic drugs or any other medications. As such, a direct association cannot be made for synapsin II expression levels in untreated patients with schizophrenia. Finally, the phosphorylated state of synapsin II also has an important role in its regulation of synaptic vesicles and is a fundamentally important question that should be addressed. However, at present time, no specific antibodies are available for the direct measure of phosphorylated synapsin II in these human post-mortem samples. Until such tools and specimens are available, a direct measure of phosphorylation in synapsin II cannot be performed.

Clinical potency of existing antipsychotic drugs has been directly correlated to the drug’s ability to antagonize dopamine D2 receptors. However, antagonism of a receptor is achieved instantaneously, whereas the beneficial effect of antipsychotic drugs shows a delayed therapeutic effect. As such, the therapeutic effect of antipsychotic drugs must be explained by a more complicated and gradual mechanism, which can account for the delayed therapeutic effects. Recent evidence suggests modification of synaptic connections and synaptic proteins by antipsychotic drugs as a more likely explanation for the therapeutic effects seen in antipsychotic drug use. Previous studies from our laboratory have shown that antipsychotic drug use induces increases in synapsin II levels in neuronal cells, through the cyclic adenosine-3',5'-monophosphate/protein kinase A-dependent mechanism involving the AP-2α transcription factor. In addition to changes in levels of synapsin II, other studies have observed synaptic plasticity and neuronal remodeling in various regions of the brain, including the prefrontal cortex and nucleus accumbens, as a result of antipsychotic drug use. Both classes of antipsychotic drugs have been found to reverse the markers of pathology in schizophrenia, by increasing brain volume, and inducing neuroplasticity by affecting synaptic rearrangements (that is, increasing synaptic connections, activity and density) and altering gene expression (that is, increasing synthesis of synaptic proteins, such as synapsin II) in the respective brain regions.

Antipsychotic drugs do not induce change in synaptic mechanisms directly, but rather modulate other neurotransmitter systems to do so, thus suggesting indirect modulation of synaptic activity. Mechanistically, antagonism of dopamine D2 receptors (in addition to other receptor types, depending on the antipsychotic drug administered) can lead to subsequent glutamatergic signaling through local circuits or long-loop neurotransmitter pathways. Glutamate receptor activity has an important role in synapse formation and stabilization. A reduced number of glutamate receptors and activity leads to the ‘synaptic disorder’ in schizophrenia, whereas an increase in this activity is directly (or indirectly) beneficiary. Antipsychotic drugs can lead to synaptic plasticity and thus an attenuation of the behavioral symptoms and biochemical markers of schizophrenia.

In conclusion, results indicate that dorsolateral prefrontal cortical synapsin IIa and IIb gene expression is specifically reduced in patients with schizophrenia and that prefrontal cortical synapsin IIa is preferentially upregulated by antipsychotic agents. Given that dorsolateral prefrontal cortical synaptic aberrations are also associated with the deficits in attention, executive function and other cognitive abilities seen in schizophrenia, our results may contribute to a better understanding of the role of synapsin II in the development of hypo-frontal activity in patients with schizophrenia. Clinical studies examining the relationship between reductions in prefrontal cortical synapsin II gene expression and deficiencies in the cognitive functions observed in schizophrenia are, however, clearly warranted. The present study also provides insight into the therapeutic and potentially adverse mechanisms of action with use of antipsychotic drugs at the synaptic level. Additional roles played by other synapsin isoforms have, however, yet to be established. Although this study alludes to a fundamental role of synapsin II in the underlying pathophysiology of schizophrenia, the exact mechanistic pathways underlying remain to be identified in future studies. The understanding of synapsin II in mediating the dynamics of synaptogenesis and synaptic vesicle regulation remains critical to unraveling the enigma of schizophrenia. However, given our understanding of abnormal synaptic function and projections in schizophrenia, and the importance of synaptic mechanisms in the treatment of schizophrenia, the development of novel antipsychotic drugs, which target synaptic remodeling, will be particularly beneficial. Results from our study support the notion that modulation of synapsin II and thus neuroplasticity, in specific brain regions, may be relevant to the design of an effective antipsychotic drug for schizophrenia. The further understanding of synapsin II in these synaptic mechanisms, at both the levels of disease production and treatment design, will facilitate the production of more specific targets and potentially quicker-acting drugs in comparison to existing antipsychotic drugs in the market.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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