Up-regulation of the D1 Dopamine Receptor–Interacting Protein, Calcyon, in Patients With Schizophrenia

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Background: The dopamine hypothesis remains a prominent influence on research into the pathogenesis of schizophrenia, yet the presence of consistent schizophrenia-linked abnormalities in the presynaptic components of the dopamine system or in dopamine receptors still remains a matter of debate. The present study focuses on a recently recognized group of dopamine receptor–interacting proteins as possible novel sites of dysfunction in schizophrenia. Specifically, we examined whether the D1 dopamine receptor–interacting protein calcyon and the D2 dopamine receptor–interacting proteins filamin-A and spinophilin are affected in the dorsolateral prefrontal cortex of patients with schizophrenia.

Methods: Slot blots of dorsolateral prefrontal cortical tissue were used to compare the levels of the 3 proteins of interest in control, schizophrenic, bipolar, and major depression groups (n=15 per group). The nonschizophrenic psychiatric groups were included to determine the specificity of the detected abnormalities.

Results: The dorsolateral prefrontal cortex in schizophrenic patients displayed nearly twice the normal levels of calcyon, whereas filamin-A and spinophilin levels were unaltered. Patients with bipolar disorder or major depression showed no changes in all 3 proteins examined.

Conclusion: Our findings provide the first evidence that abnormalities in the dopamine system of patients with schizophrenia may lie in altered levels of dopamine receptor–interacting proteins.

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The dopamine hypothesis continues to guide biological research in schizophrenia despite difficulties in identifying a clear disease-associated abnormality in the presynaptic or postsynaptic components of the brain’s dopamine system. This hypothesis is founded in large part on the therapeutic efficacy of antipsychotic medications, universally attributed to their action at D2 dopamine receptors. It has been argued that antipsychotic drug treatment may also affect D1 dopamine receptors. Furthermore, D1 receptor involvement in schizophrenia is suggested by the ability of D1 receptor–acting drugs to alter working memory performance, an abnormality that is now widely recognized to be a core feature of schizophrenia. Both suppression and overstimulation of D1 receptor activity have been shown to result in working memory deficits.

Dopamine receptors are members of the family of G-protein–linked receptors, which regulate intracellular signaling by directly interacting with these proteins. It has recently become apparent, however, that G-protein–linked receptors can also interact with multiple additional partners that modify and expand their functionality. The present study addressed the hypothesis that abnormalities in the dopamine system of patients with schizophrenia might lie in altered levels of dopamine receptor–interacting proteins. Specifically, we focused on 3 such proteins: the D1 receptor–interacting protein calcyon and the D2 receptor–interacting proteins filamin-A and spinophilin. Calcyon potentiates cross talk between G1-coupled receptors of the D1 class and heterologous coupled receptors and enables D1 receptors to stimulate the phosphoinositide signaling cascade in addition to cyclic adenosine monophosphate–associated intracellular pathways. Calcyon also regulates D1 receptor agonist affinity states. Filamin-A is known to interact with the D2 class to act as scaffolds for assembling various components of D2 dopamine receptor signaling complexes. In addition, filamin-A promotes clustering of D2 recep-
The levels of these proteins in the dorsolateral prefrontal cortex were compared in control, schizophrenic, bipolar, and major depressive individuals. The dorsolateral prefrontal cortex was chosen for analysis as one of the major brain areas implicated in the psychopathologic characteristics of schizophrenia. Tissue samples from nonschizophrenic psychiatric patients were included in the study to determine the specificity of any detected abnormalities. This study demonstrates that prefrontal cortical tissue from schizophrenic patients displays significantly increased levels of the D1 dopamine receptor–interacting protein calcyon. It is tempting to speculate that our findings may lead to identification of one of the core pathologic changes in the dopamine system of schizophrenic patients predicted by the dopamine hypothesis of this disease.

**METHODS**

**HUMAN SAMPLES**

Samples of the dorsolateral prefrontal cortex (Brodmann area 46) of control, schizophrenic, bipolar, and major depressive individuals (n=15 per group) were provided by the Stanley Foundation Neuropsychiatry Consortium (Bethesda, Md). The groups were matched for age, sex, ethnicity, brain hemisphere, tissue pH, and postmortem interval (for details see Torrey et al and Knable et al). All samples were received as sets of 6 consecutive 40-µm-thick cryostat-cut sections that remained frozen after sectioning. All cortical tissue from 5 of the sections was cut out and homogenized in 0.5 mL of ice-cold 0.5M Tris hydrochloride (pH 7.6) containing 0.1M sodium chloride and 0.1mM phenylmethylsulfonyl fluoride and then processed for quantitative analysis of specific proteins. The sixth section was stained with cresyl violet and used for estimation of the proportion of neuronal cells in the cortical samples. All of the analyses in this study were performed in a masked manner, with diagnosis of the patients being coded and held by Stanley Foundation Neuropsychiatry Consortium personnel until all data were collected.

**NONHUMAN PRIMATE SAMPLES**

Five 5-year-old rhesus monkeys received haloperidol (0.175 mg/kg) orally (in fruit treats) twice a day for 6 to 8 months. The selected dose is within the therapeutic range given to psychiatric patients during maintenance treatment. Previously, we demonstrated that this treatment results in up-regulation of D2 dopamine receptors and down-regulation of D1 receptors in the prefrontal cortex. Five age-matched monkeys receiving drug-free fruit treats for the same period served as a control group. On completion of treatment, the animals were humanely killed by overdose of pentobarbital sodium (Abbott Laboratories, North Chicago, Ill), and the dorsolateral prefrontal cortex (Brodmann area 46) was dissected out. A portion of the dissected tissue was homogenized as described in the previous subsection for the human samples. The remaining tissue was frozen, sectioned on a cryostat into 40-µm sections, and processed for cresyl violet staining.

**ANTIBODIES**

The primary antibodies used in this study were rabbit polyclonal antibodies to calcyon (created and affinity purified in-house; described by Lezcano et al), mouse monoclonal antibodies MAB1680 to filamin-A (Chemicon International, Temecula, Calif) (described by Aakhus et al), and rabbit polyclonal antibodies RU-144 to spinophilin (described by Allen et al and Lidow et al). To evaluate the specificity of these antibodies, we examined their labeling of Western blots of the dorsolateral prefrontal cortical tissue from 5 monkey and 3 randomly selected human cases used in this study. For this purpose, the tissue homogenates (3 µg per well) were loaded into ready-made 4% to 15% gradient sodium dodecyl sulfate gels (Bio-Rad Laboratories, Hercules, Calif) and run for 1.5 hours at 100 V using a Ready Gel Cell with Tris/glycine/sodium-dodecyl sulfate running buffer (Bio-Rad Laboratories). Separated proteins were transferred to polyvinylidene difluoride membranes (Osmonics, Minnetonka, Minn) for 2 hours at 100 V also using a Ready Gel Cell with Tris/glycine buffer (Bio-Rad Laboratories). Before immunostaining, membranes were preincubated for 1 hour at room temperature in blocking solution consisting of 0.1M phosphate buffer (pH 7.6), 5% fat-free dry milk, and 0.1% Tween 20. Incubation with the primary antibodies for calcyon (8 µg/mL), filamin-A (5 µg/mL), or spinophilin (5 µg/mL) in the same blocking solution was conducted overnight at 4°C. The membranes were next exposed for 1.5 hours at room temperature to horse-radish peroxidase–conjugated goat anti–rabbit or anti–mouse secondary antibodies (Sigma-Aldrich Corp, St Louis, Mo) (1 µg/mL) in the blocking solution described herein. Visualization of the labeling was conducted using a chemiluminescence substrate (Super Signal; Pierce Biotechnology, Rockford, Ill). The images were produced by opposing transparent plastic-wrapped chemiluminescence-soaked membranes to an autoradiography film (X-Omat AR; Eastman Kodak Co, Rochester, NY) for 1 to 45 minutes. In addition, antibody specificity was tested by preabsorption with peptides (100 µg/mL) against which the antibodies were raised. The preabsorbed antibodies were applied to slot blots of prefrontal cortical tissue from 5 monkeys and 3 randomly selected human cases in a manner described in the following subsections.

**SLOT BLOTS FOR PROTEIN ANALYSIS**

The levels of calcyon, filamin-A, and spinophilin in the sample homogenates were evaluated using slot blots on nitrocellulose membranes (NitroPure; Osmonics) prepared using a microfiltration apparatus (Bio-Dot SF; Bio-Rad Laboratories). For blotting, a portion of each homogenate was diluted 1:300 with 0.5M Tris hydrochloride buffer (pH 7.6) containing 0.1M sodium chloride and 0.1mM phenylmethylsulfonyl fluoride. The resultant solution was used per slot. For analysis of the human samples, 3 sets of 15 membranes (each containing blots of 12 samples in triplicate) were generated for examination of calcyon, filamin-A, and spinophilin. Within every set, each of the 60 samples used in this study was blotted on 3 different membranes. For nonhuman primate tissue, 3 membranes were produced for each of the 3 proteins examined, with every membrane containing triplicate blots of all 10 nonhuman primate samples generated in this study. In addition to sample blots, all membranes included blots of 6 dilutions of specially prepared ‘standard’ homogenate of the human prefrontal cortex containing 4200, 2100, 1050, 525, 262.5, and 131.25 ng of total protein (measured using a protein quantification kit [CBQCA; Molecular Probes Inc, Eugene, Ore] and a fluorescent plate reader [Gemini XS; Molecular Devices Corp, Sunnyvale, Calif]). The amounts of protein in these standards were selected based on our preliminary studies as providing for immuno/total protein staining in a linear range (sample blots usually contained 900-500 ng of total protein). Each membrane also included 3 blots made with pure 0.5M Tris hydrochloride (pH 7.6) buffer and 3 blots made with 2000 ng of bo-
vine serum albumin prepared as tissue samples to check background staining.

Before immunostaining, each membrane was incubated for 15 minutes with a fluorescent stain (SYPRO Rose Plus; Molecular Probes Inc), visualizing the total protein within each slot blot. Fluorescence was observed using a UV photo viewer illumination system (Ultra-Lum Inc, Claremont, Calif) at a 365-nm wavelength, and the total protein levels in each sample slot blot were determined densitometrically using universal software (Advanced American Biotechnology & Imaging, Fullerton, Calif) by comparing the intensities of its staining with those of the 6 standard blots. A typical example of a membrane stained for total protein is presented in Figure 1A.

Immunodetection of the specific proteins on the same membranes was performed as described previously for the Western blots. The result film images of the immunostained blots were digitized and analyzed using densitometric universal software (Advanced American Biotechnology & Imaging). The amounts of specific proteins in slot blots of the samples were assessed by comparing the gray value of the images of these blots with those of the 6 standard blots of the standard tissue homogenates. Levels of specific protein in each blot were then expressed in equivalents of nanograms of total protein in standard blots producing the same immunostaining intensity. A typical example of a film image of a membrane immunostained for calcyon is presented in Figure 1B.

SLOT BLOTS FOR DNA ANALYSIS

DNA slot blots were generated on a set of nylon membranes (Hybond N; Amersham Biosciences Corp, Piscataway, NJ) as described by Lidow et al.28 Before blotting, a portion of each sample homogenate was diluted 1:300 in a 90mM citrate buffer containing 0.9M sodium chloride and ribonuclease A (40 µg/mL), with 200 µL of the resultant solution being used per slot blot. As in the case of the blots for protein analysis, each sample used in this study was blotted in triplicate on 3 different membranes. All membranes also included a set of 6 standard blots of 300, 150, 75, 37.5, 18.75, and 9.37 ng of salmon sperm DNA (Sigma-Aldrich Corp), which produced staining intensities in a linear range (sample blots contained 60-30 ng of DNA). In addition, membranes included "background" blots of 2000 ng of bovine serum albumin. The blots were stained with DNA-specific blot stain (SYBR DX; Molecular Probes Inc), visualizing the total protein within each membrane resulting from calcyon-specific immunolabeling. In A and B, specimens 1 through 12 are triplicate blots from different tissue samples. Six "standard" blots were made of specially prepared human cortical homogenates with known amounts of the total protein. The "background" blots contain either pure Tris hydrochloride buffer or bovine serum albumin (BSA). C, Typical film images produced by the Western blots of the proteins examined in this study. These images show only bands with the molecular weight appropriate for the specific protein being visualized.

Determination of the proportion of neurons in the tissue

Estimation of the proportion of neurons in the cortical samples used in this study was based on 3-dimensional cell counting. The counting was performed in 1 cresyl violet–stained section per sample as outlined by Lidow et al.29 For each section, the counting was performed in 5 nonoverlapping, randomly selected counting boxes (55 × 8 µm) stretching across the entire thickness of the cerebral cortex from the pial surface to the white matter. The total number of cell nuclei and the number of neuronal nuclei were obtained for every counting box, and then the mean proportion of neurons among the total cell nuclei was calculated for each case. Neuronal nuclei were identified based on the criteria of Selemon et al.40

Expression of the data and statistical analysis

Levels of calcyon, filamin-A, and spinophilin are expressed in 2 ways. First, the levels are expressed per nanogram of the total protein in a sample, calculated by dividing the mean levels of specific proteins in slot blots from a given sample by the mean levels of the total protein in the same blots. Second, the levels of these proteins are expressed per nanogram of neuronal DNA, which stands for the expression of specific proteins per neuron in the tissue. Because it is not possible to determine precisely the number of cells in the tissue samples used for blots, the closest representation of the quantity of a given protein per
neuron is to divide the amount of this protein by the DNA amount in the same sample and to multiply by the proportion of neuronal cells in the sample tissue (we selected DNA to represent cells in this study because careful evaluation of the literature showed that DNA complement is least susceptible to alterations due to mental disease, demographic, or psychopharmacologic effects compared with all known general cellular or neuron-specific proteins). In practice, we divided the mean levels of specific proteins in the protein slot blots from a given sample by the mean levels of DNA in the DNA slot blots from this sample and multiplied by the mean proportion of neurons in a section from the same tissue.

Statistical analysis of the levels of individual proteins in our diagnostic groups was conducted using 1-way analysis of variance (ANOVA) followed by Dunnett and Bonferroni post hoc tests. One-way ANOVAs were also used to evaluate whether the levels of specific proteins were affected by the cause of death (cardiopulmonary disease, accident, or suicide) and by alcohol consumption (no or light use [<1 drink daily], moderate use [1-2 drinks daily], or heavy use [>2 drinks daily]). In addition, a 1-way ANOVA was used to compare the effects of treatments with typical, atypical, and a combination of typical and atypical antipsychotic drugs. Correlation analysis was used to examine possible effects of age, lifetime phenothiazine equivalent, postmortem interval, and brain pH. Effects of sex, right vs left hemisphere, exposure to antipsychotic drugs at the time of death, and haloperidol treatment on the levels of individual proteins in nonhuman primates were assessed using 2-tailed t tests. Because all but 4 of the patients examined were white, the effects of race were not examined. The study also included too few nonalcohol drug abusers to allow a meaningful evaluation of the possible effects of drug abuse.

**RESULTS**

**ANALYSIS OF ANTIBODY SPECIFICITY**

Immunostaining of Western blots of monkey or human prefrontal cortical tissue with calcyon-specific antibodies revealed one strong band with a molecular weight of approximately 34 kd and another much weaker band with a molecular weight of approximately 28 kd (Figure 1C). These bands correspond with those produced by calcyon modified by N-linked oligosaccharides and described previously in blots from the monkey prefrontal cortex. The filamin-A-specific antibodies also visualized 2 bands: a heavy band at approximately 280 kd and a lighter band at approximately 90 kd (Figure 1C). The first band corresponds to the molecular weight of a filamin-A monomer. The second band represents degradation product of filamin-A containing the antigen sequence for MAR1680 antibodies, which has been shown to result from calpain and trypsin proteolyses. Antibodies to spinophilin produced a single band of approximately 140 kd (Figure 1C), which corresponds to the molecular weight of this protein. In addition, the preadsorption of all 3 primary antibodies used in this study with peptides against which these antibodies were raised fully prevented immunolabeling of slot blots of human or monkey tissue (data not shown).

**ESTIMATION OF THE PROPORTION OF NEURONS IN THE SAMPLES**

Cell counting was performed solely to determine the proportions of neurons in the tissue samples needed for calculation of the levels of specific proteins per neuronal DNA. A more detailed evaluation of cell density in cortical tissue from the Stanley Foundation Neuropathology Consortium has recently been published by Cotter et al. Herein, we report that the mean±SD percentage of neuronal nuclei among all cell nuclei in sections of dorsolateral prefrontal cortex from the schizophrenic (44.62%±7.7%), bipolar (40.09%±8.9%), and major depressive (42.01%±7.1%) groups was within 5% of the mean±SD percentage of neuronal nuclei in sections from the control group (39.95%±7.4%), which is in agreement with investigations from this and other groups. In addition, in agreement with findings from previous studies, haloperidol treatment changed the percentage of neuronal cells in the monkey dorsolateral prefrontal cortex by less than 3% (mean±SD 55.3%±4.3% in control animals vs 56.6%±5.1% in haloperidol-treated animals).

**EXAMINATION OF TISSUE LEVELS OF CALCYON, FILAMIN-A, AND SPINOPHILIN**

Results of ANOVA of the dorsolateral prefrontal cortical levels of calcyon showed significant differences between diagnostic groups examined, whether these levels are expressed per total protein (P=.046) or per neuronal DNA (P=.02). Dunnett post hoc comparison between the control and disease groups revealed significant up-regulation of calcyon levels (which nearly doubled) in the schizophrenic group, with no such changes in either the bipolar or the major depression groups (Figure 2). Further analysis using the Bonferroni test not only supported this finding but also demonstrated that calcyon levels in schizophrenic patients were significantly higher than those in patients with bipolar disorder or major depression (eg, for expression per neuronal DNA, P=.047 for the difference between the schizophrenia and bipolar groups and P=.01 for the difference between the schizophrenia and major depression groups). Results of ANOVAs of the dorsolateral prefrontal cortical levels of filamin-A and spinophilin showed no significant differences between the diagnostic groups examined (for filamin-A expressed per total protein, P=.48; for filamin-A expressed per neuronal DNA, P=.21; for spinophilin expressed per total protein, P=.12; and for spinophilin expressed per neuronal DNA, P=.46) (Figure 2).

Antipsychotic drugs are the major group of medications used to treat schizophrenia. Therefore, we looked for a possible relationship between exposure to these medications at the time of death and levels of the proteins examined in this study. This evaluation showed that the patients taking antipsychotic medications at the time of death were significantly more prone to having higher levels of calcyon than the antipsychotic-free individuals (Figure 3). The latter conclusion was independent of whether the levels of calcyon were expressed per total protein or per neuronal DNA. Furthermore, there were no differences in the mean±SD levels of calcyon in patients taking typical (23.98±4.10) or atypical (26.12±5.68) antipsychotic drugs or their combination (29.88±7.56) (expressed per neuronal DNA, P=.77 by 1-way ANOVA). Because
Compared with controls are seen only in the schizophrenia group. Statistically significant increases in calcyon levels (marked by asterisks) are observed in the antipsychotic drug haloperidol-treated animals displayed a significant (∼25%) increase in the levels of spinophilin (Table 1). Only 3 patients in the schizophrenic group were drug-free, we could not perform a meaningful comparison between treated and untreated patients in the schizophrenic cohort. However, we addressed this issue in 2 important ways. First, there were sufficient numbers of nonschizophrenic antipsychotic-exposed and antipsychotic-free cases to conduct such an analysis. There were no significant differences in calcyon levels in these 2 groups (Figure 3). Second, we compared the levels of calcyon in the dorsolateral cerebral cortex of rhesus monkeys treated long term with the antipsychotic drug haloperidol with those in monkeys receiving placebo for the same period. Haloperidol was selected as a widely used representative antipsychotic drug because, as mentioned previously, we detected no differences between calcyon levels in patients taking typical vs atypical antipsychotic medications. Haloperidol treatment did not significantly affect calcyon levels (Table 1). Comparison of filamin-A and spinophilin between the human cases exposed vs unexposed to antipsychotic medications did not show any significant effects of these medications (data not shown). This result might be expected based on the present finding of a lack of difference between levels of these proteins in the drug-naive control group. The effects of exposure to antipsychotic (AP) drugs at the time of death on levels of calcyon in samples of dorsolateral prefrontal cortex from patients from the Stanley Foundation Neuropathology Consortium. Mean calcyon levels are expressed per nanogram of total protein (A) and per nanogram of neuronal DNA (B). P values show the significance of 2-tailed t tests. Asterisk indicates statistically significant differences. Comparing all patients taking AP drugs at the time of death (n=20) with individuals receiving no such medications (n=40), the differences are statistically significant. However, these differences disappear when AP-exposed (n=8) and non-AP (n=37) patients are compared in the nonschizophrenic group. Error bars represent SD.

Figure 2. Levels of calcyon, filamin-A, and spinophilin in the dorsolateral prefrontal cortex in the control (NORM), schizophrenia (SCH), bipolar (BP), and major depression (MD) groups of the Stanley Foundation Neuropathology Consortium. Each group includes 15 patients. The levels of all 3 specific proteins are expressed per nanogram of total protein and per nanogram of neuronal DNA. Circles represent data from individual samples; horizontal lines, the mean values for the group; error bars, SD. P values show the statistical significance of Dunnett comparisons after 1-way analysis of variance preceding the Dunnett tests: P = .046 for calcyon expressed per total protein, P = .02 for calcyon expressed per neuronal DNA, P = .48 for filamin-A expressed per total protein, P = .21 for filamin-A expressed per neuronal DNA, P = .12 for spinophilin expressed per total protein, and P = .46 for spinophilin expressed per neuronal DNA). Statistically significant increases in calcyon levels (marked by asterisks) compared with controls are seen only in the schizophrenia group.

Figure 3. The effects of exposure to antipsychotic (AP) drugs at the time of death on levels of calcyon in samples of dorsolateral prefrontal cortex from patients from the Stanley Foundation Neuropathology Consortium. Mean calcyon levels are expressed per nanogram of total protein (A) and per nanogram of neuronal DNA (B). P values show the significance of 2-tailed t tests. Asterisk indicates statistically significant differences. Comparing all patients taking AP drugs at the time of death (n=20) with individuals receiving no such medications (n=40), the differences are statistically significant. However, these differences disappear when AP-exposed (n=8) and non-AP (n=37) patients are compared in the nonschizophrenic group. Error bars represent SD.
We detected no correlation between the levels of any of the specific proteins selected for this study and lifetime consumption of antipsychotic medications in fluphenazine equivalents. Furthermore, the levels of these proteins were not correlated with age, duration of the postmortem period, or the value of brain pH (such an analysis for calcyon is presented in Figure 4). Finally, the levels of these proteins were not affected by sex, brain hemisphere, the 3 most often encountered causes of death (cardiopulmonary disease, accident, and suicide), or alcohol consumption (examples of these analyses for calcyon, expressed per neuronal DNA, are given in Table 2).

The main finding of this study is that the levels of the D1 dopamine receptor–interacting protein calcyon in the dorsolateral prefrontal cortex of schizophrenic patients are nearly twice those found in control subjects. The increase was specific for this group of patients and was not observed in groups of bipolar and major depressive patients. These observations were made when the data were expressed per both normalizing factors selected for this study: total protein and neuronal DNA. The fact that elevated levels of calcyon were detected in schizophrenic patients under both modes of expression, together with the absence of changes in the 2 other proteins examined in this study, rules out general changes in total protein content as an explanation for the observed increase in calcyon levels.

Because calcyon is expressed in neuronal and glial cells26 we evaluated the possibility that the elevation observed in this protein might simply reflect a shift in the relative proportion of neuronal and glial cells in the prefrontal cortex of schizophrenic patients. This does not seem to be the case, however, because the calculated alteration in the percentage of neurons among neuronal and glial cells in the prefrontal cortex of the schizophrenia group is approximately 5%, whereas the observed increase in calcyon levels is nearly 2-fold. In addition, be-

Table 1. Effects of Long-term Haloperidol Treatment on Levels of Calcyon, Filamin-A, and Spinophilin in the Dorsolateral Prefrontal Cortex of Rhesus Monkeys*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Haloperidol-Naive Monkeys (n = 5)</th>
<th>Haloperidol-Treated Monkeys (n = 5)</th>
<th>P Value (t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcyon</td>
<td>15.83 ± 3.73</td>
<td>14.70 ± 6.96</td>
<td>.57</td>
</tr>
<tr>
<td>Filamin-A</td>
<td>11.77 ± 7.12</td>
<td>9.82 ± 8.34</td>
<td>.72</td>
</tr>
<tr>
<td>Spinophilin</td>
<td>27.32 ± 4.90</td>
<td>22.28 ± 3.11</td>
<td>.02†</td>
</tr>
</tbody>
</table>

*Data are given as mean ± SD per nanogram of neuronal DNA. †Statistically significant.

Figure 4. Correlation analyses of the levels of calcyon in the dorsolateral prefrontal cortex of patients from the Stanley Foundation Neuropathology Consortium expressed per nanogram of neuronal DNA in relation to age, postmortem period (PMI), brain pH, and the lifetime dosage of antipsychotic drugs in fluphenazine hydrochloride equivalents. There are no significant correlations between calcyon levels and any of the 4 parameters examined.
cause cortical afferents display almost no calcyon, the elevated levels of this protein cannot be attributed to an increase in the number of neuronal fibers innervating the prefrontal cortex in the affected cases. It is likely, therefore, that the observed increase in calcyon levels in the schizophrenic patients reflects a true increase in the expression of this protein in individual cortical neurons.

We also considered whether elevated levels of calcyon could be affected by demographic and tissue preservation parameters, which were carefully documented by the Stanley Foundation Neuropathology Consortium. Based on this documentation, brain hemisphere, age, sex, cause of death, and alcohol use were not associated in any systematic way with calcyon levels. Likewise, there was no correlation between the levels of calcyon and the duration of the postmortem period, brain pH, and the amount of antipsychotic medication taken during the patient’s lifetime. In addition, the individuals in this study were virtually homogeneous in relation to race, and most of them were not nonalcohol substance abusers.

Aside from diagnosis, the only parameter that was predictive of an increase in prefrontal calcyon levels was exposure to antipsychotic medications at the time of death, whether patients were taking typical or atypical antipsychotic drugs or a combination of the two. It is likely, however, that this association may reflect the fact that almost all schizophrenic patients included in this study were taking these medications. Indeed, nonschizophrenic patients taking antipsychotic drugs did not display increased levels of calcyon compared with antipsychotic-free nonschizophrenic patients. We also did not detect changes in calcyon levels in nonhuman primates subjected to long-term antipsychotic treatment. Although each of the aforementioned analyses by itself may not be sufficient to prove the absence of effects of antipsychotic drugs, together they suggest that these medications are unlikely to be the cause of the alterations in the cortical levels of calcyon. It is also interesting that the levels of calcyon in haloperidol-treated monkeys did not change despite down-regulation of D1 receptors in these animals. Furthermore, Lidow et al recently found that transfection of calcyon into D1-expressing cells did not affect the levels of these receptors. These observations suggest that calcyon and D1 receptors do not regulate expression of one another. Indeed, it is still unclear which, if any, changes in the levels of prefrontal cortical D1 receptors are associated with schizophrenia, since, to date, findings from positron emission tomography have been inconsistent, reporting down-regulation,64 up-regulation,65 and no alterations46 in these sites in antipsychotic-free schizophrenic patients. Furthermore, examinations of the prefrontal cortex of patients with schizophrenia from the Stanley Foundation Neuropathology Consortium revealed normal levels of D1 receptor message. Therefore, at this time, it is too early to discuss a combination of changes in levels of calcyon and D1 receptors in schizophrenia.

In contrast to the finding in most studies of decreased expression of specific markers in schizophrenic brains, our findings point to a significant increase in calcyon levels in this disease. Such an increase may indicate that calcyon is causally involved in producing the disorganization of brain functionality in schizophrenia. Alternatively, the observed increase in calcyon expression may be a compensatory reaction of the nervous system to schizophrenia-induced deficits. Only future investigations can resolve this question.

Considering that calcyon allows D1 receptors to couple to phosphoinositide signaling, it is reasonable to expect that if calcyon levels are indeed elevated in the prefrontal cortex of schizophrenic patients, this tissue should show increased phosphoinositide hydrolysis in response to dopamine and D1 receptor agonists. Consistent with this idea, previous studies have demonstrated that dopamine and, particularly, the D1 receptor agonist SKF 38393 are capable of inducing much stronger increases in phosphoinositide hydrolysis in the prefrontal cortex of patients with schizophrenia than in normal control tissue. Another expectation is that the overexpression of calcyon in tissue from brains of schizophrenic patients results in a decrease in the proportion of the high-affinity sites for D1 receptor agonists because this protein has been shown to shift D1 receptors into a low agonist affinity state. Once again, a decrease in the proportion of the high-affinity sites for D1 receptor agonists has been demonstrated in brain tissue of individuals with schizophrenia.

The present study focused on the dorsolateral prefrontal cortex, the region commonly associated with deficits in the working memory of schizophrenic patients. Because D1 dopamine receptors are known to play an essential role in regulating the working memory circuits of this cortical region, we believe that it is significant that our study identified a schizophrenia-associated elevation in the levels of calcyon, a protein that specifically affects the function of these receptors.

Table 2. Effects of Sex, Brain Hemisphere, the Most Frequent Causes of Death, and Alcohol Consumption on Calcyon Levels in the Dorsolateral Prefrontal Cortex of Patients From the Stanley Foundation Neuropathology Consortium

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Calcyon, mean ± SD†</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M (n = 36)</td>
<td>21.62 ± 12.20</td>
<td>.38‡</td>
</tr>
<tr>
<td>F (n = 24)</td>
<td>18.62 ± 11.44</td>
<td></td>
</tr>
<tr>
<td>Side of the brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right (n = 27)</td>
<td>20.40 ± 11.52</td>
<td>.68‡</td>
</tr>
<tr>
<td>Left (n = 33)</td>
<td>19.38 ± 11.12</td>
<td></td>
</tr>
<tr>
<td>Cause of death</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiopulmonary disease (n = 32)</td>
<td>17.28 ± 7.62</td>
<td>.59§</td>
</tr>
<tr>
<td>Accident (n = 4)</td>
<td>21.28 ± 15.06</td>
<td></td>
</tr>
<tr>
<td>Suicide (n = 20)</td>
<td>21.90 ± 12.78</td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption at the time of death</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None/light (n = 48)</td>
<td>17.24 ± 12.74</td>
<td>.29§</td>
</tr>
<tr>
<td>Moderate (n = 5)</td>
<td>19.70 ± 10.90</td>
<td></td>
</tr>
<tr>
<td>Heavy (n = 7)</td>
<td>19.48 ± 12.28</td>
<td></td>
</tr>
</tbody>
</table>

*Calcyon levels are not affected by any of these parameters.
†Expressed per nanogram of neuronal DNA.
‡By t test.
§By 1-way analysis of variance.
*None/light indicates less than 1 drink daily; moderate, 1 to 2 drinks daily; and heavy, more than 2 drinks daily.
suggests that calcyon overexpression–induced alterations in D1 receptor functionality, such as the previously mentioned abnormal increase in the ability of D1 receptors to activate phosphoinositide signaling pathways, may affect cognitive processes carried out in the dorsolateral prefrontal cortex. Such a possibility is supported by a recent demonstration of substantial impairment in working memory associated with overactivation of protein kinase C.\textsuperscript{53,54} A kinase that is stimulated by the phosphoinositide signaling cascade.\textsuperscript{55}

Increased levels of calcyon in schizophrenia may not be confined to the prefrontal cortex but may be part of a much more global phenomenon, as is indicated by the recent preliminary study\textsuperscript{56} of the elevated expression of calcyon transcripts in the thalamus of patients with schizophrenia.

The fact that we did not observe changes in the D2 dopamine receptor–interacting proteins filamin-A and spinophilin in schizophrenia or in other mental disease groups examined in this study does not necessarily indicate that these or other D2 receptor–interacting molecules are unaffected in mental disorders. It is possible that significant disease-associated alterations in these proteins are present in other brain areas. Indeed, a schizophrenia-associated increase in the thalamic expression of spinophilin has been suggested by recent studies by Meadow-Woodruff et al.\textsuperscript{56} It is also possible that the prefrontal cortex of schizophrenic patients expressed changes in the levels of filamin-A, spinophilin, or both that, however, were too small to be statistically identified by our methods in the present sample sizes. Furthermore, at least for spinophilin, these changes may also be reduced by antipsychotic drug treatment, since we found that long-term haloperidol treatment produced small but significant reductions in the levels of this protein in healthy monkeys. Future studies in much larger groups of drug-naive schizophrenic cases are needed to resolve this question.

The present study points to the novel possibility that aberrations in the dopaminergic system of patients with schizophrenia, long predicted by the dopamine hypothesis of this disease, may lie in altered levels or functionality of specific dopamine receptor–associated proteins, such as calcyon. Furthermore, our findings allow the merger of the dopamine hypothesis of schizophrenia with other relevant hypotheses of second-messenger imbalance in this disease.\textsuperscript{57,58} By identifying abnormalities in a specific protein within the dopaminergic system of schizophrenic individuals, which likely leads to changes in the levels of certain second messengers in neuronal cells.

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