Antipsychotic Treatment Induces Alterations in Dendrite- and Spine-Associated Proteins in Dopamine-Rich Areas of the Primate Cerebral Cortex

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**Background:** Mounting evidence indicates that long-term treatment with antipsychotic medications can alter the morphology and connectivity of cellular processes in the cerebral cortex. The cytoskeleton plays an essential role in the maintenance of cellular morphology and is subject to regulation by intracellular pathways associated with neurotransmitter receptors targeted by antipsychotic drugs.

**Methods:** We have examined whether chronic treatment with the antipsychotic drug haloperidol interferes with phosphorylation state and tissue levels of a major dendritic cytoskeleton–stabilizing agent, microtubule-associated protein 2 (MAP2), as well as levels of the dendritic spine–associated protein spinophilin and the synaptic vesicle–associated protein synaptophysin in various regions of the cerebral cortex of rhesus monkeys.

**Results:** Among the cortical areas examined, the prefrontal, orbital, cingulate, motor, and entorhinal cortices displayed significant decreases in levels of spinophilin, and with the exception of the motor cortex, each of these regions also exhibited increases in the phosphorylation of MAP2. No changes were observed in either spinophilin levels or MAP2 phosphorylation in the primary visual cortex. Also, no statistically significant changes were found in tissue levels of MAP2 or synaptophysin in any of the cortical regions examined.

**Conclusions:** Our findings demonstrate that long-term haloperidol exposure alters neuronal cytoskeleton– and spine–associated proteins, particularly in dopamine-rich regions of the primate cerebral cortex, many of which have been implicated in the psychopathology of schizophrenia. The ability of haloperidol to regulate cytoskeletal proteins should be considered in evaluating the mechanisms of both its palliative actions and its side effects.

**Key Words:** Antipsychotic drugs, dendrite, spine, synapse, microtubule-associated protein 2, spinophilin, synaptophysin

**Introduction**

Since the introduction of antipsychotic medications nearly 40 years ago, the main emphasis of studies on the effects of these drugs in brain tissue has been on alterations in neurotransmitters and their receptors (for a review, see Csernansky 1996). There is, however, mounting evidence that antipsychotics may produce morphological changes in cellular elements in several regions of the brain, particularly in the association areas of the cerebral cortex (Benes et al 1985; Klinzova et al 1989, 1990; Meshul et al 1992; Uranova et al 1991; Vincent et al 1991, 1994). It has even been suggested that these slow-developing morphological changes might explain why the pharmacologic treatment of psychosis typically requires several weeks to attain its full effect and why it may take months to reverse this effect after cessation of treatment (Benes et al 1985). It has also been proposed that the antipsychotic-induced morphological alterations may underlie the side effects produced by these drugs (Kelley et al 1997; Seeman 1988). Interest in the effects of antipsychotic medications on the morphology of cortical cells is further reinforced by the discovery of alterations in the volume and organization of the neuropil in postmortem cortical tissue from schizophrenic patients (Anders 1978; Garey et al 1998; Glantz and Lewis 1997; Selemon et al 1996; Uranova et al 1996), raising the question as to whether these changes are associated with the disease itself or its treatment.

The ability of antipsychotic drugs to affect cell morphology can be predicted from their binding to dopaminergic and other neurotransmitter receptors (Seeman 1990), which are coupled to second messengers that regulate the activity of kinases and phosphatases (Kebabian and Greengard 1971; Roth et al 1998; Walsh et al 1972; Yurko-Mauro and Friedman 1995). These enzymes control the phosphorylation states of such proteins as...
microtubule-associated protein 2 (MAP2; Goldenring et al 1985; Sloboda et al 1975; Tsuyama et al 1986; Walaas and Nairn 1989). MAP2 contains multiple phosphorylatable residues, and the levels of phosphorylation of this protein are inversely proportional to its ability to stabilize dendritic microtubules (Cleveland and Hoffman 1991; Maccioni and Cambiazo 1995; Tsuyama et al 1986, 1987; Wiche et al 1991). As microtubules are among the major cytoskeletal constituents involved in the maintenance of dendritic processes (Cleveland and Hoffman 1991; Keith 1990; Shea and Beermann 1994; Yamada et al 1970), any changes in their stability could affect major cellular compartments of neurons—their dendrites, spines, and synapses. Despite the obvious importance of understanding the effects of antipsychotic drugs on the cytoskeleton of cortical cells, their effects on the phosphorylation of MAP2 and other cytoskeletal proteins in the cortex are not known.

During the past several years our laboratories have been involved in an analysis of the effects of chronic antipsychotic treatment on the integrity of the primate cortex. We have previously reported a significant upregulation of D2 receptors and downregulation of D1 receptors in the prefrontal and temporal cortical regions of the rhesus monkey brain following chronic treatment with several typical and atypical antipsychotic drugs (Lidow et al 1997; Lidow and Goldman-Rakic 1994). The cortex of these monkeys also exhibits increased glial density (Selemon et al 1999), although this increase was not induced by all of the antipsychotic drugs examined. In particular, haloperidol was not among the drugs that induced significant gliosis.

This study extends our investigations to the influence of chronic treatment with antipsychotic drugs—in this instance, haloperidol—on tissue levels and degree of phosphorylation of the dendritic cytoskeleton—stabilizing protein MAP2 in multiple regions of the monkey cerebral cortex. In addition, we examined the impact of this treatment on cortical levels of spinophilin, the dendritic spine–affiliated protein that has been implicated in linking plasma membrane–associated synaptic components, such as dopamine D1 and D2 receptors, to the actin cytoskeleton (Allen et al 1997; Smith et al 1999; Yan et al 1999). We also evaluated cortical levels of synaptopysin, a synaptic marker commonly associated with synaptic vesicles (Eastwood et al 1995; Masliah et al 1990) presumed to act as an exocytotic fusion pore (Bajjalieh and Scheller 1995; Edelmann et al 1995). Effects of haloperidol treatment on spinophilin and synaptopysin were examined because alterations in the density of spines and synapses containing these proteins have previously been noted in the cortex of haloperidol-exposed animals (Benes et al 1985; Klinzova et al 1989, 1990; Meshul et al 1992; Vincent et al 1991).

In this investigation we studied monkeys that were 15 years of age or older to model the effects of drug treatment on brain cells in a population of older patients. A parallel study is currently in progress on a younger cohort of monkeys. We report the findings on the older animals now because of the insight they provide for neural vulnerabilities in aged individuals as well as for possible implications for chronic medication in clinical practice.

**Methods and Materials**

**Animals**

This study included four haloperidol-treated and four drug-naive control female rhesus monkeys (*Macaca mulatta*). The monkeys in the drug-exposed group were 15, 19, 19, and 20 years of age, whereas the animals in the control group were 15, 16, 19, and 25 years of age. The animals were kept in individual cages in accordance with Yale Animal Use and Care Committee guidelines for nonhuman primates. All monkeys were fed High Protein Monkey Chow (Ralston Purina, Saint Louis), were given fruit twice a day, and had fresh water available ad libitum. The animals were also provided with standard enrichment devices: logs, dog toys, plastic chains, and mirrors.

**Drug Treatment and Tissue Collection**

Haloperidol (in powder form) was obtained from RBI (Natick, MA). For administration, a stock solution of haloperidol:sucrose (1:50) was prepared, from which daily doses were portioned out and given to animals within fruit treats such as pieces of banana, prune, apple, or marshmallow. The animals received the drug twice a day for a period of 1 year. During the first month of treatment the daily dose was 0.07 mg/kg. During the second month it was increased to 0.14 mg/kg. After that, the daily dose was increased to 0.20 and 0.27 and finally to 0.35 mg/kg at 2-week intervals. The latter dose was maintained throughout the rest of the treatment period. For one monkey, the final daily dose was 0.42 mg/kg, which was dictated by requirements of testing for cognitive impairments, also conducted in these animals. The final doses of haloperidol employed in this study fall within the therapeutic range given to psychiatric patients during maintenance treatment (Physicians’ Desk Reference 1999). The control animals received fruit treats only. During the entire period of treatment the animals displayed no signs of extrapyramidal side effects.

Between 12 and 18 hours after the last treatment, the animals were anesthetized with sodium pentobarbital. Their brains were rapidly removed, dissected, and immersed in liquid nitrogen for storage.

The female monkeys used in this study were cycling. Since we cannot exclude the possibility that the biochemical parameters examined may also fluctuate with the estrous cycle, vaginal smears were taken from all the animals for several days before the end of the treatment and the perfusions were conducted on the next day after the appearance of the menstrual bleeding. This assured a reasonable uniformity of the hormonal state of the
animals at the time of perfusion. Also, since the estrous cycles in rhesus monkeys display seasonal variations (Hutz et al 1985; Walker et al 1983), all the animals were perfused in the period from November through February, which is within the breeding season for these species.

Samples from the following cortical areas were analyzed:

- Frontal pole (cortical area 10; Walker 1940)
- Dorsolateral prefrontal cortex (area 46; Walker 1940)
- Dorsomedial prefrontal cortex (area 9; Walker 1940)
- Anterior orbital cortex (area 11; Walker 1940)
- Posterior orbital cortex (area 13; Walker 1940)
- Anterior cingulate cortex (area 24; Walker 1940)
- Prelimbic cortex (area 25; Walker 1940)
- Premotor cortex (lateral portion of area 6; Brodmann 1994)
- Primary motor cortex (area 4; Brodmann 1994)
- Primary visual cortex (area 17; Brodmann 1994)
- Entorhinal cortex (area 28; Brodmann 1994)

The cortical regions examined are shown in Figure 1. Samples of all brain regions were collected from both hemispheres, but those from each hemisphere were processed separately.

**Tissue Levels of MAP2, Spinophilin, and Synaptophysin**

**TISSUE HOMOGENATES.** Tissue samples were homogenized for 3 min on a TriR Homogenizer (Cole-Palmer, Vernon Hill, IL) in 100 volumes of ice-cold TBS buffer (0.5 mol/L Tris base [pH 8.0] containing 0.1 mol/L NaCl and 0.8 mmol/L phenylmethylsulfonyl fluoride).

**SLOT BLOTS FOR PROTEIN ANALYSIS.** Comparative levels of specific proteins in the sample homogenates were examined using slot blots on NitroPure membranes (Osmonics, Westbrough, MA) prepared with Bio-Dot SF Microfiltration Apparatus (Bio-Rad, Hercules, CA). Before the blotting, each tissue homogenate was diluted 1:100 with the TBS buffer, and 200 μL of the resultant solution were used per blot.

For immunolabeling, membranes were preincubated for 1 hour at room temperature in blocking solution containing 5% dry milk and 0.2% Tween, in PBS buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L NaH₂PO₄, and 1.4 mmol/L KH₂PO₄; pH 7.3). Incubation with protein-specific antibodies diluted in the same buffer was conducted overnight at 4°C. The dilution of MAP2 and spinophilin antibodies was 1:2500; for synaptophysin antibodies, the dilution was 1:1000. After incubation, the membranes were washed 2 × 5 min in PBS buffer and exposed for 1.5 hours to the secondary peroxidase-conjugated antibodies diluted 1:62500 in the blocking solution described above. Visualization of labeling was conducted with the Super Signal Chemiluminescence Substrate (Pierce, Rockford, IL). The images were produced by opposing transparent plastic-wrapped chemiluminescence-soaked membranes to an X-Omat AR Film (Kodak, Rochester, NY) for a period of 2–25 min. For analysis with Universal Software (Advanced American Biotechnology, Fullerston, CA), the film images of slot blots were digitized on a UC 1260 flat bed scanner (U-Max, Hsinchy, Taiwan). For examination of the levels of each specific protein, the tissue samples from the same brain region of the same hemisphere of all eight animals used in this study were always processed simultaneously on a single membrane and blots for every sample were done in triplicate (Figure 2B). This assured the similarity of the blot formation and immunolabeling allowing the gray values of the resultant film images to be used in the comparative analysis of the levels of specific proteins in individual blots, as long as the measured gray values had linear relationship to the amounts of the blotted antigen. To verify the linearity of the relationship between the amounts of antigen in the blots and the gray values of the resultant film images, each membrane also included...
controls for linearity, represented by six blots generated by a serial dilution of monkey brain prepared for this purpose (Figure 2B). The film images of the sample blots from a membrane were accepted for analysis only if all of them had gray value within the range of those produced by the controls for linearity and if the relationship between the gray values of the images of the linearity control blots and the tissue dilution in these blots was in the linear range. In addition, every membrane included a line of six blots produced by bovine serum albumin (BSA), prepared as the tissue samples, to check the level of background immunolabeling. The BSA blots produced no detectable images on any of the films examined in this study (Figure 2B).

**ANTIBODIES.** Specific proteins were labeled with the following antibodies: monoclonal antibodies to the high–molecular weight α and β isoforms of MAP2 (Sigma, Saint Louis), the rabbit polyclonal antibodies RU 144 to spinophilin (Allen et al 1997), and the mouse monoclonal antibodies EP10 to synaptophysin (StressGen Biotechnology, Victoria, Canada). The secondary goat antimouse and goat–antirabbit peroxidase–conjugated antibodies were purchased from Jackson Immunoresearch (West Grove, PA). The specificity of the primary antibodies was tested on Western blots of monkey cortical and striatal tissues. The homogenates were mixed 5:1, with the loading buffer containing 1.0 mol/L Tris-HCl (pH 6.8), 20% sodium dodecyl sulfate (SDS), 50% glycerol, 0.2% Bromophenol blue, and 4.9% DTT. Thirty microliters of this mixture were loaded per well in Ready-made 4–15% Gradient SDS Gels (Bio-Rad). The gels were run for 1.5 hours at 100 V using a Bio-Rad Ready Gel Cell with Bio-Rad Tris/Glycine/SDS Running Buffer. The transfer on a PVDF-Plus membrane (Osmonics) was performed at 100 A, overnight at 4°C also using a Bio-Rad Ready Gel Cell with Bio-Rad Tris/Glycine Buffer. Immunolabeling of the membranes was performed as described above for the slot blots. The specificity of all the antibodies used in this test was demonstrated by the observation that they produced labeling only in bands with molecular weight in the appropriate range (Figure 2A): MAP2; 280 kd (two bands of close molecular weight that represented α and β isoforms of this protein were visualized; Johnson and Jope 1992), spinophilin; 140kD (Allen et al 1997), and synaptophysin; 38 kD (Honer et al 1992).

**SLOT BLOTS FOR ANALYSIS OF THE DNA LEVELS.** The slot blots were used for measurement of the comparative levels of DNA in the sample homogenates. Similar to the blots for the protein analysis, the blots for analysis of DNA were prepared using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad, Hercules, CA). However, here it was loaded with the Hybond-N Nylon membrane (Amersham, Piscataway, NJ). Also, before blotting, the tissue homogenates were diluted 1:20 in 90 mmol/L citrate buffer (pH 7.0) containing 0.9 mol/L NaCl, 3.0 mmol/L EDTA, and 40 μg/mL RNase A. For visualization of the DNA, the dried membranes were soaked for 15 min in SYBR DX DNA-specific Blot Stain (Molecular Probe, Eugene, OR) mixed 1:1000 with 89 mmol/L Tris-HBO 3 buffer (pH 8.0) containing 10 mmol/L EDTA. The staining was observed with the UV Photo Viewer Illumination System (Ultra-Lum, Paramount, CA) at the wavelength of 454 nm. The system also digitized the images for densitometric analysis with Universal Software. A typical example of the digitized image of a membrane stained for DNA is presented in Figure 2C. As in the case of the blots for the protein analysis, samples from the same region of the same hemisphere were processed simultaneously for all eight animals, with blots for every sample done in triplicate. The controls for linearity

Figure 2. Representative examples of the images of the Western blots and slot blots generated in this study. (A) 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. (B) Film images generated by the spinophilin immunolabeling of slot blot samples from tissue homogenates of the right dorsolateral prefrontal cortex from four control and four haloperidol-treated animals. (C) Fluorescent images of DNA-specific–labeled slot blots from the same homogenates. 1–4, control animals from which the triplicate blots were made; 5–8, haloperidol-treated animals from which the triplicate blots were made. The background blots contained bovine serum albumin. The controls for linearity for spinophilin immunolabeling were six serial dilutions of the monkey brain tissue collected and stored for this purpose. The controls for linearity for DNA labeling were six serial dilutions of a herring sperm DNA.
MAP2 and spinophilin are produced by cortical neurons, since the overwhelming majority of cells in the brain generated no staining. DNA (Figure 2C). The background staining on each membrane was checked with six blots of BSA prepared in a manner identical to that of the experimental samples (Figure 2C). On all membranes used for densitometric analysis, the BSA blots generated no staining.

**DETERMINATION OF PROPORTION OF NEURONS IN THE TISSUE.** Since the overwhelming majority of cells in the brain are not in a process mitosis and have a single complement of identical DNA, it is reasonable to expect that the proportion of the neuronal DNA in a blot would be very close to the proportion of the neuronal cells in the tissue sample. Therefore, it was of interest to determine the proportions of the neuronal DNA in the samples collected for this study. For this purpose, three randomly cut slabs from each tissue sample were processed for direct three-dimensional counting of cell nuclei. The counting was performed as outlined in Selemon et al (1996, 1999) using a Macintosh-based computer system described in Williams and Rakic (1988b). For counting, the slabs were sliced into 80-μm sections on an HM 500 OM cryostat (Zeiss, Walldorf, Germany), and the sections were stained with cresyl violet. The counting was conducted on one section per slab. For each section, the counting was performed in two nonoverlapping randomly selected counting boxes (55 μm wide × 25 μm deep) stretching across the brain structure. Therefore, we examined six counting boxes for every tissue sample (two boxes × three separate sections). Neuronal nuclei were identified based on the criteria of Williams and Rakic (1988a). The proportions of neuronal nuclei among all the nuclei were calculated for every counting box. These data were then used for calculation of the mean proportions ± SEMs of neuronal nuclei for every brain region of each animal.

**MEASUREMENT OF TOTAL PROTEIN.** The measurement of total protein in the sample tissue homogenates was performed with a Modified Lowry Protein Assay Kit (Pierce) on a Hitachi U110 spectrophotometer (Hitachi USA, San Jose, CA).

**EXPRESSION OF THE DATA AND STATISTICAL ANALYSIS.** MAP2 and spinophilin are produced by cortical neurons, and synaptophysin, although situated presynaptically, still represents the synapses on cortical cells. Therefore, we wanted to evaluate the effects of the drug treatment on the levels of MAP2, spinophilin, and synaptophysin per neuron in every brain region examined. Since 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 specific protein per neuron is to divide the amount of this protein by the DNA amount in the same sample and multiply by the proportion of neuronal cells in the sample tissue. In practice, the mean gray values of the images generated by the protein-specific immunostaining of the blots from each sample were divided by the mean gray value of the images generated by the DNA staining of the blots from this sample and multiplied by the mean proportion of the neurons in the counting boxes in the sections also obtained from the same sample. In addition, we expressed the levels of all specific proteins examined (gray value of blot labeling) per unit of total protein in the sample.

While 22 cortical areas (11 areas per hemisphere) were examined in this study, the goal of the experiments did not include any comparison of protein levels between these areas. The basic analysis of the data, therefore, was a two-tailed t test comparing the levels of each protein in individual cortical area between control and treated animals. Given that four different proteins were measured in each cortical area, however, we adopted a conservative approach by applying a Bonferroni correction to the p values calculated from the t tests. Hence, the p value used in determining the statistical significance of each analysis was equal to the p value calculated from the t test divided by 4, which is the number of proteins analyzed in each cortical area.

**Analysis of Phosphorylation of MAP2**

**PROCESSING OF THE TISSUE.** The analysis of the effect of drug treatment on phosphorylation of MAP2 was conducted according to Miyamoto et al (1997) with modifications. Frozen tissue samples were homogenized for 3 min on ice (using a TriR S63C Homogenizer [Cole-Palmer, Vernon Hill, IL]) in 3 volumes of 50 mmol/L Tris-HCl buffer (pH 7.4) containing 0.8 mol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 2.5 mmol/L β-mercaptoethanol, 50 mmol/L sodium pyrophosphate, 4 mmol/L p-nitrophenyl phosphate, 1% Sigma Protease Inhibitor Cocktail for mammalian tissue, 1% Sigma Phosphatase Inhibitor Cocktail for Phosphoserine, and 1% Sigma Phosphatase Inhibitor Cocktail for Phosphothreonine and Phosphotyrosine. Immediately after homogenization, the tissue samples were centrifuged at 20000 g for 30 min at 4°C. MAP2 was precipitated from the supernatants with the IMMUNOCatcher Protein Immunoprecipitation Kit (CytoSignal, Irvine, CA). The immunoprecipitating agent (the same monoclonal antibodies that were employed in examination of the tissue levels of MAP2) was used at a concentration of 2.5 μg/100 μL of the supernatant. The resultant immunoprecipitates were mixed 1:5 with the loading buffer described above for the gels used for Western blot testing of the specificity of antibodies employed for labeling of slot blots, and boiled for 5 min. The boiled mixtures (30 μL per well) were run on a gel and blotted on a PVDF membrane as described for the Western blots used in testing of the specificity of antibodies employed for labeling of slot blots. The completeness and evenness of transfer was verified by afterstaining of gels with Comassie blue. Every run included samples of MAP2 immunoprecipitates obtained from the matching cortical regions of the same hemisphere of all eight animals used in this study. The blots were first processed for immunolabeling with antibodies for phosphoserine, phosphothreonine, or phosphotyrosine and the resultant images digitized. The membrane was then stripped of antibodies using a buffer containing 2% SDS, 100 mmol/L β-mercaptoethanol, and 62.5 mmol/L Tris (pH 6.7) at 50°C for 30 min (Wilson et al 1998) and processed for the MAP2 immunolabeling. The overall immunolabeling and signal visualization procedures were similar to those described above for the slot blots, with the exception that here we used the phosphorylated residue-free Blocking Solution from Zymed Laboratories.
Table 1. Percentages ± SEMs of Neurons in 11 Different Cortical Regions of the Right Hemisphere of the Long-Term Haloperidol-Treated and Drug-Naive Control Monkeys

<table>
<thead>
<tr>
<th>Region</th>
<th>Control animals</th>
<th>Haloperidol-treated animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal pole</td>
<td>57.4 ± 4.2</td>
<td>55.8 ± 5.2</td>
</tr>
<tr>
<td>Dorsolateral prefrontal</td>
<td>54.8 ± 4.6</td>
<td>56.1 ± 3.2</td>
</tr>
<tr>
<td>Dorsomedial prefrontal</td>
<td>53.0 ± 2.2</td>
<td>52.3 ± 3.6</td>
</tr>
<tr>
<td>Anterior orbital</td>
<td>57.0 ± 4.0</td>
<td>55.8 ± 3.6</td>
</tr>
<tr>
<td>Posterior orbital</td>
<td>56.4 ± 3.0</td>
<td>55.4 ± 4.4</td>
</tr>
<tr>
<td>Anterior cingulate</td>
<td>49.9 ± 5.6</td>
<td>51.1 ± 3.6</td>
</tr>
<tr>
<td>Prelimbic</td>
<td>52.6 ± 2.2</td>
<td>52.6 ± 1.4</td>
</tr>
<tr>
<td>Premotor</td>
<td>49.7 ± 4.0</td>
<td>49.0 ± 3.4</td>
</tr>
<tr>
<td>Primary motor</td>
<td>55.4 ± 3.8</td>
<td>56.4 ± 2.0</td>
</tr>
<tr>
<td>Primary visual</td>
<td>60.9 ± 4.2</td>
<td>59.3 ± 5.0</td>
</tr>
<tr>
<td>Entorhinal</td>
<td>58.3 ± 4.6</td>
<td>56.9 ± 6.0</td>
</tr>
</tbody>
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* t test

EXPRESSION OF THE DATA AND STATISTICAL ANALYSIS. The residue-specific phosphorylation in every blot of a given sample was expressed as the ratio of the gray value of the film images produced by the phosphoaminoacid immunolabeling and the gray value of the images produced by the immunolabeling of the immunoprecipitated protein of this blot. For every set of blots on a single membrane, the ratios obtained were then normalized to the ratio generated by the sample from the 15-year-old control animal. Since every assay was performed in six replicates (on six separate membranes), the normalized ratios from the matching samples in all six replicates (membranes) were averaged for statistical analysis. The data obtained in this way were appropriate only for comparison of the levels of residue-specific MAP2 phosphorylation in the same region of the same hemisphere of control and experimental animals. The statistic employed for this purpose was a two-tailed $t$ test. In assessing statistical significance, we used the Bonferroni correction with $p$ value divided by 3, the number of specific assays performed in each brain area.

Results

Proportion of Neuronal Cells in Multiple Cortical Regions

Our calculations of the proportions of neuronal nuclei among the cell nuclei in the samples from all the cortical regions examined in this study showed that neurons constitute approximately half of the cells in these regions (Table 1). There were no statistical differences between the proportions of neuronal nuclei in the haloperidol-treated and control animals in any of the cortical regions in either hemisphere (Table 1).

Levels of Spinophilin, MAP2, and Synaptophysin

Comparative analysis of spinophilin levels in the haloperidol-treated and control animals showed that the drug induced a statistically significant downregulation of this protein in all of the frontal and temporal cortical areas examined in both hemispheres (Figure 4). These areas included the cortex of the frontal pole, dorsolateral and dorsomedial prefrontal cortices, anterior and posterior orbital cortices, anterior cingulate and prelimbic cortices, premotor and primary motor cortices, and entorhinal cortex. Among the cortical areas
analyzed in this study, only the primary visual cortex did not display a statistically significant decline in this protein (Figure 4). The same results were obtained whether the levels of spinophilin were expressed per neuronal DNA or per total protein (Figure 4).

We also found that the haloperidol-treated monkeys had elevated levels of MAP2 in all cortical regions examined in both hemispheres, whether the levels of this protein were calculated per neuronal DNA or per total protein of the sample (Figure 4). However, this upregulation did not reach statistical significance in any region (Figure 4).

We detected no statistically significant differences in the levels of synaptophysin between the drug-treated and control animals in any cortical region examined (Figure 4).

Phosphorylation of MAP2

The analysis of MAP2 phosphorylation in the haloperidol-treated and control animals revealed a statistically significant drug-induced increase in the levels of phosphorylation of this protein on the serine residue in the cortex of the frontal pole, dorsolateral and dorsomedial prefrontal cortices, anterior and posterior orbital cortices, anterior cingulate and prelimbic cortices, and entorhinal cortex (Figure 5). Increases in MAP2 serine phosphorylation were also observed in the premotor, primary motor, and primary visual cortical areas, but these increases did not reach statistical significance (Figure 5). Increases in the levels of MAP2 phosphorylation were also observed on the threonine residue in all brain regions, but these increases were also not statistically significant (Figure 5). No significant effects on the tyrosine residue phosphorylation of MAP2 were found in the haloperidol-treated animals (Figure 5).

Discussion

Selective Regional Vulnerability of MAP2 and Spinophilin to Chronic Haloperidol Treatment

This study demonstrates that chronic haloperidol treatment significantly increases MAP2 phosphorylation on the serine residue and significantly downregulates spinophilin in specific regions of the primate cerebral cortex. Furthermore, the haloperidol-induced downregulation of spinophilin is detectable independent of whether the levels of this protein are expressed per neuronal DNA or per total protein in the tissue. This indicates that haloperidol treatment selectively affected spinophilin levels, without interfering with the proportion of neurons in the cortex, as demonstrated by the cell counts conducted in this study, and without altering the total protein content of the cortical cells. The protein specificity of the detected effects is also emphasized by the absence of statistically significant

Figure 4. Histograms showing differences in the levels of MAP2, spinophilin, and synaptophysin between the control and haloperidol-treated monkeys in 11 cortical regions examined in this study. The differences are expressed as percentages ± SEMs of the mean levels of these proteins in control samples. Note that, for each protein, one set of bar graphs shows changes expressed per neuronal DNA and another set represents changes expressed per total protein. The data expressed per DNA are from the right hemisphere, whereas the data expressed per protein are from the left hemisphere. *Statistically significant change.
changes in the levels of MAP2 and synaptophysin in the haloperidol-treated animals.

A central observation of this study is that the effects of haloperidol were detectable in such cortical areas as the frontal pole; the prefrontal, anterior cingulate, prelimbic, and entorhinal regions; and the motor and premotor cortices, all of which are known to contain extensive dopaminergic innervation (Berger et al. 1988; Nieuwenhuys 1985; Williams and Goldman-Rakic 1993). These areas, except the motor cortex, also contain the highest densities of D2 receptors in the monkey cerebral cortex (Lidow et al. 1998). Conversely, we detected no significant haloperidol-induced alterations in the aforementioned proteins in the visual cortex, which has a barely detectable dopamine innervation and is D2 receptor poor (Berger et al. 1988; Lidow et al. 1989). Interestingly, in the motor cortex, which is richly innervated by dopamine fibers (Berger et al. 1988; Williams and Goldman-Rakic 1993) but relatively poor in D2 receptors (Lidow et al. 1998), haloperidol interfered significantly only with spinophilin levels without affecting MAP2 phosphorylation. This suggests that the cortical dopaminergic system plays an important role in haloperidol’s ability to affect both the level of spinophilin and phosphorylation of MAP2 and that D2 receptors may be particularly involved in regulation of the phosphorylation of the latter protein.

Also of interest is that the cortical regions in which haloperidol treatment affected MAP2 and spinophilin have all been implicated in the psychopathology of schizophrenia (Goldman-Rakic 1999a; Harrison 1999; Weinberger et al. 1994). These regions have also been suggested as possible sites for the palliative action of antipsychotic medications (Lidow and Goldman-Rakic 1997; Lidow et al. 1998). It is possible, therefore, that haloperidol-induced changes in MAP2 phosphorylation and spinophilin levels may underlie, at least in part, the ability of antipsychotic medications to improve the quality of life of schizophrenic patients. It is important to remember, however, that the results of this study pertain to older (15–25 years of age) monkeys, and that some of the drug-induced alterations could be products of an interaction of the treatment with an aged brain.

The Molecular Biological Findings of This Study Reflect Morphological Changes in the Cortices of the Haloperidol-Treated Animals

The molecular biological data collected in this study correspond well with morphological findings from several previous studies on the effects of chronic haloperidol treatment. As mentioned earlier, the degree of MAP2 phosphorylation is inversely proportional to its ability to stabilize dendritic microtubules, which are essential for the maintenance of these neuronal processes. Therefore, it is reasonable to expect that increased MAP2 phosphorylation, which we observed in several cortical regions of haloperidol-treated animals, would result in some measure of dendritic collapse in these regions. Indeed, Benes and her colleagues (Benes et al. 1985; Vincent et al. 1991) reported a significant decrease in the number of small-caliber dendritic processes in the prefrontal cortex of haloperidol-exposed rats. Furthermore, an electron microscopic analysis (Benes et al. 1985) revealed that most of these processes were dendritic spines, whose necks have a cytoskeletal structure identical to that of the dendritic shaft proper (Bloom and Fawcett 1975). These findings are also compatible with the decrease in levels of the spine-associated protein spinophilin, which we observed in the frontal lobe of the drug-treated animals. Additionally, a decline in the volume of cortical neuropil (which is composed largely of dendritic processes), together with a reduced number of microtubules within dendritic shafts and a lower density of dendritic spines, has been found in postmortem studies of the frontal cortex of schizophrenic patients.
patients (Garey et al. 1998; Glantz and Lewis 1997; Selemon et al. 1996; Uranova 1988). Our data, along with those of Benes and her colleagues (Benes et al. 1985; Vincent et al. 1991), indicate that antipsychotic treatments may have contributed to the generation of some of the aforementioned alterations in the schizophrenic brains.

Another observation of our study is that the levels of MAP2 in the haloperidol-treated animals, though not reaching statistical significance, were consistently elevated in the same cortical areas where increases in phosphorylation of this protein were detected. This is consistent with reports that an increase in MAP2 phosphorylation not only reduces its microtubule-stabilizing capacity but also elevates its resistance to proteolysis (Alexa et al. 1996; Johnson and Foley 1993). Interestingly, studies in schizophrenic brains have suggested that this disease may be characterized by an upregulation of nonphosphorylated MAP2 and downregulation of the total MAP2 in some cortical areas (Arnold et al. 1995; Cotter et al. 1997; Dwork 1997). Our findings demonstrate that haloperidol can, at least partially, counteract these effects both by increasing the levels of MAP2 phosphorylation and by reducing the extent of MAP2 degradation in cortical cells. Consequently, this may be among the bases for the therapeutic benefits of antipsychotic drugs.

It has recently been demonstrated that spines are the main sites of D1 dopamine receptors in cortical pyramidal neurons (for a review, see Goldman-Rakic 1999b). Therefore, any decrease in the number of spines in the cortex would be expected to affect the expression of D1 receptors by these cells. This may explain a decline in the levels of D1 receptors detected after long-term neuroleptic treatment in the primate cortex (Lidow et al. 1997; Lidow and Goldman-Rakic 1994) and in the cortex of schizophrenic patients (Okubo et al. 1997; Sedvall and Farde 1996).

Several studies have suggested that haloperidol treatment is accompanied by a shift in the position of synaptic contacts from dendritic spines to dendritic shafts, without significantly changing the density of synaptic contacts per se within the cortex (Benes et al. 1985; Klinzova et al. 1989, 1990; Meshul et al. 1992). In agreement with these observations, we detected no significant changes in the levels of the synaptic marker synaptophysin in any of the cortical areas examined. Our results also support the earlier report of Nakahara et al. (1998), who found no changes in the levels of synaptophysin messenger RNA in the prefrontal cortex of rats chronically treated with haloperidol. On the other hand, Eastwood et al. (1997) detected an increase in the levels of this message in the frontoparietal cortex of haloperidol-treated rats. It should be noted, however, that the latter study characterized the parietal cortex, which was not analyzed by us nor by Nakahara et al. (1998). Finally, the lack of haloperidol-induced changes in synaptophysin levels observed in our study suggests that antipsychotic treatment may not be responsible for the decrease in this protein reported in the prefrontal and medial temporal cortical regions of schizophrenic patients (Eastwood and Harrison 1995; Glantz and Lewis 1997; Honer et al. 1999; Karson et al. 1999; Perrone-Bizzozero et al. 1996).

Possible Cellular Mechanisms of Haloperidol-Induced Alterations in the Proteins Observed in This Study

One of the major pharmacologic properties of haloperidol as an antipsychotic medication is its ability to block D2 dopaminergic receptors (for a review, see Seeman 1992). Since D2 receptors are negatively coupled to adenylyl cyclase (Hemmings et al. 1987), their blockade results in an increase in the intracellular levels of cyclic adenosine monophosphate (cAMP; Kaneko et al. 1992; Nilsson and Eriksson 1993), which may persist during long-term treatments despite D2 receptor upregulation (Lau and Gnegy 1982; Okada et al. 1996). Analysis of the literature suggests that elevated intracellular cAMP levels can lead to all of the alterations detected in the haloperidol-treated animals in this study. For example, upregulation of cAMP activates protein kinase A (PKA; Walsh et al. 1972), which is one of the major enzymes responsible for phosphorylation (Goldenring et al. 1985; Sloboda et al. 1975; Tsuyama et al. 1986, 1987; Walaas and Nairn 1989). The involvement of the cAMP–PKA pathway in increasing phosphorylation of MAP2 is supported in our study by the fact that this increase reaches statistical significance only for the serine residues, the only MAP2 residues known to be susceptible to PKA-induced MAP2 phosphorylation (Goldenring et al. 1985; Walaas and Nairn 1989).

Elevated levels of cAMP could also lead to a collapse of dendritic spines and, consequently, to decreases in levels of spinophilin in the affected neurons. This collapse might result from destabilization of the microtubule skeleton of spine necks due to the PKA-induced increase in MAP2 phosphorylation. Alternatively, increased intracellular levels of cAMP may affect the cAMP–PKA–CREB pathway, which has also been demonstrated to play an important role in the maintenance of dendritic spines (Martin and Kandel 1996; Murphy and Segal 1997).

Haloperidol may also generate the effects observed in this study by acting through nondopaminergic receptors because treatment with this drug affects multiple neurotransmitter systems in the brain, including noradrenergic and γ-aminobutyric acid–ergic circuitries (Borda et al. 1999; Bourdelais and Deutch 1994; Nalepa 1993; Sasaki et al. 1997), which also play a role in the regulation of the neuronal cytoskeleton (Lipton and Kater 1989). In this
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