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Original investigations

Study of neuropathologic changes in the striatum following 4, 8 and 12 months of treatment with fluphenazine in rats*

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Abstract. Persistent tardive dyskinesia is a serious side effect of long-term treatment with neuroleptics. Although striatal pathologic changes are believed to underlie this potentially irreversible iatrogenic syndrome, the nature of the neuroleptic-induced neuropathology is unclear. In the present study, we treated rats with either vehicle or fluphenazine decanoate (5 mg/kg, IM) every 2 weeks for 4, 8 or 12 months. Four to nine weeks after the last injection, the animals were sacrificed and the density of cells in the central part of the striatum was measured with a computerized image-analysis system. The control and experimental animals did not differ in body weight with 4 and 8 months of treatment, but the rats treated with fluphenazine for 12 months had significantly lower body weights than comparable controls. Four months of neuroleptic use produced no significant neuropathologic changes. The animals treated with fluphenazine for 8 months had a significantly lower density of the large neurons. In the 12-month-treated group, there was no significant difference between the control and experimental animals, probably because of a 'floor effect': the density of the large neurons was significantly lower in the 12-month-treated compared to the 8-month-treated control rats.

Key words: Fluphenazine – Neuroleptics – Tardive dyskinesia – Neuronometry – Striatum – Cholinergic neurons

Neuroleptic medications are the mainstay of the treatment for psychosis. In some chronic conditions, such as schizophrenia, the patients require treatment for extended periods of time. Prolonged treatment with neuroleptics is not without risk, however, because of the frequent occurrence of tardive dyskinesia (TD), a potentially irr versible syndrome of abnormal movements affecting particular, the orofacial and upper extremity muscul ture (Jeste and Wyatt 1982; Casey 1987; Kane et 1988). Some studies have suggested an association persistent TD with striatal damage (Gross and Kalte bach 1969; Jellinger 1977; Pandurangi et al. 1980; Lo et al. 1986). Interpretation of the human studies is li ited, however, because of difficulties in assessing t contribution of factors such as the type and degree psychiatric illness, as well as the age or the length a amount of the neuroleptic treatment (Lam et al. 198 Animal models have been used to circumvent those co straints and to more directly investigate possible under ing variables.

Table 1 summarizes the published histopathologic investigations of animals treated with neuroleptics f weeks to months. Despite methodologic differences, number of these studies reported abnormalities in d ferent brain areas, especially motor areas including t striatum. Thus, there is some evidence of histopatholo cal abnormalities, especially in the striatum, followi long-term neuroleptic administration. Nevertheless, t nature of the striatal pathologic changes is unclear. We controlled neuropathological studies of animals treat with neuroleptics for very long periods utilizing co puterized image-analysis techniques are lacking.

Over the past 3 decades, a number of investigato have studied the behavioral and neurochemical effects long-term administration of neuroleptics in anim (Tarsy and Baldessarini 1974; Rupniak et al. 1983; Eb and Hama 1988). For example, Clow, Rupniak a collaborators (Clow et al. 1979, 1980; Rupniak et a 1983) reported evidence of increased dopamine recep numbers and possibly increased receptor sensitivity animals treated for greater than 6 months with a vari of different neuroleptics administered in drinking wa The relevance of the striatal dopamine receptor supers sitivity to the pathogenesis of persistent TD is, howe questionable (Jeste and Wyatt 1982). Other recent stu have reported a decrease in dopamine release (Lane a

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Table 1. Neuropathologic effects of long-term neuroleptic use in animals

Authors	Species	Neuroleptic	Months	Pathologic changes in experimental animals
[illegible] et al. (1959)	Monkeys	Chlorpromazine	8	Diffuse
[illegible] and Gershon [illegible])	Guinea pigs	Chlorpromazine or reserpine	1–3	Diffuse
[illegible] (1967)	Rats	Haloperidol	4	Limbic-gliosis
[illegible] et al. (1965)	Rabbits and mice	Various	1–12	Variable
[illegible] and Quandt (1970)	Rabbits	Chlorpromazine	6	Brainstem cell death
[illegible] and Colleagues [illegible] 1974)	Rats	Perphenazine	1–2 12	Nil 20% cell loss in basal ganglia
[illegible] and Lange [illegible])	Rats	Chlorpromazine	6	Brainstem and cerebeller gliosis
[illegible] (1975)	Rats	Perphenazine	6–12	Nil
[illegible] and Lyon (1978)	Rats	Flupenthixol	6	10% cell loss in ventro-lateral but not dorso-medial striatum
[illegible] et al. (1981)	Rats	Fluphenazine	1 3/4	Nonsignificant decrease in neuron density in ventro-lateral striatum in 7-month old, but not 25-month old rats
[illegible] et al. (1983)	Rats	Haloperidol	4	Light microscopy: no change in striatal neuron density but increased neuron size. Electron microscopy: substantia nigra: axon and collateral sprouting. Striatum: increased size of neurons, dendrites, number of associated synaptic vesicles
[illegible]dik et al. (1988)	Rats	Haloperidol	1 1/2	Apparent reduction in size and number of striatal neurons and neuronal processes that stained positive for choline acetyl transferase
[illegible] and Casey (1989)	Rats	Haloperidol	1/2 month treatment then 1/2 month withdrawal	Increase in striatal, but not nucleus accumbens, synapses with perforated post-synaptic densities. Change reversed on neuroleptic-withdrawal

[illegible] (1987) and in choline acetyl transferase (ChAT) activity (Mahadik et al. 1988) in the striatum following chronic neuroleptic treatment.

In this paper, we report morphometric effects of treatment with fluphenazine for 4, 8, and 12 months in rats. We hypothesized significant decreases in striatal neuronal density with longer-term neuroleptic treatment.

Materials and methods

The subjects were 58 male Sprague-Dawley rats (obtained from [illegible] Sprague-Dawley, Inc.), weighing 150–175 g each. They were maintained on a 12-h light-dark cycle, and housed two per cage for [illegible] 2 weeks prior to starting treatment with either fluphenazine [illegible] (5 mg/kg; $n = 32$) or vehicle (sesame oil; $n = 26$) injections [illegible] every 2 weeks. Six animals from the control group and ten from the experimental group were treated for 4 months, five each for 8 months, and 15 from the control group and 17 from the experimental group for 12 months. All the animals were weighed prior to each injection.

Four weeks after the last injection (except for five control and seven experimental animals treated for 12 months, which were sacrificed 6 weeks after the last injection), the animals were sacrificed by decapitation and the brains quickly removed. One hemisphere from the brain of each animal was used for neuropathologic studies. The right hemisphere was used in 50% of the animals, the left in the other 50%. (The other hemisphere was used for neurochemical studies, to be described separately).

The tissue was blotted dry to avoid formation of ice crystals, and frozen in liquid nitrogen after being placed in a disposable test tube. The brains were then stored in a freezer at −80° C. For sectioning, the brains were mounted on a cryostat specimen disc and the temperature was allowed to rise to −20° C before sectioning. Sections (12 μ thick) were made at 60–72 μ intervals. (In the 4-month-treated group, we used 20-μ-thick sections, but because of the overlap of cells sometimes seen, decided to use thinner sections for the 8- and 12-month-treated groups. Subsequently, we added a control group of ten animals that was sacrificed at 4 months but had 12-μ-thick sections, for comparison with the 8- and 12-month-treated controls.) The slides were dried for 24 h, then post-fixed with acetone at 4° C, and air-dried in a refrigerator. The sections were stained with cresyl violet.

Measurements were performed on the IBAS 2000 image analysis system (Zeiss, Inc.). A trained neuropathology technician examined the sections blind with respect to the identity of the animal. Using the atlas of Paxinos and Watson (1986), we selected for study the section closest to the center of the striatum, and examined the central part of the striatum in this section. The areas were first located under the low power, then quantitated at 500× (40 × objective × 10 × eyepiece × 1.25 × optical disc). We measured the total number of cells per five screens in the striatum of each hemisphere.

Next, using a modified computer program on IBAS 2000, we divided the striatal cells in the 12-μ-thick sections into three categories by size. We based our measurements on the diameter of a circle of the same area as the measured cellular area. Thus, we separated the cresyl violet-stained cells into small (< 7.5 μ in diameter), medium-sized (7.5–14 μ in diameter) and large (> 14 μ in diameter). The numbers of the medium-sized and large cells per 20 contiguous screens were counted. The reason for using 20 screens (rather than five screens as for the total number of cells) was that the large cells were found to be very infrequent, usually ranging from 0 to 4 per 20 contiguous screens.

We identified the neurons manually, by staining characteristics, nuclear morphology and presence of a nucleolus. While there could be an overlap between glia and small neurons among cells < 7.5 μ in diameter, this is extremely unlikely among larger cells. Indeed, we found that more than 95% of the medium-sized and all of the large cells, identified by our automated counting procedure, also met the morphological criteria for neurons defined above. In a study of cresyl-violet stained sections from the human brains, Terry et al. (1981) found that practically all of the cells with diameter less than 7.1 μ (i.e., an area of less than 40 μ^2) were glia, while almost all of the larger cells were neurons. We used a technique similar to that of Terry et al. (1981) for determining cell size. The cell diameter (d) was computed by using the formula $d = 2\sqrt{A/\pi}$, where A = cell area.

Terry and DeTeresa (1982) have demonstrated that computerized image analysis using automated counting apparatus may yield inaccurate results if time-consuming video editing is not used. Those researchers found that a hand tally of the cells was within 3% of the count produced by an edited image analysis of the same microscopic area. We employed a similar method of video editing, and also found that the hand tallies were within 5% of the automated counts.

All the measurements were done by the same rater who was blind to the data on treatment or age. Test-retest reliability was determined by repeating the morphometry in five control and five experimental animals. The intraclass correlation coefficient for repeat measures was 0.90 ($P < 0.001$).

A neuropathologist evaluated the same sections under a light microscope, blind to other data, to comment on any obvious neuropathologic abnormalities in any of the sections.

Reliability of measurement. The IBAS 2000 was calibrated according to the manufacturers' instructions using a grid with a 10-μ separation between lines. This procedure was performed before analysis for each group of animals. In addition, the accuracy of the final measurement was manually checked, employing a reticuled ocular. The agreement between the two methods (automatic and manual) was greater than 95%.

To compare our values with those in the literature, we computed volume densities of the total number of the striatal cells in the 12-μ thick sections of the 8-month-treated controls. From the mean number of the cells per screen, and the area per viewing screen (0.02380 mm^2), the areal densities of the cells were calculated. An edge correction was applied in the determination of the cell number per screen. Using the values for areal density and the thickness of the section, we computed the volume densities (cells/mm^3). A modification of the Abercrombie split-cell correction was applied, as described by Konigsmark et al. (1969), to correct the volume densities.

Results

Changes in body weight

The mean weights of the animals in the experimental and control groups were similar (Fig. 1). The mean (with standard deviations in parentheses) weights in grams were: 423.6 (49.6) and 430.8 (23.5), respectively, following 4 months' treatment, 488.6 (30.4) and 496.2 (28.2), respectively, following 8 months' treatment, and 534.0 (54.8) and 459.7 (34.0) respectively, following 12 months' treatment. The experimental and control groups did not differ in their overall growth patterns following 4 and 8 months of treatment, but the experimental group had a significantly lower mean body weight than the control group following 12 months of treatment ($P < 0.05$, two-tailed *t*-test). Also, three animals in the 12-month-treated experimental group became severely ill and either died or had to be euthanized. (The data on these three animals were excluded form further analysis.)

Light microscopic changes

There were no significant differences between the two hemispheres on any neuronometric measures for either the control or the experimental group. Hence, we combined the data from the two hemispheres. Also, the neuropathologist who examined the brain sections independently, found no obvious neuropathological abnormalities in any of them.

After 4 months of treatment with fluphenazine, there were no differences in total striatal cell density between the controls and the fluphenazine-treated animals (Table 2).

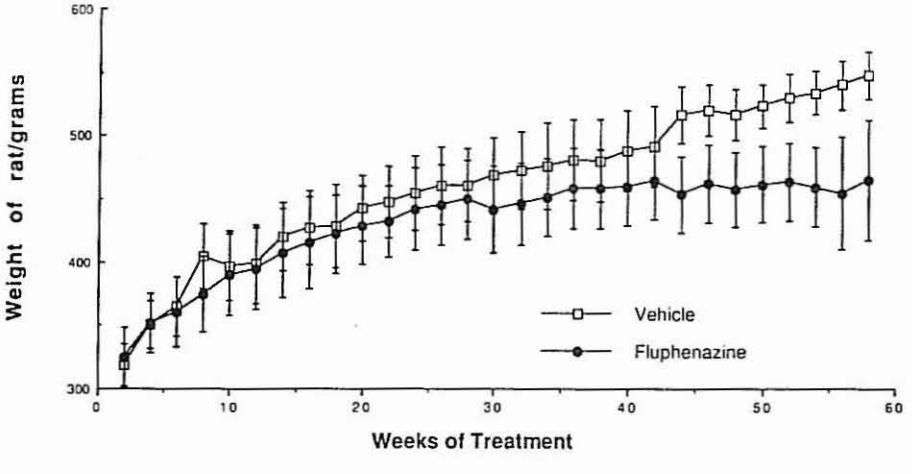

Fig. 1. The figure shows mean increases in body weight of the rats over the course of 12 months of treatment with vehicle (□) or fluphenazine (●)

Table 2. Total cell density[a] the in striatum

Length of treatment	Vehicle	Fluphenazine
8 months		
n	6	10
Mean (SD)	161.0 (40.5)	170.8 (60.4)
12 months		
n	5	5
Mean (SD)	173.8 (40.6)	151.4 (50.1)
12 months		
n	10	7
Mean (SD)	141.4 (21.7)	121.0 (23.3)

[a] Density represents the number of cells per 5 screens in the central sector of the striatum

Table 3. Density[a] of medium-sized and large neurons following 8 and 12 months' treatment

Neuronal diameter	Vehicle ($n=5$)	Fluphenazine ($n=5$)
Following 8 months' treatment		
7.5–14 μ	493.8 (76.2)	372.2 (68.7)
>14 μ	2.2 (1.7)	0.3 (0.3)[b]
Following 12 months' treatment		
7.5–14 μ	459.7 (76.2)	470.7 (35.5)
>14 μ	0.2 (0.6)[b]	0.6 (0.5)

[a] Density represent the number of neurons per 20 screens in the central sector of the striatum. The values are means (with standard deviations). Please note that (unlike Table 2) neurons with diameters less than 7.5 μ are not included in Table 3

[b] $P<0.01$, on protected t-tests for the 8-month-treated control versus experimental groups, and for the 8-month controls versus 12-month controls (large cells only)

The values for the volume density of cells in our 8-month-treated control animals generally ranged between 69000 and 70000/mm^3. We found a trend for a somewhat lower total cell density in the 8-month- and 12-month-treated experimental animals compared to the controls. Hence, we decided to measure the density of the large and medium-sized cells in these groups (Table 3). (Cells <7.5 μ in diameter were not considered because of difficulties in distinguishing between small neurons and glia in the cresyl violet-stained sections.)

To analyze the effects of the treatment as well as the age of the animals in the 8- and 12-month groups, we performed 2×2 analyses of variance (ANOVAs) followed by post-hoc protected t-tests for the total cell density, and the density of the medium-sized cells (7.5–14 μ in diameter), and of the large cells (>14 μ in diameter) in the control and experimental groups. The ANOVA revealed a significant treatment-by-age interaction for the large neurons only ($P<0.01$). The post-hoc tests showed significant differences in the density of the large neurons between the 8-month-treated control versus 8-month-treated experimental animals ($P<0.01$), and between the 8-month-treated controls versus 12-month-treated controls ($P<0.01$). Table 3 compares the densities of the medium-sized and large neurons in the control versus experimental animals (per 20 screens) following 8 and 12 months of treatment.

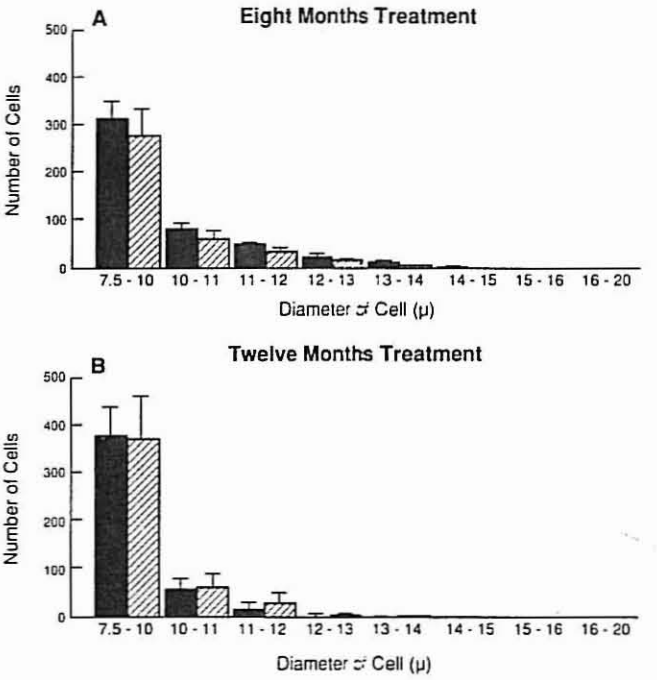

Fig. 2A, B. The figure shows frequency histograms of the densities of the striatal cells grouped according to cell size in the **A** 8- and **B** 12-month-treated control and experimental animals. For the 8-month-treated group, $n=5$ each for controls and fluphenazine-treated rats. For the 12-month-treated group, $n=10$ for the controls *(black columns)* and $n=7$ for fluphenazine-treated rats *(hatched columns)*

We also measured ten 4-month control animals using the same technique as the 8- and 12-month-treated control animals. The 4-month controls had a mean medium-sized neuron density of 497 (105) and a mean large neuron density of 3.9 (3.0). There was no significant difference in the medium-sized neuron densities among the 4-, 8-, and 12-month control groups. There was, however, a significant difference in terms of the density of the large neurons ($P<0.005$, ANOVA), with the 4-month control animals having significantly greater neuron density than the 12-month group ($P<0.01$, protected t-test), but not the 8-month group.

In both Tables 2 and 3 (and in Fig. 2) the values given for the 12-month group are those for the animals sacrificed four weeks after the last injection (similar to the 8-month group). There was, however, no significant difference between the 12-month-treated experimental animals sacrificed 4 weeks versus 9 weeks after the last injection. The same was true for the 12-month-treated control animals.

Figure 2 shows frequency histograms of the densities of the striatal cells grouped according to cell diameter (7.5–10 μ, 10–11 μ, 11–12 μ, 12–13 μ, 13–14 μ, 14–15 μ, 15–16 μ, >16 μ) in the 8- and 12-month-treated control and experimental animals. There was no significant shift to the left that could have indicated a cell shrinkage rather than a cell loss with either neuroleptic treatment or aging.

Discussion

The rationale for our study design was as follows. We chose fluphenazine decanoate because this commonly used depot neuroleptic has been shown to be associated with persistent TD at least as much as other frequently prescribed neuroleptics (Jeste and Wyatt 1982). The dose of fluphenazine selected (i.e., 5 mg/kg every 2 weeks) was higher than the usual clinical dose (but well below the LD_{50} for the species, as stated by the manufacturer and as determined from our previous studies) primarily to compensate for a more rapid rate of the metabolism of fluphenazine in the rats. We chose treatment durations of 4, 8 and 12 months because, in a rat with an average life span of 2–3 years, these lengths of time are comparable to the long durations of the neuroleptic treatment commonly noted in the patients with persistent TD.

As we were primarily interested in determining the persistent effects of neuroleptic use, we waited for at least 4 weeks after the last injection, before sacrificing the animals. Following 12 months of treatment, it seemed appropriate to have a longer period of neuroleptic withdrawal in some animals – hence, a proportion of the rats treated for 12 months were sacrificed 9 weeks after the last injection. (We did not measure the blood or brain concentrations of fluphenazine because of methodologic problems in such determinations as well as difficulties in relating these to the neuroleptic amounts in the striatum.) We must add, nonetheless, that there is not enough information in the literature to choose the "ideal" doses, durations and lengths of neuroleptic withdrawal for neuropathologic studies.

The validity of our neuronometric technique is suggested by comparing our values with those in the literature. Our classification of the striatal cells into small ($< 7.5\ \mu$), medium-sized (7.5–14 μ) and large ($> 14\ \mu$) was comparable to that reported by Kemp and Powell (1971) and by Pasik et al. (1979), who divided the neostriatal neurons into small (less than 8 μ), medium (9–18 μ) and large (20 μ or more). The exact values for the large neurons were different from ours, probably because the latter investigators used maximum rather than average diameters to compute cell size. In every case, less than 1% of all the neurons were classified as being large.

Our calculated values of approximately 69 500/mm^3 for the cell density in the 8-month-treated control group are in reasonable agreement with the published values of 60 000/mm^3 in 7-month-old controls (Brizzee et al. 1981) or 62 000/mm^3 in 6–7-month-old controls (Benes et al. 1983) in the rat striatum. Differences in the strain, age, nutritional status and tissue preparation, uncertainties regarding the exact thickness of every section, and the fact that we only measured the central striatum may account for the small differences observed from the published values.

We studied the central sector of the striatum because it is large enough to allow examinations of 20 contiguous screens, which was necessary for measuring a minimum number of the large neurons. Whether the ventrolateral sector would be affected more, as suggested by Nielsen and Lyon (1978), remains to be confirmed.

Our main findings may be summarized as follows. Four months' treatment with fluphenazine did not produce significant changes in the number of striatal neurons. However, the animals treated for 8 months had a significantly lower density, compared to the controls, of large neurons in the central part of the striatum. A similarity in the growth pattern of the 8-month-treated control and experimental animals suggests that systemic toxicity of neuroleptics was not likely to have caused the striatal pathologic changes in the experimental group. After 12 months of treatment, however, the cell densities of the treated animals were comparable to those of the controls, with the latter values for the large neurons being significantly lower than those for the 8-month-treated controls.

Because of relatively small *N*s, especially in the 8-month group, our study must be considered preliminary. Nonetheless, if confirmed using larger sample sizes, our finding of a reduction in the large striatal neurons in the animals treated with fluphenazine for 8 months is consistent with the reports of pathology of the large neurons in the striatum in patients with persistent perioral TD (Gross and Kaltenbach 1969; Jellinger 1977). Gross and Kaltenbach (1969) reported that their patient (case #1), a man who developed persistent perioral dyskinesia following neuroleptic treatment, had satellitosis and neuronophagia mostly of the large neurons in the medium part of the caudate. Jellinger (1977) reported that five of his nine patients with perioral dyskinesia, but none of the 19 non-TD controls, had swelling of the large neurons and gliosis in the rostral two-thirds of the caudate bilaterally. Obviously, caution is necessary in comparing neuropathologic findings in patients with TD to those in the rats treated long-term with neuroleptics.

The largest striatal neurons are most likely cholinergic interneurons (Kimura et al. 1980; Groves 1983). It has been known from clinical observations and neurochemical experiments that there is a dopaminergic-cholinergic interaction in the neostriatum, suggesting a direct dopaminergic control of the neostriatal cholinergic neurons (e.g., Sethy and Van Woert 1974a, b). Recently, Kubota et al. (1987) and Chang et al. (1988) provided morphological evidence that the nigrostriatal dopaminergic neurons could influence the striatal cholinergic neurons monosynaptically. Using immunocytochemical techniques, the investigators found that cholinergic neurons were in direct synaptic contact with dopaminergic axons in the striatum of rats. It is, therefore, conceivable that prolonged administration of dopamine-receptor-blocking neuroleptics could result in damage to the cholinergic neurons.

Our suggestion of cholinergic neuropathology induced by prolonged use of neuroleptics is also consistent with at least two other reports of significant decreases in striatal choline acetyl transferase (ChAT) activity following chronic administration of neuroleptics to rats (Pedata et al. 1980; Mahadik et al. 1988). Pedata et al. (1980) found significant decreases in the striated ChAT activity in rats treated with haloperidol (OS), pimozide (OS) or fluspirilene (IM) in doses of 1 mg/kg/day given for 45–60 days. There was no significant change in the striatal

ChAT activity following 1 or 12 days of fluspirilene treatment. Among the animals treated with fluspirilene for 120 days, the decrease in the ChAT was dose-dependent. Mahadik et al. (1988) reported that daily injections of haloperidol, 2 mg/kg/day IM for 40 days produced a 20% decrease in the striatal ChAT activity as well as an apparent reduction in the size and number of ChAT-positive striatal neurons and their processes in the rat. No such changes were noted following either single dose of haloperidol or chronic treatment with clozapine or imipramine. Rupniak et al. (1986) found no significant reduction in the ChAT activity in the striatum after administration of haloperidol (1.4–1.6 mg/kg/day) for up to 12 months, although the neuroleptic had been given in drinking water, rather than parenterally.

The mechanisms by which neuroleptics exert their neurotoxic effects are unclear. We (Cadet et al. 1987 and Lohr et al. 1988) have suggested that an excessive production or a reduced detoxification of free radicals may be responsible, at least in part, for the neuroleptic-induced neurotoxicity. It is also conceivable that a prolonged blockade of dopamine receptors by fluphenazine may decrease the release or interaction of an important neurotrophic factor that is essential for the survival of the large cholinergic neurons. Thus chronic neuleptic treatment may accelerate the loss of these large neurons which die naturally with aging.

The lack of a significant difference in the density of the large neurons after 12 months of treatment warrants comment. We found a significant difference in the density of the large neurons among the 4-, 8- and 12-month control rats, with the 12-month group having significantly lower values than the other two groups. This suggests a possible "floor effect", viz., a marked reduction in the density of the large neurons in the 12-month control group itself so that the effects of aging on the neuronal loss overshadowed the changes produced by 12 months of neuroleptic treatment. Such a conclusion would be consistent with the report by Brizzee et al. (1981) that older age had a far greater effect on the striatal neuropathology than did long-term neuroleptic treatment of rats.

The results of 12-month treatment are also complicated by possible systemic toxicity of fluphenazine in the experimental group (suggested by the death of three animals and an overall significant loss of body weight). We may, therefore, suggest that 8 months of neuroleptic treatment may be optimal for studying striatal neuropathologic changes in rats. An additional technique of assessing neuroleptic-induced neuropathology that will be useful is immunocytochemistry for the identification of the striatal cholinergic neurons. Finally, it will be important to relate the light microscopic findings to the interesting results of the electron microscopic studies published by Benes et al. (1983, 1985) and Meshul and Casey (1989).

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