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Evidence for Cell Loss in Corpus Striatum After Long-Term Treatment With a Neuroleptic Drug (Flupenthixol) in Rats

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Abstract. The number of nerve cells in two different areas of the corpus striatum (i.e., ventrolateral and dorsomedial) was estimated in rat brain after long-term (36 weeks) treatment with the neuroleptic flupenthixol. Nine rats were given weekly injections of 4 mg/kg flupenthixol dissolved in Viscoleo® i.m., and seven rats received Viscoleo® alone. Fourteen to 18 weeks after the last drug injection, the animals were decapitated and half of each brain was fixated with formalin for cell-count analysis and the remaining half used for a biochemical analysis (Nielsen, 1977). Separate cell counts in the ventrolateral and dorsomedial corpus striatum yielded a significant cell loss of approximately 10%, but only in the ventrolateral striatum of treated animals. These results suggest at least one concrete anatomical basis for the behavioral and biochemical deficits found in the same animals, as reported earlier. The results further suggest that persistent irreversible anatomical changes can follow long-term neuroleptic treatment. The inconsistencies of results regarding cell loss in the corpus striatum may be due to neglect of dorsal-ventral structural differences in corpus striatum.

Key words: Flupenthixol – Long-term treatment – Cell loss – Corpus striatum – Neuroleptics.

Long-term treatment with neuroleptic drugs is known to lead to various motor symptoms and disturbances, such as the tardive dyskinesias. These can be a major problem in some patients, and symptoms seem to increase in severity with continued use of drugs (Klawans, 1973; Gerlach and Thorsen, 1976; Gerlach, 1977). Furthermore, recent evidence (Weiss et al., 1977) indicates that similar disorders are reproducible in normal monkeys treated for many months with haloperidol, so that these symptoms are not merely an interaction between the psychotic state and the drug effect, but are largely due to the drug alone. Both dopaminergic hypersensitivity and cholinergic hypofunction have been implicated in the pathology of tardive dyskinesia (Gerlach et al., 1974).

Because of the implication of dopaminergic hypersensitivity, some interest has been directed toward changes in the basal ganglia (Gross and Kaltenbäck, 1968). Such observations led to a series of animal studies with long-term neuroleptic treatment. Pakkenberg et al. (1973) reported a 20% cell loss in the corpus striatum following long-term treatment with perphenazine enanthate. When this 12-month treatment period was shortened to 1–2 months in a subsequent study (Pakkenberg and Fog, 1974), no cell losses were found. In a third study (Fog et al., 1977), a six-month treatment period with a tenfold higher dose of perphenazine enanthate (40 mg/kg s.c. every second week) failed to demonstrate any significant differences from control animals in cell counts from the basal ganglia and the cortex. Similarly, negative results were reported for the long-term effect of perphenazine on the substantia nigra in rats, using both the low dose 12-month treatment and the high dose 6-month treatment, as reported in the previous studies (Gerlach, 1975).

In the above studies, cell-counting in the striatum was almost exclusively restricted to the dorsomedial region, whereas there are good reasons for suspecting that the ventrolateral corpus striatum may be more relevant for symptoms such as the tardive dyskinesias. Just as in the human brain, the rat brain is organized so that the ventrolateral frontal cortex is more closely related to the head and mouth region of the sensory motor cortex (Woolsey, 1958), and there is a topo-

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graphic distribution of projections from the cortex into the basal ganglia (Knook, 1965; Leonard, 1969; Divac, 1972, 1977).

Furthermore, within the corpus striatum in rats there are important functional and behavioral differentiations between dorsal and ventral regions (Neill et al., 1974a, b; Winocur, 1974; Neill and Linn, 1975; Livesey and Muter, 1976; Cools et al., 1976).

Further projections caudally from the corpus striatum are also differentially organized, and Bunney from their Figure 3 (see their subdivisions D, L, V on Fig. 1 below), it is apparent that the dorsomedial area used in the previous cell-counting studies is only one of these possible subdivisions. For these reasons, the following study was arranged so that cell counts could be taken both from the well-known dorsomedial area and from a ventrolateral area corresponding to the different projection regions for collaterals from the frontal cortex, and to the Bunney and Aghajanian nigral projection subdivision.

Materials and Methods

The animals in this study have been sent through various neurological and behavioral tests before sacrifice. Details on these methods and results can be found in Nielsen (1977). A brief overview of the experimental history of these animals is as follows: 16 male Wistar rats, weighing approximately 200 g each, were used. After behavioral training (DRL 15-s schedule with water reinforcement), the animals received the drug treatment schedule or control injections for 36 weeks. After a 7-week pause, the animals were tested on the behavioral schedule for 4–8 weeks. Upon completion of behavioral tests, the animals rested for 2 weeks more before sacrifice. Food was available ad libitum throughout the entire experiment. Animals were deprived of water prior to behavioral tests, but no water deprivation occurred during the drug-treatment period or the 2-week period before sacrifice. Water deprivation schedules were either 24-h deprivation before behavioral tests or a fixed amount of water intake per week, which was given as a daily water ration and amounted to approximately 75 ml/week in all. Since equal numbers of experimental animals and control animals were on these two deprivation schedules, and since no difference could be found regarding cell-count numbers between animals on the two schedules, the data in the present study have been combined for these subgroups.

Drug Treatment Schedule. After completing the training phase, experimental animals ($N = 9$) were injected weekly for 36 weeks with flupenthixol decanoate (4 mg/kg of 1.6%) dissolved in Viscoleo® (oleum vegetabile tenue, i.m.). Control animals ($N = 7$) were injected with the Viscoleo® alone. Injections were aimed at the hind leg biceps femoris muscle and alternated between right and left hind legs to avoid possible irritation.

Histological Procedure. All animals were rapidly decapitated without anesthesia; the skull was quickly opened; the dura was sectioned and a longitudinal midline cut made to separate the hemispheres. One-half of the fresh brain was carefully removed for biochemical analysis. These procedures involved measuring the dopamine metabolite homovanillic acid and the noradrenaline metabolite 3-methoxy-4-hydroxyphenylglycol (see Nielsen, 1977).

The other half of the brain was left undeformed in the skull and was fixated in several changes of formalin (Lillie's fluid) before removal for the cell-counting procedure. The rostral half of each hemisphere was embedded in synthetic paraffin (Paraplast) and sectioned transversely at 6 µm. Each 10th and 11th section was mounted, yielding two parallel series, of which one was stained with gallocyanin while the other served as a reserve for possible staining failures, etc.

Four sections were selected from each series by taking every second section, starting from a rostral plane situated at the caudal end of the insula Calleja magna. This appeared to give a good, relatively fixed, rostro-caudal starting plane. The general appearance of this section is illustrated in Figure 1 and is approximately section 15-B (A. 8380 µm) in the atlas of Kõnig and Klippel (1963).

Microscopic measurements were made using a Leitz Ortholux microscope fitted with 10 × oculars and both 10 × and 40 × objectives yielding a low magnification of 100 × and a high magnification of 400 ×. One of the oculars contained a 10 × 10 grid square, which at 400 × formed the basic counting unit (10000 µm²).

Based on the previous work mentioned above, indicating a topographical organization within the corpus striatum, we chose two different counting areas, a dorsomedial (DM) and a ventrolateral (VL) (see Fig. 1). From the appropriate starting point, the grid was moved in a regular up-and-down scanning sequence over a square counting area consisting of 6 × 6 counting units at each dorsomedial and ventrolateral position in each section. In counting cells which lay under the grid edges, cells were counted only once, even though they also came to lie under the edge of the next adjacent grid placement. The precise positioning of the dorsomedial (DM) and ventrolateral (VL) start points was made as follows. (1) For the DM point the 400 × magnification was used and the grid's lower edge was placed in line with the slight indentation made by the lateral ventricle over the septal region (see Fig. 1) and then moved horizontally until the grid was just inside the corpus striatum. The grid was then moved twice into the striatum and twice upward. If the latter movement brought the grid too near the upper edge of the striatum, it was moved downward until at least half a grid width separated its upper edge from the border. Readings were then noted from vertical and horizontal Vernier millimeter scales on the sides of the microscope stage. (2) The VL starting point was obtained by first using the 400 × magnification and setting the grid's bottom edge at the bottom of the lateral ventricle (see Fig. 1). However, since the lateral border of the striatum, which is formed by fibers of the corpus callosum, is diffi-

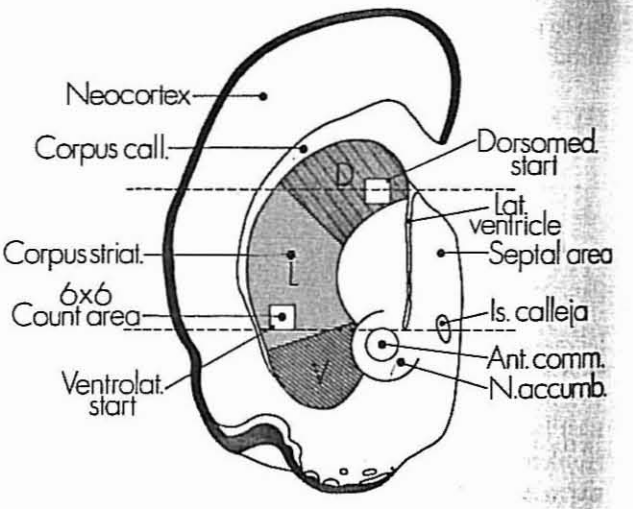

Fig. 1. Cell-counting areas in the corpus striatum

cult to identify at 400×, the lower power objective was then swung in to establish the starting point. At low magnification the grid was moved horizontally and laterally until its upper left-hand corner touched the fibers from the corpus callosu. This insured an adequate placement of the high-magnification grid within the corpus striatum when the microscope was switched back to 400× to begin counting. Coordinates for the VL point were noted from the Vernier scales as before.

The coordinates for all DM and VL start points were determined by the same person (M.L.) and were then entered on coded sheets used by the observers, who were thus unaware of the animals treatment conditions.

Once established, these coordinates allowed the two observers to find the same starting point with a minimum of difference.

Criteria for Cell-Counting. The following criteria, in order of descending importance, were used to identify neurons: (1) large size, presence of identifiable soma, nucleus and nucleolus; (2) large size, identifiable nucleus and nucleolus only, (3) large size nuclear structure alone. For medium-sized cells criterion 3 was not accepted because of possible confusion with glial cells and all smaller cells lacking the combined nucleus/nucleolus structure were not counted. To establish the criteria of cell size, the two observers were trained to make independent cell counts on the same known material until no significant difference was found between their reported counts. The observers ability to use the above individual criteria was also checked by one of us (M.L.), and they were supplied with sketches of typical neurons, glia cells, and artefacts. Using these criteria approximately 1000 cells were counted at each of the DM and VL areas for each animal.

Results

Examination of the brains revealed that one brain from the experimental group could not be used because of faulty sectioning. Cell counts were than made on the remaining material, the mean counts per observer are shown in Table 1.

Statistical analysis of the data from the two observers indicated a small but stable difference in total cells counted on the same sections. These differences and their variance were essentially constant for both experimental and control animals and for DM and VL areas, thus indicating that observers' methodological differences were unlikely to be responsible for the effects reported.

Table 1. Cell numbers in VL area as a percentage of those in DM area

	Control	Experimental	Difference between treatments
Observer 1	98.2	92.2	$t = 3.69$, $df = 7$, $P < 0.01$
Observer 2	100.5	89.5	$t = 2.67$, $df = 7$, $P < 0.05$
Differences between observers	NS	NS	

The table shows the mean VL cell counts expressed as percentages and derived from the comparisons of each animal's VL count with its DM count where DM = 100%. A t-test for matched mean differences was made between treatments and between observers

For both observers, there were no great differences in absolute cell counts between DM and VL areas in the control group (Mean difference, − 7 and + 33), while there were differences in the direction of higher cell counts in DM for the experimental group (Mean difference, + 126 and + 108).

However, due to unavoidable differences in staining, cell volume, section thickness, number of artefacts, etc., it is undesirable to place too much weight on absolute cell number comparisons *between* different animals. The best control measure for these sources of variance is to compare each animal with itself by using the same section and comparing the DM with the VL area. As shown in Table 1, this comparison supported the difference in absolute cell counts and yielded a significant difference for both observers, equivalent to about 10% cell reduction in the VL area.

Discussion

While the small number of animals makes conclusions less certain, the above results support the contention that under certain conditions long-term treatment with neuroleptic drugs in rats may result in a loss of cells in the corpus striatum and that the effect is most readily seen in the ventrolateral striatum.

The fact that this effect is clearly seen, despite its small absolute size (10%), indicates that the ventrolateral area may be much more critical in investigations of this type. All earlier studies (Pakkenberg et al., 1973; Pakkenberg and Fog, 1974; Fog et al., 1977) concentrated their cell-counting in a vaguely defined area in the dorsomedial corpus striatum. We have been fortunate to have the preparations used in these studies available for comparison (see acknowledgements). In general, the counting areas in the Pakkenberg et al. (1973) study were at or slightly rostral to our DM area and at approximately the same location.

In the latest study (Fog et al., 1977) the counting area is, with only a few exceptions, at the same dorsoventral level, but is caudal to our DM counting area. In any case, the ventrolateral frontal cortex, especially the region bordering the sulcus rhinalis, has little if any connection with this dorsomedial area of the corpus striatum (Leonard, 1969; Clavier and Corcoran, 1976; Lyon, unpublished). This may be relevant to the present study in that lesions of the sulcus rhinalis cortex, but not other dorsolateral cortex, have been shown to alter the increase in the stimulating effect of a dopamine-releasing drug (d-amphetamine), while subsequently deepening the behavioral suppression produced by a

DA-receptor blocker (Spiramide) upon conditioned behaviour in the rat (Lyon, 1975). Since flupenthixol is a potent dopamine-receptor blocker in the striatum (Iversen et al., 1976) the possible significance of the loss in conditioned behavior in the animals showing ventrolateral striatal cell loss in the present study (see Nielsen, 1977) should not be ignored. It is, however, too early to be certain whether the behavioral changes produced are primarily related to changes in dopaminergic cell function, even though this certainly plays an important role.

Further studies of this problem should include attempts to identify the intracellular elements altered by the drugs. Fog et al. (1977) did subject some of their animals (after only four months' treatment, however) to electron microscopical analysis, but this was apparently also concentrated in the dorsomedial corpus striatum, and no differences in neurones, glia cells, neuropil, or organelles in the cytoplasm of neurons were found. Unfortunately, no report of possible synaptic differences was given, since it might be expected that the postsynaptic DA-receptor blockade would provide one of the first sites of anatomical changes.

The question still remains as to why the original investigation of Pakkenberg et al. (1973) yielded such a large (20%) cell loss even though counts were carried out in the dorsomedial area, while the other investigations (Pakkenberg and Fog, 1974; Fog et al., 1977), as well as the present study, found no difference in this area. Since a six-month treatment with perphenazine enanthate at ten times the dose level used in the original one-year study did not produce any significant cell loss, it would appear that the source of such changes, in the dorsomedial area at least, depends more on time than on dosage. This argument also appears to support the possibility of very gradual changes at the synapses incurred by long-term neuroleptic treatment, with cell loss the final stage in a lengthy process. Such a gradual decline of cellular activity, with subsequent slow changes in cell size and synaptic connection, might explain the failure of these studies to find excessive glial reaction, cell debris, or other changes related to more traumatic injury. When and how this apparent deterioration occurs is the subject of continuing investigation in our laboratories.

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