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16. Septal cell suspensions were prepared by dissection of developing septal tissue from the ventral forebrain of embryonic donor rats (between the embryonic ages of days E14 and E16) of the same inbred strain. The tissue from 10 to 12 fetuses was collected in 0.6 percent glucose-saline in room temperature, incubated in 0.6 percent glucose and 0.1 percent trypsin-saline for 20 minutes at 37°C, and washed and mechanically dissociated in 0.1 ml of glucose-saline to form a milky cell suspension. Aged rats were anesthetized with 1.25 ml (per kilogram of body weight) of Rompun:Ketalar in a 1:5 ratio, and received three stereotaxic injections of 3-μl portions of the cell suspensions into the hippocampus on each side. Injection coordinates were: A (anterior) = 4.5 mm from interaural line, L (lateral) = ±3.5 mm, V (ventral) = 3.0 mm below dura; A = 3.0 mm, L = ±3.7 mm, V = 3.7 mm; and A = +3.0 mm, L = 4.8 mm, V = 5.7 mm. The incisor bar was set at 0 mm.
17. Nigral cell suspensions were prepared from E13 and E14 embryos, with tissue dissected from the ventral mesencephalic region containing the developinig substantia nigra–ventral tegmental area. Cell suspensions and surgery on the aged rats were conducted identically to the septal suspension injections. Each rat received six 2-μl injections bilaterally, stereotaxically aimed at the head of the caudate-putamen. Injection coordinates were: A = 1.0 mm from bregma, L = ±2.5 mm, and V = 3.5 and 5.2 mm below dura; A = +1.0 mm, L = ±3.5 mm, V = 5.0 and 6.5 mm; and A = −0.2 mm, L = ±3.8 mm, V = 4.0 and 6.0 mm, with the incisor bar set at 0 mm. The locations of the two rostral injections are illustrated in Fig. 2C.
18. Activity tests were conducted in a bank of six automated hole boxes. Each box was constructed of gray plastic 100 by 100 cm^2 with walls 60-cm high. The box was traversed by eight photocell beams, each 2 cm above the floor, at 20 cm intervals and parallel to each wall. Additionally, four rows of four 4-cm circular holes were cut into the floor at 20-cm intervals. Four additional photocell beams passed under the floor 1 cm below each hole to record nose pokes. Each rat received a single 60-minute test. The rat was placed in the box, and locomotor activity (interruptions of the upper photocell beams) and exploratory nose pokes (interruptions of the lower photocell beams) were automatically recorded by on-line connection to an ABC80 microprocessor. Although photocell counts were recorded in 3-minute time bins, only the 60-minute total has been included in the analysis; nose pokes were not analyzed further since young and old rats did not differ on this measure.
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Haloperidol-Induced Plasticity of Axon Terminals in Rat Substantia Nigra

Abstract. *An electron micrographic morphometric analysis of nerve endings in substantia nigra of rats repeatedly treated with haloperidol was performed. Although most parameters showed no difference, drug-treated animals exhibited a significant shift in the distribution of relative numbers of axon terminals, suggesting neuroleptic-induced axon-collateral sprouting.*

Various experimental studies have lead to the conclusion that dopamine receptor blockade is the mechanism of action of antipsychotic agents (*1*). Neuroleptic effects on the "dopamine system" include not only antipsychotic actions but also extrapyramidal movement syndromes (*2*). Permanent alterations of striatal and cortical integration following cessation of neuroleptic treatment have been inferred from clinical observations of tardive dyskinesia (*3*) and the so-called supersensitivity psychosis (*4*), respectively. These lingering effects of antipsychotic medication in some patients raise the possibility that these agents may induce structural changes in the synaptic organization of brain regions involved in dopamine-controlled mechanisms. A few studies to date investigating morphological changes associated with neuroleptic medication have used light microscopic approaches to suggest a decrease in the number of dopaminergic cells in substantia nigra (SN) (*5*). Since changes in central integration reflecting structural reorganization at the synaptic level could only be revealed by a quantitative electron microscopic (EM) analysis, the study here reported was done by the use of quantitative morphometric assessments at the EM level (*6*) to examine the synaptic relationships in SN of rats with chronic neuroleptic treatment.

Male Sprague-Dawley rats (200 ± 5 g) were used in this study. Experimental drug-treated rats were injected daily for 16 weeks with haloperidol (3 mg/kg, based on the daily body weight; McNeil Laboratories) dissolved in 0.02 m*M* lactic acid. Control animals were injected with appropriate volumes of lactic acid alone. A detailed description of handling, treatment, and behavioral changes has been presented elsewhere (*7*). At the end of the 16-week interval, all animals were anesthetized with Chloropent (1.0 ml per 300 g of body weight) and perfused intracardially (*8*). The SN, excised using a Zeiss OpMi-1 surgical microscope, was postfixed with 1.5 percent osmium tetroxide in 0.1*M* cacodylate buffer (*p*H 7.4), stained en bloc with 2 percent uranyl acetate, dehydrated with a graded series of ethanol and propylene oxide, and embedded in Spurr's epoxy resin. Thin (1-μm) sections were stained with toluidine blue to confirm the orientation of the SN. In addition, the numerical density and volume of dopaminergic and nondopaminergic neurons in the pars compacta distinguished by cytological criteria (*8*), were determined using a camera lucida projection system attached to a Zeiss light microscope. For neuron size, ten cells were obtained from each animal for a total of 40 control and 40 haloperidol-treated cell samples.

Ultrathin sections (30 to 40 nm thick, silver-gray) were counterstained with lead citrate, and viewed with a Siemens Elmiskop electron microscope. The EM morphometric analysis of SN was performed "blindly" on cross-sectional profiles of dendrites in the transitional zone between the pars compacta and the pars reticulata, which were in synaptic relationship with at least one axon terminal (Fig. 1A). A minimum of 12 dendrite cross-sectional profiles were obtained for each control ($N = 4$) and haloperidol-treated ($N = 4$) rat. The data shown in Fig. 1 represent 51 control and 48 drug-treated dendrite samples with their associated terminals ($N = 166$ and 187, respectively). The presence of a synapse was considered necessary to permit the definitive differentiation of a dendrite from glial processes. Number and area of dendrites and axon terminals, as well as the numerical density of synaptic vesicles, were determined for each sample according to a modification (*9*) of standard stereological techniques (*6*). These data were expressed as cross-sectional area of dendrites (caliber), number of axon terminals per dendrite, axon terminal volume, and number of synaptic vesicles per unit volume of axon terminal (synaptic vesicle density). A mean and standard error of the mean was obtained for each variable for the respective animals in each group, and these were used to assess the consistency of findings from animal to animal. In all cases, the values obtained for the animals in each experimental group showed good agreement.

A distribution curve for each parameter was initially plotted for experimental and control groups to determine whether

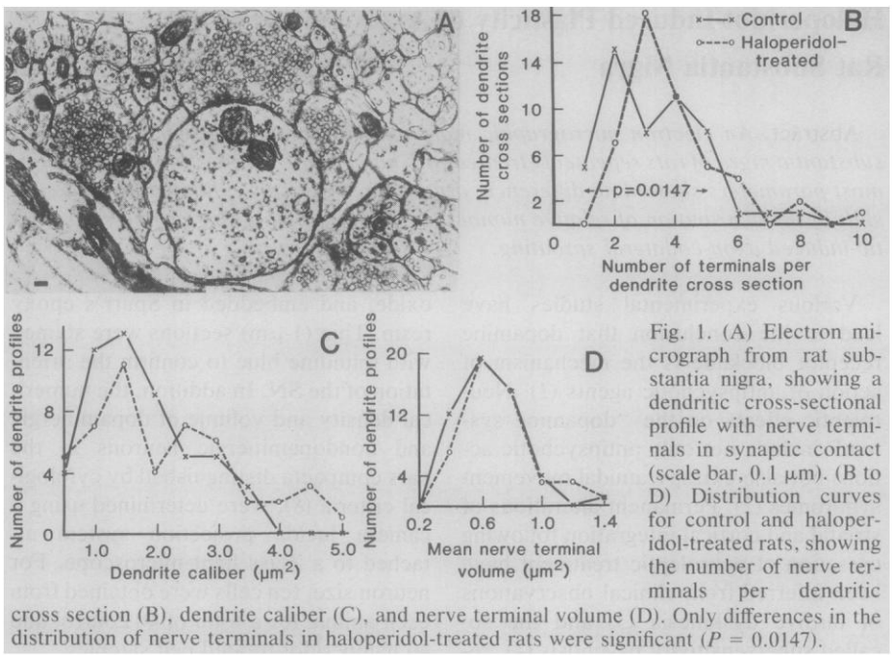

Fig. 1. (A) Electron micrograph from rat substantia nigra, showing a dendritic cross-sectional profile with nerve terminals in synaptic contact (scale bar, 0.1 μm). (B to D) Distribution curves for control and haloperidol-treated rats, showing the number of nerve terminals per dendritic cross section (B), dendrite caliber (C), and nerve terminal volume (D). Only differences in the distribution of nerve terminals in haloperidol-treated rats were significant ($P = 0.0147$).

that variable exhibited a skewed distribution. The two-tailed Student's *t*-test was used to evaluate the significance of differences for variables showing a normal distribution (neuron density and size, nerve terminal volume, and synaptic vesicle density). The Wilcoxon rank-sum test was used for those showing a nonnormal distribution (dendrite caliber), except in the case of the number of boutons per dendrite, where extensive ties between control and drug-treated animals occurred. The computation of exact probabilities for R × C contingency tables was chosen to analyze the number of boutons per dendrite instead of the Kolmogorov-Smirnov two-sample test because it provides a more detailed and comprehensive evaluation of distribution patterns.

Visual inspection of dendrites and axon terminals in the two groups did not reveal any obvious differences. Despite the apparent similarities, however, morphometric analysis showed that the number of axon terminals per dendrite profile was significantly changed in the SN of haloperidol-treated animals (Fig. 1B). Figure 1B shows that a large proportion of dendrites (39 percent) in control SN have only one or two terminal profiles. In contrast, no dendritic samples from haloperidol-treated nigra showed only one terminal; only five (10 percent) showed two nerve terminals. The experimental animals showed a sharp peak at three terminals per dendrite (38 percent of drug-treated samples) while only 16 percent of the control samples contained this same number of terminals per dendrite. It seems unlikely that these results represent random variations among animals since individual animals within each group showed similar patterns. The major difference in terminal numbers was significant in the range of one to four terminals per dendrite cross section ($P = 0.0147$). Both groups had a small proportion of dendrites with five or more terminal profiles, but no differences occurred in this range. The distributions of dendrite caliber (Fig. 1C) and nerve terminal volume (Fig. 1D) showed no significant changes in drug-treated animals. Other variables compared between control and haloperidol-treated rats, such as neuron density (60.2 ± 15.6 versus 60.8 ± 16.9), neuron size (0.16 ± 0.01 versus 0.16 ± 0.01 mm^2), and synaptic vesicle density (232 ± 8 versus 211 ± 9 vesicles per square micrometer), did not show significant differences. In addition, preliminary assessments of the numerical density (number per unit area) of dendritic profiles revealed no differences between the two groups. This latter finding argues against the possibility that the increase in the number of terminals per dendrite cross section was only an apparent one, which occurred because of spatial rearrangements of preexisting terminals. Such rearrangements could theoretically arise if a portion of the dendritic pool were resorbed and the associated terminals established new connections with remaining dendrites.

In an attempt to identify the type of terminals that may have been increased, the samples were classified by two independent "blind" raters according to four morphological classes [all containing small clear vesicles (SCV)] based on the presence or absence of symmetrical synaptic thickenings (ST) and large dense vesicles (LDV). A fifth heterogeneous group that did not fit any of these categories and accounted for less than 9 percent of the nerve terminal population included terminals with asymmetrical thickenings. The data indicate that only terminals with SCV, LDV, and no ST were doubled in haloperidol-treated animals, suggesting that each of a preexisting group of axons may have sprouted only one new axon terminal. Thus it is not likely that these terminals contain γ-aminobutyric acid since the latter have been shown to have symmetrical thickenings (*10*). Moreover, substance P nerve endings in the locus coeruleus and hypothalamus appear to have asymmetrical thickenings (*11*), while those in nigra may show both symmetrical and asymmetrical densities (*12*). It is possible that endings with no thickening could contain membrane specializations in subsequent sections, but this seems unlikely since terminals with thickenings showed no change in numbers. The identity of the neurotransmitter or peptide system associated with the sprouted fiber type cannot be determined at this time.

The proliferation of SN axon terminals in relation to haloperidol may be seen as a reflection of the pharmacologic actions of this drug, or it may parallel the development of tolerance to the neurological effects of the medication. Behavioral studies performed on the same animals revealed that substantial tolerance to the cataleptic and ptotic (sedative) effects of haloperidol may develop over time (*7*). Since tolerance to catalepsy and ptosis in these animals was appreciable within 3 to 4 weeks of initiating drug treatment (*7*), the above question might be resolved by studying the morphological effects of haloperidol at an earlier interval. A final consideration regarding the acquired axon terminals is whether they represent a reversible phenomenon. Subsequent studies can determine whether the morphological effect reported here persists after drug treatment has been terminated. The answer to this question might contribute to our understanding of the mechanism of tardive dyskinesia or other adaptive neurological responses to prolonged neuroleptic treatment.

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Neurofilament Accumulation Induced in Synapses by Leupeptin

Abstract. *The hypothesis that the usual absence of neurofilaments in synaptic terminals is due to their degradation by the calcium-activated protease present in axoplasm was tested by injecting leupeptin, which inhibits the protease, into the optic tectum of goldfish kept at 15° and at 25°C. The resulting accumulation of neurofilaments in synaptic terminals provides in vivo evidence in support of the hypothesis. The significance of these results and the potential uses of this pharmacological tool are discussed.*

Two major unsolved questions in neurobiology are: What happens to the cytoskeleton when it reaches the synaptic terminals? and How is the growth of axons regulated and what makes them stop growing when they reach their target cells? The cytoskeleton consisting of neurofilaments and microtubules is generated in the neuronal soma and transported along the axons at a rate of 1 to 4 mm per day (*1*). This process is considered to play a major role in axonal growth, which occurs at a rate that is approximately the same as the rate of delivery of cytoskeletal elements (*2*). Axoplasmic transport, however, occurs throughout the life of the neuron, even when the neuron is not growing, but neurofilaments and microtubules are usually not present in synaptic terminals. Therefore, some mechanism must exist to prevent the accumulation of neurofilaments and microtubules in synaptic terminals.

When neurofilaments are found in synaptic terminals, they are often in the form of annuli which, in sections of silver-impregnated material, are seen as dark neurofibrillary rings (*3*, *4*). Such rings occur only sporadically in the central nervous system of most vertebrates (*3*, *5*). However, in the brains of poikilotherms, such as lizards, bullfrogs, and goldfish, that have been subjected to low temperatures, the rings are numerous and quantitatively related to the environmental temperature (*6*, *7*). In seeking to explain this phenomenon in goldfish, Roots and Bondar (*8*) postulated that when the neurofilaments reached the synaptic terminals they were subjected to enzyme-mediated breakdown; during prolonged exposure to low temperatures, enzyme activity was inhibited either by a change in *p*H during acclimation or by the low temperatures, so that the breakdown of neurofilaments was slower than the delivery of neurofilaments to the terminals by axoplasmic transport. After the discovery of a calcium-activated endogenous protease in the axoplasm of invertebrate giant axons (*9*), Lasek and Hoffman (*2*) proposed a model that would explain not only the usual absence of neurofilaments in synaptic boutons but also how the growth of axons is regulated and terminated when the target cells are reached. According to the model, when the growth cone meets a target cell, differentiation of a synaptic terminal is initiated and mechanisms that degrade the cytoskeleton are activated. Lasek and Hoffman (*2*) proposed that within the axon the endogenous protease is inactive, but upon entering the axon terminal it is activated by calcium ions and disassembles the neurofilaments by cleaving the polypeptide backbone. An integral assumption in the model is that the calcium ion concentration in the axon differs from that in the terminal. The calcium ions in the axon terminals could originate from the agranular reticulum or the mitochondria and, once transmission begins, from the extracellular space, since there is evidence that calcium enters axon terminals in association with transmitter release (*10*) and membrane depolarization (*11*). Because microtubule disassembly can be brought about in vitro by low concentrations of calcium, the increased concentration of calcium in the terminals could also alter the equilibrium between polymerized microtubules and free tubulin (*12*). Axons from a wide variety of animals are now known to contain a calcium-activated protease (*13*, *14*). This enzyme is capable of disrupting neurofilaments and of cleaving neurofilament proteins in vitro. The finding that calcium-activated protease is present in various types of axons and is not restricted to invertebrate giant axons lends credence to the general applicability of the hypothesis.

The experiment described here was designed to test the hypothesis that neurofilaments are degraded in synaptic terminals in vivo by a calcium-activated protease. If the hypothesis is correct, inhibition of the protease by an inhibitor such as leupeptin would result in the accumulation of neurofilaments in the synaptic terminals.

Leupeptin was injected into the right optic tectum of goldfish maintained at 15° or at 25°C at a dose of 1.2 mg per 100 g of body weight. Depending on weight, 5 to 12 μl of a solution of leupeptin in teleost saline (50 mg/ml) (*15*) was injected. Control fish received saline

Fig. 1. Optic tectum 72 hours after injection of leupeptin in a goldfish kept at 15°C. Neurofibrillary rings are darkly stained. Scale bar, 5 μm.

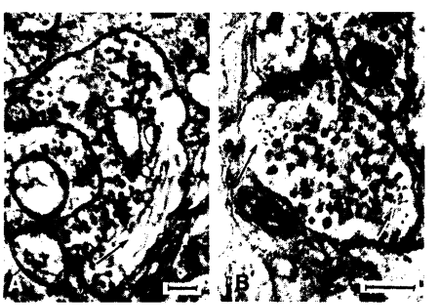

Fig. 2. Synaptic terminals in the optic tectum 48 hours after injection of leupeptin in a goldfish kept at 25°C. (A) Neurofilaments seen in a longitudinal plane (arrow). (B) Two groups of transversely sectioned neurofilaments approximately 1 μm apart (arrows). These represent a cross section of a neurofibrillary ring seen by light microscopy (Fig. 1). Scale bars, 300 nm.