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# Aging-Related Changes in Rat Striatal $D_2$ Receptor mRNA–Containing Neurons: A Quantitive Nonradioactive *in situ* Hybridization Study

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***In situ* hybridization of a digoxigenin-labeled oligonucleotide probe combined with computer-assisted image assessment was used to directly visualize $D_2$ receptor mRNA–containing neurons in rat striata, and quantify age-related changes in the sizes and relative mRNA content of these neurons. It was found that: (1) numbers of $D_2$ mRNA–containing neurons appear to decrease in striata of aged rats, (2) relative amounts of $D_2$ receptor mRNA decrease in striatal neurons of aged rats, and (3) neurons of all sizes appear to be lost, with the greatest absolute decrease in those smaller than 90 $\mu m^2$. These data confirm the well documented age-associated loss of $D_2$ dopamine receptors, and further suggest that both neuronal death and reduced transcription contribute to this decrement.**

***[Key words: aging, $D_2$ receptor mRNA–containing neurons, striatum, nonradioactive* in situ *hybridization, digoxigenin, computer-assisted image assessment]***

Loss of striatal $D_2$ dopamine receptors is one of the most robust and functionally important neurochemical markers of aging in species ranging from rodents to humans (for reviews see Lai et al., 1987; Roth and Joseph, in press). The mechanisms responsible for this decrement are both molecular and cellular (Roth and Joseph, in press). All of these changes are in the range of 30–50% over the adult lifespan.

At the molecular level, the net rate of the receptor biosynthesis has been shown to be reduced in the aged striata in a number of reports (Henry and Roth, 1984; Henry et al., 1987; Norman et al., 1987). In addition, the concentration of striatal $D_2$ receptor mRNA decreases up to 50% (Mesco et al., 1991; Della Vedova et al., 1992; Weiss et al., 1992) and is synthesized more slowly (Mesco et al., 1993) during the same period of time, suggesting that a gene transcriptional deficit might contribute to the aging-associated loss of $D_2$ receptors.

In addition to these molecular alterations, approximately 20% of the striatal neurons are lost over the same period (Han et al., 1989). The neurons involved in $D_2$ receptor loss apparently reside within a kainic acid–sensitive population (Joseph et al., 1991). *In vitro* studies using neonatal rat striatal neurons suggest that the larger $D_2$ receptor–containing neurons are most vulnerable to kainate, and that $D_2$ receptor–containing neurons in general are more vulnerable than those containing $D_1$ receptors (Mesco et al., 1992). In light of parallels between such *in vivo* and *in vitro* observations, and the fact that kainic acid toxicity is mediated through a glutamate receptor signal transduction pathway involving calcium influx and possibly oxygen free radicals (Crowder et al., 1987; Mesco et al., 1992), it is conceivable that neuronal death may occur through a related mechanism during normal aging. Thus normal dopaminergic or colocalized glutamatergic neurotransmission may eventually lead to neuronal death.

In the next step toward testing this hypothesis, it became necessary to identify actual $D_2$ receptor–containing neurons, determine their sizes and relative $D_2$ receptor mRNA content, and assess their numbers in striata of young and old rats. Since previous studies on striatal neuronal quantity measured only total cells, not neurons specifically containing the $D_2$ receptor, $D_2$ receptor–containing neurons might be lost to an even greater extent than other neurons. Therefore, it is still uncertain as to how much of the decrease in $D_2$ receptors and mRNA concentrations in the aging striata is due solely to neuronal death or whether a downregulation of gene transcription might actually result in death. In the present study, we used *in situ* hybridization of a digoxigenin-labeled oligonucleotide probe for the $D_2$ receptor mRNA and computer-assisted image assessment to directly visualize $D_2$ receptor mRNA–containing neurons and compare their density, relative amounts of mRNA, and sizes in mature and senescent rats.

## Materials and Methods

*Animals and brain tissue preparation.* Male Wistar rats aged 6 months (mature) and 25–26 months (senescent) were obtained from the colony of the Gerontology Research Center (NIA), Baltimore, MD. The older animals were generally in good health, without detectable tumors. Animals were killed by decapitation and the brains were quickly removed, frozen, and stored at −70°C. Serial 10 μm cryostat sections were cut on a freezing microtome, collected on nuclease-free slides (PGC Scientific, Gaithersburg, MD), and stored at −70°C until required.

*Nonradioactive* in situ *hybridization.* Our protocol was that of Brouwer et al. (1992), with slight modification. Briefly, an oligonucleotide probe (30-mer) complementary to nucleotides 4–33 of the rat $D_2$ cDNA (Bunzow et al., 1988) was purchased from the Midland Certified Reagent Company (Midland, TX) and labeled with digoxigenin following the manufacturer's direction (DNA tailing kit, Boehringer Mannheim, Indianapolis, IN). The slides were air dried, fixed with 4% paraformaldehyde solution (in phosphate-buffered saline, pH 7.4, 15 min), dehydrated in a graded series of ethanol up to 100%, delipidated in chloroform (10 min, RT), then partially rehydrated (95% ethanol) and air

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dried before being incubated in hybridization buffer [4× sodium (SSC)/10% dextran sulphate/50% formamide/1× Denhardt's solution/250 μg/ml$^{-1}$ sonicated salmon sperm DNA] containing the digoxigenin-labeled antisense $D_2$ oligonucleotide, at a final concentration of 0.025 pmol per microliter. Sections were hybridized overnight at 37°C in humidified chambers, after which time they were washed sequentially in 2× SSC, 1× SSC, 0.5× SSC, each 30 min at room temperature, followed by washing in 0.1 M Tris-HCL buffer, pH 7.4/0.15 M NaCl (2 × 10 min). Sections were then processed for immunological detection with alkaline phosphatase (AP)-conjugated anti-dioxigenin IgG (as described in the Dioxigenin Detection Kit, Boehringer Mannheim, Indianapolis, IN). After color developing, dehydration in ethanol, and clarification in xylene, the sections were coverslipped with Permount. The blue/purple precipitates indicated the presence of dopamine $D_2$ receptor mRNA. Nonspecific sites were determined by incubating the slides with only hybridization buffer that contained no labeled probe. In order to insure accurate, quantitative comparisons of rats of different ages, equal numbers of sections from young and old brains were processed simultaneously in the same reagents under the same conditions. In addition, the optical density of the color reaction was found to increase proportionally with total RNA concentration up to 1.7 μg/ml, as indicated by dot blot hybridization of increasing striatal RNA dilutions. The color intensity at this concentration far exceeded that in any of the sections.

*Computer-assisted image assay.* Each positive-staining neuron was measured for optical density and size under bright-field illumination (400× magnification), using the Carl Zeiss IBAS20 program (Hanover, MD), by an independent observer. This program automatically corrects for any differences in background among preparations. All measurements were made in the dorsomedial region of the striatum, except for a subset in the dorsolateral area where noted. Six microscopic fields per section and six sections per animal were sampled. Numbers in figures refer to actual individual animals used for statistical analysis.

## Results

### *Identification of $D_2$ receptor mRNA–containing neurons*

Sites of expression of $D_2$ receptor mRNA were visualized by the concentration of purple/blue AP reaction product. Areas of dense staining were observed in striosomes of the striatum, cortex, and amygdaloid area (data not shown), as previously reported by Brower et al. (1992). Staining of individual cells is particularly obvious at high magnification in striata of a young and old rat (Fig. 1, Y and O, respectively).

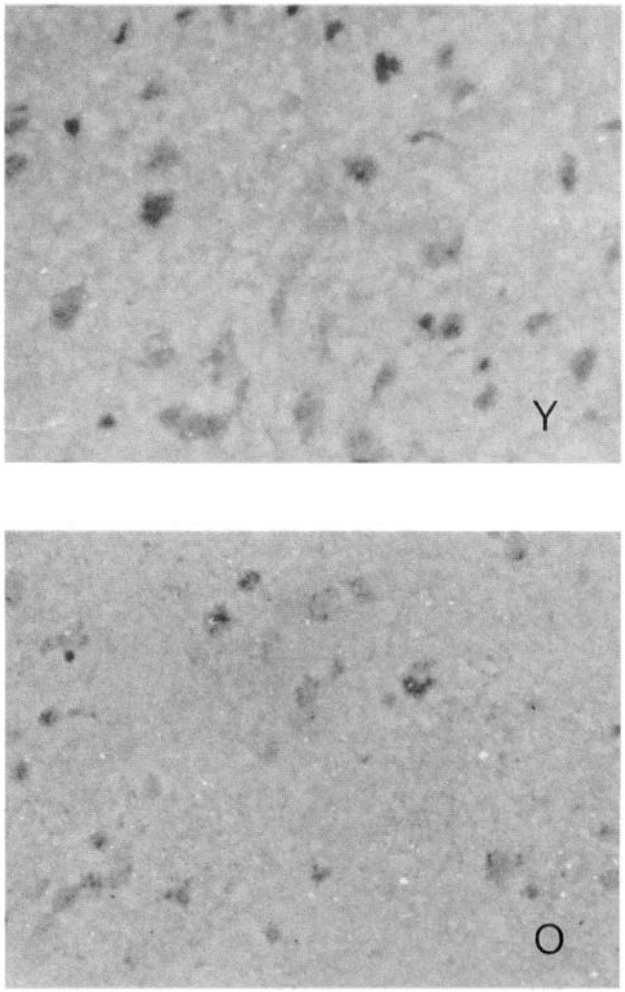



*Figure 1.* Bright-field photomicrographs of neostriatal sections, illustrating the age-related difference in the $D_2$ receptor mRNA–containing neurons. Note the relatively high cell density and darker staining neurons in the young rat (*Y*) compared to low cell density and more lighter staining neurons in the old rat (*O*) at 400× magnification of the striatal area.

### *Loss of $D_2$ mRNA–containing neurons in the striata of senescent rats*

Numbers of positive neurons per 0.5 mm$^2$ microscopic field were obtained for each striatal pair, and analyzed by the Student's *t* test, which showed a significant difference between the mature and senescent groups for the dorsomedial region (Figs. 1, Y and O, 2; $p < 0.01$). There appears to be an approximately 27% loss of $D_2$ receptor mRNA–containing neurons in the senescent rats. In order to determine whether a similar loss occurred in other areas of the striatum, the dorsolateral region was also examined, and positive neuronal numbers were 91 ± 11 and 64 ± 6 for mature and senescent animals, respectively ($p < 0.05$). This loss is 29%.

### *Decrease in $D_2$ receptor mRNA content of aged striatal neurons*

The intensities of AP reaction product reflect the relative amounts of $D_2$ receptor mRNA, which are decreased in aged striatal neurons (a typical example is shown in Fig. 1). In order to determine the pattern of $D_2$ receptor mRNA change in individual neurons during aging, the optical density of AP reaction product was measured for each positive neuron, and a frequency distribution curve was plotted for each animal. When these curves are compared, a leftward peak shift (i.e., toward lower average values per neuron) is observed in senescent rats (Fig. 3, top). For statistical comparison, the neuronal population from each animal was then separated into five optical density ranges, which were subjected to two-way ANOVA and showed a significant main effect of aging ($F[1,90] = 11.703$, $P < 0.001$) as well as an age by OD interaction ($F[4,90] = 11.963$, $P < 0.001$) (Fig. 3, bottom). A subsequent Student's *t* test for each subpopulation showed: (1) neurons with low mRNA content (ODs are less than 0.21) increased significantly in old rats by 44% ($p = 0.01$); (2) neurons with medium mRNA content (OD range from 0.21 to 0.28 and 0.28 to 0.35) decreased significantly in old rats by 47% and 55%, respectively ($p < 0.0$); and (3) neurons with high mRNA content (ODs greater than 0.35) did not change with age ($ps > 0.05$).

### *Apparent loss of $D_2$ receptor mRNA–containing neurons of all sizes in the striata of old rats*

The size of each positive neuron was measured and frequency distribution curves plotted for each animal. When distribution curves were compared as a function of age, an apparent loss of $D_2$ receptor mRNA–containing neurons of all sizes was observed

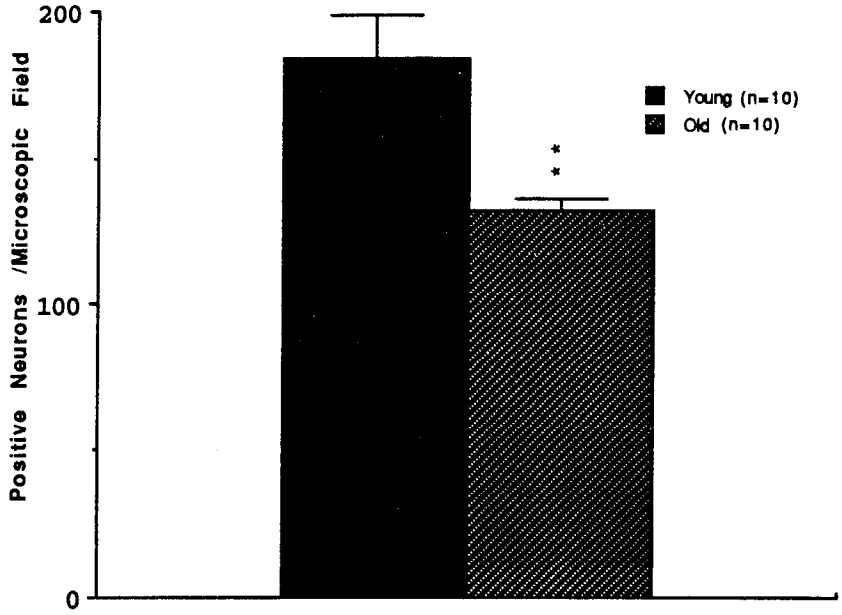



*Figure 2.* Decrease of $D_2$ receptor mRNA–containing neurons in the aged rat striata. Old (25–26 months) and young (6 months) rats were sampled to assess the number of positive-staining neurons per microscopic field (0.5 $mm^2$). The bars provide means of counts ± SEM. ** Indicates significant decrease in the aged rats ($p < 0.01$, Student's $t$ test).

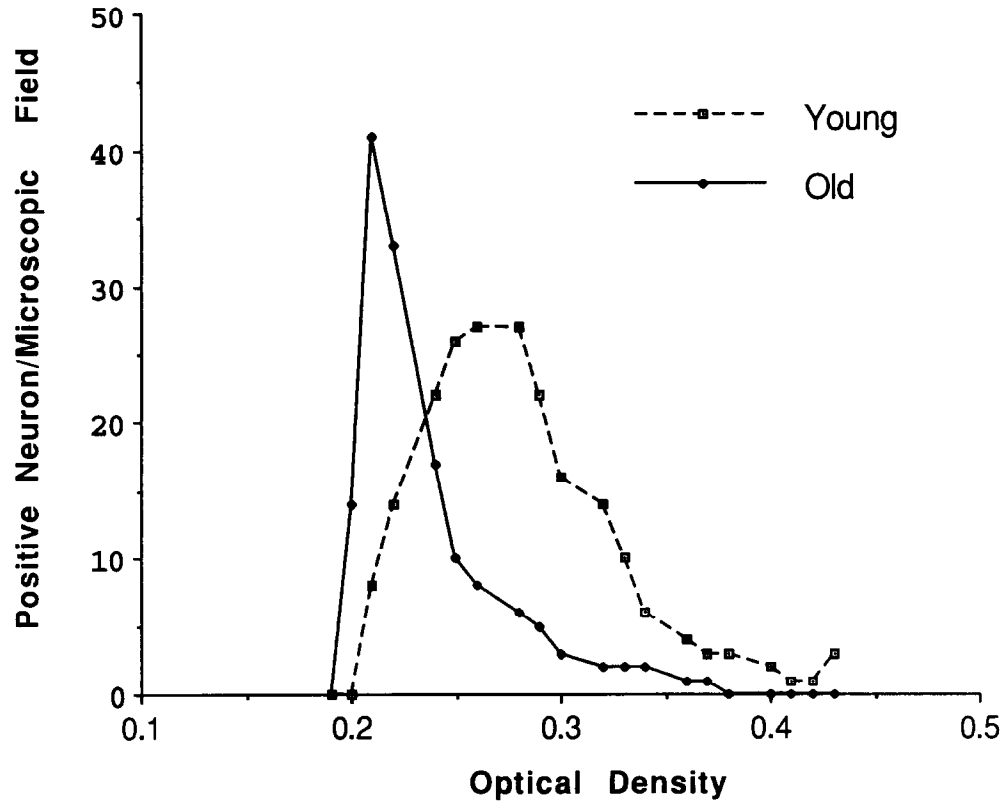
in the striata of old rats (Fig. 4, top). Two-way ANOVA showed a significant main effect of age ($F[1,90] = 40.9$, $P < 0.001$), as well as an age by size interaction ($F[4,90] = 4.2$, $P < 0.01$). Relative decreases in neuronal populations are 38% for large (>210 $\mu m^2$) and small (≤30 $\mu m^2$) neurons and 20–25% for medium-size neurons ($ps < 0.01$ by Student's $t$ test) (Fig. 4, bottom). However, since most neurons are less than 90 $\mu m^2$, absolute loss appears to be greatest in these populations.

## Discussion

The present study has combined nonradioactive *in situ* hybridization with computer-assisted image assessment to demonstrate for the first time the apparent loss of $D_2$ receptor–containing neurons from rat striata during aging. This brain region appears to be the most important in the loss of dopaminergic function (Roth and Joseph, in press), and the high concentration of positive neurons therein, as well as the anatomical distribution of other $D_2$ receptor mRNA–positive neurons, is in good agreement with the report of Brouwer et al. (1992) which employed the same hybridization technique.

Our laboratory previously reported a 19% absolute loss of rat striatal neurons between 3–6 and 24–26 months of age (Han et al., 1989). The slightly larger decreases, 27% and 29% for dorsomedial and dorsolateral striatum, respectively, observed in the present study suggest that $D_2$ receptor–containing neurons may be more vulnerable than other types of striatal neurons. Absolute positive neuronal numbers are higher in the dorsomedial region despite lower receptor densities, consistent with our earlier report (Han et al., 1989). Although several groups have reported loss of $D_1$ dopamine receptors during aging (e.g., Henry et al., 1986), these are much less consistent than those for the $D_2$ receptor (Lai et al., 1987; Roth and Joseph, in press). Our laboratory has also observed greater susceptibility of cultured neonatal rat $D_2$ neurons than $D_1$ to the toxic effects of kainic acid (Mesco et al., 1992). Moreover, the $D_2$ receptor–containing neurons apparently lost during aging appear to reside in a kainate-sensitive population based on *in vivo* toxicity studies in mature and senescent rats (Joseph et al., 1991). Since kainic acid appears to exert its effects through the glutamatergic system, utilizing calcium influx and possibly oxygen free radicals (Crowder et al., 1987; Mesco et al., 1992), it is tempting to speculate that such mechanisms may be responsible for $D_2$ receptor neuron death *in vivo*. Since neuronal colocalization of $D_2$ and glutamate receptors has not yet been conclusively established, it is also conceivable that dopamine itself may exert some toxicity (Michel and Hefti, 1990; L. Zhang, A. Ravipati, J. Joseph, G. S. Roth, unpublished observations). Thus, since our previous report counted all types of striatal neurons, loss was slightly less than the present study measuring only the more sensitive $D_2$ neurons.

Relative content of $D_2$ receptor mRNA per striatal neuron generally decreases, as indicated by the leftward shift of frequency distribution curves for old rats. This is consistent with our previous reports (Mesco et al., 1991, 1993) and those of others (Della Vedova et al., 1992; Weiss et al., 1992), demonstrating reductions of up to 50% in $D_2$ mRNA concentrations in aged animals. It is not possible to determine from the present data whether decreases in mRNA content occur in essentially all $D_2$ receptor neurons or reflect preferential loss of cells with medium mRNA concentrations. It is evident from present results that loss of neurons cannot solely explain the decreases in $D_2$ receptors and mRNA, since cells with low mRNA content actually increase during aging. It is possible that the age-related loss of $D_2$ receptor neurons might not be as great as that observed here, if $D_2$ receptor mRNA concentrations drop below detectable levels in some cells, rendering them $D_2$ negative by the present technique. It is not possible to determine from the present data whether a decrease in mRNA content is a cause or effect of neuronal death. Further insight into the causal relationship between mRNA loss and cell death might be obtained by future examination of intermediate age groups of animals or by following the chronology of molecular events following induction of neuronal death by various means (neurotoxins, etc.). If any situations exist in which either cells or mRNA are lost in the absence of loss of the other, the absent phenomenon might be presumed to be noncausal. However, even this approach would not be definitive if separate neuronal subpopulations exhibit mRNA loss and/or cell death at different times in the lifespan or under different neurotoxicity conditions.



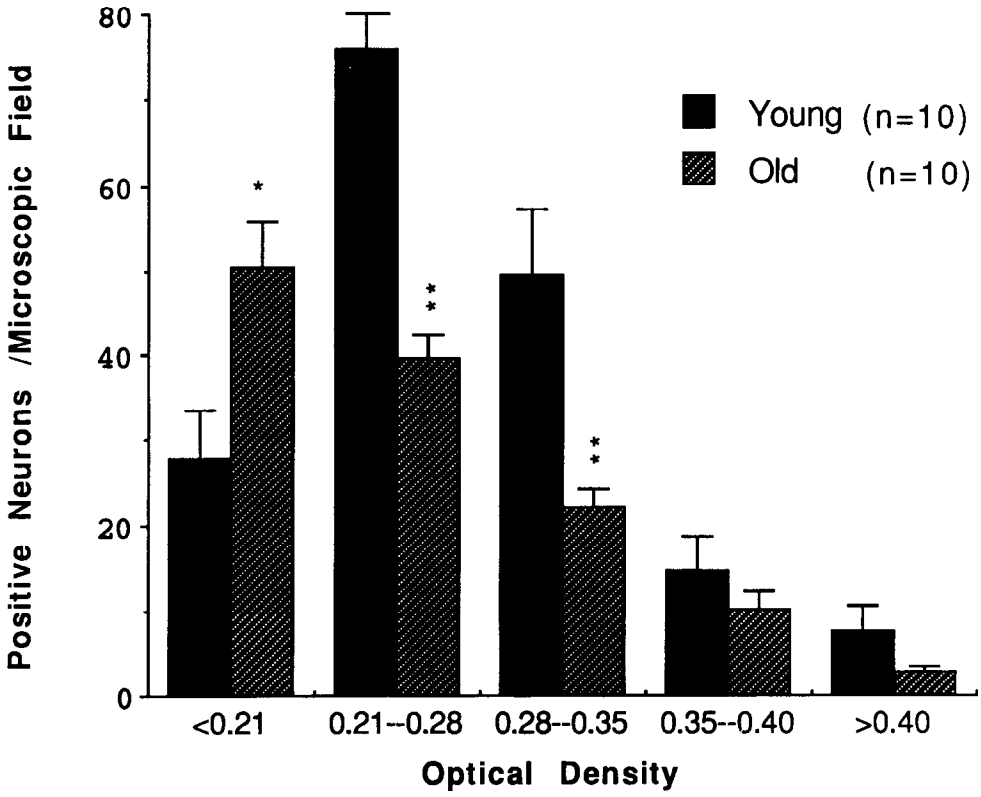
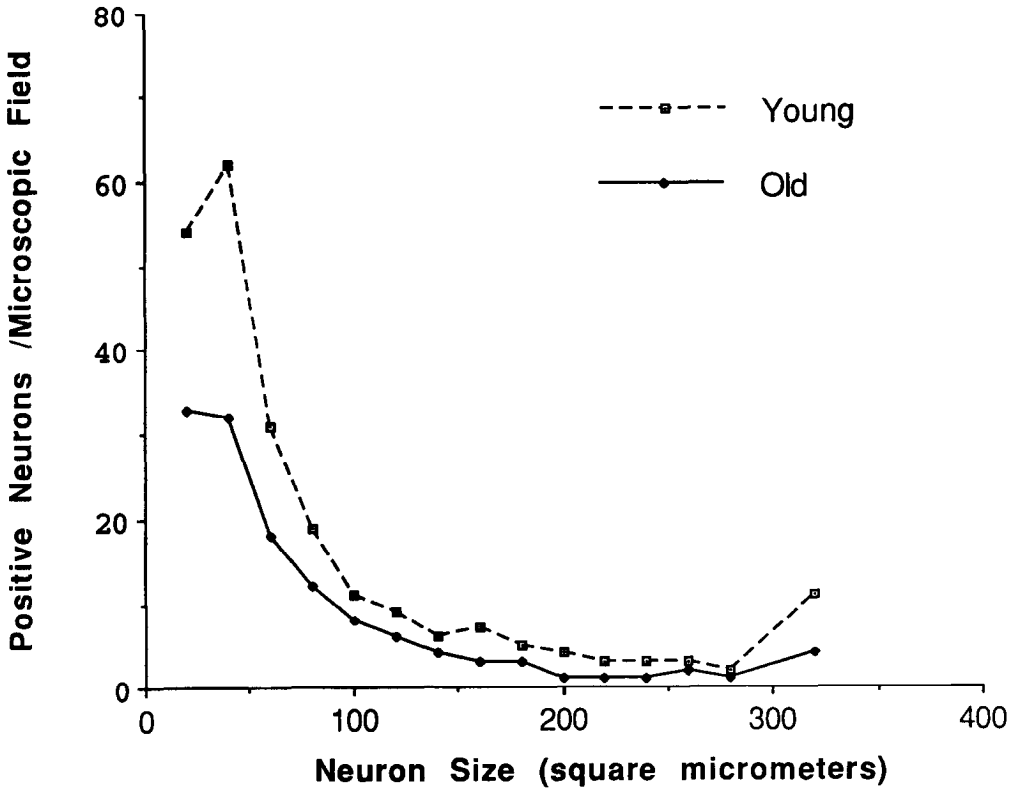




*Figure 3. Top,* Optical density (representing relative amount of mRNA) frequency distribution curves. The OD for each positive neuron was measured and plotted into a frequency distribution curve for each animal. The curves for a typical young and old rat are shown here. Note the leftward shift in the peak of the old animal. *Bottom,* Effect of aging on optical density (relative amount of mRNA) frequency distribution in neurons of different OD levels. All positive neurons for each animal were divided into five subpopulations of different OD ranges. The normalized values of positive neuron number per microscopic field were first analyzed by a two-factor Anova analysis, followed by a Student's $t$ test for each subpopulation. A 2 (age) by 5 (OD) Anova revealed a significant mean effect of age ($F[1,90] = 11.703$, $p < 0.001$) as well as an age by OD interaction ($F[4,20] = 11.9.63$, $p < 0.001$). The bars provide means of counts ± SEM. * Indicates a significant age difference in the subpopulation ($p < 0.05$, Student's $t$ test). ** Indicates $p < 0.01$.



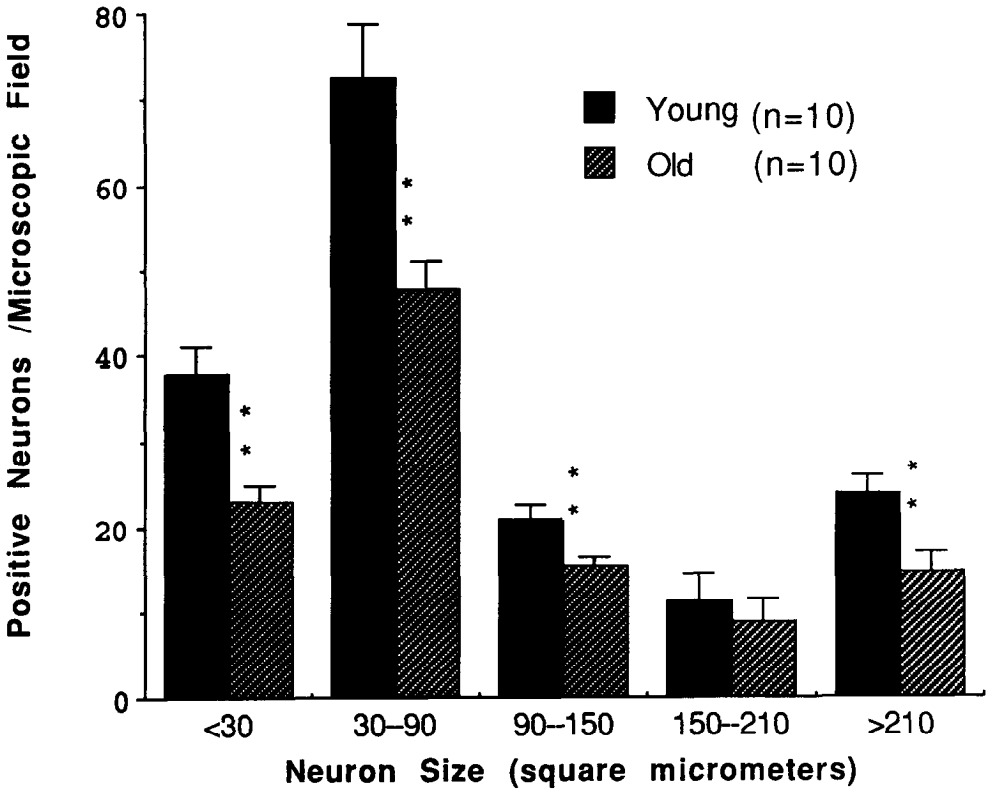




*Figure 4.* *Top,* Frequency distribution curves for $D_2$ mRNA–positive neurons as a function of size. The size of each positive neuron was measured and then a frequency distribution curve was plotted for each animal. Curves for a typical young and old rat are shown here. Note a general loss of positive neurons of all sizes, with the greatest absolute loss for those smaller than 90 $\mu m^2$. *Bottom,* Effect of aging on frequency distribution of $D_2$ mRNA–positive neurons as a function of size. Positive neurons of each animal were classified into five subpopulations of different size ranges. The normalized values of positive neurons per microscopic field were first analyzed by two-factor Anova analysis, followed by a Student's $t$ test for each subpopulation. A 2 (age) by 5 (size range) Anova revealed a significant main effect of age ($F[1,90] = 40.9$, $p < 0.001$) as well as an age by size interaction ($F[4,90] = 4.2$, $p < 0.01$). The bars provide means of counts ± SEM. ** Indicates a significant difference of age in the subpopulation ($p < 0.01$, Student's $t$ test).

$D_2$ receptor mRNA–containing neurons of all sizes appear to be lost from striata during aging, although a greater percentage of large and small cells than medium are affected. However, since most of the neuronal population is smaller than 90 $\mu m^2$, greatest absolute loss appears to occur in this size group. We previously observed a preferential loss of large $D_2$ receptor–containing neurons following kainate administration to cultures obtained from neonatal rats (Mesco et al., 1992). The latter results may not, however, be directly comparable to the present study in light of multiple systematic differences including age, *in vivo* versus *in vitro* conditions, death due to possible toxicity versus normal aging, etc. However, we have also observed that those neurons which lose $D_2$ receptors during aging reside in a population which is sensitive to kainic acid lesions *in vivo* (Joseph et al., 1991). These latter studies may provide a more appropriate comparison to those presented here.

In summary, we have demonstrated for the first time an apparent loss of identifiable $D_2$ dopamine receptor mRNA–containing neurons in striata of aged rats. The magnitude of the decrease, 27–29%, is sufficient to explain at least half of the well established loss of $D_2$ receptors during aging (Lai et al., 1987; Roth and Joseph, in press). Average content of $D_2$ receptor mRNA per neuron also decrease with age, but it is not clear whether this is a cause or effect of neuronal death. Although $D_2$ receptor mRNA–containing neurons of all sizes appear to be lost during aging, greatest absolute decrease seems to occur in those smaller than 90 $\mu m^2$. Future studies must now focus on the molecular mechanism of cell death and possible strategies to halt or prevent it.

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