Nitric Oxide Synthase in Rat Brain: Age Comparisons Quantitated with NADPH-Diaphorase Histochemistry

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We examined age-related differences in nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) containing neurons and neuropil in the striatum and hippocampus of male Fischer 344 rats at 6, 12, and 26 mo of age. NADPH-d staining is considered to be a marker for neurons and neuronal processes containing nitric oxide synthase. Rat brains were processed for NADPH-d histochemistry and analyzed morphometrically using computerized image analysis. The following NADPH-d histochemical parameters were examined: neuronal density, neuronal size, and neuropil staining optical density of selected regions. In the striatum, significant age-related declines were observed in NADPH-d-positive neuronal density and in neuropil staining, while neuronal size increased between 6 and 12 mo and then declined between 12 and 26 mo. In the hippocampus no significant age-related changes were noted in NADPH-d-positive neuronal density or size, or in the optical density of the molecular layer of the hippocampal dentate gyrus. Thus, age differences in NADPH-d histochemistry appear to be regionally specific in the Fischer 344 rat.

Age-related reductions have been reported in neuronal density and size in selected brain loci in both human (Brody, 1955; McGeer et al., 1977; Vijayashanker and Brody, 1979; Whitehouse et al., 1983; Chui et al., 1984) and experimental animals (Hornberger et al., 1995; Mesulam et al., 1987; Smith et al., 1993). In rodent models, age-related neuronal declines in specific loci have included the basal forebrain (Fischer et al.; 1989; Smith et al., 1993), the cerebellar Purkinje layer (Rogers et al., 1981), the olfactory bulb (Hinds and McNelly, 1979), hypothalamus (Hsu and Peng, 1978; Sartin and Lamperti, 1985), amygdala (Sabel and Stein, 1974), the striatum (Han et al., 1989), and the hippocampus (Landfield et al., 1977). The specificity of the neurotransmitter systems associated with neuronal loss in these regions, however, has not been well characterized in rodent models except in a few studies (e.g., Fischer et al., 1989).

Selected populations of neurons in the striatum and hippocampus stain for nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) (Vincent and Kimura, 1992). This histochemical reaction has been used to mark cells containing nitric oxide synthase (NOS) (Dawson, T.D. et al., 1991; Hope et al., 1991). Interest in NOS-containing neurons was sparked by the identification of nitric oxide (NO) as a putative retrograde messenger stimulating presynaptic release onto N-methyl-D-aspartate (NMDA) receptors (Dawson, V.L. et al., 1991; Garthwaite, 1991; Breit and Snyder, 1992). NO is a highly reactive gaseous oxyradical generated from arginine by NOS. Three distinctive types of NOS have been identified: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) (see review in Mayer, 1995). NO generated from brain NOS has been identified in the formation of long-term potentiation and learning in rodent models (Malinow and Tsien, 1990; Malenka, 1991; Chapman et al., 1992).

In addition to its putative role in normal physiological function, NO has been hypothesized to be the mechanism involved in glutamate excitotoxicity. In this light, it has been reported that neurons containing NOS are spared from NMDA and NO toxic effects (Dawson, V.L. et al., 1991). For example, it has been reported for Huntington's disease that neurons positive for NADPH-d appear to be resistant to neurodegenerative processes in the caudate nucleus (Ferrante et al., 1985) and in the hippocampal formation in Alzheimer's disease (AD) (Hyman et al., 1992). However, it was also reported that the pattern of NADPH-d staining in the neuropil of the hippocampus is reduced dramatically in AD patients (Rebeck et al., 1993). In addition, a compartmental loss of NADPH-d in the neuropil of the striatum in Huntington's disease patients has been reported (Morton et al., 1993).

Few studies have provided quantitative assessment of age-related changes in NOS neuronal populations in humans or rodents. Kawamata et al. (1990) reported that the size but not the density of NADPH-d-positive neurons in the laterodorsal tegmental nucleus of DDD mice showed a significant reduction with aging. The objective of our study was to conduct age comparisons of the density and size of NADPH-d-positive neurons in the striatum and hippocampus in male Fischer 344 (F344) rats. In addition, we examined the density of neuropil NADPH-d staining in the striatum and in the molecular layer of the hippocampal dentate gyrus. These brain regions were selected because of their functional relevance, and they also appear to be highly vulnerable to age-related change in many neurobiological parameters. The F344 rat was selected to add to the extensive data base that exists regarding age-related neurobiological changes in this model. In addition, this strain has received extensive behavioral characterization in this laboratory to document the marked age-related decline in motor and cognitive performance (Spangler et al., 1994).
MATERIALS AND METHODS

Animals and tissue preparation. — Male F344 rats obtained from Harlan Sprague-Dawley (Indianapolis, IN) were sampled from three age groups: 6-mo \((n = 12)\), 12-mo \((n = 6)\), and 26-mo \((n = 12)\). All rats had been raised in groups \((2-4)\) in a specific pathogen-free (SPF) environment. They were maintained on ad libitum feeding (NIH-31 and 07) and a 12-hr light/dark cycle.

The rats were decapitated during diurnal hours. Brains were removed promptly and immersion-fixed immediately in 4% paraformaldehyde in 0.1 M sodium phosphate-buffered saline, pH 7.4 (PBS) at 4 °C for 12 hr, transferred to cryoprotectant solution (20% sucrose in 0.1 M PBS), and stored at 4 °C for 24 hr. The brains were sectioned on a cryostat parasagittally through the striatum and hippocampus into 25-µm serial sections containing both regions.

NADPH-d histochemistry. — NADPH-d histochemistry was performed according to the protocol previously described (Kuo et al., 1994). Free-floating sections from the three age groups were incubated in 0.1 M PBS, pH 7.4, containing 0.3% Triton X-100, 0.4 mg/ml nitroblue tetrazolium, 5 mg/ml malic acid, 4 mg/ml magnesium chloride, and 0.8 mg/ml NADPH for 45 min at 37 °C. Care was taken to match sections across age groups for histochemical processing. After the reaction was terminated, the sections were rinsed quickly in PBS three times and mounted onto slides coated with gelatin, dehydrated through graded alcohol, cleared with xylene, and covered. The dried slides were kept protected from light in slide boxes before image analysis.

Histochemical analysis. — The target areas from striatum and hippocampus were selected at a specific plane (Figure 1) which is lateral 2–3 mm from midline according to the Paxinos and Watson atlas (1986). The following NADPH-d histochemical parameters were examined: (1) neuronal size, (2) neuronal counts per unit area (density), and (3) optical density of neuropil NADPH-d staining. These parameters were measured using an IBAS 20 image-analyzing computer (Zeiss, Göttingen, Germany) coupled to a Zeiss scanning camera, which was attached to a Zeiss light microscope. The analysis was conducted blindly as to the age of the sample provided. Sample sizes for the striatal analysis were 12, 6, and 12 for the 6-, 12-, and 26-mo-old rats.

Figure 1. Histochemical demonstration of NADPH-d in parasagittal sections of 12-mo-old rat brains. The striatum (A) contains evenly distributed intense NADPH-d-positive neurons and neuropil. B is an enlarged area from the box area in A and shows the different shapes of NADPH-d-positive striatal neurons and a dense fiber network of neuropil staining. The hippocampus (C) has a sparse population of NADPH-d-positive neurons and intense neuropil staining in the molecular layer of the dentate gyrus. D is the higher magnification from C showing the laminar pattern of NADPH-d neuropil staining in the molecular layer of the dentate gyrus. ac, anterior commissure; CPu, caudate putamen; G, granule cells of the dentate gyrus; GP, globus pallidus; IB, inner band of the molecular layer; OB, outer band of the molecular layer; P, pyramidal cells of the hippocampus. Scale bars = 500 µm (A and C), 30 µm (B), and 50 µm (D).
groups, respectively; for the hippocampal analysis, sample sizes were 6 for each age group.

For the measurement of neuronal size, morphometry was limited to about 100 NADPH-d-positive cells found in randomly selected microscopic fields in the dorsal striatum of six sections from each animal. In the hippocampus, about 100 NADPH-d-positive cells were selected from randomly selected microscopic fields in the CA1, CA2, and CA3 areas of six sections from each animal. The number of cells selected from CA1, CA2, and CA3 were equivalent across regions. Neuronal size measurements were based on the number of pixels occupied by the target cell multiplied by the scaled pixel area to arrive at the estimate of area. The area of each microscopic field was also assessed, and neuronal density of NADPH-d-positive neurons was expressed as the number per square unit.

For the measurement of the optical density of neuropil staining, six randomly selected areas (about 4–5 mm²) in the dorsal striatum from each animal were recorded. In the hippocampus, only the neuropil staining of the molecular layer of the dentate gyrus was examined because this area provided the most distinctive staining in the hippocampal formation. Six randomly selected areas (about 1 mm²) within the inner and outer molecular layer of the dentate gyrus from each animal were recorded (Figure 1D). To obtain a specific measurement of neuropil staining, the morphometric program on the IBAS system was designed to eliminate the measurement of the stained neuronal soma, unstained fiber tracts, and unstained blood vessels and to correct for different background densities.

Statistical analysis. — Because the data were normally distributed, statistical comparisons could be made by one-way analysis of variance (ANOVA) on all the measured parameters for the three age groups. Comparisons among age groups were conducted using the Student Newman-Keuls multiple range test. Statistical significance for all comparisons was accepted as p < .05.

RESULTS

In agreement with previous studies (Vincent et al., 1983b; Sandell et al., 1986; Hope and Vincent, 1989; Vincent and Kimura, 1992; Kuo et al., 1994), NADPH-d histochemical staining in the striatum and hippocampus revealed a distinctive blue reaction product within neuronal somata and neuropil. NADPH-d histochemistry showed similar staining and anatomical distribution patterns across all age groups. The striatum showed an evenly distributed intense NADPH-d staining with a scattered population of medium-sized neurons in the striatum (Figure 1A and B). These NADPH-d-positive cells were triangular, oval, or elongated in shape and gave rise to three or four large primary processes that frequently bifurcated into secondary processes. It is known that NADPH-d neurons in the striatum correspond to medium-sized aspiny interneurons containing both neuropeptide Y and somatostatin (Vincent et al., 1983a, 1983b).

Regarding the age comparisons, Figure 2A reveals that the mean density of NADPH-d-positive neurons in the striatum declined significantly with age in a linear fashion for a total decline of about 30% between 6 and 26 mo of age. Figure 2C reveals significant age differences in neuronal size as measured by the mean cross-sectional area of NADPH-d-positive neurons in the striatum. The neuronal size increased about 10% from 6 to 12 mo of age, and then decreased about 10% from 12 to 26 mo of age.

In addition to these NADPH-d-positive cells, numerous processes formed a dense fiber network with varicosities within the striatal neuropil. This dense neuropil staining...
was intermingled with clearly unstained fiber tracts and blood vessels (Figure 1A and B). Figure 2B reveals a significant age effect on NADPH-d staining density in the striatal neuropil. No significant difference was observed between 6- and 12-mo-old rats; however, there was a significant reduction of neuropil staining between 12 and 26 mo.

In the hippocampus, NADPH-d histochemistry revealed a sparse population of intensely stained neuronal cell bodies and widespread staining of the neuropil (Figure 1C and D). The present study focused on the NADPH-d containing cells in the CA1, CA2, and CA3 sectors of the hippocampus and on neuropil staining of the dentate gyrus. These NADPH-d-positive neurons represent a subpopulation of interneurons in the hippocampus that are co-localized with γ-aminobutyric (GABA), somatostatin, and neuropeptide Y (Vaitschanoff et al., 1993). We observed that the pattern of cell staining in CA1, CA2, and CA3 was similar. The pyramidal cell layer was largely unstained, and a number of small scattered cells of moderate staining were present in all layers of CA1, CA2, and CA3 areas. Several morphological types of stained neurons were observed, including triangular, fusiform, and oval cells.

The means for neuronal density and cross-sectional area of these stained neurons are presented in Table 1 for the three age groups. Both the size and density of the stained cells appeared to increase from 6 to 12 mo of age and then decreased at 26 mo; however, the differences among these three age groups were not statistically significant for either parameter [size: F(2,15) = 1.11, p > .05; density: F(2,15) = 5.4, p > .05].

Regarding the neuropil staining in the hippocampus, we observed that the molecular layer of the dentate gyrus demonstrated the most intense NADPH-d staining of the hippocampus. The staining within the dentate gyrus exhibited a laminar pattern. The feature of this laminar pattern of staining was that the inner band of the molecular layer was darkly stained, and the outer band was stained less intensely (Figure 1D). The optical density of both inner and outer bands of the molecular layer of the dentate gyrus was measured. We found no significant differences in the density of neuropil staining from either the inner or outer bands among three age groups [Table 1, inner layer: F(2,15) = 1.10, p > .05; outer layer: F(2,15) = 1.40, p > .05].

Discussion
Results from our previous technical study examining the correlation between neuronal counts and optical density of NADPH-d histochemistry demonstrated that neuropil staining does not represent unspecific background staining (Kuo et al., 1994). We also documented previously that the density of NADPH-d-positive neurons was independent of the optical density of the neuropil staining. Thus, the quantitative analysis of neuropil staining intensity appears more sensitive to the histochemical procedure than does analysis of NADPH-d-positive cell number in detecting the subtle changes of NADPH-d activity. For example, in the hippocampus of AD patients, it was found that NADPH-d staining in the neuropil of the molecular layer was reduced dramatically, although NADPH-d-positive neurons were relatively spared (Hyman et al., 1992; Rebeck et al., 1993). Therefore, the present study on age-related changes examined not only the number and size of NADPH-positive cells but also the optical density of neuropil staining in the striatum.

We found that the density of NADPH-d-positive neurons in the striatum declined linearly with increasing age in the F344 rat at an extrapolated rate of 1.5% per month of age between 6 and 26 mo. In addition, comparing middle-aged (12 mo) to aged rats (26 mo), we observed that striatal NADPH-d-positive neurons appeared to shrink in size, contrasted to a slight increase in neuronal size between young (6 mo) to middle-aged rats. However, these observations on neuronal size must be assessed against the finding of the 15% loss of neuronal density between 6 and 12 mo of age. These findings would indicate that the loss of NADPH-d-positive neurons in the rat striatum between 6 and 12 mo may reflect the loss of smaller neurons, which could create the appearance of an increase in the size of NADPH-d-positive neurons. This conclusion is supported by the previous finding that during aging the rat striatal neurons containing dopamine D_2 receptors of all sizes were lost, but the greatest absolute decrease was for those cells smaller than 90 μm² (Zhang et al., 1995). The degree of age-related loss of striatal neurons containing D_2 receptors appeared to be on the same magnitude (~28%) as that observed for NADPH-d-positive neurons in this brain region of male Wistar rats (Zhang et al., 1995).

Regarding NADPH-d neuropil staining in the striatum, the pattern of age-related change appeared similar to that observed for cell size. The significant age-related decline appeared between 12 and 26 mo of age with no significant difference observed between 6 and 12 mo of age. In a previous study of C57BL/6J mice, the total dendritic length of striatal medium aspiny neurons was reported to increase between 6 and 20 mo and decreased thereafter (McNeill et al., 1988). The present results of age-related differences showing the nonlinear pattern in NADPH-d staining reflecting neuronal size and neuropil density also underscore the importance in aging studies of examining middle-aged groups instead of relying upon simple comparisons of young and old groups alone (Coleman et al., 1990).

A study of aging in the human brain revealed that the corpus striatum lost 8% of its volume up to the 80th year.

Table 1. Summary of NADPH-d Staining Characteristics in Hippocampus of Fischer 344 Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Age (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>6 12 26</td>
</tr>
<tr>
<td>Number of cells per unit area</td>
<td>121.16 138.17 119.67</td>
</tr>
<tr>
<td>Outer molecular layer</td>
<td>1.88     1.61 1.87</td>
</tr>
<tr>
<td>Outer molecular layer</td>
<td>1.75     1.45 1.69</td>
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<td>Values shown are means (SEM).</td>
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both neuropeptide Y and somatostatin (Vincent et al., 1991). The present results from rats showed that both the size and the number of the NADPH-d neurons in the striatum decreased after middle age. Although we can assume that the loss of NADPH-d neurons in the striatum reflects a loss of interneurons containing both neuropeptide Y and somatostatin (Vincent et al., 1983a, 1983b), the determinants of age-related decline in the density of NADPH-d-positive neurons in the rat striatum is unclear. Previous studies revealed that NADPH-d-positive neurons were selectively spared in the striatum in Huntington’s disease patients (Ferrante et al., 1985). The present results suggest that the pattern of age-related changes in NADPH-d-positive neurons in the striatum appears different to the fate of these neurons observed in neurodegenerative diseases. Opposing findings have also been noted between human and rat brain for norepinephrine-positive neurons in the locus ceruleus. Specifically, marked neuronal loss in this locus is associated with AD in the human brain (Bondareff, 1986), but no significant age-related loss is observed in the F344 rat brain (Goldman and Coleman, 1981).

In the CA1, CA2, and CA3 subfields of the rat hippocampus, only a relatively few number of small, scattered neurons stained for NADPH-d. These cells presumably represent a subpopulation of interneurons in the hippocampus that are co-localized with GABA, somatostatin, and neuropeptide Y (Velayos et al., 1993). In the present study, no age-related differences in hippocampal NADPH-d-positive neurons in the hippocampus were found. We could not find any similar analysis of age differences in hippocampal NADPH-d-positive neurons in the hippocampus in rodent models. Although past studies of human hippocampus have noted age-related loss of neurons in particular hippocampal subfields (Mani et al., 1986), the latest stereological studies, which remove tissue shrinkage artifacts from age-comparative analysis, have noted little evidence for age-related changes in the total number of CA1, CA2, and CA3 neurons (West, 1993b).

In addition to the NADPH-d neurons, the molecular layer of the dentate gyrus of the hippocampus also demonstrated an intense laminar pattern of neuropil staining, a pattern similar to the neuropil staining in the human hippocampus attributable to efferents from entorhinal cortical (Rebeck et al., 1993). In AD patients’ neurons, NADPH-d staining of neuropil in the dentate gyrus was reported to be reduced markedly (Rebeck et al., 1993), but his observation was not repeated in our analysis of age differences in the F344 rat.

By attempting to quantitate age-related alterations in NADPH-d histochemistry, our results must be viewed in light of the new morphological studies emerging from unbiased stereological analysis (West, 1993a). Results using this technique have cast doubt over previous findings that may be subject to biased analysis due to tissue shrinkage or other artifacts, including changes in cell shapes and sizes. However, because we found regional specificity in the age differences observed, our concern about the possibility of biased results should be reduced unless we assume that the bias would also be regionally specific.

In summary, the present results demonstrated selective age-related alterations in NADPH-d histochemistry in brains of male F344 rats. Between 12 and 26 mo of age, declines in neuronal density, size, and neuropil staining were evident in the striatum but not in the hippocampus. Comparing the age-related alterations in NADPH-d histochemistry in rat brain with those in human brain associated with degenerative diseases, we conclude that NADPH-d-positive neurons and neuronal processes in this rat model manifest a different pattern of age changes than observed in Huntington’s disease (Ferrante et al., 1985) and AD (Rebeck et al, 1993). It appears that neurons and neuronal processes containing NOS undergo significant alteration in the rat striatum during aging.

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