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# Increases in TH immunoreactivity, neuromelanin and degeneration in the substantia nigra of middle aged mice

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**Abstract**

The dopaminergic (DArgic) neurons in the substantia nigra (SN) are particularly vulnerable to oxidative stress and during aging. The present study was undertaken in order to determine whether aging is associated with changes in the DA synthesizing enzyme tyrosine hydroxylase (TH) as early as middle age by comparing 7- and 50-week-old mice. Quantitative analysis, performed by measuring the density of TH-immunopositive neurons, revealed that in the older animals, the number of DArgic neurons was decreased by 10% while TH immunodensity was $24 \pm 3\%$ higher compared to the younger animals. Based on Masson-Fontana staining for neuromelanin (NM), the number of NM-containing neurons in the SN and the volume of NM per NM-positive neurons in the older animals were 5- and $11.6 \pm 0.1$-fold higher, respectively. The silver stain-positive fibers, indicative of degeneration, were higher in the SN and striatum of the older animals, with the optical density $3.3 \pm 0.1$- and $5.4 \pm 0.2$-fold of the younger animals. The present study demonstrates that aging is associated with changes in the DA synthesizing enzyme TH as early as middle age and that this is associated with dramatic increases in the number of NM-containing neurons, volume of NM per cell, and degeneration.


*Keywords:* Aging; Degeneration; Neuromelanin; Substantia nigra; Tyrosine hydroxylase

Normal aging is associated with elevated oxidative stress as a consequence of weakening of the endogenous antioxidant defense mechanisms and/or an increase in generation of pro-oxidants. Oxidative stress-associated cell damage has been described in age-related neurodegenerative disorders including Parkinson's disease (PD), in which the neuromelanin (NM)-containing dopaminergic (DArgic) neurons in the substantia nigra (SN) undergo severe degeneration [27]. DArgic neurons in the SN are particularly vulnerable to oxidative stress because they are low in antioxidant enzymes and contain molecules that contribute to generation of oxidative stress including DA [27]. In particular, elevated DA leads to the formation of DA-quinone, which can lead to modification of cellular proteins [10], generation of reactive oxygen species (ROS) [6], and formation of NM [9].

A line of evidence indicates that aging is associated with a linear decrease in the number of neurons [8,22] and increased NM [20] in the SN. However, whether this is accompanied by changes in the DA synthesis enzyme tyrosine hydroxylase (TH) has been unclear. A decrease in TH activity [21–23], no difference in TH activity or gene expression [13,17,28], and an increase in TH immunodensity [11] in very old animals have been reported. In the 6-hydroxydopamine-induced PD model, increased TH activity [38] and immunodensity [37] in the surviving SN neurons have been noted. We have recently observed that acute stress leads to DA elevation that is accompanied by NM accumulation and degeneration in the SN [18]. As the mechanism of cellular responses to aging and 6-hydroxydopamine, both associated with oxidative stress, might be similar and the decreased antioxidant capacity is observed as early as middle age of rodents [1], it is possible that TH expression in nigral neurons might be altered by midlife, which might influence the cell's survival. To our knowledge, such question has never been addressed thus far in the literature. In the present study we therefore assessed TH expression at middle age and whether this is accompanied by NM accumulation and SN degeneration. Morphological and quantitative analyses were performed on 7- and 50-week-old mice, each rep-

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resenting young adults and middle-aged animals, respectively [14,24].

All procedures were performed in compliance with the *Laboratory Animal Manual* of the University of Ulsan Asan Institute for Life Sciences, which adhered to the guidelines set forth by the Society for Neuroscience. Female ICR mice (Orient Corp., Sungnam, Korea) were maintained in a pathogen-free temperature- and humidity-controlled room with a 12-h light:12-h dark cycle and food and water available ad libitum until 7 and 50 weeks of age ($n = 10$ in each group). The brains were prepared and cut into 20 μm sections as described previously [16,18].

Tissue sections were subjected to TH immunohistochemistry as described previously [16]. We manually counted the number of TH immunoreactive neurons and quantified immunodensity using Quantity-One software (Bio-Rad, Hercules, CA) after outlining all TH cells (80–90 per SN section) in the microscopic field at 100× magnification. To account for differences in background staining intensity, four background density measurements were taken from each microscopic field in regions lacking immunoreactive profiles, the mean of which was subtracted from the immunodensity of each individual TH cell to provide the final density value. For TH immunofluorescence staining, sections were blocked with 0.3% Triton X-100 and 5% fetal bovine serum, incubated for 1 h with TH antibody (Protos, New York, NY, 1:5000), followed by incubation for 90 min with fluorescence-labeled Alexa 488 (Molecular probes, Eugene, OR, 1:1000), with rinsing in between. The sections were mounted and analyzed by confocal microscopy (TCS-ST2; Leica, Wetzlar, Germany).

Masson-Fontana staining for NM was performed as described previously [3,18]. To determine the number of NM-containing neurons in the SN, we counted the number of cells containing empty granules and those containing NM granules in 6–9 SN sections per animal at 1000× microscopic field image, making a distinction from lipofuscin based on the criteria described by Cabello et al. [2]. Using the same sections, we assessed the volume of NM granules by manually outlining and quantifying each granule using Quantity-One software. The volume of each granule was added up to obtain the total volume in each section, from which average volume per cell was calculated.

Amino-cupric-silver degeneration staining was carried out essentially by the method of de Olmos et al. [5] as previously described [18]. For quantitation, we randomly selected a total of 25 small circles (57.5 and 115 μm and in diameter for SN and striatum, respectively) from microscopic fields (200× and 100× magnifications, respectively). Their optical density was measured using Quantity One software. To account for differences in background staining intensity, four background optical density measurements were taken from each microscopic field in regions lacking silver stain profiles, the mean of which was subtracted from the optical density of silver stain positive area to provide the final optical density value.

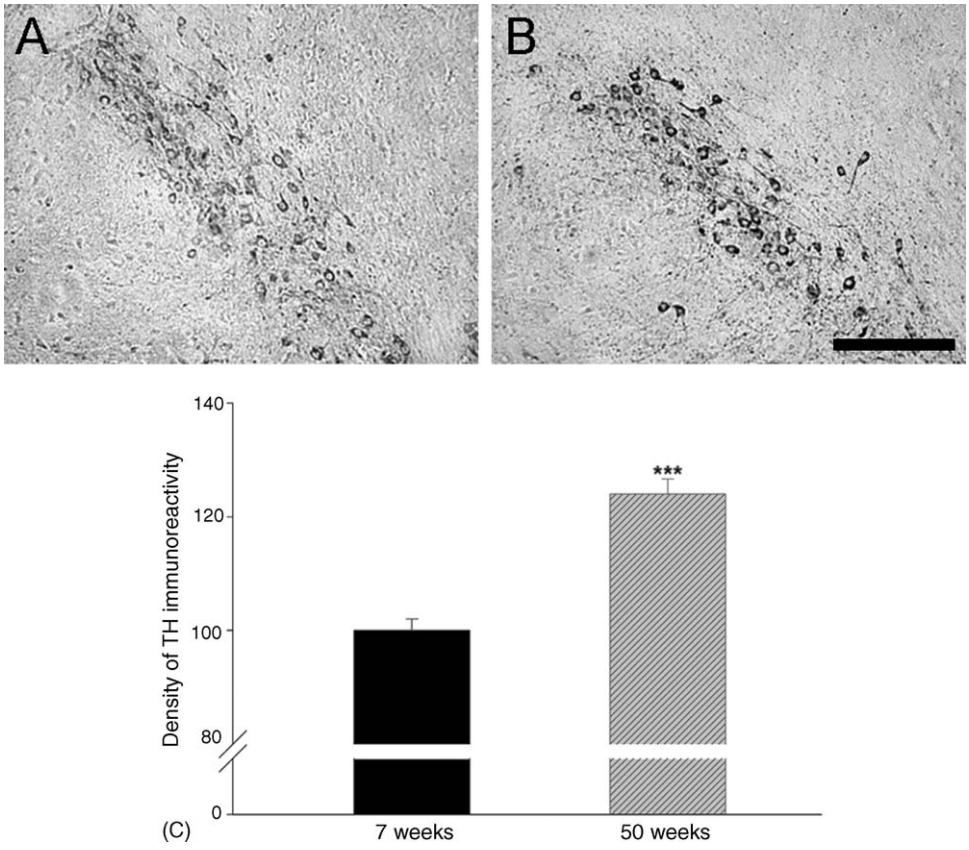


Fig. 1. Comparison of TH immunoreactivity in SN between the younger and older mice. Typical immunohistochemistry of SN against TH of (A) 7-week-old mice and (B) 50-week-old mice. Size bar = 200 μm; (C) densitometric analysis of TH immunoreactivity. Data are expressed as mean ± S.E.M. in percent of 7-week-old mice. $^{***}p < 0.001$ vs. the 7-week-old mice.