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NEUROMELANIN-CONTAINING, CATECHOLAMINERGIC NEURONS IN THE HUMAN BRAIN: ONTOGENETIC ASPECTS, DEVELOPMENT AND AGING

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The present review compiles data on the development and aging of neuromelanin (NM)-containing neurons in the central nervous system. Neuromelanin is brownish-to-black pigment that accumulates in the catecholaminergic (noradrenergic and dopaminergic) neurons and is a reliable natural marker that delineates the A1-A14 catecholaminergic groups of Dahlstrom and Fuxe in the human brain. The pigmentation of noradrenergic locus ceruleus *neurons starts earlier than that of dopaminergic* substantia nigra*, but also a considerable individual variability is present. The pigmentation is well advanced in adolescence. The data at what age the maximal pigmentation is reached are controversial, as are the data on the cell loss in the NM-containing neuronal populations by normal aging. Thus, the participation of NM in the pathogenesis of Parkinson's disease remains enigmatic.*
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INTRODUCTION

The brownish-to-black pigment neuromelanin (NM) accumulates in catecholaminergic (CAergic) neurons in the central nervous system of humans and apes, and to a lesser extent - in other primates, ungulates and carnivores (reviewed in 1). Although the majority but not all CAergic neurons contain NM (2,3), this pigment is a reliable natural marker of CAergic neurons, and several atlases are present (1,4,5) that demonstrate the A1-A14 CAergic neuronal groups of Dahlstrom and Fuxe (6) in the human brain.

The NM-containing neurons are involved in severe diseases, including motor disorders, mainly the idiopathic Parkinson's disease and in Parkinson-plus syndromes (7-12, and refs. therein) and Alzheimer's disease (13-17, and refs. therein).

Marsden (18) reasonably asks: "Neuromelanin is closely linked to Parkinson's disease, but is it the cause of the illness?" The issue is controversial and is discussed in numerous reports (19-22, and refs. therein). The data on the normal aging of NM-containing neurons are especially important, when considering the development of neurodegenerative diseases, characterized primarily by the cell loss of CAergic neurons. The present review compiles the data on the development and aging of NM-containing CAergic neuronal cells in the brain of man and other mammals.

NEUROMELANIN IN DEVELOPMENT

According to Friede (23), Stilling (24) was the first to note that pigmentation in the brain stem is absent in very young children. Pilcz (25) observed pigmentation of the human *locus ceruleus* (LC) by 11-12 months and of the *substantia nigra* (SN) by the third year of life. Calligaris (26) found no pigmentation in LC of children up to the ninth month. Like

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Pilcz, he states that the pigment formation starts at about the 11th and 12th months and is well developed by the second year. Pigmentation of SN develops approximately a year later than that in LC, and adult-like pigmentation is evident by about 10-18 years. However, Cooper (27) had noticed NM-containing SN neurons in a fetus at the 5th month of gestation (she used silver techniques known to be more efficient than the routine stainings). Mettler (28) states that the pigmentation of SN neurons begins in the third year, is well advanced by the 6th but not completed until 16th or 18th year of life. Olszewski and Baxter (29) report the presence of melanized LC neurons in five year old child but that are not seen in SN. Foley and Baxter (30,31) have examined 100 brains from fetuses, infants and children under the age of 5 years. They encounter NM in a fetal (5th month) LC, but deny its presence in SN before 18 months after birth. These significant discrepancies are probably due to natural individual variations, to the limited numbers of the examined brains, and to the different criteria in recognizing reliable melanin granules. In addition, Nieto *et al* (32), Fenichel and Bazelon (33), Mann *et al* (34), and Barden (35) stressed the limited reliability of the classical staining techniques (for the same reason large discrepancies appear in the description of changes in aging as well as in the comparative anatomy). Using the diamine silver technique of Lillie (36), Fenichel and Bazelon (33) examined 44 brains: from the 34th gestational week to 16 years of age. Their results show that great individual variability indeed exists, since they reported that SN is "routinely pigmented" at the age of 5 years, but the youngest brain to contain NM in SN was 5 months old. These authors present the only report so far of NM existence in the dorsal vagal nucleus (DVN) - in the most immature brain examined by them (34 weeks of gestation). The method used appears to be of great importance. Cowen (37) used the diamine silver technique (36) and Schmorl's ferric ferricyanide method (38) to demonstrate the melanoneurons of human cerebellum already at the 26th week of gestation. On the other hand, with routine staining methods, the brown granules in the NM-containing neurons of the *pars cerebellaris loci cerulei* were shown by Cowen only after 2,5 years after birth.

It was repeatedly shown that when NM first appears, the individual granules are smallest in size (35,39-42), least in number (31,35,41,43,44), and the pigment in the youngest individuals occupies the smallest intraperikaryal volume (34,43-46). Fenichel and Bazelon (33) describe a rapid increase of melanin content in SN, LC and DVN that reaches its maximum at 16 years of age. Thus, SN and LC can already be identified grossly at preadolescence. Although these authors investigate also adult brains (47), they have apparently concentrated their investigation mainly on brainstems of infants and children as to conclude that the maximal pigmentation is reached at puberty. Other researchers declare a longer development. According to Moses *et al* (42), the maximum is reached between 20 and 30 years of age. In a painstaking series of cytophotometric investigations, Mann and his colleagues established that the percent of strongly pigmented cells in SN and LC progressively increases from 18 months to 60 years (34,44,48,49). Similarly, Graham (45), who calculates both the perikaryal volume and its NM content in 10, 40 and 80 year old brains, shows not only a sharp increase between the first and the 4th decade, but also that the volume of the pigment in the 8th decade is twice more abundant than in the fourth decade. On the other hand, Gellerstedt (50) was the first to describe a decline in the NM content of the SN and LC in aging of people without detectable neurologic disease, while Adler (39) found the same amount of pigment in 18 and 68 year old brains. According to Moses *et al* (42), the amount and distribution of NM in SN and LC in 20-30 year old brains is comparable with 58, 70 and 82 year-old brains of people without neurological disease. The precise quantitative estimations of Mann and Yates (43,44) and Mann *et al* (34,51) demonstrate that a gradual decline takes place from 60 to 90 years in all NM-containing nuclei, and especially - in SN. These authors propose that the decline in the quantity of the pigment is due to loss of the heavily loaded with NM perikarya, and that the loss of NM is greatest in the ventromedial and dorsal SN, in contrast to the ventrolateral focus of degeneration in Parkinson's disease (7,8,43,44,52,53). The number of NM-containing nigral neurons (52,54-56), but not tyrosine hydroxylase (TH)-immunopositive neurons (57) decreases at a rate of ~ 5% *per* decade. In 36 control cases ranged in age from 21 to 91 years, Fearnley and Lees (52) reported a decline of 4,7% *per* decade, and the total decrease reached 33%. There was a significant sparing of the ventrolateral and ventrointermediate groups in SN *pars compacta* - 15% cell loss. On the other hand, exactly these groups are most severely affected by idiopathic Parkinson's disease - the heaviest cell loss involves the dorsal tier of *pars compacta* (48%), followed by *pars lateralis* (46%). Faraldi *et al* (58) estimated the amount of NM in hematoxylin-eosin-stained sections of SN *pars compacta* in 69 cases, 14 to 100 years old. The mean area of cellular NM showed a curvilinear increase from 103 μm^2 at age 14, to 600μm^2 at age 67 before dropping down to 328μm^2 at age 100. By contrast, the fraction of NM area relative to perikaryal area showed a linear increase with age. The latter and an increase of NM pigmented perikarya from 83% at age of 14 to almost 100% at age of 65 or older, accounted for the increase of NM mean area. By contrast, a decrease in the mean area of neuronal perikarya and a decrease in the number of profiles of neuronal cell bodies explained the decrease of NM mean area beyond age of 67. Faraldi *et al* (58) support the hypothesis that an overload of NM is neurotoxic and emphasize the importance of using age-matched controls in histopathologic studies of the SN. Mann *et al* (49), and Mann and Yates (48) established that the increased amount of NM correlates with a reduction of the cytoplasmic and nucleolar RNA. Mann *et al* (49) suggest that the death of neurons maximally accumulating NM is due to a reduction in protein synthesizing ability directly associated with the physical displacement and dysruption of cytoplasmic RNA. In

this regard, Barden (40) points out the impaired function of the Golgi-apparatus in NM-containing neurons in old monkeys. Unlike the interpretations of Mann's group (34,43,44,49), Graham (45,59) proposed that the atrophy and cell death of the melanized neurons is a consequence of the intraneuronal toxicity of the cytotoxic quinone precursors of NM.

CATECHOLAMINERGIC NEURONAL LOSS IN AGING

Neuronal loss in SN with aging is also verified by biochemical studies. Riederer and Wuketich (60) establish a 13% reduction of caudate nucleus dopamine content *per* decade. Carlsson *et al* (61) report a non-linear decline of striatal dopamine with little loss until the age of 60, followed by a dramatic fall in the following decades. Scherman *et al* (62) assessed striatal dopamine levels by measuring the binding of alpha-dihydro-tetrabenazine, a ligand of the vesicular monoamine transporter and found a linear decline of under 10% *per* decade. It is generally assumed that the neuronal loss in normal aging is not sufficient to cause Parkinson's disease (52 and refs. therein). In Parkinson's disease, there is a presymptomatic phase and clinical signs do not appear until 50% of nigral neurons and 80% of striatal dopamine are lost (see 63).

There is a considerable body of literature, reporting a decrease in LC neuron number with advancing age, similarly to SN (14,46,64-74, and refs. therein). Most studies have used NM as a cell marker of noradrenergic neurons, and some studies have additionally used immunohistochemical staining with antibodies against TH (46,73,75), or against dopamine-β-hydroxylase (68,73). However, the data of the total number of LC neurons at different age are contradictory in both cell number and age-dependency, and often the comparative evaluation of the data is difficult (see 74). The reported mean cell numbers range from approximately 6,000-19,000 (68,69,72,76) to about 27,000-32,000 *per* side (77,78). In brains from four young individuals (1-28 years of age), Manaye *et al* (46) found an average of 21,000 LC and *nucleus subceruleus* neurons in one hemisphere, counting both TH and NM-containing neurons. Lohr and Jeste (72), and Manaye *et al* (46) reported linear age-related changes, while other authors (14,64,66,70,79) found non-linear changes. In LC and *nucleus subceruleus*, neuronal loss of up to 40% in the 7th decade and up to 48% by the 9th decade was described (64). The authors found a mean number of approximately 14,000 ± 3,500 neurons (24 individuals between 14 and 87 years). The mean for the group under 60 years was 16,840 ± 2,320, whereas the group above 60 had a mean of 13,378 ± 2,141 neurons. Chan-Palay and Asan (73) distinguish rostral, middle and caudal portions of LC, that contain four neuronal types in different proportions. They point out that cell loss in the old adult brain shows a topographical arrangement with a distinct rostrocaudal gradient. Cell loss is highest in the rostral LC part, displaying a reduction of 54.3% - 62.9%, it is 21.6% - 32.4% in the middle part, and 1.8% - 15.4% in the caudal part, which is greatly spared. Manaye *et al* (46) investigated 17 cases (1-104 years of age) and reported that from the first to 10th decade of life there is over 50% loss of LC neurons. On the other hand, Ohm *et al* (74) find no correlation between the age of the individuals and the cell number. Their study involves 20 cases (49-98 years of age), all of which - carefully examined for absence of neurological or psychiatric disorders. These researchers report a mean number of NM-containing LC neurons per side 15,731 ± 3,408, with a quite broad range: 11,737 - 25,319. At a variance to the studies cited above, Mouton *et al* (80), state that there is no change in pigmented cell number or size in the LC of nondemented older persons as compared with that of young individuals. An important point that at least partially explains these controversies is proposed by Manaye *et al* (46). According to them, the magnitude of LC neuronal loss that occurs with aging depends upon whether the cell marker is NM or TH-immunostaining. Manaye *et al* (46) insist that although NM is a useful marker of CAergic neurons in the human brain, NM is not a reliable marker for LC neurons in brains under 50 years of age. They demonstrate that below age of 25 there are much fewer NM-containing neurons than TH-containing neurons in LC. Manaye *et al* recall the data of Graham (45), who examined the area of the LC neurons occupied by NM in the first, 4th and 8th decade - there was a doubling of cellular area occupied by NM from first to the 4th decade, and again from the 4th to the 8th decade of life. Thus, Manaye *et al* (46) proposed that in studies in which brains less than 50 years of age had been used (64,69,70,72,76), counting of pigmented cells results in an underestimation of their number.

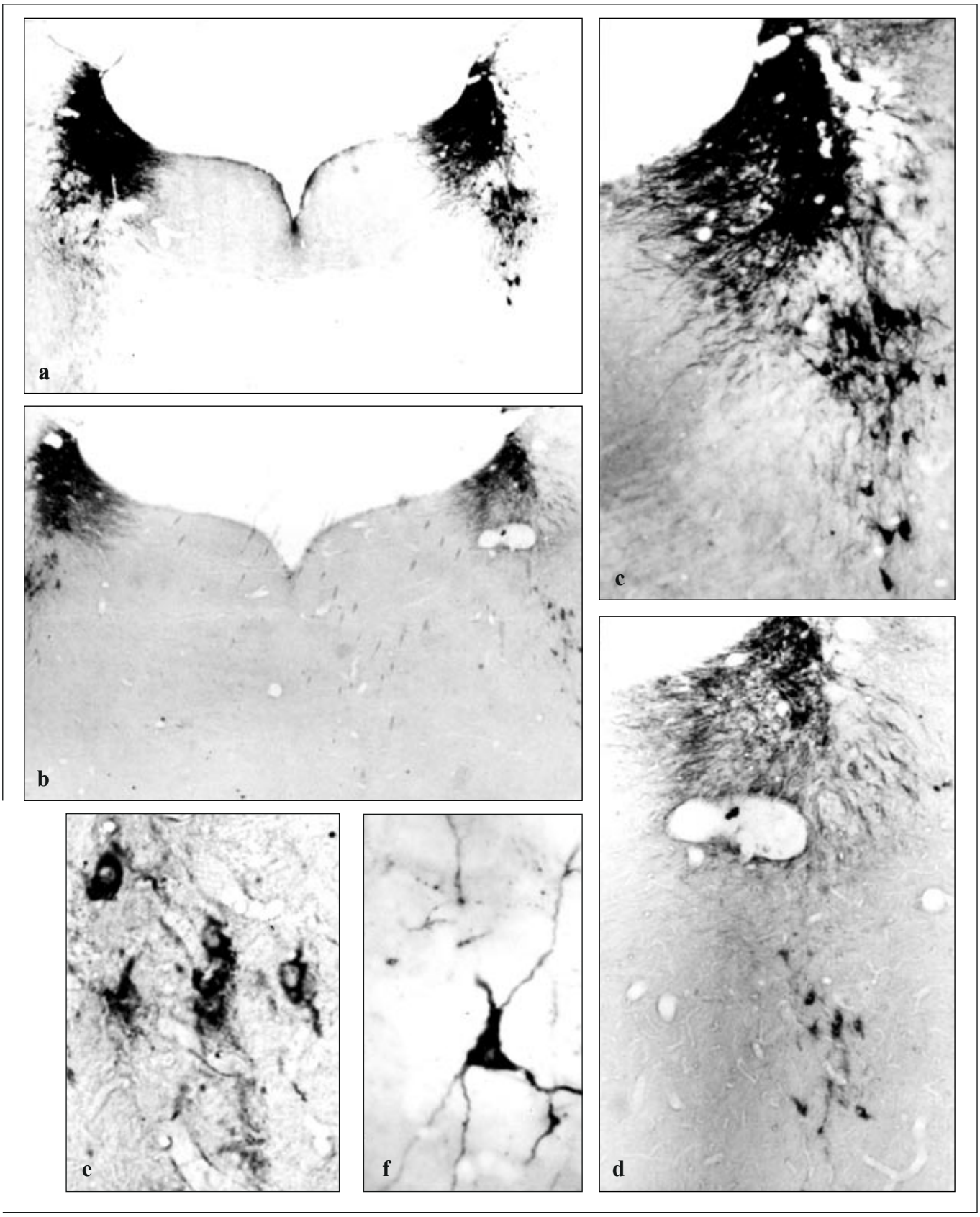
The data focused on whether aging results in a random loss of LC neurons throughout the nucleus or whether different rostrocaudal portions of the nucleus age at different rates, are also controversial. Marcyniuk *et al* (76) find a random cell loss, whilst Chan-Palay and Asan (81) establish a non-random cell loss in LC. Manaye *et al* (46) report a statistically significant interaction between age group and magnitude of rostrocaudal cell loss. They describe that in the rostral portion of LC there is a substantial age-related cell loss, whilst in the caudal portions of the nucleus little or no cell loss occurs. Recently, Kubis *et al* (82) provided unexpected data. They examined the human TH-immunostained neuronal population in SN, ventral tegmental area (VTA), peri- and retrorubral area, central gray substances and LC in 21 control subjects who died at ages 44-110 years. They found no statistically significant cell loss of TH positive neurons in the older subjects, either in the SN or in the remaining CAergic neuronal groups that degenerate to a lesser degree in Parkinson's disease. Kubis *et al* (82) concluded that from middle age to 110 years, aging in control adults is not (or is scarcely) accompanied by CA-containing cell loss in the mesencephalon, hence Parkinson's disease is probably not caused by an acceleration of a degenerative process during aging.

Although more scant, and only rarely with precise quantitative measurements, there are data for an increased

pigmentation in aged animals: chimpanzee and orangutan (83), gorilla (84), dog (85-88), cat (85,89,90), rhesus monkey (40,41,91), and horse (92). Herrero *et al* (93) estimated the distribution and number of NM-positive neurons in *Macaca fascicularis*, aged 0-13 years, by means of calculating unstained NM-containing neurons, or stained with Masson silver impregnation, and by TH-immunocytochemistry. At birth, no unstained NM-positive neurons were detected, but Masson-stained cells were observed in LC. At 8 and 13 years, unstained NM was present in Masson-positive neurons. Herrero *et al* (93) state that a differential increase in NM content with age in the neurons of mesencephalic catecholamine group is present. Irwin *et al* (94) examined young, intermediate-aged and old squirrel monkeys. Contrary to the obvious functional and neurochemical age-related changes (70% loss of dopamine in SN, and 30% in the putamen), the number of TH-immunoreactive cells did not significantly differ among the three age groups. Pakkenberg *et al* (91) examined the number of pigmented and nonpigmented neurons in the SN of young and old *Macaca mulatta* monkeys. They found that the total number of pigmented neurons was about eight times higher in old animals compared with young ones (166,000 *versus* 21,400), while the total number of non-pigmented SN neurons was less than half in old animals compared with young ones (139,000 *versus* 285,000). Siddiqi and Peters (95) point out that with age, all of the neurons in rhesus monkey SN accumulate lipofuscin, especially the small multipolar GABAergic neurons. These authors also report that although both neurons and neuroglial cells are affected with age, no entities that could be construed to be dying neurons might be encountered.

The CAergic neurons in rodents do not contain MN (90,96,97). There is a considerable body of data based on cell loss and TH-immunostaining but the reports are somewhat contradictory. In the ASH/TO mouse, a significant loss of LC cells has been observed by Sturrock and Rao (98). They found a 47% decrease in Nissl-stained LC neurons in 31-month-old mice, compared to 6- and 15-month-old mice. Shores *et al* (99) also found a LC cell loss in Brown-Norway rats but reported that the TH mRNA expression increases, which may potentially increase norepinephrine synthesis in the remaining neurons. We investigated the noradrenergic neuronal systems in young (3-month-old) and very old (26-28-month-old) Wistar rats by means of dopamine-β-hydroxylase immunostaining (Figs. 1, 2). The cell loss in LC and *nucleus subceruleus* is obvious (Fig. 1**a-d**). In young rats (Fig. 1**a, c**) the cell density in LC is so high that it is difficult to discriminate the individual immunopositive neuronal perikarya. On the other hand, the decreased cell density in aged rats improves the visualization of individual neurons (Fig. 1**b, d**). In young rats, the immunostaining is present also in distal dendrites (Fig. 1**f**). In old rats, only proximal dendritic stumps are visualized (Fig. 1**e**). Alongside the atrophy of the cell processes, the amount of dopamine-β-hydroxylase is probably also decreased. The differences between young and aged rats in noradrenergic axon numbers are drastic (Fig. 2**a, b**). The number of immunolabeled axons in the medial forebrain bundle on the territory of the lateral hypothalamus is greatly reduced in the aged rats. Also, the axonal immunostaining is considerably more pale in old animals, so that only individual axonal varicosities are comparable to those in young rats. Tatton *et al* (100) and Greenwood *et al* (101) investigated the SN and LC in C57B1 mice aged 8 to 104 weeks, and found that the neuronal loss was due to neuronal death rather than loss of TH-immunoreactivity. The cytoplasmic TH was increased by 63% in 104-week-old mice in comparisson to 8-week-old animals. McNeill *et al* (102,103) investigated the SN in young and aged C57B1/6N Nia mice. They found a progressive accumulation of cytoplasmic lipofuscin granules and a markedly reduced dopamine content *per* cell as determined by histofluorescence. Further, McNeill and Koek (104) investigated six age groups (3 - 30 months aged) of C57BL/6N mice, and reported a small decline (11%) in the total number of dopamine neurons of the SN with age, a decrease not reaching a statistical significance. Voogt *et al* (105) did not observe detectable changes in TH mRNA levels in the rat SN with age. Emerich *et al* (106) also reported a lack of reduction in number, area and length of TH-immunoreactive neurons within the A8, A9 or A10 region of aged (24-25 month old) rats. Schuligoi *et al* (107) suggested that the reduction in TH mRNA in the VTA and SN *pars compacta* in 33-month-old Sprague-Dawley rats is not due to a loss of TH mRNA expressing cells but due to a reduction in the hybridization signal *per* expressing cell. Himi *et al* (108) investigated the expression of mRNAs encoding the dopamine transporter and TH in SN of young and aged Fischer 344 rats. They found that dopamine transporter mRNA decreases by 18 months, whilst TH mRNA reduction does not occur until 24 months. Finally, De La Cruz *et al* (109) report a significant decrease of TH activity in SN in rats between 12 and 24 months of age.

***Figure 1**. Low power view of the dorsal pontine tegmentum in young (**a**) and old rat (**b**). Fig. 1**c** is a detail from Fig. 1**a**. The strong immunostaining and the great neuronal density impedes the visualization of individual neurons in LC (upper part of the figure, but individual neurons are clearly demonstrated in nucleus subceruleus dorsalis (central and lower part of the figure). Fig. 1**d** is a detail from Fig. **1b**. Both the cell loss and decreased immunostaining are obvious in the old rat. Fig. 1**e** is a detail from Fig. 1**d**. Neuronal group in the nucleus subceruleus. Only the proximal parts of the dendritic trunci are visualized. Compare with Fig. 1**f** - strongly immunostained neuron in nucleus subceruleus ventralis of a young rat. The cell nucleus is unstained but the reaction product extends also to distal portions of the dendrites. **a,b** x 40; **c,d** x 100; **e,f** x 400.*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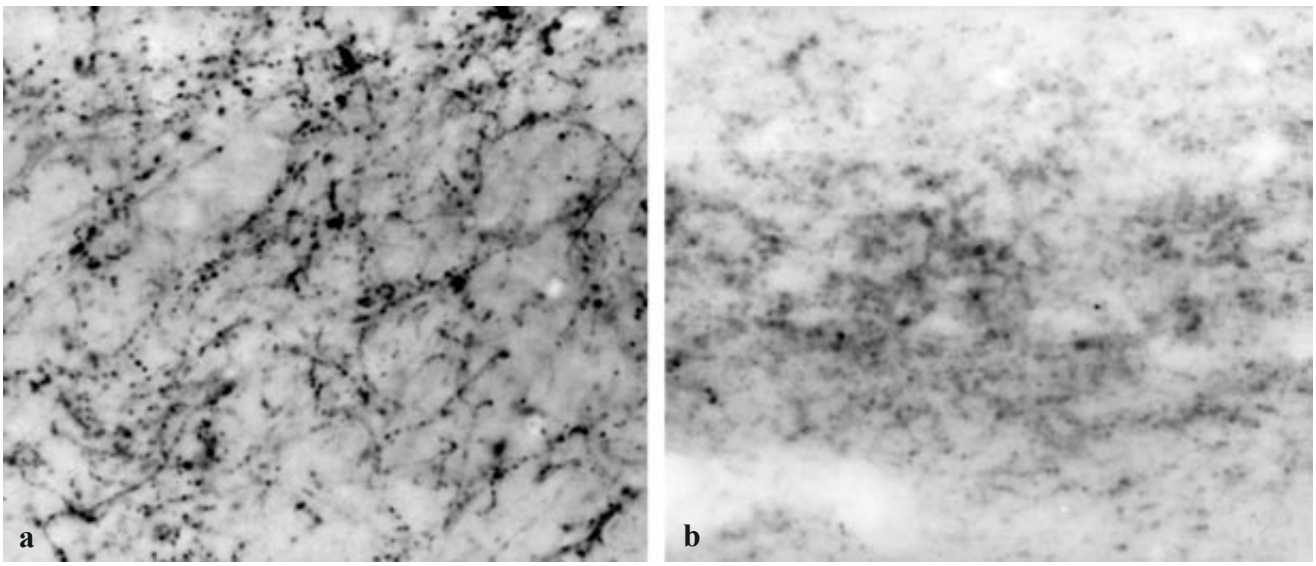

Figure 2. *Noradrenergic, dopamine-β-hydroxylase immunolabeled axons in the medial forebrain bundle of a young rat (**a**) and of an old rat (**b**). In the old animal the number of labeled axons is greatly reduced, and the immunostaining is more pale as compared to the young rat. x 400.*

CONCLUSION

NM is a pigment that accumulates in the majority primate central nervous system CAergic neurons. The NM-containing neurons are involved in severe diseases, including Parkinson's disease and Alzheimer's disease. Therefore, the data on the normal aging of NM-containing neurons are especially important. Although a subject of intensive research, the precise involvement of NM in the development of Parkinson's disease is still controversial. A better understanding of this issue could provide new strategies in the treatment of human neurological disease.

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