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AGE CHANGES IN NUMBER OF PIGMENTED NEURONS IN THE MONKEY LOCUS COERULEUS

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ABSTRACT

Using stereological counting methods, the total number of pigmented and non-pigmented nerve cells was estimated in locus coeruleus of 19 rhesus monkeys: 13 old animals (10 females, three males), and six young females. Statistically, the total cell number was not different in young and old animals: 47,700 (coefficient of variation (CV = SD/mean) = 0.30) in young and 53,200 (CV = 0.64) in old monkeys; thus, no loss of neurons was found as a function of age. Young monkeys had an average of 1,600 (CV = 1.49) pigmented neurons, while the old anmials had 10,600 (CV = 1.16) pigmented neurons in locus coeruleus, so increased pigmentation is a function of age. The number of nonpigmented cells was almost the same in the two groups (young animals 46,100 (CV = 0.39) and old animals 42,600 (CV = 0.81)). Locus coeruleus volume was the same in young and old animals, and there were no statistically significant systematic right-left differences in the number of pigmented and nonpigmented neurons. Relatively few age-related changes have previously been confirmed in primates.

Key words: brain, optical disector, pigment, total neuron number.

INTRODUCTION

Neurons that synthesize noradrenaline are restricted to the pontine and medullary tegmental regions. Seven noradrenergic cell groups, designated as A1-A7, have been described in rodents, and most of these have also been recognised in primates (Nieuwenhuys et al., 1981). Locus coeruleus (LC) is a macroscopically visible blue-black streak of tissue consisting of a considerable aggregation of closely packed pigmented cells situated in the floor of the fourth ventricle at rostral pontine levels caudally to colliculus superior and rostrally to nucleus mesencephali nervi trigemini (Bogerts, 1981). LC in humans contains about 30,000-40,000 pigmented neurons bilaterally (Mouton et al., 1994). Evidence suggests that all neurons situated in the central part of locus coeruleus are noradrenergic (Nieuwenhuys et al., 1981).

A distinction has been made between the projections of the LC and those of the remaining noradrenergic cell group. Its function is still uncertain, but its efferents constitute a major ascending pathway designated the dorsal noradrenergic bundle and such noradrenaline projections to neocortex, cerebellum, thalamus, hippocampus and basal ganglia in rats and monkeys have been described (Coull, 1994; Freedman et al., 1975; Ungerstedt, 1971). Impulse activity of individual neurons in the LC has been recorded from chair-restrained, unanesthetized cynomolgus monkeys. LC activity was closely related to the behavioral state of the animal. In alert waking, LC neurons displayed continuous, moderately irregular activity, while in contrast drowsiness was accompanied by prolonged pauses in activity (Rajkowski et al., 1994). It was concluded that LC may contribute both to maintaining tenic levels of vigilance and to phasically modulating the current vigilance level in a stimulus-dependent mode. Thus, there seems to be agreement on LC being involved in generating a generalized brain state that can be characterized as "alertness" (Foote et al., 1991). Brain stem sites previously thought to be primarily involved in cardiovascular function and anatomic regulation, including locus coeruleus/subcoeruleus, have also been demonstrated to play a role in the modulation of spinal nociceptive transmission (Jones, 1991).

The pathology of LC has been studied in aging and in several diseases, e.g. Alzheimer's disease, Parkinson's disease and schizophrenia (Karson, 1991; Lohr, 1988; Marcyniuk et al., 1989; Mouton et al., 1994; Tomlinson, 1981; Vijayashankar and Brody, 1979). In the present study we have estimated the total number of neurons in LC in young and old rhesus monkeys to evaluate the effect of age on the total number of neurons and divided the neurons into pigmented and nonpigmented. These values should serve as guidelines for future studies on the effect of disease where rhesus monkeys are used as models and increase knowledge about age-related changes in LC in a higher primate. Modern stereological cell counting methods were applied.

MATERIAL AND METHODS

Nineteen rhesus monkeys (macaca mulatta) previously used in a study of monkey substantia nigra (Pakkenberg et al., 1995) were divided into two age groups: 13 old animals (10 females and three males), average age 22.6 years, and six young females, average age 7.7 years. The old female monkeys had an average age at sacrifice of 23.3 years, and the three male monkeys an average age of 20.3 years.

The animals were immobilized with Ketamine (10 mg/kg, i.m.), intubated using the topical Cetacaine, and ventilated with atmospheric air using a Harvard Pump (TV 15 cm^3/kg, RR 12-15/min). The head was held in a fixed position using a Kopf stereotaxic frame. After administration of an anaesthetic dose of pentobarbital (20 mg/kg given intravenously), a midline sagittal scalp incision was made, the temporalis muscles were reflected laterally, and the calvarium was removed surgically, leaving the dura intact. A second lethal dose of pentobarbital (360 mg) was given intravenously. The brain was cooled rapidly by pouring large quantities of iced saline onto it. It was carefully removed within 10 min of cessation of respiration and placed on a metal dissection tray supported by a bed of ice. The brainstem was transected just above the upper border of the midbrain and immersion-fixed in formalin. The remainder of the brain was processed and frozen for biochemical studies.

The brainstem was bisected along the midline, embedded in agar and cut into 3-mm-slices perpendicular to the brainstem axis. The right and left LC was counted separately for each animal. Each tissue block containing LC was embedded in LKB Historesin and cut exhaustively into 35-µm-thick sections. Sections through the entire LC

were then sampled by taking every 15th section, starting with a random number between 1 and 15, which provided an average of 14 sections per LC. At low magnification (15.5x), the LC was identified on routine hematoxylin and eosin stained sections, and the LC area was encircled. Nucleus subcoeruleus, a nucleus located in the lateral part of the pontine tegmentum at the level of the oral pole of the superior olivary complex, and extending rostrally a further 7 mm to the level of the oral pole of the nucleus papillioformis was not included. The area of LC on each sampled section was estimated by systematic point-counting using a counting grid with a point-spacing of 6 mm x 7 mm. To each point as a pointcounting grid there is a corresponding area, a(p). An estimate of the area of LC on each section is obtained by counting the total number of points, ΣP, that hit the encircled area and multiply that number with a(p).

Total volume of LC, V(LC), was estimated from the equation: $V(LC) = k \times \bar{t}\, a(p) \times \Sigma P$, where $\bar{t}$ is the average section thickness, k = 15 is the inverse fraction of sampled sections, a(p) = 42 mm^2 is area per point on the test grid uncorrected for the magnification, and ΣP is the total number of test points that hits all LC profiles. With an average of 14 sections per LC and an average ΣP of 110, the coefficient of error = SEM/mean of the estimated V(LC) was 0.067 (Pakkenberg and Gundersen, 1988).

Over the last 10 years stereological methods for counting and volume measurements have been developed and applied (Gundersen et al., 1988). The disector is a probe which samples isolated particles with a uniform probability in 3-dimensional space, irrespective of their size, shape and orientation in the tissue (Sterio, 1984). The idea of the optical disector is to start by making only one relatively thick section, e.g. 35 µm, and then make the two or more parallel section planes for the disector as thin optical sections inside the thick one by moving the plane of focus up or down (Gundersen et al., 1988). The optical disector counting equipment consists of a BH-2 Olympus microscope with motorized stage movement, and an electronic microcator with digital readout for measuring movements in the Z-direction to the nearest 0.5 µm. High image resolution and a thin focal plane is obtained using a high numerical aperture (NA = 1.4) and 100x oil-immersion objective. Using the CAST-GRID software-package (Olympus, Denmark) and an ordinary PC, counting frames (average area 9940 μm^2) were superimposed by video camera/Genlock connection to a colour monitor where actual counting took place at a final magnification of 1760x.

A uniform sample of LC cells was made using an optimized, systematic sampling design (Gundersen and Jensen, 1987; Pakkenberg and Gundersen, 1988). Pigmented and nonpigmented neurons were counted separately. For optimization at the level of disectors, a uniform step-length, i.e. the lateral spacing between disectors, was determined in pilot cases and used for the entire study.

An estimate of the total number of neurons, N, was obtained by multiplying the reference volume, V(LC), by the global numerical density, N_V(neu/LC):

$$N(neu,LC)=N_V(neu/LC) \times V(LC) = \Sigma Q^- / (\Sigma v(dis)) \times V(LC) \qquad (1)$$

where ΣQ^- is the total number of neurons (classified as either pigmented or non-pigmented) counted in all disectors in LC, and $\Sigma v(dis)$ is the total volume of the disectors equal to the area of the test frame (in this case 9940 μm^2) multiplied by the height of the disector (in this case 15 µm) and multiplied by the total number of sampled disectors.

An average of 75 pigmented and 337 nonpigmented neurons were counted in an average of 190 disectors evenly spaced through the LC in the old female animals. In the old males an average of 104 pigmented and 360 nonpigmented neurons were counted in an average of 235 disectors, while an average of 19 pigmented neurons and 440

nonpigmented neurons were counted in an average of 240 disectors in the young animals. The nucleus of each cell was used as the counting item in the optical disector. There are two types of pigment in the nerve cells (Barden, 1969): lipofuscin is a brownish, coherent, fine-grained mass, whereas neuromelanin is brown to dark brown, isolated, with rather big grains. Thus it is possible to distinguish between neurons with neuromelanin and those with lipofuscin. Large cells characterized by a mainly centrally located nucleus, one nucleolus and a pigmented or nonpigmented cytoplasm were separated into pigmented or nonpigmented neurons on the basis of the following criteria: A minimum of 10 to 15 pigment granules were required for cells to be classified as pigmented. Some cells had lipofuscin in the cytoplasm, but only cells with regular melanin grains were counted as pigmented cells. Generally, distinction between small nerve cells and big astrocytes was not difficult in this region, but in case of doubt the cells were classified separately and not included in total numbers.

With about 80 pigmented neurons counted per animal on 10-20 sections, the coefficient of error = SEM/mean of N(pigm.neu,LC) was 0.19 (Pakkenberg and Gundersen, 1988). Because young animals had much fewer pigmented neurons, estimates had lower precision (average count of 19 neurons per animal: CE(N,pigm.) young animal = 0.40).

Statistics

Because of right-skewed distributions, group mean values and most statistical tests were performed on logarithmically transformed individual values. Geometric mean values are reported, and the measure of absolute within-group variation is the tolerance factor, TF, considered analogous with the ordinary coefficient of variation: CV = TF-1 = exp(SD/mean)-1 for the logarithmic values. Differences between groups were assessed with the two-tailed Student's unpaired t-test ($2p < 0.05$).

RESULTS

As no difference in number of neurons was found between old male and old female monkeys, they were considered as one group (Fig. 1). No difference was found in the total neuron number (nonpigmented + pigmented), in young = 47,700 (CV = 0.30) and old animals = 53,200 (CV = 0.64), $2p = 0.50$. Old female monkeys had an average of N = 10,900 pigmented neurons bilaterally, CV = 1.07, the 3 old male 9,500 (CV = 1.91) vs. young animals, N = 1,600 bilaterally, CV = 1.49, a highly statistically significant difference, $2p < 0.0005$). Note the pronounced biological variation in the total number of pigmented cells in both young and old animals, Fig. 1. The average number of nonpigmented neurons was the same in the two groups, N(nonpigmented, old animals) = 42,600, CV = 0.81 vs. N(nonpigmented, young animals) = 46,100, CV = 0.39, $2p = 0.77$. Total LC volume was not different in the two groups (6.31 mm^3, CV = 0.68, in the young, and 6.14 mm^3, CV = 0.36, in the old animals ($p = 0.91$) (Fig. 2).

There were no statistically significant systematic side-differences in the number of pigmented and nonpigmented cells.

The CE on the estimate of the mean number of pigmented neurons was 0.19 in the old animals and 0.40 in the young monkeys. At worst the ratio between the error variance ($CE_{young} = 0.40$, $CE^2 = 0.16$) and the biological variance ($CV_{young} = 1,49$, $CV^2 = 2.22$) is somewhat less than one in ten, which is a more than sufficient precision compared with the high biological variation among animals (CV_{old}, average = 1.16 and $CV_{young} = 1.49$).

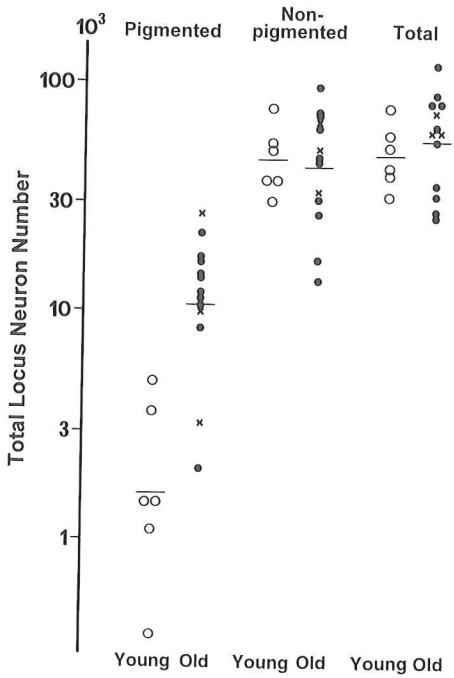

Fig. 1. The total number of pigmented and non-pigmented neurons in locus coeruleus (bilateral) is shown in young (○) and old (●) female rhesus monkeys and in old males (x) on a logarithmic scale.

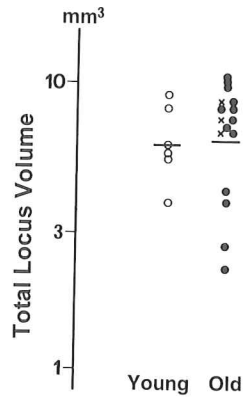

Fig. 2. The total volume of the locus coeruleus (bilateral) is shown on a logarithmic scale in young (○), old female monkeys (●), and in old males (x).

DISCUSSION

Using computer imaging procedures to map cell location in 50 μm-thick sections processed for immunocytochemistry and cresyl violet, respectively, and estimating total numbers of LC and subcoeruleus cells using a conventional correction factor (Abercrombie, 1946), Manaye et al. (1995) have reported neuromelanin pigment to be a useful marker of catecholaminergic neurons in the human brain, but indicated that neuromelanin pigment is not a reliable cell marker for catecholamine producing neurons in LC in the human brains under 50 years of age. This is not in contrast to our findings in monkeys where the total number of neurons was the same in young and old animals although there was a considerable shift in the number of pigmented neurons.

The explanation for the increase in total number of pigmented neurons as a function of age in the rhesus-monkey is most probably that nonpigmented neurons become pigmented during the life of the animal, and that this pigmentation is far from being completed by the age of seven years. This is also in agreement with previous results for substantia nigra in the same animals (Pakkenberg et al., 1995). However, in contrast to those results the number of pigmented neurons was not inversely correlated with the number of nonpigmented neurons, i.e. the increase in the number of pigmented neurons in the old animals was not followed by a similar decrease in the number of nonpigmented neurons, which may partly be explained as problems of an exact delineation of LC combined with a high biological variance.

Also in contrast to previous results in other brain areas we found no direct positive relationship between the total number of pigmented and nonpigmented neurons. In e.g. human substantia nigra (Pakkenberg et al., 1991) we found a direct, positive relationship between the total number of pigmented and nonpigmented neurons, i.e. a high number of pigmented neurons was associated with a high number of nonpigmented neurons, which was not the case in this study of monkey LC.

Pigment granules are seen in the cytoplasm of some nerve cells and are of two kinds. Dark brown or almost black particles of melanin are found in the cells of certain regions such as substantia nigra, locus coeruleus, and, sometimes, in the dorsal efferent nucleus of the vagus and in spinal and sympathetic ganglia. Other pigmentations such as lipochrome- and lipofuscin-staining granules of yellowish-green, gray, brown-orange, or organge-red colour appear in cells in the central nervous system (Crosby et al., 1962) and lipochrome has been found in the nerve cells of individuals of six years of age or older. The intensity of the pigmentation in primates is greater than in any other order and reaches maximum intensity in man. It is found in small amounts in some very old subprimates such as the basal portion of the midbrain in dogs and horses and is present in some of the highest subhuman primates (Truex and Carpenter, 1969). It is also present in albinos. Some neuromelanin pigment in substantia nigra is present at birth, but it increases substantially from the sixth to the eighth year and is then added very slowly throughout life (Crosby et al., 1962). This neuromelanin pigment, lying within the cells in the live brain, is often extracellular in postmortem material. It is noteworthy that the increase in pigmentation in the monkey model is many times higher than the one seen in humans (Manaye et al., 1995) since this could limit the use of the rhesus monkey as an experimental animal model. Evidently all animal models have their limitations when trying to understand the cause and progress of human disorders. However, monkeys rank high being a primate close to humans but should be used with increasing reservation the more they differ from humans. The functional relevance of the eight times increase in neuromelanin pigmentation is unknown.

The volume of the LC was the same in young and old monkeys. This is in agreement with our finding in substantia nigra in Parkinson patients (Pakkenberg et al., 1991) where the volume of SN was the same in both patients and controls in spite of a 65% reduction of the pigmented neurons in the Parkinson patients. No systematic right-left differences in LC were found, which means that one may choose to study just one of the paired nuclei.

Advantages of the methods used in this study include a uniform sampling design which provided unbiased estimates of total neuron numbers, counting in optical disectors improved efficiency, i.e. the counting could be done in one to two hours per locus, and the precision of the results obtained (CEs from 6 to 40%) were more than sufficient for a brain region in which the number of neurons varies much from one monkey to the other with coefficients of variation from 0.40 to 1.50.

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