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Aging is associated with a diffuse impairment of forebrain cholinergic neurons

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The study evaluates whether any degenerative changes affect forebrain cholinergic systems during natural aging. The medial septal nucleus, the nuclei of the diagonal band, the neostriatum, and the basal nucleus were studied in adult and aged Wistar rats. Butcher's technique for acetylcholinesterase allowed us to identify neurons located in these forebrain nuclei, which stained intensely or moderately for the enzyme and were putatively cholinergic. The size of forebrain regions containing stained neurons, and the number and size of stained perikarya located therein, were measured. In aged rats, the size of forebrain cholinergic nuclei was reduced by an average of 26%. The density of neurons located in these regions was also significantly lower in aged rats than in controls: intensely stained neurons displayed a mean reduction of 27.81%, while intensely and moderately stained perikarya together were reduced by 25.43%. Cross-sectional area of the stained perikarya was also reduced in aged rats by 32.87%. These data show that the number of forebrain acetylcholinesterase-containing neurons is reduced in aged rats. They are consistent with the hypothesis that natural aging brings about a diffuse and homogeneous depletion of forebrain cholinergic perikarya. Neurons which are viable, and can be selectively stained, show morphological alterations, which are likely to be related to a degenerative process.

INTRODUCTION

The anatomy of forebrain cholinergic systems has been thoroughly investigated during recent years. Cholinergic perikarya are located in the medial septum (Ch1, according to the terminology proposed by Mesulam et al.[34]), in the nuclei of the diagonal band (Ch2, Ch3), in the basal nucleus (Ch4), and in the neostriatum. With the exception of striatal interneurons, forebrain cholinergic cell bodies project preferentially to the cerebral cortex: the hippocampal archicortex is the main target of septal neurons, while cholinergic cells of the basal forebrain impinge upon most of the neocortex[18,34]. Therefore, their anatomical organization is consistent with the view that cholinergic neurons are involved in higher-order activities, such as memory and cognition.

Renewed interest on the relationship between forebrain cholinergic activity and aging has been fostered in recent years by the study of cognitive changes associated with impairment of cholinergic functions. In fact, a reduced activity of cholinergic basal forebrain neurons is found in Alzheimer's disease, a degenerative dementia characterized by some pathological features similar to natural aging[44]; this is confirmed by the observation that experimental lesions of forebrain cholinergic systems is capable to bring about a cognitive impairment in rats[8,38,41]. Based on the presence of a comparable decline of memory and cognitive capabilities, several studies have addressed the question as to whether forebrain cholinergic neurons undergo a functional decline during natural aging. Biochemical and receptor-binding studies, which have been reported so far, have consistently confirmed the presence of a cholinergic impairment in Alzheimer's disease, while they have provided contradictory results on aging. In fact, as reported by Bartus et al.[7], the number of papers describing a reduction in choline acetyltransferase (ChAT) activity in aged humans or animals, is balanced by an almost equal number of studies which have found no differences with adult controls; a similar inconsistency exists in papers based on quantification of muscarinic receptors. Furthermore, in a recent study on mice, Mesulam et al.[35] were able to find shrinkage of cholinergic perikarya, but no cell loss in aged individuals. We have recently addressed the question as to whether natural aging is associated with loss of forebrain cholinergic neurons. In a previous paper, we have demonstrated that degenerative changes indeed occur in the basal ganglia of aged rats[3]: this raised the

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question as to whether age-dependent decline diffusely affects forebrain cholinergic neurons and, in the affirmative, whether this feature is in any way comparable to the loss of forebrain perikarya that we have observed in a genetic animal model of Alzheimer's disease[2,24].

MATERIALS AND METHODS

Male Wistar rats, kept on a natural light–dark cycle, were used. The rats were housed in couples when adult, and individually after about 8 months of age. Access to food and water was free in the home cage.

Three 24-month-old and three 3-month-old animals were studied by means of Butcher's pharmacohistochemical technique for acetylcholinesterase (AChE)[11], which allows a detailed visualization of AChE-containing neurons, and is suitable for the study of putative cholinergic perikarya (see ref. 43). Eight hours prior to perfusion, the animals were poisoned with diisopropylfluorophosphate (DFP). Such a short survival after DFP was chosen in order to minimize the staining of non-cholinergic, light, AChE-containing neurons, which are located in a wide number of brain regions[11]. DFP was injected i.m. in a 0.15% peanut oil solution in the dose of 1.5 mg/kg b. wt. Then, under deep general anesthesia with sodium pentobarbital (40 mg/kg i.p.), the animals were perfused through the heart with 0.9% saline solution, followed by 10% phosphate-buffered formalin (pH 7.4). The brains were kept in the same formalin solution for 48–72 h before being transferred into cold (4 °C) 30% sucrose for an additional 48-h period. Coronal sections were cut at 30-μm intervals by means of a freezing microtome, according to a standard plane[37]. The brain of one adult rat was always stained in parallel with that of one aged individual. Every other tissue section was stained with Nissl's technique, while the alternate series was processed according to the following protocol: sections were immersed for 30 min into 30 μM *N,N'*-bis(1-methylethyl)pyrophosphoroamidic anhydride (iso-OMPA), to inhibit butyrilcholinesterase, and then incubated in the AChE incubation medium, as reported previously[1,12].

Every other AChE-stained section through the medial septum, diagonal band and basal nucleus, and every third section through the neostriatum, were charted on a drawing microscope, and then analyzed by means of a VIDS III semiautomatic image analyzer. The brains of one adult and one aged rat, belonging to a single staining session, were charted blindly by the same observer. The boundaries of areas rich in

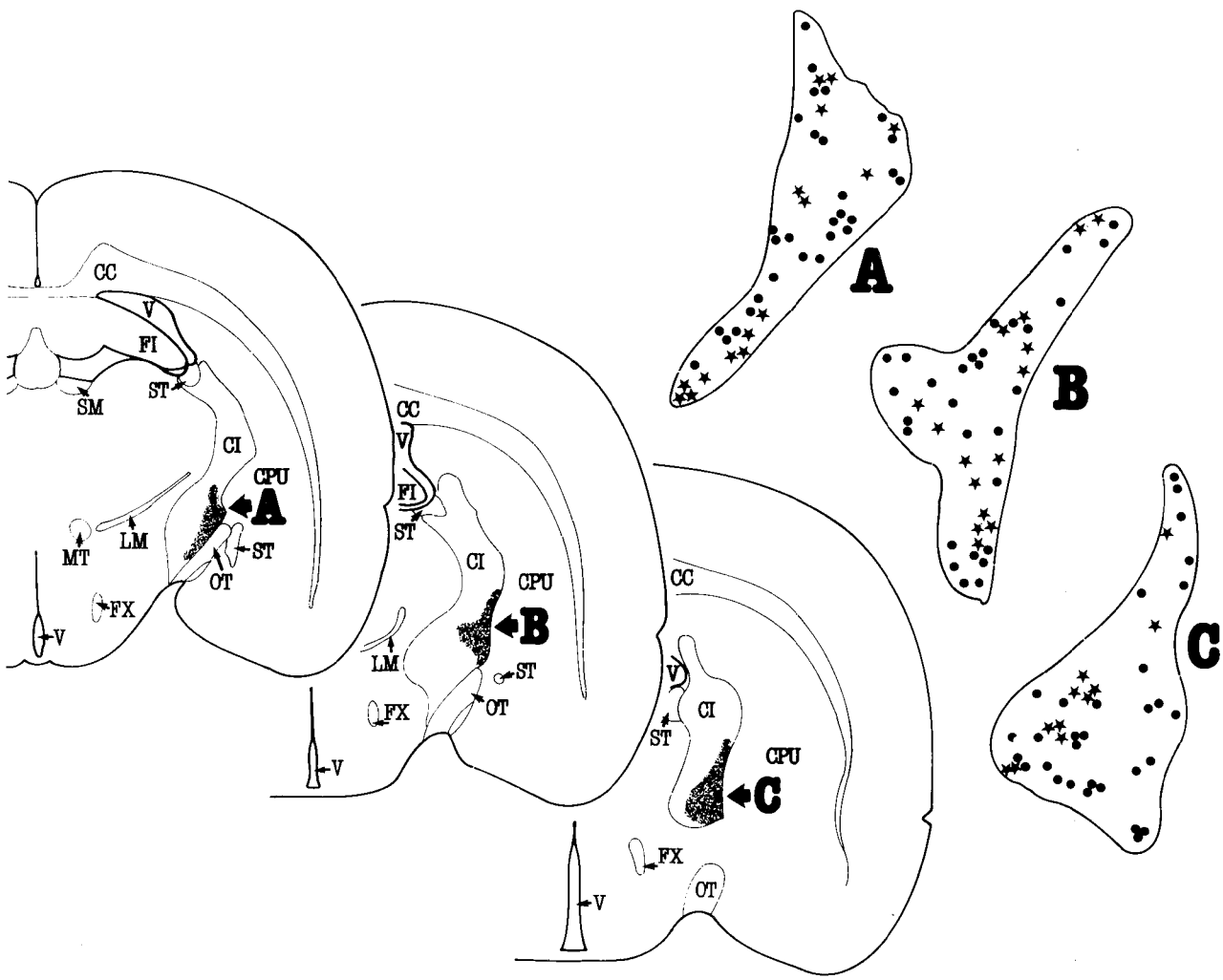


Fig. 1. Drawings of 3 representative coronal sections through the left side basal nucleus in one aged rat (D501). A, B and C represent a series of 3 different caudorostral levels, spaced by approximately 0.5 mm. Dots and stars indicate intensely and moderately stained perikarya, respectively. It can be observed that the two classes of AChE-containing cell bodies are regularly distributed throughout the nucleus. Abbreviations: CC, callosal body; CI, internal capsule; CPU, caudate-putamen; FI, fimbria; FX, fornix; LM, medial lemniscus; MT, mammillothalamic tract; OT, optic tract; SM, stria medullaris; ST, stria terminalis; V, ventricle.

cholinergic neurons were identified by comparing alternate AChE- and Nissl-stained sections (see examples in Fig. 1). Cell number, cell density, and regional area computed in the adult and aged groups were compared by means of analysis of variance. The rostrocaudal distribution of cell and area values were statistically analyzed by averaging the series of data obtained from adjacent coronal sections; in each region, the distribution of neural density was studied by means of frequency analysis. In each rat, measurements of the size of individual AChE-containing neurons were performed at random throughout the medial septum, the diagonal band nuclei, the basal nucleus, and the neostriatum. Only neurons containing a well-defined unstained nucleus were individually chosen from any segment of the observed region; they were automatically taken in by a Leitz ASBA image analyzer. Their cross-sectional areas were computed in the adult and aged groups, and were compared by means of analysis of variance.

RESULTS

In old rats, the overall distribution and the morphology of AChE-containing forebrain neurons were similar to that observed in young subjects. Thus, large, intensely stained, russet, AChE-containing neurons were seen in the neostriatum, medial septum, nuclei of the diagonal band, magnocellular preoptic nucleus, and basal nucleus. In the same regions they were intermingled with moderately stained perikarya. By contrast, lightly stained cell bodies, which are located in several other brain regions[13], could barely be seen. In our comparative study, only

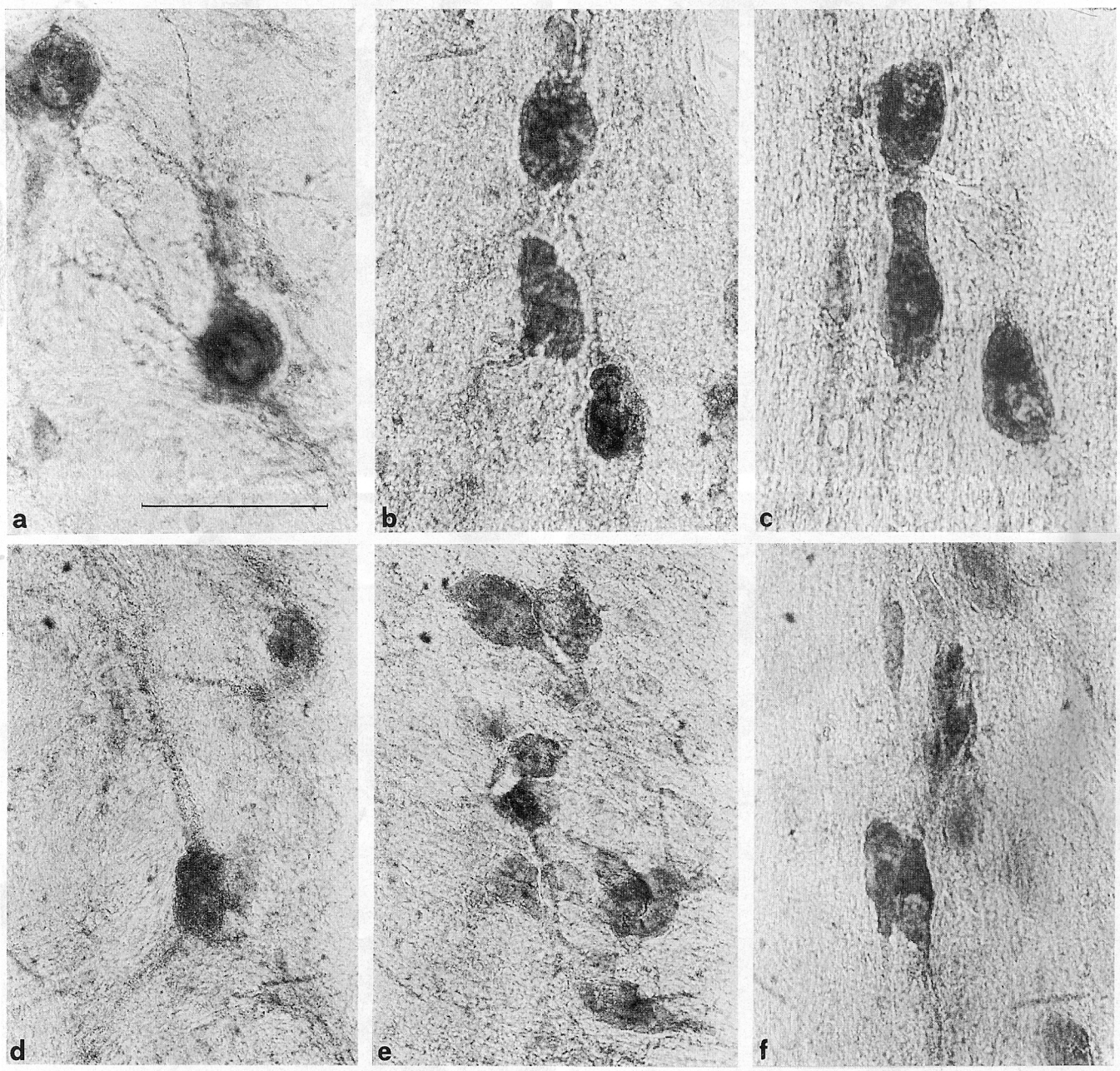
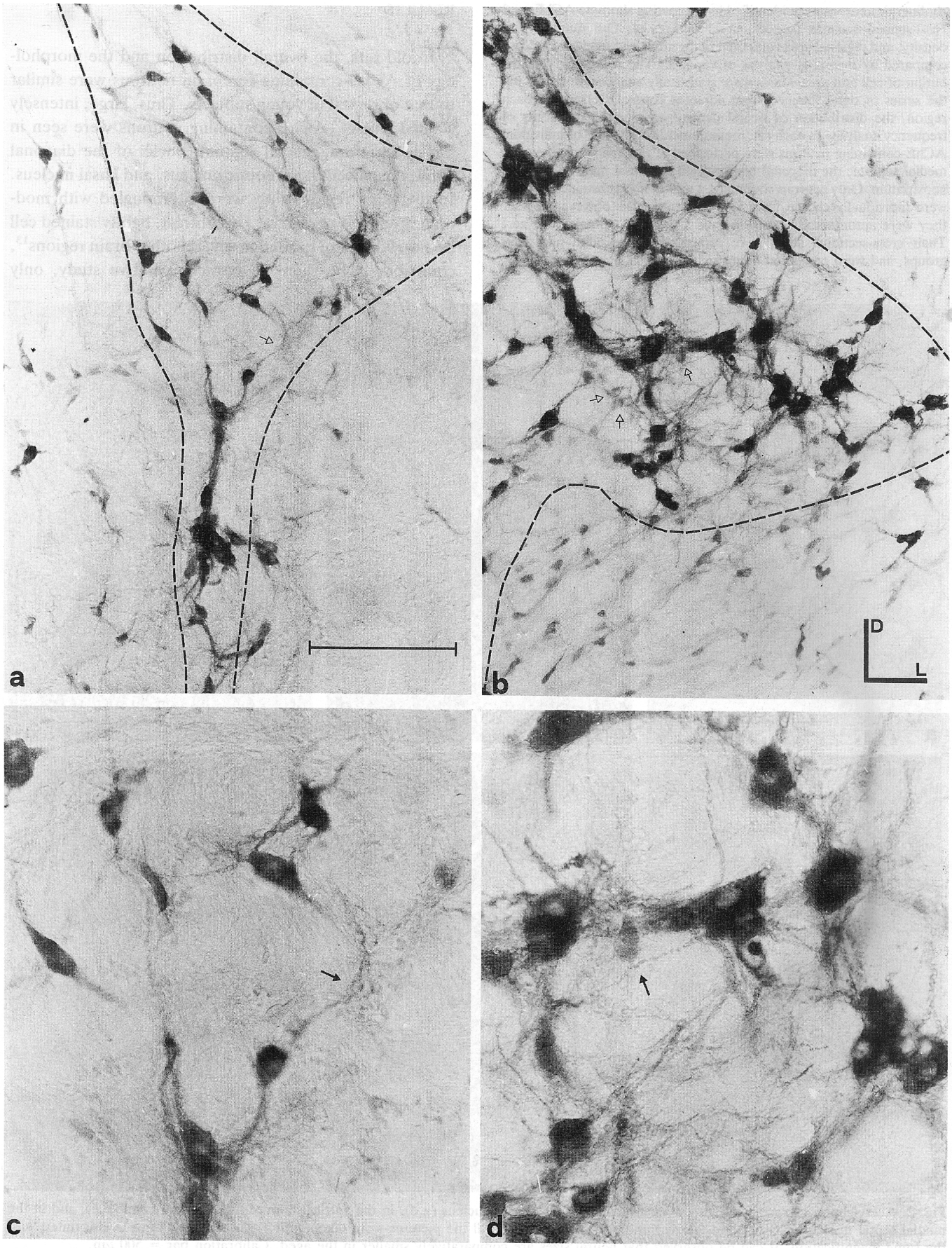


Fig. 2. Acetylcholinesterase-containing neurons located in the basal nucleus (a,d), in the vertical limb of the diagonal band (b,e), and in the medial septal nucleus (c,f) of adult (above) and aged rats (below). All the pictures were taken with a 63× lens and have been printed with the same magnification. It can be observed that neural sizes are comparatively smaller in the aged. Calibration bar = 500 μm.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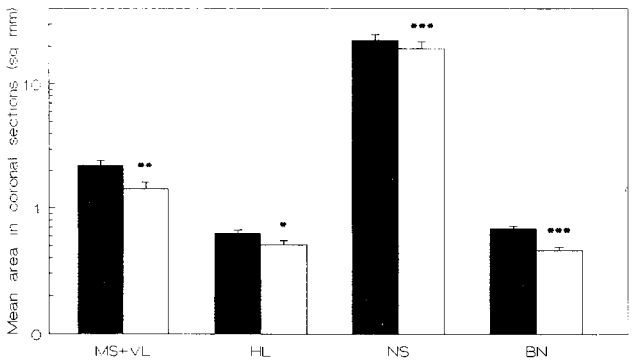

Fig. 4. Age-dependent reduction in the size of forebrain nuclei containing cholinergic perikarya, as observed in coronal sections. Mean areas (and S.E.M.) are plotted in adult (black bars) and aged rats (white bars) for the medial septal nucleus and the nucleus of the vertical limb (MS + VL), the nucleus of the horizontal limb (HL), the neostriatum (NS), and the basal nucleus (BN). Statistical comparison of the two age groups is significant for all values ($n = 3$): $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

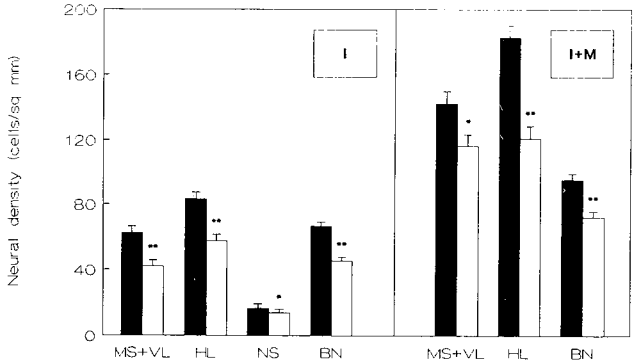

Fig. 5. Density of AChE-positive neurons in adult (black bars) and aged rats (white bars). Mean values (and S.E.M.) of intensely (I), and intensely plus moderately stained cells (I + M) are plotted for the medial septal nucleus and the nucleus of the vertical limb (MS + VL), the nucleus of the horizontal limb (HL), the neostriatum (NS), and the basal nucleus (BN). Statistical comparison of the two age groups is significant for all values ($n = 3$): $^{*}P < 0.01$; $^{**}P < 0.0001$.

intensely AChE-stained neurons were charted in the neostriatum, while both intensely and moderately stained perikarya were charted in the Ch1–Ch4 nuclei (Figs. 2, 3). Cell bodies were distributed in the neostriatal matrix with a typical patchy arrangement, matching an anatomical and biochemical mosaic, which is a feature of this territory[25]. In the medial septal nucleus, AChE-rich neurons were aggregated along the midline and sparsely distributed on each side; they merged ventrally with neurons of the vertical limb. At more caudal levels, cell bodies located in the vertical and horizontal limb nuclei abutted the ventral surface of the brain. Thus, in the Ch1–Ch3 nuclei, AChE-containing neurons constituted a continuous collection of cells, so that boundaries between different anatomical structures were sometimes difficult to draw. This, in particular, was the case for the medial septal nucleus and the nucleus of the vertical limb, which in the present study have been analyzed together. As observed in coronal sections, AChE-containing cell bodies located in the basal nucleus constituted a well-demarcated grouping of large perikarya, displaying a typical triangular or lentiform shape (Figs. 1, 3).

Upon examination of AChE-stained material, some differences between adult and aged brains were quite clear. In fact, independent observers, who had no knowledge of which age group they were looking at, could consistently spot the brains of aged rats by observing reduced neural sizes and numbers. This was confirmed, in fact, by morphometric analysis, which revealed clear-cut age-dependent differences in all the forebrain cholinergic nuclei. First, the mean area of cholinergic nuclei was reduced in aged rats by approximately 26%: the size of neostriatum by 14.48%, that of basal nucleus by 33.33%, that of the nucleus of the horizontal limb by 19.05%, that of the medial septum and vertical limb by 36.32% (Fig. 4). Second, the number of AChE-containing neurons was also reduced in the same regions, by a larger degree. Intensely stained neurons showed a mean reduction of about 47% in aged rats: 27.56% in neostriatum, 56.30% in the basal nucleus, 44.22% in the nucleus of the horizontal limb, and 60.25% in the medial septum and vertical limb (Table I). Intensely and moderately stained cells together underwent a mean reduction of approximately 47% in the aged (51.18% in the basal nucleus, 43.01% in the nucleus of the horizontal limb, and 45.57% in the medial septum and vertical limb). As a consequence, the age-dependent decrease in the density of intensely stained cells was 27.81%, that of intensely and moderately stained cells was 25.43% (Fig. 5).

In each animal, a comparison of morphometric data obtained in the right and left hemispheres was performed in all regions, except the medial septum and the vertical limb. No right–left asymmetries were observed, and,

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Fig. 3. Acetylcholinesterase-containing neurons located in the basal nucleus of one aged (left; case D500) and one adult rat (right; case D502). This composite figure shows two frontal sections at corresponding rostrocaudal levels: boundaries of the basal nucleus can be seen in lower power pictures (a,b); their core is shown at higher magnifications in c and d, respectively. It can be observed that most neurons in the basal nucleus stain intensely for the enzyme, and that sparse moderately stained neurons can also be seen. In fact, arrows in a and b point to 4 representative moderately stained AChE-containing neurons; two of these perikarya are also indicated by arrows in c (slightly out of plane) and d. Abbreviations: D, dorsoventral intersection; L, mediolateral intersection. Calibration bar = 0.2 mm for a and b; 800 μm for c and d.

TABLE I

Intensely stained AChE-containing neurons in the forebrain of adult and aged rats

Cell values indicate the mean number of cells (± S.E.M) in each section; density is evaluated as the average number of cells (± S.E.M.) per mm^2. In each adult and aged rat, the number of sections is 14 and 16 (respectively) in the medial septum and vertical limb, 17 and 17 in the horizontal limb, 34 and 31 in the neostriatum, and 14 and 13 in the basal nucleus. These data are based on 3 adult and 3 aged rats.

	Adult	*Aged*	*P*	*Variation*
Medial septum and vertical limb				
Cells	116.54 ± 7.36	47.52 ± 7.00	< 0.0001	−60.25%
Density	62.37 ± 4.08	41.88 ± 3.88	< 0.001	−32.85%
Horizontal limb				
Cells	49.68 ± 2.90	27.71 ± 2.90	< 0.0001	−44.22%
Density	83.06 ± 4.22	57.40 ± 4.22	< 0.0001	−30.89%
Neostriatum				
Cells	367.70 ± 63.90	266.35 ± 54.01	< 0.001	−27.56%
Density	16.43 ± 2.78	13.87 ± 2.08	< 0.01	−15.58%
Basal nucleus				
Cells	44.07 ± 1.92	19.26 ± 1.66	< 0.0001	−56.30%
Density	66.60 ± 2.70	45.33 ± 2.34	< 0.0001	−31.94%

therefore, measurements of the right and left side were considered as belonging to a homogeneous population. The analysis of a rostrocaudal series of coronal sections did not reveal qualitative differences in the distribution of AChE-containing cell bodies. Thus, aged rats simply had a reduced number of AChE-positive neurons, which were distributed in a pattern similar to that of adult controls. This is exemplified in Fig. 6, where the rostrocaudal distribution of cell densities in the left side basal nucleus of case D503 (adult) is compared to that of the homologous structure of case D500 (aged). It can be observed that values measured in the aged rat are constantly lower than those of the adult animal. In both cases, however, there is a comparable rostrocaudal increase of cell densities, so that plots obtained in the aged animal have a slope similar to those of the adult rat. Also, no age-dependent differences were observed in the 3-dimensional shape of cholinergic regions, as observed in the rostrocaudal series of coronal sections: in fact, volumetric reduction occurring in aged animals was evenly distributed in mediolateral and rostrocaudal diameters.

Upon observation, age-dependent differences in the appearance of individual AChE-containing neurons were evident. In aged animals, both nuclei and perikarya showed an irregular contour, the nuclei being often displayed to the periphery of the cytoplasm. In every region, quantitative image analysis showed that intensely stained AChE-containing neurons were clearly smaller in the aged. The average reduction of the cross-sectional area of perikaryon was 32.87%, accounting for a 22.49% reduction in the medial septum and vertical limb nuclei,

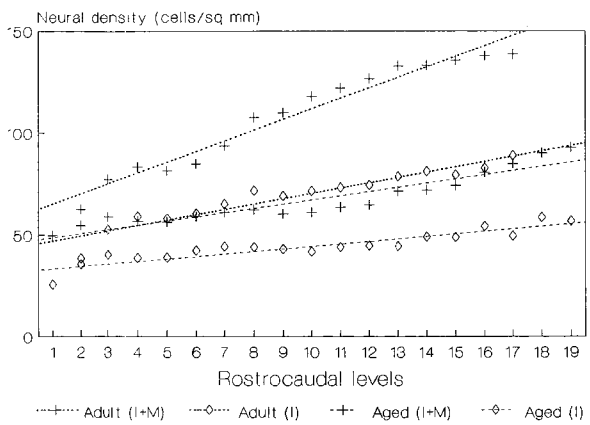

Fig. 6. Rostrocaudal variations in AChE neuron density, in one adult (D503) and one aged rat (D500), as exemplified by comparison of the left side basal nucleus in each case. A rostrocaudal series of coronal sections is plotted against values of cell density: it can be observed that, in the adult animal, measures of intensely and moderately stained (I + M), or just of intensely stained AChE perikarya (I), have larger values than the corresponding measures obtained in the aged. A rostrocaudal increase of cell density in the basal nucleus has been observed in all cases.

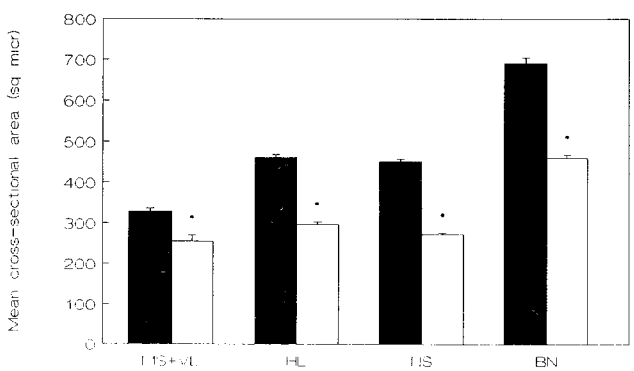

Fig. 7. Cross-sectional area of intensely stained AChE-containing neurons in adult (black bars) and aged rats (white bars). Mean values (and S.E.M.) are shown for the medial septal nucleus and the nucleus of the vertical limb (MS + VL), the nucleus of the horizontal limb (HL), the neostriatum (NS), and the basal nucleus (BN). The number of samples is 339 and 53 (in adult and aged rats, respectively) in the medial septum, 392 and 254 in the horizontal limb, 318 and 320 in the neostriatum, and 207 and 216 in the basal nucleus. Statistical comparison of the two age groups is significant in all cases ($n = 3$): $^*P < 0.001$.

35.72% in the horizontal limb nucleus, 39.68% in the neostriatum, and 33.58% in the basal nucleus (Fig. 7).

DISCUSSION

Age-dependent cell loss and decrements in brain volume and weight have been the object of several studies. As reviewed by Creasey and Rapoport[17], the number of glial cells is usually unaffected by aging, while neural loss is widespread, but not haphazard. In brain regions where it occurs, a decrease of neural density may also be observed, depending on the severity of regressive changes affecting the neuropil and extra-neural compartments. In the present study, we have seen that an age-dependent decrease in the number of AChE-containing neurons is a diffuse process, affecting all the forebrain cholinergic regions. Quantitative analysis has indicated that a marked decrease in the number of neurons is associated with a volumetric reduction of territories containing cholinergic perikarya. The volumetric decrement is conspicuous in nuclei where cholinergic neurons are the majority (e.g. in the basal nucleus), and it is less evident in regions where the relative proportion of other neurochemical classes of neurons is higher. The neostriatum is one example of the latter case: here, the mean decrement in the cross-sectional area of coronal sections is less than half that observed in the basal nucleus or in the medial septum and vertical limb. This would support the hypothesis that non-cholinergic neostriatal neurons may be poorly affected by age-dependent decline. However, since the neostriatum of rodents contains a large number of fibers-of-passage, it can be argued that they can interfere with a correct measurement of its volume. A quantification of age-dependent white matter variations, or the study of higher animal species, would be required in order to obtain exact measures of the neostriatal age-dependent volumetric decrement. Still, the regional analysis of age-dependent cell loss in the neostriatum[3], and earlier data indicating that both gray and white matter atrophy occurs in relation to age[5,36], support the overall reliability of our analysis in the neostriatum. We have previously seen that age-dependent loss of cholinergic perikarya preferentially affects the ventromedial part of the neostriatum[3]. Due to their size and shape, the other forebrain regions containing cholinergic neurons were unsuitable for a similar analysis.

The present study shows that the number of AChE-containing perikarya is decreased in all forebrain cholinergic nuclei of aged Wistar rats. This is in keeping with earlier biochemical observations indicating an age-dependent reduction of markers for cholinergic activity (see refs. 7, 30, 39), and reflects a specific impairment of cholinergic neurons during aging. The observation that AChE staining is lost or decreased does not necessarily mean that unstained neurons are lost or dead. As shown by Lams et al.[28], the expression of AChE and ChAT can be regulated independently of the survival of cholinergic neurons following an injury. Thus, in our case it is not possible to rule out that the observed age-dependent decrease in the number of AChE-stained neurons may actually reflect an impairment of AChE synthesis or a poor recovery from DFP poisoning, rather than a reduced viability of cholinergic nerve cells. Still, our observation that the size of AChE-containing cells is markedly reduced in aged rats would favor the latter hypothesis, as it indicates that neurons which have not degenerated are unhealthy. This is also in keeping with data reporting age-dependent impairment in impulse-conduction properties of cortically projecting neurons located in the basal nucleus[6].

Direct comparison of AChE- and ChAT-staining patterns in the same regions has shown that intensely stained AChE-containing neurons are invariably cholinergic[19,29,43]. In the neostriatum there is a direct 1/1 relationship between intensely AChE-stained and ChAT figures; therefore, the number of intensely stained AChE neurons gives a good estimate of the actual number of cholinergic perikarya in this region. Similarly, a close relationship between AChE and ChAT staining has been also shown in the basal nucleus of man[33]. However, as shown by Wainer et al.[43], the number of intensely stained AChE-positive cell bodies of the Ch1–Ch4 forebrain cholinergic nuclei slightly underestimates the amount of cholinergic perikarya in rodents. Therefore, in order to minimize errors and still have morphological specimens comparable to earlier work from this laboratory, in the present study we have chosen to measure either the number of intensely stained or that of intensely plus moderately stained AChE-containing perikarya (see Fig. 5). In either case, the age-dependent decrease of AChE-containing neurons was significant and of a comparable degree. A survival interval of 8 h after DFP poisoning has allowed us to differentiate AChE-containing neurons into 3 main categories, according to their weak-, moderate-, or intense-staining intensity (see ref. 13). For the purpose of this study, the intensity of AChE staining has been identified by direct observation (e.g. see ref. 1), rather than by means of quantitative histochemistry, as described in an earlier report from this laboratory[27].

The use of Butcher's pharmacohistochemical procedure for AChE has allowed us to obtain data comparable to earlier information from this laboratory. We have recently shown[2,4,24] that C57BL/6 mice suffer from a genetically dependent consistent loss (20–23%) of forebrain cholinergic perikarya. Our recent unpublished

observations show that these inbred mice constitute a genetic mutant with an abnormal development of forebrain cholinergic perikarya. The present data show that aging in the Wistar rat is indeed associated with a loss of cholinergic perikarya in the forebrain, which is comparable to that observed in C57BL/6 mice. Hornberger et al.[26] have shown that in C57BL/6NNIA mice no decline in the number, but only a 6–17% reduction in the size of basal forebrain AChE-containing neurons occurs from 7 to 53 months of age. However, they have only measured intensely stained neurons, and have just studied C57BL/6 mice, which suffer from a genetically determined impairment of cholinergic functions. In a more recent work based on choline acetyltransferase immunocytochemistry, Mesulam et al.[35] found no decline in the number of cholinergic perikarya located in the basal forebrain and in the dorsomedial caudate-putamen of CD-1 mice, although they observed a 21–48% reduction in their mean cross-sectional area. By contrast, biochemical and morphological studies performed on some rat strains by different independent groups have consistently demonstrated that natural aging is associated with loss of forebrain cholinergic neurons[9,20,21,23], and a decrease of cholinergic markers in their terminal sites[42]. Therefore, it must be assumed that, while in some strains of mice cell shrinkage but not staining loss occurs as an age-dependent process, both events happen to be detectable in some other rat strains.

The picture is even more complicated in man. Earlier studies performed on human brains have not consistently confirmed a depletion of forebrain cholinergic cell bodies. Chui et al.[14] have shown with classic techniques that the number of neurons contained in high density clusters of the basal nucleus do not change with age. However, Mann et al.[31], using similar techniques, reported that the total number of nucleolated nerve cells, located in the most extensive portion of the basal nucleus, are reduced by 30% by 90 years of age. McGeer et al.[32] have observed that the number of ChAT-containing neurons located in the basal nucleus undergoes a progressive age-dependent decline. Recently, Bigl et al.[10] have approached the same problem by using classic morphological techniques: they have observed no age-dependent decline of the number of nucleolated cells located in the human basal nucleus, up to the age of 60 years. However, in none of these studies has the size of neurons located in the basal forebrain been measured. Age-dependent decline of cholinergic activity in the human forebrain is also supported by analysis of postsynaptic cholinergic sites, as it has been recently demonstrated that muscarinic receptors are reduced in the frontal cortex, hippocampus, caudate nucleus, and putamen of the aged[40]. Inconsistencies of data collected from human material may depend upon the heterogeneity of either the methodologies or the samples employed (see review by Coleman and Flood[15]). In fact, it must be considered that: (1) data obtained by means of classic, non-specific, staining techniques cannot be directly compared to those based upon chemically specific staining; and (2) the human subjects under study are likely to belong to a heterogeneous population. This does not apply to animal studies, as individuals of a single breed are housed in a similar way and simply allowed to have different life spans. Therefore, animal studies differ from human studies also because aging occurs in an environment deprived of natural stimuli. The latter argument must not be understated, as both neurobiological and clinical data stress the role played by environmental factors on brain activity in the elderly (see Gainotti[22]). The environment can also affect morphological parameters in the brain: as shown by Connor et al.[16], socially isolated old rats lose a greater number of dendritic spines in the occipital cortex than age-matched rats living in an enriched environment.

The reason why cholinergic neurons may be particularly sensitive to aging is still unknown. It can be postulated that some specific neurotrophic factors may gradually decrease with age; in addition, the susceptibility to the expression of such variations may be regulated genetically. Although the possibilities to act at the genetic level are currently quite poor, the search for replacement therapies with neurotrophic factors (e.g. see ref. 21) may represent a promising avenue in clinical neurology of the aged.

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