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Age-dependent loss of cholinergic neurones in basal ganglia of rats

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A morphometric study of the striatal complex has been performed in adult and aged rats, by means of Butcher's acetylcholinesterase (AChE) staining. The size of neostriatum, and the number of putatively cholinergic AChE-positive perikarya, are significantly reduced in two-year-old rats. Consequently, mean cell density is decreased in the striatal complex by 15.58%; age-dependent loss is more pronounced: (1) in the dorsal and lateral parts of neostriatum, which in adults have a higher neural density; and (2) in the ventromedial part, which in the aged becomes almost void of neurones. It is concluded that depletion of cholinergic perikarya is a specific feature of natural ageing, which affects diffusely the neostriatum, and particularly its ventromedial territories.

Based on the presence of a decline in cognitive capabilities, several studies have addressed the question as to whether forebrain cholinergic neurones undergo a loss of function during natural ageing. Biochemical and receptor binding studies so far have consistently confirmed the presence of a cholinergic impairment in senile and presenile dementia, while they have provided contradictory results on natural ageing. 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 (see review in ref. 3). Therefore, we have addressed the question as to whether natural ageing is associated with loss of forebrain cholinergic neurones and, in the affirmative, whether morphological data in the aged are comparable to what has been observed in C57BL/6 mice, which are characterised by poor open field activity and learning abilities, and suffer from untimely loss of forebrain cholinergic neurones[1,10].

Until the availability of reliable immunocytochemical techniques for ChAT, Butcher's[5] procedure for acetylcholinesterase (AChE) has been effectively used to stain putative cholinergic neurones in the brain. Later studies comparing the staining pattern obtained by using this technique and ChAT immunocytochemistry have shown that Butcher's procedure reliably stains neostriatal cholinergic neurones[17,19]. Therefore, this technique has been used in the present study as a means to analyse the morphometric arrangement of cholinergic neurones in the neostriatum of aged rats and young adult controls.

Four male Wistar rats, including two two-year-old and two 3-month-old animals, were studied by means of Butcher's technique. Eight hours prior to perfusion, the animals were poisoned with di-isopropylfluorophosphate (DFP). This irreversible AChE inhibitor was injected intramuscularly as 0.15% arachid oil solution in the dose of 1.5 mg/kg b.wt. Then, under deep general anaesthesia 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 immersed in the same formalin solution for 48–72 h before being transferred into cold (4 °C) 30% sucrose for an additional 48-h period. Sections were cut coronally at 30-μm intervals according to a

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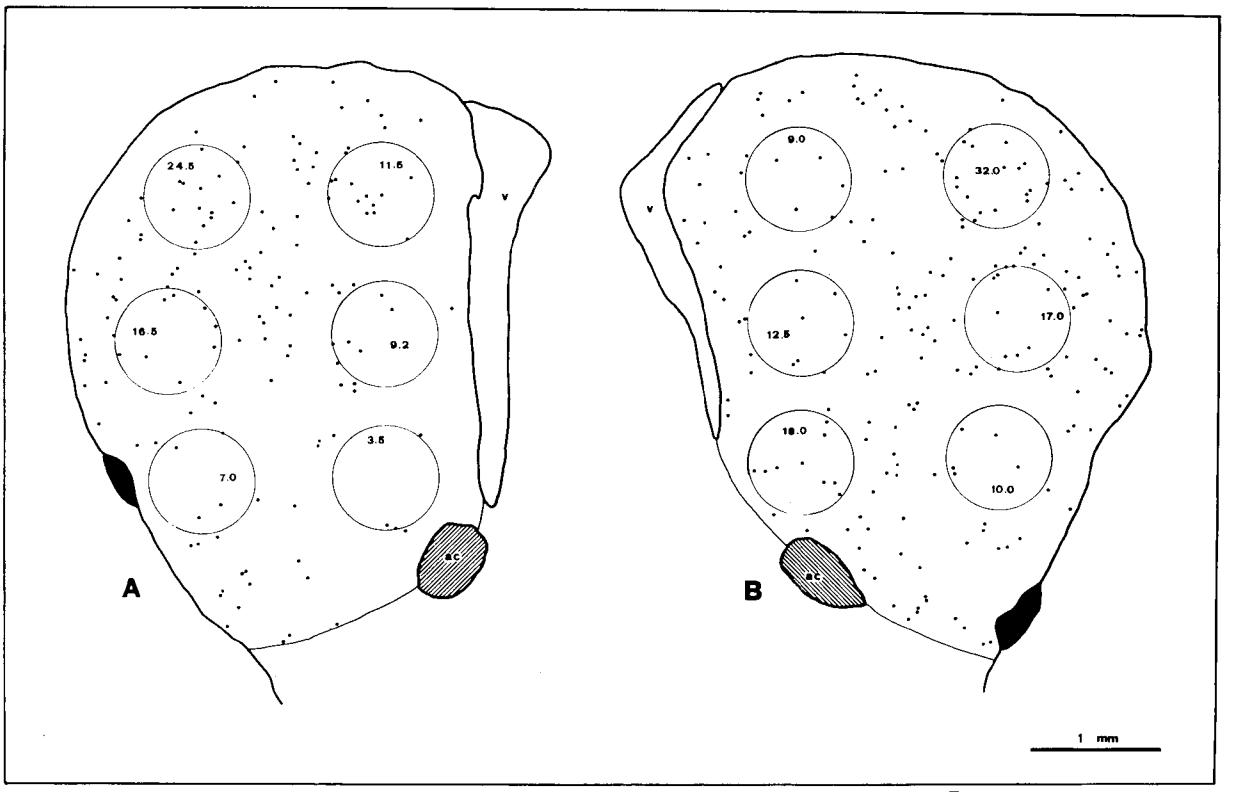

Fig. 1. Camera lucida drawings of coronal sections through the neostriatum in aged rat D500 (A) and in control rat D502 (B). The two sections are about at the same level. Circles indicate 0.5 mm^2 sample areas, where regional cell density (which is shown by figures) has been measured. ac, anterior commissure; v, lateral ventricle.

standard plane[15]. 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[6]. Every other section through the neostriatum, was charted on a drawing microscope, and then analysed by means of a custom-made microcomputer programme for semiautomatic image analysis. The brains of one adult and one aged rat, belonging to a single staining session, were charted blindly by the same observer. Ventral boundaries of the neostriatum were identified by comparing alternate AChE- and Nissl-stained sections (see examples in Fig. 1). In every third chart, sample areas (about 0.5 mm^2) located in the dorsolateral, dorsomedial, lateral, medial, ventrolateral, and ventromedial part of the nucleus were analysed. Total cell number and density, total neostriatal area, and cell density in sample areas were computed in the adult and aged groups, and compared by means of analysis of variance. Measurements of the size of AChE-containing neurones were performed at random throughout the neostriatum in adult and aged rats. Only neurones containing a well-defined unstained nucleus were individually chosen from any neostriatal segment; they were automatically taken in by a Leitz ASBA image analyser. Cross-sectional areas of these neurones were computed in the adult and aged groups.

In adult rats, large, intensely stained, russet, AChE-containing neurones were seen in the caudatum-putamen, medial septum, nuclei of the diagonal band, magnocellular preoptic nucleus, and basal nucleus of Meynert. In the same regions they were intermingled with moderately stained perikarya. In addition, lightly stained cell bodies were observed in a wide number of areas (see atlas by Butcher and Woolf[7]). In this study only intensely AChE-stained neurones were charted in the neostriatum. Cell bodies were distributed in the neostriatal matrix with

TABLE I

Summary of mean values for morphometry of AChE-containing neurones in the neostriatum of adult and aged rats

	Adults (mean ± S.E.M.)	*Aged (mean ± S.E.M.)*	*P*	*Variation in the aged*
Cells*	367.70±63.90	266.35±54.01	<0.001	−27.56%
Area (mm^2)	22.44±2.42	19.19±2.62	<0.001	−14.48%
Density**	16.43±2.78	13.87±2.08	<0.01	−15.58%

* Number of cells per each section (68 sections in adults, 62 in the aged); ** Number of cells per mm^2.

a typical patchy arrangement, matching a hodological and biochemical mosaic, which is a feature of this territory[11]. In rostral sections, AChE-containing cell bodies were densely aggregated in the dorsal part of the striatal complex, while in the caudal two-thirds of the nucleus cell aggregates were predominantly distributed in the dorsolateral sectors.

Observation of old rats indicated that the overall distribution of AChE-containing striatal neurones is similar to that of adult subjects, while their number appears to be reduced. This was confirmed by morphometric analysis, which has revealed, in fact, clear-cut age-dependent differences in the observed animals (Table I). Firstly, the mean area of neostriatum was decreased in aged rats by approximately 14.48%; secondly, the number of AChE-containing neurons was also reduced, in the same region, by 27.56%. As a consequence, the age-dependent decrease in the density of AChE-stained cells averaged 15.58%. A comparison of morphometric data obtained in the right and left hemispheres was performed for all sections, in each rat. No right–left asymmetries were observed, and, therefore, measurements of the right and left side were considered as belonging to a homogeneous population. The analysis of rostrocaudal series of coronal sections did not reveal qualitative differences in the distribution of AChE-containing cell bodies. Analysis of measures obtained from sample areas showed that, either in the aged or in controls, AChE-containing cell bodies were predominantly distributed in the dorsomedial, dorsolateral, and lateral parts of the striatal complex (Table II). Cell depletion in the aged was clearly quite pronounced in the dorsolateral and lateral regions (15.16% and 29.59%, respectively), and it reached the highest proportion in samples located in the ventromedial part (38.33%).

TABLE II

Mean density of AChE-containing neurones in sample areas located in different segments of the striatal complex

Figures indicate cells/mm^2. Number of samples for each variable: 22 in adults, 20 in the aged.

Sample location	*Adults (mean ± S.E.M.)*	*Aged (mean ± S.E.M.)*	*P*	*Variation in the aged*
Dorsolateral	17.15±1.11	14.55±1.82	<0.5	−15.16%
Dorsomedial	14.20±2.03	14.39±2.08	ns	
Lateral	18.01±1.45	12.68±1.20	<0.01	−29.59%
Medial	9.52±1.49	10.70±2.22	ns	
Ventrolateral	12.07±1.01	11.08±0.89	<0.05	− 8.20%
Ventromedial	11.14±1.52	6.87±1.19	<0.05	−38.33%

Observation also showed age-dependent differences in the appearance of individual AChE-containing neurones located in the neostriatum. In fact, in aged animals, both perikarya and nuclei showed an irregular contour, the nuclei being often displaced to the periphery of the cytoplasm. Quantitative image analysis confirmed a clear-cut 39.68% reduction of the area of perikaryon, as observed in cross-sections of AChE-containing cell bodies (Table III).

The present study shows that the number of AChE-containing perikarya is decreased in the neostriatum of aged rats. This is in keeping with earlier biochemical observations indicating age-dependent reduction of markers for cholinergic activity[3,12,16], and reflects the actual disappearance of cholinergic neurones during ageing. In fact, direct comparison of AChE and ChAT staining patterns in one and the same region has shown that intensely stained AChE-containing neurones are invariably cholinergic[19]. In the neostriatum there is a direct 1/1 relationship between intensely AChE-stained and ChAT figures[7,19]; therefore, the number of intensely stained AChE neurones gives a good estimate of the actual number of cholinergic perikarya in this region.

The use of Butcher's procedure for AChE has allowed us to obtain data comparable to earlier information from this laboratory. It has been recently reported[1,2,10] that C57BL/6 mice undergo a consistent loss (20–32%) of forebrain cholinergic perikarya in a period ranging between 21 and 60 days of postnatal age. Since the development of forebrain cholinergic perikarya in this inbred strain is normal, we have proposed that C57BL/6 mice belong to a genetic mutant

TABLE III

Mean cross-sectional area of AChE-containing neurones in the neostriatum of adult and aged rats

Number of samples is 318 for adults, 320 for the aged.

	Adults (mean ± S.E.M.)	*Aged (mean ± S.E.M.)*	*P*	*Variation in the aged*
Area (μm^2)	464.93±5.13	280.43±5.12	<0.0001	−39.68%

characterised by early senescence of the cholinergic cell systems[10]. The present data corroborate such hypotheses, as they show that ageing is indeed associated with loss of cholinergic cell bodies in the forebrain. With just one exception[8], all studies performed on the human basal nucleus are in keeping with our data on rats. In fact, a morphometric analysis based on classic techniques[13] has shown 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. Finally, it has been observed[14] that the number of ChAT-containing neurones located in the basal nucleus undergoes a progressive age-dependent decline.

As shown by the present data, cell depletion affecting aged rats is widespread, but not haphazard; it chiefly affects striatal territories which are richer in cholinergic perikarya. However, it is of particular interest to note that cell loss is massive in the ventromedial region, which in adults is not very rich in AChE-containing perikarya. On anatomicofunctional grounds, the striatal complex can be subdivided into a dorsolateral *extrapyramidal* part and a ventromedial *limbic* component. As reviewed by Björklund and Lindvall[4], the dorsolateral division mainly receives afferent projections from the pars compacta of substantia nigra and from the neocortex, while the lateral is mainly impinged upon by the ventral tegmental area, the prefrontal cortex and the amygdala. Cholinergic striatal interneurones are thought to receive direct monosynaptic input from midbrain dopaminergic neurones (e.g. see ref. 9); therefore, it is worthy of interest that cell bodies preferentially connected with the ventral tegmental area are peculiarly sensitive to age-dependent decline. This raises the question as to whether loss of cholinergic perikarya is primary or secondary to degeneration of other neurochemical classes of neurones. Indeed, it has been shown[18] that dopaminergic binding sites in the human basal ganglia decrease with age.

Morphometric analysis of individual AChE-containing cell bodies has allowed us to show that age-dependent loss of cholinergic striatal perikarya is associated with degenerative features. This indicates that age-dependent decline is a process specifically affecting the cholinergic neostriatal population, which suffers of trophic derangement before actually disappearing. Therefore, the present data raise some quite important questions. First, is age-dependent decrease of cholinergic neurones a diffuse process affecting all cholinergic regions in the forebrain? Second, is such decrease dependent on some metabolic or trophic substrate specific to cholinergic neurones? Answers will be provided by subsequent studies. We are currently analysing the remainder of forebrain cholinergic territories; our preliminary data indicate that age-dependent loss of cholinergic cell bodies is a diffuse process. If this is confirmed, the search for a metabolic cause will become a primary task, together with the evaluation of replacement therapies.

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