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Chronic Excess of Corticosterone Increases Serotonergic Fibre Degeneration in Aged Rats

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Key words: serotonergic and cholinergic fibres, degeneration, ageing, corticosterone excess.

Abstract

Evidence is presented for the potentiating role of corticosterone on axonal degeneration of serotonergic neurones during ageing. Aged rats, 24 months old, were implanted subcutaneously with 2×100 mg pellets of corticosterone. Serotonergic and cholinergic (ChAT- and NADPHd-positive) fibre degenerations in the anteroventral thalamic nucleus (AVT) were measured 2 months after corticosterone implantation. Numbers of immunoreactive serotonergic raphe and mesolimbic cholinergic neurones were also quantified. Basal plasma corticosterone and adrenocorticotropin (ACTH) concentrations were assayed at 2, 4, 6, and 8 weeks after implantation in the plasma and at 1, 2, 4 and 6 weeks in urine. The degree of serotonergic fibre aberrations in the AVT increased significantly after corticosterone exposure, while that of ChAT-positive and NADPHd-stained axon aberrations showed a modest but nonsignificant increase. A positive correlation between the magnitudes of serotonergic and cholinergic fibre aberrations appeared in the AVT, but only in the corticosterone-treated rats. The number of serotonin immunopositive neurones in the raphe nuclei after corticosterone decreased marginally, while that of mesopontine ChAT-positive neurones was not influenced. Measurements of basal plasma corticosterone and ACTH, as well as urine corticosterone, revealed that the steroid implantation increased the plasma corticosterone level for at least 4 weeks and decreased ACTH level for at least 6 weeks. By the week 8, the pituitary–adrenal function was apparently restored. However, at sacrifice, both the weight of adrenal glands and that of thymus remained reduced, indicating the long-lasting effects of corticosterone on target tissues. It is concluded that the raphe serotonergic neurones and their projecting fibres are sensitive to corticosterone excess in aged rats and become more vulnerable to degeneration processes than under normal ageing conditions. Cholinergic neurones of brainstem origin, which also express massive NADPHd activity, are more resistant against corticosterone, but their axon degeneration correlates to serotonergic fibre degeneration.

Ageing is associated with a number of hormonal changes after the cessation of the reproductive age. Gonadal hormones decline, as well as many other hormones, including growth hormone, thyroid hormones, vasopressin and melatonin (1, 2). The most prominent exception is the glucocorticoid hormones because their level progressively increases in the circulation during the course of the ageing process in a number of specified conditions (3–5). This hypercorticism has been attributed to a deficient receptor efficacy of the central glucocorticoid receptors, which results in an attenuated negative-feedback regulation (6). Another prominent causal factor is the decreased hippocampal inhibition towards the hypothalamic–pituitary–adrenocortical (HPA) axis (3). Chronic stress or the excess of glucocorticoids is associated with an accelerated neuronal dysfunction in the ageing brain (4, 7). In this respect, most of the previous studies focused on the vulnerability of hippocampal neurones as brain glucocorticoid targets (4–7). From these studies, it was concluded that long-term excess of glucocorticoids during old age is considered as a risk factor for accelerated normal neuronal ageing and for neuronal survival in both human and rodents.

Ageing is associated with the decline of viability of neurones, which may well be selective to one or to another cell types, especially under pathological conditions. Serotonergic (8–10) as well as cholinergic (11, 12) neurones show degenerative features during ageing, especially in response to noxious stimuli, which manifests itself in fibre varicosity swelling and other aberrations such as fibre thickening and clustering. Serotonergic fibres might degenerate at younger ages too, but only under pathological

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conditions similar to those in response to toxic injury caused by chronic fenfluramine treatment (13, 14) or hypoxia/anoxia (15).

We have reported earlier that prenatal chronic hypoxia, while causing a hyperfunctioning HPA system during ageing, enhances serotonergic fibre degeneration in the hippocampus of aged rats (16). However, in this study, it was not clear whether the serotonergic fibre degeneration was enhanced by the chronic HPA hyperactivity or by a brain maldevelopment process caused by the hypoxia in the fetal age, or by both. In the present study, we studied the direct action of corticosterone excess on serotonergic neurodegeneration in aged rats. For comparison, we selected the cholinergic neurones as another neurotransmitter system, and followed the fibre degeneration of the two transmitter systems in parallel. One of the reasons for the parallel investigation of these neurotransmitter systems is that the functional decline of forebrain and mesopontine cholinergic neurones during ageing was reported to be closely related to that of serotonergic neurones (17, 18). For example, serotonergic neurones through 5-HT_{1A} receptors exert a neuroprotection on forebrain cholinergic neurones against overexcitation (19). Further support for the trophic interaction is that serotonergic lesion of median raphe nucleus increases the vulnerability of septohippocampal cholinergic neurones to the cholinotoxin ethylcholine aziridinium (20).

The degeneration of serotonergic fibres through the brain of ageing rats is widespread (8). Recently, we observed that cholinergic fibres coming from the mesopontine nuclei (Ch5 and Ch6) (21), can profoundly become degenerated during ageing (unpublished observation). The most intensively innervated target area by these cholinergic cells is the anterior thalamus, and especially the anteroventral thalamic nucleus (AVT) (22). This area of thalamus also receives a massive serotonergic innervation, making the AVT nucleus a useful brain region for the simultaneous study of serotonergic as well as cholinergic fibre degeneration within the same brain area. Therefore, we investigated the action of chronic excess amount of corticosterone on the degenerative process of both fibre types in the AVT. To assess the impact of corticosterone on cell bodies of projection neurones, the numbers of immunoreactive cholinergic and serotonergic cells in the mesopontine brainstem areas were also measured. By this method, we aimed to answer the question of whether there is any relationship between the identified number of brainstem neurones and the magnitude of fibre degenerations in the selected AVT target area. Comparing the steroid effects on the two neurotransmitter systems, the serotonergic neurones proved to be sensitive to the excess of corticosterone and showed signs of an enhanced degeneration, while degeneration of cholinergic fibres did not reach significance.

Materials and methods

Animals and treatment

Male Wistar rats, aged 24-months, were implanted subcutaneously either with two pellets of corticosterone (100 mg each, RBI, Natick, MA, USA, n = 16) or with two 100 mg pellets of cholesterol (controls, n = 18) in the interscapular region at the back under pentobarbital anaesthesia. In our previous study, we found that 2×150 mg corticosterone pellets were too high a dose to support neurodegeneration (23). To prepare the steroid and the cholesterol pellets, the protocol of Meyer *et al.* (24) was followed. Briefly, 100 mg corticosterone or cholesterol was melted in a small stainless steel spoon over a low gas flame and then poured into a drilled hole of 6 mm diameter in a candle wax block. The solidified pellet was removed from the wax and weight. The pellets were paired in such a way that their total weight was approximately 200 mg. The rats were originally obtained from Charles River (Budapest, Hungary) and subsequently bred in our laboratory. The principles of laboratory animal care met the standards set by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were in agreement with the decision of Ethical Committee of Semmelweis University, Budapest. The animals were kept 2–3 per cage under a standard 12 : 12 h light/dark cycle (lights on at 7.00 h and off at 19.00 h), and food and water were available *ad libitum*. Both the corticosterone- and cholesterol-implanted rats were divided into two groups: one group was used for hormonal measurements in the plasma and urine (seven corticosterone and nine cholesterol animals). The second group, which was left undisturbed for 2 months, was subjected to the histological examinations (nine rats in each group).

Brain tissue processing

The rats were transcardially perfused under deep pentobarbital anaesthesia with a fixative solution of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 after a short prerinse with heparinized saline at 26 months (i.e. 2 months after implantation). The brains were removed, cryoprotected by 30% sucrose for 3 nights (72 ± 2 h) and sectioned on a cryostat microtome at a thickness of 20 µm. At the level of AVT, starting the sectioning in front of the full appearance of hippocampal commissure at Bregma −1.30 mm (25), all sections were preserved and collected into three consecutive separate series (i.e. for serotonin, ChAT and NADPHd staining). Each cup (10 ml multipurpose container) processed for immunocytochemical and histochemical stainings contained a maximum of 10 free floating sections. Brainstem sectioning started at the anteroposterior level of 13.81 according to the atlas of Swanson (26), and every other section was kept for either serotonin or ChAT immunostainings. In this case, one cup contained a maximum of 15 sections.

Immunocytochemical stainings

Immunostaining procedures were applied to visualize 5-hydroxytryptamine (serotonin, 5-HT) positive serotonergic and choline acetyltransferase (ChAT) positive cholinergic neurones in the brainstem and their axon ramifications in the target brain areas. Serotonin (27) and ChAT immunostaining (12) was described by us previosuly. Briefly, for primary antibodies polyclonal rabbit anti-5-HT (Zymed, San Francisco, CA, USA) and polyclonal goat anti ChAT (28, kindly donated by Dr L. B. Hersh) antibodies were used in a dilution rate of 1 : 200 and 1 : 2000, respectively. The stainings were completed after peroxidase-antiperoxidase (PAP) complex formation with diaminobenzidine reaction in the presence of H_2O_2. Staining intensity probably was strong enough to visualize all fibre aberrations in the innervated target areas because the aberrations accumulated large amounts of 5-HT or ChAT and they were stained very intensively (i.e. much more intensively than the normal fibres). Aberrant fibres were defined as single or clustered and, in most cases, as thickened fibres with swollen varicosities.

NADPHd histochemical staining

Because the mesopontine cholinergic cells are nitric oxide synthase (NOS) positive neurones (29), NADPH diaphorase positivity was used as an additional marker to visualize the putative cholinergic fibre aberrations. The β-nicotinamide adenine dinucleotide phosphate (reduced form, NADPH, Sigma, St Louis, MO, USA) diaphorase (NADPHd) activity was visualized in brain sections incubated in the presence of 0.01% nitroblue tetrazolium and 0.1% β-NADPH for 2 h at room temperature as described previously (30).

Quantification of fibre aberrations

Swollen fibre varicosities and enlarged axon fragments were selected for quantification of fibre aberrations by means of a computer-assisted image analysis (Quantimet 600H, Leica, Rijkswijk, the Netherlands). Using 20 × primary objective magnification and no. 600 emission filter, and following shading correction and background subtraction, an optimal threshold level was selected, which was kept constant at every separate area measurement throughout the entire quantification procedure. Following unbiased manual delineation of areas within the AVT infiltrated by clusters of swollen fibre varicosities or by enlarged axon fragments (called infiltrated area), the quantitative determination of the surface area of structural malformations (i.e. fibre aberrations) was performed. With the computer program, the net area of the aberrations were highlighted and computed in calibrated μm^2 while the normal thinner fibres remained unselected. Using a double-blind experimental approach, three sections at selected coordinates were employed for measurements at both left and right sides. The sections were serial sections comparable to the levels of Bregma −1.30 and −1.40 (25) and were

collected as described above. Therefore, three sections were counted per animal and the results were expressed as averaged area of aberrations and averaged infiltrated area within the AVT per single brain section. The 'area of aberration' values were corrected for background measurements, which were obtained in 'normal' AVT areas of each animal in which practically no fibre aberrations were present, but apparently only normal fibres. This type of background measurement became possible because of the inhomogeneous, patchy-like infiltration of AVT by aberrant fibres.

Quantification of mesopontine cell bodies

The number of ChAT-stained cholinergic cell bodies was counted in the brainstem at three selected levels of the mesopontine nuclei. At each level, three brain sections with a standard cross sectional distance of 40 μm were processed for measurements and the results were averaged at each level. The three selected levels were: (i) pedunculopontine nucleus (PPN) anterior to the appearance of laterodorsal tegmental nucleus (LDT), at the anteroposterior level of 14.17 according to the atlas of Swanson, 1992 (26); (ii) PPN-parabrachial nucleus (PBN) and LDT at anterior level of 14.69; and (iii) PPN–PBN and LDT at an intermediate anteroposterior level of 14.93. The 5-HT positive cell bodies were counted in the middle anteroposterior level of the dorsal and median raphe nuclei around the anteroposterior coordinate of 14.37 in triplicate. The averaged cell number expressed per single brain section was processed for statistical analysis.

Hormonal and organ weight measurements

The action of corticosterone excess on the function of pituitary-adrenocortical system was followed by measuring plasma adrenocorticotropin (ACTH) and corticosterone concentrations. Basal plasma levels of these hormones were measured at several time points after the steroid implantation (i.e. at 2, 4, 6 and 8 weeks). Blood samples were taken from the tail vein within 1.5 min after touching and fully awaking the rat in the home cage at 09.00–10.00 h. Plasma ACTH and total corticosterone concentrations were determined by radioimmunoassay methods in triplicate using rabbit antisera as described previously (31). Free corticosterone concentration in the urine was also determined. After two 4-h long habituation periods spent in the individual metabolic cages suitable to collect urine selectively, 24-h urine samples were collected at 1, 2, 4 and 6 weeks after implantation from six animals per group. At the conclusion of the experiment, the weights of different organs related to the function of pituitary–adrenocortical system were measured and compared to the body weight.

Statistical analysis

Statistical evaluation of paired morphometric data was carried out with Student's t-test. The analysis of variance (ANOVA) for repeated measures was used for additional evaluation of changes in cell number. Assessing correlations between two variables, the Spearman nonparametric test was applied. $P < 0.05$ was considered statistically significant. Results are expressed as means ± SEMs.

Results

HPA hormone levels and organ weights

Implantation of 2×100 mg corticosterone pellets resulted in a long-term elevation of circulating corticosterone. The steroid-implanted rats displayed higher basal plasma corticosterone levels at least up to 4 weeks after implantation (Fig. 1). Morning plasma ACTH content was markedly suppressed, which lasted at least up to 6 weeks (lower panel of Fig. 1). The urinary excretion of corticosterone was also increased (Table 1). At the end of the first week, the corticosterone concentration in the urine was 5.5-fold higher than in controls. By the second week, it decreased significantly and a further decrement could be detected by the week 6. Similar to basal corticosterone measurement in the urine, the corticosterone concentration was elevated at least up to week 4. The dynamic profile in the change of urinary corticosterone concentrations showed that the most marked corticosterone exposure occurred at the beginning of the 8-week survival period.

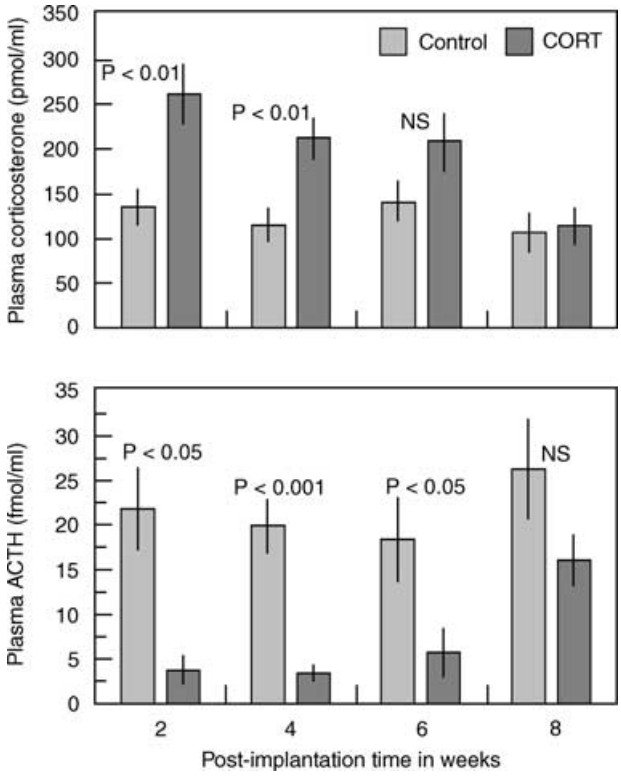

FIG. 1. Resting plasma corticosterone and adrenocorticotropin (ACTH) concentrations at different periods after corticosterone (CORT) and cholesterol (Control) implantation into 24-month-old rats. Means ± SEMs are shows for seven CORT and nine Control rats. Significant differences between groups are represented by P-value; NS, not significant.

TABLE 1. Effect of Corticosterone Implants (2×100 mg) on Corticosterone Concentration in the Urine (pmol/ml)

Weeks after implantation	Corticosterone	(%)	Cholesterol	t	P
1	1732 ± 230	550	315 ± 46	9.05	0.001
2	904 ± 194*	340	267 ± 27	4.20	0.001
4	626 ± 45	300	208 ± 40	2.76	0.01
6	533 ± 131†	205	259 ± 47	1.83	0.1

Means ± SEMs are shown, 't' post-hoc t-test value. Samples are from 24-h urine collections. Both groups contained six rats, *$P < 0.001$ versus first week corticosterone; †$P < 0.05$ versus second week corticosterone.

TABLE 2. Effect of Corticosterone Excess on the Weights of Organs Related to the Function of Pituitary-Adrenal Axis

	Corticosterone		Cholesterol	
	(n = 9)	%	(n = 9)	P
Body weight (g)	656 ± 21	102	641 ± 34	NS
Brain weight (mg)	2061 ± 28	96	2156 ± 49	NS
Brain w./100 g b.w. (mg)	321 ± 18	92	349 ± 24	NS
Pituitary (mg)	24.4 ± 1.39	111	22.0 ± 1.88	NS
Adrenal gland (mg)	69.9 ± 7.2	73	95.6 ± 7.8	0.05
Adrenal/100 g b.w. (mg)	10.7 ± 1.4	72	14.9 ± 1.0	0.05
Thymus (mg)	156 ± 21	65	239 ± 33	0.05
Thymus/100 g b.w. (mg)	23.8 ± 3.3	64	37.3 ± 5.3	0.05

Means ± SEMs are shown.

Restoration of the pituitary ACTH production under baseline condition had been reached by week 8 after implantation (i.e. by the time of sacrifice and brain fixation) (Fig. 1). The weights of brains and those organs which are under influence of the function of the pituitary–adrenocortical system are summarized in Table 2. Brain weight and size of the pituitary gland were not different between the two groups. Adrenal and thymus weights decreased as a result of corticosterone excess.

Fibre aberrations at the thalamic projection area (AVT)

Figures 2 and 3 show examples for serotonergic and cholinergic axon degeneration in the two parts of the AVT: dorsomedial (AVDM) and ventrolateral (AVVL) subdivisions. The lateral part of the nucleus (AVVL) contained much more abundant cholinergic fibre aberrations as compared to the medial part (AVDM, Fig. 3A,C). The ratio of the amount of ChAT-positive

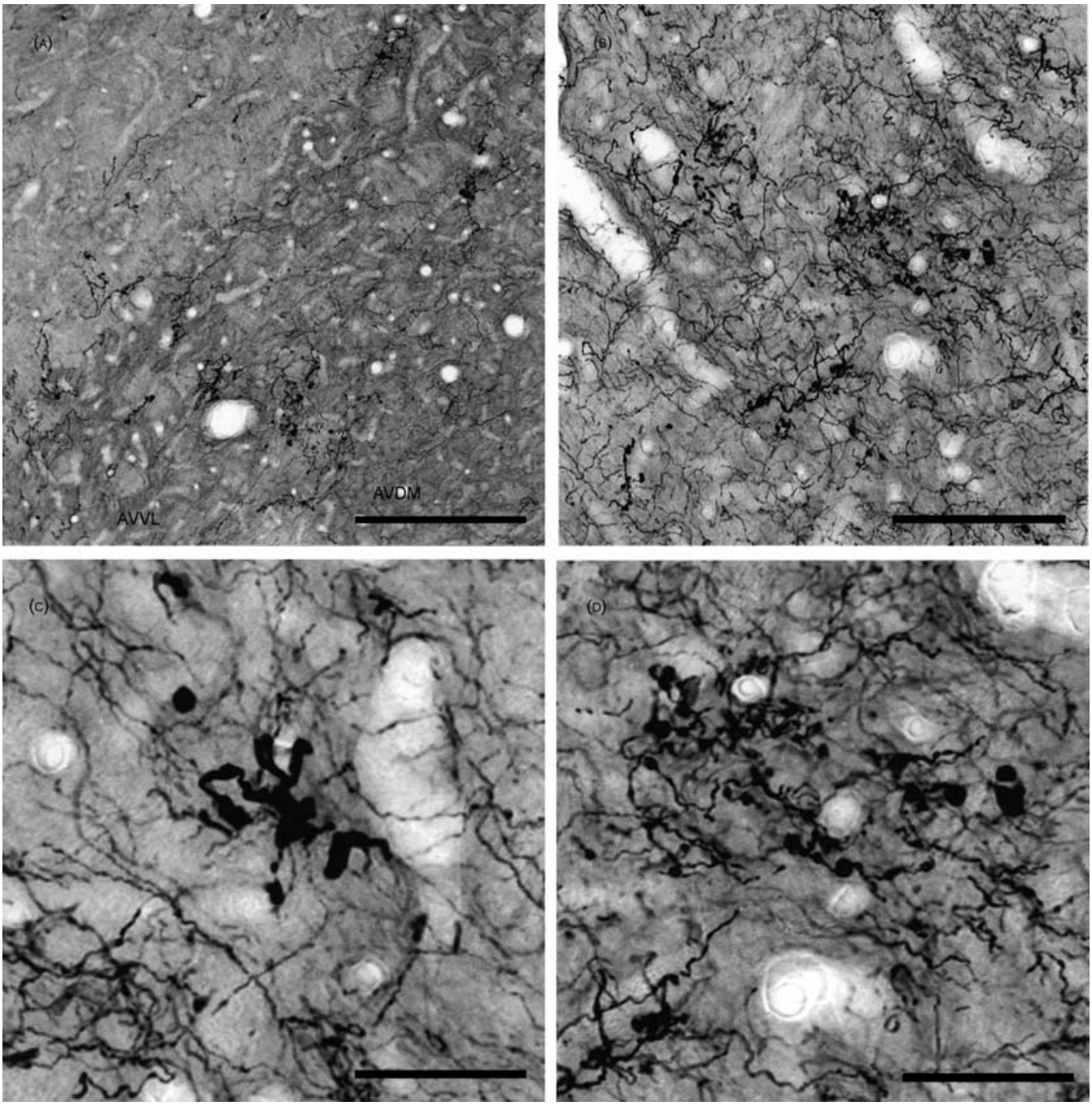

FIG. 2. Photomicrographs of serotonin (5-HT)-positive degenerative structures in the anteroventral thalamic nucleus (AVT) of rat aged 26 months. Fragments of the two subdivisions of the AVT nucleus are shown in (A) at lower magnification (scale bar = 400 μm): AVVL, Ventrolateral part of AVT, AVDM, dorsomedial part of AVT. Clusters of enlarged swellings and thickened fibres are distributed evenly within the subdivisions of the nucleus. (B) Showing with higher magnification (bar = 200 μm) the grape-like clusters of swellings; a distinction may be made between the sharply stained and thickened degenerative fibres and the more faintly stained thinner normal axonal arborizations and varicosities. Part of (B) is enlarged in (D) where the scale bar is 85 μm. A cluster of enlarged swellings accompanying markedly thickened axonal fragments is shown in (C) (scale bar = 60 μm).

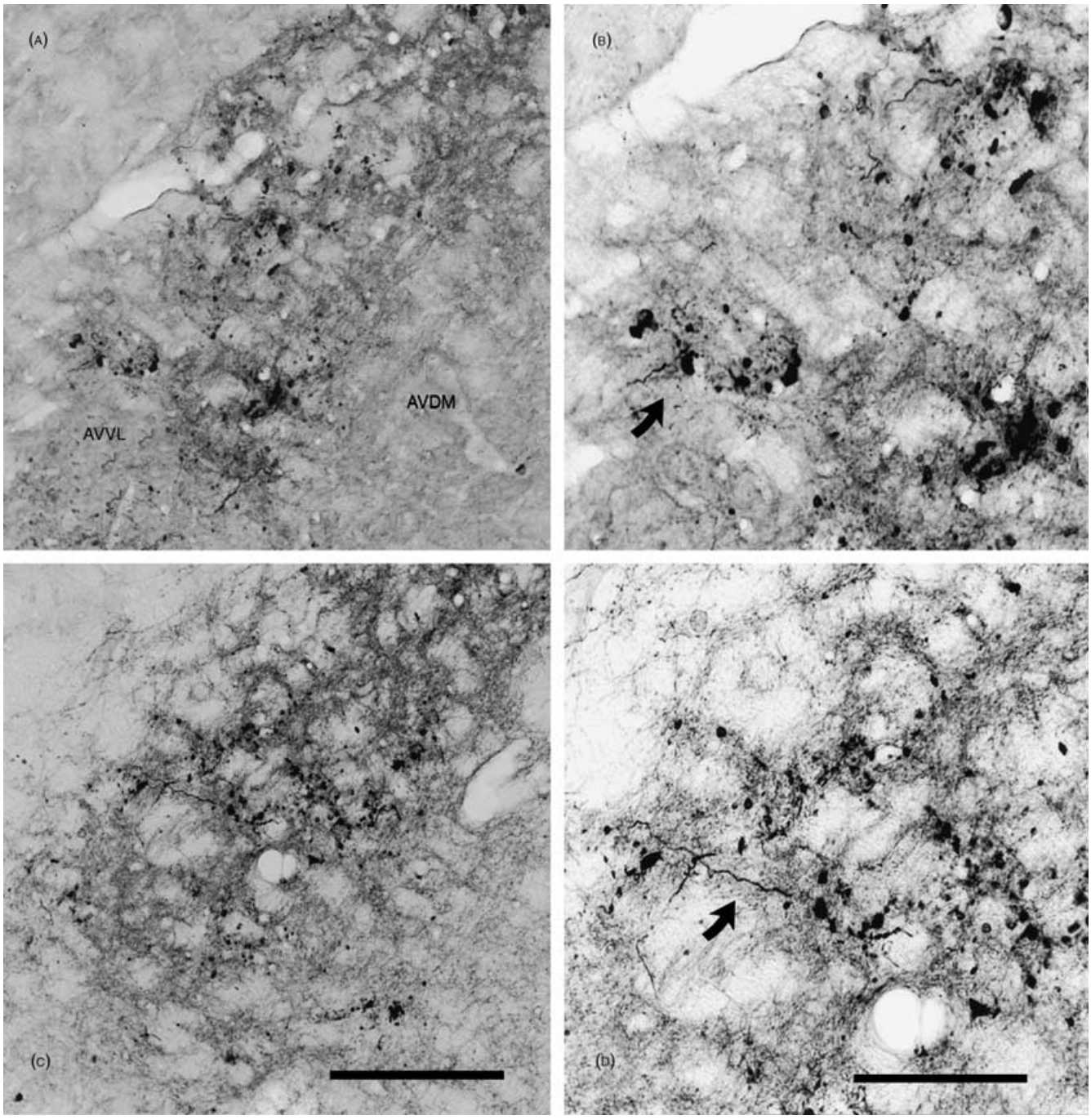

FIG. 3. Photomicrographs of choline acetyltransferase (ChAT)-positive structures and fibre aberrations in the anteroventral thalamic nucleus (AVT) of rats aged 26 months. (A) and (B), and (C) and (D), are from different animals. In (A) and (C), the two nuclear subdivisions are clearly distinguishable (AVVL, ventrolateral part of AVT, AVDM, dorsomedial part of AVT, scale bar = 400 μm). Much more fibre aberrations are present in the AVVL. In the AVDM subdivision of the nucleus, much less fibre aberrations can be observed (A,C). Lot of swellings, but relatively few thickened fibres, are visible [see arrows at the higher magnification in (B) and (D) indicating thick fibres]. In (B) and (D), the AVVL areas of (A) and (C) are enlarged, respectively, for a better observation of the microstructure (scale bar = 200 μm).

degeneration profiles between these two subdivisions appears to reflect the intensity of original cholinergic innervation of the two parts of the nucleus (22). In the case of serotonergic aberrations, there was no clear distinction between the two nuclear subdivisions (Fig. 2A). This corresponds to a relatively even distribution of invading serotonergic fibres within the nucleus. Axon aberrations consisted of large swellings and thickened fibres, which generally clustered together. Several examples of thickened fibres are indicated by arrows in Fig. 3(B,D). Normal fibres exposed a much finer and delicate appearance and could be easily distinguished under microscope and also by the densitometric computer program, which selected with high certainty and reproducibility only the enlarged and darkened aberrations (Fig. 2B–D).

Corticosterone excess clearly and significantly increased 5-HT fibre degeneration in the AVT compared to control rats ($t = 3.12$,

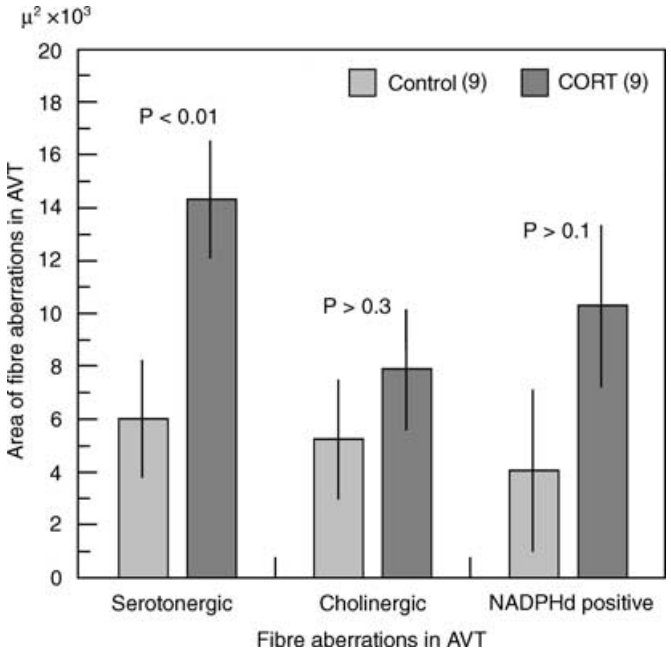

FIG. 4. Surface areas of fibre aberrations in the anteroventral thalamic nucleus (AVT) stained serotonin (5-HT)-positive (serotonergic), choline acetyltransferase (ChAT)-positive and NADPHd-positive in 26-month-old rats implanted with corticosterone pellets (CORT) or cholesterol pellets (Control) 2 months before sacrifice. The area of serotonergic fibre aberrations increased significantly after corticosterone treatment ($P < 0.01$), while there were no significant increments in the measures of cholinergic and NADPHd-positive degenerative structures.

d.f. = 16, $P < 0.01$) (Fig. 4). Statistical analysis demonstrated that ChAT-positive and NADPHd-labelled fibre degenerations were not significantly elevated by corticosterone. In control animals, a certain level of axon aberrations was also present (Fig. 4). The degree of axonal degeneration showed wide individual differences. This was especially marked in cases of cholinergic fibre degeneration using NADPHd as a putative marker.

The results obtained by comparing the extent of aberrant fibres-infiltrated areas of the AVT are shown in Table 3. AVT areas innervated by aberrant 5-HT fibres were larger in the corticosterone-treated rats. Although aberrant cholinergic fibres, both ChAT- and NADPHd-positive, invaded larger areas in the steroid-treated rats, the increments were not statistically significant.

Raphe serotonergic and mesopontine cholinergic neurones

Next to quantifying the degree of fibre aberrations in the target area, the number of projecting neurones were also counted in the corticosterone-treated and control rats. Serotonergic neurones in the dorsal and median raphe nuclei were counted at the intermediate level of the anteroposterior dimension of the nucleus. The selected pontine level is indicated in Fig. 5(A). In Fig. 5(B), NADPHd positive neurones are visible at the same anteroposterior level. Relatively few neurones stained positively for NADPHd in the raphe areas. The staining intensity was light, suggesting that these positive neurones probably will not transport the enzyme molecules into their axons to such a level or concentration that NADPHd fibre staining will represent detectable fibres in the projection areas. It is also unclear whether is there any colocalization between the two markers in the raphe nuclei. Even if there is some overlap, the staining patterns are clearly very different: there are large numbers of densely stained serotonergic cells, and relatively few faintly stained NADPHd positive neuronal cell bodies are visible in the raphe nuclei.

TABLE 3. Effect of Corticosterone Excess on the Extent of Infiltrated Area by Aberrant Fibres Within the Anteroventral Thalamic Nucleus

Aberrant fibres	Corticosterone (n = 9)	Cholesterol (n = 9)	t	P
Serotonergic	210 ± 21	135 ± 21	2.53	0.021
Cholinergic				
ChAT-immunoreactive	302 ± 73	192 ± 68	1.10	0.287
NADPHd-positive	240 ± 49	151 ± 51	1.25	0.229

Means ± SEMs are shown. Values are mm^2 per 10^3.

Corticosterone excess decreased the number of 5-HT-immunoreactive neurones in the raphe nuclei (Table 4). Measurements at the dorsal nucleus did not result in significant difference but, at the median raphe nucleus, the effect of corticosterone reached significance. Analysing the total number of counted cells per section resulted in a significant suppression ($P < 0.05$). The decrease in the cell number in response to corticosterone exposure reached approximately 13% (Table 4). Statistical analysis of dorsal and median raphe nuclei with repeated measures ANOVA also resulted in a significant difference [$F(1,15) = 6.12$, $P < 0.05$], confirming that corticosterone excess indeed lowered the number of 5-HT-immunoreactive neurones.

By contrast to 5-HT cells, the number of ChAT-immunoreactive cholinergic cell bodies was not influenced by the corticosterone excess (Table 5). Cell numbers in the PPN–parabrachial nucleus (PBL) regions did not change while, in the LDT nuclei, they showed a 10% decrement. However, even this 10% decrement proved to be nonsignificant ($P > 0.05$). The mesopontine cholinergic nuclei were studied at three selected levels. All three anteroposterior levels contained cholinergic neurones at PPN-PBL nuclei, while two out of the three also contained LDT cholinergic neurones. The most caudal level (third level) selected for cell counting is represented in Fig. 6. Figure 6(A) shows the ChAT-positive neurones, while Fig. 6(B) shows the NADPHd-positive ones. The stained neurones are grouped into two areas: PPN–PBL and LDT. The overlap between the two markers is so remarkable that it is not possible to distinguish the two staining by visual inspection. NADPHd staining of these neurones was extremely strong, in sharp contrast to the staining intensity in the raphe cells (Figs 5B). Figure 6 strongly suggests that cholinergic neurones in the brainstem contain NOS (NADPHd-positivity) and the degree of colocalization should be very high, if not complete. It should be noted that the projecting cholinergic pathway, Mlf, contains ChAT positive axon swellings (Fig. 6A, open arrow).

Table 6 summarizes some representative correlations between the variables measured in the corticosterone- and cholesterol-implanted rats separately. The magnitude of 5-HT versus ChAT-positive fibre aberrations correlated to each other in the corticosterone-treated rats. There was also a positive correlation between the two cholinergic markers at the fibre aberration level. The correlations between the immunoreactive neurone numbers and the corresponding other parameters did not reach significance, not even in the corticosterone-treated rats (Table 6). However, the tendencies are meaningful after corticosterone treatment. More than eight individual cases are necessary to find a definite answer to the existence of these correlations.

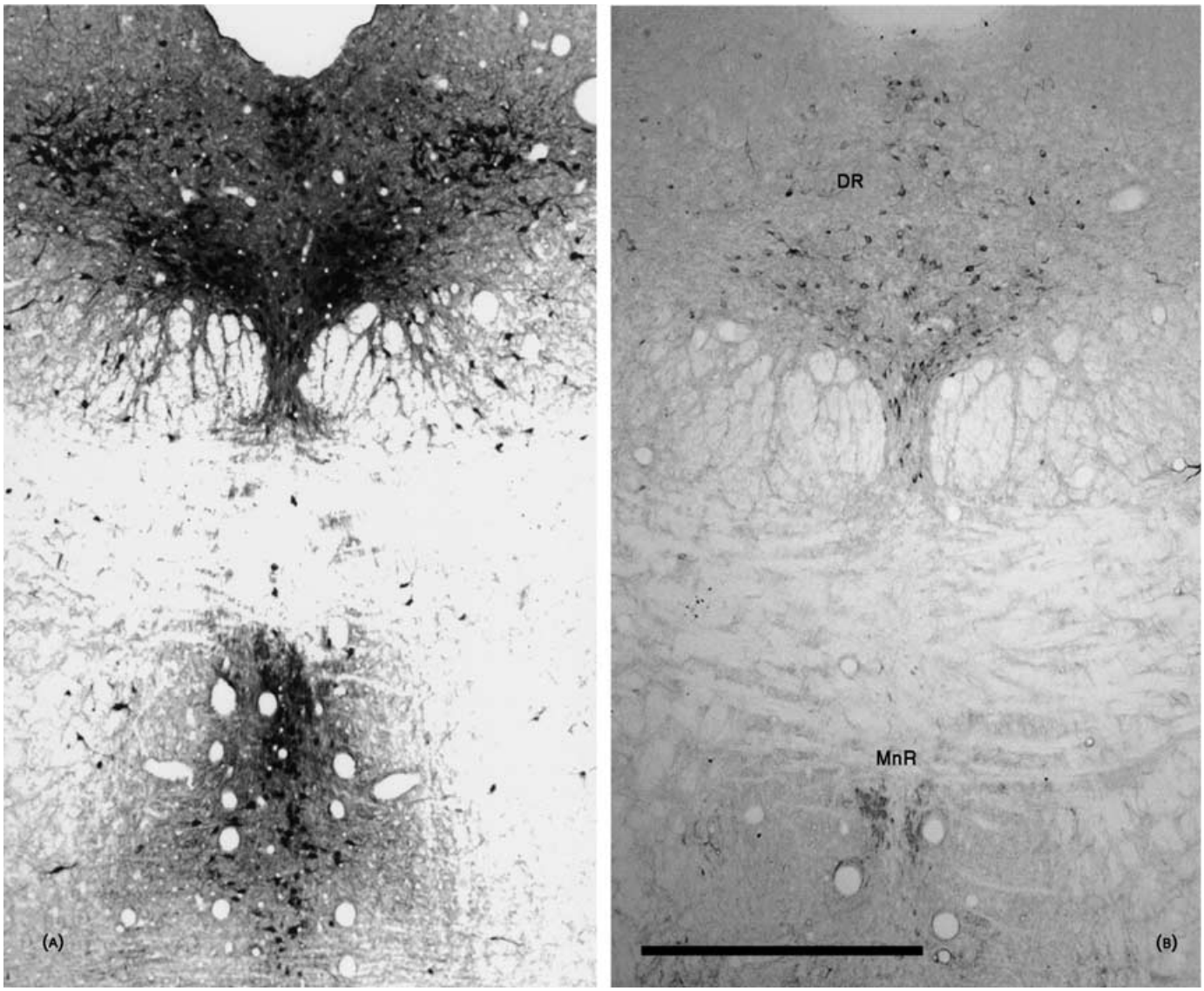

FIG. 5. The left photomicrograph shows serotonin (5-HT)-immunoreactive cell bodies in the raphe nuclei (A) at anteroposterior coordinate 14.37 (26). The upper part corresponds to the butterfly shaped dorsal (DR) and the lowed midline arrangement to the median (MnR) raphe nuclei. (B) Showing slightly stained NADPHd-positive neurones in the raphe areas. Scale bar = 500 μm.

TABLE 4. Effect of Corticosterone Excess on the Number of Immunoreactive Serotonergic Neurones in the Raphe Nuclei of Aged Rats

Treatment	n	Dorsal raphe	(%)	Median raphe	(%)	Total	(%)
Corticosterone	8	161.6 ± 9.0	88	44.1 ± 2.6	86	205.8 ± 9.6	87
Cholesterol	9	182.9 ± 5.3	100	51.4 ± 2.3	100	237.8 ± 5.5	100
Student's t		2.09		2.11		2.47	
P		0.051		0.050		0.025	

Means ± SEMs are shown. Values correspond to number of neurones per a single brain section.

TABLE 5. Effect of Corticosterone Excess on the Number of Brainstem ChAT-Immunoreactive Cholinergic Cells in Aged Rats

Treatment	n	PPN-PBL	(%)	LDT	(%)	Total	(%)
Corticosterone	8	252.3 ± 13.7	97	150.5 ± 7.4	90	402.8 ± 19.3	94
Cholesterol	9	259.9 ± 14.2	100	167.1 ± 6.1	100	427.0 ± 17.6	100
Student's t		0.147		1.75		0.868	
P		0.706 (NS)		0.098 (NS)		0.369 (NS)	

PPN–PBL pedunculopontine nucleus, Lateral part of parabrachial nucleus (the averaged cell numbers per section counted at the three different anteroposterior levels are pooled). LDT, Laterodorsal tegmental nucleus (the averaged measurements per section obtained at two different anteroposterior levels are pooled). Total, Total number of cholinergic cells at the two regions (PPN − PBL + LDT). Means ± SEMs are shown. NS, not significant.

Discussion

The main findings of this study are: (i) corticosterone excess in the advanced ageing period selectively enhances serotonergic fibre degeneration compared to cholinergic fibre abnormalities in the AVT; (ii) corticosterone excess results in a slight decrement in the number of immunoreactive serotonergic raphe neurones but does not influence the number of mesopontine cholinergic cells; and (iii) an interaction between the serotonergic-cholinergic neuronal projections may exist, because a correlation has been found

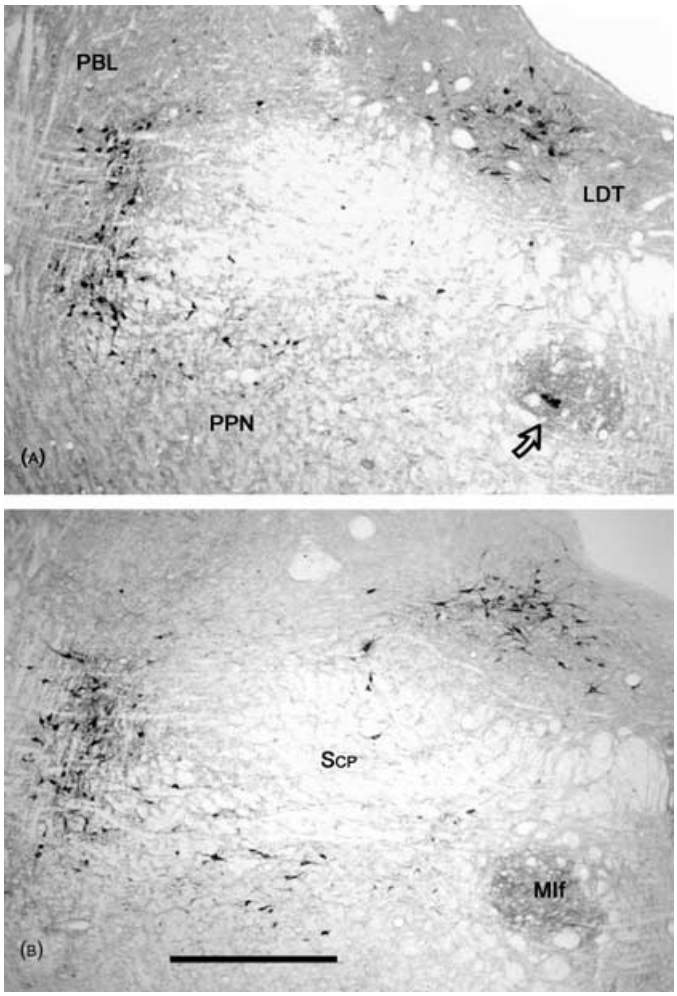

FIG. 6. Photomicrographs of choline acetyltransferase (ChAT)-immunoreactive (A) and NADPHd-positive (B) neurones at upper pontine level represented unilaterally, anteroposterior level of 15.13 (26). Note the remarkable coincidence between the two pictures. LDT, Laterodorsal tegmental nucleus; PPN, pedunculopontine nucleus; PBL, lateral part of parabrachial nucleus; Mlf, medial longitudinal fasciculus; Scp, superior cerebellar peduncle. Scale bar = 500 µm.

TABLE 6. Correlations Between the Magnitudes of Fibre Aberrations and the Numbers of Brainstem Immunoreactive Cholinergic and Serotonergic Neurones in Corticosterone- and Cholesterol-Treated Rats

		Corticosterone		Cholesterol	
Variable 1	Variable 2	r	P	r	P
Comparing fibre aberrations[a]					
5-HT fibre	ChAT fibre	(+) 0.683	0.050	(+) 0.412	0.237
5-HT fibre	NADPHd fibre	(+) 0.667	0.056	(+) 0.450	0.200
ChAT fibre	NADPHd fibre	(+) 0.683	0.045	(+) 0.483	0.168
Comparing neurone to neurone and fibre aberration to neurone number[b]					
5-HT neurone	ChAT neurone*	(+) 0.613	0.101	(–) 0.338	0.342
5-HT fibre	5-HT neurone	(–) 0.498	0.188	(–) 0.233	0.517
ChAT fibre	ChAT neurone*	(–) 0.667	0.074	(+) 0.063	0.837
NADPHd fibre	ChAT neurone*	(–) 0.548	0.143	(+) 0.404	0.252

Spearman correlation (R); [a]nine, and [b]eight plots were evaluated. *Number of ChAT immunoreactive neurones in LDT.

between the degrees of individual axon aberrations in the corticosterone-treated rats.

In the present study, the impact of an excess amount of corticosterone was investigated on the serotonergic and cholinergic axon degenerations and neuronal survival rate in aged rats. Corticosterone selectively enhanced 5-HT axon degeneration compared to the cholinergic fibre system. Although the degree of cholinergic fibre degeneration assayed on ChAT- and NADPHd-stained sections was higher in corticosterone-treated animals, the increment did not reach statistical significance. However, there was a positive correlation between the degree of serotonergic and that of cholinergic fibre aberrations in individual steroid-treated animals. It should be noted that this correlation, together with the tendencies for a slight increment in the cholinergic fibre aberrations, did not allow us to completely rule out the possibility that the cholinergic axons were also involved in the corticosterone-induced axon degeneration process. It might be possible that a higher dose of corticosterone or a longer treatment schedule with 200 mg corticosterone would have influenced the cholinergic neurones significantly. Further studies may answer the question of how sensitive the cholinergic neurones are during ageing to the excess amount of circulating corticosterone. One conclusion certainly may be drawn: the serotonergic neurones are more susceptible to corticosterone excess than the cholinergic neurones in terms of accelerating fibre aberrations during ageing.

In the brainstem nuclei, where the cell bodies of the projection neurones are located, only the number of immunostained serotonergic cells was influenced by the corticosterone treatment. It decreased by 13% in the raphe nuclei and this decrement reached a level of significance. Immunostaining of cell bodies implies that perikarya might escape detection because of the decrement in the production of immunostained material (serotonin in the present case). To solve this dilemma, we need to know more about the nature of the formation of fibre aberrations and degeneration. The exact nature of axon degeneration is not yet clarified, but it might be that axonal transport processes are compromised, resulting in large swellings and thickened fibre fragments along the axon. Another causal factor might be the progression of an axon degeneration process taking place distal to the aberrations. Both of these assumptions are in agreement with an inhibited 5-HT synthesizing capacity of serotonergic neurones for the advanced stage of their dysfunction.

The cell numbers in the mesopontine tegmental cholinergic nuclei did not change notably. We may conclude that the excess corticosterone, in the way it was used in the present study, did not influence cholinergic cell number in the brainstem. However, a strong correlation at the individual level for the degree of serotonergic and cholinergic axonal degeneration suggests the existence of interneuronal cooperation at the brain target level. It might also be that both neurotransmitter systems are influenced simultaneously by a common independent third factor, such as retrogradely acting neurotrophic factors.

Serotonergic raphe nuclei projecting to different forebrain structures, including the thalamus, are located in the midbrain and upper pons (32) and contain large amount of glucocorticoid receptors (33). Counting of cholinergic cell bodies was performed in the pedunculo-pontine tegmental and LDT nuclei, including cholinergic cells in the lateral part of PBL. These nuclei correspond to the Ch5 and Ch6 cholinergic subdivisions of the mesopontine tegmental cholinergic system (21) and project to the

thalamus as well (22). Our results show that the mesopontine cholinergic neurones contained large amounts of NADPHd, while the serotonergic neurones did not stain for NADPHd. Therefore, the NADPHd-positive fibre aberrations principally corresponded to fibres of a cholinergic nature (29).

The function of serotonergic neurones is coupled to regulation of mood, anxiety and depression (34), and declines with ageing (35). The mesopontine cholinergic neurones, and especially those located in the PPN, are claimed to participate in attention and learning behaviours and their degeneration in human neurodegenerative disorders is characterized by attentional and/or mnemonic deficit (36).

Ageing, even under normal conditions, is associated with the structural and functional disintegration of neurones, which results in a decrease in the number of synapses (37). At the same time, plastic changes may compensate for the structural loss (38). It is not yet known how dendritic plasticity is maintained during the ageing process and how it is influenced by glucocorticoid excess during ageing. In young adults, a marked dendritic reorganization takes place in the pyramidal neurones in the medial prefrontal cortex after chronic corticosterone administration (39). Furthermore, in young rats, an apical dendritic atrophy of hippocampal CA3 pyramidal neurones has been reported after excess glucocorticoid treatment (40). Moreover, a strong positive correlation exists between the circulating level of glucocorticoids and the degree of hippocampal atrophy in demented patients (5), or the corticosterone level and the morphological derangement of hippocampal pyramidal cells in aged rats (41). In conclusion, there is good evidence that corticosterone exerts a profound influence on the regulation of structural neuritic integrity in the cortex and hippocampus.

Two times 100 mg of corticosterone was implanted at the age of 24 months, which evoked a chronic increment in plasma and urine corticosterone concentrations. It is probable that the corticosterone excess was much more pronounced a short time after implantation, similarly to that previously described (42). The time-dependent decline of corticosterone excess in the body is reflected by the high corticosterone concentration in the urine 1 week after implantation and the subsequent marked decline. The corticosterone excess lasted at least for 4 weeks and corresponded to a moderate stress response level from the second week on. The basal plasma ACTH content was extremely low, and lasted for at least 6 weeks after implantation, indicating that pituitary ACTH production was severely impaired. At the conclusion of the experiment (i.e. 8 weeks after implantation), the pituitary-adrenocortical hormone productions had been normalized. However, the weights of adrenal glands and thymus were still lower in the corticosterone group, indicating a prolonged action of corticosterone on peripheral target tissues, including the endocrine gland, an endogenous source of corticosterone. These results suggest that, in addition to corticosterone hormone, other hormonal factors, including ACTH peptides, might be contributing factors in the initiation and regulation of the axonal degeneration process. The nature and the time-associated dynamic aspect of the complex neuroendocrine regulation of fibre degeneration during ageing remains to be clarified. It may be that the effect of corticosterone on neuronal structure and function is mediated through neurotrophic factors. Adrenal steroids are intimately involved in the regulation of the expression of neurotrophins such as brain-derived neurotrophic factor, neurotrophin-3 or basic fibroblast growth factor (43).

Glucocorticoids regulate both the serotonergic and the cholinergic neurones. Interactions between glucocorticoids and serotonergic neurones are likely to be of importance for understanding the mechanism underlying affective dysfunctions in response to adrenal hypercorticism in humans. Glucocorticoid excess corresponding to stress level and occupying the larger part of glucocorticoid receptors increases the activity of the raphe-hippocampal system and 5-HT neurotransmission (44). Under pathological conditions of chronically elevated corticosteroid concentration, 5-HT neurotransmission is impaired (44). Furthermore, corticosterone tonically inhibits the genetic expression and function of 5-HT_{1A} receptor (45). Our notion is that corticosterone excess during ageing may exhaust 5-HT neurones and dysbalance the function of their receptors. This assumption is supported by the finding of Venero *et al.* (10), who found an increased 5-HT turnover in the hippocampus of aged rats parallel to the appearance of aberrant 5-HT fibres, and concluded that the high 5-HT turnover in aged rats is a signal of neuronal degeneration. Similar to 5-HT neurones, cholinergic neuronal functions are also influenced by corticosterone. A high dose of corticosterone enhances the degeneration of basal forebrain cholinergic neurones in response to NMDA overexcitation (23) and potentiates cholinergic degeneration in rat hippocampus induced by ethylcholine aziridinium (46).

Ageing rats are more susceptible than younger animals to the effects of stress or glucocorticoids (47). Chronic infusion of dexamethasone reduces 5-HT transporter expression in the brain of aged, but not of young rats (48). Even mid-aged rats proved to be more sensitive to corticosterone excess compared to young rats tested in a Morris water maze learning paradigm (49). These findings support the notion that susceptibility to elevated corticosterone is increased during ageing, probably due to a progressive desinhibition of HPA axis and a subsequent enhanced sensitivity to corticosterone-induced neuritic degeneration processes, notably of serotonergic neurones and their innervation targets.

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