Journal of Neuroendocrinology, 1994, Vol. 6, 673–679

Activation of Vasopressin Neurons in Aging and Alzheimer's Disease

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Key words: human hypothalamus, aging, Alzheimer's disease, Golgi Apparatus, supraoptic nucleus.

Abstract

The supraoptic (SON) and paraventricular nuclei (PVN) of the human hypothalamus are production sites of vasopressin (AVP) and oxytocin (OXT). Although the hypothalamus is affected in Alzheimer's disease (AD), previous work has not only shown that in these two nuclei no neurons are lost, neither during aging nor in AD, but that the number of AVP-expressing neurons and their nucleolar size had even increased with age. These observations indicated that the peptide synthesis of the AVP neurons was activated in the oldest age-groups. Recently published, qualitative observations, using the area of the Golgi Apparatus (GA) as a sensitive parameter for neurosecretory activity, confirmed the activation of SON and PVN neurons with age in human; however, in this report the neurons were not identified according to their neuropeptide content. In the present quantitative study we determined whether the AVP neurons were indeed activated as a result of the aging process in controls and AD patients. We applied a polyclonal antiserum directed against the medial cisternae of the GA on formalin-fixed, paraffin-embedded tissue sections taken from the dorsolateral SON (dl-SON) of 10 controls and 10 AD patients, and performed our measurements in this area that is known to be predominantly occupied (90–95%) by AVP neurons. In addition, the sparse OXT cells present in the area of study, were excluded from the measurements on the basis of alternative sections stained for OXT. In the dl-SON, the area occupied by the GA and the cellular profile area per patient were quantified by means of image analysis. The results show a significant increase in GA area with age in controls and in AD, demonstrating an activation of the AVP neurons in the dl-SON of the human hypothalamus in these two conditions. No changes were observed in the cellular profile areas with age, neither in the controls nor in AD, suggesting that the GA area is a much more sensitive parameter for monitoring activity changes in post-mortem material than neuronal size. It is proposed that this activation of AVP cells with age, which has been suggested to be a compensatory response to the age-related loss of AVP receptors in the kidney, might be the basis of the stability of these neurons in aging and AD.

In the hypothalamus, the neurohormones vasopressin (AVP) and oxytocin (OXT) are produced by magnocellular neurons of both the supraoptic (SON) and paraventricular nucleus (PVN). After synthesis at the endoplasmic reticulum and subsequent processing by the Golgi Apparatus (GA), they are packaged in neurosecretory granules which are transported to the posterior pituitary. The two peptides are then released into the general circulation and play a key role in the regulation of water metabolism and reproduction (1, 2). Parvocellular AVP- and OXT- containing neurons of the PVN also project into the brain and are involved in central functions (3, 4, 5). Earlier animal studies demonstrated that the neuron number of the SON and PVN remains stable throughout life (6, 7, 8, 9) and that the neuronal activity of these neurons was shown to be increased in old rats as appeared from the larger area of the GA in old rats demonstrated by enzymehistochemistry (10), and from the increased 24 h excretion of AVP and OXT with age (11, 12). The hyperactivity of the SON and PVN that occurs with age has been suggested to be due to an age-related loss of kidney AVP receptors (10, 11, 12, 13, 14, 15).

In human SON and PVN too, an absence of cell loss in aging and Alzheimer's disease (AD) has generally been reported (16, 17, 18, 19). The AVP neuronal size and nucleolar size were increased in the oldest age group, indicating an activation of these neurons in human aging as well (20, 21). This is in accordance with the age-related increases in plasma AVP and neurophysin levels in human (22, 23, 24).

Earlier studies have shown neuronal profile area, nuclear volume, nucleolar profile area and GA area to be sensitive parameters for detecting experimentally induced or age- or pathology-related changes in neurosecretory and cellular activity in both animal and human tissues (21, 25, 26, 27, 28, 29, 30, 31, 32, 33). Recently, using immunocytochemistry, our group has observed qualitatively an increase in GA area in human SON and PVN neurons irrespective of their peptide content, indicating an activation of these nuclei with age and in AD (34).

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Furthermore, the number of AVP-expressing neurons in the PVN and the dorsolateral part of the SON (dl-SON) was recently shown to be increased with aging (19), reinforcing the idea that in particular the AVP neurons in these nuclei might be activated. In contrast, the number of neurons in the human PVN expressing OXT was found to remain constant with age (18), which is in accordance with the lack of activational signs of these cells in rat and human senescence observed earlier (10, 20, 21). The aim of the present study was, therefore, to determine whether AVP neurons are indeed activated in the SON. In order to do so, we quantified—by means of an image analysis system—the GA area and cellular profile area in neurons of the dl-SON of controls and Alzheimer patients with respect to age. The dl-SON has been reported to contain in excess of 90% AVP cells (20, 35). Adjacent sections were stained for OXT in order to exclude areas in which a few positive OXT neurons were still present.

Results

Immunostaining

Using the antibody against MG-160 in combination with the earlier described microwave pretreatment of tissue sections (28, 34), we obtained a clear staining of the GA in the dl-SON, displaying the characteristic granular cytoplasmic pattern with a perinuclear distribution in dl-SON neurons of all patients studied, except for AD patient no. 86001, whose dl-SON showed considerably smaller and vaguely stained GA fragments. In Fig. 1, the characteristic differences in GA size between young and old control and AD patients are depicted.

Adjacent sections that were stained for OXT generally displayed only a few positive cells with a preferential localization of the immunostain in the outer border of the medial and dorsal zone of the nucleus, whereas in the dl-SON of patients nos. 81064, 86001, 88252, 86364.5 and 82175 hardly any OXT cells were present. During the IBAS procedures, the OXT neuron containing areas could easily be excluded from the manually made outline of the cells in the sections stained for the GA.

Effects of age

The increase in GA area in SON and PVN neurons with age, observed earlier in a non-identified neuronal population by means of qualitative measurement, was confirmed in the present study in a quantitative way in AVP neurons of the dl-SON. Results from the image analysis measurements are summarized in Table 1 and depicted in Figs. 2, 3 and 4. A significant difference in mean area of the GA emerged between the young (<65 years of age)

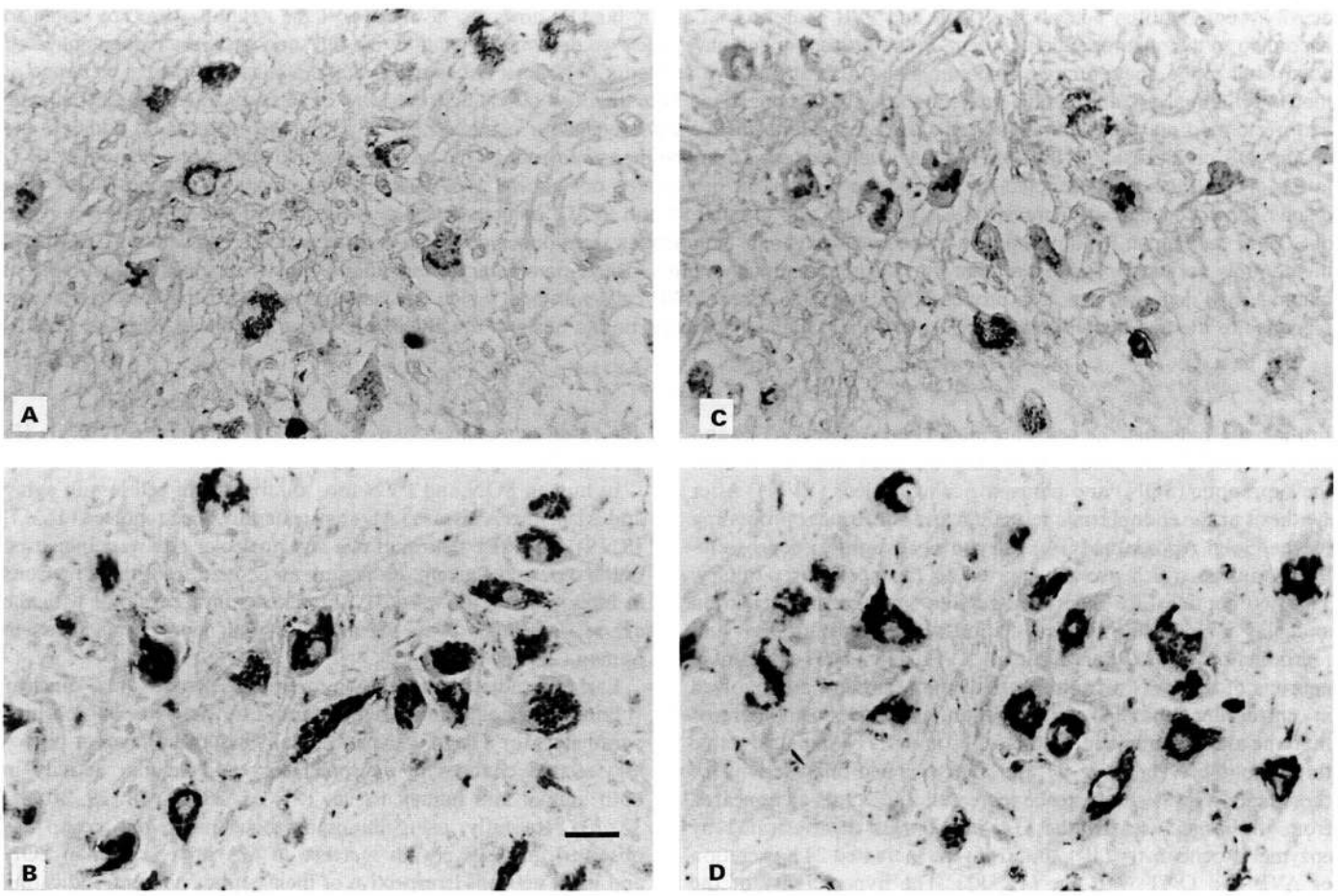

FIG. 1. Photomicrograph depicting Golgi apparatus (MG-160) staining in vasopressinergic SON neurons of the hypothalamus of (A): a 43-year-old control subject (Table 1, 81267), (B): an 82-year-old control subject (Table 1, 90–75), (C): a 49-year-old Alzheimer patient (Table 1, 89503.2) and (D): an 81-year-old Alzheimer patient (Table 1, 88237). Bar represents 28 μm.

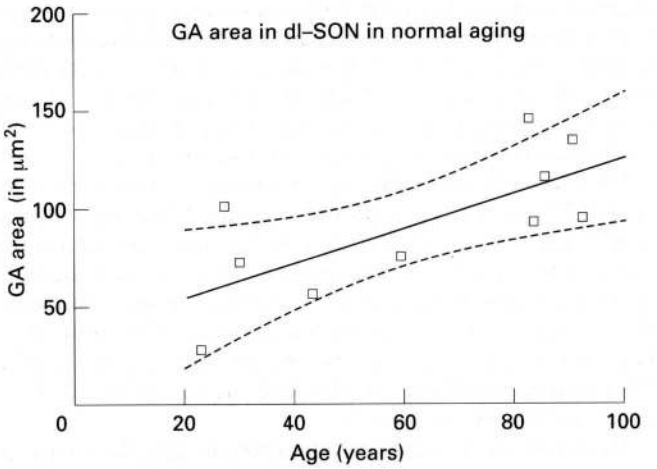

FIG. 2. Graph depicting mean Golgi Apparatus (GA) area per neuron in young and old controls as measured in the maximal vasopressinergic area of the dorsolateral supraoptic nucleus (dl-SON). A significant correlation with age is present ($Y=35.87+0.90X$; $r=0.708$; $P=0.021$; P-Pearson's test). The dashed line depicts the 95% confidence interval.

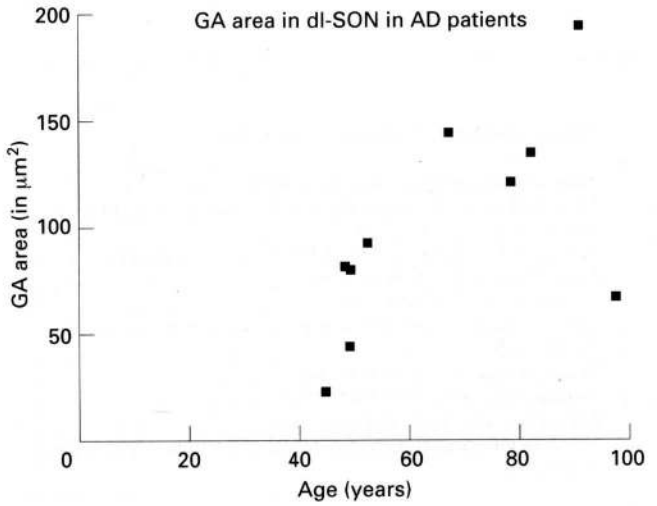

FIG. 3. Graph depicting mean Golgi Apparatus (GA) area per neuron in presenile (<65 years of age) and senile (≥65 years of age) Alzheimer (AD) patients as measured in neurons of the maximal vasopressinergic area of the dorsolateral supraoptic nucleus (dl-SON). A significant difference between the young and old AD group is present ($P=0.01$, Student's t-test). Only a non-significant trend with age was found ($Y=-4.87+1.57X$; $r=0.599$; $P=0.066$; P-Pearson's test) due to one low value (Patient no. 86001, 97 years).

and old control group (≥65 years of age) (Student's t-test; $P=0.006$) (Table 2). A significant difference was also found for the presenile (<65 years of age) versus the senile (≥65 years of age) AD group ($P=0.01$). The GA areas in the old control and old Alzheimer group did not differ from each other significantly ($P=0.50$), nor did the young control and young Alzheimer group differ significantly in this respect ($P=0.88$). Using P-Pearson's test, a significant positive correlation with age was observed in the control group ($Y=35.87+0.90X$; $r=0.708$; $P=0.021$) (Fig. 2). In the Alzheimer group only a non-significant trend with age was present ($Y=-4.87+1.57X$; $r=0.599$; $P=0.066$) (Fig. 3).

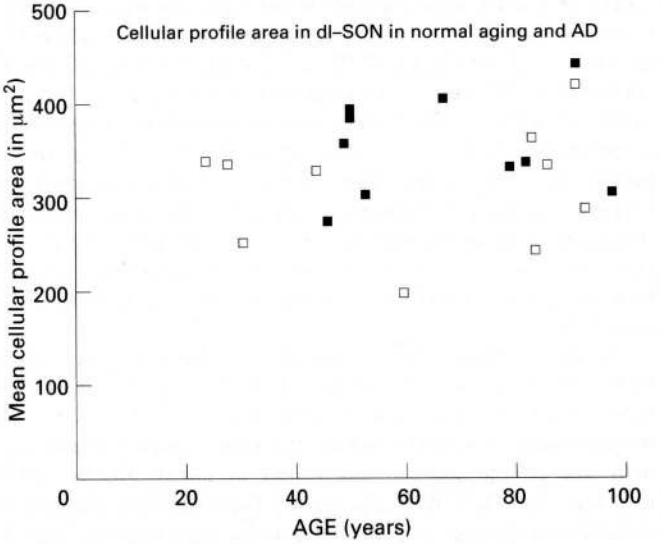

FIG. 4. Graph depicting mean cellular profile area per neuron in various age groups. No difference between the young and old groups was found in the controls ($P=0.33$, Student's t-test) or in the AD group ($P=0.50$). Filled squares represent AD patients. Open squares represent control subjects.

As far as the mean cellular profile area is concerned, between the young and old groups no differences were found for this parameter, neither in the control group (Student's t-test; $P=0.33$) nor in the AD group ($P=0.50$) (Fig. 4). In addition, no significant correlation with age was present in these groups (controls: $Y=287.33+0.39X$; $r=0.169$; $P=0.64$; Alzheimer patients: $Y=328+0.41X$; $r=0.152$; $P=0.67$).

Discussion

In the present study, we confirmed earlier qualitative observations concerning an increase in the GA area with age in controls and AD patients in unidentified SON and PVN neurons, but this time in a quantitative way in the AVP population of dl-SON neurons. The contribution of OXT neurons to the GA measurements were minimized in the present study by selecting the maximal vasopressinergic area of the dl-SON and by excluding areas that still contained some OXT cells as they appeared in alternating sections stained for OXT. In this way, the contribution of OXT neurons to the measurements was negligible.

Our data are in agreement with those of Van der Woude *et al.* (19), who reported in normal aging an increase in the number of AVP-expressing neurons in the PVN. Such an increase was not found in AD patients. Their observation of an increased nuclear diameter of both PVN and dl-SON neurons in both, old controls and in old AD patients indicated, however, that in AD patients the AVP neurons were also activated during aging although apparently to a lesser extent than in controls, which is also in agreement with our data on the size of the GA. In the present study, after dividing the Alzheimer patients in a presenile and a senile group, the differences in GA areas between the young and old AD groups was statistically significant, but the correlation with age just failed to reach significance ($P=0.066$). In controls, in addition to a significant difference of GA areas between the

young and old group, the correlation with age was also highly significant ($P=0.021$). Alzheimer patient no. 86001, whose GA area was small for her age of 97 years, appeared to be responsible for the lack of a significant correlation between age and GA area in the AD group; if this patient was excluded from the study, the correlation with age was highly significant ($P=0.0012$). In this patient, the GA staining pattern consisted of many small and vaguely stained GA fragments, resembling to some degree the 'fragmented' GA of motor neurons in amyotrophic lateral sclerosis (36). The patient's clinical data or neuropathology findings, however, gave no indication of a neurodegenerative process other than AD.

The mean cellular profile areas, measured in the present study by the IBAS system, were found to be in the same range as those reported earlier using a different technique (20). However, in the present study, no correlation of the mean cellular profile area with age of the patient was found in either groups, which illustrates that the GA area is a much more sensitive measure for determining changes in neuronal activity than neuronal size. In addition, patient no. 82181 displayed relatively high values for what was to be expected on the basis of his age (Table 1 and 2; Fig. 2). This patient was reported to have suffered from serious decompensatio cordis (Table 1), which led us to expect an activation of the SON and PVN. Although chronic activational changes such as an increased size of the GA are not likely to occur within hours, the contribution of dehydration and osmotic changes around the moment of death on the activity of human SON and PVN neurons should be a subject for future studies.

With respect to the other major peptide present in the SON, i.e. OXT, a similar activation with age and AD as was observed in the present study for AVP is, however, not expected as other markers for cellular activity indicate that this population of neurons remains stable instead of activated in these conditions (10, 18, 20, 21). In view of the heterogenous distribution of the OXT neurons, the only way to measure the GA in identified OXT neurons would be to double-stain these neurons, which was so far, technically not possible.

Data from the literature indicate generally that the number of SON and PVN cells remains constant during aging (16, 17, 37). There is one single exception (38) for which no explanation is currently at hand, in which a decrease in SON and PVN cell number in old AD patients was reported. On the basis of, e.g. nucleolar size (21), AVP and neurophysin blood levels (22, 23, 24) and the number of neurons expressing AVP (19), it has been

TABLE 1. Brain Material Used.

Pat.no.	Sex	Age (y)	PMD (h)	BW (g)	Fix (d)	Clinico-pathological diagnosis, cause of death
Controls						
87110	M	23	13	1310	40	Subacute encephalitis, cerebral oedema
82181	M	27	nd	1560	40	Drug addiction, *decompensatio cordis*, sepsis, respiratory insufficiency
81255	F	30	24	1460	39	Intramural dissecting haematoma of coronary artery
81267	M	43	23	1260	53	Non-Hodgkin lymphoma, sepsis
80087	F	59	24	1110	183	Acute monoblastic leukemia
90–75	M	82	5h20′	1268	nd	Hydronephrosis, cachexia, urothelia carcinoma, respiratory insufficiency
81064	M	83	26	1280	42	Diverticulitis, myocard infarction
82175	M	85	16	1400	44	Chronic myelocytic leukemia, bronchopneumonia
81033	F	90	13	1110	48	Femur fraction, bronchopneumonia
93–12	M	92	12h30′	1038	32	Emphysema, pneumonia, myocardal infarction, a few cerebellar infarctions
Mean		61.4	16.9	1280	57.8	
±SEM		±8.9	±2.2	±52	±15.7	
Alzheimer patients						
86364.5	M	45	4	1130	119	presenile AD
90–45	M	48	5h40′	1435	27	Fam. presenile AD, non-treated pneumonia, uremia
89503.2	F	49	5	1260	28	AD, septic shock, epilepsy, anaemia
90–262	M	49	4h25′	1426	28	Fam. AD, epilepsy, diabetes mellitus
893453	M	52	23	880	102	Fam. AD, bronchopneumonia
88252	M	66	3h15′	1256	30	AD
88330	M	78	nd	1240	nd	AD
88237	F	81	2	1020	nd	AD
84050	F	90	2	950	33	AD, anaemia
86001	F	97	5	1200	40	AD
Mean		65.5	6*	1180	50.8	
±SEM		±6.2	±2.2	±58.8	±13.2	

Using Student's *t*-test for unpaired data, no significant differences between the control and Alzheimer group emerged with regard to age ($P=0.70$), brain weight ($P=0.22$) or fixation time ($P=0.74$). There was a significant difference for the PMD between the control and Alzheimer group (*$P=0.002$, unpaired Student's *t*-test). However, earlier work had already shown that no correlation with the PMD for AVP or OXT immunostaining was present (19, 20, 46). Also, no such correlation between PMD and GA staining has been found (28, 34, 41), so that the differences in PMD will not have influenced our results. Abbreviations used: AD: Alzheimer's Disease, y: years, PMD: post mortem delay (in hours), BW: brain weight (in grams), Fix: fixation time (in days), nd: not determined, Fam: familiar; SEM: standard error of the mean.

TABLE 2. Golgi Apparatus (GA) Area and Mean Cellular Profile (MA) Area in Dorsolateral SON (dL-SON) Neurons in Young (<65 years) and Old (>65 years) Control Subjects and Alzheimer's Disease (AD) Patients.

	Age	Young Control GA	Young Control MA	Age	Young AD GA	Young AD MA	Age	Old Control GA	Old Control MA	Age	Old AD GA	Old AD MA
	23	27.2	339.0	45	22.8	275.5	82	146.4	366.9	66	145.3	409.0
	27	101.3	336.0	48	80.7	360.1	83	92.9	246.0	78	121.2	335.6
	30	72.2	252.3	49	44.4	388.0	85	116.6	337.8	81	135.6	340.0
	46	56.4	330.8	49	79.2	394.4	90	135.1	422.0	90	195.4	447.0
	59	75.3	198.9	52	92.6	304.5	92	95.6	291.8	97	67.4	310.0
Mean	37.0	66.5	291.4	48.6	63.9	344.5	86.4	117.3*	332.9	82.4	132.9*	368.3
SEM	6.7	12.2	28.2	1.1	13.0	23.4	1.9	10.5	30.3	5.3	20.6	25.6

Abbreviations used: SEM: standard error of the mean. Statistical significance *$P < 0.01$ (unpaired Student's t-test) different from the young group.

presumed that the SON and PVN neurons are activated during the course of aging. The present study shows that this is indeed the case and illustrates, in addition, that, so far, measurement of the GA area is the most sensitive parameter as far as evaluating changes in neuronal activity in human post-mortem material is concerned. It has been hypothesized that the hyperactivity of these neurons renders them less susceptible to degenerative changes in aging and AD (39, 40).

Materials and methods

Tissue collection

Brains from 10 control subjects without a primary neurological or psychiatric disease ranging in age from 23 to 92 years and from 10 presenile and senile Alzheimer patients ranging in age from 44 to 97 years were obtained at autopsy (see Table 1 for details). Neuropathological investigation of the controls and confirmation of the AD diagnosis was performed by Drs W. Kamphorst (Free University), F.C. Stam (Netherlands Brain Bank) or D. Troost (Academic Medical Center), all in Amsterdam. After the brain had been weighed, the hypothalamus was dissected and fixed in 4% formaldehyde in phosphate buffered saline (pH 7.4) at room temperature, generally for about 40 days (see Table 1 for details). The tissue was subsequently dehydrated and embedded in paraffin. Serial 6 μm coronal sections were cut and mounted on chrome-alum-coated glass slides. For anatomical orientation, every 50th section was stained with 0.1% thionine in acetate buffer (pH 4.0).

Morphometry

Control and AD subjects were subdivided into 2 age groups, one younger and one older than the arbitrary chosen age of 65; ages in the group designated 'young' ranged from 23 to 59 and those in the group designated 'old' ranged from 66 to 92 years.

On the basis of thionine stained serial sections throughout the hypothalamus of these patients in which the dl-SON could be discerned, sections 300 μm apart were stained immunocytochemically for AVP as described before (18, 19). Subsequently, cross-sectional vasopressinergic areas of the dl-SON were determined using a Calcomp 200 digitizer connected to an HP 9000/835 computer using a Zeiss microscope with a PLAN 2.5× objective and PLAN 12.5× oculars as described before (16, 18, 19). On the basis of these data, two sections in the central part of the nucleus, i.e. at the level of the maximal vasopressinergic area in the dl-SON, were subsequently mounted, one of which was stained later for the GA and the other, adjacent one, for OXT.

Immunocytochemistry

In order to be able to detect the GA, a polyclonal organelle specific antibody was used that was raised against immunoaffinity purified MG-160, a sialoglycoprotein of the medial cisternae of rat neuronal GA (160 KD). The specificity of this antibody has been established previously by light and immunoelectron microscopy (41, 42). Sections on which this antibody was applied were microwave pretreated if the tissue was fixed for prolonged periods of time in formalin and embedded in paraffin (34) in order to be a reliable reagent for the immunocytochemical visualization of the GA (28, 41).

Staining for MG-160 was performed as described earlier (28, 34). In brief, mounted sections were hydrated and stained using the following procedure: (1) Incubation of the sections in a microwave oven twice for 5 min in a solution of 1% $ZNSO_4$; (2) pretreatment of the sections in 0.5% saponin (Merck)/3% fish gelatin (Merck) and in 10% horse serum in Tris Buffered Saline; (3) incubation with the primary antibody (MG-160) 1:800 in phosphate buffered saline (pH 7.6); (4) incubations with biotinylated goat anti rabbit IgG (Vector) 1:500, and Avidin Biotin-HRP (Vector elite kit) 1:1500 in a solution of 0.05 M Tris, 0.15 M NaCl, 0.25% Gelatin and 0.5% Triton X-100 (Sigma), and 5) subsequent incubation in 0.05 mg/ml 3.3′ diaminobenzidine (DAB; Sigma) in 0.05 M Tris/HCL (pH 7.6) containing 5 μl H_2O_2 30% and 0.035 g ammonium nickel sulphate at room temperature for 10 min, (6) followed by dehydration in graded ethanol and xylene and subsequent coverslipping with Entellan (Merck).

For staining of OXT in adjacent sections, antiserum O-2-T, (batch 29-1-89) was used. This antiserum was raised as described before (43, 44) and purified by preabsorbing it twice with AVP-glutaraldehyde-coupled sepharose beads according to Pool *et al.* (45). Antisera have been checked for cross-reactivity with AVP in alternating 6 μm sections of the PVN and appeared to stain either for OXT or for AVP after purification, as described (18). After rehydration, adjacent sections were: 1) incubated with purified OXT-antiserum O-2-T 1:1000 in Tris Buffered Saline (pH 7.6) and 0.5% Triton X-100; 2) incubated with goat-anti-rabbit IgG serum (Betsy) 1:100 in TBS, and 3) peroxidase-anti-peroxidase (PAP) 1:500 in TBS and subsequently incubated with 0.5 mg/ml 3-3′-diaminobenzidine (DAB) (Sigma) in 0.05 M Tris HCl containing 0.01% H_2O_2 followed by dehydration in graded ethanols and xylene and coverslipping with Entellan.

Image analysis

Quantification of the surface of the GA and the cellular profile area was performed using an IBAS-KAT image analysis system (Kontron KAT based system) connected to a Bosch TYK9B TV camera as has been described in detail earlier (28). All measurements were performed using a 560 nm small band DEPAL filter (Schott, Germany) which coincides with the maximum absorption of the diaminobenzidine/nickel sulphate precipitate in the sections. In brief, sampling procedures and selection of neurons consisted of: (a) outlining of the dl-SON and (b) determining the total GA area per cellular profile in those neurons in which a nucleolus was visible. Area selection and sampling of the neurons were performed as follows: in each GA stained section an image of the dl-SON area (using the 2.5× objective of the microscope) was loaded into the IBAS and displayed on the image analysis monitor. The position of the section under the microscope was subsequently stored using the X-Y coordinates of the scanning stage of the microscope. In this image, the contour of the dl-SON was outlined manually, but excluding areas that contained positive neurons in the adjacent OXT-stained section. In order to select the fields for cell sampling in the dl-SON, the outlined area was subdivided in fields corresponding to the image size at 500× magnification (40× objective) (cf. 28). Twenty fields were then selected systematically on the basis of an ad random starting point and their positions were again

expressed in X-Y coordinates of the scanning stage. On the basis of these coordinates, the selected fields were later retrieved at 500 × magnification and the measurements of GA and cellular profiles were performed in these fields.

In order to determine the total GA area per cell profile, the 40 × objective was positioned in the microscope and the scanning stage moved to the previously defined X-Y coordinates of the selected fields. At each position, a 768 × 512 pixel large image was loaded and stored. Subsequently, a mask of the GA area in the selected cells in all stored images was calculated and again stored. Each of the grey value images with the calculated GA masks were then retrieved under program control and only of those cellular profiles that displayed a nucleolus, the borders were outlined manually and stored together with the Golgi masks. On average 3 to 4 neurons per field were outlined, yielding a total of 60 to 80 neurons measured per patient. Finally, of the outlined neurons in the selected fields, mean GA area and mean cellular profile area were calculated and data were stored per patient and per group.

Statistical methods

Differences in mean values of the GA area and mean cellular profile area per neuron per patient per group between young and old groups from the control and AD group were tested using Student's *t*-test for unpaired data. Furthermore, the P-Pearson's test was used in order to determine correlations of the mean GA area and mean cellular profile area values with age. It was decided that a P-value smaller than 0.05 was significant.

Acknowledgements

We thank the Netherlands Brain Bank for providing us with human brain material (Coordinator: Dr R. Ravid). Furthermore, we thank Dr P. van der Woude for providing morphometric data on the human SON, Dr M. A. Hofman for his statistical advice and for his comments to the manuscript and Ms W. Verweij for correcting the English.

This work was supported by grants from the Netherlands Organisation for Scientific Research (NWO grant 900-552-083) (P.J.L.), by a grant from the Shahid Beheshti University of Medical Sciences, Tehran, Iran (A.S.), by an award of the Amyotrophic Lateral Sclerosis Association, USA (N.K.G.) and by a grant from the National Institutes of Health (NS 05572) (N.K.G.).

Accepted 4 August 1994

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