Oxytocin Cell Number in the Human Paraventricular Nucleus Remains Constant With Aging and in Alzheimer's Disease

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WIERDA, M., E. GOUDSMIT, P. F. VAN DER WOUDE, J. S. PURBA, M. A. HOFMAN, H. BOGTE AND D. F. SWAAB. *Oxytocin cell number in the human paraventricular nucleus remains constant with aging and in Alzheimer's disease.* NEUROBIOL AGING 12(5) 511–516, 1991.—Total cell numbers in the paraventricular nucleus (PVN) were previously shown to remain unaltered with aging and in AD. The aim of the present study was to determine the aging pattern of the oxytocin (OXT) cell population in the PVN. For this purpose, the number of immunocytochemically identified oxytocin cells was determined in the PVN of the human hypothalamus in 20 control subjects ranging in age from 15 to 90 years and in 10 Alzheimer's disease (AD) patients aged 46 to 97 years. The results show that the number of OXT cells in the PVN is similar in males and females and remains unaltered in senescence and AD. It is concluded that the remarkable stability of the PVN in these conditions also applies for the subpopulation of OXT cells in this nucleus and that reports in the literature on diminished OXT secretion in AD do not seem to be based on a decrease in the number of OXT expressing neurons from the PVN.

Oxytocin Paraventricular nucleus Aging Alzheimer's disease Immunocytochemistry Hypothalamus
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THE nonapeptides vasopressin (AVP) and oxytocin (OXT) are synthesized in neurons of the supraoptic (SON) and paraventricular (PVN) nuclei in the hypothalamus. AVP and OXT cells in these nuclei project to the posterior lobe of the pituitary, constituting the hypothalamo-neurohypophyseal system (HNS). OXT is involved in labor and lactation (29). In addition, AVP and OXT cells in the PVN project to central areas of the brain (5). Central OXT projection might be involved in maternal behavior (36).

Cell counts in the SON and PVN have demonstrated the absence of cell loss in these nuclei during both rodent and human aging (8, 10, 13, 16, 31, 25). The marked stability of the SON and PVN with aging and in Alzheimer's disease (AD) is in sharp contrast with degenerative changes observed in other hypothalamic nuclei (viz. the suprachiasmatic nucleus and the sexually dimorphic nucleus) in these conditions (14, 30–32) and this stability was recently proposed to be related to the increased activity of AVP cells, which was previously observed in the SON and PVN in senescence and AD (7, 15, 28). However, until now no distinction was made between numbers of neurons and glial cells in the human PVN with aging and in AD (10). Therefore, the aging pattern of the immunocytochemically defined population of OXT neurons in the PVN was investigated in the present study.

In addition to the normal aging process, AD patients were

studied. The OXT cell population in the PVN in AD patients is of special interest in view of reports on reduced plasma levels of estrogen-stimulated neurophysin in AD (3). Reduced levels of OXT were also reported in cerebrospinal fluid (CSF) of AD patients (34).

METHOD

Brains from 20 subjects (10 males and 10 females) ranging in age from 15 to 90 years without neurological or psychiatric diagnosis were obtained at autopsy. In addition, brains of 10 subjects (4 males and 6 females, ranging in age from 46 to 97 years) with a clinical diagnosis of Alzheimer's disease were studied. AD patients showed abundant neocortical and hippocampal senile plaques and tangles. The areas examined were cortical areas: 4, 10, 17–18, 20, 38 and 42, corpus striatum, gyrus brevis of the insula, hippocampus and mesencephalon including substantia nigra, medulla oblongata, thalamus, locus coeruleus and cerebellum. Brains were weighed and fixed for about 6 weeks in 10% formalin at room temperature (for details see Table 1). The hypothalamic area containing the PVN was dissected, dehydrated in graded ethanol and embedded in paraffin. Six- μ m frontal sections were cut on a Leitz microtome and mounted on chrome-alum-coated object slides. Every 50th section was stained with thionine in order to locate the PVN before immunocytochemical staining.

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TABLE 1
POSTMORTEM INTERVAL AND DURATION OF FIXATION IN YOUNG
AND OLD CONTROL SUBJECTS AND IN ALZHEIMER CASES

Group	Postmortem Interval (hours)	(n)*	Fixation Time (days)	(n)*
Controls				
≤60	22 ± 4	(10)	47 ± 13	(12)
>60	22 ± 5	(7)	42 ± 4	(7)
AD cases				
≤60	4; 24	(2)	48; 119	(2)
>60	17 ± 7	(6)	41 ± 4	(7)†

Data are given as mean ± S.E.M.

*Postmortem interval and duration of fixation were not available in all cases studied.

†One AD patient had an extremely long fixation time (292 days) and is not included in the mean value.

Immunocytochemistry

A series of sections taken at regular 300- μ m intervals throughout the region in which the PVN could be discerned in the

FIG. 1. Relation between first antiserum dilution and absorbance (peroxidase activity) for AVP beads incubated with anti-OXT serum; anti-OXT serum preadsorbed twice with VP beads (anti-OXT-2 $\times$ AVP), and preimmune serum (control serum). The reaction product was obtained using a horseradish peroxidase conjugate stained with H₂O₂/ortho-phenyldiamine. For coupling and adsorption procedures see Pool et al. (22). A major cross-reaction of the nonpurified anti-OXT serum is present with AVP. This reaction is abolished if the purified anti-OXT-2 $\times$ AVP serum is used. The purified anti-OXT-2 $\times$ AVP serum still has a high affinity for OXT.

thionine-stained material, was stained immunocytochemically for OXT.

Purification of antisera. In order to remove cross-reactivity with AVP, the OXT antiserum (O-1-V, 4-4-75) was preadsorbed twice with AVP-glutaraldehyde-coupled sepharose beads (22). The second incubation resulted in complete removal of cross-reactivity (Fig. 1). In addition, cross-reactivity was checked in alternating 6- μ m sections of the PVN which were stained with purified OXT- and AVP-antisera, respectively. No cells were detected which stained with both antisera.

Immunocytochemical staining. Mounted sections were hydrated and stained using the following procedure: 1) Incubation with AVP-adsorbed OXT antiserum (anti-OXT-2 $\times$ AVP) 1:250 in 0.05 M Tris containing 0.9% NaCl (TBS, pH 7.6) with 0.5% Triton X-100. Sections of two additional AD patients were incubated with an antiserum against OXT-neurophysin (1:2000). Incubations were performed for 1 hour at room temperature and subsequently overnight at -4°C in plastic boxes to prevent evaporation; 2) washing in TBS (2 $\times$ 10 min); 3) incubation with goat-anti-rabbit IgG serum (Betsy) 1:100 in TBS at room temperature for 30 min; 4) washing in TBS (2 $\times$ 10 min); 5) incubation with peroxidase-anti-peroxidase (PAP) 1:500 in TBS at room temperature for 30 min; 6) washing in TBS (2 $\times$ 10 min); 7) rinsing in 0.05 M Tris-HCl (pH 7.6); 8) incubation with 0.5 mg/ml 3-3'-diaminobenzidine (DAB; Sigma) in 0.05 M Tris-HCl containing 0.01% H₂O₂ at room temperature for 10 min; 9) rinsing in aquadest followed by dehydration in graded ethanol at room temperature. Sections were embedded in Entellan (Merck).

Morphometry

In contrast to the rat, the human PVN cannot readily be divided into magnocellular and parvocellular compartments, but rather appears to consist of intermixed populations of larger and smaller neurons (20). OXT cells have been found to be scattered among the AVP cells (6). Cross-sectional areas of the PVN in OXT-stained sections were measured with a Calcomp 2000 digitizer connected to a HP 9000/835 computer using a Zeiss microscope with a PLAN 4.0 $\times$ objective and PLAN 12.5 $\times$ oculars. Islands of OXT cells between the SON and the PVN [accessory neurosecretory nuclei (6)] were not included. If the cross-sectional area of the PVN extended beyond the field of vision in a particular section, this area was measured step-wise using a quadrangular grid in one of the oculars as a reference. The PVN was measured on the right-hand side of the brain with the exception of three cases in which the nucleus was not completely present within the dissected tissue on that side.

The volume of the OXT cell population in the PVN was determined by integrating the area measurements of all sections containing OXT cells (35). An average number of 17 sections per PVN was measured.

Numerical OXT cell density in the PVN was estimated by counting the total number of nuclear profiles in immunoreactive neurons per unit area followed by a discrete "unfolding" procedure (37) with the modification proposed by Cruz-Orive (4) and a correction for section thickness (6 μ m). For this purpose nuclear profile areas of all OXT cells were measured in every 200th section throughout the nucleus (i.e., at 1200- μ m intervals) in a "random systematic" way, using the equipment described above with a PLAN 40 $\times$ objective. Measurements of controls and AD cases were performed on coded sections in order to avoid observer's bias.

The total number of OXT cells in the PVN was computed by multiplying the numerical cell density with the volume of the OXT cell population.

TABLE 2
MEAN AGES AND BRAIN WEIGHTS FOR MALE AND FEMALE SUBJECTS

Group	Males			Females		
	Mean Age (years)	(n)	Brain Weight (grams)	Mean Age (years)	(n)	Brain Weight (grams)
Controls						
≤60	37 ± 5	(6)	1363 ± 42	40 ± 7	(6)	1297 ± 57
>60	80 ± 6	(4)	1366 ± 30	83 ± 8	(4)	1152 ± 31*
total	54 ± 8	(10)	1364 ± 27	57 ± 8	(10)	1239 ± 42*
AD cases						
≤60	46	(1)	1130	56	(1)	1180
>60	75 ± 8	(3)	1252 ± 7†	86 ± 5	(5)	1114 ± 49*
total	68 ± 9	(4)	1221 ± 31‡	82 ± 6	(6)	1125 ± 41

Data are given as mean ± S.E.M.

*Lower than in male subjects (M-W: $p < 0.05$).

†Reduced as compared to male control subjects in the same age-group (M-W: $p < 0.05$).

‡Idem; $p < 0.01$.

Statistics

Subjects were divided into two age-groups as in our former study (10), one group with ages ranging from 15 to 60 years (designated "young") and the other ranging in age from 61 to 97 years (designated "old"). In addition, controls and AD cases were treated as separate groups. Sex differences and differences in brain weight, postmortem delay, duration of fixation and the various parameters determined for the OXT cell population in the PVN were tested using the Mann-Whitney U-test (M-W). If no sex differences were observed, data of both sexes were pooled for further analysis. Relationships between age, postmortem delay, duration of fixation and the number of OXT cells in the PVN were determined using linear regression analysis and Pearson's r (27).

Bivariate linear models in which the strength of the relationship is reflected by Pearson's r were used to study relationships between OXT cell numbers and brain weight and the other morphological parameters measured.

A 0.05 level of significance was used in all statistical tests.

RESULTS

Postmortem delay and duration of fixation did not differ between young and old controls (M-W: $p > 0.49$), nor between females and males ($p > 0.33$), nor between controls and Alzheimer patients ($p > 0.39$; Table 1). Postmortem delay and duration of fixation showed no significant correlations with any of the morphological parameters determined in the PVN ($p > 0.12$).

Average brain weight in female control subjects (1239 ± 42 g; mean $\pm$ S.E.M.) was significantly lower than in male control subjects (1364 ± 27 g) (M-W: $p < 0.05$; Table 2). If young and old controls were considered separately this sex difference reached statistical significance only in the old subjects (M-W: $p < 0.05$; Table 2). A decrease in brain weight with aging, which almost reached statistical significance (M-W: $p = 0.09$), was observed in female controls (Table 2). No such tendency was observed in male controls (M-W: $p = 0.45$). Average brain weight of AD patients was reduced as compared to controls (M-W: $p < 0.01$). If the sexes were considered separately this difference reached statistical significance in males only (M-W: $p < 0.01$; Table 2).

The OXT cells in the PVN formed a circumscribed population

which could be easily delineated, and which showed good staining in all control subjects (Fig. 2). No sex differences were observed in any of the morphological parameters determined in the PVN in young nor in old control subjects (M-W, $p > 0.20$). Therefore, data of both sexes were pooled for further analysis.

The volume of the OXT cell population in the PVN showed no differences between young and old subjects, nor between controls and AD cases (Table 3). OXT cell densities ranged from an average of 5234 ± 142 cells/mm³ (young AD patients) to 6968 ± 538 cells/mm³ (young control subjects), and showed no significant differences between the various groups (M-W: $p > 0.20$).

The number of OXT cells showed no significant difference between young and old subjects, nor between old AD cases and old controls (M-W: $p = 0.17$, $p = 0.83$, respectively; Table 3, Fig. 3). In addition, no significant correlations were found between age and OXT cell number in control subjects ($r = -0.29$, $p = 0.21$), nor in AD cases ($r = 0.34$, $p = 0.34$). No significant correlations were observed between brain weight and OXT cell number in controls ($p > 0.54$).

The diameter of the nuclei of OXT cells in the PVN did not change with normal aging, ($p = 0.76$) but, in contrast, showed a trend towards an increase in Alzheimer patients (M-W: $p = 0.09$, Table 3).

In addition to the 10 AD patients which were examined in the present experiment, two AD patients (one 66-year-old male and one 81-year-old female) were studied who did not show any staining with the OXT antiserum. Postmortem delay and duration of fixation of these brains were within the range of the other AD cases, which stained well for OXT. Incubations with OXT-neurophysin antiserum resulted in good staining of neurons in the PVN of these two patients.

DISCUSSION

The results of the present immunocytochemical investigation support our previous study which showed absence of changes in total cell numbers in the human PVN with aging and AD in conventionally stained tissue (10). The present results indicate a similar stability of the immunocytochemically identified OXT cell population in the PVN in these conditions. In view of earlier estimations of the total neuron number in the PVN, which ranged from $51,320 \pm 4910$ (11) to $55,500$ (20), our figures of $25,373 \pm 1149$ OXT cells in this nucleus suggest that about half

FIG. 2. Frontal section (6 μm) of the paraventricular nucleus stained with purified anti-OXT serum. PVN, paraventricular nucleus; V, third ventricle. Bar represents 250 μm .

TABLE 3

CELL NUMBER, VOLUME AND MEAN NUCLEAR DIAMETER OF THE OXT CELL POPULATION IN THE PARAVENTRICULAR NUCLEUS IN YOUNG AND OLD CONTROLS AND AD PATIENTS

Group	OXT Cell Number	Volume (mm ³)	Mean Nuclear Diameter (μm)
Controls			
≤60 (12)	26466 ± 1487	3.979 ± 0.290	8.38 ± 0.18
>60 (8)	23733 ± 1763	4.139 ± 0.318	8.28 ± 0.13
AD			
≤60 (2)	21716 ± 933	4.157 ± 0.291	8.37 ± 0.42
>60 (8)	26373 ± 3766	3.924 ± 0.342	8.81 ± 0.26

Data are means ± S.E.M.

of the neurons in the human PVN contain OXT. This is well in line with data reported by Fliers et al. (7).

An effect of differences in postmortem delay or fixation time on our results seems improbable in view of the lack of significant differences between the age groups for these parameters and the absence of significant correlations between postmortem delay and fixation time with any of the morphological parameters studied.

The more pronounced decrease in brain weight in females than in males during normal aging confirms earlier observations (10,18). On the other hand, the additional reduction in brain weight in AD appears to be relatively moderate in females as compared to males. Apparently, a reduction in brain weight per se is not a reliable characteristic of AD. The absence of significant correlations between OXT cell number and brain weight in control subjects suggests that aging patterns in the PVN are independent of changes in other parts of the brain and supports the idea of regional differences in cell loss with aging [cf. (12)].

The absence of changes in the number of OXT cells in the human PVN during normal aging is in line with the stability of total cell numbers in this nucleus in senescence (10). Previous studies have shown that the OXT cells in the PVN show no signs of increased activity in senescence and AD as opposed to the AVP cells in this nucleus (7,15). The lack of age-related changes in nuclear diameter of the OXT cells in the human PVN, as reported in the present study, supports these observations. Therefore, both the morphology and the physiological activity of the OXT cell population in the human PVN appear to remain unaltered in senescence. In contrast, urinary OXT excretion was found to be elevated along with AVP excretion in the senescent rat, suggesting a species difference in this respect (9). In view of our hypothesis that activation of neurons might prevent cell loss during the process of aging (28), one might speculate that the level of activity which is maintained in the OXT cells in the human PVN throughout the life span is sufficient to prevent degenerative changes in this population.

Although Alzheimer-type neuropathological changes were reported in several hypothalamic nuclei in AD, the PVN seems to be little affected in this disease (17, 24, 26). Total cell counts in this nucleus in AD patients were also within the range of controls (10). The absence of changes in the number of OXT neurons in the PVN of Alzheimer patients reported in the present study supports these observations. OXT neurons were still present in two AD patients who did not show OXT immunoreactivity at all, as revealed by the neurophysin staining. This latter finding might suggest a change in the balance between neuropeptide synthesis, transport, release and degradation or, alternatively, a

FIG. 3. Linear regression between OXT cell number in the PVN and age. Data of male (Δ) and female ($\circ$) control patients did not differ and were pooled. No statistically significant correlations were observed in either young or old control subjects. Values of male ($\blacktriangle$) and female ($\bullet$) AD patients are delineated by a minimum convex polygon and were within the range of the controls (further details are given in Table 3).

change in the processing of the precursor molecule in these two cases. Such functional changes might explain the decreases in estrogen-stimulated neurophysin in plasma of AD patients as reported by Christie et al. (3).

OXT concentration in CSF was also found to be reduced in AD (34), but others reported unchanged levels (23). In contrast, elevated levels of OXT were reported in human hippocampus and temporal cortex (19). Animal experiments indicate that extrahypothalamic OXT is derived from a subgroup of neurons in the PVN (5,33). In this respect, the recent report by Calza et al. (2), who found a decrease in the number of parvocellular, i.e., centrally projecting, but not in the number of magnocellular OXT neurons in the rat PVN with aging, is of interest. However, the proportion of centrally projecting OXT cells in the human PVN has not yet been determined.

In conclusion, the results of the present study indicate that the OXT cell population in the human PVN remains stable with aging and in AD. Therefore, reports on diminished OXT secretion in AD patients do not seem to be based upon a loss of OXT cells in this disease. The stability of the OXT cell population in senescence and AD in both males and females suggests that OXT might not only play a role in labor and lactation (29), but also in other, as yet undisclosed functions. In this respect, the early observation by Bodansky and Engel (1) that OXT may have a life-prolonging effect in the rat might be of significance. Therefore, the search for additional functions of OXT, in particular in relation to the aging process appears to be of great interest.

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