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Age-Related Increase in the Total Number of Corticotropin-Releasing Hormone Neurons in the Human Paraventricular Nucleus in Controls and Alzheimer's Disease: Comparison of the Disector With an Unfolding Method

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ABSTRACT

It has been hypothesized that the corticotropin-releasing hormone (CRH) neurons of the hypothalamic paraventricular nucleus (PVN) become hyperactive with age, and even more so in Alzheimer's disease. This hyperactivity could be due to an increased production of CRH per neuron, or an increased number of PVN neurons producing CRH, or both. As a first step in elucidating which of these biological mechanisms might be operative, we have estimated the absolute number of CRH immunoreactive neurons in the PVN of 10 human control subjects between 36 and 91 years of age and 10 Alzheimer patients between 40 and 97 years of age. CRH neurons were immunocytochemically detected in 6 μ m paraffin sections with the aid of a highly specific monoclonal antibody to CRH. The antibody signal was amplified by the biotin-streptavidin and alkaline phosphatase methods. The absolute number of CRH neurons in the PVN was obtained by multiplying the number of CRH neurons in a unit volume (N_V) by the total volume of the PVN. Two different methods were used to estimate the N_V : an unfolding method and a disector method (about three times more time-consuming). Compared to the disector, the unfolding method consistently yielded a lower cell number for all patients by 38% ($\pm 2.8\%$; mean $\pm$ SEM). However, both methods yielded an increase in the absolute number of CRH neurons in control and Alzheimer patients with age. No statistically significant difference in the absolute number of CRH neurons was found between control and Alzheimer patients with both methods. The age-dependent increase in the absolute number of CRH neurons within the PVN of both control and Alzheimer patients is interpreted as a sign of activation of the CRH neurons with age. © 1994 Wiley-Liss, Inc.

Key words: CRH, absolute neuron number, human hypothalamus, dementia, stereology

Corticotropin-releasing hormone (CRH) is a 41 amino acid peptide that has a potent stimulatory effect on the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland (Vale et al., '81; '83). The effect on ACTH release is potentiated by vasopressin (Gillies et al., '82; Rivier and Vale, '83). Immunocytochemical localization of CRH has been described in neuronal cell bodies in the human hypothalamic paraventricular nucleus (PVN), as

well as in axons in the PVN, median eminence (ME), pituitary stalk, and posterior pituitary gland (Pelletier et al., '83; Takahashi et al., '89). Within both the rat and human PVN, the CRH neurons are parvicellular. In the rat,

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these CRH neurons are localized in a well partitioned subnucleus of the PVN (Merchenthaler et al., '82; Swanson et al., '86; Sawchenko and Swanson, '90), while in the human they are spread throughout the entire PVN, except for the most rostral part where they are not found (Raadsheer et al., '93).

The CRH-triggered release of ACTH leads in turn to the release of glucocorticoid hormones, particularly cortisol, from the adrenal cortex. The corticosteroids enhance the body's capacity to cope with stress by affecting the immune, energy producing, cardiovascular and gastrointestinal systems (as first discovered by Selye ['36] and recently reviewed by Swanson et al. ['86] and Bissette ['90]). CRH neurons of the PVN, therefore, have a key role in the stress response via the hypothalamo-pituitary-adrenal (HPA) neuroendocrine axis.

One current hypothesis of biological aging proposes that aging results from alterations in neuroendocrine functions (Hayflick, '85). The experimental work of Sapolsky et al. ('86) indeed demonstrated prolonged glucocorticoid secretion upon stress in old rats compared to young control rats. The prolonged increased levels of corticosteroids are hypothesized to damage brain structures containing high concentrations of corticosteroid receptors, notably the hippocampus (Sapolsky et al., '86). These findings have subsequently been substantiated in human studies, with increased cortisol levels being detected during aging in plasma (Weiner et al., '87) and postmortem cerebrospinal fluid (CSF; Swaab et al., '93). Furthermore, Alzheimer's disease (AD) patients exhibit prolonged ACTH secretion (Dodt et al., '91) and increased urinary, post-dexamethasone plasma and post-mortem CSF cortisol levels (Gurevich et al., '90; Martignoni et al., '90; Maeda et al., '91; Swaab et al., '93).

Since glucocorticoid secretion is a result of activation of the HPA-axis by CRH neurons, it is possible that these elevated glucocorticoid levels reflect hyperactivity of the CRH neurons within the PVN. This hyperactivity could be due to an increased production of CRH per neuron, an increased number of PVN neurons producing CRH, or both. To elucidate which mechanisms might be operative, it was imperative to start by estimating the absolute number of CRH neurons during aging and in AD (cf. Braendgaard and Gundersen, '86; Swaab and Uylings, '87; Pakkenburg and Gundersen, '88; West and Gundersen, '90; Oorschot et al., '91). Numbers of CRH neurons in postmortem tissue of control subjects and AD patients have been estimated by other workers (i.e., Pearson and Jennes, '87; Powers et al., '87; Kelley and Kowall, '89), but not under the right conditions and by means of qualitative estimates of neuronal number.

We therefore estimated the absolute numbers of immunoreactive CRH neurons within the PVN of the human hypothalamus. Two different methods were used to determine cell number per volume (N_V), namely the deconvolution or unfolding method (Cruz-Orive, '78; Weibel, '79) and the disector method (Sterio, '84). In combination with the total PVN volume estimated by Cavalieri's method, absolute number estimates were obtained. The disector method yields unbiased results when the general requirement of accurate cell identification in two adjacent sections is fulfilled (Gundersen, '86). By contrast, the unfolding method is only unbiased under much more restricted conditions. These include a convex shape and random orientation of the neuronal population under investigation (Weibel '79). The reason for comparing both methods is to find out which

one is most appropriate and efficient for estimating the absolute number of CRH neurons in our collection of human hypothalami with our immunocytochemical procedure (Raadsheer et al., '93). We found systematic differences between the outcomes of the two methods; the cell numbers estimated by the unfolding method were $38\% \pm 2.8\%$ (mean $\pm$ SEM) lower than those found by the disector method. Irrespective of the method used, the results demonstrate an increase in the total number of CRH neurons during aging.

MATERIALS AND METHODS

Human brain material

Hypothalami from 10 clinically well-defined Alzheimer's disease patients and 10 control subjects without a primary neurologic or psychiatric disease were obtained at autopsy according to the dissection protocol of the Netherlands Brain Bank (for clinical information, see Table 1). The disease cases were clinically diagnosed as having "probable Alzheimer's disease" according to NINCDS-ADRDA criteria (McKahn et al., '84), and all had a neuropathological confirmation based on the numbers and distribution of senile plaques and neurofibrillary tangles, as determined by using conventional histopathological staining techniques on formalin-fixed tissue. AD patients and controls were age matched (mean age in years $\pm$ SEM: AD = 65.7 ± 5.6 ; contr. = 65.7 ± 6.0). The hypothalami were fixed in 4% w/v formaldehyde (for 41.1 ± 6.6 days at room temperature), after which they were dehydrated in graded ethanol and embedded in paraffin (Histowax, melting point 56–58°C, Histo-Lab L.T.D., Göteborg, Sweden). Serial 6 μ m frontal sections were cut on a Leitz microtome and stored at room temperature.

Mounting the sections

To apply the disector method, a systematic series of two adjacent sections is required. It is also necessary for the neurons to be accurately identified within both sections. To achieve this, it is important to reproducibly stretch and mount the adjacent sections. This was achieved by using a procedure based on Pakkenberg and Gundersen ('88). A series of section pairs, from which the first pair was randomly chosen, were taken at 300 μ m intervals throughout the complete PVN region. Sections were transferred to a 10°C water bath. After 60 seconds, the sections were transferred to a 47–48°C 0.5% w/v bovine serum albumin (BSA)-bath. The stretched sections were removed after 60 seconds from the BSA solution and mounted on chrome-alum coated object slides. They were then dried at 37°C overnight and stored at room temperature until immunostaining. From each pair of sections, one section was also used for the unfolding procedure.

Immunocytochemical staining

Before staining, the sections were deparaffinized in xylene and rehydrated through a graded ethanol series. To diminish potential volume changes during the rehydration procedure, the time in each bath was standardized for all sections. After the final rehydration in 50% ethanol, CRH was immunocytochemically stained by a modification of the method of Raadsheer et al. ('93). After washing (3×10 minutes) in Tris-buffered saline (TBS, consisting of 0.05 M Tris and 0.15 M NaCl, pH 7.6), the sections were incubated for one hour with staining buffer (TBS with 0.5% v/v

TABLE 1. Clinical and pathological data of cases studied¹

Patient #	Sex	Age (yr)	BW (g)	PMD (hr)	Fix (d)	Clinical diagnoses
<i>Control subjects</i>						
1. 91009	F	36	1348	< 72	61	Acute death, duodenal perforation
2. 88311.4	M	49	1309	< 24	49	Overdose barbiturates
3. 82109	F	50	1360	53	nd	Bronchopneumonia, tumor carotis interna
4. 88378	M	59	1180	< 24	29	Renal insufficiency
5. 81014	F	64	1090	8	44	Hypovolemic shock
6. 82086	M	68	1300	6h30	nd	Cardiac failure
7. 80118	M	70	1165	nd	nd	Pneumonia
8. 82176	M	83	1400	nd	nd	Bronchopneumonia
9. 83158	F	87	1140	7	34	Myocardial failure
10. 83179	F	91	1060	36	28	Respiratory insufficiency
<i>Alzheimer's disease patients</i>						
11. 89166	M	40	1410	2h50	28	AD, cachexia
12. 86364.52	M	45	1027	4	119	AD
13. 90262	M	49	1426	4h25	nd	AD
14. 91091	F	61	1060	3h45	29	AD
15. 90-41	M	63	1350	5	28	AD
16. 88325	F	64	800	2h50	31	AD, cachexia, dehydration
17. 88252	M	66	1250	3h15	31	AD, cachexia, septic shock
18. 88272	F	84	1000	4h30	30	AD, cachexia, septic shock
19. 8950	F	88	950	2h45	28	AD, diabetes mellitus
20. 86001	M	97	1200	5	40	AD

¹Note that AD patient #8950 received Imipramine (3x 20 mg per day). Long term administration of this drug to rats decreased CRH mRNA levels by 37% in the PVN (Brady et al., '91b). None of the other AD patients showed symptoms of depression according to the patient documentation or were treated with any (antidepressant) drug from which is known that it influences CRH neurons in the rat PVN (c.f. Brady et al., '91a; Veith et al., '93). yr, years; BW, brain weight; g, grams; PMD, postmortem delay; hr, hours and minutes; Fix, fixation time; d, days; F, female; M, male; nd, not determined; AD, Alzheimer's disease.

Triton X-100 and 0.25% w/v gelatin) containing 1% normal goat serum in order to reduce aspecific background staining. Thereafter, the sections were washed in TBS and incubated with a highly specific monoclonal rat anti-CRH antibody (PFU 83, Van Oers et al., '89; for specificity controls, see Raadsheer et al., '93), diluted 1:100,000 in staining buffer. The sections were incubated in this primary antibody for 30 minutes at room temperature and subsequently overnight at 4°C. During this incubation, the sections were enclosed within humidified trays to prevent evaporation of the antibody solution. Immunocytochemical control sections were always processed alongside the experimental sections. These consisted of one positive control section from a subject known from previous work (Raadsheer et al., '93) to have strongly immunoreactive CRH neurons and two negative control sections from which the primary antibody was omitted (these control sections were extra experimental sections from the subject whose sections were being processed).

The following day, the sections were washed in TBS (3 x 10 minutes) and then incubated for 1 hour at room temperature with a biotinylated sheep anti-rat antibody (Amersham, U.K.), diluted 1:200 in staining buffer. After further washing (3 x 10 min TBS), the sections were incubated for 1 hour at room temperature with streptavidine-conjugated alkaline phosphatase (KPL), diluted 1:100 in staining buffer. Thereafter, the sections were washed twice in TBS (10 minutes each), then rinsed for 10 minutes in 0.05 M Tris/HCl (pH 8.5). The phosphatase activity was demonstrated by incubating the sections for 10 minutes at room temperature with a filtered suspension of 2 mg Fast Blue BB base (Sigma) in 10 ml 0.1 M Tris (pH 8.5)

containing 2 mg Naphthol-AS-AX phosphate (Sigma), 0.2 ml N,N-dimethylformamide and 3 mg levamisole (Sigma). The sections were then rinsed in TBS and histochemically counter-stained with neutral red (1% in H₂O for 6 seconds) to stain the cytoplasm of non-immunoreactive cells. Counter-staining was necessary to delineate the boundary of the PVN for volume estimations. Finally, the sections were rinsed in TBS, coverslipped with Kaiser's glycerin gelatin (Merck), sealed with nail varnish, and stored at 4°C.

Estimation of the volume of the PVN

The volume of the PVN (necessary for the calculation of the absolute number of CRH neurons, see further) was determined using Cavalieri's direct volume estimate (Sterio, '84; Gundersen and Jensen, '87). The cross-sectional area of the PVN was measured in every 50th serial section (from which the first one was randomly chosen) that contained the PVN (i.e., every section separated by a nominal distance, *d*, of about 300 µm [see below for final values for the mean section thickness, *t*, which is multiplied by 50 to obtain *d*]). These areas were summed and multiplied by the distance *d*.

The cross-sectional area of the PVN was measured by a method reported previously by Goudsmit et al. ('90). Every 50th section containing the PVN was projected onto a Calcomp 2000 digitizer (using a Zeiss light microscope with a 2.0× Plan objective, 12.5× Plan oculars, and a 1.8× drawing tube). The projected boundary of the PVN in each section was then traced with a digitizing pen. The digitized areas were stored on an HP9000/835 computer connected to the digitizing pad. If the cross-sectional PVN area extended beyond the field of vision in a particular section, it was measured step-wise using a quadrangular grid in one of the oculars as a reference. Each area was then summed and converted to real area by correcting for magnification. The PVN volume was then estimated as outlined above.

Estimation of the numerical density, or *N_V*, of PVN CRH neurons

Two different methods were used to estimate the numerical density (*N_V*) of PVN CRH neurons: the deconvolution or unfolding method (Cruz-Orive, '78; Weibel, '79) and the disector method (Sterio, '84). For both methods, every 50th section or section pair throughout the entire PVN was sampled. This resulted in 9–25 sections or section pairs being used per subject.

For the deconvolution or unfolding method, the *N_V* of CRH neurons in the PVN was estimated by using the same (single) sections and the same equipment with a 40× Plan objective as described for the estimation of the PVN volume. Each section was systematically and carefully scanned through its entire PVN for all blue immunoreactive CRH neurons that contained a white nucleus. The area of each nuclear profile was traced onto the digitizer pad, and the digitized areas of all CRH neuronal nuclei for each patient were stored in an HP9000/835 computer connected to the digitizing pad. These areas were corrected for magnification and "unfolded" (Weibel, '79) by a computer program written by one of us. This program incorporates a modification for the class centres of the observed profile distribution proposed by Cruz-Orive (1978) and contains a correction for section thickness (*t*, see below).

For a particular density (*N_V*) of spherical cells, the number of profiles per area (*N_A*) found in single sections depends on the section thickness (*t*) and the cell size (*D*). If

the cells have one size, N_V can be estimated from N_A using the correction of Abercrombie ('46): $N_V = N_A/(t + D)$, where D equals the maximum of the observed profile diameters. However, if the cells have different sizes, D is not available and an unfolding procedure is necessary, which involves repeated application of Abercrombie's formula. By applying Abercrombie's formula once, with the mean diameter substituted for D , we run the risk of underestimating D and overestimating N_V .

For the disector method, the boundary of the PVN in every 50th section (i.e., the first or reference section of each disector pair, which was the same as used for the PVN estimation and the unfolding method) was accurately drawn onto A4 sheets of paper using a Zeiss light microscope with a $3.2\times$ Plan objective, $10\times$ Plan oculars, and a $1.8\times$ drawing tube. The sections were systematically and carefully scanned for all immunoreactive CRH neurons inside the PVN boundaries. Accurate identification of all immunoreactive neuronal profiles was ensured by the use of $10\times$ and $40\times$ objectives as well. CRH neurons were counted as disector neurons for the reference section if their nucleus was present in only the reference section, but not in the adjacent look-up section (Sterio, '84; Gundersen et al., '88). The numerical density was computed from $N_V = \Sigma Q^- / \Sigma A \times t$, in which ΣQ^- = sum of the total number of disector neurons, ΣA = summed area of the PVN within the reference sections, and t = the mean section thickness.

Estimation of the absolute number of CRH neurons

Estimation of the absolute number of CRH neurons in the PVN was computed by multiplying the N_V of the unfolding procedure and of the disector procedure by the PVN volume. For all subjects, these parameters were estimated for the left PVN, with the exception of four subjects in which the PVN was not completely present on that side.

Estimation of mean section thickness

Section thickness was estimated by the differential focusing technique (Uylings et al., '86), using a linear displacement device (Sony Magnescale LH20-RS-232C) attached to a Zeiss microscope with a Plan $100\times$ oil-immersion objective (with a numerical aperture of 1.25) and Plan $10\times$ oculars. In every 50th section, the thickness within the PVN was measured at least twice in two to three separate areas. The refractive indexes of the glass slide, the section in the glycerin/gelatin, the coverslip, and the immersion oil were all very similar (i.e., 1.48–1.51). The measured distance between the focused upper and lower faces of the sections was corrected for depth of vision (Uylings et al., '86).

Statistics

Data analysis requires non-parametric statistics, since the data are discrete and unlikely to be normally distributed.

The difference in outcome of the two estimation methods for the absolute number of CRH neurons was tested by the Wilcoxon signed rank test for two paired samples. Estimation methods were considered to disagree if they differed statistically at $P < 0.05$.

Subjects were divided into two age groups according to the literature (Dodt et al., '91; Swaab et al., '93). The young group ranged from 36 to 64 years of age; the old group, from

65 to 97. Since this preconceived subdivision was inappropriate to show clear differences in the absolute number of CRH neurons between young and old subjects, we also made a post-facto subdivision with the boundary between young and old at 70 years of age.

Potential differences in the absolute number of CRH neurons, PVN volume, and section thickness for control and Alzheimer patients, young and old subjects, and male and female patients, were analyzed by the Mann-Whitney U test using SPSS-X (SPSS Inc., Chicago) followed by a two-tailed probability of Z .

Relations between the number of CRH neurons in the human PVN and factors like age, brain weight, postmortem delay, and fixation time were determined using linear regression and Spearman's rho based on a two-tailed test (SPSS-X analyses). The results were considered statistically significant when $P < 0.05$.

RESULTS

Immunocytochemical staining

For all 20 subjects, immunocytochemical staining for CRH revealed blue immunoreactive neurons within a lightly red histochemically counter-stained PVN (Fig. 1). The immunocytochemical reaction product was distributed over the entire cytoplasm. No CRH cells were observed outside the PVN, which could easily be delineated by the neutral red counter-staining. All positive control sections showed strong immunoreactivity for CRH, while all negative control sections did not.

Estimation of the volume of the PVN

PVN volumes from Alzheimer patients ($8.2 \pm 0.6 \text{ mm}^3$) did not statistically significantly differ from PVN volumes from control subjects ($8.1 \pm 1.0 \text{ mm}^3$, $P = 0.631$). Of course, these data provide no information on whether these values are indicative of the in vivo volume of the PVN because differential shrinkage during paraffin embedding as a result of age and/or dementia was not measured. For the determination of the absolute cell number, shrinkage is not of importance.

Estimation of the absolute number of CRH neurons in the human PVN

In all patients, unfolding estimates of the absolute number of CRH neurons in the human PVN were systematically $38\% \pm 2.8\%$ (mean $\pm$ SEM) times lower than disector estimates (Fig. 2), resulting in a statistical disagreement (Wilcoxon, $P < 0.001$) between both methods. However, this disagreement had no consequences for biological effects on the CRH cell number because of a very strong correlation between the results obtained by the two methods (Spearman's rho = 0.981, $P < 0.001$, Fig. 2).

A positive correlation was found between age and the absolute number of CRH neurons in the PVN for both the unfolding (Fig. 3a) and the disector (Fig. 3b) methods. This age effect was statistically significant for control subjects (disector: rho = 0.66, $P = 0.02$; unfolding: rho = 0.62, $P = 0.03$), but in AD patients the age effect was almost significant for the disector data (rho = 0.53, $P = 0.06$), but not for the unfolding data (rho = 0.41, $P = 0.12$).

Although a statistically significant positive correlation between age and absolute number of CRH neurons in control subjects was present (see above), there was no

Fig. 1. Frontal adjacent paraffin sections (6 μ m) through a human hypothalamic-paraventricular nucleus (PVN) immunocytochemically stained for corticotropin-releasing hormone (CRH) and histochemically counter-stained with neutral red. The CRH containing neurons are stained blue (black cells with arrows), whereas the CRH depleted cells are faintly stained red (light gray cells). The profile of the CRH neuron (1) in the reference section (A) contains a nucleus, whereas in the look-up section (B) no nucleus is present. Therefore, this cell is considered as a disector neuron of the reference section. The profile of the CRH neuron (2) in the reference section does not show a nucleus, although a nucleus is present in the look-up section. Therefore, this cell is considered to be a disector cell for the look-up section. (C) Negative control section showing no CRH positive staining. Bar = 25 μ m.

Fig. 2. The absolute number of CRH neurons in the human PVN determined by both the unfolding method and the disector method. The regression line shows that there is, per patient, a high correlation between methods (Spearman's $\rho = 0.981$, $P < 0.001$). However, note that in all patients, unfolding estimates are systematically $38\% \pm 2.8\%$ (mean $\pm$ SEM) times lower than disector estimates, resulting in a statistical disagreement (Wilcoxon, $P < 0.001$) between both methods. Filled circles indicate control subjects and open circles show Alzheimer (AD) patients.

statistically significant difference in absolute CRH neuron number between young and old control subjects (for both the disector and unfolding procedures, $P = 0.15$ and $P = 0.22$, Fig. 4B, E, respectively) when the boundary between young and old was defined at the age of 65 years (which is common in the literature; see, for example, Dodt et al., '91; Swaab et al., '93). However, if the boundary was defined at 70 years (Fig. 4C, F), a statistically significant difference was found (disector: $P = 0.02$; unfolding: $P = 0.04$). For the AD patients, who showed a trend toward a significant positive correlation between age and CRH cell number, the disector method showed a significant difference between patients younger and older than 65 years ($P = 0.02$, Fig. 4B), whereas the unfolding method showed only a tendency toward a difference ($P = 0.07$, Fig. 4E). After defining the boundary at 70 years (Fig. 4C, F), the difference between young and old was statistically significant for both estimation methods ($P = 0.02$).

When the absolute number of CRH neurons in the PVN of control and Alzheimer patients were compared, there were no statistically significant differences (disector: $P = 0.09$, Fig. 4A; unfolding: $P = 0.11$, Fig. 4D). There were also no statistically significant differences between control subjects and AD patients at a young age (< 65 years, disector and unfolding: $P = 0.13$; < 70 years, disector and unfolding: $P = 0.73$), and at an old age (≥ 65 years, disector: $P = 0.41$, and unfolding: $P = 0.73$; ≥ 70 years, disector and unfolding: $P = 0.86$).

Gender did not influence the absolute number of CRH neurons (disector and unfolding: $P = 0.71$). The absolute number of CRH neurons was also not correlated to factors

Fig. 3. Linear regression between age and absolute CRH cell number in the PVN estimated by the disector method (A) and the unfolding method (B). Filled circles and solid lines indicate control subjects; open circles and dashed lines indicate Alzheimer's disease patients. A significant correlation was found between age and absolute CRH cell number for control subjects with both the disector and unfolding methods (respectively: $\rho = 0.66$, $P = 0.02$; $\rho = 0.62$, $P = 0.03$). However, in AD patients, the age effect was almost significant for the disector data ($\rho = 0.53$, $P = 0.06$) but not for the unfolding data ($\rho = 0.41$, $P = 0.12$).

such as brain weight (disector: $P = 0.31$; unfolding: $P = 0.23$), postmortem delay (disector: $P = 0.25$; unfolding: $P = 0.27$), or fixation time of the material (disector: $P = 0.17$; unfolding: $P = 0.15$).

Section thickness measurements

In all sections, the upper and lower focal planes for the blue and the neutral red stained neurons were identical. For each group, the mean section thickness was as follows: young control, $5.95 \pm 0.42 \mu\text{m}$ (mean $\pm$ SEM); old control, 7.04 ± 0.59 ; young AD, 6.14 ± 0.15 ; old AD, 6.09 ± 0.39 .

Fig. 4. Absolute number of CRH neurons in the PVN. Bars represent means and SEM; n, number of subjects per group. When the absolute number of CRH neurons was compared between control subjects and Alzheimer's patients, both the unfolding (A) and disector (D) methods gave no statistically significant differences. Subdivision into age groups (B, C, E, F) did not result in a significant difference between control subjects and Alzheimer patients. No statistically significant difference between young and old control subjects was present with both the disector ($P = 0.15$) and unfolding ($P = 0.22$) methods when the age boundary was defined at 65 years (B and E). But

when the border was chosen at 70 years (C and F), significant differences were evident between young and old control subjects with the disector ($P = 0.02$) and the unfolding ($p = 0.04$) methods. With the unfolding method, a tendency ($P = 0.07$) toward a statistically significant difference between young (< 65 years) and old (≥ 65 years) Alzheimer's patients was present (E). This difference was statistically significant with the disector method (B), and also when the boundary between young and old was defined at 70 years (C and F) with both methods ($P = 0.02$). A bar with an asterisk is significantly ($0.01 < P < 0.05$) higher than other age groups.

The section thickness was not statistically significantly different between groups. The variability within and between each section was estimated by the coefficient of error (or CE = SEM/mean) and was always < 0.10 , as is desirable (Gundersen, '86).

DISCUSSION

Biological considerations

The results of this study show that the absolute number of CRH immunoreactive neurons in the human PVN increases with age (Fig. 3). This holds true for both the control subjects and the AD patients. According to the generally accepted principle that adult neurons do not divide, this increment likely indicates that, with age, increasing numbers of parvicellular PVN neurons become active in terms of CRH production and storage. There is good evidence from studies in human and rat PVN that the peptide production of both the parvicellular and magnocellular neurons increase with age. Recently, we reported (Raadsheer et al., '93) an age-dependent increase in the

co-localization of vasopressin (AVP) in the parvicellular CRH neurons in the human PVN. Co-localization was increasingly noted in older control subjects (ages 43–91 years), while no co-localization was evident in younger subjects (ages 23–37 years). Based on rat experiments, in which stress was found to activate parvicellular CRH neurons to produce and costore AVP (Kiss et al., '84; De Goeij et al., '91, '92a,b), the increase in co-localization of AVP with CRH in the human PVN has been interpreted as an index of increased activity with age. The present findings add to this view and show that, with age, increasing numbers of parvicellular neurons are able, or are being induced, to produce CRH in immunodetectable quantities (i.e., without immediately transporting and releasing it). A recent study on the AVP-producing neurons of the human PVN has also shown a statistically significant increase in the absolute number of these neurons during normal aging (Van der Woude et al., '93). In this study, the mean absolute AVP cell number ($17,039 \pm 2,082$, $\pm$ SEM) in young (≤ 60 years of age) control subjects was significantly lower than in old (> 60 years of age) controls ($26,621 \pm 2,368$). A part of

this increase in AVP cell number could be attributed to the increase in CRH cell number, from which the majority show co-localization with AVP at old age (Raadsheer et al., '93).

These findings are in agreement with various studies indicating an activation of the HPA-axis during aging (Weiner et al., '87; Dodt et al., '91). Hyperactivity of the HPA-axis might have severe long-term consequences, since increased levels of corticosteroids are hypothesized to lead to irreversible hippocampal damage (Sapolsky et al., '86). Hippocampal lesions may then lead to a hyperactivity of CRH neurons (Herman et al., '89), thus starting a positive feedback loop leading to more CRH activation and more brain damage. Whether such a mechanism is indeed operating in the human brain in aging and AD remains, however, to be proven.

When the absolute number of CRH neurons in the PVN of AD patients is compared to age-matched controls, there is no significant increase in AD (Fig. 4). These findings seemingly contradict the hypotheses that there is an even higher activation of the HPA-axis in dementia than during aging (Gurevich et al., '90; Maeda et al., '91) and that the CRH neurons are instrumental in this activation (Von Bardeleben and Holsboer, '90). However, the possibility remains that the CRH neurons in AD patients are more active than the CRH neurons in controls, but that this activity is not represented in an increase in the absolute number of CRH neurons. A higher activation of the HPA-axis can, for example, also result from an increased number of CRH neurons that co-localize AVP with CRH, since AVP potentiates the effect of CRH on ACTH release (Gillies et al., '82; Rivier and Vale, '83). This possibility will be investigated by double-labeling of these two neuropeptides in AD. In addition, hyperactivity can result from increased CRH production and release per neuron. This potential source of hyperactivity is currently studied by *in situ* hybridization. By this method, the intensity of CRH mRNA staining per neuron, and the amount of mRNA per population, will be used as a measure of the level of CRH synthesising activity of the CRH neurons.

At the other hand, the results of our present study seem at first to conflict with reports in which CSF CRH concentrations did not correlate with age or were even decreased in Alzheimer's disease (reviewed by Bisette and Nemeroff, '90). However, the PVN is not the only possible source of CSF CRH. Since also CRH neurons from cortical, limbic, and brainstem regions, which are all in close proximity to the ventricular system, will probably contribute to the CSF CRH pool (Owens and Nemeroff, '93), CSF CRH determinations do not provide direct information on the activity of the paraventricular CRH neurons. Because it is known that CRH immunoreactivity in the cortex decreases in Alzheimer's disease (De Souza et al., '90), the cortical CRH neurons might be mainly responsible for the lower CSF CRH in AD patients. The finding of the group of De Souza (1990) that in postmortem hypothalami from control patients and those with histologically confirmed Alzheimer's disease, the CRH-like immunoreactivity measured by radio-immunoassay was not significantly different, agrees fully with our observations of the number of CRH neurons.

Because of the lack of statistically significant differences between control subjects and Alzheimer patients, we also determined the correlation between age and the absolute number of CRH neurons in both groups together. This appeared to be highly significant (disector: $P = 0.001$; unfolding: $P = 0.004$), which again demonstrates the

age-dependant increase in the absolute number of CRH neurons.

Stereological considerations

The absolute number results discussed above were estimated by both the unfolding or deconvolution method (Cruz-Orive, '78; Weibel, '79) and the disector method (Sterio, '84).

In a recent review, Colonnier and Beaulieu ('85) compared several formulae for the quantification of synapses and cell number. The methods they received, however, do not incorporate section thickness, which in our material is of the same order as the mean diameter of the cell nucleus. Consequently, they cannot be applied to our material.

Although the trend of increasing numbers detected by both the unfolding and disector method is highly correlated, a statistical disagreement between the actual numbers obtained by each method is evident. The absolute number of CRH neurons in the PVN estimated by the unfolding method is consistently 38% lower for all groups than the absolute number of CRH neurons estimated by the disector method (Fig. 2). This difference between the two methods in the actual absolute number of CRH neurons was due to the significantly lower unfolding N_V as compared to the disector N_V . In principle, the disector technique is unbiased because no assumptions between cell shape, size, and orientation need to be made (Sterio, '84), whereas the unfolding method is only unbiased under strict conditions (Weibel, '79). However, in our material, some practical problems are associated with both methods. Thus, the difference between both methods may result from an underestimation by the unfolding method and/or an overestimation by the disector method.

An underestimation of the N_V by the deconvolution method could be explained by not fulfilling the general requirements necessary for this number estimator. The exact contributions of the various factors can not be given, however. One key assumption in the unfolding method is that the measured profiles all originate from spheres (Weibel, '79). However, in this study, oval nuclei were seen in many CRH neurons. Therefore, there is a tendency to measure the area (and thus derive the diameter) through the major axis of the nucleus, which overestimates the mean diameter, D , of the nucleus. This led to an underestimation of N_V , since D is in the denominator of the N_V calculation [$N_V = N_A / (t + D)$]. The presence of the relatively high number of oval nuclei sectioned through their major axis in these frontal (i.e., coronal) sections is probably a result of the non-random orientation of the CRH neurons, since CRH neurons are projecting to the median eminence, pituitary stalk, and pituitary gland.

For the unfolding method, it is also necessary to measure a reasonable number of nuclear diameters (e.g., 320–440, or even more if there is a deviation from a spherical shape, Mackay and Bedi, '87; Bedi et al., '89). This is because larger profiles have a greater probability of being sampled when only one section is used. Thus, if the measured number is low (which it consistently was in this study, with only 64 ± 13 [mean $\pm$ SEM] profiles being counted in the sections for the 20 subjects), then the measured D is likely to be an overestimate of the real population D (because smaller profiles have not been adequately sampled). The measured D is then corrected upwards (i.e. slightly increased) during the unfolding procedure because not all the measured D s are based on the maximum diameter of each

profile (Cruz-Orive, '78). Thus, the original overestimation is increased a little bit more. As described in the preceding paragraph, this will lead to an underestimation of the final unfolded N_V .

An overestimation of the N_V by the disector method could result from violating the general requirement that the distance between the lower face of the reference section and the lower face of the look-up section must be less than the minimum projected height of the smallest particle (4.4 μm in our case). It is best to use a section thickness that is one-quarter to one-third of the mean diameter of the profile being measured (Gundersen et al., '88). However, in this study the ratio was closer to or even larger than 1:1, with a mean section thickness of 6.31 μm (± 0.21 , SEM). If the particles to be measured are sparsely distributed, slightly thicker sections can be used (Gundersen et al., '88). For this study, the CRH neurons are indeed present at a very low density. However, the section thickness is more than slightly thicker, and it therefore seems likely that an overestimation of N_V by the disector procedure in this study may be occurring, although its incidence and magnitude is probably low.

Another requirement for the disector method is equivalent stretching of adjacent sections in order to facilitate accurate cell identification. When this is achieved (Fig. 1), profiles of the same cell can be identified in both reference plane and look-up plane. However, this could not always be unambiguously determined. This is because parts of the adjacent sections were not evenly stretched, and the counterstaining could not provide enough information about the profiles with respect to each other. If the tissue was damaged (i.e., by little cracks, etc.), it was even more difficult to identify corresponding profiles in the reference section and in the look-up section. In all cases in which there was doubt whether or not a profile in the look-up section corresponded to a profile in the reference profile, this profile was counted as a positive disector cell. This could contribute to an overestimation of the disector N_V , although the incidence of uneven stretching of adjacent sections, and of tissue damage, was low.

The measurement of N_V with the disector showed a high variability between sections. For example, for each old (i.e., ≥ 65 years) control subject, the coefficient of error ($\text{CE} = \text{SEM}/\text{mean}$) ranged from 0.17 to 0.29, which was above the desirable CE of <0.10 (Gundersen, '86). However, the observed CE^2 within patients was less than half the observed coefficient of variation squared (CV^2 , $\text{CV} = \text{SD}/\text{mean}$) between patients, which meant that enough neurons in each patient were counted (Gundersen, '86). In the old control group, for example, the observed $\text{CE}^2(N_V)$ was 0.01, while the observed $\text{CV}^2(N_V)$ was 0.09. Furthermore, the high $\text{CE}(N_V)$ is due to the inhomogeneous distribution of CRH neurons in the PVN, since they are found only in the central and caudal parts (Raadsheer et al., '93). However, as also described by Pakkenberg et al. (1991), this precision is adequate for the purpose of comparing young and old, and control and demented, subjects with respect to CRH neurons in the PVN.

Thus, from the comparison of the unfolding method with the disector method, it seems very likely that the unfolding method is indeed underestimating the N_V for these CRH neurons, because only a small number of non-randomly oriented non-spherical profiles of nuclei from CRH neurons have been measured. The disector may be overestimating the N_V due to the relatively very large section thickness as

compared to the profiles of the CRH neuron nuclei, and due to uneven stretching of, and tissue damage to, adjacent reference and look-up sections. The degree of overestimation due to these technical shortcomings, which were rather rare events, is probably small. When comparing the disector method with another unfolding method in estimating the number of nerve cells in the substantia nigra in normal subjects and patients with Parkinson's disease, Pakkenberg et al. (1991) also found a difference in N_V between methods by 20 to 40%.

Is there then a preferable method to use? Considering all these previous points, this is hard to tell. In the present study, the real absolute number of CRH neurons will lie somewhere between the estimates derived by the unfolding method and the disector method, with conceivably a leaning towards the disector values because their possible overestimation is perhaps a smaller deviation from the real number than the degree of underestimation by the unfolding method. However, it needs to be acknowledged that, for our material, the disector method is approximately three times more time-consuming performing the stereological analyses. This is an argument in favour of using the unfolding method. The disector also requires twice as many sections, and thus twice the volume of immunoreagents. But this would also be needed to improve the number of CRH profiles measured for the unfolding analysis. Since the methods reveal the same biological results, it could be argued that they both seem appropriate for answering the question that was raised. However, in other work, the assumptions inherent to the unfolding analysis do not always hold for both the control and treated conditions (see Mendis-Handagama and Ewing, '90). Since it is not usually known until the end of a stereological experiment whether the unfolding analysis is appropriate, it seems preferable to use the disector method, in spite of its time-consuming nature. The material of our collection of hypothalami was unfortunately not suitable for the application of other unbiased stereological tools, such as the optical disector (Gundersen, '86). In addition, preparing the material in such a way that the requirements for this stereological method are fulfilled (e.g. thicker sections, other embedding material) might well interfere with the requirements for the immunocytochemical procedure (i.e., a full penetration of the antibody; cf. Piekut and Casey, '83).

It is also worth noting that, while meaningful comparisons can be made between different methods for estimating N_V for the same tissue, it is unlikely that meaningful comparisons of the N_V and PVN volume can be made between patients, between young and old, and between control and demented groups. This is because the numerous dimensional changes of tissue during the agonal/postmortem phases, during fixation, dehydration, paraffin embedding, and sectioning, as well as the likelihood that the degree of distortion varies with age and according to disease state, make it likely that the estimations do not represent the real *in vivo* values (see Braendgaard and Gundersen, '86; Swaab and Uylings, '87; Pakkenberg and Gundersen, '88; Oorschot et al., '91). By contrast, the absolute number of CRH neurons is independent of all these factors, thereby underscoring its value in making meaningful biological comparisons—and thus meaningful biological conclusions—between groups.

In conclusion, the absolute number of CRH neurons in the human PVN increases gradually during aging, both in control subjects and Alzheimer's disease patients. The

age-dependent increase in the absolute number of CRH neurons is interpreted as a rise in activation of these neurons. In this particular study, it is of no significance which number estimation procedure is used in order to detect the age-dependent increase in the absolute number of CRH neurons.

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Cover Illustration: Transmission electron micrograph of a chicken cochlear nucleus neuron from an animal sacrificed 6 hours after removal of the ipsilateral cochlea. Cochlea removal immediately silences eighth nerve input to these neurons and within hours causes a transient proliferation of mitochondria. Tissue was stained for cytochrome oxidase histochemistry prior to embedding. See Hyde and Durham, Rapid increase in mitochondrial volume in nucleus magnocellularis neurons following cochlea removal. The Journal of Comparative Neurology, Volume 339, Number 1, pages 27-48.

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