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Localization of corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus of the human hypothalamus; age-dependent colocalization with vasopressin

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Immunocytochemical staining, using a monoclonal antibody against corticotropin-releasing hormone, was performed on hypothalami of 13 human subjects between 23 and 91 years of age who had not suffered from a primary neurological or psychiatric disease. Corticotropin-releasing hormone (CRH) immunoreactivity was present in neurons of the paraventricular nucleus (PVN) and in their fibers running to the median eminence. The CRH-positive neurons were scattered throughout the PVN, but in the rostral part relatively few cells were present. There were large individual differences in the number and staining intensity of CRH neurons in the PVN and in the staining intensity of the median eminence. These differences seemed not to be attributed to age, sex, postmortem delay, fixation time or hour of death. In the rat, too, no relationship was found between a postmortem delay of up to 24 h and CRH staining intensity of the median eminence. Since the distribution of CRH-immunoreactive neurons in the human PVN strongly overlap with vasopressin, colocalization of these peptides was investigated in a double label study and indeed found in subjects ranging between 43 and 91 years of age. However, cells staining for only one of the peptides were also observed. The vasopressin cells had a mean cellular profile area which was 2.3 times as large as the CRH cells and 2.2 times as large as the CRH and vasopressin containing neurons. In younger subjects (23–37 years of age) no colocalization of the two peptides was seen. The age-dependent colocalization of CRH with vasopressin is interpreted as a sign of increased activation of the CRH neurons with age.

INTRODUCTION

Immunocytochemical localization of corticotropin-releasing hormone (CRH) in the human hypothalamus has been described in the paraventricular nucleus (PVN), median eminence (ME), pituitary stalk and posterior pituitary gland^{24,38}. However, it has so far not been investigated whether the CRH neurons in the human PVN are localized in a subnucleus of the PVN, as has been described for the rat³⁷. In the rat PVN, CRH neurons are situated in the lateral part of the medial parvocellular zone and project via the paraventriculo-neurohypophyseal tract to the portal capillaries of the ME, where the peptide is released. After excretion, the 41-aminoacid peptide CRH has a potent stimulatory effect on adrenocorticotrophic hormone (ACTH) release from the pituitary^{27,40,41}. In addition,

subpopulations of CRH neurons project to the posterior pituitary and to autonomic centers of the brainstem and spinal cord^{29,36,37}, on which its central effects are based^{4,11}.

CRH plays a key role in the function of the hypothalamo-pituitary-adrenal (HPA) axis, the involvement of which in the stress response was first discovered by Selye³¹ (for a recent review see ref. 3). In addition, CRH is thought to be involved in various stress-related human pathologies, e.g. depression, Cushing's syndrome, alcoholism, anorexia nervosa and Alzheimer's disease⁴⁶.

In addition to CRH also vasopressin (AVP) plays a crucial role in the control of the release of ACTH from the pituitary gland^{21,28}. AVP and CRH potentiate each other's action on ACTH release^{16,28}. Therefore, it is of interest that CRH and AVP are at least partly synthe-

sized and released in close concert^{2,39}. In normal rats, colocalization of AVP and CRH is visualized in a few neurons²⁹, but in the adrenalectomized rat, AVP is demonstrable in the majority of CRH-containing PVN cells²⁰. Moreover, stress-induced activation of CRH neurons enhances AVP colocalization with CRH both in parvicellular cell bodies and in terminals in the ME^{7,8}. Thus, AVP colocalization in CRH neurons seems to be a sign of hyperactivity of these neurons.

The ultimate aim of our line of research is to establish whether alterations take place in the number or activity of CRH neurons in the PVN in relation to aging and various human pathologies. As a prerequisite for such quantitative studies, the localization of CRH neurons in the human PVN had to be established, as well as the effect of factors like sex, postmortem delay, fixation time and hour and date of death on the stainability of these neurons. So far, only the presence of CRH in the human PVN is mentioned^{24,38}, without providing the above details required for further studies.

We studied, therefore, immunocytochemically the distribution of CRH neurons and the colocalization of AVP with CRH in the human PVN of 13 subjects between 23 and 91 years of age who had not died from a primary neurological or a psychiatric disease. In

addition, in the rat, the effect of a postmortem delay of up to 24 h was investigated experimentally on CRH staining of the ME.

In the present paper we show that in the human hypothalamus (a) CRH neurons are present only in the PVN. They are not localized in a well-defined subnucleus of the PVN, but rather scattered all over the PVN except for the small most ventrorostral part where only few cells were present. (b) CRH neurons are parvicellular, independently of the colocalization of AVP in these cells and (c) CRH was colocalized with AVP in an age-dependent way, indicating activation of CRH neurons during the course of aging.

MATERIALS AND METHODS

Human brain material

Brains of 13 subjects (23–91 years of age) who had not suffered from neurological or psychiatric disorders were obtained by autopsy (for clinical information see Table I). The brains were fixed in 4% w/v formaldehyde at room temperature (RT). After 5–6 weeks (37.5 ± 3.3 days, mean $\pm$ S.E.M.) the hypothalamus was dissected, dehydrated in graded ethanol and embedded in paraffin (Histowax, melting point 56–58°C, Histo-Lab. LTD, Göteborg, Sweden). Serial 6- μ m frontal sections were cut on a Leitz microtome, mounted on chrome-alum-coated object slides, and stored at RT. Before use, the sections were deparaffinized in xylene and rehydrated through graded ethanol. Every 50th section was stained with thionine (0.1% w/v thionine in acetate buffer, pH 4) in order to localize the PVN before immunocytochemical staining.

TABLE I

Clinical and pathological data of cases studied

Pat Nr.: patient number; yr: years; BW: brain weight; g: grams; PMD: postmortem delay; d: days; Fix: fixation time; h: hours; f: female; m: male; nd: not determined.

Case	Pat Nr.	Sex	Age (yr)	BW (g)	PMD (h)	Fix (d)	Clinical diagnoses	hour and date of death	
1)	87110	m	23	1310	13	11	encephalitis	11.00	March 26
2)	82181	m	27	1560	24	40	pneumonia, sepsis	nd	July 27
3)	81255	f	30	1460	24	39	acute heart death	11.40	Nov. 8
4)	84248	m	37	1370	39	35	bronchopneumonia pleuritis	1.35	Okt. 28
5)	81267	m	43	1260	23	53	sepsis, icterus non-Hodgkin lymphoma	12.25	Nov. 18
6)	82165	f	52	1370	5	33	bronchopneumonia medullar mammary carcinoma	5.40	July 14
7)	82086	m	68	1300	6.5	nd	cardiac failure	20.30	Dec. 28
8)	4725	f	72	1200	21	53	endocarditis lenta glomerulonephritis renal insufficiency cerebral infarction in right frontal lobe	16.45	Sept. 17
9)	81032	m	74	1410	13	48	bronchopneumonia decompensatio cordis 0	5.15	April 8
10)	81064	m	83	1280	22	42	coronary sclerosis myocardial infarction pulmonary emphysema prostatitis	7.00	March 15
11)	81100	f	88	1030	11	35	decompensatio cordis	24.00	May 3
12)	8538	f	90	1300	6	33	mammatur, struma	9.00	Dec. 15
13)	83179	f	91	1060	36	28	respiratory insufficiency	15.35	June 18

Localization of CRH in the human hypothalamus

Immunocytochemical staining for CRH was done on a series of sections taken at 600- μ m intervals throughout the region in which the PVN was present (as determined from the thionine-stained material). This part of the hypothalamus also contained parts of the supraoptic nucleus (SON)¹⁷, suprachiasmatic nucleus³⁴ and sexually dimorphic nucleus³³.

The monoclonal rat anti-CRH 'PFU 83' (IgG2a subclass)⁴⁴ was used that was directed to the C-terminal part (amino acids 38–39) of rat/human CRH. A radioimmunoassay (RIA) has proved it to be highly specific, with no significant binding of pituitary hormones and hypothalamic peptides other than CRH⁴⁴. Immunocytochemical staining with PFU 83 in rat hypothalamus showed staining patterns that were qualitatively similar to those obtained with a conventional CRH antiserum used earlier⁷.

Using a modification of an immunocytochemical double staining⁵, CRH was stained blue by alkaline phosphatase (AP). The sections were washed (i.e. 3 $\times$ 10 min) in 0.05 M Tris containing 0.15 M NaCl (TBS, pH 7.6). Incubations with PFU 83, diluted 1:100,000 in staining buffer (TBS with 0.5% v/v Triton X-100 and 0.25% w/v gelatin), were kept at RT for 30 min and subsequently at 4°C overnight in plastic boxes to prevent evaporation. The following day, the sections were washed and incubated for 30 min with an AP-labeled goat-anti-rat IgG (H+L) serum (KPL, Maryland) and diluted 50 times in staining buffer. Thereafter, the sections were washed and rinsed in 0.05 M Tris (pH 8.5). The AP activity was demonstrated by incubating exactly 10 min at RT with a filtered suspension of 2 mg Fast blue BB base (Sigma, St. Louis) in 10 ml 0.1 M Tris (pH 8.5) containing 2 mg naphthol-AS-AX phosphate (Sigma, St. Louis), 0.2 ml *N,N*-dimethylformamide and 3 mg levamisole (Sigma, St. Louis). Longer incubation did not result in further intensification of the blue cells but only in higher background staining. Finally the sections were washed and coverslipped with Kaisers Glycerin-gelatin (Merck).

The rostro-caudal distribution of CRH cells in the PVN was determined on the right side of the brain (except in case Nr. 87110, where the tissue was not completely present on that side and the left side was used). The total PVN length was defined as the distance between the most rostral and most caudal thionine-stained section showing a cluster of at least 5 magnocellular neurons. The length of the PVN that contained CRH cells was defined as the distance between the most rostral and most caudal section which contained one or more CRH positive cells. This criterion of only one cell was used because of the low number of CRH neurons in some of the cases investigated. The length of the CRH-containing part of the PVN was expressed as a percentage of the total PVN length for each subject.

Colocalization of AVP and CRH

Immunocytochemical double staining was performed on a second series of sections, taken at 600- μ m intervals, in order to demonstrate colocalization of CRH and AVP. The staining was a modification of the method of Claassen et al.⁵, resulting in blue CRH cells, red AVP cells, and purple CRH and AVP cells. In addition to the CRH staining described above, AVP was stained by a peroxidase-anti-peroxidase (PAP). For immunostaining of AVP a rabbit antiserum 'Truus'⁴² was used. From this AVP antiserum oxytocin binding antibodies were removed by preadsorbing twice with oxytocin-glutaraldehyde-coupled sepharose beads (cf. ref. 25). For the first antibody incubation, Truus and PFU 83 were diluted in staining buffer, respectively, 1:300 and 1:100,000. The following day the sections were washed and incubated with goat anti-rabbit IgG serum, diluted 1:50 in staining buffer (1 h at RT). After washing, the sections were incubated with both an AP-labeled goat-anti-rat IgG (H+L) serum (K.P.L., Maryland, USA) and a rabbit PAP, diluted 1:50 and 1:1,000, respectively, in TBS. Thereafter, the sections were washed, and rinsed in 0.05 M Tris (pH 8.5). The AP activity was demonstrated as described above. The sections were subsequently washed and incubated (10 min, RT) in filtered PAP-substrate prepared by dissolving 25 mg 3-amino-9-ethylcarbazole (Sigma, St. Louis) in 2.5 ml *N,N*-dimethylformamide, which was added to 50 ml 50 mM Na-acetate (pH 5.0) and activated by 20 μ l 30% H₂O₂. Longer incubation did not result in more intensified cells. Only the back-

ground staining became higher. Finally, the sections were washed and coverslipped as described above.

Cellular profile area measurements

In immunocytochemically double-stained sections of the PVN, profile areas of AVP, CRH and both AVP and CRH containing cells (red, blue and purple, respectively) were measured by means of a Calcomp 2000 digitizer, using a Zeiss microscope with a PLAN 40 $\times$ objective and PLAN 12.5 $\times$ oculars. The profile area was measured only in neurons containing a cell nucleus.

Antibody specificity

Immunocytochemical control stainings in the double staining were performed with anti-CRH (PFU 83), preincubated with 10⁻⁵ M CRH, and the AVP antibody preadsorbed twice with AVP. Pread-sorption was performed as the removal of cross-reacting antibodies (cf. ref. 25). Neither these controls, nor pre-immune serum or normal rat serum as first antibody revealed any staining (Fig. 3D).

A spot-blotting experiment, based upon the press-blotting procedure of Van der Sluis et al.⁴², was performed in order to investigate possible cross-reactivities of the above antisera with the following peptides: synthetic rat CRH (Peninsula), three fragments of rat/human CRH consisting of aminoacids 1–20, 6–33, and 21–41 (kindly obtained from Dr. J. Rivier), AVP, oxytocin (both from Sigma, St. Louis), neurophysin-A (kindly obtained from Dr. B.T. Pickering, Bristol, UK), and a glycopeptide fragment (22–39) (kindly provided by Dr. J. van Nispen, Organon, Oss, The Netherlands). Spots containing 100 ng and a step-wise 1:3 dilution from 10 ng to 1.5 μ g of the above mentioned peptides were applied to a gelatin coated nitrocellulose (NC) membrane in 1 μ l spot buffer (i.e. 10% v/v dimethylformamide (BDH, Poole, UK) 1% v/v glycerol (BDH), 2.5% v/v Nonidet P-40 (Sigma, St. Louis), 2% w/v Servalyt 2–11 (Serva, Heidelberg, Germany) in aquadest), instead of the isoelectric focussing gel as described in the original paper⁴². Finally the peptides were fixed with 4% w/v formaldehyde to the gelatin coated NC membranes.

For the immunocytochemical staining of the NC membranes a PAP procedure⁴² was used with either the purified AVP antibody (Truus, diluted 1:500), or the CRH antibody (PFU 83, diluted 1:10⁵) or three rabbit antisera (diluted 1:1,000); anti-neurophysin-A (kindly obtained from Dr. A.G. Robinson, Pittsburgh, USA), anti-glycopeptide 22–39^{30,43}, and anti-oxytocin (O-1-V³⁵). As secondary antibodies for the CRH antibody we used a biotinylated anti-rat antiserum (Amersham, UK) and for the other primary antibodies a goat-anti-rabbit antiserum (Betsie), diluted 1:200 and 1:1,000, respectively, in staining buffer. For the peroxidase reaction an ABC-peroxidase kit (Vector Lab. Inc., Burlingame) or a PAP-labeled goat anti rabbit was used. Stainings for oxytocin, glycopeptide (22–39) and neurophysin-A with their homologous antisera were performed in order to control for the presence of these peptides on the NC membrane.

Quantification of the immunocytochemically stained NC-membranes was performed according to Van der Sluis et al.⁴². From each spot, the area and optical density were measured. For setting a 100% transmission, a gelatin-coated NC membrane without peptide spots was used. The corrected integrated optical density was obtained by multiplying area and optical density values and correcting for the blank value (spot buffer without peptide).

Effect of postmortem delay (PMD) on CRH staining in rats

Nine male Wistar rats (strain: Cpb:WU, age: $\pm$ 7 weeks, weight: 225–249 g, Harlan Cpb, Zeist, The Netherlands) were sacrificed at 10.00 a.m. by decapitation. The brains of three of these rats were immediately dissected and immersion-fixed in 4% w/v paraformaldehyde (PMD 0 h). The brains of the other two groups of three rats were removed from the skull and fixed after storage of the intact skulls for 6 or 24 h at 4°C (PMD 6 and 24 h, respectively).

After 4 weeks of fixation at 4°C, all rat brains were embedded in paraffin, cut and prepared for immunocytochemistry in the same way as described for the human hypothalamus. The PAP procedure described in the spot blot experiment was used for immunocytochemistry.

Statistics

Profile areas (Table IV) from CRH cells, AVP cells and cells containing both CRH and AVP were analyzed by one way analysis of variance followed by a two-tailed *t*-test.

Analysis of semi-quantitative data

In order to investigate whether differences in the number and staining intensity of CRH-neurons in the PVN and in the staining intensity of the ME could be attributed to age, postmortem delay, fixation time or hour of death, Spearman correlation coefficients were determined after ranking the semi-quantitative data from Table II ($\pm = 1$, $++ = 2$, $+++ = 3$, $++++ = 4$), followed by a one-tailed significance test for correlation (Conover, 1980). The age-dependency of the colocalization of AVP with CRH was analysed by determining Spearman correlation coefficients of age vs. the ranked ratio of the number of purple cells with the number of purple and blue cells together, also followed by a one-tailed significance test for correlation (Conover, 1980).

Differences in the number and staining intensity of CRH neurons in the PVN and in the staining intensity of the ME between sexes, and differences in the ratio of purple/(purple+blue) cells between young subjects (< 65 years of age) and old subjects (≥ 65 years of age), were analysed with the Mann-Whitney test followed by a two-tailed probability of *Z* corrected for ties. Correlation coefficients and significance values are only mentioned in the text if they reached significance.

For all statistical analyses SPSS-X (SPSS Inc, Chicago, USA) was used. Significance was defined at the 0.05 level.

Evaluation of the statistical procedures of the semi-quantitative data is presented in the discussion.

RESULTS

Localization of CRH in the human hypothalamus

Immunocytochemical staining for CRH revealed immunoreactive neurons and nerve fibers in the PVN of all 13 subjects. In the cell bodies the reaction product was distributed over the entire cytoplasm (Fig. 1). No CRH cells were observed outside the PVN, i.e. neither in the suprachiasmatic nucleus (SCN), nor in the sexually dimorphic nucleus (SDN), nor in the supraoptic nucleus (SON). In only one section a single CRH cell was seen in the SON (Nr. 4725) and one CRH cell was found lateral to the fornix (Nr. 81064).

The number of CRH neurons (a semi-quantitative estimate of the number of cells over the entire length of the PVN) appeared to vary greatly between the subjects (Table II). These differences have not yet been quantified. The CRH staining intensity of PVN cell bodies and ME nerve fibers showed also inter-individual variation (Table II). Factors such as age, sex, fixation time, PMD, hour of death and date of death

Fig. 1. Frontal paraffin sections (6 μ m) through a human PVN stained (A) for thionine and (B) immunocytochemically for CRH. Note in (A) the clustering of the magnocellular cells forming the nucleus, and in (B) the cytoplasmic staining of the parvocellular CRH neurons, the staining of CRH fibers in this nucleus and the unstained magnocellular neuron (arrow). Bar represents 25 μ m.

seemed not to influence CRH staining intensity and CRH cell number.

The rostro-caudal distribution of CRH cells in the PVN revealed that in only the small most ventro-rostral part of the PVN generally no CRH cells were present. In one case (Nr. 81100), a few CRH cells were found in the most rostral part. More caudally, the first CRH neurons appear (Fig. 2) in the central part of the PVN (Fig. 2A). In the central part of the PVN the lateral boundaries for CRH neurons were more or less identical to those obtained after thionine staining. The same distribution was found in the caudal part of the PVN, although in almost half of the subjects the number of CRH cells was too low to clearly establish the boundaries of the PVN area that contained CRH cells. The median length of the PVN part that contained CRH cells was 67% of the total PVN length (Table II). The CRH cells were spread evenly over this part of the PVN.

Colocalization of AVP and CRH

In the human PVN, anti-AVP, anti-CRH and anti-CRH/AVP staining cells were visualized as red, blue and purple, respectively (Fig. 3). The reaction products were distributed over the entire cytoplasm. The red cells were clearly stained, the majority of blue cells were faintly stained and the purple cells were darkly stained (Fig. 3, Table III), whereas the background staining was very low. Also in this double staining protocol, the staining intensity of the CRH cells and the number of CRH cells varied greatly among different subjects (Table III). In the SON, only red stained (AVP) cells were observed (Fig. 3)

Colocalization of CRH and AVP in the PVN was only found in subjects over about the age of 40 years (Table III and IV). The majority of CRH neurons were stained purple (CRH plus AVP) from the age of 52 years onwards, except the subjects Nr. 82086 and Nr. 83179 (68 and 91 years of age, respectively), in which the majority of CRH neurons were blue (only CRH). These results are based upon two independent but identical assessments by two researchers (F.C.R. and D.F.S.)

TABLE II

CRH staining in human hypothalami

Cell number: $\pm$ very low; + low; ++ high; +++ very high
Staining intensity: $\pm$ faint; ++ intense; + good; +++ very intense.

Case	Total PVN length (μ m)	CRH-PVN length (μ m)	Ratio of CRH-PVN and total PVN length	CRH cell number in PVN	Intensity of	
					CRH cells in PVN	fibers median eminence
1)	4800	1200	25%	$\pm$	$\pm$	++
2)	4800	3600	75%	+++	++	+++
3)	3000	600	20%	+	$\pm$	+++
4)	4200	3600	75%	++	++	+++
5)	3600	2400	67%	++	+	++
6)	4200	1800	43%	+	++	+
7)	4200	1800	43%	+	++	++
8)	5400	3600	67%	+++	+++	+++
9)	3600	2400	67%	+++	+++	++
10)	5400	3600	67%	+	$\pm$	++
11)	3000	3000	100%	+++	++	+++
12)	4200	3000	70%	+++	+++	+++
13)	4800	2400	50%	+	$\pm$	++

Cellular profile area measurements

Measurements of the profile areas from the three different cell types revealed that the mean profile area of the red (AVP) cells ($327.0 \mu\text{m}^2$, ± 16.3) was 2.2 times as large as the mean profile area of the purple (AVP plus CRH) cells ($148.5 \mu\text{m}^2$, ± 10.8) and 2.3 times as large as the mean profile area of the blue (CRH) cells ($140.6 \mu\text{m}^2$, ± 7.4). The red (AVP) cells were significantly larger than the purple (AVP and CRH) and blue (CRH) cells ($P < 0.001$).

Antibody specificity

Quantification of the immunocytochemically stained NC-membranes showed binding of the CRH antibody 'PFU 83' to intact CRH and fragment 21-41 (Fig. 4). PFU 83 did not bind to spots containing the CRH fragments 1-20 or 6-33, AVP, neurophysin-A, glycopeptide (22-39) or oxytocin. The AVP antiserum 'Truus', which had been purified to avoid cross-reaction with oxytocin, only reacted with AVP and not with oxytocin, neurophysin-A, glycopeptide (22-39), CRH or any of the CRH fragments (Fig. 4). The control blots, which contained neurophysin-A, glycopeptide

Fig. 2. A: topography of CRH cells in the human PVN from rostral (left) to caudal (right). F, fornix; OT, optic tract; III, third ventricle; PVN, paraventricular nucleus; CRH, the part of the PVN which contained CRH cells. B: length of the part of the PVN containing CRH cells expressed as percentage of the total length of the PVN. The total PVN length is represented by the upper horizontal line and put on 100% for each subject. The rest of the horizontal lines indicates the length (in %) and localization of the part of the PVN containing CRH neurons. C: projection of the CRH cells on the mediosagittal plane of the PVN in the hypothalamus. Scheme after Diericks and Vandesande⁹. 1, PVN; 1a, part of PVN which contains CRH; 2, dorsolateral part of SON; 3, dorsomedial part of SON; 4, ventromedial part of SON; 5, SCN; 6, optic chiasm; 7, optic nerve; 8, origin of pituitary stalk; 9, mammillary body; 10, lamina terminalis; 11, anterior commissure; 12, axis of the fornix; 13, hypothalamic sulcus. Note that CRH neurons are lacking in the small most ventro-rostral PVN in all cases but one.

TABLE III

Immunocytochemical double staining for AVP and CRH in the human PVN

CRH cells are blue, AVP cells red and CRH and AVP cells purple. Cell profiles per section: — none; $\pm$ 1–5; + 6–9; ++ 10–49; +++ 50–99; ++++ $\geq$ 100. Staining intensity: — deficient; $\pm$ very faint; + faint; ++ normal; +++ intense; ++++ very intense.

Case	Majority of CRH cells	Cell profiles			Staining intensity of		
		blue cells	purple cells	red cells	blue cells	purple cells	red cells
1)	blue	++	—	++++	$\pm$	—	++
2)	blue	+++	—	+++	++	—	+
3)	blue	++	—	+++	$\pm$	—	+
4)	blue	+++	—	++++	++	—	+++
5)	blue	++	$\pm$	++	+	+	+
6)	purple	+	++	++	++	++	++
7)	blue	+	$\pm$	+++	+	+	+
8)	purple	$\pm$	+++	+++	$\pm$	+++	+
9)	purple	++	+++	++++	$\pm$	+++	++
10)	purple	$\pm$	+	++	$\pm$	+	+
11)	purple	++	++++	++++	+	+++	++
12)	purple	$\pm$	+++	+++	$\pm$	+++	+
13)	blue	+	$\pm$	++	+	+	++

(22–39) or oxytocin, showed immunoreactivity after staining with their homologous antisera (Fig. 4), proving that the peptides were indeed bound to the NC membrane.

Effect of postmortem delay (PMD) on CRH staining in rats

In the rat hypothalamus, no CRH immunoreactivity was seen in the PVN, SON or SCN after a PMD of 0, 6 or 24 h. However, intense CRH immunoreactivity was found in the external zone of the ME in all cases (Fig. 5). The different PMDs did not result in obvious differences in CRH staining intensities of the ME (Table V).

DISCUSSION

In the human PVN, cell bodies can be found that stain very clearly for CRH^{24,38} (this article). In untreated rats, these cells can not be observed and colchicine administration or adrenalectomy are required in order to visualize CRH-producing cell bodies²⁹. The long PMD of the human subjects as compared to rats is unlikely to be responsible for the species differences in stainability of cell bodies, since no CRH staining was evident in the PVN of rats even at 24 h postmortem. It is not clear at present whether this species difference is due to increased production,

decreased transport rate or a different processing of the precursor in human CRH neurons.

We found CRH neurons in all 13 subjects investigated, in spite of the fact that we incubated mounted sections from paraffin-embedded material, which illustrates the sensitivity of our immunocytochemical procedure.

TABLE IV

Mean cellular profile areas of CRH cells, AVP cells and AVP and CRH cells in the human PVN

area = mean cellular profile area (μm^2); S.E.M. = standard error of the mean; n = number of measured cells.

Case	Blue cells (only CRH)			Purple cells (CRH and AVP)			Red cells (only AVP)		
	area	$\pm$ sem	n	area	$\pm$ sem	n	area	$\pm$ sem	n
1)	161.34	12.81	18	—	—	—	445.84	12.73	100
2)	139.07	4.24	94	—	—	—	356.33	12.44	65
3)	96.42	5.75	15	—	—	—	230.33	6.67	62
4)	137.10	4.39	119	—	—	—	357.97	9.38	101
5)	134.52	6.16	37	90.68	16.56	3	281.44	15.89	21
6)	100.08	7.88	14	126.68	9.75	23	238.50	2.73	41
7)	178.08	9.34	21	120.00	2.34	2	275.96	8.11	56
8)	165.94	2.73	2	151.00	5.77	74	355.64	12.85	60
9)	128.37	9.68	20	155.85	4.93	98	315.96	6.57	143
10)	113.15	14.21	5	149.68	18.24	10	342.55	15.04	38
11)	172.07	10.96	16	169.16	4.76	123	356.48	9.06	132
12)	136.45	14.01	6	200.94	5.72	132	366.92	12.09	66
13)	164.58	13.91	11	172.63	8.69	2	326.66	9.35	55
mean	140.55	7.43	13	148.51	10.83	9	326.97	16.30	13

Fig. 3. Immunocytochemical double staining on frontal paraffin sections (6 μm) through the human hypothalamus. CRH cells stained blue, AVP cells red and CRH and AVP containing cells purple. A: section through the PVN of a young patient (Nr. 84248), not showing colocalization of AVP and CRH; B: section through the PVN of an old patient (Nr. 81032), showing red (*), blue ($\uparrow$) and purple cells ($\blacktriangle$). (For profile areas of the different cell types, see Table IV). C: section through the SON of patient Nr. 81032, showing only red AVP cells. D: staining by AVP-adsorbed anti-AVP and with 10^{-5} M preincubated CRH anti-CRH of a section through the PVN of patient Nr. 81032, showing no immunoreactive cells. Bar = 25 μm .

ANTIBODY SPECIFICITY TEST

Fig. 4. Corrected integrated optical density (IODC) values of peptide spots blotted on gelatine coated nitrocellulose (NC). The IODC was corrected for (1) the optical density of gelatine coated NC without peptide (= 100% transmission); (2) the optical density of the blank; and (3) the area of the spot. Different quantities (blank, 1.5 pg, 4.5 pg, 13 pg, 41 pg, 123 pg, 370 pg, 1.1 ng, 10 ng and 100 ng) of peptide were applied to the NC in a volume of 1 μ l of spot buffer. The blank consists of spot buffer without peptide. The upper, middle and lower parts of the figure show the results from, respectively, the spot blot stained by the CRH antibody PFU 83, the spot blot stained by the AVP antibody Truus, which was purified for crossreactivity with oxytocin, and three different blots stained with their homologous antibodies.

ture if we compare this to the literature. In mounted paraffin-embedded material, Ohtani et al.²³ could detect CRH immunoreactivity in only 5 out of the 23 cases. Therefore, they had to increase the sensitivity of the CRH staining in human material by using free-floating sections, which resulted in CRH positive cells and nerve fibers in all 7 cases examined³⁸.

In spite of the sensitivity of our method and the standardized way we performed it, considerable inter-individual differences in CRH staining intensity and cell number (based upon semi-quantitative observations), were present in our material (Table II). These differences seem not to correlate ($P > 0.06$) with factors such as age, sex, PMD, fixation time and hour of death. Only the effect of PMD on CRH immunoreactivity in the hypothalamus has been investigated earlier^{1,38}. The first group could not find with a RIA an influence of PMD on CRH contents. However, they only investigated three subjects. The second group suggested the use of short PMDs after finding good CRH staining in tissue obtained within 3 h post-mortem. However, they have not investigated longer PMDs. Since our group showed that a longer PMD resulted in even better staining of AVP positive cell bodies in the rat SCN⁴⁵, the need of short PMDs for peptide immunocytochemistry in general can be questioned. Indeed, our human hypothalamic CRH data (Table I) do not point to an effect of the PMD on CRH staining, neither in PVN cell bodies nor in ME fibers. Since it is difficult if not impossible to study one factor in isolation in human material, we also investigated the effect of PMD on CRH staining in the ME of the rat (Table V). This structure is of interest, since in the human material the intensity of the CRH staining of both, the ME and PVN cell bodies varied in a similar way (Table II). From the rat data it also seems that the CRH staining intensity is not affected by differences in postmortem delay.

Like postmortem delay, also different fixation times could theoretically lead to immunocytochemical staining differences. In order to gain some information about the possible influence of these factors together with factors like age and sex on the immunocytochemical results we obtained in this paper, we tried to analyse the data in a non-parametric way. However, the limited material does not allow very strong conclusions.

The same restriction holds for the possible influence of the time of death on the stainability of CRH-neurons, which is of interest since circadian changes of CRH are found in cerebrospinal fluid (e.g. Ref. 14). This restriction does not hold for the clear age dependent colocalization of AVP with CRH, because the

correlation of age vs. the ranked ratio's of purple cells/(purple + blue cells) is very high ($\rho = 0.8$, $P = 0.001$), and the difference in ratio between young and old subjects is also very significant ($P = 0.006$).

In the SON of the human hypothalamus we did not find CRH cells (see also refs. 24, 38), whereas in the rat the presence of CRH cells in the SON has often been described²² (for review see ref. 29). However, one

Fig. 5. Immunocytochemical CRH staining of 6 μm paraffin sections through the median eminence of rats. The external zone of the median eminence is stained. A: no postmortem delay (PMD); B: PMD of 6 h; and C: PMD of 24 h. No difference in staining intensity was found. Bar = 25 μm .

TABLE V

Effect of postmortem delay (PMD) on the intensity of CRH staining in the median eminence of 9 rats

+ good staining; ++ intense staining; +++ very intense staining.

PMD 0 h	PMD 6 h	PMD 24 h
+++	+++	+++
+	++	++
+++	++	+++

patient with a high CRH cell number and very intense CRH staining in both ME and PVN showed one single CRH cell in the SON. This may indicate that in the human SON CRH-producing cells occur and can be made detectable following activation of the CRH system. The presence of radioimmunoassayable CRH in extracts of the human SON²⁶, favour this hypothesis. A more likely explanation, however, is that the content of CRH fibers running along the SON to the neurohypophysis have been included by the dissection procedure.

The CRH neurons in the human PVN were all parvocellular (Table III). They were dispersed all over the central and caudal part of the PVN, which is in contrast to the localization in a distinct subnucleus as in the rat^{22,29,37}. The species difference in the localization of CRH neurons is in agreement with species differences found previously in the basic structure and topographical arrangement of AVP and oxytocin neurons in the PVN. In contrast to rats, in humans, cows, cats and guinea pigs no arrangement of the PVN into clear subnuclei was found³². In this respect it seems that the clear partition of the rat PVN into well defined subnuclei is the exception rather than the rule.

In the present study, the colocalization of AVP and CRH had been investigated for two reasons. Firstly, the distribution of the CRH neurons in the human PVN showed a large overlap with that of the AVP neurons, which could indicate that CRH and AVP are colocalized in a similar way as has been described for the rat (for review see ref. 29). Secondly, colocalization of AVP in CRH neurons in the rat was suggested to be a marker for the activity of CRH neurons^{7,8}. In order to investigate the colocalization of AVP and CRH, we used an immunocytochemical double staining procedure. This procedure has a major advantage to the alternative of staining adjacent paraffin sections with a single staining procedure because it is often difficult in paraffin-embedded tissue to recognize with certainty the same cell in the adjacent section because of the non-uniform way sections stretch. Immunocytochemical double staining for AVP and CRH indeed showed

colocalization of CRH and AVP in the human PVN, but only in cases of over about 40 years of age (Table III). This observation supports the view that the activity of the CRH neurons increases with aging, which is in accordance with findings demonstrating hyperactivity of the human hypothalamo-pituitary-adrenal axis during aging^{10,13,47}. Activation of AVP neurons in the human PVN during aging has also been described (i) on the basis of changes in cell size¹² and nucleolus size¹⁹, but only in the age group of 80–100 years, and (ii) on the basis of the number of cells expressing AVP¹⁸, which is higher in subjects older than 60 years of age. It might well be that in humans the parvocellular CRH cells which start to synthesize AVP during the course of aging contribute to a considerable degree to the increase found in AVP cell numbers during aging¹⁸. At the other hand, this possibility is not supported by the finding that the mean cellular profile area of AVP cells increases during aging¹². Our data (Table IV) do not show a significantly larger mean cellular profile area for AVP cells in the PVN of patients older than 80 years of age, but this group concerns only 4 patients.

The observation that CRH neurons are activated during the course of aging by coexpressing higher levels of AVP can not be confirmed by RIA because it is not possible to isolate PVN cells for assay without dissecting at the same time fibers containing either one or both of the peptides. Even if it were possible to isolate PVN cells, differences in AVP levels caused by the colocalization of AVP with CRH in the parvocellular neurons would be masked by the high AVP levels obtained from the majority of magnocellular AVP neurons present in the PVN. However, our observations can be confirmed by *in situ* hybridization.

Since in the human PVN, CRH neurons and neurons containing CRH and AVP were both found to be parvocellular (Table IV), and because at a younger age only CRH neurons were present (Table III), one may wonder whether beyond the age of 43 years of age CRH neurons may start to synthesize AVP or alternatively parvocellular AVP neurons may begin to make CRH. The first possibility would fit with results obtained in the rat. In this species, two types of CRH neurons have been found after colchicine treatment and adrenalectomy, i.e. AVP deficient and AVP expressing ones⁴⁸. The AVP-containing subset of CRH was selectively activated by stress, resulting in (i) depletion of neurosecretory vesicles from the axonal swellings in the external zone of the median eminence⁴⁹, (ii) increased AVP stores and colocalization in CRH nerve terminals⁸, and (iii) a five-fold elevation in number of the AVP containing CRH cell bodies⁷. However, there is a difference with the data we obtained in the human

hypothalamus where no colocalization of CRH and AVP was found before activation of the CRH neurons before the age of 43 years. This species difference may be due to the colchicine treatment, resulting in a high AVP content in the CRH cell bodies, whereas in humans the AVP content of the CRH neurons before the age of 43 years might be too low for immunocytochemical detection. The second possibility can at present also not be excluded.

The colocalization of CRH and AVP did not result from cross-reactivity of the antibodies, as was verified from the specificity tests for the antibodies. Since in the spot blot experiment the antigen is fixed in a similar way as in the tissue sections, it may give a good impression about the recognition of the antigen by the antibody in tissue sections during immunocytochemistry. The observation that purple (CRH- and AVP-containing) cells were never present in double-stained sections of the human SON, whereas they were abundant in the PVN, also indicates that the anti-CRH antibody does not cross-react with AVP or parts of its precursor.

In the present study we make it clear that it will indeed be possible to study the CRH neurons in the PVN during disease states. However, one should consider that (1) the CRH cells are not localized in a distinct subnucleus and (2) the colocalization of CRH and AVP is an age-dependent phenomenon, which is possibly related to its activity status.

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