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Increased Numbers of Corticotropin-Releasing Hormone Expressing Neurons in the Hypothalamic Paraventricular Nucleus of Depressed Patients

Key Words

Corticotropin-releasing hormone
Immunocytochemistry
Human
Hypothalamus
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Hypothalamo-pituitary-adrenal axis
Depression
Clinical neuroendocrinology

Abstract

The hypothalamo-pituitary-adrenal (HPA) axis is known to be activated in depressed patients. Although direct evidence is lacking, this activation is hypothesized to be due to hyperactivity of corticotropin-releasing hormone (CRH) neurons of the hypothalamic paraventricular nucleus (PVN). Recent immunocytochemical studies in experimental animals and in humans showed that the number of CRH-expressing neurons correlated with the activity of these neurons. In addition, colocalization of AVP in CRH neurons has been shown to be an index for the secretory activity. Therefore, we estimated the total number of CRH-immunoreactive neurons and their fraction showing colocalization with AVP in the PVN of 10 control subjects and of 6 depressed patients who were diagnosed to be suffering from a major depression or a bipolar disorder. The mean total number of CRH-expressing neurons of the 6 depressed patients was four times higher, and the number of CRH neurons co-expressing AVP was almost three times higher than those in the control group. We also determined the two activity parameters of CRH neurons in the PVN of 2 subjects with a depressive organic mood syndrome or a depressive disorder not otherwise specified. In these two 'non-major depressed' subjects, the activity parameters of CRH neurons were comparable to those of control subjects. Our observations strongly support the hypothesis that CRH neurons in the PVN are hyperactivated in major depressed patients. This hyperactivity might be causally related to at least part of the symptomatology of depression.

Introduction

Corticotropin-releasing hormone (CRH) is a 41-amino acid peptide with a high capacity of releasing adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland [1, 2]. The effect on ACTH release is potentiated by vasopressin (AVP) [3, 4]. ACTH, in turn, induces the production and secretion of cortisol from the adrenal cortex. Accordingly, the hypothalamic paraventricular CRH neurons play a key role in the hypothalamo-

pituitary-adrenal axis. The effect on ACTH release is potentiated by vasopressin (AVP) [3, 4]. ACTH, in turn, induces the production and secretion of cortisol from the adrenal cortex. Accordingly, the hypothalamic paraventricular CRH neurons play a key role in the hypothalamo-

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pituitary-adrenal (HPA) response to stress [5]. In addition, CRH neurons have been hypothesized to be involved in the pathophysiological response of the HPA axis in various stress-related human pathologies, e.g. depression [6, 7]. This hypothesis is largely based on reports showing that in depressed patients: (i) 24-hour cortisol excretion was elevated [8]; (ii) the HPA axis was often unable to respond appropriately to exogenous corticosteroids, as measured by the dexamethasone suppression test [9], and (iii) ACTH responses to test doses of ovine CRH [10–15] or human CRH [16–18] were blunted. These blunted responses in depressed patients have been interpreted as an index of CRH hyperexposure because rats that were chronically exposed to CRH develop a reduced CRH receptor efficacy, resulting in blunted ACTH responses to a CRH challenge [19]. Furthermore, elevated CRH levels have been reported in the cerebrospinal fluid (CSF) of patients with depression [20–24]. It should be noted that this CRH may not originate just from paraventricular CRH neurons controlling ACTH secretion, since these neurons represent only one element of an extensive system of CRH neurons and projections throughout the brain [25].

In the human hypothalamus, the CRH neurons are localized in the paraventricular nucleus (PVN) [26–29], especially in the central and caudal parts [30]. Recently, we found that the total numbers of CRH-expressing neurons increased with age and that Alzheimer patients did not differ in this respect [31]. Increased numbers of immunoreactive neurons were interpreted as an index of hyperactivity, since in experimental animals adrenalectomy and chronic stress have been reported to increase the numbers of CRH immunoreactive neurons in the PVN [25, 32–35].

In addition, colocalization of AVP in CRH neurons could serve as another index of secretory activity of CRH neurons, since chronic hyperactivity of CRH neurons in rats induces coproduction of AVP in these neurons as has been demonstrated after repeated hypoglycemia, immobilization, psychosocial stress and adrenalectomy [32–36]. Accordingly, the age-related increase in the numbers of CRH immunoreactive neurons in humans are likely to reflect an age-related increase in the activity of these neurons [31] which is supported by our findings that also the fraction of CRH neurons that coproduces AVP increases with age [30, 37].

In the present paper, we used the two above-mentioned measures of secretory activity of CRH neurons to test the hypothesis of CRH hyperactivity during depression.

Materials and Methods

Human Brain Material

Brains were collected according to the dissection protocol of the Netherlands Brain Bank from 6 clinically well-defined depressed patients and 2 subjects with a depressive organic mood syndrome or a depressive disorder not otherwise specified (for clinical information see table 1). The clinical diagnosis of depression was based upon DSM-III-R criteria [38]. The reference group consisted of 10 age-matched control subjects without a primary neurological or psychiatric disease (table 1). The data on total numbers of CRH neurons, the absence of a sex difference, and the presence of colocalization with AVP of this reference group have been described before [31, 37]. The absence of neuropathological changes, both in the depressed patients and in the controls, was confirmed by systematic neuropathological investigation by Prof. F.C. Stam (Netherlands Brain Bank), Dr. W. Kamphorst (Free University) or Dr. D. Troost (Academic Medical Centre), all in Amsterdam. Neuropathology revealed, in particular, the absence of cell loss and the occurrence of abnormal numbers and/or distribution patterns of senile plaques, neurofibrillary tangles or a disorganized fibre pattern (dystrophic neurites) in Bodian and Congo stains of hippocampus and cortical areas [39]. In addition, both controls and depressed patients showed no abnormal Alz-50 immunoreactivity [39]. The hypothalami were dissected and fixed in 0.1 M phosphate-buffered 4% w/v formaldehyde (pH 7.2) for 28–69 days. Tissues were dehydrated in graded ethanol, embedded in paraffin and serially cut in frontal sections (6 µm). A series of three adjacent sections, from which the first sections were randomly chosen, was taken at 300-µm intervals throughout the complete PVN region and reproducibly stretched and mounted on chrome-alum-coated object slides [31].

Immunocytochemical Staining of CRH

Series of two adjacent sections were deparaffinized in xylene, rehydrated through a graded ethanol series and immunocytochemically stained for CRH using a procedure that has been described earlier [31]. A highly specific monoclonal rat anti-CRH antibody ('PFU 83') [40, 30] was used in this procedure. In immunocytochemical control stainings the CRH antibody was preincubated with 10^{-5} M CRH. A biotinylated sheep antirat antibody (Amersham, UK; diluted 1:200) was used as a secondary antibody. The sections were then incubated in streptavidin-conjugated alkaline phosphatase (K.P.L., USA) and the phosphatase activity was demonstrated, resulting in blue CRH neurons [31]. The sections were counterstained with neutral red in order to delineate the boundary of the PVN for volume estimations. Finally, the sections were coverslipped with Kaisers glycerin gelatin (Merck) and sealed with nail varnish.

Immunocytochemical Double Staining of CRH and AVP

Colocalization of CRH and AVP was determined in a second series of sections, also taken at 300-µm intervals, using an immunocytochemical double staining procedure described before [30]. In this procedure, the CRH neurons were stained blue, the AVP neurons red and the neurons containing both peptides purple. For immunostaining of AVP a rabbit antiserum 'Truus' [41] was used from which oxytoxin-binding antibodies were removed [42]. For immunostaining of CRH we used again the rat monoclonal antibody 'PFU 83'. As secondary antibody for 'Truus', a goat antirabbit IgG serum was used, after which the sections were incubated with an alkaline phosphatase-labelled goat anti-rat IgG (H+L) serum (K.P.L., USA) and a rab-

Table 1. Clinical and pathological data of patients studied

Patient No.	Sex	Age years	BW g	PMD h	Fixation time, days	Clinical diagnosis, cause of death	Clinical diagnosis (DSM-III-R)	Antidepressive Medication in last month (dose per day)
<i>Control subjects</i>								
91082	F	36	1,348	<72	61	duodenal perforation		
883114	M	49	1,309	<24	49	overdose barbiturates		
82109	F	50	1,360	53	n.d.	bronchopneumonia, tumor carotis interna		
88378	M	59	1,180	<24	29	renal insufficiency		
81014	F	64	1,090	8	44	hypovolemic shock		
82086	M	68	1,300	6.5	n.d.	cardiac failure		
80118	M	70	1,165	n.d.	n.d.	pneumonia		
82176	M	83	1,400	n.d.	n.d.	bronchopneumonia		
83158	F	87	1,140	7	34	myocardic failure		
83179	F	91	1,060	36	28	respiratory insufficiency		
<i>Depressed patients</i>								
93076	M	21	1,492	24	33	overdose opiates	major depressive episode	amitriptyline
93090	M	39	1,220	48	30	respiratory insufficiency, overdose paroxetine	bipolar disorder	moclobemide (450 mg)
91042	F	55	1,320	6.8	30	cardiac failure	major depressive episode	flufenazine lithium carbonate maprotiline (150 mg) oxazepam (100 mg) flupentixol (5 mg)
93054	M	63	1,210	24	33	cardiac failure	bipolar disorder	haloperidol (15 mg) temazepam (20 mg) diazepam (40 mg) levomepromazine/ promethazine (100/100 mg)
90028	M	70	n.d.	48	28	cardiac failure	major depressive episode recurrent with psychotic features	fluvoxamine (250 mg) lorazepam
90031	F	80	1,300	24	69	bronchopneumonia	bipolar disorder	sulpiride (800 mg) lithium carbonate haloperidol amantadine
<i>'Non-major depressed' subjects</i>								
93021	F	73	1,032	96	28	cardiac failure	organic mood syndrome, depressive	tranylcypromine (70 mg) temazepam (20 mg) oxazepam (10 mg)
90032	F	85	1,230	74	49	bronchopneumonia	depressive disorder NAO	trazodon

BW = Brain weight; PMD = postmortem delay; n.d. = not determined; NAO = not otherwise specified.

bit peroxidase-antiperoxidase (PAP). The phosphatase activity was stained blue and the PAP-activity red [30]. Finally, the sections were coverslipped as described above. The fraction of CRH neurons that colocalized AVP (i.e. purple neurons) was estimated as a percentage of all CRH neurons (i.e. purple and blue neurons) per patient by systematically scanning the sections for all CRH-immunoreactive neuron profiles with a cell nucleus. The actual number of CRH neurons showing AVP colocalization is calculated from the total number of CRH neurons and the fraction of these neurons showing colocalization with AVP (table 2).

Estimation of the Volume of the PVN

The cross-sectional area of the PVN was measured in serial sections 300 μ m apart as described previously [31]. The boundary of the PVN, which was visualized by the neutral-red staining, was traced with a digitizing pen in the projection of the section onto a Calcomp 2000 digitizer. If the cross-sectional PVN are extended beyond the field of vision in a particular section, it was measured stepwise using a quadrangular grid in one of the oculars as a reference. Each area was converted to real area by correcting for magnification. The PVN volume (necessary for the calculation of the absolute number of CRH

Table 2. Fraction/numbers of CRH neurons showing colocalization of AVP

Patient No.	Total number of CRH neurons	Colocalization fraction %	Number of CRH neurons showing AVP colocalization
<i>Controls</i>			
91082	46	32.6	15
883114	2,941	5.6	163
82109	3,280	34.4	1,130
88378	2,402	55.6	1,334
81014	1,910	61.1	1,166
82086	1,272	79.2	1,007
80118	6,079	81.3	4,942
82176	5,685	52.4	2,978
83158	2,998	67.8	2,032
83179	7,520	63.5	4,772
Mean	3,413	53.4	1,954
± SEM	736	7.4	552
<i>Depressed patients</i>			
93076	6,060	17.1	1,036
93090	12,400	84.7	10,503
91042	24,095	13.1	3,156
93054	11,758	28.7	3,375
90028	13,793	76.1	10,496
90031	13,955	35.5	4,954
Mean	13,677	42.5	5,587
± SEM	2,394	12.5	1,635
<i>'Non-major depressed' subjects</i>			
93021	2,625	23.7	622
90032	5,415	12.1	1,655

neurons, see further) was then determined using Cavalieri's direct volume estimate [43, 44] by summation of the measured areas and multiplying the outcome by the separation distance between sections.

Estimation of the Numerical Density of PVN CRH Neurons

The numerical density of the PVN CRH neurons was estimated by means of the disector method [43], the application of which on human hypothalamic material had been described in great detail before [31]. In short, the first or reference section of each disector pair was systematically scanned for all immunoreactive CRH neurons inside the counterstained PVN boundaries using a Zeiss light microscope with a 3.2 × Plan objective and 10 × Plan oculars. Accurate identification of all immunoreactive neuronal profiles was ensured by the use of 10 × and 40 × objectives as well. CRH neurons were counted as disector neurons if their nucleus was present in the reference section alone, so *not* in the adjacent look-up section (fig. 1) [45]. The numerical density (N_V) was then 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 Total Number of CRH Neurons

The total number of CRH neurons in the PVN was estimated by multiplying the numerical density by the PVN volume. For all subjects these parameters were estimated for the left-hand side PVN, with the exception of two subjects (No. 90031, No. 93076) whose PVNs were not completely present on that side.

Statistics

Data analysis was performed with nonparametric statistics since the data were discrete and unlikely to be normally distributed.

Potential differences between the absolute numbers of CRH neurons in controls and depressed patients were analyzed by the Mann-Whitney U test using SPSS-X (SPSS Inc., Chicago, Ill., USA) followed by a two-tailed probability of Z.

Relations between the number of CRH neurons in the human PVN and factors such as age, postmortem delay and fixation time were determined using linear regression and Spearman's rho based on a two-tailed test (SPSS-X analyses, SPSS Inc.).

Significance was defined at the 0.05 level and values are expressed as mean ± SEM.

Results

Immunocytochemical Staining

For all depressed patients, immunocytochemical staining for CRH revealed blue immunoreactive neurons within a lightly red histochemically counterstained PVN (fig. 1a, b). The intensity of the staining and the distribution of the immunocytochemical reaction product over the entire cytoplasm was similar to the results of the control cases (fig. 1c). The cell size of the CRH-immunoreactive neurons in depressed patients was also similar to that of control cases. No CRH cells were observed outside the PVN, which could easily be delineated by the neutral red counterstaining.

Volume of the PVN

PVN volumes (unilateral) from depressed patients ($10.1 \pm 1.1 \text{ mm}^3$) did not differ significantly from PVN volumes of control subjects ($8.1 \pm 1.0 \text{ mm}^3$, $p = 0.16$). The volumes of the PVN of the subject with the 'depressive organic mood syndrome' and the subject with the 'depressive episode not otherwise specified' were also in the normal range (9.8 and 11.3 mm^3 , respectively). Of course, these data provide no information on the exact *in vivo* volume of the PVN. However, for the determination of the total number of CRH neurons in the PVN this point is of no importance.

Total Numbers of CRH Neurons in the PVN

The PVN of depressed patients ($n = 6$) contained 4 times as many CRH neurons as that of the control sub-

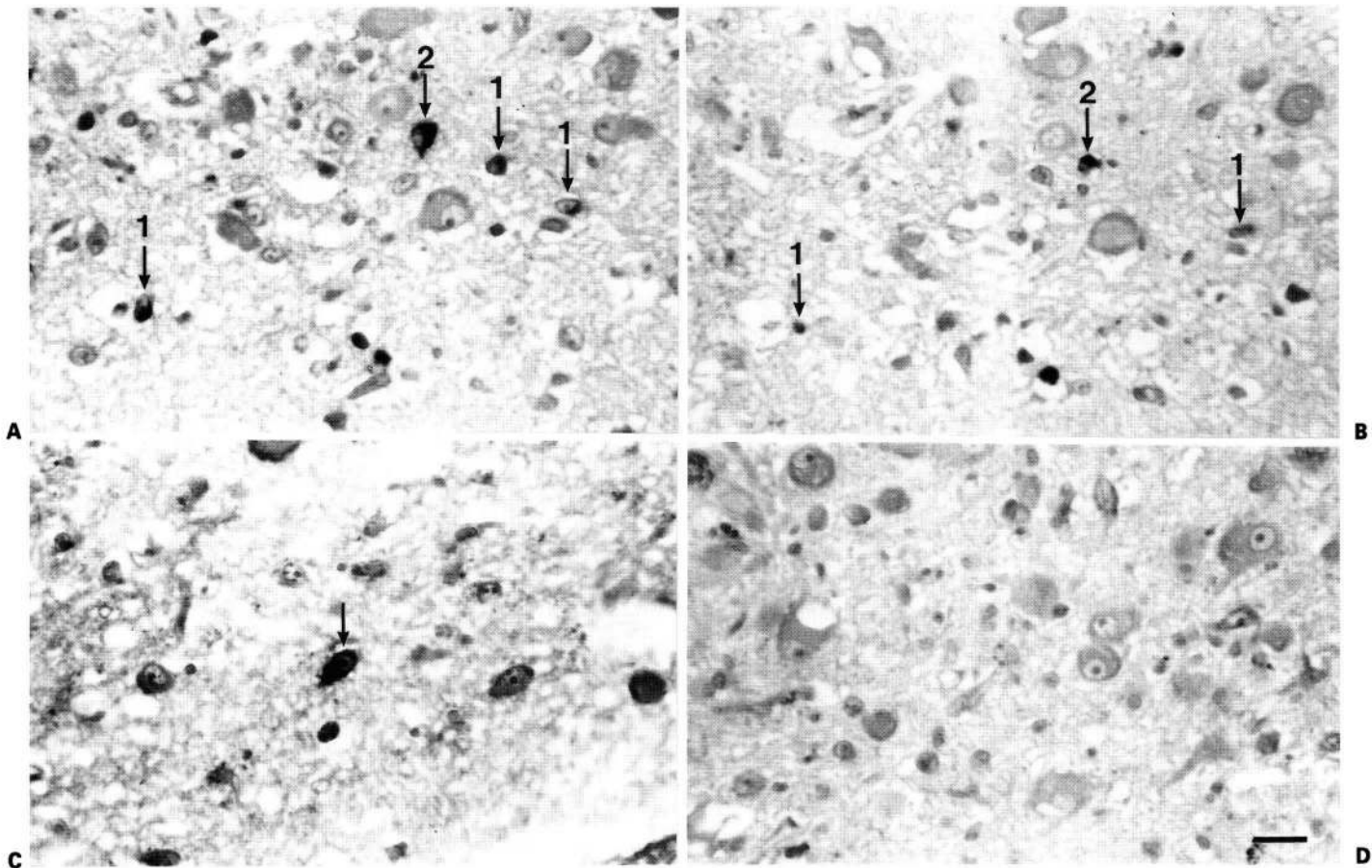


Fig. 1. Frontal sections (6 μm) of human PVN immunocytochemically stained for CRH and counterstained with neutral red. In both depressed (**A, B**) and control subjects (**C**), the CRH-containing neurons are stained blue (black cells with arrows), whereas CRH-negative cells are lightly stained red (light-gray cells). The profiles of the CRH neurons (1) in the reference section (**A**) contain a nucleus, whereas in the adjacent look-up section (**B**) no nucleus is present. Therefore, these profiles are considered as 'disector neurons'. The profile of the CRH neuron (2) contains a nucleus in both the reference section and the look-up section and is, therefore, not considered to be a 'disector neuron'. **D** Negative control section of the depressed patient showing no CRH-positive staining. Bar = 25 μm .

jects ($n = 10$) (fig. 2). This difference in the total number of CRH neurons of depressed patients ($13,676.5 \pm 2,394.2$) and control subjects ($3,413.3 \pm 736.0$) was highly significant ($p < 0.001$). The 2 subjects with a depressive organic mood syndrome or a depressive disorder not otherwise specified showed numbers of CRH-immunoreactive neurons in the PVN that were comparable to control cases ($p = 0.83$; fig. 2).

The 6 depressed patients did not show a significant age-dependent increase in the total number of CRH neurons ($p = 0.13$), in contrast to the control subjects ($p = 0.02$). The total number of CRH neurons of depressed patients was not influenced by gender ($p = 1.00$), nor was

it correlated to brain weight ($p = 0.50$), postmortem delay of the tissue ($p = 0.28$), or fixation time of the tissue ($p = 0.35$). The total number of CRH neurons in control subjects was also unaffected by these factors ($p = 0.71$, $p = 0.31$, $p = 0.27$ and $p = 0.17$, respectively [31]).

Colocalization of AVP in CRH Neurons

The AVP-, CRH- and CRH/AVP-immunoreactive neurons were visualized as red, blue and purple, respectively. The intensity and the distribution of the reaction products was comparable in all subjects and very similar to the results reported previously [30, 37]. The CRH neurons, whether or not colocalizing AVP, were parvocellu-

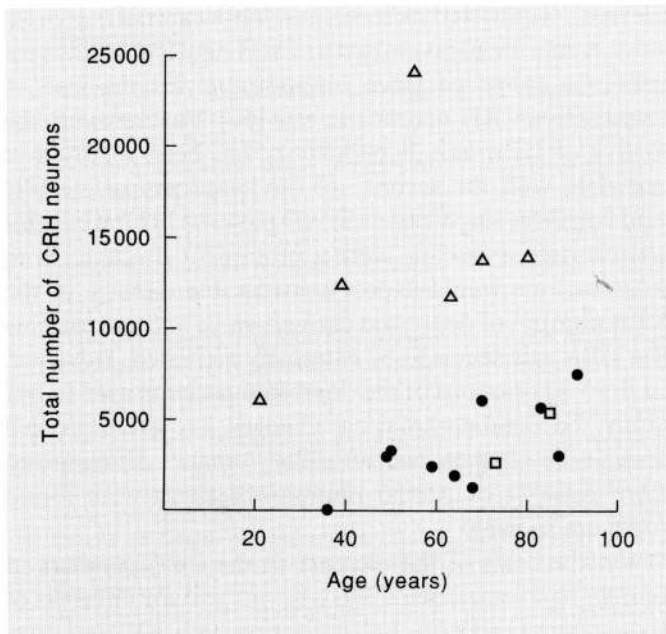


Fig. 2. The total number of CRH neurons in the PVN of human subjects at different ages. ● = 10 control subjects; △ = 6 bipolar or major depressed patients; □ = 2 'non-major depressed' subjects with either an organic mood syndrome or a depressive episode not otherwise specified.

lar, whereas the only AVP-expressing neurons were, in general, magnocellular. Although the percentage of CRH neurons colocalizing AVP was lower in depressed patients than in control cases (i.e. 42.5 vs. 53.4%), the mean number of CRH neurons colocalizing AVP was 2.9 times higher ($p = 0.04$) in the 6 depressed patients than in the controls (table 2).

Discussion

In the present study, total numbers of CRH-immunoreactive neurons in the PVN of depressed patients and control subjects were estimated by the disector method [31, 43]. The outcome of this stereologic method is independent of potential age- and/or disease-state-related differences in neuronal shape and tissue dimensions that might originate during the agonal or postmortem phases, fixation, dehydration, paraffin embedding, or sectioning [46, 47]. Therefore, meaningful biological comparisons can be made between the two groups.

The major results of the present study are the fourfold increase in the total numbers of CRH neurons in the PVN

of depressed patients as compared to those in control subjects (fig. 2), and the almost threefold increase in the numbers of CRH neurons showing AVP colocalization (table 2). According to the generally accepted principle that adult neurons do not divide, it is likely that the increment in the number of CRH-immunoreactive neurons is due to an increase in the expression of CRH by the parvocellular CRH neurons in the PVN in depression. Another sign of increased activation of the neurosecretory CRH neurons in depression is the increase in the numbers of these neurons coproducing AVP. These findings are in agreement with literature that has so far provided various indirect indications of the presence of an increased CRH peptide production in depression (see 'Introduction'). Our study, however, is the first to deal with the state of activation of the human paraventricular CRH neurons directly in the hypothalamus.

The hyperactivity of CRH neurons in depressed patients might be causally related to symptoms of this disorder, since it is known from animal experiments that intracerebroventricular administration of CRH not only stimulates the HPA axis but also leads to symptoms of depression, e.g. decreased food intake [48–50], decreased sexual activity [51], disturbed sleep and motor behavior [52], and increased anxiety [53]. The neuroanatomical context for the interactions between CRH-expressing neurons from the PVN and symptoms of depression is very complex and might include projections to brainstem and spinal cord [6, 54].

The increased activity of the paraventricular CRH neurons in the depressed patients is unlikely to be the result of the chronic administration of antidepressants (table 1), since these kinds of drugs have been found to attenuate rather than enhance activity of CRH neurons. In rats, stress-evoked stimulation of CRH neurons is reduced by tianeptine [55] and chronic treatment with amitriptyline attenuates the activity of the HPA system [56]. Long-term treatment of rats with imipramine, fluoxetine, idazoxan and phenelzine has been shown to decrease CRH-mRNA levels in the PVN [57, 58]. In healthy volunteers, the antidepressant desipramine has been shown to reduce CRH concentrations in CSF [59]. Thus, if antidepressants would interfere with our measurements it would lead to an underestimation of the observed difference between controls and depressed patients in the state of activity of their CRH-expressing neurons in the PVN.

In the colocalization study we found almost three times more paraventricular CRH neurons coexpressing AVP in depressed patients than in control subjects, as was postulated before, by Von Bardeleben and Holsboer [64].

This was in accordance with the expected activity of CRH neurons in depression. Since the total number of CRH neurons showed a fourfold increase in depressed patients, it seems that the number of non-AVP-expressing CRH neurons increased more than the number of AVP-expressing CRH neurons. This could mean that different phenotypic subtypes of CRH neurons are present and are activated in a different way in depressed patients. This view is supported by the observation of two subtypes of CRH neurons in the PVN of experimental animals [60–63]. One type can coproduce AVP and projects to the median eminence [60–63] and the other type does not coproduce AVP and projects to the brainstem and spinal cord [54]. Although in the rat these non-neuroendocrine neurons represent only a minor subpopulation of the CRH neurons in the PVN [54], it seems from our data that this fraction is considerably larger in humans and even larger than the fraction of CRH neurons projecting to the median eminence in depressed patients. It should be noted, however, that this speculation needs to be further investigated by a combined retrograde transport-immunocytochemical study. Accordingly, it seems also from our data that non-neurosecretory CRH neurons are more hyperactive than the neurosecretory CRH neurons in the PVN of depressed patients, and we hypothesize, therefore, that especially the part of the CRH neurons that does not project to the median eminence is involved in depression. It is interesting to note that the dexamethasone suppression test will not monitor alterations in this important population of CRH neurons.

Previous studies on the total number of CRH neurons in the human PVN and AVP coproduction in these neurons showed an age-dependent activation of the CRH neurons in control subjects and in Alzheimer patients [30, 31, 37]. However, the numbers of CRH neurons in the PVN of depressed patients showed no correlation with age ($p = 0.13$). Whether this is related to the limited group size or a genuine characteristic of depressed patients remains to be established.

The results from the two subjects with the ‘organic mood disorder’ and ‘the depressive disorder not otherwise specified’ may suggest that CRH neurons are not hyperactivated in these ‘non-major depressed’ patients.

Our neuropathological results of the depressed patients with hyperactive CRH neurons do not seem to support the ‘glucocorticoid cascade hypothesis’ [65]. Following this hypothesis, the combination of corticosterone-mediated hippocampal damage with the capacity of the normal hippocampus to inhibit corticosterone secretion (via the HPA axis) should lead to a feed-forward cascade of

elevated corticosterone levels and hippocampal degeneration. A role for paraventricular CRH neurons in this process was proposed since hippocampal lesions lead to hyperactive CRH neurons in rats [66]. Furthermore, the degree of HPA axis hyperactivity has been reported to correlate with the severity of the hippocampal atrophy and cognitive impairment in AD patients [67, 68]. If this glucocorticoid cascade were a prominent mechanism in humans, one would expect considerable damage in the hippocampus of depressed patients since in these patients the HPA axis seems to be extremely activated. However, in the hippocampus of the depressed patients used in our study, no neuropathological changes, i.e. excessive cell loss, senile plaques, neurofibrillary tangles, disorganized fibre patterns or Alz-50 immunoreactivity were found [data not shown].

On the basis of the increase in the total numbers of human paraventricular CRH neurons and the numbers of these neurons showing colocalization with AVP, we conclude that these neurons are activated in depression. On the basis of animal experimental data on intracerebroventricular CRH injections, we propose that the hyperactivity of CRH neurons may be causally related to the symptomatology of depression. Whether hyperactive CRH neurons play a role in the pathology of the disease via activation of the HPA axis and/or through direct projections to other brain areas needs to be further investigated.

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