Colocalization of corticotropin-releasing hormone and oestrogen receptor-\(\alpha\) in the paraventricular nucleus of the hypothalamus in mood disorders

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Summary
Oestrogens may modulate the activity of the hypothalamic–pituitary–adrenal (HPA) axis. The present study was to investigate whether the activity of the HPA axis in mood disorders might be directly modulated by oestrogens via oestrogen receptors (ORs) in the corticotropin-releasing hormone (CRH) neurons of the human hypothalamic paraventricular nucleus (PVN). Brains of 13 subjects ranging in age between 45 and 79 years suffering from major depression/major depressive disorder (eight cases) or bipolar disorder (five cases) and of 13 controls, matched for sex, age, brain weight, post-mortem delay, fixation time and season and clock time at death, were studied with double-label immunocytochemistry. The total number of CRH-immunoreactive (IR) neurons, CRH neurons that localized OR\(\alpha\) in the neuronal nucleus and the number of only nuclear OR\(\alpha\)-containing neurons in the PVN were measured using an image analysis system. In addition, the volume of the PVN delineated on the basis of CRH neurons was determined. It was found that the total number of CRH-IR neurons in patients with mood disorders was nearly 1.7 times higher than in controls (\(P = 0.034\)). A novel finding was that the total number of CRH-IR neurons and the number of CRH-nuclear OR\(\alpha\) double-staining neurons in the PVN were strongly correlated both in controls and in patients with mood disorders (\(P < 0.001\) and \(P = 0.022\), respectively). The ratio of the CRH-nuclear-OR\(\alpha\) double-staining neurons to the total CRH-IR neurons in patients with mood disorders was similar to that in the controls (\(P = 0.448\)). The volume of the sub-region of the PVN that was delineated on the basis of CRH neurons was significantly larger in patients with mood disorders than in controls (\(P = 0.022\)). Another novel finding was the large population of extra-hypothalamic CRH neurons that was found in the thalamus. In summary, oestrogens may directly influence CRH neurons in the human PVN. The increased numbers of neurons expressing CRH in mood disorders is accompanied by increased OR\(\alpha\) colocalization in the nucleus of these neurons. These changes seem to be trait- rather than state-related.

Keywords: hypothalamic-pituitary-adrenal axis; corticotropin-releasing hormone; oestrogen receptor-\(\alpha\); paraventricular nucleus; mood disorders

Abbreviations: BD = bipolar disorder; CRH = corticotropin-releasing hormone; HPA = hypothalamic–pituitary–adrenal; HPG = hypothalamic–pituitary–gonadal; MD = major depression; MDD = major depressive disorder; OR = oestrogen receptor; ORE = oestrogen responsive element; PTN = paratenial thalamic nucleus; PVN = paraventricular thalamic nucleus; TBS = Tris-buffered saline


Introduction
A number of studies have pointed to the possible involvement of sex hormones in mood, cognition and a variety of psychiatric disorders. Epidemiological studies have shown that the lifetime prevalence of major depression is twice as high in women as in men (Lehtinen and Joukamaa, 1994; Pearlstein et al., 1997). The prevalence of major depression increases
during the reproductive years, especially during times of changes in gonadal hormone levels, e.g. the premenstrual and postpartum periods and during the transition to the menopause (Paykel, 1991; Lehtinen and Joukamaa, 1994; Pearlstein et al., 1997; Young and Korszun, 2002). There is also some evidence that hormone replacement therapy could improve and prevent postpartum depression (Sichel et al., 1995; Gregoire et al., 1996). Moreover, it was found in males that testosterone levels were lower in severely depressed patients (Heuser, 2002) and that older men with lower available testosterone levels were found to be more frequently depressed (Barrett-Connor et al., 1999).

The effects of oestrogens on the brain are, at least partly, mediated by oestrogen receptors (ORs) of which two subtypes exist, i.e. ORa and ORb. Both OR subtypes are extensively localized throughout the brain (Pfaff and Keiner, 1973; Donahue et al., 2000; Osterlund et al., 2000a,b; Kruijver et al., 2002, 2003), where they may mediate the effects of oestrogens on emotion, cognition and procreation. It is also well known that conversion of androgens into oestrogens by the enzyme aromatase is a key mechanism by which testosterone regulates many physiological and behavioural processes (Balthazart and Ball, 1998). The highest levels of brain aromatase activity have been found in various limbic regions, such as the preoptic and hypothalamic regions, which overlap extensively with brain regions containing oestrogen receptors (Steimer and Hutchison, 1981; Roselli et al., 1985; Schumacher and Balthazart, 1987; Vockel et al., 1990).

It is, therefore, logical to hypothesize that the ORs may be involved in the pathogenesis of mood disorders. However, although endocrine studies have shown that oestrogens modulate a variety of neurotransmitter systems [e.g. the norepinephrine, dopamine and serotonin system (Clark et al., 1985; Blaustein et al., 1986)] that have been strongly implicated in the aetiology of mood disorders (Asberg et al., 1976; van Praag, 1983; Clark et al., 1985; Blaustein et al., 1986; Pryor and Sulser, 1991), so far the mechanism and the putative sites of oestrogen action in the human brain has not been studied in post-mortem material in relation to mood disorders (Osterlund and Hurd, 2001). The hypothalamic–pituitary–adrenal (HPA) axis—the key system in the stress response—has been extensively studied in affective diseases and is considered by many to be the final common pathway for a number of signs and symptoms of depression (Holsboer and Barden, 1996; Swaab et al., 2000). The corticotropin-releasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus (PVN) are the central driving force for the HPA response to stress (Bissette, 1990). There is a strong increase in the activity of the CRH neurons in major depression (Raadsheer et al., 1994, 1995) and a CRH-overproducing transgenic mouse model shows symptoms related to major depression, i.e. an anxious phenotype, that can be counteracted by a CRH antagonist (Stenzel-Poore et al., 1994; Muller et al., 2004). Moreover, signs and symptoms of depression can be induced in experimental animals by intracerebroventricular injection of CRH (Holsboer and Barden, 1996; Richardson et al., 2000) and antidepressant drugs attenuate the synthesis of CRH (Fischer et al., 1990; Nemeroff, 1996; Reus et al., 1997).

The presence of a close functional interaction between the HPA axis and the hypothalamic–pituitary–gonadal (HPG) axis has been known for many years. A link of infertile periods to increased levels of adrenal steroids was observed in animals (Christian, 1971), while studies in a number of species have documented inhibitory effects of CRH on gonadotropin-releasing hormone and luteinizing hormone secretion (Ferin and Vande Wiele, 1984; Gambacciani et al., 1986; Olster and Ferin, 1987; Petraglia et al., 1987; Dudas and Merchenthaler, 2002). Diminished luteinizing hormone response to gonadotropin-releasing hormone following long-term prednisolone treatment has been found in women (Sakakura et al., 1975) and, in Cushing’s disease, the presence of an irregular menstrual cycle was found related to hypercortisolemia (Lado-Abeal et al., 1998). Moreover, oestrogen-responsive elements (OREs) are found in the CRH gene promoter region and oestrogen has been shown to have modulatory actions on CRH gene expression in rodents (Vamvakopoulos and Chrousos, 1993).

On the basis of the observations mentioned above, the present study was aimed at determining whether the activity of the HPA axis in mood disorders might at least be partly modulated by oestrogens via ORs and, more specifically, whether this would affect the CRH neurons in the human PVN directly or whether it would be mediated by interneurons.

Material and methods

Subjects

Twenty-six autopsied brain samples were studied, of which 13 were patients clinically followed for mood disorders with an age range of 45–79 years and 13 were control subjects matched for sex, age, season and clock time at death, brain weight, post-mortem delay, fixation time and storage time in paraffin. Brain material was obtained from the Netherlands Brain Bank (coordinator Dr R. Ravid) following permission from the patient or next of kin for a brain autopsy and for the use of the brain material and clinical data for research purposes.

DSM-III/DSM-IV criteria were used for the diagnosis of major depression (MD)/major depressive disorder (MDD) or bipolar disorder (BD) at any time during life. The criteria for the presence, duration and severity of symptoms of either MD/MDD or BD, as well as the exclusion of other psychiatric and neurological disorders, were systematically scored by two qualified psychiatrists (Dr W.J.G. Hoogendijk or Dr E. Vermette). Eight patients fulfilled the criteria for MD/MDD and five fulfilled the criteria for BD. For detailed information, see Table 1. An overview of the medication in the past and in the last month before death for patients with mood disorders and control subjects is also given in Table 1. The medical records did not reveal any alcohol or other drug abuse among subjects of either group unless stated otherwise (C1, CS). The absence of neuropathological changes, both in the patients with mood disorders and in the controls, was confirmed by systematic neuropathological investigation (van de Nes et al., 1998) by Dr W. Kamphorst (Free University Amsterdam, The Netherlands).
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Human brain material

The hypothalami were dissected and fixed in 0.1 M phosphate buffered 4% w/v formaldehyde (pH 7.2) for 1-2 months (Table 1). Tissues were dehydrated in graded ethanol, embedded in paraffin and serially cut in frontal sections (6 µm) on a Leitz microtome and stored at room temperature. Every 100th section was stained with thionine (0.1% w/v thionine in acetate buffer, pH 4) in order to localize and orient the PVN before immunocytochemical staining.

Immunocytochemistry

The monoclonal rat anti-CRH antibody ‘PFU 83’ (IgG2a subclass) (kindly donated by Professor F.J.H Tilders) was aimed at the C-terminal part (amino acids 38-39) of rat/human CRH. For immunocytochemical staining of ORα, the polyclonal rabbit anti-ORα antibody (MC-20), which recognized the C-terminal epitope of the ORα (SantaCruz Biotechnology, Santa Cruz, CA, USA; catalogue no. sc-542) was used. The specificity of these two antibodies has been confirmed previously in our laboratory (Raadsheer et al., 1993; Ishunina et al., 2000; Kruijver et al., 2002). The staining was a modification of the method used by Erkut et al. (1995) and Huitinga et al. (2000), resulting in blue cytoplasmic CRH staining by alkaline phosphatase, red ORα nuclear or cytoplasmic staining by peroxidase, and purple CRH-ORα double staining cytoplasm.

We did perform a pilot study of ORβ staining according to the protocol of Ishunina et al. (2000) and looked for CRH-ORβ double-staining in these subjects. However, we found virtually no ORβ immunocytochemical staining in the PVN of these subjects. This was in agreement with previous findings that, in contrast to ORα, mRNA expression are very low in this hypothalamic area in humans (Osterlund and Hurd, 2001; Kruijver et al., 2002, 2003). The high ORα/β ratio seems to be a species-specific phenomena, since ORβ is abundantly present in the PVN in rat and mouse, while ORα is absent or barely expressed (Simonian and Herbison, 1997; Alves et al., 1998; Hrabovszky et al., 1998). However, ORβ is also barely present in CRH neurons of the PVN in mouse (Nomura et al., 2002).

The double staining for CRH and ORα was performed on every 100th section throughout the complete PVN region, stretched and mounted on SuperFrost/Plus slides (Menzel, Braunschweig, Germany). The rostral-caudal length of the PVN was defined as the distance between the most rostral and most caudal section that contained three or more CRH or ORα positive cells. Both criteria gave similar results. Run-effects were counteracted by a design in which sections from equal numbers of depressive patient(s) and control(s) were stained in the same run while the investigator was blind to the identities of the subjects. Staining was performed as follows:

(i) deparaffinized and rehydrated using xylene and decreasing grades of ethanol;
(ii) placed in 0.05 M Tris–HCl buffer (pH 7.6) and microwave-treated (for antigen retrieval) at 90°C for 30 min;
(iii) cooled to room temperature for 30 min and rinsed 3× for 10 min in 0.05 M Tris-buffered saline (TBS) (pH 7.6);
(iv) incubated in milk (elk, campina)-TBS (pH 7.6) for 60 min to reduce non-specific staining;
(v) incubated overnight at 4°C with rabbit anti-ORα 1:100 and rat PFU83 1:100 000 diluted in semi-milk (0.25% gelatin and 0.5 ml Triton X-100 and 5% of milk powder in 100 ml TBS, pH 7.6);
(vi) rinsed 3× 10 min in TBS and incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlington, CA, USA) 1:200 and anti-rat IgG conjugated with alkaline phosphatase (K.P.L., Maryland, USA) 1:50 in semi-milk at room temperature for 60 min;
(vii) rinsed 3× 10 min in TBS and incubated with ABC elite kit (1:400, Vector Laboratories) diluted in TBS for 60 min;
(viii) rinsed 3× 10 min in TBS and incubated with biotinylated tyramide diluted 1:750 in TBS plus 0.01% hydrogen peroxide for 15 min to amplify the immunocytochemical reaction of the ORα antigen (Adams, 1992);
(ix) repeat step (vii);
(x) rinsed 3× 10 min in TBS and incubated for 5 min in 0.2 M Tris–HCl buffer pH 8.8;
(xi) incubated with a filtered suspension of 2 mg Fast Blue BB base (Sigma, St.Louis, MO, USA) in 10 ml 0.2 M Tris–HCl buffer (pH 8.8) containing 2 mg Naphtol-AS-AX phosphate (Sigma) and 3 mg levamisole (Sigma);
(xii) rinsed 3× 10 min in TBS and incubated for 5 min with 0.2 M acetic acid buffer pH 5;
(xiii) incubated with a filtered suspension of 5 mg of 3-amino-9-ethylcarbazole (AEC) in 10 ml 0.2 M acetic acid buffer (pH 5) containing 0.5 ml N,N-dimethylformamide and 5 µl 30% hydrogen peroxide;
(xiv) rinsed 3× 10 min in TBS and coverslipped with Kaisers glycérin-gelatin (Merck, Darmstadt, Germany).

Since cells showing only ORα and no CRH cytoplasmic staining generally comprised <10% of all of the cell profiles, we did not distinguish this as a separate cell population. The total numbers of the three main classes of cell profiles were determined, i.e. (i) CRH single or CRH-ORα cytoplasmic double-staining cells, (ii) ORα nuclear single-staining cells and (iii) cytoplasmic CRH-ORα nuclear double-staining cells.

Volume measurements

The volume of the PVN delineated by CRH neurons was estimated unilaterally by measuring the cross-sectional area delineated by immunoreactive CRH neurons in every 100th section using 2.5× objective (Plan-Neofluar) on a Zeiss (Oberkochen, Germany) Axiostkop microscope mounted with a Sony (Tokyo, Japan) black/white CCD camera (model XC77CE), connected to an IBAS image analysis system (Kontron-IBAS, Munich, Germany). The total volume of the PVN delineated by CRH neurons was calculated according to the Cavalieri principle (Gundersen et al., 1988). A pilot study had shown that the sampled volume delineated either by CRH or ORα neurons gave similar results.

Estimation of total neuronal number

Cross-sectional digital images (every 100th section) were made using a 2.5× objective (Plan-Neofluar) on a Zeiss Axiostkop microscope, mounted with a Sony black/white CCD camera (model XC77CE) that was connected to an IBAS imaging analysis system. The contour of the entire PVN field occupied by CRH and/or ORα-immunostained neurons was manually outlined by the operator with the cursor, with a final on-the-monitor magnification of 90× (when the objective was 2.5×); subsequently, the imaging analysis system overlaid a grid of rectangular fields within the outlined cross-sectional area. Each field was equal in size to the area displayed by the camera at 63× objective (Plan-APOCHROMAT).
Table 1  *Brain material of patients with mood disorders and control subjects*

<table>
<thead>
<tr>
<th>Mood disorder patients</th>
<th>Sex</th>
<th>Age at death/age of onset (years)</th>
<th>Brain weight (gram)</th>
<th>Post-mortem delay (hours: minutes)</th>
<th>Fixation time (days)</th>
<th>Storage time in paraffin (months)</th>
<th>Clock time at death</th>
<th>Month of death</th>
<th>Clinicopathological information</th>
<th>Suicide attempt</th>
<th>No. of episodes</th>
<th>Medication taken in the past</th>
<th>Medication taken in the last month</th>
<th>Died during depressive episode?</th>
</tr>
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<td>M</td>
<td>45/32</td>
<td>1427</td>
<td>54</td>
<td>7</td>
<td>2:30</td>
<td>6</td>
<td>6</td>
<td>brain haemorrhage (pons)</td>
<td>No</td>
<td>1 SSRI</td>
<td>SSRI</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>D2 F 55/40 1320 4:54</td>
<td>F</td>
<td>55/40</td>
<td>1320</td>
<td>52</td>
<td>92</td>
<td>7:45</td>
<td>11</td>
<td>1</td>
<td>heart failure, urosepsis</td>
<td>Yes</td>
<td>2 MAP, BZD, TCA, PHT</td>
<td>SSRI, BRO</td>
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<tr>
<td>D3 M 61/50 1424 40:20</td>
<td>M</td>
<td>61/50</td>
<td>1424</td>
<td>42</td>
<td>98</td>
<td>4:40</td>
<td>10</td>
<td>10</td>
<td>diabetes, pneumonia, infarction right hemisphere</td>
<td>No</td>
<td>1 TCA</td>
<td>PHT</td>
<td>Probably yes</td>
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<tr>
<td>D4 M 71/53 975 16:15</td>
<td>M</td>
<td>71/53</td>
<td>975</td>
<td>38</td>
<td>105</td>
<td>16:15</td>
<td>2</td>
<td>2</td>
<td>cerebral ischemia, pneumonia mesothelioma, pneumonia</td>
<td>Yes</td>
<td>4 BZD, MAOI TCA</td>
<td>None</td>
<td>Yes</td>
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<tr>
<td>D5 F 72/54 1287 22:00</td>
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<td>72/54</td>
<td>1287</td>
<td>39</td>
<td>87</td>
<td>19:00</td>
<td>1</td>
<td>1</td>
<td>heart failure, septic shock, pyelonephritis</td>
<td>No</td>
<td>3 MAP</td>
<td>BZD</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>D6 F 72/53 1116 28:25</td>
<td>F</td>
<td>72/53</td>
<td>1116</td>
<td>35</td>
<td>12</td>
<td>4:20</td>
<td>4</td>
<td>4</td>
<td>Strangulation (suicide)</td>
<td>No</td>
<td>4 MIA, TCA</td>
<td>BZD</td>
<td>Yes</td>
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<td>74/74</td>
<td>1444</td>
<td>100</td>
<td>17:05</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>suicide</td>
<td>subdural haematomata following fall</td>
<td>Yes</td>
<td>1 None</td>
<td>ZUC, SSRI, BZD</td>
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<td>D8 M 70/ND 1530 21:10</td>
<td>M</td>
<td>70/ND</td>
<td>1530</td>
<td>101</td>
<td>17:50</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>cardiogenic ischaemia, cardiac arrest, ileus due to intestinal haemorrhage cachexia, dehydration</td>
<td>Yes</td>
<td>ND None</td>
<td>SSRI, MAOI</td>
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<td></td>
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<tr>
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<td>M</td>
<td>68/48</td>
<td>1424</td>
<td>30</td>
<td>4</td>
<td>2:30-9:45</td>
<td>2</td>
<td>2</td>
<td>Strangulation (suicide)</td>
<td>No</td>
<td>4 Li</td>
<td>MAOI</td>
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<tr>
<td>D10 b M 68/32 1204 5:55</td>
<td>M</td>
<td>68/32</td>
<td>1204</td>
<td>53</td>
<td>23:15</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>cardiogenic ischaemia, cardiac arrest, ileus due to intestinal haemorrhage cachexia, dehydration</td>
<td>Yes</td>
<td>4 None</td>
<td>Li, TCA</td>
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<td>70/35</td>
<td>1490</td>
<td>43</td>
<td>32</td>
<td>2:45</td>
<td>10</td>
<td>10</td>
<td>CVA left, acute abdomen secondary to perforation either stomach or intestines</td>
<td>No</td>
<td>8 Li</td>
<td>Mantle</td>
<td>Yes</td>
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<td>D12 b M 73/28 1260 5:15</td>
<td>M</td>
<td>73/28</td>
<td>1260</td>
<td>36</td>
<td>20</td>
<td>9:30</td>
<td>1</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
<td>9 Mo, Li</td>
<td>SSRI, MAOI</td>
<td>Yes</td>
<td>Mantle</td>
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<tr>
<td>D13 b F 75/40 1123 4:00</td>
<td>F</td>
<td>75/40</td>
<td>1123</td>
<td>38</td>
<td>23</td>
<td>20:45</td>
<td>1</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
<td>3 SSRI, NSAID</td>
<td>SSRI, MAOI</td>
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<td>Median</td>
<td>71/44</td>
<td>1320</td>
<td>12:00</td>
<td>38</td>
<td>12:00</td>
<td>38</td>
<td>6</td>
<td>6</td>
<td>1309.5 ± 167.7</td>
<td>18.54 ± 17.28</td>
<td>38.7 ± 7.6</td>
<td>56.5 ± 4.2</td>
<td>0.4 ± 2.2 *</td>
<td>23.48 ± 4.40*</td>
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<tr>
<td>Range</td>
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<td>555</td>
<td>58.55</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>6</td>
<td>6</td>
<td>1309.5 ± 167.7</td>
<td>18.54 ± 17.28</td>
<td>38.7 ± 7.6</td>
<td>56.5 ± 4.2</td>
<td>0.4 ± 2.2 *</td>
<td>23.48 ± 4.40*</td>
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<tr>
<td>C1</td>
<td>F</td>
<td>43</td>
<td>1345</td>
<td>92.00</td>
<td>63</td>
<td>30</td>
<td>23:00</td>
<td>7 myocardial infarction, (alcohol abuse)</td>
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<tr>
<td>C2</td>
<td>M</td>
<td>44</td>
<td>1565</td>
<td>10.00</td>
<td>149</td>
<td>56</td>
<td>7.00</td>
<td>7 acute myocardial infarction</td>
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<tr>
<td>C3</td>
<td>M</td>
<td>47</td>
<td>1368</td>
<td>29.13</td>
<td>68</td>
<td>112</td>
<td>11:47</td>
<td>12 hypercholesterolemia, acute myocardial infarction</td>
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<tr>
<td>C4</td>
<td>F</td>
<td>61</td>
<td>1296</td>
<td>17.45</td>
<td>119</td>
<td>55</td>
<td>23:15</td>
<td>12 acute heart failure</td>
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<td>C5</td>
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<tr>
<td>C6</td>
<td>M</td>
<td>69</td>
<td>1352</td>
<td>19.15</td>
<td>ND</td>
<td>28</td>
<td>23:00-3:30</td>
<td>8 pneumonia, sudden death during sleep (delirium)</td>
<td></td>
<td></td>
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<td>11 myocardial infarction, septic shock, metastasized pancreas carcinoma</td>
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<td>Mean ± SD</td>
<td>66.5 ± 14.0</td>
<td>1322.8 ± 123.4</td>
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<td>54.3 ± 40.1</td>
<td>56.5 ± 30.0</td>
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*Temazepam was started 3 days and Haloperidol 2 days before death for a state of delirium. *Circular mean and SD of the clock time at death or the month of death. Neither the clock time or month of death differed significantly between the groups as tested with the Mardia-Watson-Wheeler test. βB = beta-blocker; BRO = bromperidol; BZD = benzodiazepine; F = female; Hal = haloperidol; Li = lithium; MAOI = monoamine oxidase inhibitor; M = male; MAP = maprotiline; MIA = mianserin; Mo = morphine; ND = no data; None = no medication; NSAID = non-steroidal anti-inflammatory drug; PG = prostaglandin synthetase inhibitor; PHT = phenothiazine; SSRI = selective serotonin reuptake inhibitor; TCA = tricyclic antidepressant; To1 = Tolvon; ZUC = zuclopenthixol.
For analysis, 10% of the rectangular fields (each field covering at least 10% of the outlined area) for the ORα nuclear single-staining, 60% for the CRH single-staining or for the CRH-ORα cytoplasmic double-staining was selected by a random systematic sampling procedure. A pilot study had shown that these counting percentages yielded a good estimation of the total numbers of cell profiles. To prevent double counting, only neurons containing a nucleolus (~2 μm diameter) were counted. This counting procedure, which was judged to be the best for the thin (6 μm) section used, is based on the principle that nucleoli can be considered as hard particles that will not be sectioned by a microtome knife but, instead, are pushed either in or out of the paraffin when hit the knife (Jones, 1937; Cammermeyer, 1967; Koningsmark, 1970; Braendgaard and Gundersen, 1986; Huitinga et al., 2000; Chung et al., 2002). No double nucleoli were observed, confirming the observation of Huitinga et al. (2000) for the PVN. All visible neurons with a nucleus containing a nucleolus, within the exclusion lines, were counted using a 63× objective with a final on-the-monitor magnification of 2400×. By dividing the total number of determined cell profiles by the sampled volume, an estimate of the cellular density was calculated for each PVN area and the total number of the cell profiles was calculated by multiplying this volume with the cellular density. The measurements were made without knowledge of the identity of the sections.

A test of the precision of the method for estimating CRH neurons was performed in eight subjects whose sections were stained and counted in two separate runs by one investigator (I. Huitinga; data from Huitinga et al., 2000), who was blind to the subjects’ identities. This test revealed highly significantly correlated values (p = 0.905, P = 0.002).

Statistical analysis

Differences among the groups were statistically evaluated by the nonparametric Mann–Whitney U-test, since the data were discrete and not normally distributed. The association between the number of total CRH cells and the number of CRH-ORα nuclear-colocalizing cells and the association between the duration of mood disorders with the volume of PVN and CRH data were examined using Spearman’s correlation coefficient. Differences in clock time of death and month of death (circular parameters) between controls and patients with mood disorders were tested with the Mardia–Watson–Wheeler test (Batschelet, 1981). Tests were two-tailed. Values of P < 0.05 were considered to be significant.

**Results**

The sex, age (z = −0.103, P = 0.920), season and clock time at death (F = 2, χ² = 0.553, P = 0.766; and F = 2, χ² = 3.320, P = 0.190; respectively), brain weight (z = −0.026, P = 1.000), post-mortem delay (z = −0.0359, P = 0.724), fixation time (z = −0.821, P = 0.418) and storage time in paraffin (z = −0.333, P = 0.762) were well matched for the mood disorders and control group. The intensity of the staining, the distribution of immunocytochemical reaction product and the appearance of the immunoreactive neurons in patients with mood disorders were similar to the results of the control cases, except that there were more CRH neurons present in some of the patients with mood disorders. Scattered CRH neurons were found in the PVN (our main area of interest), but also in the hypothalamic infundibular (= arcuate) nucleus. CRH fibres were found in the area of the suprachiasmatic nucleus. A surprisingly large population of CRH neurons was found in the thalamus, i.e. in the paraventricular thalamic nucleus (PVN) and paratenial thalamic nucleus (PTN), extending to the anteromedial thalamic nucleus and the anterior principle nucleus (for an example, see Fig. 1A). Clear CRH-staining in the thalamus was present, both in controls and in patients with mood disorders. No obvious characteristic differences were found between the CRH neurons in the thalamus and those in the PVN (Fig. 2), except that the staining intensity of the CRH neurons in the thalamus was generally weaker than those in the PVN.

The total population of CRH neurons (including the only CRH-containing and CRH-ORα nuclear staining neurons) was significantly larger than in controls (Fig. 3). The median of the total number of CRH neurons (i.e. 15 298 in the patients with mood disorders) was ~1.7 times higher than that found in the controls (z = −2.128, P = 0.034) (Fig. 3A). The median of the total number of CRH-ORα nuclear double-staining neurons was 5594, which was ~1.2 times higher than found in the controls—a trend that did not reach significance (z = −1.769, P = 0.081). The median proportion of the number of CRH-ORα nuclear double-staining neurons of the total number of CRH neurons in patients with mood disorders was

![Fig. 1](image_url) A large population of CRH neurons in the thalamic anterior principal nucleus: a frontal section (6 μm) of the human thalamus of a control subject (C11) (A,B). (B) represents a 12.5× higher magnification of (A) and shows a cytoplasmic CRH (blue)–ORα (red) nuclear double-staining neuron.
similar to that in the controls (48% versus 37%) (z = −0.795, P = 0.448). Significant correlations between the number of CRH cells and the number of CRH-ORα nuclear double-staining neurons in the PVN were found both in control and mood disorders groups (ρ = 0.852, P < 0.001 and ρ = 0.626, P = 0.022, respectively) (Fig. 4). The same holds for the two combined groups (ρ = 0.801, P < 0.001). The total number of ORα single nuclear staining neurons in patients with mood disorders (56 018) was not significantly higher than that in controls (44 433) (z = −1.308, P = 0.204).

Fig. 2 Frontal section of the PVN in a control (C12) (A,B) and a patient with mood disorder (D10) (C,D) stained for CRH (blue) and ORα (red). (B) and (D) represent a 4 × higher magnification of (A) and (C). The arrows, solid and hollow arrowheads in (A,B) and (C,D) indicate the same place in the preparation to facilitate comparison. Both sections show the central part (mid-level) of the PVN and contain the largest number of stained neurons. It is clear by comparing (A) with (C) and (B) with (D), that the number of stained neurons is markedly increased in this patient of mood disorder. III: the third ventricle. The arrow points to an ORα nuclear single-staining cell; the solid arrowhead points to a cytoplasmic CRH-ORα nuclear double-staining cell and the hollow arrowhead points to a CRH single-staining cell.
The median of the PVN volume delineated on the basis of CRH neurons in mood disorders (20.88 mm³) was found to be significantly larger than that in the controls (13.74 mm³) (z = -2.282, P = 0.022) (Fig. 3B). This significance did not depend on the highest value in the mood disorders group (D1, who was depressive 12 years earlier, refused therapy). When this value was left out, the difference between the groups remained significant (z = 2.067, P = 0.040).

No significant differences were found between MD/MDD (n = 8) and BD subjects (n = 5) (P = 0.127). Similar values were obtained in patients that died during a depressed state (n = 9) or not (n = 3) (P ≈ 0.260) or in a manic state (one patient) in any one of the parameters mentioned above, i.e. the total population of CRH neurons, the total number of CRH-ORα nuclear double-staining neurons, the proportion of the number of CRH-ORα nuclear double-staining neurons...
of the total number of CRH neurons, correlations between the number of CRH cells and the number of CRH-ORα nuclear double-staining neurons in the PVN, the total number of ORα single nuclear staining neurons and the median of the PVN volume delineated on the basis of CRH neurons. There was no difference between the patients with mood disorders with a history of suicide attempt and those without such a history (n = 7 and n = 6, respectively; P = 0.138) in all these parameters. In addition, we did not find differences in any one of these parameters between females and males, either within the control group (n = 4 and n = 9, respectively; P = 0.148), or in the mood disorders group (n = 4 and n = 9, respectively; P = 0.199). It should be noted, however, that the clinical data in these sub-groups of patients were not perfectly matched. Some trends of differences may exist in the statistics mentioned above that did not achieve significant levels due to the small sample size in each sub-group.

When we checked the clinical pathological data of the two patients with mood disorders with the largest numbers of CRH-IR neurons, no other common characteristics presented besides the common diagnosis of MD/MDD. There were no significant correlations between: (i) the total number of CRH neurons, ORα nuclear single staining neurons, or the volume of the PVN delineated on the basis of CRH neurons; and (ii) the post-mortem delay, fixation time or storage time in paraffin (P = 0.289) in the control group, nor did these parameters have significant correlations with the duration of the mood disorders (from <1 year to 45 years) (P = 0.114) in the mood disorders group. A significantly negative correlation was found in the mood disorders group between the number of single ORα nuclear staining neurons and the post-mortem delay (ρ = −0.775, P = 0.002). However, use of ANOVA (analysis of variance) controlling for differences in post-mortem delay between the groups showed that there was no significant difference in the number of single ORα nuclear staining neurons between the depressive and the control subjects (P = 0.078). No significant correlations were found between age and the number of immunostained cell profiles in either the control or the mood disorders group (P = 0.627 and P = 0.589, respectively). It was noticed that control subject C1, a 43-year-old female with liver cirrhosis due to alcohol abuse, had a much higher total number of CRH (21 410), CRH-ORα nuclear double-staining neurons (10 749) and volume of the PVN delineated by the presence of CRH neurons (19.15 mm³) than the rest of the controls. Activation of the HPA axis is, indeed, a well-known characteristic of alcoholism (see Discussion). The significances found above—comparison of the total number of CRH neurons with the volume of PVN delineated on the basis of CRH neurons between the patients with mood disorders and controls—were reinforced (z = −2.339, P = 0.019 and z = −2.393, P = 0.016, respectively) and the trend in the number of CRH-ORα nuclear double-staining neurons between the patients with mood disorders and controls reached statistical significance when the subject with alcohol abuse was left out of the analysis (z = −2.067, P = 0.040).

Discussion

The present study demonstrates for the first time that ORα is co-localized with CRH in ~40% of total CRH neurons in the human PVN and that increased numbers of CRH neurons in mood disorders are accompanied by a similar increase in nuclear ORα staining in these neurons. Oestrogens are thus capable of influencing CRH neurons directly, as is clear from the colocalization of ORα in these neurons. This finding agrees fully with the presence of OREs in the CRH promoter region (Torry et al., 1997). The increased total number of CRH neurons in mood disorders confirmed earlier studies by our group on the protein and mRNA level (Raadsheer et al., 1994, 1995). Previous studies of our group did not find a difference in the PVN volume delineated by a conventional staining (Raadsheer et al., 1994) nor in the PVN volume as stained for oxytocin or vasopressin neurons (Purba et al., 1996), which agrees with the finding of Bernstein et al. (1998). To our knowledge, our study is the first to find a significantly larger volume of the sub-region of the PVN, which is delineated on the basis of CRH neurons, in patients with mood disorders than in controls. This indicates that, during activation, more cells in the periphery that do not stain in controls are ‘recruited’ to produce CRH. Since in mood disorders some 7000 more cells express CRH and the median value of single ORα nuclear expressing neurons goes up from 44 433 to 56 018 (see Results), it seems that cells expressing neither CRH nor ORα in controls are recruited in mood disorders. The observed larger volume of the CRH sub-region of the PVN in patients with mood disorders is consistent with the presence of a permanent hyperactivity of the HPA axis. As has been described before, depressive illness is presumed to be the result of an interaction between the effects of environmental stress and genetic and developmental predisposition (Holsboer and Barden, 1996; Swaab et al., 2000). The set point of the HPA-axis activity is not only programmed by genotype, but can be changed to another level by early life events (De Kloet et al., 1997; Swaab et al., 2000; Heim and Nemeroff, 2001). Stressful life events during development may predispose individuals to adult-onset depression by a permanent hyperactivity and hypersensitivity of the HPA axis (Levitan et al., 1998; De Bellis et al., 1999; Weiss et al., 1999; Swaab et al., 2000). Interestingly, prenatal oestrogen administration may also increase the risk of affective disorders, as appeared from a study on individuals who, during foetal life, were exposed to diethylstilbestrol, a synthetic non-steroidal oestrogen (Meyer-Bahlburg, 1987). Since the HPA axis is considered to be the ‘final common pathway’ in the stress responsive and depressive symptomatology (Holsboer and Barden, 1996; Swaab et al., 2000), this observation (Meyer-Bahlburg, 1987) and our results raise the possibility that oestrogens are involved in prenatal programming of the level of activity of CRH neurons, thus influencing the increased risk of mood disorders. This hypothesis should be further investigated.

Oestrogen receptors, like many of the other members of the nuclear receptor superfamily, act as ligand-activated
transcription factors. The human CRH gene contains five perfect half-palindromic OREs and estradiol may enhance the HPA-axis activity by stimulation of CRH gene transcription (Torpy et al., 1997). Our finding of the close relationship of CRH with ORα nuclear double-staining in the PVN is consistent with these data. Upregulation of ORα seems to accompany activation of CRH neurons in mood disorders. The similar ratios of the amount of CRH with ORs nuclear double-staining neurons to the total number of CRH neurons in patients with mood disorders (48%) and control subjects (37%) suggest that oestrogens may contribute to CRH activation via OR. However, the exact mechanism of the action of oestrogens on CRH neurons should be a topic for further study.

The finding that there virtually no ORβ immunochemical staining in the PVN of these subjects was in agreement with previous reports showing that, in contrast to ORα, ORβ and ORβ mRNA expression are very low in this hypothalamic area in human (Osterlund and Hurd, 2001; Kruijver et al., 2002, 2003) and that the high ORα/β ratio seems to be a species- and area-specific phenomena (Simonian and Herbison, 1997; Alves et al., 1998; Hrabovszky et al., 1998; Nomura et al., 2002; Kruijver et al., 2003). Indeed, it was previously shown that there are antagonistic effects mediated by ORα and ORβ in HeLa cells in vitro, where ORα mediated the activation and ORβ the inhibition of transcription (Paech et al., 1997). Previous studies by our group have proposed that ORβ mediates inhibition and that ORα mediates stimulation—also of AVP cells in human supraoptic nucleus—by oestrogens acting on the genomic level (Ishunina and Swaab, 1999; Ishunina et al., 1999, 2000). It would certainly be of interest to investigate whether ORα mediates activation of CRH neurons in the human PVN.

The increased activity of the PVN CRH neurons in the patients with mood disorders is unlikely to be the result of the duration of mood disorders or the chronic administration of antidepressants (Table 1), since it has been found that both the chronicity of the disease and antidepressants may attenuate rather than enhance the activity of CRH neurons (Fischer et al., 1990; Vingerhoets et al., 1996; Nemeroff, 1997; Oldehinkel et al., 2001). Thus, if antidepressants interfered with our measurements, this would have led to an underestimation of the observed difference between controls and patients with mood disorders in the state of activity of their CRH-expressing neurons in the PVN. Moreover, no significant differences were found between subjects taking lithium or mood stabilizing drugs and subjects taking antidepressants or other psychoactive drugs. However, we did not include this point in Results because the statistical analyses were doubtful due to the small sub-samples and the overlap in the use of these drugs among these patients. A proper comparison between the effects of those drugs on the HPA and/or the HPG axes deserves further study. No relationship was found between the total number of CRH neurons and the duration of mood disorders in the present study. The finding that MD/MDD and BD patients showed similar activities of the HPA axis is also consistent with previous studies (Raadsheer et al., 1995; Zhou et al., 2001). It was not surprising to find an extremely high total number of CRH neurons in control subject C1, since it is known that chronic alcohol abuse is associated with activation of the HPA axis and resistance to dexamethasone (Wand, 1999; Nurnberger et al., 2001).

Some limitations of the present post-mortem study should be mentioned. There were not enough data available on the CSF pH as an indicator for agonal changes (Swaab, 2003). However, it is unlikely that a difference in the activity of the PVN CRH neurons is related to agonal state, since a previous study by our group found that agonal differences do not significantly affect the activity of the HPA axis as indicated by CSF cortisol levels (Erkut et al., 2004). In addition, the mood disorders group was made up of a heterogeneous patient sample, i.e. patients diagnosed with MD/MDD or BD who had died during a depressed state or not, or in a manic state. Although no significant differences were found between these sub-groups, the numbers of each sub-group are too small to allow definite conclusions. Some trends do exist in these sub-analyses, suggesting that, possibly, no significant differences were achieved due to the small sample size in each sub-group. Moreover, the patients’ data were not perfectly matched in these sub-analyses. The increased expression of CRH and ORα in mood disorders seems to be related to trait rather than state, as no differences were found between those who died during a depressive episode or when not in a depressed state. Future studies with larger post-mortem samples of particular diagnostic entity are thus needed for the various aspects mentioned above.

The large CRH neuron population we found in the human thalamus opens a new topic for further study. The exact nature of these CRH-containing neurons and whether their function changes in physiological or pathological conditions is not known. The question if whether these neurons may contribute—as an extrahypothalamic source—to the CRH levels in the CSF also deserves further study. These CSF-CRH levels probably do not reflect the activity of the PVN, but rather reflect fluctuations in extrahypothalamic CRH (Mitchell, 1998; Arborelius et al., 1999; Beyer, 2000; Vythilingam et al., 2000), e.g. from the neocortex, limbic and brainstem regions and from the thalamus (this study). The present study could not answer the question how ORα is upregulated in the PVN in mood disorders. Since a similar pattern of upregulation was found in males and females, the role of circulating levels of oestrogens in the regulation of ORs in CRH neurons is doubtful. Whether a higher local production of oestrogens—produced via the enzyme aromatase from adrenal or brain-derived androgens—may act as ligand and upregulate ORα in the brain (Kroboth et al., 1999) and/or whether the upregulation of ORα is modulated by neurotransmitters such as norepinephrine, dopamine, serotonin—all of which are involved in the central stress responses and mood disorders (Asberg et al., 1976; Blaustein et al., 1986)—deserves further study in human.
Acknowledgements

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