Increased activity of hypothalamic corticotropin-releasing hormone neurons in multiple sclerosis

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Abstract

Clinical observations and animal studies suggest that the hypothalamo-pituitary-adrenal (HPA) axis plays a role in the susceptibility to and the recovery from multiple sclerosis (MS). Since the HPA-axis is under the control of corticotropin-releasing hormone (CRH) neurons of the hypothalamus, we determined 2 parameters for activation of the CRH neurons in the hypothalamic paraventricular nucleus (PVN) in MS patients. Since the HPA-axis is more activated in MS, we expected an increased activity of CRH neurons. We also expected to see an age-related increase in CRH activity, because of the possible role of the HPA-axis in the age-related decrease in susceptibility to MS. The number of CRH cell profiles and the proportion of CRH neurons co-expressing vasopressin were used as parameters for activity. CRH cell population became more activated both in control and MS patients, from 40 years of age onwards, when the prevalence of MS starts to decrease in the population. The CRH neurons showed a significantly higher level of activation in MS patients than in controls, as appeared from the 3-fold increase in CRH cell number and the 4.5-fold increase in cells co-expressing CRH and vasopressin (AVP).

Keywords: Multiple sclerosis; Hypothalamo-pituitary-adrenal axis; Paraventricular nucleus; Corticotropin-releasing hormone; Vasopressin; Human hypothalamus

1. Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating disease of the central nervous system (CNS). It is generally a disease of young adulthood, with the peak age of onset between 25 and 30 years of age (Limburg, 1950). MS is an immune-mediated disease, resulting in myelin breakdown and CNS damage, and is characterized by demyelination plaques of the white matter, disseminated in time and space (Raine, 1994). Experimental allergic encephalomyelitis (EAE), the most commonly used animal experimental model for MS, shows neuropathological changes and involvement of the immune system during the relapsing phase, similar to those of MS (Rodriguez and Scheithover, 1994; McDonald, 1994; Sobel et al., 1988; Pender, 1987; Polman et al., 1986). Studies performed with different rat strains showed that having a higher activity level of the hypothalamo-pituitary-adrenal (HPA)-axis decreases their susceptibility to EAE, and, once the disease is established, helps to further recovery from the symptoms (MacPhee et al., 1989; Mason et al., 1990; Kuroda et al., 1994; Villas et al., 1991). Administration of corticosteroids may induce suppression of the symptoms both in EAE (Levine and Strebel, 1969; Mason et al., 1990) and in MS (Frezquin et al., 1994; Miller et al., 1992; Milligan et al., 1987; Wenning et al., 1994). Endocrine studies in MS patients indicate an increase in HPA-axis activity in this disease, as appears from increased levels of baseline plasma cortisol, urinary free cortisol, plasma adrenocorticotropic hormone (ACTH) (Michelson et al., 1994) and adrenal gland size (Reder et al., 1987,1994).

Recently, Purba et al. (1995) showed a significant 2.5-fold increase in the number of CRH-expressing neurons in the paraventricular nucleus (PVN) of the hypothalamus of MS patients. In aging, Alzheimer’s disease and depression, the CRH neurons appeared to show different modes of activation, depending on the clinical disorder, i.e. (i) more neurons expressing CRH, (ii) more CRH neurons co-expressing AVP and/or (iii) increased CRH mRNA levels in humans (Raadsheer et al., 1993, 1994a,b, 1995).
Following up the observations concerning an increase in the number of CRH-expressing neurons (Purba et al., 1995), the intention of the present study was to determine in MS, 1) the number of CRH-containing cell profiles in the PVN, and 2) the ratio of CRH neurons co-expressing AVP, as a new measure for activation of CRH neurons (DeBold et al., 1984; De Goeij et al., 1991; Liu et al., 1983). The latter parameter appeared to be a reliable measure of activation of CRH neurons both in human (Raadsheer et al., 1993, 1994a,b) and in rat, as judged from the significant enhancement of the co-expression of AVP and CRH in the hypothalamic PVN cells and in the median eminence, under stressful conditions (De Goeij et al., 1991, 1992, 1993). We expected an increased activity of hypothalamic CRH cell population in MS, as well as an age-related increase in CRH activity in controls, indicating a possible relationship with the age-related resistance to MS.

2. Materials and methods

2.1. Human brain material

We studied the hypothalami of 8 MS patients, 4 males and 4 females, who had been clinically diagnosed to suffer from MS according to the Poser criteria (Poser et al., 1983), and who had not been recently treated with corticosteroids. The clinical diagnosis MS (by Prof. Dr. C.H. Polman, Free Univ., Amsterdam) was confirmed by neuropsychological examination (by Dr. W. Kamphorst, Free Univ., Amsterdam). A group of 8 subjects without a primary neurological, neuroendocrine or psychiatric disease, who had not undergone recent steroid treatment, with a similar sex and age distribution, postmortem delay, fixation time and clock-time of death, served as controls.

To be able to refer to the results of Purba et al. (1995), we used, whenever possible, the same subjects as they did. Thus all MS patients and 4 (from the total of 8) controls were the same subjects that were used for the study of Purba et al. The 4 new control subjects were chosen either to have a better match for the variables age, fixation time and postmortem delay between the groups, or to replace the subjects of whom we did not have enough hypothalamic sections left. None of the MS patients had an acute relapse at the time of death. For detailed clinicopathological information on the subjects, see Table 1.

Preparation of the brain material

The hypothalami were obtained from the Netherlands Brain Bank (NBB). The brains were weighed and the hypothalami were immediately dissected out and fixed in 4% w/v formaldehyde at room temperature for about one month. The tissue was subsequently dehydrated in graded ethanol and embedded in paraffin (Histowax, melting point 56–58°C, Histo-Lab LTD, Sweden).

Serial 6 μm frontal sections were cut on a microtome, mounted on chrome-alum-coated object slides and stored at 37°C for 24 h, followed by deparaffinization in xylene and redehydration through a graded ethanol series. Every 100th section was stained conventionally with thionine to localize the boundaries of the PVN before the immunocytochemical (ICC) staining.

2.2. Immunocytochemistry

After the PVN was localized in the hypothalamus with thionine staining, every 50th section through the PVN was subjected to ICC staining and used for counting. The procedure of Raadsheer et al. (1993) was followed for the ICC double-staining for CRH and AVP. Briefly; the sections were washed (3 × 10 min) in Tris-buffered saline (TBS; 0.05 mol/1 Tris (hydroxymethyl amino-methane/ SAGMA, USA) and 0.15 mol/1 NaCl (Merck), pH = 7.6), and incubated with a highly specific monoclonal rat anti-CRH antibody (‘PUF-83’) (Van Oers et al., 1989), diluted 1:100,000, and rabbit AVP anti-serum (‘Truus’, 29.01.1986) diluted 1:300 in staining buffer (‘super mix’; 0.5% v/v Triton X-100 (Sigma) and 0.25% w/v gelatin (Merck) in TBS, pH = 7.6. This staining buffer is used for all dilutions unless otherwise specified), for 1 h at room temperature (RT) and subsequently at 4°C overnight. The rabbit AVP-antiserum was purified for oxytocin-binding antibodies by adsorbing it twice with oxytocin-glutaraldehyde Sepharose beads (Pool et al., 1984).

The second day, the sections were washed in TBS 3 × 10 min and incubated with goat anti-rabbit IgG serum (‘Betsie’) diluted 1:50, for 1 h at RT. Then, after a TBS wash, the sections were incubated with alkaline phosphatase labelled goat anti-rat IgG (H + L) serum (KPL, USA) diluted 1:50, and with rabbit peroxidase-antiperoxidase (PAP) diluted 1:1000, for 30 min at RT. Following a wash in TBS, the sections were rinsed in 0.05 mol/1 Tris (pH = 8.5), and incubated for 10 min with a filtered suspension of 0.5 mmol/1 Fast Blue BB Base (Sigma) in 0.1 mol/1 Tris (pH = 8.5), containing 1.25 mmol/1 levamisole (Sigma), and 0.5 mmol/1 Naphthol-AS-AX phosphate (Sigma) dissolved in 2% of N,N-dimethylformamide (DMF) (DBH, UK).

The sections were then washed in TBS and incubated for 10 min with a filtered PAP substrate, by dissolving 2.25 mmol/1 3-aminio-9 ethyl carbazole (Sigma) in 5% DMF, and adding it to the sodium acetate buffer (pH = 5.0), activating it with 0.01% H2O2 (30%). After washing in TBS, the sections were covered in Kaiser’s glycérin gelatin (Merck) and stored at 4°C, protected from light.

During the staining sessions, paired series of MS and control subjects were run together and a section from a separate control subject (no. 8538), who was previously known to have high numbers of CRH neurons, was added to each staining session, to serve as a positive control for the procedure.
Table 1
Clinicopathological data of the subjects

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>pmt (h)</th>
<th>fxt (d)</th>
<th>brw (g)</th>
<th>Clock-time of death</th>
<th>Diagnosis and clinico-pathological information</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>91–112</td>
<td>m</td>
<td>33</td>
<td>52</td>
<td>65</td>
<td>nd</td>
<td>04:00</td>
<td>23 SP 30–32 Bronchopneumonia</td>
</tr>
<tr>
<td>90–246</td>
<td>f</td>
<td>41</td>
<td>5</td>
<td>142</td>
<td>1080</td>
<td>01:00</td>
<td>12 SP nr Cachexia due to refusal of food, dehydration, amnestic syndrome</td>
</tr>
<tr>
<td>91–175</td>
<td>f</td>
<td>47</td>
<td>5</td>
<td>33</td>
<td>1010</td>
<td>17:45</td>
<td>21 SP nr Pneumonia, drop out of hippocampal neurons</td>
</tr>
<tr>
<td>93–128</td>
<td>m</td>
<td>48</td>
<td>8</td>
<td>33</td>
<td>1193</td>
<td>16:00</td>
<td>31 SP nr Respiratory insufficiency</td>
</tr>
<tr>
<td>90–317</td>
<td>m</td>
<td>55</td>
<td>9</td>
<td>30</td>
<td>1377</td>
<td>06:30</td>
<td>48 SP nr Respiratory problems due to brain stem metastases from unknown origin</td>
</tr>
<tr>
<td>92–056</td>
<td>f</td>
<td>59</td>
<td>8</td>
<td>30</td>
<td>1275</td>
<td>19:35</td>
<td>36 PP nr Septic shock due to urinary tract infection, cardiac insufficiency</td>
</tr>
<tr>
<td>91–307</td>
<td>m</td>
<td>62</td>
<td>10</td>
<td>29</td>
<td>1050</td>
<td>06:00</td>
<td>30 SP nr Bilateral multiple pulmonary embolism and lung infarction</td>
</tr>
<tr>
<td>91–215</td>
<td>f</td>
<td>63</td>
<td>6</td>
<td>30</td>
<td>1071</td>
<td>09:40</td>
<td>23 PP nr Severe renal impairment, cachexia</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>51.0</td>
<td>12.9</td>
<td>49.0</td>
<td>1150.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>± S.E.M.</td>
<td></td>
<td>3.8</td>
<td>5.6</td>
<td>14.0</td>
<td>51.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90–901</td>
<td>m</td>
<td>30</td>
<td>4</td>
<td>46</td>
<td>1325</td>
<td>18:00</td>
<td>18:00 Fallot’s tetralogy, arrhythmia, bacterial endocarditis</td>
</tr>
<tr>
<td>87–260</td>
<td>m</td>
<td>37</td>
<td>36</td>
<td>46</td>
<td>1370</td>
<td>05:40</td>
<td>05:40 Alcohol and benzodiazepine intoxication, cerebral edema</td>
</tr>
<tr>
<td>82–165</td>
<td>f</td>
<td>52</td>
<td>5</td>
<td>33</td>
<td>1370</td>
<td>05:40</td>
<td>05:40 Metastatic mamma carcinoma, multiple cerebral and meningeal metastases, bronchopneumonia, polychemotherapy and radiotherapy, morphine course</td>
</tr>
<tr>
<td>86–403</td>
<td>f</td>
<td>53</td>
<td>24</td>
<td>17</td>
<td>1410</td>
<td>14:00</td>
<td>14:00 Chronic myeloid leukemia with dura matter metastases, morphine course</td>
</tr>
<tr>
<td>92–046</td>
<td>f</td>
<td>54</td>
<td>13</td>
<td>nd</td>
<td>1080</td>
<td>nd</td>
<td>1080 nd Traffic accident</td>
</tr>
<tr>
<td>92–047</td>
<td>m</td>
<td>54</td>
<td>14</td>
<td>31</td>
<td>1410</td>
<td>nd</td>
<td>1410 nd Bronchogenic carcinoma</td>
</tr>
<tr>
<td>82–086</td>
<td>m</td>
<td>68</td>
<td>6</td>
<td>50</td>
<td>1300</td>
<td>05:15</td>
<td>05:15 Arteriosclerosis, glomerulosclerosis of kidney, myocardial infarction and coma; dopamine and catecholamine therapy</td>
</tr>
<tr>
<td>93–041</td>
<td>f</td>
<td>73</td>
<td>9</td>
<td>34</td>
<td>1344</td>
<td>09:10</td>
<td>09:10 Lung emphysema, bronchopneumonia, sepsis, hypertension, arteriosclerosis, cardiac and septic shock</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>52.6</td>
<td>13.9</td>
<td>36.7</td>
<td>1343.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>± S.E.M.</td>
<td></td>
<td>5.0</td>
<td>3.9</td>
<td>4.3</td>
<td>44.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations used: brw, brain weight (grams); C.S. c., the age in years, at which the patient had last corticosteroid course; d.t., disease type; f, female; fxt, fixation time (days); m, male; nd, not determined; nr, not reported; pmt, postmortem time (h); PP, primary progressive; SP, secondary progressive.
Immunocytochemical control stainings were performed with anti-CRH (PFU-83), preincubated with $10^{-7}$ M CRH and the AVP antibody preadsorbed with AVP (for details see Raadsheer et al., 1993). Neither these controls, nor the pre-immune serum or normal rat serum as first antibody revealed any staining.

2.3. Counting of the cell profiles and statistical evaluation

Those profiles of CRH-expressing neurons in the PVN that showed a nucleus, and expressed either AVP co-expression (purple) or only CRH (blue), were counted under a Zeiss light microscope using a 10 × ocular and 20 × or 40 × objectives. All cell profiles at both sides of the hypothalamus were systematically scanned, unless only one side was available (subjects #93–128, #91–215, #87–260, #92–046 and #92–047). When the cell profiles were counted at both sides of the hypothalamus, the mean of the two sides was used as the average unilateral cell profile number of the subject. The ratios between the two types of CRH neurons were based upon a mean of $142 ± 48$ (SEM) cell profiles per patient. Since the size distribution of the parvicular CRH neurons that co-express AVP and those that only express CRH is similar (Raadsheer, 1994c), corrections for the ratios were not necessary. The total cell number was estimated from the cell profile counts and the distance between the sections.

Differences in age, postmortem delay, fixation time, cell profile counts and the ratios between the control subjects and MS patients were tested two-tailed, using the Mann-Whitney U test. Linear regression analyses were performed using Pearson’s correlation test (GraphPad InPlot, California, USA). For multiple regression analysis, the SPSS program was used (SPSS, Chicago, USA).

3. Results

There were no differences between the MS and control groups as far as the variables age, postmortem delay and fixation time were concerned ($P > 0.9$; $P > 0.6$; $P > 0.6$ respectively), while the brain weights were significantly lower in the MS group in comparison with the controls ($P = 0.014$) (Table 1). The distribution of CRH and AVP neurons were as described before (Raadsheer et al., 1993; Van der Woude et al., 1995). The results of estimated cell profile counts and ratios are summarized in Table 2.

3.1. Percentage of CRH neurons co-expressing AVP

The percentage of hypothalamic CRH neurons co-expressing AVP was significantly increased ($P = 0.038$) in MS (Table 2). There were no linear relations between that percentage and the duration of the disease ($r = 0.21$; $P > 0.6$) or the age at onset ($r = 0.55$; $P > 0.1$), while there was a significant age-related increase in the MS patients ($r = 0.78$; $P = 0.023$), but not for controls ($r = 0.48$; $P > 0.2$) (Fig. 1). Multiple regression analysis of sex, age, disease state (MS or control), age at onset of the disease, duration of the disease and total cell profile count as independent variables, in relation to the percentage of CRH-neurons co-expressing AVP, as the dependent variable showed that only age and disease state were associated with this ratio. Thus, the multiple regression analysis performed with age and disease state as independent variables, showed a significant contribution of these two variables to the percentage of CRH neurons co-expressing AVP ($F_{2,13} = 6.28, P = 0.012$) with $t = 2.66 (P = 0.020)$ for age and $t = 2.15 (P = 0.026)$ for the disease state.

<table>
<thead>
<tr>
<th>CRH cells which do not co-express AVP</th>
<th>CRH cells co-expressing AVP</th>
<th>Total CRH cells</th>
<th>% of CRH cells co-expressing AVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS ($n = 8$)</td>
<td>$1509 \pm 439$</td>
<td>$9063 \pm 4365$</td>
<td>$10547 \pm 467$</td>
</tr>
<tr>
<td>Controls ($n = 8$)</td>
<td>$1540 \pm 733$</td>
<td>$2025 \pm 817$</td>
<td>$3566 \pm 1524$</td>
</tr>
<tr>
<td>$P = 0.878$</td>
<td>$P = 0.049$</td>
<td></td>
<td>$P = 0.105$</td>
</tr>
</tbody>
</table>

The values are given as mean $\pm$ SEM. * Significant at $P < 0.05$ level.
3.2. Cell profile counts

Cell profile counts showed no linear relation with age of onset \((r = 0.47; \ P > 0.2)\) or duration of the disease \((r = -0.06; \ P > 0.8)\); controls tended to show an age-related increase \((r = 0.68; \ P = 0.064)\), but not MS patients \((r = 0.43; \ P > 0.2)\) (Fig. 2). There was a non-significant 3-fold increase in total CRH cell profile number \((P < 0.1)\) and a significant 4.5-fold increase in the AVP co-expressing CRH neurons \((P < 0.05)\) (Fig. 3). Multiple regression analysis performed with sex, age, disease state, age at onset of the disease, duration of the disease and co-localization percentage as independent variables, showed that none of them made a significant contribution to the total number of cell profiles.

We did not have enough subjects to test any circadian pattern of CRH activation for either the cell numbers or AVP co-localization.

4. Discussion

The significant difference between the brain weights of MS patients and controls were in agreement with the literature (Jellife and White, 1935).

Although we did not use the unfolding method, as Purba et al. (1995) did to estimate the total CRH cell number in PVN, but just counted cell profiles, we found a 3-fold increase in MS patients, similar to the 2.5-fold increase reported by Purba et al. However, our results only indicated a tendency, due to the high variance in our study.

The new measure for CRH cell activation used in the present study was the proportion of CRH neurons that showed co-localization with AVP. It has been shown that AVP potentiates CRH in its action on ACTH secretion. The combination of CRH and AVP may be much more effective than a 30-fold increase in the amount of CRH alone (DeBold et al., 1984; Liu et al., 1983). AVP co-expression in CRH neurons was indeed found to be increased in animals with a hyperactive HPA-axis (De Goeij et al., 1991,1992). In humans the number of CRH neurons co-expressing AVP increased with aging (Raadsheer et al., 1994a) and in depression (Raadsheer et al., 1994b, 1995). In the present study we found a significant 4.5-fold increase in the proportion of CRH neurons co-expressing AVP in MS patients. This means that the increased activation of the hypothalamic CRH neurons in MS patients is now suggested by two different parameters; the increase in CRH cell number (Purba et al., 1995) and the increase in CRH cell activity as judged from the increase in proportion of AVP-CRH co-localization. The entire increase in CRH cell numbers in MS was due to an increase in those CRH neurons co-localizing AVP (Fig. 3). While 54% of the CRH neurons showed AVP co-localization in controls, this proportion increased to 76% in MS patients \((P = 0.038)\), agreeing with the interpretation of an hyperactive state of the CRH neurons in MS.

These data are consistent with the observations of Michelson et al. (1994) who concluded on the basis of provocative tests for the HPA-axis in MS patients that there was increased relative activity of AVP in the regulation of the HPA-axis of these patients. In a rat model for chronic inflammatory stress, Harbuz et al. (1992) also found an increased AVP release. It should be noted though that in both this model for arthritis and in EAE, decreased CRH-mRNA was observed in the rat PVN (Harbuz et al., 1992,1993a,b).

Although we do not know how specific this CRH response is for MS, it does not seem to be just an indication of a non-specific stress response to the disease state of the patients. Most of the control subjects in this study also suffered from serious and often chronic diseases
including endocarditis and sepsis (Table 1), and in the study of Purba et al. (1995) from polymyalgia rheumatica. In addition, Alzheimer’s disease patients and depressed patients did not show a difference in the proportion of AVP-CRH co-localized neurons (Raadsheer et al., 1994a,b), while in depressed Parkinson patients the number of CRH-expressing neurons was not altered (Purba et al., unpublished results). In rheumatoid arthritis patients, CRH neurons even seemed to be deficient (Chikanza et al., 1992). This observation agrees with those on rat model for this disease, i.e. adjuvant induced arthritis, where CRH mRNA in the PVN is reduced (Harbuz et al., 1993b). These observations suggest that the CRH response observed in MS is quite specific for this disease.

It should be noted that both the proportion of AVP co-expressing CRH neurons and the total CRH cell number showed an age-dependent increase in controls from the 4th decade of life onwards, an age at which MS prevalence starts to decline in the population. A similar age-dependent increase for the number of neurons expressing CRH in controls was also observed by Raadsheer et al. (1994a,b).

Since it has been shown in animal experiments that an increased HPA-axis activity may lead to decreased susceptibility to EAE, the age-related increase of the activity of CRH neurons agrees with the hypothesis that this may be an important factor leading the prevalence of MS to decrease with age.

Although the present study and the paper from Purba et al. (1995) show an activation of the hypothalamic CRH system in MS, the endogenous HPA-axis does not seem to be effective enough to bring the corticosteroid levels up to a level where they suppress the immune system sufficiently, to quell the symptoms of MS at relapse. Despite the strong increase in AVP co-expressing CRH cells, the number of CRH neurons which do not express AVP was the same for controls and MS patients. Animal experiments showed that the majority of AVP co-localizing CRH cells send their projections to the median eminence for the regulation of ACTH secretion (Sawchenko and Swanson, 1985; Sawchenko, 1987; Whitnall et al., 1987; Whitnall, 1990). It has also been shown that a part of the CRH neurons in the PVN that do not co-express AVP (but may possibly contain other peptides), send their projections to the other parts of the CNS (Sawchenko and Swanson, 1985; Sawchenko, 1987) where they are supposed to play a role in central processes. Our finding of an increase in the number of CRH neurons co-expressing AVP in MS thus suggests that the alterations in this disorder occur in the CRH cell population participating in the HPA-axis, rather than in centrally terminating CRH cells. However, before we can draw any final conclusions about the exact nature of the involvement of the two types of CRH neurons in MS, it should be investigated whether the activity level of CRH neurons with or without AVP co-expression is changed, e.g. by measuring the mRNA levels of these CRH neurons.

Our future plans therefore include investigation of the CRH mRNA levels of neurons with and without AVP co-expression in the hypothalamus of MS patients and control subjects in order to draw further conclusions concerning the exact nature of HPA-axis activation at the hypothalamic level.

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