The hypothalamic paraventricular nucleus in two types of Wistar rats with different stress responses. II. Differential Fos-expression

W.H.A.M. Mulders a,*, J. Meek a, E.D. Schmidt c, T.G.M. Hafmans a, A.R. Cools b

a Department of Anatomy and Embryology, PO Box 9101, 6500 HB Nijmegen, The Netherlands
b Department of Psychoneuropharmacology, University of Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands
c Department of Pharmacology, Free University, PO Box 7161, 1007 MC Amsterdam, The Netherlands

Accepted 18 April 1995

Abstract

The present study investigates the role of corticotropin-releasing hormone (CRH) neurons in stress regulation by a comparison of stress induced Fos-immunoreactivity and CRH-immunoreactivity in the hypothalamic paraventricular nucleus (PVH) of APO-SUS (apomorphine-susceptible), APO-UNSUS (apomorphine-unsusceptible), normal Wistar and adrenalectomized Wistar (ADX) rats. The first two types represent a good model to study the role of the PVH in stress regulation, since they show different stress responses and a differential synaptic organization of the PVH. After placement on an open field for 15 min all rats showed an increase in the number of Fos-immunoreactive nuclei compared to control handling. Interestingly, open field stress, but not control handling, induces significantly fewer Fos-immunoreactive nuclei in the PVH of APO-SUS rats (1255 ± 49) compared to APO-UNSUS rats (1832 ± 201). Experiments with ADX rats revealed that 93% of the CRH-immunoreactive neurons contained a Fos-immunoreactive nucleus, which suggests that the differential Fos-expression in APO-SUS and APO-UNSUS rats represents a differential activation of the CRH neurons. This hypothesis is discussed in relation to reported differences in stress responses, stress-induced ACTH levels and synaptic organization of the PVH.

Keywords: Hypothalamic paraventricular nucleus; Corticotropin-releasing hormone; Fos-immunoreactivity; Pharmacogenetics; Selective breeding; Stress; Wistar rat

1. Introduction

The hypothalamic paraventricular nucleus (PVH) plays an important role in the regulation of stress responses. It contains corticotropin-releasing hormone (CRH) producing paravascular neurons, which induce adrenocorticotropic hormone (ACTH) release from the corticotrope cells in the anterior pituitary [2]. ACTH subsequently regulates corticosteroid production in the adrenal cortex [45]. Corticosteroids are involved in several stress reactions and exert a negative feedback on CRH and ACTH release [21]. Consequently, removal of the circulating corticosteroids by adrenalectomy (ADX) induces an increase of CRH mRNA in the short term and an increase of CRH in the long term [1,31].

Several stimuli evoke stress responses, and for some of these the expression of c-fos mRNA or Fos-like immunoreactivity (Fos-IR) has been used to demonstrate the involvement of the PVH. c-fos mRNA or Fos are often used as activation markers [24,27,37], since c-fos is responsible for the synthesis of the nuclear protein Fos, which, together with other factors, makes complexes with DNA at AP-1 binding sites [11] to regulate transcription [24,34]. Accordingly, the expression of c-fos and the accumulation of the protein Fos are associated with cell activation in response to a variety of stimuli [14]. Stressful stimuli that have been used to induce c-fos or Fos-IR in the PVH include intraperitoneal hypertonic saline injections [36], immobilization stress [5,19], pain [35] and swim stress [15].

A new model to study stress responses is presented by two lines of Wistar rats, which have been pharmacogenetically selected on the basis of their gnawing responses after an identical, subcutaneous dose of apomorphine [6–8].
They are indicated as APO-SUS (apomorphine susceptible) and APO-UNSUS (apomorphine unsusceptible) rats, showing a high and low gnawing response, respectively [6]. Other interline differences can be observed on an open field, where APO-SUS rats show more locomotor activity and edge-hugging behavior than APO-UNSUS rats. In the so-called defeat test, in which the rat is confronted with a much larger rat, APO-SUS rats show fleeing behavior, whereas APO-UNSUS rats exhibit freezing [6,8]. Furthermore, a conditioned emotional stress stimulus evokes higher plasma ACTH levels in APO-SUS rats than in APO-UNSUS rats [26,42].

The present paper investigates the stress induced expression of Fos-IR in the PVH of APO-SUS and APO-UNSUS rats. For this purpose, we quantified Fos-IR in the PVH after a mild novelty stress in both APO-SUS and APO-UNSUS rats and compared this with Fos-IR in the PVH of normal Wistar rats after the same stress. Novelty-induced stress is known to modulate differentially the hippocampal content of dynorphine [7] as well to affect differentially the release of ACTH and corticosteroids in APO-SUS and APO-UNSUS rats [26]. We investigated whether the novelty-induced Fos-IR is expressed by the CRH-cell population using ADX rats, since untreated rats do not show distinct CRH-IR in the PVH. The differences in Fos-expression between APO-SUS and APO-UNSUS rats will be correlated with the reported differences in synaptic densities in the PVH [25] and differential stress induced ACTH levels in both types of rats [42].

2. Materials and methods

2.1. Animals

The present study is based on 44 male Wistar rats (weighing 200–330 g), including 6 APO-SUS and 8 APO-UNSUS rats. All rats were bred in our Animal Laboratory and originally housed in groups of 2–3 animals per cage (36 × 24 × 25 cm) in a room with a constant temperature (20 ± 2°C) and a 06.00–18.00 h light period. Food and water were given ad libitum. All rats (except for 3 rats used for CRH-immunocytochemistry) were isolated in separate cages three days before the experimental procedure. Bilateral adrenalectomy (ADX) was performed in a number of rats under ether anaesthesia 4 weeks before the experimental procedure (rats weighing 170 ± 10 g at time of ADX). The ADX rats were given saline (0.9% sodium chloride in aqua dest) instead of water.

2.2. Experimental procedures

In order to investigate whether Fos-immunoreactivity (Fos-IR) in the PVH shows interline variation between APO-SUS and APO-UNSUS rats and whether this Fos-IR is expressed by the CRH-cell population, we applied different experimental procedures to 5 groups of rats. We investigated Fos-IR after open field stress in the PVH of normal Wistar rats (group A), as well as in the PVH of APO-SUS and APO-UNSUS rats (group B). The relation between Fos-IR and CRH-IR was studied in ADX rats, since untreated rats do not show distinct CRH-IR in the PVH. The PVH of ADX Wistar rats was stained for either Fos (group C) or CRH (group D), or double-stained for Fos and CRH (group E). In more detail the experimental groups were treated as follows.

Group A: three Wistar rats were placed for 15 min on an open field (open field stress; see Cools et al. [6]) and then returned to their home cages. 60 min later these experimental rats were perfused and processed for Fos-IR (see below). Three control Wistar rats were handled for 15 s, returned to their home cages for 15 min, handled again for 15 s and left in their home cages for the next 60 min. Subsequently, they were perfused and processed in the same way as group A.

Group B: three APO-SUS (F14 generation, 240–330 g) and five APO-UNSUS rats (F14 generation 200–330 g) were treated similarly as the experimental rats in group A. Three APO-SUS (F18 generation, 190–230 g) and three APO-UNSUS (F18 generation, 220–250 g) control rats were treated similarly as the controls of group A.

Group C: three ADX rats were treated similarly as the experimental rats in group A, and three ADX rats were treated similarly as the control rats of group A.

Group D: three ADX rats were perfused and processed for CRH-IR (see below), without open field stress.

Group E: three ADX rats were treated similarly as group C, but the sections obtained from the PVH were double-stained for Fos and CRH (see below).

2.3. Tissue processing

For perfusion, rats were deeply anaesthetized with pentobarbital (6 mg/100 g b.wt.) and transcardially perfused with 100 ml saline (0.9% sodium chloride) followed by 450 ml 4% paraformaldehyde (PF, group D and E) or 2% PF (group A, B, and C) in 0.1 M phosphate-buffered saline (PBS; pH 7.3). Immediately after perfusion, they were decapitated, and the dorsal part of the skull was removed. The heads were placed in a stereotactic device, and a transverse incision was made to allow sectioning of all brains in the same transversal plane. After removal of the brains out of the skull, they were placed overnight in the same fixative as used for perfusion.

The procedure just described resulted from comparing different fixation protocols after intraperitoneal injections of a hypertonic salt solution (NaCl, 1.5 M, 1 ml/100 g b.wt.), a stimulus inducing intense Fos-IR in the PVH [44]. For this purpose rats were perfused transcardially with 2% or 4% PF in 0.1 M PBS (pH 7.3), followed by a postfixation overnight (18 h) or for 42 h. Quantitative analysis showed that 2% PF with 18 h postfixation yields 1876 ±
693 Fos-IR nuclei in the PVH, 2% PF with 42 h postfixation 1766 ± 292 Fos-IR nuclei, 4% PF with 18 h postfixation 1057 ± 143 Fos-IR nuclei and 4% PF with 42 h postfixation only 735 ± 204 Fos-IR nuclei. Consequently, we decided to use 2% PF with a postfixation overnight, since a further increase of PF concentration as well as a fixation time results in a reduction of Fos-IR.

For visualization of Fos-IR, sections of 75 μm were cut on a vibratome in PBS (pH 7.3). After rinsing (one hour in PBS), sections were pre-incubated with 5% normal horse serum, 0.5% Triton X-100 and 0.1% bovine serum albumin (BSA) in PBS for one hour. Subsequently, the sections were incubated overnight at room temperature with a sheep polyclonal Fos antiserum, diluted 1:2000 (Cambridge Research Biochemicals Inc., Wilmington). After rinsing, sections were incubated for 90 min with a horse anti-sheep antibody (1:100 in PBS) and rinsed again. Next, the sections were treated for 90 min with sheep peroxidase-anti-peroxidase (sh-PAP; Nordic) diluted 1:600 in PBS. After additional rinsing, sections were preincubated for 10 min in 0.02% 3,3’-diaminobenzidine • 4HC1 (DAB, Sigma), 0.3% nickel ammonium sulphate in a 0.05 M tris(hydroxymethyl)-aminomethane solution (pH 7.6), followed by an incubation for 10 min in the same solution containing 25 μl of H2O2 (DAB reaction). Subsequently, the sections were mounted on gelatin-coated slides, dried overnight in a stove of 37°C, dehydrated and coverslipped with Entellan.

For visualization of CRH-IR, vibratome sections (75 μm) were pre-incubated with normal goat serum, 0.5% Triton X-100 and 0.1% BSA in PBS for 1 hour and subsequently incubated overnight at room temperature with a rabbit polyclonal CRH antiserum diluted 1:1000 (for characterization see below). After rinsing, sections were incubated for 90 min with a goat anti-rabbit antibody (1:100 in PBS) and rinsed again. Next, the sections were treated for 90 min with sheep peroxidase-anti-peroxidase (sh-PAP; Nordic) diluted 1:600 in PBS. After additional rinsing, sections were preincubated for 10 min in 0.02% 3,3’-diaminobenzidine • 4HC1 (DAB, Sigma), 0.3% nickel ammonium sulphate in a 0.05 M tris(hydroxymethyl)-aminomethane solution (pH 7.6), followed by an incubation for 10 min in the same solution containing 25 μl of H2O2 (DAB reaction). Subsequently, the sections were mounted on gelatin-coated slides, dried overnight in a stove of 37°C, dehydrated and coverslipped with Entellan.

2.4. Characterization of the CRH-antiserum

The antiserum (8Bo) was raised in a rabbit against rCRH1-41 conjugated to thyroglobulin. For immunization 25 μg equivalent of CRH (ca. 160 μl of CRH-conjugate) plus 340 μl of PBS was mixed with 500 μl of Freund’s complete adjuvant (FCA) and injected intramuscularly and subcutaneously. After 1 month the rabbit received a boost injection with CRH-conjugate plus FCA as above. The antiserum was characterized by ‘immunospotting’ and immunocytochemistry.

Immunospotting: r/HCRh1-41 solutions of 10⁻⁹ to 10⁻⁴ M in distilled water were spotted (1 μl) on nitrocellulose filter (pore size 0.45 μm; Schleicher and Schuell). After drying (5 min), the filters were fixed in freshly prepared 4% PF in 0.1 M PB (pH 7.6). After washing (3 X) in 0.01 M PBS (pH 7.6) the filters were incubated for 1 h with 8Bo diluted 1/100 or 1/500 in PBS containing 0.5% BSA and 0.1% Tween 20 (incubation buffer). After washing (3 X) in PBS with 0.1% Tween 20 (PBS-Tween), the filters were incubated for 30 min with goat anti-rabbit antiserum (Nordic) in incubation buffer. The filters were washed (3 X PBS-Tween) and incubated for 30 min with rabbit PAP-complex (DAKO) in incubation buffer, washed (2 X PBS-Tween and 1 X 0.1 M Tris-HCl buffer) and subsequently stained with 0.1 M DAB in Tris-HCl containing 0.05% H2O2. All washes and incubations were performed at 21 ± 1°C. 8Bo showed an antibody-concentration dependent staining of CRH. Spots of CRH showed a concentration dependent staining from 10⁻⁸ to 10⁻⁵ M CRH; staining of 10⁻⁶ M CRH was completely abolished by preincubation of 8Bo with 10⁻⁶ M CRH (2 h 37°C). No staining was observed with vasopressin, oxytocin or αMSH spots (up to 10⁻³ M).

Immunocytochemistry: vibratome sections (50 μm) of immersion or perfusion fixed tissues showed an excellent signal-to-background ratio with 8Bo dilutions of 1/400 to 1/800 (incubations overnight at 4°C in a 0.1 M Tris buffer, pH 7.6, containing 0.2% BSA, 0.2% NGS, 0.1% NaN₃ and 0.1% Triton X-100) and subsequent PAP/DAB procedures. After colchicine treatment (50 μg of colchicine in 10 μl of saline, injected i.c.v., survival 24 h) strong CRH immunostaining was found in the PVH, the external layer of the median eminence (ZEME), the stria terminalis (ST) and the central amygdala (CA). Weak to moderate staining was observed in neurons of the nucleus supraopticus (SON), the periventricular nucleus (NP), the bed nucleus of the stria terminalis (BNST) and in some scattered cells in the lateral hypothalamic area (LHA). The observed staining patterns are similar to those reported for several other CRH antibodies [10,33,41], and correlate well with reported distribution of CRH mRNA in hypothalamic nu-
clei [4]. All immunostaining is completely blocked by pre-incubation of 8Bo with 10^-5 \( \text{r/hCRH}_{1-41} \) (2 h, 37°C), whereas no inhibition was seen after pre-incubation with upto 10^-3 M vasopressin or \( \alpha \text{MSH} \). No immunostaining was found in control sections in which the first antibody was omitted.

2.5. Quantitative and statistic analysis

All Fos-IR nuclei and CRH-IR neurons within the left PVH of the experimental and control animals were drawn with the aid of a Zeiss light microscope and drawing tube (magnification used for single stained sections: 125 X; for double stained sections: 312.5 X). To determine their number and distribution within the PVH, the immunopositive cells were plotted in an atlas containing 8 levels of the PVH (Fig. 2). Subdivisions of the PVH were delineated on the basis of our previous morphometric results [25]. Since darkly as well as lightly stained Fos-IR nuclei and/or CRH-IR cells were plotted without distinction, the quantitative results reflect only the numbers of neurons and not the intensity of staining. Statistical analysis was performed using the Mann-Whitney \( U \)-test.

3. Results

The numbers of Fos-IR nuclei and CRH-IR neurons observed in the left PVH of the different experimental and control groups are summarized in table 1. Details on their distribution and intensity of staining are described in detail in the following paragraphs and are visualized in Figs. 1-3.

3.1. Fos-IR after open field stress

In normal Wistar rats that were exposed to open field stress, 1360 ± 21 Fos-IR nuclei were counted in the PVH (group A), whereas in the control group only 261 ± 74 Fos-IR nuclei were found. This statistically significant difference (\( P < 0.05 \)) occurs in all subdivisions of the PVH with the exception of the ventral parvocellular part (PCv) (Table 1, Fig. 1C; for delineation of PVH subdivisions; see Mulders et al. [25]). The Fos-IR nuclei are not

<table>
<thead>
<tr>
<th>Exp. group</th>
<th>PVH</th>
<th>PV</th>
<th>MC</th>
<th>PCC</th>
<th>PCD</th>
<th>PCV</th>
<th>PCp</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Wistar open field</td>
<td>n = 3</td>
<td>1360 ± 21</td>
<td>135 ± 8</td>
<td>128 ± 21</td>
<td>858 ± 50</td>
<td>51 ± 9</td>
<td>68 ± 15</td>
</tr>
<tr>
<td>Normal Wistar control</td>
<td>n = 3</td>
<td>261 ± 74</td>
<td>50 ± 25</td>
<td>39 ± 8</td>
<td>109 ± 31</td>
<td>15 ± 8</td>
<td>20 ± 13</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APO-SUS open field</td>
<td>n = 3</td>
<td>1255 ± 49</td>
<td>140 ± 10</td>
<td>89 ± 8</td>
<td>695 ± 37</td>
<td>56 ± 5</td>
<td>80 ± 14</td>
</tr>
<tr>
<td>APO-SUS control</td>
<td>n = 3</td>
<td>249 ± 62</td>
<td>49 ± 16</td>
<td>18 ± 1</td>
<td>75 ± 16</td>
<td>22 ± 3</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>APO-UNSUS open field</td>
<td>n = 5</td>
<td>1832 ± 201</td>
<td>170 ± 21</td>
<td>140 ± 15</td>
<td>1157 ± 152</td>
<td>82 ± 11</td>
<td>106 ± 12</td>
</tr>
<tr>
<td>APO-UNSUS control</td>
<td>n = 3</td>
<td>227 ± 39</td>
<td>48 ± 4</td>
<td>18 ± 2</td>
<td>82 ± 25</td>
<td>18 ± 2</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX open field</td>
<td>n = 3</td>
<td>1209 ± 178</td>
<td>137 ± 11</td>
<td>64 ± 4</td>
<td>686 ± 119</td>
<td>41 ± 6</td>
<td>70 ± 12</td>
</tr>
<tr>
<td>ADX control</td>
<td>n = 3</td>
<td>440 ± 169</td>
<td>49 ± 25</td>
<td>37 ± 10</td>
<td>242 ± 100</td>
<td>20 ± 5</td>
<td>23 ± 7</td>
</tr>
<tr>
<td>CRH-IR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX control</td>
<td>n = 3</td>
<td>846 ± 152</td>
<td>32 ± 9</td>
<td>52 ± 9</td>
<td>605 ± 102</td>
<td>42 ± 10</td>
<td>36 ± 12</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fox-IR + CRH-IR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX open field</td>
<td>n = 3</td>
<td>457 ± 131</td>
<td>15 ± 5</td>
<td>33 ± 1</td>
<td>359 ± 64</td>
<td>32 ± 6</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Single Fox-IR open field</td>
<td>n = 3</td>
<td>1661 ± 234</td>
<td>266 ± 20</td>
<td>120 ± 19</td>
<td>942 ± 154</td>
<td>82 ± 21</td>
<td>87 ± 11</td>
</tr>
<tr>
<td>Single CRH-IR open field</td>
<td>n = 3</td>
<td>489 ± 70</td>
<td>15 ± 6</td>
<td>38 ± 2</td>
<td>380 ± 60</td>
<td>37 ± 6</td>
<td>6 ± 2</td>
</tr>
</tbody>
</table>
Fig. 3. Photomicrographs of vibratome (75 μm) sections of the left PVH showing Fos-IR of a control rat (A), and an APO-SUS (B) as well as an APO-UNSUS rat (C) after open field stress. Magnification 89×.
homogeneously distributed over the different subdivisions in the PVH. The central parvocellular part (PCc) contains about 63% of the total number of Fos-IR nuclei, the periventricular part (PV) 10%, the magnocellular part (MC) 9%, the parvocellular dorsal part (PCd) 4%, the PCv 5% and the parvocellular posterior part (PCp) 9% (Fig. 1C). The control group shows a somewhat different distribution: the PCc contains 42%, the PV and MC 17%, the PCd 6% and the PCv and PCp, respectively 5% and 13% of the total number of Fos-IR nuclei (Fig. 1C).

Similar to normal Wistar rats, APO-SUS and APO-UNSUS rats show a statistically significant (P < 0.05) increase in the number of Fos-IR nuclei after open field stress (APO-SUS: 1255 ± 49 vs. 249 ± 62 in controls, APO-UNSUS: 1832 ± 201 vs. 227 ± 39 in controls). This statistically significant difference is present in all subdivisions of the PVH (Table 1, Fig. 1A and B). Moreover, the PVH of APO-UNSUS rats contains significantly more Fos-IR nuclei after open field stress than the PVH of APO-SUS rats (P < 0.05; Fig. 1A, B and Fig. 2), but not after control handling. The numerical difference of Fos-IR after open field stress is corroborated by the higher intensity of the Fos-IR in APO-UNSUS rats compared to APO-SUS rats (Fig. 3). The difference in Fos-IR between APO-SUS and APO-UNSUS rats finds its origin predominantly in the PCc (Fig. 1A, B, Figs. 2 and 3), the only subdivision of the PVH that shows a statistically significant difference in the number of Fos-IR nuclei between the two rat lines: It contains 1157 ± 152 Fos-IR nuclei in APO-UNSUS rats but only 695 ± 37 Fos-IR nuclei in APO-SUS rats (P < 0.05), numbers representing 63% and 56% of the total number of Fos-IR nuclei in the PVH, respectively (Fig. 1A and B).

The total number of Fos-IR nuclei in the PVH after open field stress and control handling of both APO-SUS as well as APO-UNSUS rats is not statistically significant from that of the overall population of Wistar rats (Table 1). Likewise, the distribution of Fos-IR nuclei over the different subdivisions of the PVH after open field stress and control handling is basically similar in normal Wistar rats and APO-UNSUS rats (cf. Fig. 1A and C). In contrast, APO-SUS rats show some statistically significant differences in the number of Fos-IR nuclei after open field stress compared to normal Wistar rats: the number of Fos-IR nuclei in their PCc is slightly but significantly smaller (APO-SUS: 695 ± 37; normal Wistar: 858 ± 50; P < 0.05) and in their PCp slightly but significantly larger (APO-SUS: 194 ± 18; normal Wistar: 119 ± 18; P < 0.05).

Control ADX rats have a similar number and distribution of the Fos-IR nuclei in the PVH as control Wistar rats (ADX Wistar: 440 ± 169; intact Wistar: 261 ± 74). Likewise, ADX rats that have been subjected to open field stress (group C) show a similar Fos-IR in the PVH (1209 ± 178) as the normal Wistar rats (1360 ± 21) after open field stress. Apparently, the increase of Fos-IR in the PVH of ADX rats induced by open field stress (from 440 ± 169 to 1209 ± 178), is similar to that induced in normal Wistar rats (cf. Fig. 1C and D).

### 3.2. CRH-IR and Fos-IR after ADX

Four weeks after ADX a large number of darkly stained CRH-IR neurons can be observed in the PVH (846 ± 152; Table 1, group D). Most of these CRH-IR neurons (72%) are located in the PCc (605 ± 102; Fig. 1E). The PV, MC, PCd, PCv and PCp contain 32 ± 9 (4%), 52 ± 9 (6%), 42 ± 10 (5%), 36 ± 12 (4%) and 79 ± 34 (9%) CRH-IR neurons, respectively. The total number of CRH-IR neurons in the PVH after ADX is significantly lower than the number of Fos-IR nuclei in ADX rats after an open field test (1209 ± 178; P < 0.05). However, their distribution over the different subdivisions is similar (cf. Fig. 1D and E).

Double staining for Fos and CRH in the PVH of ADX rats after exposure to open field stress (group E), revealed 1661 ± 234 Fos-IR nuclei and 489 ± 70 CRH-IR neurons of which 457 ± 131 were double stained (Table 1, Fig. 1F). So, 93% of the CRH-IR neurons contained a Fos-IR nucleus after double staining, but only 28% of the total number of Fos-IR nuclei was located in a CRH neuron. The most extensive double labeling was found in the PCc (38%) and PCd (38%). In the double-staining experimental group the total number of Fos-IR nuclei is substantially higher than in single stained ADX rats after open field stress, and the number of CRH-IR neurons is considerably lower than in the control ADX animals, although both not statistically significant. Most likely, technical aspects of the double-staining procedure are involved in these differences.

### 4. Discussion

The present study investigates the role of CRH neurons in stress regulation by a comparison of stress-induced Fos-IR and CRH-IR in the PVH of normal Wistar, APO-SUS, APO-UNSUS and ADX rats. To evaluate the functional significance of the results, they will first be compared with previous studies that determined Fos-IR in the PVH after stressful stimuli and secondly with previous studies on the number and distribution of CRH cells in the PVH. Finally, the significance of the differences in Fos-IR between APO-SUS and APO-UNSUS rats will be discussed in relation to the reported differences in stress responses, stress-induced ACTH levels and the synaptic organization of the PVH.

#### 4.1. Stress-induced Fos-IR

Open field stress proves to be an adequate stimulus to induce Fos-IR in the PVH of normal Wistar, APO-SUS and APO-UNSUS rats since a 5–8-fold increase in Fos-IR
nuclei was found compared to controls, yielding 1200–1800 Fos-IR nuclei per PVH (Table 1). This response is stronger than observed previously after different stressful stimuli. Duncan et al. [15] counted 840 Fos-IR cells in rats subjected to swim stress and 36 Fos-IR cells in controls. Other stimuli that have been applied to induce Fos-IR in the PVH are immobilization and painful stress, inducing 1093 and 958 Fos-IR cells, respectively, in the PVH [35]. These numbers are lower than counted in the present study, which is surprising since an open field is considered to be a mild stressor compared to immobilization and painful stress. However, the differences with other studies are most probably due to histotechnical factors such as the use of different perfusion fluids and postfixation periods, which are known to influence immunohistochemical staining. We have shown that higher concentrations of paraformaldehyde and increased postfixation periods decrease the number of Fos-IR nuclei (see section 2).

In all rats used in the present paper the majority of Fos-IR nuclei in the PVH is situated in the PCc, both after open field stress (63%) and after control handling (43%, Fig. 1C). This agrees with previous studies, which report a majority of Fos-IR neurons in the dorsal medial parvocellular part after immobilization or pain stimulation [5,35]. This subdivision is comparable with our PCc [25].

To study the colocalization of Fos-IR and CRH-IR we used ADX rats, since untreated rats show little or no visible CRH-IR in the PVH. ADX rats show a similar response as normal Wistar rats with respect to the distribution of Fos-IR nuclei in the PVH both after control handling and after open field stress (cf. Fig. 1C and D). This is in agreement with the study of Wintrip et al. [46], who found induction of Fos-IR in the parvo cellular part of the PVH 4 h after ADX, but little or no Fos-IR 24 h after ADX. Jacobson et al. [20] report that Fos-IR in the PVH is enlarged up to 7 days after ADX.

4.2. Comparison of Fos-IR and CRH-IR

Four weeks after ADX we found a similar number of CRH-IR neurons in the PVH (846 ± 152) as Swanson et al. [41], who counted ±750 CRH neurons in the PVH of ADX rats. In agreement with previous studies [31,41], we observed that the majority of CRH-IR neurons, about 72%, is localized in the PCc (Table 1; Fig. 1E).

The present study shows that the distribution of CRH-IR neurons after ADX is similar to that of Fos-IR nuclei after an open field stress (cf. Fig. 1C and D). Most Fos-IR nuclei as well as CRH-IR neurons are observed in the PCc (63% and 72%, respectively) and only a minor portion in the other subdivisions. This suggests that the Fos-IR evoked in the PVH after open field stress is predominantly localized in the CRH-IR neurons, since other types of neurons (e.g. vasopressinergic, oxytocinergic, etc.) have different distributions in the PVH [13,22,23]. Double-staining corroborated a relationship between Fos and CRH, since 93% of the CRH-IR neurons contained a Fos-IR nucleus (Table 1), which implies that almost all neurons that display CRH-IR after ADX are activated by open field stress. This agrees with previous studies showing colocalization of Fos and CRH, or c-fos mRNA and CRH mRNA after immobilization stress [4,19].

In addition to the CRH neurons that are detectable after ADX, other neurons appear to be activated as well by open field stress, since after double-labeling only 28% of all Fos-IR nuclei in the PVH (and 38% in the PCc) is localized in a CRH-IR neuron and consequently 72% of all Fos-IR was observed in other neurons (Fig. 1F). It is presently uncertain whether these latter neurons represent CRH neurons not responding to ADX, or belong to other types of peptidergic or non-peptidergic PVH neurons.

4.3. APO-SUS versus APO-UNSUS rats

The most remarkable finding of present study is that open field stress yields substantially fewer Fos-IR nuclei in the PVH of APO-SUS rats than in the PVH of APO-UNSUS rats (Table 1, Figs. 2 and 3). This difference originates mainly from the PCc, the only subdivision that shows a significant difference \( (P < 0.05) \) between the APO-SUS (695 ± 37) and the APO-UNSUS rats (1157 ± 152) (Fig. 1A and B).

The reduced Fos activation in the PVH of APO-SUS rats is correlated with an increased synaptic density [25], which suggests that this increased synaptic density has an increased (direct or indirect) inhibitory effect on CRH neurons. An important source of (indirect) inhibitory influences on the PVH is the hippocampus [9,18,30]. These influences are probably exerted via the bed nucleus of the stria terminalis (BNST), since the hippocampus projects to the GABAergic neurons in the BNST, which in turn are known to project to the PVH [9]. So, the reduced Fos-IR in APO-SUS rats could point to an increased (inhibitory) hippocampal-BNST influence on the PVH in APO-SUS rats, compared with APO-UNSUS rats. The observation that the hippocampus of APO-SUS rats contains 50% more mineralocorticosteroid receptors than the hippocampus of APO-UNSUS rats [6,8,12,39] is in line with this suggestion, but other structures and neurotransmitters may be involved as well. The PVH is known to receive an inhibitory input from serotonin-containing neurons in the midbrain raphe nuclei [29,32] and an important noradrenergic input [16,40], arising mainly from the brainstem [28]. The influence of noradrenaline on the PVH may also be inhibitory [38], although stimulatory effects on the hypothalamo-pituitary-adrenal axis have also been described [3].

Remarkably, the reduced Fos-IR in the PVH of APO-SUS rats after open field stress is at variance with the observation that a conditioned emotional stimulus induces a higher plasma ACTH level in APO-SUS rats compared with APO-UNSUS rats [26,42], and with the higher re-
sponsiveness of the hypothalamic-pituitary-adrenal axis to stress in the APO-SUS rats [6,8,26]. So, stressed APO-SUS rats seem to combine a reduced activity of CRH neurons with an increased ACTH release compared with APO-UNSUS rats, which is surprising in view of the stimulatory effect of CRH on ACTH release. Several mechanisms may be involved in this discrepancy. For example, in APO-SUS rats the negative feedback of corticosteroids on the ACTH release may be weaker than in APO-UNSUS rats, as may be due to differences in number and/or properties of hypothalamic corticosteroid receptors [26]. Likewise, there may be a differential regulation of synthesis and release of CRH in APO-SUS and APO-UNSUS rats. Increased synthesis, which is visualized by Fos-IR [24,34], does not necessarily indicate a simultaneously increased release, as has been shown for several peptides [17,43]. Further research has to be carried out to investigate the differential stress regulation in APO-SUS and APO-UNSUS rats in more detail.

Acknowledgements

We are grateful to Prof. F. Tilders and Dr. J. Veening for their advice and stimulating discussions on the set up of our experiments and Mr. D. Heeren for statistical advice.

References


