Immunocytochemical changes in hypothalamic and pituitary hormones after acute and prolonged stressful stimuli in the anestrous ewe

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Abstract. We studied the effect of short (acute (20 min/h, for 4 h) and intermittent, long-term (20 min/h for 9 h on 3 consecutive days) electric foot shocks on the immunocytochemical localization of CRH and SRIH in the hypothalamus and of ACTH, beta-endorphin, GH and PRL in the pituitary of the anestrous ewe. Acute stress greatly reduced immunoreactive (ir) CRH in the median eminence and cellular irACTH, beta-endorphin and PRL, as well as the proportion of these cell types in the pituitary. A slight reduction of irSRIH in the median eminence was also observed. After long-term stress, reduction of irCRH in the median eminence was still observed. However, ACTH/beta-endorphin cells in the pituitary gland displayed increased secretory activity, manifested by hypertrophy and hyperplasia. A marked depletion of irSRIH in the nerve terminals of the median eminence was observed. The proportion of PRL cells but not their ir content returned to control levels. No effects were observed on the features of the GH cells. This study indicates that there are differences in the effect of short- and long-term stressful stimuli on the activity of hormonal systems in the anestrous ewe. Short-term stress immediately activates the CRH/ACTH/beta-endorphin axis. Prolonged stress appears to augment the activation of the SRIH hypothalamic system and probably has a restraining effect on ACTH/beta-endorphin release.

Numerous observations have shown that the endocrine response to stress caused by various stimuli is not limited to the activation of the hypothalamo-pituitary-adrenal axis but involves other hypothalamic and pituitary hormones too (Euker et al. 1975; Johnson et al. 1985; Rivier & Vale 1985). It is also known that the hormonal response may vary according to the length of time during which the stressor is applied (Du Ruisseau et al. 1978; Tache et al. 1978). In rats, the response to short-term (acute) stress is quite different from that resulting from repeated long-term exposure to the same stimuli (Young & Akil 1985). It has been found in our laboratory that prolonged electric foot shocks produce a biphasic pattern in the secretion of cortisol in the anestrous ewe, with a rise in mean daily plasma cortisol concentrations throughout the 3 days of stimulation and a decrease after stimulation. Since a normal rise in plasma cortisol concentrations is observed in response to exogenous ACTH in these animals, it has been postulated that some central mechanism may mediate these changes (Przekop et al. 1985). The present study was designed to examine the effects of acute and prolonged stressful stimuli at the levels of the hypothalamus and the pituitary gland in the anestrous ewe. The immunoreactive (ir) content of CRH and SRIH was studied in discrete areas of the hypothalamus and correlated temporally with the pituitary content of irACTH, beta-endorphin, GH and PRL.
Materials and Methods

Ten 2-year-old anestrous Polish Merino ewes weighing 40 ± 5 kg were studied. One group (3 animals) was subjected to short stress, a second (3 animals) to prolonged intermittent stress, and 4 animals acted as controls. The stress was induced by applying a series of weak electrical pulses (3 mA) over a 20-min period followed by a 40-min period with no stimulation. This pattern continued for 4.5 h in the first group, and for 9 h daily (from 9.00 to 18.00 h) for 3 days in the second group. The details of stimulation were as described previously (Przekop et al. 1985). The experimental and control animals were always slaughtered at the same time of the day.

Immunocytochemistry

The brains of the slaughtered ewes were immediately perfused via both carotid arteries with 0.1 mol/l phosphate buffered saline (PBS) and subsequently with paraformaldehyde-picric acid, buffered with 0.1 mol/l PBS. The hypothalami and pituitaries were dissected 30 min after the onset of perfusion and postfixed for 72 h by immersion in the same fixative. The preparations were washed with 0.01 mol/l PBS, dehydrated in graded alcohols and embedded in paraplast. Sections of the hypothalami and pituitaries were cut in the coronal plane at 5µm thickness. Hormones were localized by the peroxidase labelled antibody method (Nakane & Pierce 1986) according to the procedure described by Polkowski (1986). The following antisera were used: anti-oCRH (SV22) and anti-SRIH (19608) at a dilution of 1:1000 with incubation for 72 h at 4°C; anti-ACTH1-24, anti-ACTH17-39, anti-beta-endorphin, anti-GH and anti-PRL at a dilution of 1:100-1:200 with incubation for 24 h at 4°C. All antibodies except anti-oCRF were prepared by Dr M. P. Dubois (INRA, Nouzilly, France). Details of their preparation and of specificity studies were described by Begeot et al. (1978), Dubois & Barry (1974) and Dubois (1971a,b). The anti-oCRF serum was kindly provided by Dr S. Vigh (Medical School, Pécs, Hungary). Details of the preparation were described by Vigh et al. (1982). Some sections were also stained using the intensification method of Liposits et al. (1983). As control reaction, the inhibition of anti-hormone serum with its homologous antigen was used. The cross-reactivities of antisera were blocked by 4–10 µg of antigen per 1 ml antiserum diluted 1:100 (pituitary hormones) or 1:500 (hypothalamic hormones). The antigens and antisera were mixed and pre-incubated for 48 h at 4°C before use.

Statistical analysis

The percentage of 4 types of cells in the population of about 7000 cells in each pituitary gland was determined. Four, three and three pituitary glands were examined in the control group and the groups of animals subjected to short-term and long-term stress, respectively. Differences between the percentage of respective cell types in the pars distalis of control and stressed animals were analysed on the basis of Student's t-test with the significance level set at the 0.01. This analysis followed the ANOVA performed on the basis of Fischer's criteria.

Fig. 1.

ir CRH in the central part of the ME of the control (a), acutely (b) and chronically (c) stressed ewes. × 28. Note the conspicuous reduction of irCRH material in the acutely stressed ewe.
Results

The hypothalamus

In control ewes, a high density of irCRH was observed in the central and caudal parts of the median eminence (ME) (Fig. 1a). No irCRH perikarya were observed in any of the animals examined (control and experimental). Acute stressful stimuli caused a dramatic reduction of irCRH in ME (Fig. 1b). After long-term stressful stimuli, a slight increase in irCRH in the ME was observed as compared with the acute stress group (Fig. 1c). Abundant irSRIH material was seen in the central and caudal parts of the ME of control ewes (Fig. 2a). Numerous, heavily stained irSRIH perikarya were localized in the periventricular zone of the nucleus suprachiasmaticus and paraventricularis of the hypothalamus (Fig. 3a). Acute stressful stimuli caused only a moderate reduction of irSRIH material in the ME (Fig. 2b) and no changes in the appearance of immunocytochemically stained SRIH perikarya. After long-term stress, the density of irSRIH material stored in the ME sharply decreased as compared with the acute stress group (Fig. 2c), and the number of irSRIH perikarya and their staining intensity also markedly diminished (Fig. 3b).

The pituitary gland

ACTH/beta-endorphin, GH and PRL cells in the pars distalis of the adenohypophysis were studied. Changes after stress exposure were recognized by different staining intensity, hypertrophy and changes in the proportion of cell types present. The most prominent changes were observed in the appearance and proportion of the ACTH and beta-endorphin producing cells. In the control animals they comprised about 13% of total anterior pituitary cells and were replete with irACTH and/or beta-endorphin material. After acute stress, there was a 3-fold decrease in the proportion of these cells when compared with controls (Table 1) and most of the visible cells contained only small amounts of immunoreactive material (Fig. 4a,b). In contrast, prolonged exposure to stress caused a significant rise in the proportion of ACTH/beta-endorphin cells in comparison with the controls (Table 1); the cells were packed with the immunoprotein and very often hypertrophied (Fig. 4c). No morphological changes were seen in the GH cells and the proportion of these cells was constant in the material from the experimental animals (Table 1). PRL cells represented almost one half of the pituitary cell population in control ewes. After acute stressful stimuli they displayed sparse immunostaining and there was a 2-fold proportional decrease in this cell type (Fig. 5a,b, Table 1). Although the percentage of PRL cells after prolonged stress was almost the same as in controls, the intensity of staining remained very week (Fig. 5c, Table 1).
Table 1.
Pituitary cell types expressed as percentage of total cells in the pars distalis. Means ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Short-term stress</th>
<th>Long-term stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of pituitary glands</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>ACTH</td>
<td>12.7 ± 0.3</td>
<td>3.6 ± 0.2*</td>
<td>19.0 ± 0.6*</td>
</tr>
<tr>
<td>Beta-endorphin</td>
<td>12.8 ± 0.4</td>
<td>3.8 ± 0.1*</td>
<td>15.8 ± 0.4</td>
</tr>
<tr>
<td>PRL</td>
<td>47.6 ± 0.7</td>
<td>20.9 ± 0.6*</td>
<td>45.0 ± 0.7</td>
</tr>
<tr>
<td>GH</td>
<td>21.0 ± 0.5</td>
<td>20.1 ± 0.4</td>
<td>19.9 ± 0.4</td>
</tr>
</tbody>
</table>

* Significantly different from controls at \( P < 0.01 \).

Discussion

The present study describes changes in cellular content of CRH, ACTH and beta-endorphin and of SRIH and GH, and of PRL during short- and long-term stress in anestrous ewe.

Despite the small number of animals studied we found significant changes in number of cells containing ACTH, beta-endorphin and PRL after short-term stress and in number of cells containing ACTH after long-term stress.

The depletion of irCRH material from the nerve terminals of the ME observed in response to short-term foot shocks can be interpreted as a rapid release of this hormone. Also the decrease in the percentage and in the staining intensity of the pituitary ACTH and beta-endorphin cells probably reflects degranulation of the cells after release of stored hormones. Similar changes in the morphology and proportion of corticotropes have been observed in the rat after in vivo CRH infusion (Westlund et al. 1985). The above results in the sheep are also consistent with data from rats in which a significant reduction of hypothalamic CRH and large increments in plasma ACTH and beta-endorphin from the anterior pituitary pools are observed after acute stress (Johnson et al. 1985; Suemaru et al. 1985). The concomitant depletion of ACTH and beta-endorphin from the

Fig. 3.
irSRIH perikarya in the periventricular area of the nucleus suprachiasmaticus of the control (a) and chronically (b) stressed ewes. \( × \) 70. The reduction of stain intensity is seen in the SRIH perikarya of the experimental ewe.
irACTH cells in the adenohypophysis of control (a), acutely (b) and chronically (c) stressed ewes. × 244. Note the sharp reduction of the cell numbers after acute stress and marked increase in the number and size of cells after prolonged stress.

irPRL cells in the adenohypophysis of the control (a), acutely (b) and chronically (c) stressed ewes. × 224. A great depletion of ir material in acutely and its restoration in chronically stressed ewes in seen.
same pituitary cells suggests that the same hypothalamic mechanism controls the release of both hormones and these hormones are also released concomitantly during acute stress in rats (Tillders et al. 1985). The activation of CRH and ACTH/beta-endorphin release after acute stress in the present study is also consistent with our previous results in which a rise of cortisol secretion was found in the anestrous, short-term stressed ewe (Przekop et al. 1985).

In animals subjected to the long-term stressful stimuli, some unexpected events were observed in the immunocytochemistry of the hormones, including a dissociation between changes in hypothalamic irCRH and pituitary irACTH/beta-endorphin. Prolonged stress elicits less depletion of irCRH material from the nerve terminals of the ME than does short-term stress. This phenomenon may be explained by the direct central negative feedback action of corticosteroids released under prolonged stress conditions, as has been found in rats (Plotsky et al. 1986). The increasing proportion and hypertrophy of ACTH/beta-endorphin cells, with a concomitant increase in irACTH and beta-endorphin material, suggest that some changes in the secretory activity of these hormones occur at the pituitary level. These changes, interpreted as an impairment of the releasability of ACTH/beta-endorphin from the pituitary gland, may be responsible for the hypocortisolemia which develops in ewes subjected to prolonged stress (Przekop et al. 1985). This assumption is further supported by the fact that these animals have a normal responsiveness in cortisol secretion to exogenous ACTH. Also in rats, chronic stress elevates the ACTH content of the anterior pituitary gland without changes in ACTH and corticosteroid levels in plasma (Young & Akil 1985). It has been documented recently that an increase in ACTH/beta-endorphin stores in the pituitary gland of chronically stressed rats is due to an increase in the biosynthesis of these hormones and a decrease of the rate of their processing (Shiomi et al. 1986). Our suggestion of a similar impairment of ACTH/beta-endorphin release during prolonged stress in sheep needs confirmation with studies involving the direct measurement of ACTH secretion during treatment.

The small depletion of irSRIH material from the nerve terminals of the ME in the sheep subjected to short-term stress, indicates that stressful stimuli may also exert an excitatory effect on the release of this hormone; this effect was greatly intensified in the long-term stressed animals. The disappearance of immunoreactivity from the irSRIH perikarya in the anterior periventricular area of the hypothalamus also suggests increased secretion from the SRIH neurons and particularly an acceleration of SRIH transport along neuronal fibres in long-term stressed ewes. This interpretation can be supported by the converse evidence that the inhibition of axonal transport by colchicine unmarks neuronal perikarya by increasing their secretory product (Sétaló et al. 1976). A rapid increment in SRIH secretion after severe stressful stimuli has been documented in experiments on rats (Arancibia et al. 1984; cf. Fukata et al. 1985). Although the release of somatostatin during stress conditions can be responsible for suppression of GH secretion in rats (Crichtlow et al. 1978), no changes in the morphology and percentage of GH cells were observed in the stressed ewes. It is possible that the duration of the experiment was too short to provoke morphological changes in this type of adenohypophysial cell, as we have been unable to observe changes in the immunocytoology of GH cells indicating an inhibition of the release of this hormone, until after 12 days of long-term stress (Polkowska, unpublished).

Short foot shocks activate PRL cells by eliciting the rapid degranulation of these cells (which is interpreted as a rapid release of prolactin) and a proportional decrease in these cells in the adenohypophysis. After long-term stress, the proportion of PRL cells returned to the control state, but the content of ir material remained low. Since a highly significant rise in plasma prolactin concentration was observed in ewes during all 3 days of stimulation (Wolinska-Witort et al. 1986), our results suggest that under prolonged stress conditions, the synthesis as well as release of this hormone are increasing.

In conclusion, it may be postulated that stress induces alterations in hypothalamic and pituitary activity as evidenced by their immunoreactive content. Acute stress strongly activates the release of CRH, ACTH, beta-endorphin, PRL and, to a lesser extent, of SRIH. During longer exposure to the same stressful stimuli, the release of CRH, ACTH and beta-endorphin is attenuated, whilst secretion of somatostatin and prolactin seems to be augmented.
Acknowledgments

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