

THE EFFECTS OF PRENATAL STRESS ON THE DEVELOPMENT OF HYPOTHALAMIC PARAVENTRICULAR NEURONS IN FETAL RATS

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Abstract—The present experiments focused on the influence of prenatal stress on the development of neurons of the hypothalamic paraventricular nucleus in the fetal rat, including corticotropin-releasing factor-containing neurons. Prenatal stress was administered by restraining pregnant rats in a small cage for either 30 (30-min stress group) or 240 min (240-min stress group) daily for three days from embryonic day 15 to 17, and the fetal brains were taken on embryonic day 18 for later analysis. Golgi-impregnated neurons of the paraventricular nucleus in the 240-min stress group revealed that the total length of the processes was significantly shorter than in the control (unstressed) and 30-min stress groups. In addition, the 240-min stress group showed an increase in the number of apoptotic cells in the fetal paraventricular nucleus. On the other hand, Golgi-impregnated neurons of the paraventricular nucleus in the 30-min stress group had a greater degree of cell differentiation as manifested by an increase in both the number of branch points and the total length of the processes from the cell body. Furthermore, the fetal paraventricular nucleus in the 30-min stress group showed enhanced corticotropin-releasing factor messenger RNA expression, while the varicosities of corticotropin-releasing factor-containing axons at the median eminence revealed more matured morphology such as shorter intervals between the varicosities.

These findings suggest the duration-dependent effects of prenatal stress on the development of fetal hypothalamic paraventricular nucleus neurons, including corticotropin-releasing factor-containing neurons: long-lasting stress causes neurotoxic changes of fetal paraventricular nucleus neurons, whereas short-lasting stress facilitates the development of these fetal brain neurons. These morphological changes induced by prenatal stress may contribute to behavioral changes of the offspring after birth. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: prenatal stress, fetal hypothalamic paraventricular nucleus, development, Golgi impregnation, apoptosis, corticotropin-releasing factor.

Stress produces a variety of physiological responses, such as activation of the hypothalamic–pituitary–adrenal (HPA) axis and sympathetic nervous system. In addition, recent studies have demonstrated that neuropathological changes occur in brain neurons following repeated stress. Uno *et al.* have presented evidence that sustained social stress can cause neuronal degeneration and cell loss in the hippocampus of primates.⁵³ Furthermore, in rats and tree shrews neurotoxic changes of hippocampal pyramidal neurons, such as dendritic atrophy, also occur during physical and psychosocial stress treatment.^{9,17,22,24,25,30,55} The stress-induced

neuropathological alterations in the hippocampus have been proposed as one of the mechanisms of age- and stress-related cognitive impairments, because the hippocampus is an important structure for learning and memory.^{20,29,31,42,43,49}

Most previous studies concerning the stress-induced changes in neuronal morphology have been explored for adult animals. A considerable number of experiments have demonstrated that stress during early developmental stages affects the behavior of adult offspring. The behavioral changes after prenatal stress include enhanced emotional reactivity, hyperanxiety and impaired social behavior.^{1,7,8,33,57} Several of these stress-induced behavioral changes of the offspring are thought to be closely related to changes in the regulation of the HPA axis, including altered activity of corticotropin-releasing factor (CRF)-containing neurons in the paraventricular nucleus (PVN) of the hypothalamus.^{3,16,21,26,36,50,56} However, the mechanism of the functional changes of the HPA axis following prenatal stress remains to be elucidated. Furthermore,

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Abbreviations: BDNF, brain-derived neurotrophic factor; CRF, corticotropin-releasing factor; DAB, diaminobenzidine; DTT, dithiothreitol; E, embryonic day; EDTA, ethylenediaminetetraacetate; HPA, hypothalamic–pituitary–adrenal; IR, immunoreactive; ME, median eminence; PBS, phosphate-buffered saline; PVN, paraventricular nucleus; SSC, standard saline citrate; TUNEL, terminal dUTP nick-end labeling.

the possibility that the stress-induced changes of HPA axis function involve modifications of neuronal morphology of the developing brain has not been examined.

The present experiments focused on the influence of prenatal stress on the development of the morphology of neurons in the fetal rat PVN, including CRF-containing neurons. Before examining the effects of prenatal stress on the development of CRF-containing neurons in the fetal PVN, we studied the ontogeny of these neurons. In addition, we examined short- and long-lasting stress to see whether the effects of prenatal stress on the development of fetal PVN neurons depend on the duration of stress treatment.

A portion of the present results has been reported in abstract form.¹⁰

EXPERIMENTAL PROCEDURES

Animals

Sprague–Dawley rats (Clea Japan, Japan) were housed with free access to food and water, at a constant 22°C, under a 12:12 h light:dark cycle (light on at 8.00 a.m., off at 8.00 p.m.). After female rats (eight- to 10-weeks-old) were kept with adult male rats in the same cage overnight for mating, the vaginal smear was examined on the next morning. The day on which the smear was sperm-positive was taken as embryonic day 0 (E0). Then each pregnant rat was separated in a plastic home cage (40 × 25 × 20 cm) with woodchip bedding for later experiments. The number of pregnant rats for each group was 12. This experiment was reviewed by the Committee of the Ethics on Animal Experiment in Yamaguchi University School of Medicine and carried out under the control of the Guideline for Animal Experiments at Yamaguchi University School of Medicine and in accordance with Japanese Federal Law (No.105) and Notification (No.6) of the Japanese Government.

Procedures

For restraint stress treatment, a pregnant rat was put in a small cylindrical cage made of steel wire (Ø7 × 18 cm). The stressed rats in the restraint cage were kept in their home cages during the stress treatment, while control pregnant rats remained in their home cages. From E15 to E17, the stress treatment was performed daily from 1.30 p.m. to 2.00 p.m. for 30-min restraint (30-min stress group) or from 10.00 a.m. to 2.00 p.m. for 240-min restraint (240-min stress group). On E18, fetuses in each group were obtained by Cesarean section at 2.00 p.m. In all experiments the dams were anesthetized with Nembutal (50 mg/kg, i.p.), and in some experiments (electron microscopy and *in situ* hybridization histochemistry experiments) diethyl ether was also used. Two or three fetuses which were randomly obtained from each dam were preserved for morphological experiments, while the other fetuses were measured for whole body, brain and adrenal weights.

Golgi staining

Fetuses were perfused transcardially with a solution of 0.1 M phosphate buffer containing 4% paraformaldehyde (pH 7.4). After the intact fetal forebrains were removed, they were incubated in 1% potassium dichromate, 1% mercury chloride, 0.8% potassium chromate, and 0.5% potassium tungstate in distilled water at 20°C for two weeks. After rinsing the brains with distilled water, they

were incubated in 1% lithium hydroxide and 15% potassium nitrate in distilled water at 20°C for two days. Forebrains were cut for analysis in 200-µm-thick sections with a cryostat microtome set at -15°C. Camera lucida tracings were obtained from selected neurons (See "Morphological Analysis").

The modified terminal dUTP nick-end labeling method

To investigate the effects of prenatal stress on apoptosis in the fetal PVN, *in situ* DNA fragmentation was examined by the modified terminal dUTP nick-end labeling (TUNEL) method proposed by Gavrieli *et al.*¹² Fetuses were perfused transcardially with a solution of 0.1 M phosphate buffer containing 4% paraformaldehyde (pH 7.4). After the whole fetal brains were removed, they were postfixed with the same paraformaldehyde solution and dehydrated in 10–30% sucrose solution. Serial coronal sections of the fetal forebrains were cut at 12 µm with a cryostat microtome set at -26°C, and then mounted on gelatin-coated slides. After blocking endogenous peroxidase with methanol containing 0.3% H₂O₂, the sections were incubated with a solution containing digoxigenin–nucleotide complex and terminal deoxynucleotidyl transferase (Oncor Inc., U.S.A.) at 37°C for 60 min. The reaction was stopped in buffer at 37°C for 30 min. After washing, they were incubated with anti-digoxigenin antibody-conjugated peroxidase (Oncor Inc., U.S.A.) for 30 min. Immunoreaction was visualized using 0.01% diaminobenzidine (DAB) as the chromogen. Nickel ammonium sulfate (0.6%) was used for enhancing the reaction.

Electron microscopy

Fetuses removed from the uterus were perfused with approximately 0.3 ml of a 2% glutaraldehyde and 2% paraformaldehyde mixture in 0.1 M cacodylate buffer (pH 7.4) via the left ventricle. Immediately after perfusion, the brains were removed carefully from the skull and then immersed in the same fixative at 4°C for 1 h. In order to identify the regions, the brains were sliced with a microslicer (Dosaka EM Co., Japan) at a thickness of 200 µm. The slices were selected as to contain the paraventricular portion of the hypothalamic field using a dissection microscope and then postfixed with a 2% osmic acid solution for 2 h. They were dehydrated with graded acetone and embedded in an Epon 812 (TAAB, U.K.). Ultrathin sections were cut with an ultramicrotome (ULTRACUT, Reichert-Jung, Germany) at a thickness of 100 nm, stained with uranyl acetate and lead citrate and examined with a JEOL 200CX transmission electron microscope (JEOL, Japan). One micrometer epoxy sections were stained with a 1% Methylene Blue and 1% Azure II mixture for light microscopy.

Immunohistochemistry

For study of the effect of prenatal stress on fetal CRF neurons, fetal brain sections were prepared in the same way as in the apoptosis experiments. After blocking endogenous peroxidase with methanol containing 0.3% H₂O₂ and non-specific binding with normal goat serum (1:20), the brain sections were incubated overnight with rabbit anti-serum against rat-CRF⁵ (1:10,000). After washing, sections were incubated with biotinylated goat anti-rabbit IgG(H + L) (1:200, Vector Labs, U.S.A.) for 60 min, and streptavidin-conjugated peroxidase for 60 min. Immunoreaction was visualized using 0.01% DAB as chromogen. Nickel ammonium sulfate (0.6%) was used for enhancing the reaction.

In situ hybridization histochemistry

Fetal brains were taken out on ice without perfusion and

quickly frozen by using liquid nitrogen. Serial sections mounted on slides were made in the same way as for the immunohistochemistry procedure. The sections were fixed in 0.1 M phosphate-buffered saline (PBS) containing 4% paraformaldehyde (pH 7.4) for 1 h. After washing in PBS, the sections were treated with 10 µg/ml Proteinase K solution for 10 min in a waterbath incubator set at 37°C. Glycine (2 mg/ml) dissolved in PBS was used to block the effect of excessive paraformaldehyde solution. Rinsing off the PBS, the sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min for the purpose of decreasing the non-specific hybridization of the probe. After dehydration with 70–100% ethanol, the sections were delipidated in chloroform for 5 min. Before using the probe, the sections were pretreated with prehybridization buffer containing 50% formamide, 4× standard saline citrate (SSC) solution (0.15 M NaCl, 0.015 M sodium citrate) and 10 mM dithiothreitol (DTT). A synthetic oligonucleotide of CRF mRNA (48 mer, Du Pont, NEN, U.S.A.) was used as a probe, labeled with ³⁵S-dideoxyadenosine-5'-α-thiotriphosphate (Du Pont, NEN, U.S.A.) by means of 3'-end labeling method. For each section mounted on a slide, 2.0×10⁶ c.p.m. of the labeled oligonucleotide probe was used. The probe was dissolved in 140 µl of hybridization buffer containing 4×SSC, 50% formamide, 2.7× Denhardt's solution, 10 mM EDTA, 33 µg/ml poly A, 200 µg/ml heparin, 20 mM DTT, 250 µg/ml yeast total RNA, 10% dextran sulfate and 5% salmon sperm DNA. The sections mounted on slides were incubated with the probe solution at 37°C for 18 h. After hybridization, the slides were washed in 2×SSC with 10 mM DTT and 2×SSC containing 50% formamide, 10 mM DTT at 40°C and dehydrated. The slides were dipped in photographic emulsion (NTB-2, Kodak, U.S.A.) and exposed for three weeks. After exposure, sections were developed in a D-19 developer and counterstained with Hematoxylin and Eosin.

Morphological analysis

The results of these experiments were analysed by the National Institutes of Health-Image software (Wayne Rasband, NIH, U.S.A.) in an Apple Macintosh computer system after scanning the slides containing brain sections by a microscope (Nikon, Japan) with a CCD camera (Teli Co., Japan). All slides were coded prior to quantitative analysis. The code was not broken until the analysis was completed. In order to be selected for analysis of Golgi-impregnated neurons in the PVN, the method used by McEwen *et al.*^{6,13} was modified. Only Golgi-impregnated neurons which possessed the following characteristics were selected for analysis: (i) location in the paraventricular portion of the hypothalamic field, which was confirmed by comparing the section containing CRF-immunoreactive (IR) neurons of the PVN; (ii) dark and consistent impregnation throughout the extent of all of the processes; (iii) relative isolation from neighboring impregnated neurons that could interfere with analysis. For each brain, 30 PVN neurons were selected from one or two slices. From selected neurons, the total length of the processes and the number of their branch points were measured. For the apoptosis experiments, immunohistochemical staining of CRF-IR neurons in the fetal PVN was performed every two coronal sections. After confirming a section showing the maximal number of CRF-IR neurons in the PVN, the next serial section was used for counting the number of apoptotic cells in the fetal PVN. For evaluation of the differentiation of CRF-IR axons, the intervals between the varicosities of these fibers were measured on one representative section, in which the layer of the median eminence (ME) comprising CRF-IR fibers is the thickest. To make the measurements easier and more accurate, relatively isolated CRF-IR fibers were chosen. The total number of the intervals measured for each animal was approximately 200. To assess the expression of

CRF mRNA in the fetal PVN, the optical integrated density of CRF mRNA for each animal was measured on a coronal section, in which CRF mRNA expression is maximum. The optical integrated density was obtained as an average density over the PVN area, after subtraction of background values, using a computer-assisted densitometric analysis system (NIH-Image software, U.S.A.).

For statistical analysis of the results from the morphological studies as demonstrated in Tables 2, 3, 4 and 5, each group comprised six fetuses obtained from six different dams. All data are expressed as mean ± S.E.M. and were analysed by one-way factorial analysis of variance (ANOVA) followed by the Scheffé's *F*-test. The level of significance for all analysis was set at *P* < 0.05 and 0.01.

RESULTS

Effects of maternal stress on the weights of fetal and maternal tissues

Prenatal stress in this experiment caused no apparent change in the growth of fetal tissues including the whole body, brain, and bilateral adrenals (Table 1). However, the weight of the maternal adrenals was significantly heavier in the 240-min stress group (44.3 ± 5.3 mg, *n* = 4) than that in the control (32.1 ± 1.9 mg, *n* = 6) ($F_{2,14} = 4.51$, *P* < 0.01) and 30-min stress groups (35.2 ± 1.8 mg, *n* = 7) (*P* < 0.05). Gastric ulcer was not observed in any dams in the control and stress groups, except one dam in the 240-min stress group who had gastritis-like erosion in a small region of the gastric wall.

Maternal plasma corticosterone levels were measured on pregnant day 20 immediately after acute 30-min and 240-min restraint stress treatment. The level of maternal plasma corticosterone in the 240-min stress group (777.0 ± 117.8 ng/ml, *n* = 3) was significantly higher than that in the control group (382.8 ± 49.4 ng/ml, *n* = 5) ($F_{2,10} = 6.52$, *P* < 0.05). The 30-min stress group (656.6 ± 80.3 ng/ml, *n* = 5) also showed higher corticosterone level as compared to the control, though the difference between the two groups was not statistically significant.

Golgi study

To see whether prenatal stress could alter development of the morphology of fetal PVN neurons, Golgi-impregnated neurons in the fetal PVN were examined. Figure 1 shows camera lucida drawings illustrating the morphology of representative fetal PVN Golgi-impregnated neurons in the control, 30-min stress, and 240-min stress groups. PVN neurons of the 240-min stress group exhibited extremely short processes protruding from the cell body, while those of the 30-min stress group had more differentiated arborization as compared to the control and 240-min stress groups.

The total length of the processes of Golgi-impregnated neurons in the fetal PVN was significantly shorter in the 240-min stress (148.7 ± 7.4 µm, *n* = 180 neurons, six animals) than in the control

Table 1. The weight of fetal and maternal tissues in the control and stress groups

| Group | Fetal body (g) | Fetal brain (mg) | Fetal adrenals (mg) | Maternal adrenal (mg) |
|----------------|-----------------|------------------|---------------------|-----------------------|
| Control | 1.40 ± 0.02(24) | 92.8 ± 1.2(20) | 1.16 ± 0.06(33) | 32.1 ± 1.9(6) |
| 30-min stress | 1.42 ± 0.02(26) | 93.0 ± 1.1(21) | 1.24 ± 0.06(30) | 35.2 ± 1.8(7) |
| 240-min stress | 1.36 ± 0.02(14) | 92.6 ± 0.9(9) | 1.22 ± 0.08(16) | 44.3 ± 5.3*(4) |

Values represent the mean ± S.E.M. (number of animals).

* $P < 0.05$ as compared to the control and 30-min stress groups.

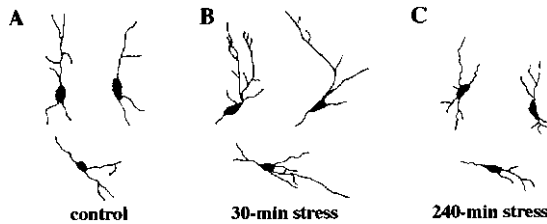


Fig. 1. Camera lucida drawings of representative Golgi-impregnated PVN neurons from control and stress-treated fetuses. (A) Control, (B) 30-min stress and (C) 240-min stress groups. Golgi-impregnated neurons in the 30-min stress group showed an increased branching pattern, whereas PVN neurons in the 240-min stress group had less extended branching compared to the control group.

Table 2. Effect of prenatal stress on morphology of Golgi-impregnated neurons in the fetal paraventricular nucleus

| Group | Total length of processes (μm) | Number of branch points |
|----------------|---|-------------------------|
| Control | 181.8 ± 8.2 | 4.2 ± 0.1 |
| 30-min stress | 212.1 ± 9.9* ** | 5.2 ± 0.2*** |
| 240-min stress | 148.7 ± 7.4* | 4.0 ± 0.1 |

Values represent the mean ± S.E.M. ($n = 6$).

* $P < 0.05$ as compared to the control group. ** $P < 0.01$ as compared to the 240-min stress group. *** $P < 0.01$ as compared to the control and 240-min stress groups.

(181.8 ± 8.2 μm , $n = 180$ neurons, six animals) ($F_{2,537} = 13.71$, $P < 0.05$) and 30-min stress groups (212.1 ± 9.9 μm , $n = 180$ neurons, six animals) ($P < 0.01$) (Table 2). In addition, the total dendritic length of fetal PVN neurons in the 30-min stress group was significantly longer than in the control

group ($P < 0.05$). Fetal PVN neurons in the 30-min stress group also showed a significantly higher number of total branch points of the processes (5.2 ± 0.2/cell, $n = 180$ neurons, six animals) as compared to the control (4.2 ± 0.1/cell, $n = 180$ neurons, six animals) ($F_{2,537} = 18.04$, $P < 0.01$) and 240-min stress groups (4.0 ± 0.1/cell, $n = 180$ neurons, six animals) ($P < 0.01$).

Apoptotic cell death by prenatal stress

The occurrence of apoptotic cell death following prenatal stress was demonstrated by two methods; light and electron microscopic study. TUNEL-positive cells revealing apoptotic cells were easily identified under light microscopy (Fig. 2). In the normal fetal PVN on E18, only a few cells were apoptotic (0.8 ± 0.3/section, $n = 6$). The number of apoptotic cells was markedly increased in the fetal PVN following the 240-stress treatment (18.3 ± 1.7/section, $n = 6$) as compared to the control ($F_{2,15} = 80.17$, $P < 0.01$) and 30-min stress groups (3.0 ± 0.6/section, $n = 6$) ($P < 0.01$). No significant change was observed between the 30-min stress and the control group (Table 3).

Electron microscopic studies demonstrated that many neurons in the 240-min stress group had a dense or breaking nucleus, which contained heterogeneous clusters of chromatin with different electron density (Fig. 3). Their cytoplasm was very scarce and contained a few organelles with a normal morphology, while plasma membrane was preserved. The morphological appearance was in agreement with the characteristics of apoptotic neurons.

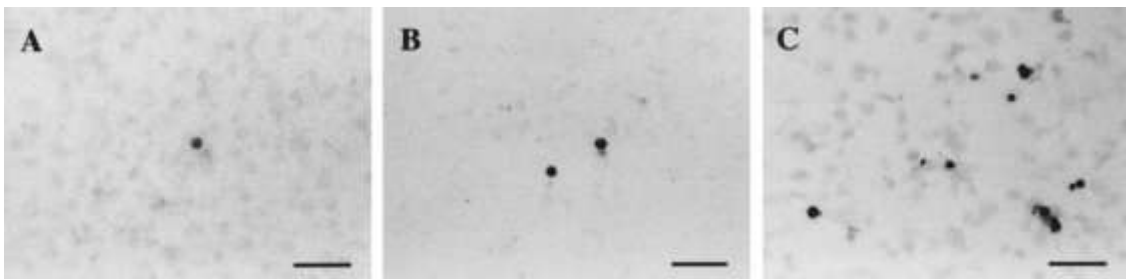


Fig. 2. Effects of prenatal stress on apoptosis of fetal PVN cells. (A) Control, (B) 30-min stress and (C) 240-min stress groups. In the 240-min stress group, many apoptotic cells appeared in the fetal PVN, whereas only a small number of apoptotic cells were expressed in the control and 30-min stress group. Scale bars = 20 μm .

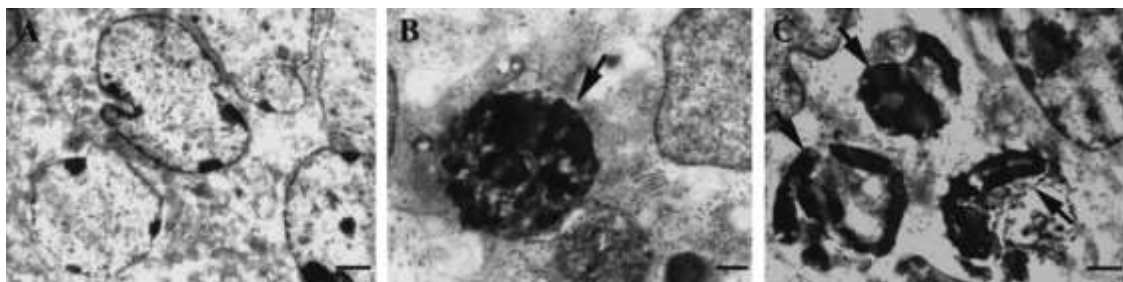


Fig. 3. Electron microscopic visualization of apoptotic cells in the fetal PVN. (A) A neuron in the control group has morphologically normal nuclei. (B, C) Apoptotic PVN neurons in the 240-min stress group were indicated as arrows. (B) A round-shaped nucleus containing heterogeneous clusters of chromatin with different electron density and (C) a dense nucleus broken in the cytoplasm of a 240-min stressed neuron. Scale bars = 1 μ m.

Table 3. Effect of prenatal stress on apoptosis in the fetal paraventricular nucleus

| Group | Number of TUNEL-positive cells |
|----------------|--------------------------------|
| Control | 0.8 \pm 0.3 |
| 30-min stress | 3.0 \pm 0.6 |
| 240-min stress | 18.3 \pm 1.7* |

Values represent the mean \pm S.E.M. ($n = 6$).

* $P < 0.01$ as compared to the control and 30-min stress groups.

are first detectable between E15 and E16.^{5,14} In addition, the number of CRF-IR neurons bearing multiple processes increased markedly during the period of E17 and E18.

CRF-IR fibers were first detectable at the ME on E16, without an apparent formation of axonal varicosities. The varicosities were first detectable on E17. With development, the varicosities became smaller in size and the intervals between the varicosities shortened.

Ontogeny of corticotropin-releasing factor-containing neurons in the paraventricular nucleus

Immunohistochemical and *in situ* hybridization studies revealed that CRF-IR neurons and CRF mRNA expression first appeared in the PVN on E15. The number of CRF-IR neurons increased rapidly from E15 to E17. These findings are in accord with the results of previous experiments in which CRF-IR neurons and CRF mRNA in the PVN

Effects of maternal stress on the development of corticotropin-releasing factor-containing neurons

The most notable finding was that most PVN neurons in the 240-min stress revealed weak CRF immunoreactivity compared to the control and 30-min stress group (Fig. 4). It was also noted that the distribution of CRF-IR neurons in the 30-min stress group extended more widely to the lateral and ventral direction in the PVN than that in the control

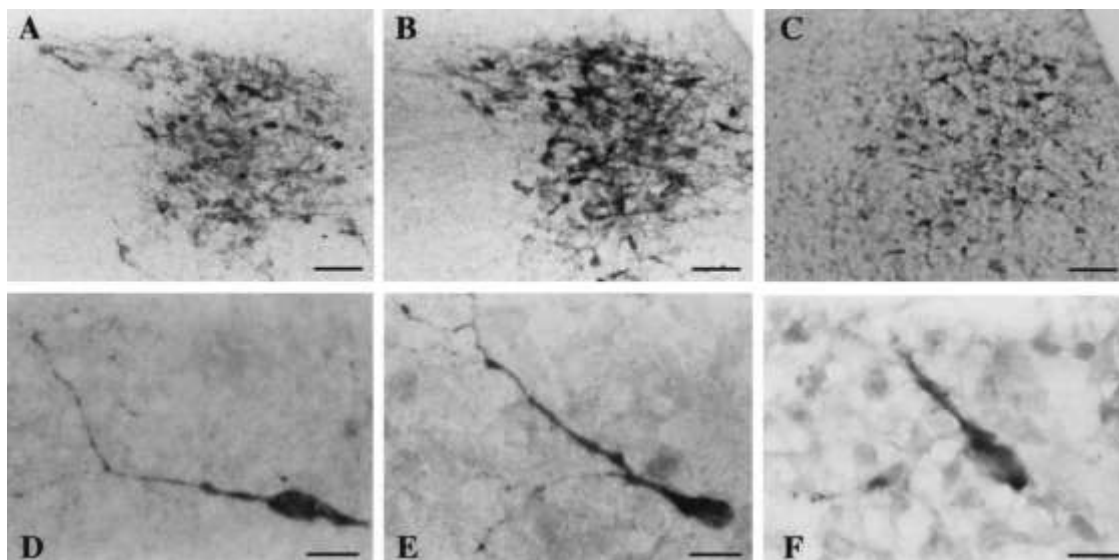


Fig. 4. CRF-IR neurons in the fetal PVN in the control and stressed animals. (A, D) Control, (B, E) 30-min stress and (C, F) 240-min stress groups. The CRF-IR neurons in the 30-min stress group appeared to possess more extended arborization as compared to the control and 240-min stress groups, whereas many fetal PVN neurons in the 240-min stress group revealed weak CRF immunoreactivity. Scale bars = 50 μ m (A–C), 10 μ m (D–F).

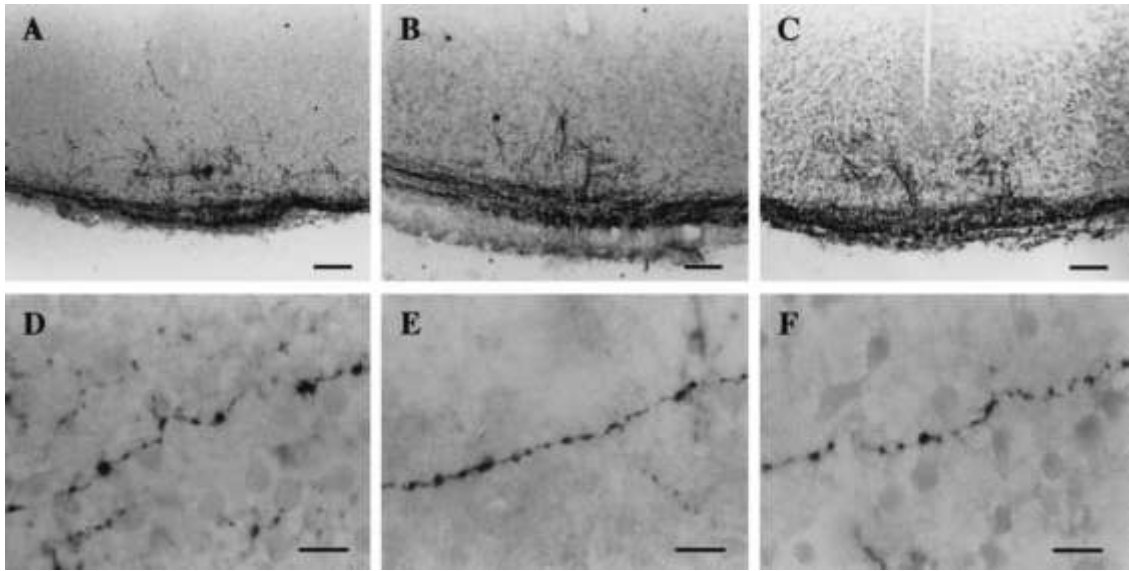


Fig. 5. Effects of prenatal stress on development of CRF-IR fibers at the ME. (A, D) Control, (B, E) 30-min stress and (C, F) 240-min stress groups. The intervals between varicosities were shorter in the 30-min stress group than in the other groups. Scale bars = 50 μm (A–C), 10 μm (D–F).

Table 4. Effect of prenatal stress on corticotropin-releasing factor-immunoreactive fibers at the fetal median eminence

| Group | Intervals between varicosities (μm) |
|----------------|--|
| Control | 7.6 ± 0.3 |
| 30-min stress | $6.0 \pm 0.1^{*,**}$ |
| 240-min stress | 7.1 ± 0.3 |

Values represent the mean \pm S.E.M. ($n = 6$).

* $P < 0.01$ as compared to the control group. ** $P < 0.05$ as compared to the 240-min stress group.

and 240-min stress group. In addition, the number of CRF-IR neurons bearing multiple processes appeared to increase in the 30-min stress group as compared to the control and 240-min stress groups, though quantitative analysis could not be made.

At the ME, the intervals between the varicosities of CRF-IR fibers were significantly shorter in the 30-min stress group ($6.0 \pm 0.1 \mu\text{m}$, 200 intervals/animal, six animals) than in the control ($7.6 \pm 0.3 \mu\text{m}$, 200 intervals/animal, six animals) ($F_{2,15} = 11.48$, $P < 0.01$) and 240-min stress groups

($7.1 \pm 0.3 \mu\text{m}$, 200 intervals/animal, six animals) ($P < 0.05$), though the latter two groups were not different from each other (Fig. 5 and Table 4).

The amount of CRF mRNA expression in the 30-min stress group was approximately twice that in the control and 240-min stress groups ($F_{2,15} = 5.76$, $P < 0.05$, $n = 6$ animals/group) (Fig. 6 and Table 5). The expression of CRF mRNA in the 30-min stress group was observed more extensively in the PVN as compared to the other groups. In the 240-min stress group, CRF mRNA expressed in the fetal PVN was nearly the same as that in the control group.

DISCUSSION

Previous experiments have indicated that prenatal stress can modify structures in the brain. In studies of sexual differentiation in the brain, it has been shown that prenatal stress can affect the size of the sexually dimorphic nucleus of the preoptic area² and the rostral anterior commissure in rats.¹⁸ However, no attempts have been made to demonstrate the

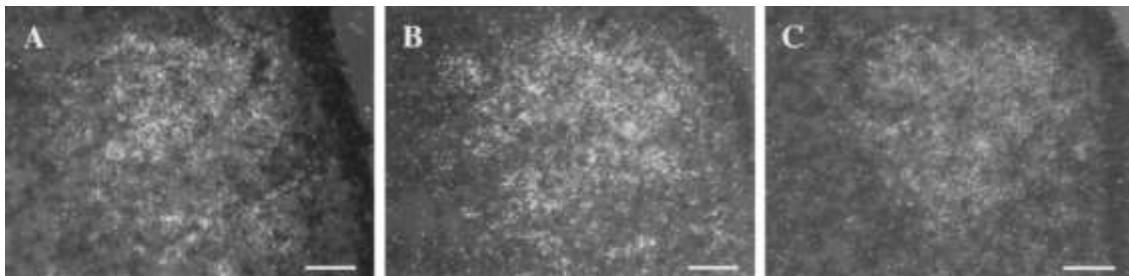


Fig. 6. Effects of prenatal stress on expression of CRF mRNA in the fetal PVN. (A) Control, (B) 30-min stress and (C) 240-min stress groups. Expression of CRF mRNA in the fetal PVN was more remarkable in the 30-min stress group than in the control and 240-min stress groups. Scale bars = 50 μm .

Table 5. Effect of prenatal stress on the expression of corticotropin-releasing factor messenger RNA in the fetal paraventricular nucleus

| Group | The optical integrated density |
|----------------|--------------------------------|
| Control | 458.3 ± 92.5 |
| 30-min stress | 846.5 ± 76.3* |
| 240-min stress | 476.8 ± 102.9 |

Values represent the mean ± S.E.M. ($n = 6$).

* $P < 0.05$ as compared to the control and 240-min stress groups.

effects of prenatal stress on the development of fetal brain neurons with special reference to a specific neurotransmitter or neurohormone.

The present experiments have demonstrated for the first time that maternal stress can modify the morphology of fetal brain neurons, including CRF-containing neurons in the PVN. In the adult brain, it has been shown that chronic stress induces degeneration and cell loss in the brain.^{9,17,22,24,25,30,53,55} Most of these reports have focused on hippocampal neurons that are known to be extremely vulnerable to environmental challenges. The present experiments have shown that neurotoxic alterations in brain neurons also occur in the fetal brain during maternal stress as demonstrated in PVN neurons in the 240-min stress group. The PVN neurons in this stress group revealed shorter, less complex processes. Of particular interest is the finding that the 240-min stress group showed an increase in the number of TUNEL-positive cells revealing apoptotic cells in the fetal PVN.

The 240-min stress group showed no apparent change in CRF mRNA expression, despite the fact that many cells in the fetal PVN in the 240-min stress group revealed a great reduction in CRF immunoreactivity. It is most likely that CRF is depleted rapidly in CRF-containing neurons in this stress group, although these neurons may produce as much CRF as fetal PVN neurons from non-stressed dams can.

The present experiments suggested that a short-duration stress during prenatal periods could facilitate the development of fetal PVN neurons. The evidence for facilitatory effects of prenatal stress on PVN neurons is supported by the following findings: (i) PVN neurons in the 30-min stress group had a greater degree of cell differentiation as manifested by an increase in both the number of branch points and the total length of the processes from the cell body; and (ii) the fetal PVN in the 30-min stress group showed enhanced CRF mRNA expression, while the varicosities of CRF-containing axons at the ME revealed more differentiated morphology in the 30-min stress group.

The effects of maternal stress observed in the present experiments were dependent on the duration of the stress treatment: maternal stress of short duration (restraint for 30 min daily for three days)

produced a facilitatory influence upon the development of CRF-containing neurons in the fetal PVN, whereas long-duration stress (restraint for 240 min daily for three days) revealed neurotoxic action. A previous experiment in our laboratory has shown that duration-dependent effects of stress occur in axonal projections of noradrenergic locus coeruleus neurons projecting to the cerebral cortex in adult rats.^{35,39} In that experiment, using antidromic stimulation technique, the coeruleo-cortical projection was found to increase in the animals restrained for 1 h daily for two weeks, but to decrease in those restrained for 6 h daily for two weeks.³⁹ The present experiment is the first to present morphological evidence for duration-dependent alterations in the development of brain neurons during prenatal periods.

The mechanisms for the duration-dependent effects of stress on the development of CRF-containing neurons in the fetal PVN remain to be investigated. The neuropathological changes caused by the long-lasting stress treatment may be due to neurotoxic action of excessive amounts of glucocorticoid, which is secreted from the dam's adrenal glands during maternal stress and enters the fetal blood circulation through the placenta.^{3,60} Since in the adult brain, neuronal degeneration and cell loss in the hippocampus following repeated stress can be mimicked by prolonged glucocorticoid administration, glucocorticoids secreted during stress are thought to be responsible for the stress-induced neurotoxicity in the hippocampus.^{23,27,28,40,41,44-46,58} The involvement of glucocorticoids in the stress-induced damage of hippocampal neurons is also supported by the fact that this brain region contains high concentrations of glucocorticoid receptors.^{32,38} In fact, excessive glucocorticoid administration showed toxic effects on brain neurons during embryonic periods.⁵² Since it has been shown that mRNAs for glucocorticoid receptors are first expressed at E16 in the rat PVN,^{4,59} the stress-induced neurotoxicity in CRF-containing neurons in the fetal PVN may occur in the same mechanism as that proposed for adult hippocampal neurons. In addition, the marked increase of TUNEL-positive cells in the PVN following the 240-min stress treatment may be also caused by glucocorticoid, because glucocorticoid has been suggested to induce apoptosis in the hippocampus in adult animals.^{15,37}

Glucocorticoid released from the dam's adrenal glands may also play a role, at least in part, in the development of CRF-containing neurons in the fetal PVN, explaining the facilitatory effects of the short-lasting stress. Trejo *et al.* have shown that glucocorticoids are required for cell maturation in the cerebral cortex of fetal rats.⁵¹ In their experiments, pregnant rats bilaterally adrenalectomized on the first day of gestation resulted in fetuses with a marked increase in the number of cells and a lower degree of cell maturation in the fetal cerebral cortex

in later embryonic days, while glucocorticoid administration could prevent the lower cell maturation observed in the adrenalectomized group. Thus it is likely that lower levels of glucocorticoids secreted during short-lasting maternal stress accelerate the maturation of CRF-containing neurons in the fetal PVN. Furthermore, it is likely that neurotrophins play a role in this facilitatory effect of prenatal stress. In the developing brain (postnatal days 9–10), Miranda *et al.* have found that mRNAs for brain-derived neurotrophic factor (BDNF) and its receptors (TrkB) are co-expressed by individual neurons in the hypothalamic PVN.³⁴ Co-expression of mRNAs for other neurotrophins (nerve growth factor and neurotrophin-3) and their receptors in the PVN is much less than that of BDNF and TrkB mRNAs.¹⁹ On the other hand, *in vivo* and *in vitro* experiments have presented substantial evidence for neurotrophic action of BDNF on neurons in the developing brain.^{11,19,47,54} These findings suggest that in the fetal PVN, BDNF exerts neurotrophic action on the development and differentiation of CRF-containing neurons via autocrine mechanisms. In the adult brain, mRNA expressions of neurotrophins including BDNF are reported to increase following stress treatments.⁴⁸ We have found that in immunohistochemical studies of BDNF localization, the number of BDNF-positive cells increased in the fetal PVN in the 30-min and 240-min stress group. Thus BDNF may be responsible, at least in

part, for the facilitatory effects of the prenatal stress on the development of fetal PVN neurons.

CONCLUSIONS

The present results provide evidence for the duration-dependent effects of maternal stress on the development of fetal CRF-containing neurons. At present, it is not clear whether these morphological changes of the fetal brain neurons induced by maternal stress occur temporarily during early developmental stages or persist through adulthood. However, our findings imply that dysregulation of the HPA axis caused by prenatal stress could be related, at least in part, to the morphological alterations of CRF-containing neurons in the hypothalamic PVN. In future experiments, it should be clarified whether behavioral changes induced by prenatal stress, including hyperactivity and altered social behaviors, are associated with these observed changes in the development of CRF-containing neurons.

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