MATERNO-FETAL COORDINATION OF STRESS-INDUCED FOS EXPRESSION IN THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS DURING PREGNANCY

T. FUJIOKA, A. FUJIOKA, H. ENDOH, Y. SAKATA, S. FURUKAWA AND S. NAKAMURA

*Department of Neuroscience, Yamaguchi University School of Medicine, Ube, Yamaguchi 755-8505, Japan

Abstract—This study investigates whether maternal stress during pregnancy induces maternal and fetal hypothalamic paraventricular nucleus (PVN) neuronal activation and the effects of maternal stress on fetal hypothalamic and PVN brain-derived neurotrophic factor (BDNF) expression. Pregnant rats were exposed to three types of maternal stress with varying severity (restraint, forced walking and immobilization) for 30 min on gestational day 21. Severity of stress was assessed by measurement of maternal plasma corticosterone 30 min following the stimulus. Maternal plasma corticosterone increased in each stress response group (immobilization > forced walking > restraint). Further, the expression of Fos protein, a marker of neuronal activation, increased in the fetal and maternal PVN in direct relation to the severity of stress treatments. Forced walking and immobilized stress, but not restraint stress, significantly increased BDNF expression in the fetal hypothalamus.

These findings suggest that the fetal hypothalamic–pituitary–adrenal (HPA) response following maternal stress mirrors maternal HPA activation. In addition, BDNF may play a role in protecting fetal brain neurons from damage caused by severe stress. © 2003 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: Fos, corticosterone, brain-derived neurotrophic factor, paraventricular nucleus, walking, restraint.

Stress during pregnancy can permanently alter fetal brain function (Weinstock, 2001; Welberg and Seckl, 2001), including the regulation of hypothalamic–pituitary–adrenal (HPA) axis (Fride et al., 1986; Henry et al., 1994; McCormick et al., 1995; Peters, 1982; Takahashi et al., 1988; Weinstock et al., 1992) and learning and memory performance (Lordi et al., 1997; Smith et al., 1981; Szuran et al., 1994; Vallee et al., 1999). We have recently demonstrated that prenatal stress exerts a duration-dependent neurotoxic or neurotrophic effect on fetal hypothalamic neurons (Fujioka et al., 1999). Prolonged restraint stress causes neurotoxic changes in fetal neurons, whereas short-duration stress facilitates development of neurons. In adult offspring of these prenatally stressed rats, corticosterone secretion in response to stress was augmented in the prolonged prenatal stress group (Hashimoto et al., 2001), and cognitive performance was enhanced in the short-duration stress group (Fujioka et al., 2001). Thus, the nature of the effects of maternal stress during pregnancy on fetal brain neurons and cognitive performance of the adult offspring may depend on the duration or severity of stress.

Neuronal Fos immunoreactivity is used as a marker of activation (Bullitt, 1990; Chen et al., 1993; Chowdhury et al., 2000; Dragunow and Faull, 1989; Duman, 1995; Sagar et al., 1988). HPA axis activation in response to acute stress increases the number of Fos-positive neurons in the hypothalamic paraventricular nucleus (PVN) (Chowdhury et al., 2000; Helmreich et al., 1996; Mansi et al., 1998; Morgan and Curran, 1991; Senba et al., 1994; Sharp et al., 1991), with the number increasing even further in response to severity of stress (Chowdhury et al., 2000). Thus, investigators have speculated that the PVN plays a role in the perception of stress severity.

Acute maternal immobilization stress at the end of gestation increases fetal plasma adrenocorticotropic hormone (ACTH) and decreases the contents of corticotropin-releasing hormone in the hypothalamus (Ohkawa et al., 1991). This finding suggests that maternal stress activates the fetal HPA axis. The present study investigates whether the severity of maternal stress is transmitted to the fetal HPA axis by assessing the number of Fos-positive cells in the PVN in both the dam and fetus following maternal stress of varying severity (restraint, forced walking, immobilization). Although stress alters the expression of neurotrophic factors in the brain (Smith et al., 1995a,b; Ueyama et al., 1997), little is known about whether maternal stress can cause changes in the expression of fetal neurotrophic factor. Therefore, we examine the changes in expression of brain-derived neurotrophic factor (BDNF) in the fetal hypothalamus with differing degrees of maternal stress.

**EXPERIMENTAL PROCEDURES**

**Animals**

Sprague–Dawley rats (Clea Japan, Japan) were housed at 22 °C with free access to food and water, with a 12-h light/dark cycle (light on at 8 a.m., off at 8 p.m.). Female rats (8–10 weeks old) were kept with adult male rats in the same cage overnight for mating, and vaginal smears were examined the following morning.
Embryonic day 0 was established as the day on which the smear was sperm-positive. Each pregnant rat was housed separately in a plastic home cage (40\texttimes{}25\texttimes{}20 cm) with woodchip bedding. All efforts were made to minimize the number of animals used and their suffering. Experimental conditions and procedures were reviewed by the Committee of the Ethics on Animal Experiments at Yamaguchi University School of Medicine and carried out under the control of the Guideline for Animal Experiments in Yamaguchi University School of Medicine and in accordance with Japanese Federal Law (No.105) and Notification (No.6) of the Japanese Government.

**Stress procedures**

Each maternal stress treatment was performed for 30 min on gestational day 21 (n=5 for each group). For restraint stress, a pregnant rat was placed in a small cylindrical cage made of steel wire (φ 7\texttimes{}18 cm) as previously described (Fujikawa et al., 1999). Immobilization stress was performed by tapeing the limbs and tail to a board and covering the rat with metal mesh (Chowdhury et al., 2000). Forced-walking stress was carried out on a treadmill, at a velocity of 6 m/min. Non-stressed control dams remained in their home cages.

**Corticosterone assay**

Thirty minutes after stress treatment (n=5 for each group), pregnant dams were anesthetized with Nembutal (50 mg/kg, i.p.), and in addition diethyl ether was also used to maintain deep anesthetic condition. Blood samples for measurements of plasma corticosterone were obtained by transecardially withdrawing 0.5 ml of blood into a tube containing EDTA solution (1 mg/ml blood) with subsequent centrifugation at 2000 r.p.m. for 15 min at 4 °C. Plasma was transferred into a fresh tube and stored at −40 °C until needed for corticosterone measurement. Plasma corticosterone level was determined using a commercial corticosterone radioimmunoassay kit (Diagnostic Products Corporation, CA, USA). The corticosterone antiserum was highly specific for rat corticosterone, with extremely low cross-reactivity (<0.9%) to other compounds, such as cortisol and progesterone.

**Immunohistochemistry**

Thirty minutes after stress treatment, pregnant dams were anesthetized with Nembutal (50 mg/kg, i.p.) and diethyl ether. Dams (n=3 for each group) were perfused transecardially with a solution of 0.1 M phosphate buffer containing 4% paraformaldehyde (pH 7.4). One or two fetuses were randomly obtained from each dam. Fetuses (n=6 for each group) were perfused transecardially with the same solution. The whole brains of dams and fetuses were removed and post-fixed with the same paraformaldehyde solution and dehydrated in 10–30% sucrose solution. Serial coronal sections of the frozen maternal brains were cut in 45 \mu m thickness with a microtome and collected in cryoprotectant solution. Serial coronal sections of the fetal brains were cut in 12 \mu m thickness with a cryostat microtome at −26 °C and mounted on gelatin-coated slides. The immunohistochemical procedures for the maternal and fetal brain sections were performed as previously described (Fujikawa et al., 1999, 2001). In brief, 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 antiserum against rat Fos protein (dam: 1:20,000, fetus: 1:10,000, Ab-5, Oncogene Research Products, CA, USA). Sections were washed with phosphate-buffered saline containing 0.3% Triton X-100 and incubated with biotinylated goat anti-rabbit IgG (H+L) antibody (1:1000, Promega, WI, USA) conjugated with horseradish peroxidase for 30 min. After washing six times for 5 min each, the antibody-antigen complex was visualized by enhanced chemiluminescent detection reagent (Pierce Biotechnology, IL, USA). The intensity of the protein signal was quantified using computerized NIH Image software (NIH). For each immunoblotting experiment, expression of BDNF in the stress groups (restraint, immobilization, forced walking) was compared with the non-stressed control group. The amount of BDNF in each stress group was calculated as percentage taking expression of BDNF in the non-stressed control group as 100%.

**Morphological analysis**

Immunoreactively stained brain sections were scanned and analyzed using the National Institutes of Health Image software (NIH, USA) on an Apple Macintosh system. For quantitative analysis of Fos-immunoreactive cells in the fetal and dam PVN, modified methods of previous studies were used (Chowdhury et al., 2000). Fos-immunohistochemical staining was performed on serial or every fourth section for the maternal and fetal PVN, respectively. To compare the number of Fos-immunoreactive cells of each group, a representative coronal section in which the number of Fos-immunoreactive cells was maximal was chosen from all sections. In the adult PVN, Fos-immunoreactive nuclei were counted bilaterally in a region of 6.25\texttimes{}10^4 \mu m^2 area in each side. In the fetal PVN, all Fos-immunoreactive nuclei within the nucleus were counted bilaterally. The demarcation of the fetal PVN was usually distinct. In the cases in which the demarcation was not clear, counting was performed under a phase-contrast microscope. All slides were coded prior to quantitative analysis, and codes were not revealed until the analysis was completed.

**Measurement of BDNF by immunoblotting**

One to three fetuses were randomly obtained from each dam. Frozen tissue samples (n=5 for each group) of the fetal hypothalamus, including the PVN, were homogenized on ice for 1 min in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na3VO4, 1 g/ml leupeptin). Homogenates were centrifuged at 4 °C at 15,000 r.p.m. for 30 min, and supernatants were collected. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, CA, USA) using bovine serum albumin as the standard protein and absorbance at 595 nm. Protein samples were separated by electrophoresis using 12.5% polyacrylamide gel in the presence of SDS. Proteins were transferred by electrophoresis to a 0.45 \mu m nitrocellulose membrane (Amersham Biosciences, NJ, USA); transfer was confirmed by protein visualization with Ponceau S solution (Sigma, MO, USA). Blots were washed with Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and incubated for 30 min with blocking solution (5% skim milk powder in TBS-T). Blots were incubated overnight with rabbit antiserum against BDNF (Fukumitsu et al., 1998) (1:200) in blocking solution. Blots were then rinsed three times for 10 min with TBS-T and incubated with goat anti-rabbit IgG (H+L) antibody (1:10,000, Promega, WI, USA) conjugated with horseradish peroxidase for 30 min. After washing six times for 5 min each, the antibody-antigen complex was visualized by enhanced chemiluminescent detection reagent (Pierce Biotechnology, IL, USA). The intensity of the protein signal was quantified using computerized NIH Image software (NIH). For each immunoblotting experiment, expression of BDNF in the stress groups (restraint, immobilization, forced walking) was compared with the non-stressed control group. The amount of BDNF in each stress group was calculated as percentage taking expression of BDNF in the non-stressed control group as 100%.

**Statistical analysis**

All data are expressed as mean±S.E.M. and were analyzed by one-way factorial analysis of variance followed by the Fisher’s PLSD test. The level of significance for all analysis was set at P<0.05.

**RESULTS**

**Corticosterone secretion**

The severity of three types of maternal stress (restraint, forced walking and immobilization) was assessed by mea-
suring maternal plasma corticosterone 30 min after the stimulus. Dams from all stress groups showed significant increases in plasma corticosterone levels compared with the non-stressed dams (F3,16 = 13.72, P < 0.01). Maternal plasma corticosterone was highest for the immobilization group, followed by the forced walking group, and then the restraint group (Table 1).

**Stress-induced Fos expression in dam and fetal PVN**

Similar to the stress-induced increases in corticosterone levels, the number of Fos-positive cells in the maternal PVN was highest in the immobilization stress group, and the lowest expression was observed in the restraint group (F3,8 = 78.74, P < 0.01) (Fig. 1 and Table 2). All stress treatments induced marked increases in Fos expression in the fetal PVN (F3,20 = 27.05, P < 0.01) (Fig. 2 and Table 2). Among the stress groups, the number of Fos-positive cells in the fetal PVN was significantly higher in the immobilization stress group (143 ± 15) (P < 0.01) than those in the restraint (59 ± 7) and forced-walking groups (93 ± 9). Fos expression in the forced-walking group was higher than that in the restraint stress group (P < 0.01). Fos expression in the fetal PVN induced by the maternal stress was associated with increased number of Fos-positive cells in the dam PVN as the severity of stress increased (RI=0.89) (Fig. 3).

**Induction of BDNF in the fetal hypothalamus by maternal stress**

The induction of BDNF following maternal stress was examined by immunoblot analysis with BDNF antibody. Significant increases in the expression of BDNF were observed in the fetal hypothalamus following maternal stress treatments with forced walking and immobilization (Fig. 4). However, maternal restraint stress resulted in no change in fetal hypothalamic BDNF expression.

### Table 1. Concentrations of maternal corticosterone in the control, restraint, forced walking and immobilization stress groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Restraint</th>
<th>Forced walking</th>
<th>Immobilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>261.5±47.6</td>
<td>454.0±50.7*</td>
<td>572.9±40.1*</td>
<td>626.3±34.3*†</td>
</tr>
</tbody>
</table>

Values represents the mean±S.E.M. * P<0.05 as compared to the control group. † P<0.05 as compared to the restraint.

---

**Fig. 1.** Fos-immunoreactive cells in the maternal paraventricular nucleus (PVN) following varying severity of stress. The number of Fos-reactive cells increased with severity of stress treatments (restraint [B], forced walking [C] and immobilization [D]). Fos expression in the PVN in the non-stress control group is shown in A. Scale bars=100 μm.
DISCUSSION

Three types of maternal stress were utilized in this experiment, the severity of which was assessed by measuring maternal plasma corticosterone levels. Maternal plasma corticosterone was highest in the immobilization group, while the forced walking group revealed higher levels of maternal plasma corticosterone than the restraint group. Maternal stress treatments increased the number of Fos-positive cells in both the fetal and maternal PVN. There was a significant correlation between the numbers of fetal and maternal Fos-positive cells. Furthermore, immobilization and forced walking resulted in a significant increase in the expression of BDNF in the fetal hypothalamus.

Maternal stress during pregnancy increases fetal plasma ACTH and corticosterone (Erisman et al., 1990; Ohkawa et al., 1991; Takahashi et al., 1998; Ward and Weisz, 1984; Weinstock et al., 1988). Since maternal ACTH does not cross the placenta (Dupouy et al., 1980; Milkovic and Milkovic, 1961), increases in fetal ACTH must be of fetal origin that results from increased maternal stress. The mechanism by which maternal stress increases fetal neuronal activity is not clear. Maternal corticosterone can cross the placenta (Arishima et al., 1978; Zarrow et al., 1970) and, thus, could contribute to the increases in Fos-positive cells in the fetal PVN. However, adrenalectomy did not alter the magnitude of the c-fos mRNA response to stress in the adult PVN (Helmreich et al., 1996; Jacobson et al., 1990). Furthermore, the negative feedback mechanism of the fetal HPA axis following an increase in plasma corticosterone operates in the last week of pregnancy (D’Angelo et al., 1973; Dupouy and Chatelain, 1984; Dupouy et al., 1987). Thus, corticosterone seems unlikely to play a role in stress-induced fetal PVN Fos expression.

### Table 2. The number of Fos-immunoreactive cells in the maternal and fetal hypothalamic paraventricular nucleus following maternal stress treatments

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Restraint</th>
<th>Forced walking</th>
<th>Immobilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dam</td>
<td>173±16</td>
<td>618±59*</td>
<td>868±32†</td>
<td>985±42*</td>
</tr>
<tr>
<td>Fetus</td>
<td>25±5</td>
<td>59±7*</td>
<td>93±9†</td>
<td>143±15††</td>
</tr>
</tbody>
</table>

Values represents the mean ± S.E.M. * P<0.05 as compared to the control group. † P<0.05 as compared to the restraint group. ‡ P<0.05 as compared to the forced walking group.

**Fig. 2.** Fos-immunoreactive cells in the fetal PVN following the same maternal stress treatments as shown in Fig. 1. The number of Fos-immunoreactive cells in the fetal PVN increased with severity of the maternal stress treatments (restraint [B], forced walking [C] and immobilization [D]). Fos expression in the PVN in the non-stressed control group is shown in A. Scale bars=50 μm.
Morishima et al. 1979 reported that uterine blood flow decreased in response to maternal stress, subsequently resulting in fetal hypoxia. Breen et al. (1997) demonstrated that fetal hypoxia increased Fos expression in fetal sheep brainstem, including several nuclei in the medulla and pons. Therefore, changes in maternofetal circulation may contribute to increased Fos expression in the fetal PVN following maternal stress. However, Jensen et al. (1991) demonstrated that oxygen delivery to the fetal brain was maintained even if oxygen content in the fetal blood was reduced after decreases in uterine blood flow. Further studies are needed to elucidate the mechanisms involved in maternal stress-induced increases in fetal PVN Fos expression.

The present study demonstrated increased fetal brain BDNF expression in response to severe maternal stress.
(forced walking and immobilization), but not following mild maternal stress (restraint). In the adult brain, immobilization increases BDNF mRNA expression in the PVN and decreases BDNF mRNA expression in the hippocampus (Smith et al., 1995a,b; Ueyama et al., 1997). The present data show that severe stress appears to increase BDNF expression in both the fetal and adult PVN, but the mechanism by which this occurs is not clear. Increased BDNF expression induced by severe stress suggests that BDNF may play a role in protecting brain neurons from the neurotoxic effects of severe stress.

Previous studies demonstrated that prenatal-stressed rats showed augmentation (Fride et al., 1986; Hashimoto et al., 2001; McCormick et al., 1995; Peters, 1982; Weinstock et al., 1992) or no change (Fujioke et al., 2001; Henry et al., 1994; Takahashi et al., 1992) in basal HPA activity. The differential effects of prenatal stress on offspring HPA activity may be attributable, at least in part, to differences in expression of Fos and BDNF in fetal PVN following maternal stress. Furthermore, prenatal stress exerts neurotoxic or neurotrophic effects on fetal PVN neurons depending on the duration of stress treatment (Fujioke et al., 1999). In addition, adult offspring that experienced mild prenatal stress demonstrated enhanced cognitive performance (Fujioke et al., 2001). The relationship between these phenotypic changes and the expression of Fos and BDNF in the fetal PVN is not clear and would benefit from further study.

CONCLUSIONS

The present study suggests that the severity of maternal stress is transmitted to the fetal HPA axis. In addition, BDNF may play a role in protecting fetal brain neurons from damage caused by severe stress.

Acknowledgements—The authors thank Mr. H. Endo and Ms M. Takao for their technical assistance and Ms K. Tanaka and M. Ogura for secretarial work. The work was partly supported by the Grants-in-aid for Science Research (No.10877167 and 12770603) from the Ministry of Education, Science and Culture of Japan and Grants-in-aid for Science Research (No.10877167 and 12770603) from the Ministry of Health and Welfare.

REFERENCES


Milkovic S, Milkovic K (1961) Reactiveness of fetal pituitary to stressful