Bull Yamaguchi Med Sch 58(3-4):19-30, 2011

The Effects of Three Types of Stress on Fos Expression in the Hypothalamic Paraventricular Nucleus, Hippocampus and Amygdala in Female Rats at Different Stages of Pregnancy

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(Received April 15, 2011, accepted December 15, 2011)

Abstract Using immunohistochemistry to reveal the Fos protein (a marker of neuronal activation), the present experiments examined whether there were differences in the responses of the paraventricular nucleus (PVN), hippocampus, and amygdala of pregnant rats exposed to three types of stressors (restraint, immobilization, and communication-box stress), all having inherently different severities, at three pregnancy stages (6 days into pregnancy, or P6, early-pregnancy), P12 (mid-pregnancy) and P18 (late-pregnancy). The parvocellular PVN was activated by all three stressors almost equally at all pregnancy stages. The magnocellular PVN appeared to become more active without stressors in mid-pregnancy than in early-pregnancy. The stress responses of the hippocampal dentate gyrus to immobilization and communication-box stress, and that of the basolateral amygdala to immobilization stress in pregnant females, were greater in mid-pregnancy than at the other pregnancy stages. The magnocellular PVN was more sensitive to communication-box stress in late-pregnancy than at the other pregnancy stages, while the hippocampal CA3 and medial amygdala were less sensitive to communication-box stress and to restraint stress in late-pregnancy than at the other pregnancy stages, respectively.

These results suggest that the responses of the PVN, hippocampus, and amygdala in pregnant female rats to different stressors were variable at different pregnancy stages.

Key words: maternal stress, psychological stress, physical stress, mid-pregnancy, pregnancy stage

Introduction

Acute stress induces a variety of neural responses, including activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system. Previous studies have shown that activation of the HPA axis by acute stressors involves increased plasma levels of adrenocorticotropic hormone (ACTH)[1-4] and corticosterone (CORT).[3,5] Endocrine responses to acute stressors are affected by different systemic hormone levels. It has been shown that in female rats, the responsiveness of the HPA axis to acute stress varies during the reproductive cycle, and particularly during pregnancy.[6]

The hypothalamic paraventricular nucleus (PVN) is the brain region that is most responsible for the activation of the HPA axis in response to acute stressors. The PVN consists of the parvocellular (pPVN) and magnocellular (mPVN) divisions. A previous study has shown that stress responses of the PVN vary during pregnancy and lactation.[6]

In that study, the activation of PVN neurons was assessed by using c-fos mRNA as a marker of neuronal activation,[7-11] and it was found that the expression of c-fos mRNA due to restraint stress was reduced during late pregnancy and lactation.[6] There is also a report indicating that the response of the HPA axis to stressors changed at around the fifteenth day of pregnancy in rats.[12] However, the response of the PVN to different types of stress during different stages of pregnancy has not yet been examined.

Neuronal Fos immunoreactivity can be used as a marker of neuronal activation.[7,9,13] In the present experiments, by counting the number of Fos-immunoreactive nuclei, we assessed the expression of the Fos protein in the PVN (pPVN and mPVN) in response to acute stressors at various stages of pregnancy: at 6 days (P6), 12 days (P12) and 18 days (P18). In addition to the PVN, we also examined Fos expression in the hippocampus (HIP) and amygdala (AMY), since they are known to be important brain sites in stress responses.[1,4,14-19] However, stress-induced Fos expression in various brain regions depends on the nature and severity of the stressors.[13,20] We thus employed three types of stress treatment, restraint, immobilization, and communication box stress, to determine how the nature and severity of the stressors could affect Fos expression in the PVN, HIP and AMY in female rats at different stages of pregnancy.

Materials and Methods

Animals

Sprague-Dawley rats (Clea Japan, Tokyo, Japan) were housed in plastic cages (40×25×25 cm; length × width × depth) with woodchip bedding, maintained at 22 ℃, and exposed to a 12 h/12 h light/dark cycle (lights coming on at 8:00 A.M. and going off at 8:00 P.M.). The relative humidity was kept at 50 ±20%. Animals had *ad libitum* access to food pellets and water. Female rats (8-10 weeks old) were mated with mature males. After mating, females were observed daily, and the day on which the seminal plug was observed was taken as day 0 of pregnancy (P0), with the day of expected parturition being day 21 (P21). Each pregnant rat was housed separately after the confirmation of pregnancy. Experiments were performed during early pregnancy (6 days of pregnancy, P6), mid-pregnancy (P12), and near-late pregnancy (P18). All efforts were made to minimize the number of animals used and their suffering. Experimental conditions and procedures were reviewed by the Committee of Ethics on Animal Experiments at Yamaguchi University Graduate School of Medicine and carried out following the Guidelines for Animal Experiments in Yamaguchi University Graduate School of Medicine; these guidelines are in accordance with Japanese Federal Law (No. 105) and the Notification Law (No. 6) of the Japanese Government, and with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 85-23) revised in 1996.

Experimental procedures

We carried out all the experiments for each group of 4 maternal female rats. All the experiments were carried out between 10:00 A.M. and 2:00 P.M. All stress treatments were administered to pregnant rats for 30 min. Control pregnant animals (non-stressed) were left undisturbed in their home cages.

Restraint stress

Pregnant rats were restrained in a small cylindrical cage made of steel wire (7 cm in diameter and 18 cm long) and were placed in their home cages during stress treatment.[13,21-25] Rats were able to move around within the restraining cylinders; this stress treatment causes mild physical and psychological stress.[4]

Immobilization stress

Pregnant rats were immobilized by taping their limbs and tails to a wooden board on their backs as described previously.[4,13,23,24,26,27] This stress treatment causes severe physical and psychological stress.[4]

Communication box stress

Psychological stress was induced using a communication box as described in a previous study.[28] A communication box divided into five compartments (each compartment measuring 19×18×57 cm) was used for this

study. One rat was placed into each of the five compartments. An electric footshock generator (Model NS-SG01, Neuroscience Inc., Tokyo, Japan) was used to produce an electrical current (2 mA, 1 sec duration) to deliver footshocks randomly (approximately four per min) through floor grids. A pregnant rat was placed in the central compartment (in which footshocks were not applied) and four non-pregnant female rats were placed in the remaining four adjacent compartments (in which footshocks were applied). The pregnant rat (psychological stress group) placed in the central compartment, in which the grid was covered by an insulating acrylic sheet to prevent the rat from receiving footshocks, was exposed to the psychological stress of being surrounded by rats receiving footshocks in the four adjacent boxes. The pregnant individual could see, hear, and smell the responses of the four neighboring rats via transparent acrylic dividing panels.

Fos immunohistochemistry

The present study was performed using a methodology based on a previous study of acute stress,[23] in which Fos expression in the fetus and maternal PVN was assessed 30 min after a 30 min period of stress treatment. This procedure was found to result in significant increases in Fos expression in the PVN in pregnant rats. Therefore, in the present experiments, we used the same stress durations and timings prior to Fos response measurement in the pregnant rat PVN, HIP, and AMY.

At 30 min after the end of each stress treatment (which lasted 30 min), the pregnant dams were anesthetized with an overdose of pentobarbital (60 mg/kg, i.p.), and were perfused transcardially with a normal saline solution (0.9%) followed by a solution of chilled 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (pH 7.4). The brain was immediately removed and postfixed in the same fixative at 4 ℃ overnight, and then placed in 10%-30% sucrose with 0.1 M PB for 72 hours at 4 ℃. Several coronal sections of the brains were cut at 50 μm with a cryostat microtome set at -20 ℃. The sections were collected in cryoprotectant solution containing 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol with 0.067 M Millonig buffer (1.285% sodium dihydrogenphosphate dihydrate, 0.26% sodium hydroxide, and 0.327% glucose) and kept at -20 ℃. The method of free floating for Fos immunohistochemistry was followed as described previously.[13,24] Briefly, after blocking endogenous peroxidase with methanol containing 0.3% H_2O_2 and non-specific binding with normal goat serum (diluted 1:20), the brain sections were incubated overnight with rabbit polyclonal anti-Fos protein antiserum (Ab-5, Oncogene Research Products, Cambridge, MA, USA) (1:20000). The sections were then washed with phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and incubated with biotinylated goat anti-rabbit IgG (H+L) (Vector Labs, Burlingame, CA, USA) (1:200) for 60 min, and streptavidin-conjugated peroxidase for 60 min. The immunoreactivity was visualized using 0.01% DAB as choromogen. Nickel ammonium sulfate (0.6%) was used for enhancing the reaction. Sections were mounted on gelatin-coated glass slides, dehydrated with ethanol and xylene, and then coverslipped.

Cell counting

Choosing one section per animal brain, we used four sections from each group, with or without exposure to stress treatment. Fos-immunoreactive nuclei were manually counted using light microscopy (Olympus, BX50, Tokyo, Japan). The number of Fos-positive cells among the control and stress groups was evaluated in the PVN, HIP and AMY, using a modified version of the method used previously.[13,24] The experimenter was unaware of the treatment given while counting the Fos-immunoreactive nuclei. In the pPVN, medial AMY (MeA), central AMY (CeA), and basolateral AMY (BLA), Fos-immunoreactive nuclei were counted individually and the number expressed per 6.25×10 μm^2 of slice area. In the mPVN, cells were counted in an area of 2.25×10 μm^2, which was smaller than that of the pPVN due to the smaller size of the mPVN. The planes of the sections were standardized as much as possible in accordance with the Paxinos and Watson atlas.[29] In order to compare the number of Fos-immunoreactive nuclei in each group, a

representative coronal section was chosen, in which the number of Fos-immunoreactive nuclei was maximal.[23,24] Cells in the PVN were counted at the mid-hypothalamus level, 1.8 mm posterior to the bregma. The CA1 area was counted 3 mm from the CA2 (the demarcation between the CA1 and CA2 could be easily recognized microscopically), whereas all immunoreactive nuclei of the CA2, CA3 and dentate gyrus (DG) were counted in an area stretching from 3.3 to 4.2 mm posterior to the bregma. As hippocampal neurons are present within a thin layer, we could not employ the same counting method as was used for the PVN and AMY, in which cell counting was performed over a certain area. For the CA1 area, cells were counted within a region stretching 3 mm from the border between the CA1 and CA2 (as mentioned before, the demarcation between the CA1 and CA2 areas was clear under the microscope). Immunoreactive cells in the MeA lateral to the optic tract, the CeA dorsolateral to the MeA, and the BLA anterolateral to the CeA were counted within the region lying from 2.3 to 2.8 mm posterior to bregma. The counts were comprised of all those nuclear immunoreactive signals that could be clearly distinguished from the background.

Statistical analysis

The data were expressed as mean values ± S.E.M. Statistical analysis was performed by SPSS statistical software (version 16.0, SPSS Inc., Illinois, USA). Immunohistochemistry was carried out on sections from the brains of four females in each treatment group. In order to extract the full significance of the data, we performed a detailed, step-wise statistical analysis. There were three levels of comparison: stress treatment (restraint, immobilization, and communication box stress), reproductive state (P6, P12, and P18) and regional specificity of response (pPVN, mPVN, CA1, CA2, CA3, DG, MeA, CeA and BLA). We therefore first performed a three-way factorial ANOVA to determine whether there were any main effects and/or interactions between stress treatment, reproductive state, and regional specificity of response. The three-way factorial ANOVA showed no significant interactions between reproductive state and the other two factors; therefore we performed a two-way factorial ANOVA to determine whether there were any main effects and/or interactions between stress and the regional specificity of response. Finally, at each pregnancy stage, a one-way factorial ANOVA was followed by Scheffe's F-test for post-hoc comparison for each brain region. The data were considered significantly different at the $p < 0.05$ level.

Results

Changes in the stress-induced Fos expression were quantitatively analyzed in the PVN, HIP and AMY in maternal female rats. From three-way factorial analyses of variance, significant effects were observed in reproductive state [$F(2,324) = 3.494$, $p < 0.05$], stress treatment [$F(3,324) = 84.151$, $p < 0.001$], and regional specificity of response [$F(8,324) = 149.397$, $p < 0.001$]. The analyses of variance also revealed significant interactions between stress treatment and regional specificity of response [$F(24,324) = 14.507$, $p < 0.001$]. Because there were no significant interactions between reproductive state and the other two factors, we performed two-way factorial analyses of variance for each reproductive state, and observed significant overall effects of stress [P6: $F(3,108) = 21.596$, $p < 0.001$; P12: $F(3,108) = 30.791$, $p < 0.001$; P18: $F(3,108) = 33.458$, $p < 0.001$] and regions [P6: $F(8,108) = 41.124$, $p < 0.001$; P12: $F(8,108) = 47.062$, $p < 0.001$; P18: $F(8,108) = 60.676$, $p < 0.001$]. Additionally, interactions between stress treatment and regional specificity of response were significant for each reproductive state [P6: $F(24,108) = 6.864$, $p < 0.001$; P12: $F(24,108) = 3.966$, $p < 0.001$; P18: $F(24,108) = 5.850$, $p < 0.001$].

The Stages of pregnancy

In all the brain regions except the mPVN, there was no significant change in Fos expression in non-stressed, control groups at the different pregnancy stages. In the mPVN, Fos expression in control (non-stressed) pregnant rats was significantly greater at P12 than at P6 [$F(2,9) = 5.351$, $p < 0.05$]. In addition, in all the brain regions, any stress treatment induced no significant difference in

Fos expression at different pregnancy stages.

Brain regions

The following results were obtained using one-way analyses of variance, followed by Scheffe's F-tests for each brain region.

Paraventricular nucleus (PVN)

Fos expression in the PVN was analyzed separately in the parvocellular and magnocellular regions (Fig. 1). For Fos expression in the parvocellular region, a two-way factorial ANOVA showed a significant stressor effect [$F(3,36) = 40.256$, $p < 0.001$]. At each pregnancy stage, significant effects were observed after stress treatment [P6: $F(3,12) = 15.468$, $p < 0.001$; P12: $F(3,12) = 8.880$, $p < 0.01$; P18: $F(3,12) = 24.250$, $p < 0.001$]. At all pregnancy stages, all stress treatments induced significantly greater Fos expression as compared to the non-stressed pregnant control group (Fig. 2).

Regarding Fos expression in the magnocellular region, analysis showed a significant stressor effect [$F(3,36) = 22.194$, $p < 0.001$]. In each reproductive state, significant effects were observed after stress treatment [P6: $F(3,12) = 11.445$, $p < 0.001$; P12: $F(3,12) = 3.916$, $p < 0.05$; P18: $F(3,12) = 10.120$, $p < 0.01$] (Fig. 2). At all stages of pregnancy, Fos expression in the magnocellular region was significantly increased in the immobilization stress group compared to the controls. At P18, Fos expression was significantly greater in the communication box stress (psychological stress) group than in the control group.

Hippocampus (HIP)

Fos expression was examined in various regions of the HIP, including the CA1, CA2, CA3, and DG regions (Fig. 3). For Fos expression in the CA1 region, a two-way factorial ANOVA showed a significant stressor effect [$F(3,36) = 13.549$, $p < 0.001$]. In each reproductive state, significant effects were observed after stress treatment [P6: $F(3,12) = 5.888$, $p < 0.05$; P12: $F(3,12) = 4.850$, $p < 0.05$; P18: $F(3,12) = 5.109$, $p < 0.05$] (Fig. 4). At all stages of pregnancy, the psychological stress group showed significantly greater Fos expression compared to the controls ($p < 0.05$). For Fos expression in the CA3 region, a two-way factorial ANOVA showed a significant stressor effect [$F(3,36) = 10.903$, $p < 0.001$]. At the early (P6) and middle (P12) stages of pregnancy, significant effects on Fos expression were observed with stress treatment [P6: $F(3,12) = 4.888$, $p < 0.05$; P12: $F(3,12) = 5.394$, $p < 0.05$]. At both the early and mid-pregnancy stages, Fos expression in the CA3 region was significantly greater in the psychological stress group than in the control group. For Fos expression in the DG region, a two-way factorial ANOVA showed a significant effect exerted by the stressor [$F(3,36) = 8.400$, $p < 0.001$]. In the mid-pregnancy stage, a significant effect was observed with stress treatment [P12: $F(3,12) = 17.703$, $p < 0.001$]. The

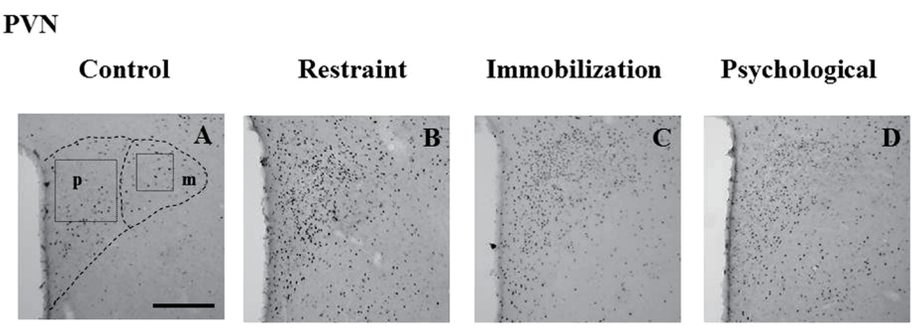

Fig. 1 Fos-immunoreactive cells in the paraventricular nucleus (PVN) following restraint (B), immobilization (C), and psychological (communication box) stress (D) at P12 (mid-pregnancy) (A, control=non-stressed pregnant animals; m=magnocellular region; p=parvocellular region). The squares indicate regions in which the number of Fos-immunoreactive cells was counted (same regions counted under all conditions). Scale bar=250 μm

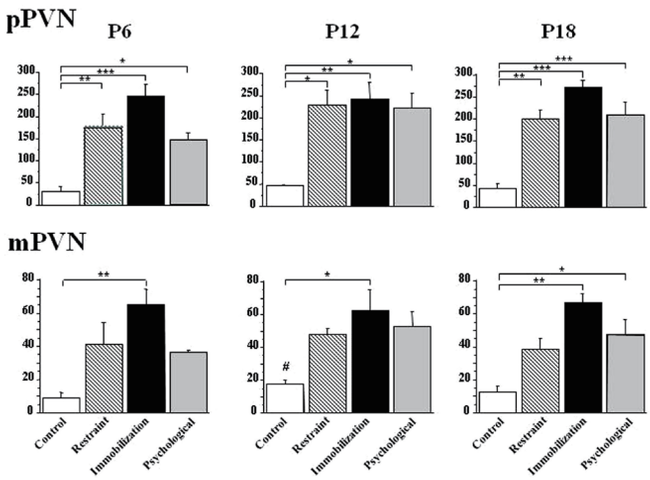

Fig. 2 The mean number of Fos-positive cells in the parvocellular (pPVN) and magnocellular (mPVN) regions of the PVN following restraint, immobilization, and psychological (communication box) stress at each stage of pregnancy (control=non-stressed pregnant animals). There were four pregnant animals in each group. Significance is shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with pregnant animals not subjected to stress treatment, and # indicates $p < 0.05$ compared with P6 animals.

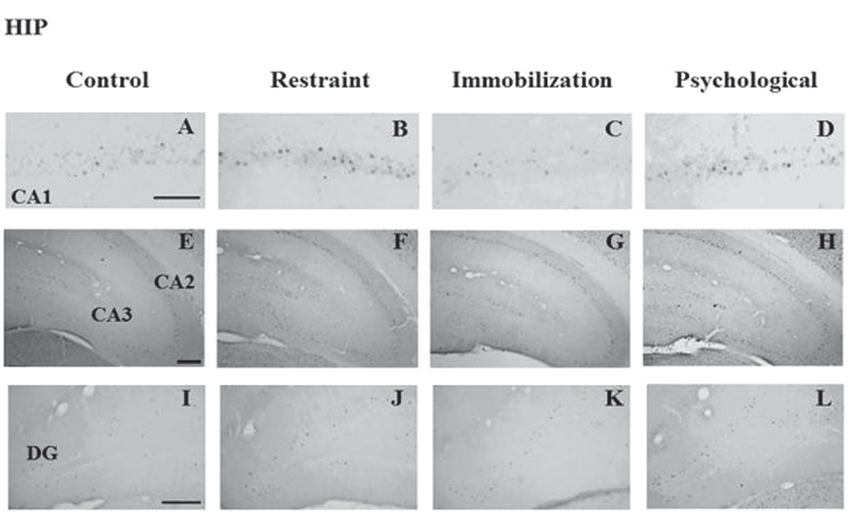

Fig. 3 Fos-immunoreactive cells in the hippocampus (HIP) following restraint (B, F, J), immobilization (C, G, K), and psychological (communication box) stress (D, H, L) at P12 (mid-pregnancy) (A & E & I, control=non-stressed pregnant animals; upper panels, CA1 (A-D); middle panels, CA2 and CA3 (E-H); lower panels, dentate gyrus (DG) (I-L). Scale bar for CA1 (A-D) =100 μm; CA2 and CA3 (E-H) =250 μm; DG (I-L) =250 μm.

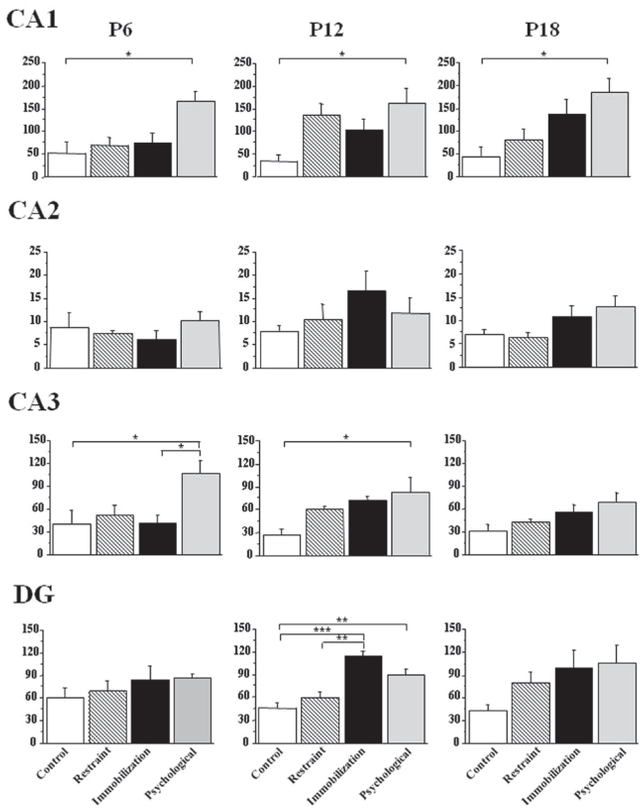

Fig. 4 The mean number of Fos-positive cells in the CA1, CA2 CA3 and DG regions of the HIP following restraint, immobilization, and psychological (communication box) stress at each stage of pregnancy (control=non-stressed pregnant animals). There were four pregnant animals in each group. Significance shown as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

levels of Fos expression in the rats exposed to immobilization and psychological stress at the mid-pregnancy stage were significantly greater compared to the control group. In the CA2 region, there were no significant differences in Fos expression at any of the three stages of pregnancy; this was so in all three stress groups.

Amygdala (AMY)

We examined three subdivisions of the amygdaloid nucleus (MeA, CeA, and BLA) (Fig. 5). For Fos expression in the MeA, the analysis showed a significant stressor effect [$F(3,36) = 41.266$, $p < 0.001$]. At each stage of pregnancy, significant effects were observed with stress treatment [P6: $F(3,12) = 20.332$, $p < 0.001$; P12: $F(3,12) = 15.070$, $p < 0.001$; P18: $F(3,12) = 11.045$, $p < 0.001$] (Fig. 6). With the exception of restraint stress at the late stage of pregnancy (P18), all other stages of pregnancy exhibited increased Fos expression in the MeA in all three stress groups when compared to the control group. The changes were significant except at the late pregnancy stage in the restraint stress group. Analysis showed a significant stressor effect on Fos expression in the CeA [$F(3,36) = 3.285$, $p < 0.05$]. However, there were no significant effects observed with stress treatment at any stage of pregnancy. Analysis showed a significant stressor effect on Fos expression in the BLA [$F(3,36) = 6.240$, $p < 0.01$]. A significant effect was observed in stress treatment only at the mid-pregnancy stage [P12: $F(3,12) = 6.373$, $p < 0.01$]. In the BLA, the immobilization group exhibited a significantly higher level of Fos expression at the mid-pregnancy stage as compared to the control group ($p < 0.05$).

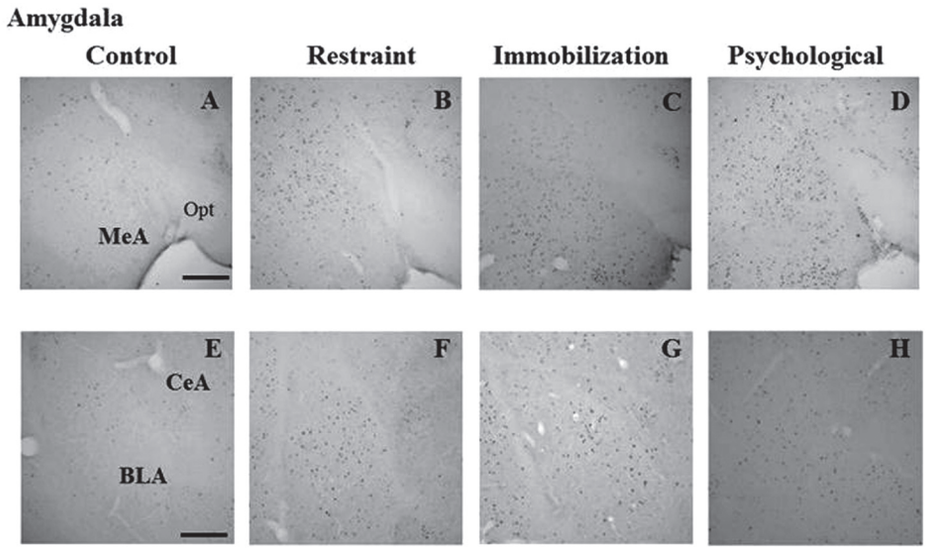

Fig. 5 Fos-immunoreactive cells in the medial (MeA) (A-D), central (CeA) (E-H) and basolateral (BLA) (E-H) regions of the amygdala (AMY) at P12 (mid-pregnancy) (A, control=non-stressed pregnant animals; scale bar=250 μm). B & F, restraint stress; C & G, immobilization stress; D & H, psychological stress.

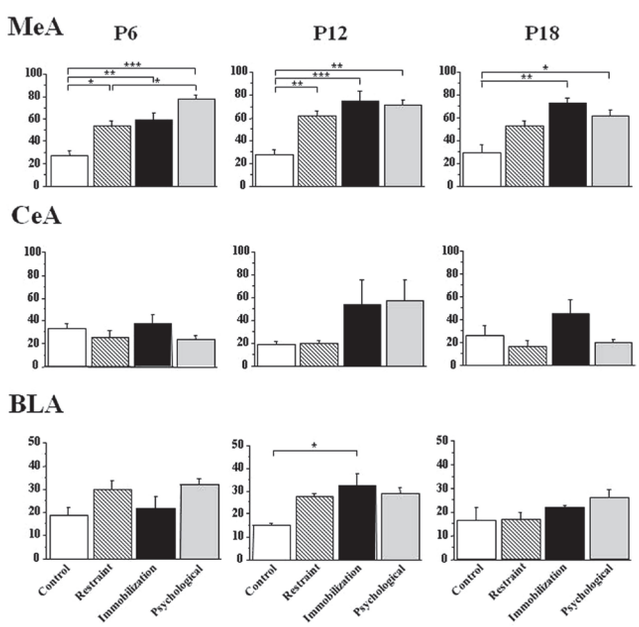

Fig. 6 The mean number of Fos-positive cells in the MeA, CeA and BLA regions of the AMY following restraint, immobilization, and psychological (communication box) stress at each stage of pregnancy (control=non-stressed pregnant animals). There were four pregnant animals in each group. Significance shown as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Discussion

Using neuronal Fos immunoreactivity as a marker of neuronal activation, the present experiments examined whether Fos expression in the PVN, HIP, and AMY could be differentially affected in pregnant female rats by the restraint, immobilization, or communication box forms of stress induction. In the pPVN, all the stress treatments significantly increased Fos expression at all pregnancy stages. In the mPVN, Fos expression in control (non-stressed) pregnant rats was significantly greater at P12 than at P6. The mPVN showed significant increases in Fos expression at all pregnancy stages following immobilization stress, and at P18 following communication box stress. In the hippocampus, communication box stress significantly increased Fos expression in the CA1 at all pregnancy stages, and in the CA3 at P6 and P12. Fos expression in the DG following immobilization and communication box stress was greater at P12 than at the other pregnancy stages. The MeA exhibited significant increases in Fos expression at all pregnancy stages following all three forms of stress, except in dams exposed to restraint stress at P18. In the BLA, only P12 rats showed an increase in Fos expression following immobilization stress.

In the present experiments, we did not use virgin rats or ovariectomized, estradiol-substituted rats as control animals. The reason for this was that, given the nature of the estrous cycle, four groups of animals at the four different stages of the estrous cycle (proestrus, estrus, metestrus, and diestrus) would be required as controls, so the present experiments employed pregnant rats as the control group. However, without comparison to unfertilized control animals undergoing normal estrus cycles, we could not determine whether the stress responsiveness of pregnant animals would be attenuated or would become more sensitive at certain stages of pregnancy. If virgin rats or ovariectomized, estradiol-substituted rats were used as control animals, we would clearly be able to confirm changes in Fos expression associated directly with pregnancy itself and with the stages of pregnancy. Therefore, the absence of these control animals limits the interpretation of the results and conclusions.

Stress-induced Fos expression in the PVN

A previous paper reported that c-fos mRNA expression in the pPVN induced by restraint stress was reduced during late pregnancy (P19-21), as was lactation, when compared to virgin females.[6] This is consistent with the finding that the stress response of the HPA axis is reduced during lactation.[30] In the present study, in which we used P18 rats, all the stress treatments increased Fos expression at all stages of pregnancy (P6, P12, and P18). The failure to detect reduced Fos expression during late pregnancy in the present work may be due to the differences between measurements made in the late stages of pregnancy in the experiments of da Costa et al. (P19-P21) and the late stage of the present experiments (corresponding to P19 in the experiments of da Costa et al.).[6] It is likely that the late stage in our experiments (in which the P20 and P21 measurements described in the experiments of da Costa et al. were not performed) was not sufficiently late to detect reduced Fos expression in the pPVN clearly. Furthermore, in the experiments of da Costa et al.,[6] ten pregnant animals were held in the same cage, while in our experiments each pregnant rat was housed separately after the confirmation of pregnancy. It is possible that these differences in housing might affect the responsiveness of pregnant animals to stressors.

Shibasaki et al.[28] reported that the levels of plasma ACTH in male rats exposed to psychological stress (30 min in duration) using a communication box similar to that used in the present study, were not significantly different from those in control animals. Most recently, the same research group has demonstrated that there are gender differences in ACTH and CORT secretion and corticotrophin-releasing factor (CRF) mRNA expression in the PVN in response to communication box stress. Females in both proestrus and diestrus showed significant increases in plasma ACTH, CORT, and CRF mRNA expression in the PVN in response to communication box stress, while no significant responses in the HPA axis to psychological

stress were found in males.[31] These findings are consistent with the results of the present study, in that the same psychological stress technique induced significant increases in Fos expression in the pPVN in female rats at all stages of pregnancy. However, it remains unclear whether the responsiveness of pregnant rats to this type of psychological stress is different from that of virgin control rats. It is essential to have information about the HPA axis responses of pregnant animals to interpret the Fos data from the pPVN.

Stress-induced Fos expression in the HIP

In a previous study, restraint stress during late pregnancy and lactation did not bring about significant changes in c-fos mRNA expression in the HIP.[6] However, previous studies using male rats have demonstrated that restraint and immobilization stress increase c-fos mRNA or Fos protein expression in the HIP.[13,32,33] Thus, it appears that there is a distinct difference in the response of the HIP to physical stress between males and females. Figueiredo et al.[34] have clearly demonstrated that c-fos mRNA expression in the HIP induced by restraint stress varies with sex and estrous cycle phase. In the HIP, the magnitude of c-fos mRNA expression induced by restraint stress was markedly lower in proestrous and estrous females compared to males and diestrous females. There is no evidence in this study that little changes in Fos expression in the HIP induced by physical stress were due to a difference in sex or due to hormonal changes during pregnancy.

The present study suggests that Fos expression in the HIP is more sensitive to psychological than physical stress. Since there are no reports in which the same type of psychological stress is used to study Fos expression in the HIP as was used in this study, it remains unclear whether the HIP is sensitive to psychological stress in non-pregnant females and males. There is a possibility that since the HIP is associated with spatial learning and memory,[35] spatial information originating from the auditory and olfactory senses about the four directions in the communication box may play a role in increasing Fos expression in the HIP following this type of psychological stress.

Stress-induced Fos expression in the AMY

The present finding is consistent with that of a previous study in which a 30-min restraint and immobilization stress technique significantly increased Fos expression in the MeA, but not in the CeA or BLA, in male rats.[13] Thus the stress-induced Fos expression in the MeA in pregnant rats may not be associated with pregnancy. In males, neither the BLA nor the DG showed any change in Fos expression following 30 min of restraint and immobilization stress.[13] Although no definitive conclusion can be drawn without virgin control rats, the stress-induced Fos expression in the BLA and hippocampal DG seen in rats in mid-pregnancy is suggested to occur in association with pregnancy, specifically its middle phase.

Conclusion

These results suggest that the responses of the PVN, hippocampus, and amygdala in pregnant female rats to different stressors were variable at different pregnancy stages.

Acknowledgements

We are grateful to Dr. Leslie Sargent Jones for reviewing the manuscript and Mrs. Kazuko Tanaka for her excellent secretarial assistance.

Conflict of Interest

The authors state no conflict of interest.

References

1. Stern, J.M., Goldman, L. and Levine, S.: Pituitary-adrenal responsiveness during lactation in rats. *Neuroendocrinology,* **12**: 179-191, 1973.
2. Gibbs, D.M.: Dissociation of oxytocin, vasopressin and corticotropin secretion during different types of stress. *Life Science,* **35**: 487-491, 1984.
3. Hanger, R.L., Millan, M.A., Lorang, M., Harwood, J.P. and Aguilera, G.: Corticotropin-releasing factor receptors and pituitary adrenal responses during immobilization stress. *Endocrinology,* **123**:

396-405, 1988.
4. Ueyama, T., Umemoto, S. and Senba, E.: Immobilization stress induced c-fos and c-jun immediate early gene expression in the heart. *Life Science,* **59**: 339-347, 1996.
5. Lightman, S.L. and Young, III, W.S.: Corticotrophin-releasing factor, vasopressin and pro-opiomelanocortin mRNA responses to stress and opiates in the rat. *J. Physiol.,* **403**: 511-523, 1988.
6. da Costa, A.P.C., Wood, S., Ingram, C.D. and Lightman, S.L.: Region-specific reduction in stress-induced c-fos mRNA expression during pregnancy and lactation. *Brain Res.,* **742**: 177-184, 1990.
7. Sagar, S., Sharp, F.R. and Curran, T.: Expression of c-fos protein in brain: metabolic mapping at the cellular level. S*cience,* **240**: 1328-1331, 1988.
8. Dragunow, M. and Faull, R.: The use of c-fos as a metabolic marker in neuronal pathway tracing. *J. Neurosci. Methods,* **29**: 261-265, 1989.
9. Bullitt, E.: Expression of c-Fos-like protein as a marker for neural activity following noxious stimulation in the rat. *J. Comp. Neurol.,* **296**: 517-530, 1990.
10. Chan, R.K., Broun, E.R., Ericsson, A., Kovacs, K.J. and Sawchenko, P.E.: A comparison of two immediate-early genes, c-fos and NGFI-B, as markers for functional activation in stress-related neuroendocrine circuitry. *J. Neurosci.,* **13**: 5126-5138, 1993.
11. Duman, R.S.: Regulation of intercellular signal transduction and gene expression by stress. *In* Friedmen, M.J., Charnery, D.S. and Deuch, Y.A. (eds.), *Neurobiological and Clinical Consequences of stress: from normal adaptation to PTSD,* Lippinjcott-Ravan Publishers, Philadelphia, 1995, pp.27-43.
12. Neumann, I.D., Johnstone, H., Hatzinger, M., Liebsch, G., Shipston, M., Russell, J.A., Landgraf, R. and Douglas, A.J.: Attenuated neuroendocrine responses to emotional and physical stressors in pregnant rats involve adenohypophysial changes. *J. Physiol.,* **508**: 289-300, 1998.
13. Chowdhury, G.M.I., Fujioka,T. and Nakamura, S.: Induction and adaptation of Fos expression in the rat brain by two types of acute restraint stress. *Brain Res. Bull.,* **52**: 171-182, 2000.
14. Herman, J.P., Schafer, M.K., Young, E.A., Thompson, R., Douglass, J., Akil, H. and Watoson, S.J.: Evidence for hippocampal regulation of neuroendocrine neurons of the hypothalamo-pituitary-adrenocortical axis. *J. Neurosci.,* **9**: 3072-3082, 1989.
15. Lightman, S.L. and Young, III, W.S.: Lactation inhibits stress-mediated secretion of corticosterone oxytocin and hypothalamic accumulation of corticotropin-releasing factor and enkephalin messenger ribonucleic acids. *Endocrinology,* **124**: 2358-2364, 1989.
16. Atkinson, H.C. and Waddell, B.J.: The hypothalamic-pituitary-adrenal axis in rat pregnancy and lactation: circadian variation and interrelationship of plasma adrenocorticotropin and corticosterone. *Endocrinology,* **136**: 512-520, 1995.
17. Fischer, D., Patchev, V.K., Hellbach, S., Hassan, A.H. and Almeida, O.F.: Lactation as a model of naturally reversible hypercorticalism plasticity in the mechanisms governing hypothalamo-pituitary-adrenocortical activity in rats. *J. Clin. Invest.,* **96**: 1208-1215, 1995.
18. Smith, M.A., Makino, S., Kvetnansky, R. and Post, R.M.: Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J. Neurosci.,* **15**: 1758-1777, 1995.
19. Herman, J.P., Ostrander, M.M., Mueller, N.K. and Figueiredo, H.: Limbic system mechanisms of stress regulation: Hypothalamo-pituitary-adrenocortical axis. *Prog. Neuropsychopharmacol. Biol. Psychiatry,* **29**: 1201-1213, 2005.
20. Emmert, M.H. and Herman, J.P.: Differential forebrain c-fos mRNA induction by ether inhalation and novelty: evidence for distinctive stress pathways. *Brain Res.,* **845**: 60-67, 1999.
21. Fujioka, T., Sakata, Y., Yamaguchi, K., Shibasaki, T., Kato, H. and Nakamura, S.: The effects of prenatal stress on the development of hypothalamic paraventricular neurons in fetal rats. *Neuroscience,* **92**: 1079-1088, 1999.

22. Fujioka, T., Fujioka, A., Tan, N., Chowdhury, G.M.I., Mouri, H., Sakata, Y. and Nakamura, S.: Mild prenatal stress enhances learning performance in the non-adopted rat offspring. *Neuroscience,* **103**: 301-307, 2001.
23. Fujioka, T., Fujioka, A., Endoh, H., Sakata, Y., Furukawa, S. and Nakamura, S.: Materno-fetal coordination of stress-induced Fos expression in the hypothalamic paraventricular nucleus during pregnancy. *Neuroscience,* **118**: 409-415, 2003.
24. Tobe, I., Ishida, Y., Tanaka, M., Endo, H., Fujioka, T. and Nakamura, S.: Effects of repeated maternal stress on Fos expression in the hypothalamic paraventricular nucleus of fetal rats. *Neuroscience,* **134**: 387-395, 2005.
25. Fujioka, A., Fujioka, T., Ishida, Y., Maekawa, T. and Nakamura, S.: Differential effects of prenatal stress on the morphological maturation of hippocampal neurons. *Neuroscience,* **141**: 907-915, 2006.
26. Schreiber, S.S., Tocco, G., Shors, T.J. and Thimpson, R.F.: Activation of immediate early genes after acute stress. *Neuroreport,* **2**: 7-20, 1991.
27. Senba, E. and Ueyama, T.: Stress-induced expression of immediate early genes in the brain and peripheral organs of the rat. *Neurosci. Res.,* **29**: 183-207, 1997.
28. Shibasaki, T., Imaki, T., Hotta, M., Ling, N. and Demura, H.: Psychological stress increases arousal through brain corticotropin-releasing hormone without significant increase in adrenocorticotropin and catecholamine secretion. *Brain Res.,* **618**: 71-75, 1993.
29. Paxinos, G. and Watson, C.: *The Rat Brain in Stereotaxic Coordianate,* Academic Press, New York, 1997.
30. Lightman, S.L.: Alterations in hypothalamic pituitary responsiveness during lactation. *Ann. N. Y. Acad. Sci.,* **652**: 340-346, 1992.
31. Iwasaki-Sekino, A., Mano-Otagiri, A., Ohata, H., Yamauchi, N. and Shibasaki, T.: Gender differences in corticotropin and corticosterone secretion and corticotropin-releasing factor mRNA expression in the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala in response to footshock stress or psychological stress in rats. *Psychoneuroendocrinology,* **34**: 226-237, 2009.
32. Imaki, T., Shibasaki, T., Hotta, M. and Demura, H.: Intracerebroventricular administrasion of corticotropin-releasing factor induced c-fos mRNA expression in brain regions related to stress responses: comparison with pattern of c-fos mRNA induction after stress. *Brain Res.,* **616**: 114-125, 1993.
33. Cullinan, W.E., Herman, J.P., Battaglia, D.F., Akil, H. and Watson, S.J.: Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience,* **64**: 477-505, 1995.
34. Figueiredo, H.F., Dolgas, C.M. and Herman, J.P.: Stress activation of cortex and hippocampus is modulated by sex and stage of estrus. *Endocrinology,* **143**: 2534-2540, 2002.
35. O'Keefe, J. and Nadel, L.: Spatial behavior. *In* O'Keefe, J. and Nadel, L. (eds.), *The hippocampus as a cognitive map,* Clarendon Press, Oxford, 1978, pp.62-101.